Cucurbit[n]uril-Dye Conjugate for Biorelevant Analytes Recognition and Distinction in Aqueous Media and Biofluids

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I hereby declare that I have completed my dissertation independently, without using any other resources other than indicated. This dissertation has not yet been submitted to any examination authority or for grading at other academic institutions.

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1. Abstract

As a nature-inspired and nature-derived scientific field, supramolecular chemistry has contributed dramatically to achievements in various scientific fields ranging from catalysis to drug delivery and accelerated the advancement of the entire subject of chemistry. In this context, supramolecular chemists use non-covalent intermolecular interactions, which can be dynamic, to design molecular aggregates with unique physicochemical properties. Such non-covalent interactions are, for example, the hydrophobic interaction, π-π interaction, hydrogen bonding, electrostatic interaction, and the solvent effect. As synthetic molecular recognition systems and macrocyclic host-guest chemistry have advanced, more and more artificial synthetic host molecules with a recognition motif for specific guest(s) have been discovered and extensively investigated. Following the example of the naturally occurring host molecule cyclodextrin, the new generation of artificial synthetic host molecules is represented by cryptands, cucurbit[n]urils, calix[n]arenes, pillar[n]arenes, to name a few. More than a hundred years after their first synthesis, cucurbit[n]urils play a prominent role among macrocyclic hosts due to their outstanding binding affinities for guest molecules, resulting in the formation of host-guest complexes to be exploited in a variety of fields ranging from catalysis, materials chemistry and drug delivery applications.

Cucurbit[n]uril-based chemosensing systems have also been widely utilized in medical diagnostics to meet the requirement of biorelevant analytes recognition and detection by taking advantage of high binding affinities with drugs or metabolites. In combination with the indicators that equip the chemosensor with photophysical responsiveness, the reliable indicator displacement assay (IDA)-based chemosensing system has been researched by many supramolecular scientists for monitoring biophysical and enzymatic processes. However, besides their solubility limitations, the development of cucurbit[n]uril-based chemosensor that predictably binds to guests in biological buffers or fluids remains a challenge because ubiquitous salts play a negative role in the host-guest system, resulting in the dissociation of the non-covalent complex.

In this thesis, the novel cucurbit[7]uril IDA-based unimolecular chemosensors were synthesized, and the sensing strategies for biorelevant analytes recognition with salt-resistance in biological media were designed. In the first section of this work, a high-affinity indicator, berberine, linked CB7 unimolecular conjugate through a short ethylene glycol linker (CB7-BC) was developed and fully characterized. The chemosensors displayed exceptional resistance to dilution and salt effect other than traditional non-covalent reporter CBN pairs – an important aspect when it comes to the application of chemosensors in a real-world scenario. This chemosensor can selectively and quantitatively respond to Parkinson’s drug amantadine (at the micromolar range) in a physiologically buffered media and genuine biofluids (e.g., urine and saliva) even in the presence of potential competitors. The second section introduced an innovative salt-induced adaptive strategy to detect biorelevant analytes using a nitrobenzoxadiazole-cucurbit[7]uril chemosensor (CB7-NBD), allowing discrimination of multiple analytes in real biofluids such as urine, saliva, and human blood serum in the micromolar range. The readout fluorescent signal is individual and unique for each analyte due to the varied salt-adaptive behaviors of chemosensor and
analyte complexes following the addition of different salts. This information can be translated into an intuitive pairwise difference using a data-driven approach. In addition, ion mobility measurements combined with density functional theory (DFT) calculation were used to investigate the binding mechanism and assess the potential conformation of complexes. Notably, this salt-adaptive system was exploited for sensing applications in real biofluids such as urine, saliva, and blood serum.

In summary, novel cucurbit[n]uril-based conjugated chemosensors were given as a practical solution to overcome the inevitable disintegration and dysfunction of traditional non-covalent host-guest chemosensors in the buffered medium with high salinity. These unimolecular conjugated chemosensors can realize the detection and recognition of biologically relevant analytes, and it opens up a constructive suggestion for the design of future functional macrocyclic chemosensors.
Kurzzusammenfassung / Abstract in German


Kurzzusammenfassung

selektiv und quantitativ auf das Parkinson-Medikament Amantadin (im mikromolaren Bereich), in physiologisch gepufferten Medien und echten Bioflüssigkeiten (z. B. Urin und Speichel), sogar im Beisein von Kompetitoren.


2. General Introduction

2.1. Supramolecular host-guest chemistry

In contrast to the more traditional covalent chemistry, supramolecular chemistry is generally defined as ‘chemistry beyond the molecule’ and based on non-covalent intermolecular forces.\(^1\) The importance of supramolecular chemistry was accentuated by the 1987 Nobel Prize for Chemistry, recognizing the development and investigations of crown ether, host-guest, and molecular recognition chemistries through the outstanding contributions of Charles J. Pedersen, Jean-Marie Lehn, and Donald J. Cram.\(^5\)\(^-\)\(^7\)

Actually, the exploration of supramolecular chemistry has been underway for a century. Johannes Diderik van der Waals first postulated the existence of intermolecular forces in 1873. Emil Fischer hypothesized the fundamental principles of molecular recognition and host-guest chemistry, which took the form of a ‘lock and key’ model imitated from enzyme and substrate interaction in 1894.\(^8\)

Latimer and Roderbush described the hydrogen bond in 1920. The supramolecular chemistry originated from our environmental living biological processes, such as the substrate binding to the receptor, assembling nucleic acid and protein complex, forming cellular membranes, signal induction by neurotransmitters, and various biological processes.\(^9\)

Nowadays, supramolecular interactions are rationalized as tools for developing structures with specific functions. In 2016, Jean-Pierre Sauvage, Sir J. Fraser Stoddart, and Bernard L. Feringa shared the Nobel Prize in Chemistry for the design and synthesis of molecular machines,\(^10\)\(^-\)\(^12\) and their work ranged from the reliable synthesis of catenanes and rotaxanes to molecule knots, rotary motors, shuttles, elevators, and even a nanocar. Meanwhile, more supramolecular chemists, \(e.g.,\) Bert Meijer (E. W. Meijer), Takuzo Aida, Samuel I. Stupp, \(etc.,\) explored supramolecular applications and expand the integration with other chemical fields.

After nearly half a century, supramolecular chemistry has moved from the conceptual stage into various practical application fields.\(^13\)\(^-\)\(^17\) The key feature of supramolecular chemistry is the dynamic character of construction and exchange of components spontaneously.\(^18\) This property endows the supramolecular entities to adjust to external stimuli, such as temperature, ion strength, pH, redox, and light.\(^19\)\(^-\)\(^23\)

Among a series of non-covalent interactions (hydrogen bonding, electrostatic interaction, Van der Waals force, \(\pi-\pi\) stacking, metal-ligand coordination, hydrophobic interaction, \(etc.,\)),\(^24\) host-guest chemistry, as a branch of supramolecular chemistry, describes that the complexes are composed of two or more molecules that are held together in a unique structural relationship by forces other than covalent bonds.\(^25\)\(^,\)\(^26\) Host-guest systems are currently researched for various applications such as drug delivery,\(^27\) detection and sensing,\(^30\)\(^,\)\(^31\) environmental monitoring,\(^32\)\(^-\)\(^35\) and the preparation of functional materials.\(^36\)\(^-\)\(^38\)

Typically, the hosts are equipped with a cavity corresponding to specific guest molecules with suitable structural properties. Macrocyclic components have been extensively studied and investigated in recent years, including cyclodextrin, cucurbit[n]uril, pillar[n]arene, calix[n]arene, \(etc.\) (Figure 2.1). They all exhibit the dynamically reversible complexation capabilities for a wide range of guest components.
General Introduction

2.2. Cucurbit[n]uril in host-guest system

2.2.1. Historical development of cucurbit[n]uril

Among the numerous natural and artificial synthetic macrocyclic host molecules, Cucurbit[n]urils are widely employed in various research areas. In 1904, Eberhard Meyer firstly synthesized by chance CB6 by the condensation reaction between glycoluril and formaldehyde, then the results were published by Behrend and co-workers in the following year. However, it was only in 1981 that the cyclic structure of CB6 was experimentally confirmed by Freeman and coworkers when X-ray crystal data revealed the presence of six glycoluril units linked by pairs of methylene bridges (Figure 2.2 (a)). Due to a general resemblance of this product to a gourd or pumpkin (family Cucurbitaceae), they proposed the trivial name cucurbituril for it. After another 20 years of development, new cucurbituril homologues containing five, seven, eight, and ten glycoluril units, were synthesized and characterized by the groups of Kim and Day. Since their discovery, the range of applications of cucurbituril has been expanded, as the larger cavity of larger CBn allows them to associate with a variety of guest molecules of different sizes and polarity (Figure 2.2 (b)). Also, higher CBn homologues were discovered gradually in the following time, such as inverted CBn, nor-seco-CBn, twisted CB14, and others, CB5-CB8 have been studied and explored in a wide range of scientific research and fundamental applications.

![Chemical structures of typical macrocyclic hosts.](image)

**Figure 2.1** Chemical structures of typical macrocyclic hosts.

![Chemical structure and schematic illustration of cucurbit[n]uril.](image)

**Figure 2.2** (a) Chemical structure and schematic illustration of cucurbit[n]uril. (b) Structural parameters of CB5 to CB8.
2.2.2. Synthesis and properties of cucurbit[n]uril

The synthesis of cucurbituril still follows the early acid-catalyzed condensation reaction of glycoluril and formaldehyde (Figure 2.3). According to Kim and Day’s research, the yield ratio of each homologue can be controllable by adjusting the reaction conditions. The temperature of reaction and the acid concentration significantly impact the formation of CBₙ. The final distribution of each CBₙ homologue in the mixture is decided by the cyclization of precursor oligomers. For instance, almost no CB7 and CB8 are formed when the condensation reaction is done at high dilution because the high-dilution experimental conditions kinetically limited the rate that oligomers achieved a length beyond 5 or 6 units before they condensed. In another case, no CBₙ formed in concentrated HCl after 1 month at room temperature, but CB6 was almost quantitatively produced after 2 months in concentration H₂SO₄ due to the dehydrating ability of concentrated H₂SO₄.

Figure 2.3 CBₙ synthesis by acid-catalyzed condensation reaction

CBₙ, particularly CB6, is characterized by excellent thermal stability and chemical inertness. The beginning of CBₙ decomposition is around 250°C, and the foremost step of decomposition can even reach 425°C. Moreover, CBₙ is fully adapted in the whole pH range or in the presence of strong redox reagents. The non-toxic and photochemical inert properties of CBₙ make it an interesting candidate for biological and spectroscopic sensing applications. The evaluation of the biocompatibility of CBₙ has been carried out by many researchers in mice and in blood cells, which exhibited excellent profiles and no apparent damage to the cell directly.

The solubility of CBₙ is commonly low in the water compared to the similarly-sized cyclodextrin homologues in size relatively, except for CB₅ and CB₇ (20–30 mM). However, all of CBₙ homologues are well soluble in acidic water as well as the in aqueous alkali metal ion solution.

In contrast to the well-known macrocyclic host of cyclodextrin, the structural features of CBₙ are characterized by the symmetric and barrel-shaped structure with highly electron-density carbonyl portals and a hydrophobic cavity. Notably, the portal diameter of CBₙ is almost approximately 2 Å narrower than the cavity diameter, which results in significant steric barriers to guest association and dissociation. Thus, the size-selectivity of CBₙ to guests exceeds those for cyclodextrin. Except for the analytes with a negative charge, almost organic molecules with the fit size to the CBₙ cavity are bound with an appreciable affinity. Due to the ion-dipole interaction with carbonyl portals, positively charged guests show a relatively higher affinity than their neutral counterparts. Taking advantage of the relatively
rigid structure, CBn can provide a great size- and charge-selectivity for many biologically and medicinally relevant analytes in aqueous media.

As shown in the electrostatic potential map (Figure 2.4 (a)), a more negative electrostatic potential (blue-colored) is observed at the portals of CB7 than β-cyclodextrin (β-CD)\(^{53}\), and this feature is also prevalent in other CB homologues. (Figure 2.4 (b)).\(^{59}\) In contrast to the high electronegative CBn portals, the inner cavity of CBn is significantly hydrophobic without any functional groups or electron pairs. This combined feature endows CBn the high affinity and selectivity for specific classes of guest molecules, e.g., protonated hydrophobic amino acids, alkylated ammonium, and imidazolium. Furthermore, the electrostatic effects and hydrophobic interactions play an essential role in the binding properties of CBn homologues.

![Figure 2.4](image)

**Figure 2.4** Electrostatic potential surfaces of CB7 and β-CD. Reprinted by permission from ref.53. Copyright © 2003, American Chemical Society. (b) Calculated electrostatic potential for CB5, CB6, CB7, CB8. Reprinted by permission from ref.59. Copyright © 2012, American Chemical Society.

Additionally, the size selectivity is also an essential factor for determining the binding affinity of guests to CBn. The packing coefficient (PC) is frequently used to estimate the fit goodness of inclusion complexes.\(^{60}\) As shown in equation 2.1, the PC of a compound is defined as the ratio between the sum \((V_{\text{w}})\) of the van der Waals volumes \((v_{\text{w}})\) of the \(n\) molecules in given volume \((V)\) and the volume \((V)\). A PC value of around 55 ± 8% is considered the best binding affinity between host and guest species according to the Mcozzi-Rebek rule.\(^{61}\) The higher or lower PC values would accompany a lower affinity. Nau and coworkers reported that the PC value analysis for known guests with hydrophobic binding motifs revealed average values of 47% for CB5, 58% for CB6, 52% for CB7, and 53% for CB8, which are well in agreement with ‘the 55% solution’ rules.\(^{60}\)

\[
PC = \frac{\sum_{i=1}^{n} v_{\text{w}}^i}{V} = \frac{V_{\text{w}}}{V} \tag{2.1}
\]

In addition to size selectivity (e.g., PC value), hydrophobic effects and ion-dipole interactions are the main effects responsible for the strong binding affinities observed in CBn, and the release of ‘high-energy’
water as the primary driving force is also one of the most potent interpretations (Figure 2.5). In general, due to the water competing strongly for hydrogen bonds and solvating charged species, the complexation binding affinity of other non-covalent systems is typically weakened in the presence of water compared to the organic environment. However, in the case of complexation with neutral guests by CBn, the release of the confined water in the cavity is a critical determinant in the binding process.\textsuperscript{62-64} The substantial enthalpic gain caused by these confined water molecule releases, namely ‘high-energy water release’, plays a primary driving force upon guests binding and the expelled water then engages in more robust H-bonding networks in aqueous bulk. The smaller cavity volume will limit the number of high-energy water molecules (e.g., CB5 and CB6), but the larger volume of the cavity will allow the H-bonding among the cavity water (e.g., CB8). This principle explained that these rigid macrocycles display a solid affinity to uncharged guests, and CB7 shows a higher binding affinity in comparison with smaller homologues CB5/CB6 and larger CB8.

![Figure 2.5](image.png)

**Figure 2.5** Schematic illustration of the release of high-energy water molecules upon guest-binding. Reprinted by permission from ref.63. Copyright © 2012, American Chemical Society

### 2.2.3. Specific Binding properties for each cucurbit\([n]\)uril homologues

CB5, as the current smallest among the CBn homologues, is limited to encapsulating guests by a size-dependent parameter and binds guests, which fit in its relatively small cavity volume (82 Å). Nevertheless, the electronegative carbonyl portals of CB5 can form complexes with cationic species, a feature observed for other CBn homologues.\textsuperscript{65} This finding also explains why the solubility of CBn is increased in acidic aqueous media or in the presence of cationic species. CB5 and its derivatives have been reported to form the portal complexes with alkali, alkaline earth, ammonium cations, and multi-charged cation.\textsuperscript{66, 67} Apart from this, CB5 has been demonstrated to encapsulate with gas molecules such as N\textsubscript{2}, O\textsubscript{2}, Ar, N\textsubscript{2}O, CO, CO\textsubscript{2}, noble gases, CH\textsubscript{4}, ethane, as well as solvent molecules such as methanol and acetonitrile.\textsuperscript{68-71} Interestingly, the selective encapsulation of nitrate and chloride ions by CB5 was observed by crystallography.\textsuperscript{72-74} Due to the lack of suitable signal transduction ways, CB5 and analogues are still not useable as gas sensors.

As the highest-yielding component from the acid-catalyzed condensation reaction, CB6 is well-known to form high-affinity complexes with aliphatic amines and small aromatic hydrocarbons.\textsuperscript{60, 75} Especially for protonated alkylammonium, alkylidiammonium, and their derivatives,\textsuperscript{57, 76, 77} CB6 exhibits ultra-high affinities due to the complementary size selectivity and strong interactions between positively charged amines and electronegative carbonyl portals. Meanwhile, taking advantage of the binding properties of CB6 with imidazolium-based guests, the poor solubility of CB6 in water has been dramatically improved\textsuperscript{78-80} and consequently used for CB6 functionalization or separation.\textsuperscript{81, 82} Notably, CB6 also can
be a recognition receptor for lanthanide ions,\cite{83, 84} noble gases molecule (e.g., $^{129}$Xe) for NMR/MRI studies,\cite{85, 87} and alkali ions at the portals for exploring the coordinative binding behaviors with other guests.\cite{88, 89}

CB7 plays a predominant role among the cucurbit[n]uril family, owing to its relatively large cavity volume of 279 Å$^3$ and its much better water solubility (20 - 30 mM) than that of CB6 and CB8 (< 0.1 mM). Compared to the limited cavity volume of CB6, a more comprehensive range of guests can be encapsulated into CB7 with high binding affinities from 1$0^7$ M$^{-1}$ to 10$^{17}$ M$^{-1}$\cite{90} The latter value is even equal to that of the natural biotin-avidin system.\cite{91, 93} Due to the maximal high-energy water release driving force for CB7 than other homologues, CB7 exhibits the largest binding affinities and enthalpies,\cite{63} exemplified by the complexation of ferrocene and adamantlylamine derivatives. Owing mainly to the excellent water solubility and high binding affinity observed for CB7, its use in supramolecular and materials science has become popular.

In addition to encapsulating all guests that fit into the CB7 cavity, CB8, equipped with 1.7 times larger volume than CB7, is unique because of its ability to form 2:1 or 1:1:1 ternary complexation with two identical or different guests.\cite{94, 96} Most CB8-based sensing application for aromatic recognition is designed and established according to this specific binding property.\cite{97, 98} Besides, CB8 can also form complexes with other macrocyclic guests for catalysis application or with two functional moieties guests to yield supramolecular polymeric materials.\cite{99, 102} However, it is noteworthy that the solubility of CB8 is much poor than other CB$n$, which restricts the applications of CB8 when high concentrations are required.

### 2.3. Application of covalently functionalized cucurbit[n]uril

The primary challenge for CB$n$ is the water solubility, which is a key point for the implementation of utilizing CB$n$-based material and exhibiting the binding functions in aqueous media. In 1992, the first relevant substituted CB$n$ work was reported by Stoddart and coworkers, in which decamethylcucurbit[5]uril (Me$_4$CB5) was synthesized from dimethylglycoluril with formaldehyde under acidic conditions.\cite{103} Later, Kim and coworkers isolated the fully substituted cyclohexanecucurbit[n]urils (Cy$_2$H$_2$CB5 and Cy$_2$H$_6$CB6) and reported their good water solubility (up to 200 mM).\cite{104} In both cases mentioned above, although CB$n$ was substituted with the non-polar alkyl group, the increasing solubility presumably comes from the perturbation of stable packing in the solid-state.\cite{103} Since then, a variously of symmetrical or unsymmetrical alkyl-substituted CB$n$ were reported (Figure 2.6),\cite{105} such as diphenylcucurbit[6]uril (Ph$_2$CB6),\cite{106} partially cyclopentane-substituted cucurbit[6]uril (CyP$_2$CB6, n = 2, 3, 4),\cite{107} dimethyl-substituted cucurbit[7]uril (Me$_2$CB7), fully cyclopentane-substituted cucurbit[n]uril (CyP$_2$CB$n$, n = 5, 6, 7),\cite{108} tetramethyl-substituted cucurbit[8]uril (Me$_4$CB8) and dicyclohexanecucurbit[8]uril (Cy$_2$CB8),\cite{109} hemimethyl-substituted cucurbit[7]uril ((HMe)-CB7),\cite{110} fully cyclobutene or cyclobutane-substituted cucurbit[n]uril (CyB$_n$CB$n$, n = 5, 6, 7, 8),\cite{111} and dodecamethyl-substituted cucurbit[6]uril (Me$_2$CB6).\cite{112} In principle, these alkyl-substituted CB$n$ derivatives generally are better soluble than their corresponding parent CB$n$ and expand the horizons of CB$n$ synthesis.
approaches. However, the second gap between CB$n$ to their practical applications is still existing due to the difficulty of functionalization.

![Chemical structures of substituted CB$n$.]

**2.3.1. Approaches for the synthesis of functionalized cucurbit[n]uril**

The installment of reactive groups on CB$n$ is the first step for further functionalization. In 2003, Kim and coworkers firstly reported a facile direct functionalization of CB$n$ by reactions with K$_2$S$_2$O$_8$ in water. The obtained perhydroxylCB$n$ (CB$n$-(OH)$_{2n}$) can be modified to provide tailored CB$n$ derivatives with further functional groups. A photochemical method using H$_2$O$_2$ as the oxidant and UV light irradiation to synthesize the hydroxyl-substituted CB$n$ was later reported by Ouari and co-workers. The authors obtained a high yield and purity of monohydroxylated CB$n$ (n = 5-8), and also first isolated the CB$8$ derivatives and revealed the mechanism for the difficulty of functionalization of CB$8$ derivatives through the DFT method. The isolation of monohydroxylated CB$7$ in this thesis referred to this work, and the detailed experimental procedure will be presented in the following sections.

In contrast to ‘post-modification’ with oxidizing, inspired by the above approaches for alkyl-substituted CB$n$ synthesis, Isaacs and coworkers presented a building-block approach for monofunctionalized CB$n$ fabrication. Firstly, they reported the templated synthesis of methylene bridged glycoluril hexamer and this hexamer was reacted with substituted phthalaldehydes in the presence of p-xylalenediammonium ion to yield a CB$6$ with a mono-naphthalene fluorophore (Figure 2.7). The fluorophore equipped CB$6$ binding with metal ion as a ‘turn-on’ probe was used for specific analytes detection (e.g., nitrosamines and amino acids) via the responses of fluorescent intensity. Moreover, a similar approach was utilized to synthesize the monofunctionalized CB$6$ with a ‘clickable’ alkyne for further versatile functionalization (Figure 2.7). This strategy also created a new entrance for the synthesis of a series of monofunctionalized CB$7$ derivatives or substituted CB$8$ derivatives by reacting the pre-functionalized glycoluril with the glycoluril hexamer (Figure 2.7).
2.3.2. Biorelevant imaging and bioprocess monitoring

Compared to a non-covalent IDA chemosensor, the covalent connection of the indicator ensured that the host could be observed directly for cellular imaging and avoid the random diffusion of the displaced indicator by analytes in cells. Monofunctionalized CB$n$ enables the preparation of their respective dye conjugates, representing an exciting class of unimolecular host-guest systems that can be explored for bioimaging applications in solution or living cells and organisms. In 2016, Urbach and coworkers designed and synthesized a CB7-tetramethylrhodamine conjugate (Q7R), which displayed a concentration-dependent fluorescent response upon binding to different biorelevant guests.\textsuperscript{121} The preparation of Q7R was accomplished through a 1,3-dipolar cycloaddition reaction between azide and alkyne.

As the potential live-cell probes, the endocytosis and excretion pathways of two dye-conjugated CB7, CB7-cyanine 3 conjugate (CB7-Cy3) and CB7-rhodamine X conjugate (CB7-ROX), was studied \textit{via} a series of experiments with different cellular process inhibitors and the results revealed the moiety of CB7 has a substantial influence on both cellular processes.\textsuperscript{122}

The fluorescence resonance energy transfer (FRET) signal was also utilized for specific cellular process imaging and monitoring. In 2017, Park and coworkers reported an ultra-stable binding pair to visualize different organelles and fusion processes in live cells.\textsuperscript{123} As shown in Figure 2.8 (a), CB7-Cy3 and adamantylamine-cyanine 5 conjugate (Ada-Cy5) as the supramolecular FRET donor and acceptor, respectively, translocated into mitochondria and lysosomes. A time-dependent increasing FRET signal indicated the occurrence of fusion \textit{via} the binding of CB7-Cy3 with Ada-Cy5. These supramolecular FRET pairs have also been utilized for monitoring the flickering dynamics of the fusion pore using
neuronal SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor)-reconstituted vesicles. Recently, a visualization of organelle-specific autophagy events was reported, which is also monitored by the FRET pair consisting of CB7-Cy3 and borondipyrromethene-adamantylammonium conjugate (BDP-Ada)(Figure 2.8 (b)). Furthermore, they demonstrate this supramolecular FRET pair as a latching system for bio-orthogonal anchoring of small molecules in a live animal model (C. elegans) and also showed a potential application on in vivo cancer imaging by Ada-Cy5 and pre-targeted CB7-antibody conjugate (CB7-Erbitux) in a live mouse model (Figure 2.8 (c)). This supramolecular latching system was also utilized for protein imaging of cell surface via a pre-labelled ultra-stable binding pair, like adamantly- and ferrocenyl-ammonium, on the cell membrane. Mainly, the internalized proteins into cells can be visualized and spatially controllable by temperature.

Likewise, taking advantage of the ultra-high affinity between CB7 and adamantylamine, Agasti and coworkers introduced a biorthogonal assembly strategy based on the supramolecular interaction between CB7 with the primary targeting agent and ADA conjugated fluorophore, which enabled simultaneous biorthogonal labelling and multiplexed imaging in cells as well as tissue sections with high imaging resolution for visualizing structure rather than traditional biotin-avidin based labelling platform. Later, they demonstrated that fluorescently labelled hexamethylenediamine (HMD) forming the dynamic complexation with CB7 could provide the fluorescent blinking with prescribed brightness and frequency.
to enable a super-resolution imaging with 10-folded better resolution than the diffraction-limited imaging.\textsuperscript{129}

Different from the traditional fluorescent dyes that are subjected to aggregation-caused quenching (ACQ), Wang and coworkers synthesized and reported an aggregation-induced emission (AIE) fluorophores tethered CB7 (CB7-AIE) conjugate for subcellular bioimaging and versatile theranostic applications.\textsuperscript{130} In a weakly acidic aqueous environment, this conjugate can spontaneously self-assemble into a compact nanoaggregation, which leads to the enhancement of fluorescent emission and generation of singlet oxygen. Besides, the CB7-AIE nanoaggregates can be enriched explicitly on lysosomes and display enhanced photodynamic cytotoxicity against cancer cells \textit{in vitro}. Based on supramolecular host-guest interaction between CB7 and drugs, this conjugate also exhibits the potential application in supramolecular chemotherapy.

\textbf{2.3.3. Nanostructure and drug delivery system}

The noncovalent approach for the preparation of CBn-based functionalized nanostructure or drug delivery system has been extensively researched and received increasing attention. Compared with the conventional covalent modification, the noncovalent approach is more flexible and provides a versatile method to endow the vesicles with new properties and functions.

In 2005, a fully substituted amphiphilic CB6 was synthesized through a reaction of CB6-(allyloxy)\textsubscript{12} with mPEG\textsubscript{3}-SH and reported that this CB6 derivative could form assembly vesicles in aqueous media.\textsuperscript{131} Through the high binding affinity between CB6 and polyamine (log$K_a$ > 6), the different tags-polyamine conjugates (\textit{e.g.}, mannose-spermidine, FITC-spermidine, and galactose-spermidine) were easily modified onto the surface and provide the vesicles with a specific function (Figure 2.9). Fully allyloxylated CB6 offered a versatile platform for a variety of functionalization with different building units and further allowed the applications in stimuli-responsive drug delivery systems and targeted bioimaging.\textsuperscript{132-137}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2_9.png}
\caption{Vesicle formed by an amphiphilic CB6 derivative and post-decoration with functional tags via host-guest interactions. Reprinted by permission from ref.131. Copyright © 2005, American Chemical Society.}
\end{figure}

In 2018, the research group discovered that mono-allyloxylated CB7 (CB7-OAl) acts as an unconventional amphiphile that could also directly self-assemble into a vesicle (a diameter of 200 nm with a membrane thickness of 3 nm) in water and exhibit a light-responsive morphology change by reaction with thiol molecule \textit{via} thiol-ene conjugation.\textsuperscript{138} Similarly, mono-hexanoate conjugated CB7
(CB7-OC₃COO), which is exceptionally high water-soluble (600 mg/mL) in contrast to parent CB7, exhibiting a specific property of well-defined pH responses on intermolecular assembly. Based on this ability, the capsulated guest molecules, such as drugs, can be controllably released upon changing pH value. A monofunctionalized CB7 with a hydrophobic alkane tail (n = 7, 9), synthesized and reported by Isaacs and coworkers, showed a self-inclusion behaviour. After being mixed with tetracationic and zwitterionic p-xylylene derivatives guests, the complexes acted as supramolecular amphiphile resulting in the formation of vesicle-type assemblies and is capable of loading the fluorescent dye as a drug model. Upon addition of surfactant (e.g., Triton X-100), the disassembly of structure can be achieved and show a potential stimuli responsiveness with other competitive guests.

As one of the most practical application directions, targeted delivery of chemotherapy drugs complexed in CB₇n to cancer cells, was extensively and deeply researched. In 2013, a biotin monofunctionalized CB7 (CB7-biotin) which maintained the ability to encapsulate guests, was synthesized and exhibited a specific targeting property to cell lines that overexpress biotin receptors. The complex of CB7-biotin with clinical antitumor drug (e.g., oxaliplatin) displayed approximately an order of magnitude higher bioactivity in the MTS bioactivity assay with the above cell line in vivo than oxaliplatin alone. Apart from oxaliplatin, doxorubicin (DOX) as a drug model was delivered by a metal-organic polyhedron with covalently functionalized CB7, which enabled the stimuli-responsive release of the encapsulated drug upon the addition of competitive binders. Meanwhile, this vesicle can also release the drug by (1) a dual pH-chemical stimulus through adamantane carboxylate in different protonation states or by (2) a dual pH-photochemical stimulus through stilbene derivative in different photoisomerization states. Similar to the aforementioned ‘Plug and Play’ CB6-based vesicle, Wang and coworkers proposed a ‘Lego-likewise’ functionalization strategy on nanoparticles surface via the CB7 binding to its corresponding guests labelled with functional units, such as folate, polyethylene glycol, and FITC. This CB7-decorated PLA/PLGA nanoparticle can also load the drug inside the vesicle or into the CB7 cavity and provide a multi-functionalized opportunity for combination drug therapy (Figure 2.10).
Figure 2.10 Synthesis route of CB7-decorated PLA and schematic illustration of CB7-decorated PLA/PLGA nanoparticle with a non-covalent tailorable surface for post-modification of functional units. Reprinted by permission from ref. 143. Copyright © 2018, American Chemical Society.

A similar idea was applied for synergistic photodynamic therapy and hypoxia-activated chemotherapy. Hydrophobic chlorin e6 (Ce6) as photosensitizer conjugated with CB7 through the PEG linker (CB7-PEG-Ce6) can spontaneously form supramolecular micelles. Banoxantrone (AQ4N) as a hypoxia-responsive prodrug and folate-amantadine (Ada-FA) as a tumor-targeting molecule were loaded onto the vesicle surface via strong host-guest interaction. After the internalization by cancer cells, the AQ4N can be released in response to the competitive spermine, which is overproduced in tumor cells, and the enhanced hypoxic environment by PDT can activate AQ4N to chemotherapeutic AQ4.144

2.3.4. Surface functionalization

One of the application directions of CBn functionalization is to graft the CBn onto the material surface or interfaces through covalent modification so that the surface can be non-covalently further bound to other components via the unoccupied cavity of CBn.145, 146 The earliest report referring to covalently grafting CBn onto the surface also came from Kim’s group in 2003, when they first proposed the hydroxylation of CBn with K2S2O8 as an oxidant. Allyloxylated CB6 was anchored on a patterned thiol-terminated glass substrate by irradiating UV light. After immersion of the modified glass into fluorophore-labelled spermine aqueous solution, the appearance of the corresponding fluorescent pattern indicated that CB6 was successfully modified on the surface.114 Based on this concept, the same group developed the protein immobilized surface as a biosensor via the anchored CB7 and ferrocene (Fc) labelled proteins, which demonstrated good performance for glucose sensing.147 Additionally, a supramolecular Velcro based on the high affinity of CB7 and Fc offered a reversible adhesion strategy without any external curing agents in a water environment, and the redox-active of Fc moiety is potential for the application of electroactive responsive adhesive material.148 In biorelevant analytes sensing and detection, the groups of Hirtz and Biedermann demonstrated a patterned immobilization CBn/indicator chemosensors into microarray for multiplexed and sensitive analytes detection based on IDA, which is in combination with the technique of microchannel cantilever spotting (μCS). The automated μCS process
controls the dot size within a narrow distribution (<10 μm for average dot radius). A series of alkyne-functionalized CBn (CB6, CB7, and CB8) was linked on the thiol-functionalized silica surface to obtain the specific microarrays (Figure 2.11). After encapsulating corresponding indicators, the multiplexed microarrays with multi-CBn/indicator pairs demonstrated the differentially responding to different analytes, e.g., spermine, cadaverine, and amantadine. The CBn/indicator microarrays possess a sensitivity in nanomolar range.

![Figure 2.11](image)

**Figure 2.11** Schematic illustration of CBn-immobilized sensor array for analyte detection based on IDA and the chemical structures of propargylated CBn and analytes. Reprinted by permission from ref.149. Copyright © 2021, American Chemical Society.

Apart from the covalent immobilization of CBn on the surface, many strategies vary from electrostatic adsorption generating a self-assembled monolayer (SAM) of parent CBn on gold surface or guest-mediated immobilization via noncovalent host-guest chemistry.

The immobilization of functionalized CBn onto the surface has also been adapted to expand microbeads or nanoparticles' applications. Taking the advantages of ultra-high affinity between CB7 and Fc derivatives, Kim and coworkers established CB7 conjugated Sepharose microbeads by treating NHS beads with hydroxylated CB7. These functionalized CB7 beads enabled to selectively capture of the membrane proteins labelled with Fc moiety from heterogeneous protein mixtures, and then the captured proteins could be recovered and collected by treatment with a stronger binding Fc derivative. With different labelling high-affinity guests, e.g., adamantane, they implemented and enriched specific intracellular protein from cell lysate using the CB7 beads via the host-guest interaction. CBn (mainly CB7 and CB8) have also been shown to bind selectively and predictably to the N-terminal aromatic residue phenylalanine (Phe), tyrosine (Tyr), and tryptophan (Trp). In 2011, Urbach and coworkers investigated that CB7 binds to the N-terminal Phe residue of the insulin B-chain with a 1000-fold preference over other aromatic sites (Figure 2.12 (a)). This result offered that the identity of the terminal residue is sufficient to prescribe the selective recognition of proteins. Similar to the approach of CB7 immobilization on sepharose resin through NHS esterification, the azidobutyl CB7 was coupled to the alkyne modified resin via Cu¹-mediated click reaction (Figure 2.12 (b)). After incubation with the proteins mixture and washed with diethylamine (DEA) or N, N'-diethyl-1,6-diaminohexane (DEDAH) solution, the results from SDA-PAGE demonstrated that the insulin and human growth hormone (hGH) which equipped with an N-terminal Phe residue are bound to the CB7-resin and can be displaced by competitive
General Introduction

high-affinity guests (Figure 2.12 (c)). This is the first example of native proteins recognition by synthetic host molecule in the complex biological mixture.

**Figure 2.12** (a) Crystal structure of CB7 and insulin complex. The terminal Phe on B-chain is bound inside CB7 cavity. Reprinted by permission from ref.163. Copyright © 2011, American Chemical Society
(b) Preparation of CB7 decorated resin via click reaction. (c) Presentative SDS-PAGE gel of protein mixture before and after treatment with CB7-resin and resulting resin after treatment with DEA and DEDAH elution. Reprinted by permission from ref.164. Copyright © 2016, Royal Chemical Society.

### 2.3.5. Other applications of functionalized CBn

Owing to the high binding affinity between CB7 and N-Phe of insulin, a platform approach to biopharmaceutical modification of insulin was developed. A series of poly (ethylene glycol) (PEG) conjugated CB7 (CB7-PEG) were shown to significantly increase the stability of protein drugs and limit aggregation for biopharmaceutical formulations. This strategy can endow the protein drugs with the benefits of PEGylating without necessitating covalent modification or even other functional molecules.165

In 2018, Nau and coworkers reported that a DNA quantitative sensing system consisting of a host-guest FRET pair, CB7 labelled with carboxyfluorescein (CB7-CF) as an acceptor and the nucleic stain 4’,6-diamidino-2-phenylindole (DAPI) as a donor, was established via the supramolecular interaction.166 This DAPI/CB7-CF system shows a great larger linear-response range than commercial DNA stain.

CB7 grafted hyaluronic acid (HA) (CB7-HA) involved a supramolecular strategy to induce the mitochondrial aggregation, and fusion was developed for potential treatment to address mitochondrial fission/fusion imbalance related disease.167 Dually tagged PEG with triphenylphosphonium (TPP) and adamantane (Ada) (TPP-PEG-Ada) was introduced into the cell and targeted the surface of mitochondria. Then, the addition of CB7-HA induced the supramolecular aggregation of mitochondria via the high affinity between CB7 and Ada located on the mitochondria. This strategy successfully protected the cells and zebrafish neurons under chemical stress *in vitro* and *in vivo*. CBn grafted hyaluronic acid was also utilized to prepare supramolecular hydrogel as artificial extracellular matrices (ECMs), which demonstrated the spatio-temporal control of engineered mesenchymal stem cells (eMSCs) for transgene expression.168

In summary, functionalized CBn has broad application prospects in various aspects, and it sets up a convenient ‘non-covalent’ bridge between the substrates and functional components. More importantly, numerous successful reported cases have greatly expanded the vision and strategies of functional material development and enriched the synthetic methods for CBn functionalization.
2.4. Cucurbit[n]uril-based supramolecular chemosensors

In contrast to the conventional biological detection strategy based on an expensive antigen-antibody or aptamer system, CBn is a macrocyclic artificial receptor with a kilogram level of production. As the primary candidate among the numerous macrocyclic hosts, CBn can be utilized for a wide range of components sensing or biosensing owing to its high affinity, fast binding kinetics and biocompatibility.56, 169-171

2.4.1. Strategies for cucurbit[n]uril-based sensing assay

Because the pristine CBn does not absorb or emit light in the visible range of the electromagnetic spectrum, CBn hosts are restricted to sensing inherently photophysical transparent analytes. In order to generate the readable signals, using of indicator displacement assays (IDA),172-177 associative binding assays (ABA),59, 94, 178, 179 and guests displacement assays (GDA)180, 181 were introduced to widen the scope of analytes detection by CBn-based chemosensors.

For chromophoric and emissive guest molecules, the complexation with CBn can usually result in significant photophysical signal changes (Figure 2.13 (a)).182 Basically, the differences in the polarity of the local environment between bulk water and the CBn cavity will cause a significant change in emission intensity or hypochromic/hyperchromic shift in the absorbance spectrum of the indicator molecules. The different signals readout on guests caused by changes in the microenvironment are also applicable to other characterization technologies, such as NMR/MRI, CD and MS.

In many circumstances, however, guests are spectroscopically silent, and their corresponding complexation is also without typical absorbance or emission signal responses. In order to meet the requirement of binding detection under this case, an additive indicator dye with spectroscopically active was introduced, and an indicator displacement assay (IDA) was established accordingly. In the presence of guests, the indicator is displaced from the hosts resulting in distinctly different photophysical properties for its bound and unbound form. (Figure 2.13 (b))

For guests with poor solubility in aqueous media, the traditional IDA is limited by the difficulty of avoiding the introduction of other non-polar solvents and cosolvents but ensuring sufficient concentrations of guests as well. Guest displacement assay (GDA) proposes the addition of indicator dye into a re-equilibrated host-guest complexation solution. As the complementary approach to IDA, GDA method is superior for insoluble and weakly binding guests. (Figure 2.13 (c))

Compared with the above assays, associative binding assays (ABA) is a unique guest detection mode based on CB8. The formation of a 1:1:1 heteroternary complex for CB8 with other two aromatic compounds facilitate the charge transfer (CT) from electron-rich molecules to electron-poor molecules that do not occur in the same concentration range in the absence of CB8. In combination with a chromophoric dye with CB8 in many ABA cases, the large space in the CB8 cavity allowed for the simultaneous binding of a second guest with a high affinity and a sensitive absorbance/ emission response. ABA is more sensitive to analytes sensing than IDA in the energetic aspect. (Figure 2.13 (d))
2.4.2. Metal ions sensing

As introduced in the previous chapters, the entire family of CBₙ and its derivatives bind (metal) cations via the ion-dipole interaction. This property is generally the cause of buffered medium dependence of CBₙ-guests binding constants. Mostly, the binding affinity between CBₙ and cations increases with the larger cation radius and increasing valence of ions. Taking advantage of this feature, CBₙ-based system can be utilized for sensing Al³⁺, Hg²⁺, Fe³⁺, Ag⁺, Ba²⁺, Pb²⁺, Cd²⁺, and other metal cations by employing the electrochemical and photophysical techniques.

2.4.3. Amino acids, peptides and proteins sensing

The detection and recognition of biologically relevant small molecules (e.g., amino acids, peptides, and metabolites) is essential to evaluate physical conditions and diagnose diseases. Due to the unique binding properties of CBₙ (size-selective, charge-selective and hydrophobic) and feasible sensing strategies (IDA, GDA, or ABA), CBₙ has come out as a special macrocyclic receptor for biorelevant molecule sensing. Among the 20 common amino acids for protein building, hydrophobic and electroneutral amino acids are all potential binding guests for CBₙ but have differences in binding affinity, respectively. The amino acids primarily used for the research of CBₙ binding include the ones equipped with an aromatic group, phenylalanine (Phe), tyrosine (Tyr), and tryptophan (Trp). The binding constant of typical amino acids is listed in Table 2 with their corresponding CBₙ receptor and experimental information. Summarizing from the reported information, these three aromatic amino acids always bind to CB₇ in the form of a 1:1 model and CB₈ in the form of a 1:2 with an auxiliary guest (indicator or two identical homogeneous guests) but cannot form a stable complex with CB₆. Notably, Isaacs and coworkers synthesized and reported a ‘CB₆-analogue’, which show a higher affinity to the above amino acids than CB₈. Except for the classic cyclic CBₙ members, acyclic cucurbit[n]uril is a famous member of the CBₙ family which is rid of the size restrictions for guests binding and minimized the difficulty of functionalization in comparison with other typical CBₙ.
Including the recognition of amino acids,\textsuperscript{215, 216} acyclic cucurbit[\(n\)]uril displays the general binding behavior to cations as well,\textsuperscript{117} but bind much larger entities, such as single-walled nanotubes (SWNTs).\textsuperscript{217} Additionally, the binding interactions of the asymmetrical inverted CB\(n\) (iQ[7]) with essential amino acids were also explored by Xiao and coworkers,\textsuperscript{218} which deeply investigated the unique binding performance and expanded the horizon of molecular recognition by CB\(n\).

Table 2.1 Binding constant of common amino acids to CB\(n\)

<table>
<thead>
<tr>
<th>Receptor</th>
<th>(K_a) (M(^{-1}))</th>
<th>Strategy</th>
<th>Indicator</th>
<th>Medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenylalanine</td>
<td>1.4 \times 10^5</td>
<td>Direct</td>
<td>-</td>
<td>50 % (v/v) aqueous formic acid</td>
</tr>
<tr>
<td>Phenylalanine</td>
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<td>ABA</td>
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<td>10 mM sodium phosphate, pH 7.0</td>
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<td>Direct</td>
<td>-</td>
<td>water</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>1.5 \times 10^5</td>
<td>IDA</td>
<td>TMSp</td>
<td>water</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>1.8 \times 10^5</td>
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<td>-</td>
<td>water</td>
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<tr>
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<td>ABA</td>
<td>DPT</td>
<td>-</td>
</tr>
<tr>
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<td>water</td>
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<td>Methyl viologen</td>
<td>10 mM sodium phosphate, pH 7.0</td>
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<td>Direct</td>
<td>-</td>
<td>water</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>2.2 \times 10^4</td>
<td>Direct</td>
<td>-</td>
<td>10 mM NH_2OAc buffer at pH 6.0</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>2.4 \times 10^4</td>
<td>IDA</td>
<td>Dapoxyl</td>
<td>10 mM NH_2OAc buffer at pH 6.0</td>
</tr>
</tbody>
</table>

\(\text{CB6}\) shows a
\(\text{CB7}\) shows a

\(\text{TSMP}\) - 3-(trimethylsilyl)propionic-2,2,3,3-\(d_4\) acid; \(\text{DPT}\) - 2,7-dimethylazaphenanthrenium; \(\text{MBBI}\) - tetramethylbenzobis(imidazolium)

Compared to amino acids, peptides, such as oligoamide or polyamide, consist of the amino acid chain. Taking into account the binding characters to CB\(n\) with amino acids, the features of peptides create both challenges and opportunities for molecular recognition. Early reports about peptides binding by CB\(n\) were focused on oligopeptides (dipeptides or tripeptides). Remarkably, the type of amino acid at the N-terminal position is always decisive for the binding behaviors of the entire peptide because of its exposed free amino group with less spatial constrictions and no -COOH groups nearby. Like the above, CB6 shows a
small affinity to peptides that usually does not exceed the range up to $10^4$ M$^{-1}$.\textsuperscript{219-221} In recent years, more and more attention has been paid to the mild affinity between CB6 or CB6 analogues with lysine.\textsuperscript{161,222} Especially for an alkyl substituted CB6, hemimethyl-substituted cucurbit[6]uril ((HMe)$_6$CB6) reported by Zhu and coworkers, showed the binding affinity to lysine up to $4.5 \times 10^6$ M$^{-1}$ determined by fluorescent IDA.\textsuperscript{222} However, the widely applied one is the inclusion complex of CB7 to N-terminal phenylalanine (Phe) peptide systems. Taking the advantages of 1) hydrophobic interaction between terminal Phe residues and CB7 cavity, 2) ion-dipole interaction, and 3) H-bond interaction between terminal ammonium group and carbonyl portal, CB7 can form strong complexes with N-terminal Phe residue of peptide and protein up to the $K_a$ of $\sim 10^7$ M$^{-1}$ in water.\textsuperscript{223} This feature was utilized for protein analysis,\textsuperscript{224} site-selective modification,\textsuperscript{225} sequence-specific inhibition of protease,\textsuperscript{226} and modulation of protein fibrillation or aggregation.\textsuperscript{227-230} The most known case of protein recognition by CB7 with human insulin exhibits a 100- even 1000-fold affinity than the manual peptides or proteins in the absence of N-Phe.\textsuperscript{163} Additionally, methylated lysine (methyllysine) also showed a high affinity to CB7 and was studied for specific protein purification and recognition applications.\textsuperscript{231-233} In analogy with the high affinity between CB7 and N-terminal Phe, CB8 equipped with one equivalent methyl viologen (MV) also displays a particular affinity to the peptide with N-terminal tryptophan (Trp) (Figure 2.14 (a)). The system demonstrated sequence-specific peptide recognition by CB$n$ receptors, \textit{e.g.}, $K_a$ of $1.3 \times 10^5$ M$^{-1}$ for TrpGlyGly (WGG) tripeptide but $K_a$ only $2.1 \times 10^4$ M$^{-1}$ for GWG and $3.1 \times 10^3$ M$^{-1}$ for GGW sequence, respectively.\textsuperscript{159} Furthermore, CB8 with MBBI system for peptides selectively recognition was reported as having a similar advantageous binding property and better stability and intense fluorescence.\textsuperscript{209} Apart from the detection by the change of emission signal, Biedermann and coworkers utilized the circular dichroism (CD) and fluorescence-detected circular dichroism (FDCD) with supramolecular host-guest system for the detection and chiral sensing of chiral amino acids and proteins, as well as for reaction monitoring.\textsuperscript{215,234} Likewise, CB8 binding with two identical amino acids at the stoichiometry at 1:2 is also appropriate for peptides. Urbach and coworkers discovered that CB8 can bind to two PheGlyGly (FGG) with the $K_a$ value of $1.5 \times 10^{11}$ M$^{-2}$, and this selectivity even is larger than WGG owing to an enthalpic advantage (Figure 2.13 (b)).\textsuperscript{160} According to this concept, Brunsveld and coworkers reported a series of works about N-terminal FGG peptide motif modified protein dimerization induced by interaction with CB8 (Figure 2.13 (c)).\textsuperscript{235,236}
General Introduction

Figure 2.14 (a) Energy minimized computer model of CB8 bound to MV and Trp showing both aromatic groups stacked face-to-face inside the cavity. (b) Crystal structures of CB8 are bound to two equivalents of FGG. Reprinted by permission from ref.159. Copyright © 2011 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim. (c) Schematic representation of two YFP proteins equipped with N-terminal FGG motif induced by interactions with CB8. Reprinted by permission from ref.235. Copyright © 2010 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

2.4.4. Metabolites and drugs sensing

Except for recognizing amino acids and peptides, CBn host-guest chemistry found its analytical applications in detecting biorelevant metabolites and drugs. Acetylcholine (ACh\(^+\)), an important neurotransmitter involved in numerous nervous system activities, showed a selectively binding affinity to CB6. In 2012, ion-selective electrodes (ISEs) for ACh\(^+\) detection by using their fully allyloxylated CB6 (CB6-(allyloxy)$_{12}$) as an ionophore were prepared. These ISEs exhibited an extraordinary selectivity to ACh\(^+\) over its metabolite choline (Ch\(^+\)) in the presence of other interfering quaternary ammoniums. Afterwards, this strategy was transformed onto the field-effect transistor platform and amplified the detection capacity of ACh\(^+\) down to the limit of $1 \times 10^{-12}$ M. In addition to the recognition of ACh\(^+\) by CBn systems, CBn exhibits the high affinities to polyamines in aqueous media as well. The formation of their pseudoreotaxane structure was widely studied and applied to the construction of supramolecular complexes in early reports. However, the biogenic polyamines also play an essential role in some physiological processes as primary metabolites, such as cell growth, proliferation, and differentiation. Determination of polyamines in biofluids has a practical clinical significance. Kim and coworkers demonstrated an array of CBn and dye complexes as sensor elements for pattern recognition of several polyamines (e.g., putrescine, cadaverine, spermidine, spermine, etc.). In combination with PCA data analysis, this approach displayed the potential to detect biomarkers of diseases. Other CBn-based
probes for polyamines detection systems associated with the aggregation-induced emission (AIE) effect were also reported as a sensitive and selective tool in biorelevant fluids.\textsuperscript{246, 247} CBN combined with mass spectrometry techniques also offered a highly sensitive detection of polyamines.\textsuperscript{248, 249} However, it is no doubt that the reports of polyamine detection based on fluorescent IDA were still dominant.\textsuperscript{250-253}

In the aspect of the drugs capsulation and recognition, hundreds of candidates have been investigated on their binding behaviors and sensing performance with CBN systems, for instance, methamphetamine,\textsuperscript{254-256} opiates and their metabolites,\textsuperscript{257} alkaloids,\textsuperscript{258} fluoroquinolone,\textsuperscript{259} illegal stimulants,\textsuperscript{260} addictive over-the-counter drugs,\textsuperscript{261} etc.

\subsection*{2.4.5. Supramolecular tandem enzyme assays}

Enzymatic reaction monitoring is a sensing application by the host-guest systems in combination with the IDA strategy. In 2007, Nau and coworkers first introduced a label-free continuous enzyme assay to monitor decarboxylase’s enzymatic process with several amine acids (Figure 2.15). According to the transformation of substrate charge (for instance, from lysine to cadaverine), macrocyclic receptors would be predisposed to binding to guests with the complementary size and charge (cadaverine). Thus, the signal changes indirectly reflect the information on enzymatic kinetic.\textsuperscript{262} Since then, different chemosensor pairs, substrates, enzymes, and required co-factors were further studied and reported.\textsuperscript{177, 179, 263-266}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{tandem_assays.png}
\caption{Product- \textit{versus} substrate-selective tandem assays for monitoring enzymatic activity. Reprinted by permission from ref.247. Copyright © 2009, American Chemical Society.}
\end{figure}

\subsection*{2.4.6. Supramolecular tandem membrane assays}

The efficient monitoring of membrane translocation or permeability for drugs and biomolecules was allowed \textit{via} the fluorescent-based supramolecular tandem membrane assays. This method is feasible to continuously follow the changes in concentration of analytes, for instance, the accumulation of analytes inside the compartmentalized structures.\textsuperscript{266} In 2013, Nau and coworkers firstly introduced this concept for the screening of diverse classes of analytes, channel proteins, and modulators.\textsuperscript{267} As shown in Figure 2.16, the analytes transported through the lipid bilayer will bind to a macrocycle and result in a displacement of dye in line with IDA. Thus, monitoring of analytes or related bioprocesses can be tracked by fluorescent in real-time. Afterwards, this strategy was utilized for monitoring the membrane transport
of cell-penetrating peptides (CPPs) inside large unilamellar vesicles (LUVs) and giant unilamellar vesicles (GUVs).\textsuperscript{268}

Figure 2.16 Schematic illustration of host-guest complex inside liposome before and after translocation of the analytes through a channel protein or the membrane directly. Reprinted by permission from ref.250. Copyright © 2014 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

Likewise, they established a real-time parallel artificial membrane permeability assay, namely RT-PAMPA, in combination with the fluorescent IDA to monitor and evaluate drug permeability across the biomembrane systems in a high-throughput screening format.\textsuperscript{269} Additionally, to improve the limited scope to strongly binding and slowly translocating analytes based on IDA, Biedermann and Nau introduced another model for spatiotemporally resolved monitoring of biomembrane permeability referred to as fluorescent artificial receptor-based membrane assay (FARMA), which exploited ABA instead of IDA strategy. With FAMRA, the permeation of hundred compounds through membranes has been monitored in real-time with high sensitivity.\textsuperscript{270}
3. Aim of this thesis

Cucurbit[\textit{n}]uril (CB\textit{n})-based host-guest supramolecular chemistry provides a versatile platform for the chemosensors design owing to its superior chemical properties and numerous successful cases of CB\textit{n} functionalization as aforementioned. However, CB\textit{n} binding to a wide range of guests with the structural matched conditions (\textit{e.g.}, packing coefficient, ionization state, \textit{etc}) seems to be a constraint for the selectivity of CB\textit{n} as a versatile chemosensor. In general, the non-negligible affinity of metal cations to the carbonyl-fringed CB\textit{n} portals, which induces the dissociation between CB\textit{n} and guest molecules (such as in biorelevant media, normal saline, and biofluids), makes the recognition of analytes by chemosensors only in pure water or low salt concentration buffer environment (Figure 3.1). The dilution is prone to induce the dissociation of non-covalent weak reporter pairs as well. For some indicators with high affinity to CB\textit{n}, the competition with cation is alleviated, but the binding of analytes is restricted at the same time. After that, CB\textit{n}-based chemosensors have been limited due to a lack of analyte selectivity for a long time. Unlike the ‘lock and key’ type chemosensor, there is not always a significantly different detection response on certain analytes by CB\textit{n}-based chemosensor. However, the increasing development of differential sensing provides a promising solution to this open challenge.

![Schematic illustration](image)

**Figure 3.1** Schematic illustration of (a) the inevitable cation-competition induced dissociation of the non-covalent reporter dyes with macrocyclic receptors in high salinity medium or real biofluids. (b) The inefficiency of analytes selectivity by non-covalent chemosensor in the presence of varied analytes with uncertain concentration.

Inspired by Urbach’s work about mono-tetramethylrhodamine labelled CB7 for cell imaging\textsuperscript{121} and Isaacs’s work about naphthalene modified CB6 derivatives for drugs detection in human urine,\textsuperscript{261} my alternative design strategy aims to overcome dilution issues and reduce the effects of salts on the chemosensor performance in biofluids by integrating the indicator and receptor into a single, non-dissociable unimolecular chemosensor. Converting intermolecular supramolecular interactions into
intramolecular supramolecular interactions is likely to change the original properties and applicability of the CB\textit{n}-based chemosensor. Due to CB7’s exceptionally high binding affinity for many hydrophobic or cationic bioactive molecules and the better water solubility compared to other CB\textit{n} homologues, unimolecular host-dye conjugates based on cucurbit[7]uril macrocycles can be promising chemosensors for analyte detection in the biologically relevant environments in the presence of competitive cations.

**Figure 3.2** (a) Schematic representation of analyte-induced conformational changes of CB7-dye conjugates that can be detected by emission spectroscopy; (b) Schematic representation of the equilibrium between free- and bound-formation of CB7-dye with/without linker in the presence of a high concentration of interferents.

Taking this idea as the starting point, the synthetic method to connect the CB7 with a matched indicator is the first issue for constructing a unimolecular chemosensor. Learning from the numerous established CB\textit{n}-functionalization related literature and detailed synthesis information, the subsequent modification of the functional moiety onto CB7 through the click reaction should be the most convenient and reliable route. It is not only because of referring to the similar successful cases but also in line with my experimental conditions and existing experiences on CB\textit{n} synthesis. Considering further sensing applications that require enough conformational freedom for the inclusion of indicators into conjugated CB\textit{n} in aqueous media, the modifiable hydrophilic ethylene glycol with a certain length, \textit{e.g.,} tetraethylene glycol or more units, is a promising candidate as the suitable linker. The fluorescent emission-based assay is commonly regarded as the most sensitive and intuitive analytical method for selecting indicators, so I decided to utilize the fluorescent dye as an indicator with the noticeable intensity.
change between bound and unbound CB7 in aqueous media. A deeper description of the underlying ideas for selecting the dye will be introduced in the following sections.

In order to verify the definite conformation of unimolecular conjugated chemosensors, NMR, mass spectrometry (MS), and ion mobility experiments were used to help characterize the geometry of conjugates or complexes with binding guests. Meanwhile, density functional theory (DFT) calculation was utilized for understanding and predicting the possible conformations from an energetically perspective.

In the main section of measurements with analytes, absorption and emission spectrometry were used as the primary techniques to reflect the interactions between analytes and chemosensors in aqueous media through the changes in spectra or intensity. A fluorescent plate reader format was used for the cases with multiple samples and repetitions.

The ultimate purpose of my thesis was to achieve the analytes sensing by the conjugated chemosensor in biofluids, that is in real human urine, saliva and serum.

This chapter is derived from the context of the following publication: Hu, C.; Grimm, L.; Prabodh, A.; Baksi, A.; Siennicka, A.; Levkin, P. A.; Kappes, M. M.; Biedermann, F. Chem. Sci. 2020, 11, 11142-11153. The synthesis, analysis, and data organization were carried out by me under the supervision of Dr. Frank Biedermann. The co-authors contributed by draft revision: Laura Grimm, Prof. Dr. Manfred Kappes, and Prof. Dr. Pavel Levkin. Other detailed contributions will be stated in the corresponding sections. Sections in this chapter have been reproduced from the published work[27] with permission from the Royal Society of Chemistry.

Non-covalent cucurbit[n]uril (CBn) based chemosensors have been widely used in proof-of-concept sensing applications. However, as aforementioned, the disintegration of CBn and indicators in the saline medium is hard to avoid. In this chapter, it is described that covalent cucurbit[7]uril-indicator dye (berberine, BC) conjugates with different length linkers are stable in high salinity media and allow for selective sensing of Parkinson’s drug amantadine in biofluids (e.g., human urine and saliva), where the traditional non-covalent CB7⊃dye complex is dysfunctional. A detailed analysis of the covalent host–dye conjugates in the gas-phase and deionized versus saline aqueous media revealed structural, thermodynamic, and kinetic effects relevant to the design of CBn-based supramolecular chemosensors. This research also presents a new high-affinity indicator dye for CB7, revealing one of the most significant shortcomings of indicator displacement assays (IDA) with high Ka values, namely an impractically slow equilibration time. Different from non-covalent CBn⊃dye reporter pairs, the conjugate chemosensors can also work via an SN2-type guest–dye exchange mechanism, shortening test durations and allowing for novel avenues for tailoring analyte-selectivity.

4.1. Introduction

In recent decades, emission-based detection and analysis employing supramolecular assemblies, such as host-guest systems, have been a popular study topic. Supramolecular complexes were extensively investigated regarding their use in drug detection[14, 25, 272] and enzymatic reaction monitoring.[177, 273] Although CBn-based chemosensors possess advantageous high binding affinities and fast binding kinetics for many biorelevant small molecules in deionized water,[75, 91, 274] the non-covalent interaction between CBn and guest is strongly modulated by salt due to competitive[65] (or cooperative)[275] cation binding to the carbonyl-fringed CBn portals. Furthermore, non-covalent CBn⊃dye reporter pairs, like any other bimolecular non-covalent complex, are inherently prone to dissociate when diluted. As a result, many reported CBn-based chemosensors are operational for sensing applications in deionized water or “minimal buffers” but are often not suitable for saline media or biofluids, especially if their salt concentration varies from sample to sample (matrix effects).[276, 277] Biedermann and coworkers recently demonstrated that using a high-affinity dye for CB8 enabled the detection of the drug memantine by IDA in blood serum.[278] However, this approach can have serious fundamental limitations, e.g., for CB7-based assays, which are described further below.
As a result, combining the indicator and receptor into a single, non-dissociable unimolecular chemosensor appears to be a promising alternative design method for overcoming dilution problems and reducing the impact of salts on the chemosensor performance in biofluids. For example, emissive naphthalene units were modified on cavity walls in de novo cucurbit[n]uril-derivatives, functional in human urine to detect addictive over-the-counter drugs.261

Because of CB7’s exceptionally high binding affinity for many hydrophobic or cationic bioactive molecules such as steroids and polyamines,91,279 can reach up to astonishing ~10^{15} M^{-1} for adamantane derivatives,260 unimolecular host-dye conjugates based on CB7 macrocycles can be promising chemosensors. In this chapter, unimolecular CB7-based chemosensors with flexible and hydrophilic linkers that allow self-encapsulating of the indicator dye in the host cavity (Figure 3.2) were presented.

4.2. Results

4.2.1. Design and synthesis of CB7-dye conjugates

Synthetic advances have enabled mono-functionalized CB7115,120,138,281-283 to be derivatized with chemically reactive groups covalently attached to dye molecule. For instance, CB7 was covalently tethered to tetramethylrhodamine, for example, and the resulting conjugate displayed CB7-like binding properties for typical CB7-guests.121 However, such CB7-dye conjugates were connected by short linkers, which cannot enable the self-encapsulation of the dye into the CB7 cavity.

Several fluorescent reporter dyes are known for CB7,284 out of which many are pKa shift-dyes, e.g., acridine dyes285 and quinone-imine dyes.286 These dyes are commonly protonated and will result in the enhancement of emission intensity upon inclusion in the CB7 cavity that is well-known to stabilize positively charged species. In buffered aqueous conditions with a set pH, such pKa shift dyes perform well. When biological fluids with changing pH, e.g., urine, are employed, however, undesired matrix-dependent signal changes are observed. In this section, berberine was chosen as an indicator dye because it shows a considerable intensity enhancement on inclusion in CB7,287 a high binding constant of 10^{7} M^{-1} in deionized water,288 and because it can be chemically functionalized by a demethylation-alkylation method (Figure 4.1 (a)).289 Ethylene glycol-based linkers were chosen as relatively hydrophilic and flexible connectors between CB7 and berberine moieties.290 Chemical modelling suggested that a linker length of at least four ethylene glycol units is necessary to enter and exit the berberine dye from the CB7 cavity. In order to evaluate the effect of the linker-length, hexaethylene glycol (HEG) and tetaethylene glycol (TEG) were utilized as linkers.

The host-dye conjugates cucurbit[7]uril-HEG-berberine (chemosensor 1) and cucurbit[7]uril-TEG-berberine (chemosensor 2) were prepared through a convergent synthetic route shown in Figure 4.1. For the purpose of minimising the number of synthetic steps with CB7-species, the HEG or TEG linkers were connected to berberine, resulting in azide-ended berberine-hexaethylene glycol-azide (BC-HEG-N3) or berberine-tetraethylene glycol-azide (BC-TEG-N3) conjugates (Figure 4.1 (a)). A Huisgen 1,3-dipolar
Monofunctionalized cucurbit[7]uril-berberine conjugate for drug detection in biofluids

cycloaddition was used to couple the conjugated to alkyne-functionalized CB7 (Figure 4.1 (c)), which was chosen for its known high reactivity and lack of side products. Particularly, BC-HEG-N\textsubscript{3} and BC-TEG-N\textsubscript{3} were prepared through a three-step procedure from commercial berberine hydrochloride (see the experimental section for details) in an overall yield of 54\% on a 50 mg scale, and mono-propargyloxylated CB7 (CB7-(Opr)) was synthesized through a two-step hydroxylation-alkylation procedure with an overall yield of 5\% on a 20 mg scale. The modest yield is in line with the literature but fortunately not problematic because CB7 can be readily prepared at a gram scale. Flash column chromatography on silica was used to purify the BC-HEG-N\textsubscript{3} and BC-TEG-N\textsubscript{3} dye-linker conjugates, and mono-propargyloxylated CB7 was obtained via the reaction between propargyl bromide and mono-hydroxylated CB7, which was purified using CHP20P resin columns. The coupling of BC-HEG-N\textsubscript{3} or BC-TEG-N\textsubscript{3} with BC7-(Opr) was then carried out in the presence of CuSO\textsubscript{4} and L-ascorbate in DMSO/H\textsubscript{2}O (v/v = 55/45). To eliminate unreacted starting material and catalyst, CB7-HEG-BC and CB7-TEG-BC were purified using a preparative HPLC column and a solvent of acetonitrile and 0.1\% trifluoroacetic acid (TFA) aqueous (v/v = 1/3) mixture.

**Figure 4.1** Synthesis routes of (a) berberine-HEG/TEG-azide, (b) mono-propargyl cucurbit[7]uril, (c) chemosensor 1 and 2. (d) Chemical structure of all guests whose binding affinities were investigated in this work.
4.2.2. Characterization and conformation of CB7-dye conjugates

The part of mass spectrometry and ion mobilogram experiments in this section was done in cooperation with Dr. Ananya Baski and Prof. Manfred M. Kappes.

The supramolecular behavior of the CB7-linker-BC conjugates was characterized by mass spectrometry experiments, $^1$H NMR, absorbance, and emission spectroscopy to determine if the CB7-linker-BC conjugates adopt the expected self-bound, “folded” conformation (Figure 3.2), or if the linkers impose constraints. Furthermore, ion mobility spectrometry was performed to get an insight into the inherent conformation of complex 1 in the absence of solvents. For chemosensor 1, the peak appeared at m/z 925.3130 as dicationic species, which can be assigned as $(1 + \text{Na})^{2+}$ (Figure 4.2 (a)). In the ion mobilogram, this species had a drift time of 5.62 ms, resulting in a cross-section of 410 Å$^2$ (Figure 4.2 (c)).

**Figure 4.2** ESI-MS of (a) chemosensor 1 and (b) chemosensor 2 in a mixture of water and acetonitrile (v/v =2/1) in the positive model. ESI-MS of (b) chemosensor 1 and (d) chemosensor 2 in the presence of 1-adamantanol as the guest in a mixture of water and acetonitrile (v/v =2/1) in the positive model. Ion mobilogram of (c) chemosensor 1 and (f) chemosensor 2 in the presence of 1-adamantanol showing a significant increase in collision cross-section (CCS) for the inclusion complex, confirming the successful inclusion of the guest.

Only one defined conformation, *i.e.*, the bound or unbound state, occurs in the gas phase, but not a mixture, as evidenced by the uniform, symmetric curve shape. Upon addition of the guest, 1-adamantanol, the characteristic complex was found at m/z 1001.368 and was assigned as $((1 + 1$-adamantanol) + Na)$^{2+}$ (Figure 4.2 (b)). This species now has a substantially longer drift time at 5.94 ms and a corresponding collision cross-section of 424 Å$^2$ in the ion mobilogram. The relatively large increase in the collision cross-section upon guest binding can be understood by the displacement of the bound BC moiety, which results in the unfolding of the conformation. For comparison, if chemosensor 1 had occurred in an
Monofunctionalized cucurbit[7]uril-berberine conjugate for drug detection in biofluids

unfolded conformation in the gas phase, guest inclusion into the cavity of CB7 would not have affected the BC moiety, and the drift time would have remained the same. The ion mobilogram for chemosensor 2 with the shorter TEG-linker exhibits two peaks in drift time (4.87 and 5.17 ms, respectively) that likely both adopt folded conformations but differ in the penetration depth of the BC into the CB7 cavity (Figure 4.2 (f)). The unfolding of chemosensor 2 occurs with the addition of 1-adamantanol (Figure 4.2 (d) and (e)), as demonstrated by the appearance of a single peak at a substantially longer drift time (5.66 ms) (Figure 4.2 (f)).

Table 4.1 Assigned mass peaks with formula, experimental mass, drift time and CCS.

<table>
<thead>
<tr>
<th>Ion</th>
<th>Formula</th>
<th>m/z</th>
<th>Drift time (ms)</th>
<th>CCS (Å²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1 + Na)²⁺</td>
<td>C₇₆H₈₃O₂₄N₃₂NaNa</td>
<td>925.299</td>
<td>5.62</td>
<td>412</td>
</tr>
<tr>
<td>((1 + Guest) + Na)²⁺</td>
<td>C₈₆H₉₉O₂₅N₃₂NaNa</td>
<td>1001.368</td>
<td>5.94</td>
<td>424</td>
</tr>
<tr>
<td>(2 + Na)²⁺</td>
<td>C₇₂H₇₅O₂₂N₃₂NaNa</td>
<td>881.289</td>
<td>4.87/5.17</td>
<td>397</td>
</tr>
<tr>
<td>((2 + Guest) + Na)²⁺</td>
<td>C₸₂H₹₂O₂₃N₃₂NaNa</td>
<td>957.324</td>
<td>5.66</td>
<td>414</td>
</tr>
</tbody>
</table>

Experiments in D₂O were conducted to see if unimolecular, self-folding of the CB7-dye conjugates happened in solution as well. The aromatic peaks from the BC moiety (indicated with red numbers) to the CB7 host and the HEG linker are attributed to three sets of peak areas in the ¹H NMR spectrum of 1 in D₂O (Figure 4.3). The singlet at 8.10 ppm (indicated with a green square) confirms the formation of a triazole moiety by the click reaction. Furthermore, when compared to the ¹H NMR spectrum of the corresponding BC-HEG-N₃, it becomes clear that the aromatic protons of 1 exhibited both substantial upfield and downfield shifts. In analogy to literature reports, this can be interpreted by the inclusion of the 1,3-benzodioxole of BC into the CB7 cavity (shielding region of CBn), while the isoquinoline moiety resides in the deshielding carbonyl-fringed portal region of CB7.
Monofunctionalized cucurbit[7]uril-berberine conjugate for drug detection in biofluids

Figure 4.3 Overlay of $^1$H NMR (500 MHz, D$_2$O) spectra of BC-HEG-N$_3$ (bottom), chemosensor 1 (middle) and chemosensor 1 with the same equivalent of amantadine (top).

Investigation of chemosensor 2 leads to similar conclusions (Figure 4.5). Notably, the $^1$H NMR spectra of chemosensor 2 show broader peaks in the folded conformation than those of 1, indicating that the distribution of conformers of 2 is present in the solution. This finding agrees with the conformer distribution seen in the ion mobilogram of folded 2. After adding amantadine to the solution of chemosensor 1 or 2, the aromatic protons of the berberine moiety were shifted to indicate displacement of the BC moiety from the host cavity (Figure 4.3 and 4.4). Note the characteristic differences in the chemical shifts of the linker ethylene glycol proton signals between the folded and unfolded structure, particularly the sharpening of the $^1$H NMR peaks of chemosensor 2 upon unfolding.

Figure 4.4 Overlay of $^1$H NMR spectra (500 MHz, D$_2$O) of BC-TEG-N$_3$ (bottom), chemosensor 2 (middle) and chemosensor 2 upon the addition of the same equivalent of amantadine (top).

Additionally, absorbance and emission spectroscopy were utilized because they allow for probing the supramolecular behavior of the chemosensors at many orders of magnitude lower concentrations than NMR. The UV-Vis spectra of chemosensor 1 in water show an indicative 5 nm bathochromic shift at 350 nm compared to BC-HEG-N$_3$ (Figure 4.5 (a)), which in analogy to literature reports$^{287}$ shows that the
BC chromophore is included in the CB7 cavity at the micromolar concentration range as well. The emission spectra of chemosensor 1 show a maximum at 540 nm by excitation at 350 nm (Figure 4.5 (b)).

Because the BC fluorophore emits a higher emission intensity inside the hydrophobic CB7 cavity than in water, the addition of amantadine leads to a strong decrease in emission intensity, indicating that the BC moiety is displaced from the cavity of chemosensor 1 (Figure 4.6).  

**Figure 4.5** (a) UV-Vis absorption spectra of BC-HEG-N₃ and chemosensor 1, each 5 μM in water. (b) Fluorescence emission spectrum (λₑₓ = 350 nm) of 1 μM chemosensor 1 in water. (c) UV-Vis absorption spectra of BC-TEG-N₃ and chemosensor 2, each 5 μM in water. (d) Fluorescence emission spectrum (λₑₓ = 350 nm) of 1 μM chemosensor 2 in water.

**Figure 4.6** Fluorescence emission spectra (λₑₓ = 350 nm) of 1 μM chemosensor 1 before and after addition of 1.5 eq. amantadine in water.
Similarly, the covalently bound CB7-dye conjugate is suitable as an indicator if the BC chromophore is still encapsulated by the host upon dilution. Indeed, it was shown that a highly linear curve of the emission intensity versus the chemosensor 1 or 2 even after several times of dilution down to 30 nM (Figure 4.7, solid line). Conversely, the intensity-concentration plot for the non-covalent bimolecular CB7⊃BC complex had a convex curved shape (see dotted line in Figure 4.7) because complex dissociation occurred at a low concentration. (In both experiments, excess NaCl was used to weaken the unimolecular chemosensors' binding strength). Thus, photophysical experiments also indicate that chemosensor 1 and 2 adopts a unimolecular, folded structure, as is graphically depicted in Figure 3.2.

![Figure 4.7](image-url) Plot of normalized emission intensity at 540 nm in 5 mM phosphate buffer containing 140 mM NaCl versus concentration of CB7⊃BC complex (dashed line) and (a) chemosensor 1 or (b) chemosensor 2 (solid line), λ<sub>ex</sub> = 350 nm.

### 4.2.3. Stability of CB7⊃dye complexes versus CB7-BC conjugates in saline media

The stability of CB7-BC conjugates was examined as a foundation for subsequent analyte detection experiments in saline media. Typically, the stability of many CB<sub>n</sub>⊃dye complexes falls dramatically in the presence of salts, which reduces their utility for IDA sensing applications. 54, 65, 279, 287, 292-294 Indeed, as shown in Figure 4.8 (black line), the presence of approximate 100 mM sodium chloride leads to complete decomposition of the bimolecular CB7⊃BC assembly, which is consistent with the prediction of competitive binding of sodium cations to CB<sub>n</sub> portals (<i>K<sub>a</sub></i> = 2.57 × 10<sup>3</sup> M<sup>-1</sup> for CB7 with Na<sup>+</sup>). 65 Clearly, the bimolecular CB7⊃BC complex will dissociate in biorelevant media such as biofluids with typically high salt concentrations, e.g., 20-40 mM Na<sup>+</sup> in urine and 135-145 mM Na<sup>+</sup> in plasma for healthy humans. 295 In contrast, the residual fluorescence intensity for CB7-BC conjugate remains at around 70% even at 800 mM NaCl (Figure 4.8, red line). Note that the moderately reduced emission intensity of 1 and 2 in saline media likely results from the formation of CB7-BC⊃Na<sup>+</sup>, in which the BC moiety remains encapsulated inside the CB7 cavity while the metal cation co-binding to the opposite CB7-portal. 275 (ESI-MS experiments gave additional evidence that the binary complex of CB7-BC conjugate with Na<sup>+</sup> is prominently present also in the gas phase, and which was the species used for the ion-mobility studies.)
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**Figure 4.8** Plot of emission intensity at 540 nm of 1 μM CB7⊃BC complex (black), 1 μM chemosensor 1 (red) and 1 μM chemosensor 2 (blue) in water versus concentration of NaCl, $\lambda_{ex} = 350$ nm

### 4.2.4. Binding affinities of biorelevant analytes with the chemosensor 1 and 2

The host-guest binding characteristics of chemosensors 1 and 2 were studied in water versus saline media. Several adamantyl derivatives (amantadine, 1-adamantanol), polyamines (cadaverine, spermine, spermidine) and steroids (nandrolone) were chosen as representative guests because of their known high binding affinities for CB7.

When competitively binding guests, the BC moiety was displaced from the cavity of both chemosensors 1 and 2 respond with emission quenching; the difference in emission intensity is slightly larger for 2 than for 1, demonstrating that the linker length has a minor but considerable effect on structures adopted in solution. Interestingly, the binding curve of amantadine with 1 (Figure 4.9 (b)) or 2 (Figure 4.9 (d)) remains very steep even in 10X PBS, and binding may be even stronger than in deionized water (Figure 4.9 (a) and (c)).
Figure 4.9 Fitting plots of normalized emission intensity at 540 nm of 1 μM chemosensor 1 in water (a) and 10X PBS (b), 1 μM chemosensor 2 in water (c) and 10X PBS (d) upon addition of amantadine ($\lambda_{ex} = 350$ nm) at 25°C. Intervals between titration steps: 500 seconds in water, 200 seconds in 10X PBS.

Likewise, for the non-charged steroid nandrolone, a very similar binding affinity with chemosensor 2 was found in 10X PBS and water (Table 4.2, Figure 4.10).
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Figure 4.10 Fitting plots of normalized emission intensity at 540 nm of 1 μM chemosensor 1 in water (a) and 10X PBS (b), 1 μM chemosensor 2 in water (c) and 10X PBS (d) at 25°C, upon addition of nandrolone, λ<sub>ex</sub> = 350 nm. Intervals between titration steps: 1000 seconds in water, 200 seconds in 10X PBS.

However, in deionized or minimal buffers, CB7 is a known high-affinity binder of polyamines such as spermidine (Table 4.2). Indeed, chemosensor 1 is also responsive to polyamines in deionized water (see details in the supplementary data) and is shown that binding affinity in a range of 10<sup>3</sup> to 10<sup>5</sup> M<sup>-1</sup> in the order spermidine > cadaverine ≥ spermine (Table 4.2). However, unlike amantadine or non-charged guest, in the saline 10X PBS the polycationic guests, cadaverine, spermine, and spermidine do not bind at all to CB7-BC chemosensors.
Table 4.2 Summary of the binding constants determined by fluorescence titration experiments with chemosensors 1 and 2 at 25°C. The estimated error of log$K_a$ is 0.2.

<table>
<thead>
<tr>
<th>guest</th>
<th>medium</th>
<th>log$K_a$</th>
<th>chemosensor 1</th>
<th>chemosensor 2</th>
<th>CB7</th>
</tr>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>amantadine</td>
<td>water</td>
<td>7.4</td>
<td>7.9</td>
<td>12.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1X PBS</td>
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<td>$&gt; 8^a$</td>
<td>-</td>
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<td>$&gt; 8^a$</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>surine</td>
<td>$&gt; 8^a$</td>
<td>$&gt; 8^a$</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>cadaverine</td>
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<td>4.0</td>
<td>8.4$^d$</td>
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</tr>
<tr>
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<td>3.7</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10X PBS</td>
<td>$&lt; 1^b$</td>
<td>$&lt; 1^b$</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>spermine</td>
<td>water</td>
<td>3.6</td>
<td>3.9</td>
<td>7.4$^e$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1X PBS</td>
<td>3.4</td>
<td>3.6</td>
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<td></td>
<td>10X PBS</td>
<td>$&lt; 1^b$</td>
<td>$&lt; 1^b$</td>
<td>-</td>
<td></td>
</tr>
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<td>water</td>
<td>4.6</td>
<td>4.5</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10X PBS</td>
<td>$&lt; 1^b$</td>
<td>$&lt; 1^b$</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>l-adamantanol</td>
<td>10X PBS</td>
<td>$&gt; 8^a$</td>
<td>7.5</td>
<td>-</td>
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</tr>
<tr>
<td>nandrolone</td>
<td>water</td>
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<td>4.5</td>
<td>7.1$^d$</td>
<td></td>
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<tr>
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<td>4.4</td>
<td>6.6$^f$</td>
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</tr>
<tr>
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<td>10X PBS</td>
<td>4.1</td>
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<td>-</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Lower estimate on the binding affinity. $^b$ The binding affinity is too small to be fitted by fluorescence titration. $^c$ Determined by NMR. $^d$ Determined with berberine as indicator. $^e$ Determined with cyanostilbene as indicator. $^f$ Determined by isothermal titration calorimetry (ITC). 1X PBS is consisting of 137 mM NaCl, 2.7 mM KCl, 10 mM Na$_2$HPO$_4$ and 1.8 mM KH$_2$PO$_4$.

Furthermore, the binding titrations revealed that TEG-linker-based 2 forms a more strained folded conformation than HEG-linker-based 1, as evidenced by the fact that chemosensor 2 had somewhat greater binding affinities than chemosensor 1 for studied guests.

4.2.5. Binding kinetics of biorelevant analytes with the chemosensors 1 and 2

The binding kinetics of chemosensors revealed some interesting characteristics: While amantadine, 1-adamantanol and the polycationic amines are all relatively strongly bound to chemosensors in water, the equilibration times follow the order polyamines (n$^+$) > amantadine (1$^+$) >> 1-adamantanol/nandrolone, ranging from seconds (polyamines, amantadine) to thousands of seconds for non-charged guests, indicating a charge-accelerating effect on the binding kinetics, see details in the supplementary data. Positively charged guests can form exclusion complexes with CB7 prior to a flip-flop mechanism into the cavity, speeding up the binding kinetics. In fact, the binding kinetics of 1-adamantanol were so slow in desalinated water that no binding isotherms for determining $K_a$ value determination could be obtained in reasonable measurement times (Figure 4.11 (a)). In PBS, the binding kinetics of amantadine
binding is much faster than in water (Figure 4.11), providing a practically convenient way to shorten assay times by adding salts. Notably, amantadine and 1-adamantanol may still be readily differentiated according to their kinetic features, allowing for selective sensing of amantadine in the presence of other non-charged high-affinity competitors (Figure 4.11 (b) and (c)).

\[
K_{CB7\text{-guest, saline media}} = \frac{K_{CB7\text{-guest, water}}}{1 + K_{CB7\text{-dye[M^+]}_0}} \quad \text{for} \quad [M^+]_0 >> [CB7]_0 \quad (4.1)
\]

For instance, in the presence of 220 mM Na\(^+\), the affinity of CB7 for amantadine still is expected as \(\sim 10^{12} \text{ M}^{-1}\), with \(K_{CB7\text{-Na}^+} = 2.6 \times 10^7 \text{ M}^{-1}\) for the competitive interaction of Na\(^+\) with CB7.\(^{65}\) Following the analysis for the design of a CB8\text{-dye IDA assay in biofluids reported by Biedermann’s group, one would conclude that indicator dyes within an affinity range of \(K_{CB7\text{-dye, saline media}} = 10^7\text{-}10^{11} \text{ M}^{-1}\) is ideally suitable for selectively detecting amantadine in the presence of salts and other weaker binding interferents, e.g., biogenic amines. However, this assumption turned out to be impractical:

Figure 4.11 Fluorescence-based kinetic traces at 540 nm of 1.2 μM chemosensor 2 upon addition of 2.09 μM amantadine (red) or 1-adamantanol (black) in (a) water, (b) 1X PBS and (c) 10X PBS (\(\lambda_{ex}=350\text{ nm}\)) at 25°C; Kinetic traces based on fluorescence at 454 nm of 1.8 μM MDAP, 1.2 μM CB7 upon addition of 2.09 μM amantadine (red) or 1-adamantanol in (d) water, (e) 1X PBS and (f) 10X PBS at 25°C, \(\lambda_{ex}=339\text{ nm}\).
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![Chemical structures of MDAP and DASAP](image)

**Figure 4.12** Chemical structures of MDAP and DASAP

1) The lack of pH-unresponsive, high-affinity indicator dyes for CB7 is the first “trivial” hindrance to developing IDA-based amantadine sensing assays in biofluids. MDAP is one indicator dye for CB7 with the highest $K_a$ value, $2.7 \times 10^9 \text{M}^{-1}$ in deionized water (see Figure 4.12 for structure). With increasing salt content, its affinity to CB7 strongly drops, reaching $1.8 \times 10^6 \text{M}^{-1}$ in 1X PBS (Figure 4.13 (a)). In 10X PBS, its $K_a$ value became so weak ($K_a = 4.2 \times 10^3 \text{M}^{-1}$) that the CB7⊃MDAP reporter pair could not be used in the micromolar concentration range (Figure 4.13 (c)). As a result, the bimolecular CB7⊃MDAP complex can be used to detect amantadine in saline media if the salt concentration does not exceed that of 1X PBS. However, matrix effects will be encountered if the salinity differs significantly between the biofluid samples.

![Normalized fitting plot](image)

**Figure 4.13** Normalized fitting plot of emission intensity at 454 nm of 2.5 μM MDAP in 1X PBS (a) and 10X PBS (c) upon addition of CB7 ($\lambda_{ex} = 339 \text{ nm}$) at 25°C. Interval time between titration steps: 100 seconds; Normalized fitting plot of kinetic traces based on fluorescence of 1.8 μM MDAP with 1.1 μM CB7 in 1X PBS (b) and 2.7 μM MDAP with 54.1 μM CB7 in 10X PBS (d) ($\lambda_{ex} = 339 \text{ nm}$) at 25°C. The excess CB7 addition in the case of 10X PBS was used to ensure a significant intensity change.
The CB7⊃MDAP reporter pair exhibits the ability of covalent chemosensors to kinetically distinguish the positively charged amantadine from the non-charged analogue 1-adamantanol (Figure 4.11 (d)) in deionized water. However, the distinguishability of amantadine and 1-adamantanol by CB7⊃MDAP is worsened by increasing salinity to 1X PBS (Figure 4.11 (e)). In 10X PBS, the detection of amantadine through CB7⊃MDAP was not possible anymore due to the disintegration of the non-covalent host-dye complex (Figure 4.11 (f)). Furthermore, the amantadine equilibration with the CB7⊃MDAP complex takes substantially longer than with the unimolecular chemosensors, resulting in longer assay times.

2). To overcome the affinity limitations of the CB7⊃MDAP host-dye complex that leads to its susceptibility to salts, a novel, pH-unresponsive, high-affinity indicator dye for CB7 was developed. Given the strong binding affinities of adamantane for CB7,56, 75, 274 and the favorable photophysical properties of stilbene-type dyes,65, 284 DASAP (Figure 4.12) was regarded as a promising indicator dye, which could be synthesized in 2 steps in 17.0% yield (see the experimental part). DASAP showed ideal properties as an indicator dye for CB7, with a 10-fold increase in its emission intensity upon binding, a substantial Stokes shift (125 nm), a more red-shifted absorption and emission spectra than MDAP, and a high binding affinity with CB7 both in deionized water and in 10X PBS (Figure 4.14). Even in 10X PBS, its binding affinity for CB7 was estimated to exceed 10^7 M^-1. At first sight, it appears that the desired non-pH-dependent, high-affinity dye for CB7 was discovered. However, its use proved to be completely impractical in an IDA format;

**Figure 4.14** UV-Vis absorption spectra of 10 μM DASAP in water (a) and 10X PBS (c) upon addition of CB7 at 25°C. Inset: plot of normalized absorption intensity at 500 nm. Emission spectra of 10 μM DASAP upon addition of CB7 in water (b) and 10X PBS (d) at 25°C, λ_ex = 450 nm. Inset: plot of normalized emission intensity at 575 nm. The Interval time between each titration step is 50 seconds.
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No appreciable change in the emission intensity of the CB7⊃DASAP complex dissolved in water or 10X PBS was observed within 500 seconds of assay time even upon the addition of 100X excess of amantadine, demonstrating that the CB7⊃DASAP complex is kinetically inert on the practically relevant time scale (Figure 4.15 (a) and (b)). Likewise, attempts to set up a guest-displacement-assay\[sup]180\] by addition of DASAP to the pre-equilibrated CB7⊃amantadine complex failed, again due to slow unbinding kinetics for high-affinity guests from CB7 (Figure 4.15 (c) and (d)).

![Normalized kinetic traces based on fluorescence monitored at 575 nm of 5 μM CB7⊃DASAP and 500 μM amantadine (100 equivalents) in water (a) and 10X PBS (b) (λ<sub>ex</sub> = 450 nm) at 25°C. (c) Kinetic traces based on fluorescence at 600 nm of 10 μM DASAP into water (red) and 1 μM CB7⊃amantadine (0.1 eq.) in water (black) (λ<sub>ex</sub> = 530 nm); (d) Kinetic traces based on fluorescence at 600 nm of 10 μM DASAP into 10X PBS (red) and 1 μM CB7⊃amantadine (0.1 eq.) in 10X PBS (black) (λ<sub>ex</sub> = 530 nm).](image)

**Figure 4.15**

4.2.7. Quantitative determination of amantadine in biofluids

Encouraged by the ability of chemosensors to detect amantadine even in ultra-high concentrated saline media (Figure 4.9, about 1.37 M NaCl) in comparison with the ineffectiveness of CB7⊃BC complexes in such environments, the binding studies were carried out in synthetic urine (“surine”) as a well-defined media and then to real human urine and saliva.

Amantadine is usually recommended in 100-200 mg daily doses to Parkinson’s patients and is typically excreted by 15-50% unchanged in the urine.\[sup]300, 301\] Thus, amantadine concentration of ≥ 40 μM will be found for up to 24 h in urine. Following established routines for spectroscopic urine diagnostics,\[sup]276\]
urine samples were diluted with water in a 1:4 ratio to reduce the absorbance of real urine and avoid inner filter effects. Because the analyte concentration in urine samples is usually unknown, a new method was devised for using chemosensor 1 or 2 for quantifying amantadine in real urine samples. In brief, chemosensors 1 and 2 were utilized as titrants to determine the amantadine concentration in several real urine samples (Figure 4.16 (a)). In the negative control samples (urine from a healthy donor who is not receiving any drug treatment), the proportional increase of the chemosensor concentration results in a linear curve when chemosensor 1 is added to the assay medium (Figure 4.16 (b)). As a result, the unfolding of the chemosensor does not occur in the negative control media, showing that the components present in this urine sample do not affect chemosensor 1. Indeed, polyamines such as cadaverine and spermidine present in human urine at a low micromolar concentration did not unfold the chemosensor, which is in accordance with their low binding affinity for the chemosensors in the saline medium.

In order to simulate urine from patients taking amantadine, urine samples from three healthy donors were each spiked with 10 μM amantadine and subsequently diluted by water to reach a final concentration of 2 μM amantadine at the biological relevant concentration. As shown in Figure 4.16 (c-e), the fluorescence intensity showed a significant two-stage distribution as a function of chemosensor concentration. The amantadine concentration crosses the two straight lines described by two sets of data points: (1) those acquired with titrant concentrations under 1.0 equivalent and (2) those obtained with titrant concentrations greater than 1.0 equivalent. After plotting the two stages together, the intersection was found at around 2 μM amantadine corresponded to the spiking amantadine concentration of 2 μM in 4:1 diluted urine medium (and hence 10 μM amantadine in urine). Experiments were implemented in three different urine samples to probe the influence of sample-to-sample matrix variations and gave in each instance the expected result (Figure 4.16 (c-e)). The data for chemosensor 2 is similar, as shown in Figure 4.17.
Figure 4.16 (a) Schematic representation of the analyte detection with CB7-dye conjugate chemosensors in real urine media; (b) The normalized fitting plot of emission intensity at 540 nm versus concentration of chemosensor 1 in 20% urine sample I; The normalized fitting plot of emission intensity at 540 nm versus concentration of chemosensor 1 in 20% urine (c) sample I, (d) sample II and (e) sample III spiked with 2 μM amantadine at 25°C.
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Figure 4.17 (a) Fitting plot of the normalized emission intensity at 540 nm versus concentration of chemosensor 2 in 20% urine sample I; Fitting plot of the normalized emission intensity at 540 nm versus concentration of chemosensor 2 in 20% urine (b) sample I, (c) sample II and (d) sample III with a final concentration of 2 μM amantadine at 25°C.

It should be emphasized that amantadine detection in urine was also possible using CB7⊃MDAP reporter pair, and the presence of biogenic amines simulated in the experiment only caused little interference (Figures 4.18 (b) and (d)). The amantadine binding to CB7⊃MDAP, on the other hand, is slower than for the unimolecular chemosensors, and its susceptibility to interference by salts or other CB7-binding compounds is higher. Thus, it remains to be observed whether the CB7⊃MDAP-based assay is reliable under practical conditions, such as in real urine with varying concentrations of salts and organic components (e.g., other drugs).
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Figure 4.18 Fitting plot of the normalized emission intensity at 454 nm versus CB7⊃MDAP (a) in surine and (b) surine spiked with 2 μM amantadine at 25°C, respectively (λex = 339 nm). Interval time between titration steps: 200 seconds. The fitting plot of the normalized emission intensity versus concentration of (c) 2 at 540 nm, λex = 350 nm and (d) CB7⊃MDAP at 454 nm in surine spiked with 2 μM amantadine and 20 μM spermine at 25°C (λex = 339 nm). Interval time between titration steps: 50 seconds for 2 and 200 seconds for CB7⊃MDAP.

After oral administration, amantadine is found in different body fluids, such as nasal, saliva and cerebrospinal fluid (CSF).\textsuperscript{303, 304} For instance, the levels of amantadine in saliva equal approximately those in the serum in the range of 0.3 μg/mL to 0.8 μg/mL (around 2 μM to 5 μM).\textsuperscript{305} However, saliva contains much higher concentrations of biogenic amines (up to 400 μM cadaverine)\textsuperscript{306} than urine while being less saline, causing unwanted interferences for indicator displacement assays with non-covalent CB7⊃dye reporter pairs. Fortunately, chemosensors 1 and 2 were discovered to work in human saliva as well: For qualitative testing, saliva samples from healthy donors were spiked with 5 μM chemosensor 1 and the emission was measured using a microplate reader prior and after spiking with 5 μM amantadine (Figure 4.19 (a) and (c)), resulting in the expected emission intensity decrease caused by unfolding of the chemosensor after complexing with amantadine. This assay can be performed directly in non-diluted saliva. The determination of the amantadine concentration in non-diluted saliva was set and 2 μM of drug spiking was used as a presentative showcase, similar to the assay type introduced for quantification of amantadine in urine.
Figure 4.19 Normalized emission intensity at 540 nm of 5 μM chemosensor 1 (a) and chemosensor 2 (c) in human saliva prior to and after the addition of 5 μM amantadine. Error bars represent the standard deviation of the mean (n = 3). b) Fitting plot of the normalized emission intensity at 540 nm obtained by the stepwise addition of chemosensor 1 (b) and chemosensor 2 (d) to saliva that was spiked with 2 μM amantadine.

The predicted amantadine concentration was returned from the assay in three different saliva samples by using chemosensors 1 and 2 (Figure 4.19 (b) and (d)). The performance of the reporter pair CB7⊃MDAP in detecting amantadine in artificial saliva was comparable to that of the chemosensors. However, the simulated presence of cadaverine that can reach high micromolar concentrations in the saliva for some patients showed a stronger impact on CB7⊃MDAP than on 2 (Figure 4.20).
The design of unimolecular chemosensor is compared to the frequently adopted use of non-covalent biomolecular reporter pairs in the following:

The increase of the dye’s affinity for the host through covalent tethering, resulting in a higher degree of complexation in the face of disruptive competitors, enables the employment of intrinsically weakly binding indicator dyes with acceptable photophysical properties. Due to the disintegration of the non-covalent reporter pair complex, non-charged or monocationic CB7-indicator dyes like berberine (BC) cannot be used in biofluids, e.g., $K_a = 2.4 \times 10^7 \text{ M}^{-1}$ for $\text{CB7} \supseteq \text{BC}$ in water but less $1.9 \times 10^5 \text{ M}^{-1}$ in the presence of 50 mM NaCl.\textsuperscript{287} The choice of indicators was limited to the few known high-affinity dyes for CB7, mostly dicationic aromatic species such as MDAP\textsuperscript{275, 299} and 2,7-dimethyl diazaphenanthrenium (MDPT)\textsuperscript{178}, due to their electron-poor and symmetric structures, which have rather unimpressive photophysical properties. For example, this section demonstrated that the $\text{CB7} \supseteq \text{MDAP}$ reporter pair is functional for indicator displacement assays in a typical biorelevant saline buffer such as 1X PBS while maintaining a high $K_a$ value of $1.8 \times 10^6 \text{ M}^{-1}$. Higher salinity, on the other hand, was not tolerated by this reporter pair. From a photophysical standpoint, MDAP is an applicable fluorophore ($\lambda_{\text{em. max}} = 454 \text{ nm}$), which absorb and emit in the visible region of the electromagnetic spectrum. The host-dye conjugation

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**Figure 4.20** Fitting plot of the normalized emission intensity at 540 nm versus concentration of chemosensor 2 in artificial saliva spiked with 2 μM amantadine (a) and additional 100 μM cadaverine (b) at 25°C ($\lambda_{\text{ex}} = 350 \text{ nm}$); Fitting plot of the normalized emission intensity at 454 nm versus concentration of $\text{CB7} \supseteq \text{MDAP}$, $\lambda_{\text{ex}} = 339 \text{ nm}$ in artificial saliva spiked with 2 μM amantadine (c) and additional 100 μM cadaverine (d) at 25°C. Interval time between titration steps: 50 seconds for chemosensor 2 and 200 seconds for $\text{CB7} \supseteq \text{MDAP}$.

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strategy widens the range of functional chemosensors that can be prepared in (saline) aqueous media by lifting the previous constraints on searching for high-affinity dyes with suitable photophysical properties.

Inspired by the reported adamantly-BODIPY conjugate developed as a pK_a shift indicator dye for CB7, a novel pH-independent push-pull type high-affinity indicator dye (DASAP) for CB7 that functions both in water and in strongly saline buffers were developed. At first, this design appeared to overcome the affinity and the photophysical limits encountered by MDAP, but practice uncovered a fundamental restriction of indicator displacement assay-based sensing for CB7⊃dye reporter pairs. Most indicator dyes attain a critical length such that upon inclusion of complex formation with CB7, both portals of the hosts are occupied by the dye, as seen in Figure 4.21. This structural feature is also adopted by the high-affinity CB7⊃dye complexes with dicationic dyes and has important implications on the equilibration times for sensing assay times. Basically, the guest-exchange process for host-guest complexes can occur through an S_N1-type or S_N2-type-mechanism. Considering the situation of non-covalent CB7⊃dye complexes, the S_N1 mechanism appears to be the major pathway, proceeding through the exit of the dye from the host as the first and rate-limiting step (Figure 4.21 (a)).

Conversely, an S_N2-type dye-guest exchange mechanism would require the formation of a guest•CB7•dye transient complex, which is hampered by the protruding indicator dye from the CB7 portal. (BC may be one exception as the dye does not enter the whole cavity, allowing simultaneous binding with cationic species, e.g., metal ions. However, the CB7⊃BC complex is salt labile and cannot be used in biofluids, see above).

![Figure 4.21](chart)

**Figure 4.21** (a) Schematic representation of an S_N1-type-mechanism for the guest-exchange process on analyte-induced conformational changes of non-covalent CB7⊃dye reporter pair complexes and (b) an S_N2-type-mechanism for the guest-exchange process on analyte-induced conformational changes of CB7-dye conjugates.

With most CB7⊃dye reporter pair complexes restricted to an S_N1-type dye-guest exchange mechanism, it becomes clear that the higher affinity of the dye to the host, the lower rate constant for the exit of the dyes from the host because \( k_{\text{out}} = k_{\text{in}} K_a^{-1} \) and \( k_{\text{in}} \) are relatively similar among different guests for the same
CBn homologue. These counteracting effects result in a severe limitation for applying CBn-based IDA with non-covalent reporter pairs for sensing applications in biofluids. On the one hand, the affinity between the host and the dye needs to be high enough to avoid the disintegration of the CBn>dye reporter pair in the presence of other competitively binding salts or interferents. On the other hand, ultra-high-affinity dyes cause overall slow exchange kinetics, which results in prolonged assay times. Note that a similar rationale holds for guest-displacement assays (GDA), which likewise show impractically slow equilibration times for detecting high-affinity guests such as amantadine.

Fortunately, the thermodynamic and kinetic parameters of the CB7>MDAP complex are counterbalanced ($K_a = 2.7 \times 10^9$ M$^{-1}$, $k_{in} = 2.4 \times 10^7$ M$^{-1}$ s$^{-1}$, $k_{out} = 9.0 \times 10^{-3}$ s$^{-1}$ in water), so it can be practically used for sensing applications in saline media and biofluids. The high-affinity CB7>DASAP complex was impracticable as a reporter pair for amantadine detection despite its excellent salt stability and favorable photophysical properties. Similarly, the previously reported CB8>MPCP pair ($K_a = 3.9 \times 10^{12}$ M$^{-1}$, $k_{in} = 2.0 \times 10^7$ M$^{-1}$ s$^{-1}$, $k_{out} = 5.1 \times 10^6$ s$^{-1}$ in water) was also limited and resulting in assay times of several minutes even the accelerating effect of salts present in biofluids. It is worth mentioning that CB8 has a lower inherent affinity to biorelevant metal cations such as Na$^+$, K$^+$ and Ca$^{2+}$ than CB7. Therefore, CB8>dye reporter pairs are less influenced by salts and allowed the use of the dye with a lower affinity than for the analogous CB7>dye pairs in saline media. On the contrary, CB6-based chemosensing assays will not be operational in biofluids because the ingestion rates are relatively low for CB6 complexes due to the constricted portal region (thus limiting the acceptable affinity for the CB6>dye pairs). On the other hand, CB6 has a sizeable affinity for Na$^+$, K$^+$ and Ca$^{2+}$. The design of a covalent chemosensor overcomes these issues and will be particularly attractive for CB6-based chemosensors.

For chemosensors 1 and 2, the conjugated indicator (berberine) does not occupy both CB7 portals but remains one portal accessible, as confirmed by $^1$H NMR experiments and molecular modeling. The situation is graphically depicted in Figure 4.21 (b) that berberine as the indicator dye does not fully penetrate the CB7 cavity and from the restricted linker lengths. The accessible CB7-portal region can form transient complexes with positively charged guests via cation-carbonyl interactions. In this case, the $S_N2$ dye-guest exchange mechanism can be adopted by positively charged guests such as amantadine, explaining why much faster equilibration of chemosensors 1 and 2 than CB7>MDAP with cationic target analyte and the kinetic differences observed between amantadine and 1-adamantanol binding. The covalent chemosensor design strategy opens new opportunities for increasing the distinguishability of different analytes via their characteristic binding kinetics.

The research also revealed new supramolecular effects that the significantly attenuated binding affinity of the chemosensors for polyamines can be observed in saline buffer, in contrast to the modest attenuated binding affinity for neutral and mono-charged guests as salinity increases in the medium. Thus, an increase in the salinity of the medium can improve practical assay performance, such as reducing the equilibration times. However, a similar strategy cannot be applied to the CB7>MDAP (or CB7>BC)
reporter pair-based assays because the bimolecular host-dye complex dissociates in high salinity media. In addition, the characteristic effects on the thermodynamics and kinetics of analyte binding to CBn through the addition of salts to the assay medium will be useful for pattern recognition-based differential sensing of organic analytes.

4.3. Conclusion

In this chapter, two novel CB7-based unimolecular chemosensors were designed and synthesized. Their conformation as a back-folded unimolecular structure was confirmed in the gas phase and solution and reported on their interaction with biologically relevant molecules. Both conjugates displayed exceptional resistance to dilution and salt effects and remained functional chemosensors even in \( \sim 10^4 \) times excess NaCl. In biologically relevant buffered saline and real urine, the chemosensors displayed a great selectivity for Parkinson’s drug amantadine in potential interferents such as polyamines (low-affinity binding in saline media) or hydrophobic, non-charged guests such as the steroid nortestosterone (slow binding). To the best of my knowledge, this is the first CBn-based unimolecular chemosensor that can be utilized to detect a blockbuster drug, i.e., amantadine, in the medically relevant concentration range in human urine and saliva.\(^{310}\) While the non-covalent CB7⊃MDAP reporter pair may also be a viable option for amantadine detection in biofluids, the investigation nevertheless uncovered important and general shortcomings of using high-affinity host-guest. It appears promising that the covalent chemosensor design strategy can overcome many of these limitations and increase the selectivity for analyte-detection by offering access to an \( \text{S}_2\text{N}_2 \)-type guest exchange mechanism.

The “chemosensor-standard-addition-method” used here for amantadine quantification is particularly advantageous since it circumvents the otherwise often observed matrix-to-matrix effects between different biofluid specimens, such as differences in color, emission backgrounds or salinity of urine samples. The simplicity and speed of the supramolecular analytical approach proposed may find future use in established diagnostic laboratories or point-of-care applications.

4.4. Experimental part

4.4.1. Material and method

4.4.1.1. Material

All purchased chemicals and solvents were used as received from suppliers without any further purification. 2,7-dimethyl-diazapyrenium diiodide (MDAP)\(^{311}\) and 1,1’-(octane-1,8-diyl)bis(3-methyl-1H-imidazol-3-ium) bromide (\(\text{C}_8\text{mimBr} \))\(^{312}\) was synthesized according to established literature methods. The buffer solutions of 1X (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄ and 1.8 mM KH₂PO₄) and 10X (1.37 M NaCl, 27 mM KCl, 100 mM Na₂HPO₄ and 18 mM KH₂PO₄) phosphate-buffered saline (PBS) at pH 7.45 were prepared from GibcoTM PBS tablets by dissolving a tablet in 500 mL or 50 mL of distilled water, respectively. The artificial saliva was ordered from Pickering Laboratories and used as received. Surine was ordered from Cerilliant and used as received. MDAP, CB7, chemosensor 1 and chemosensor 2 stock solutions were prepared in Milli-Q water and then diluted in 1X or 10X PBS. The concentration of berberine hydrochloride (BC), MDAP, and nortestosterone stock solutions were
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accurately determined according to their molar absorption coefficients (BC: 22300 M⁻¹cm⁻¹ at 344 nm, MDAP: 7800 M⁻¹cm⁻¹ at 393 nm, and nortestosterone: 15320 M⁻¹cm⁻¹ at 248 nm) by UV-Vis absorption titration measurements in Milli-Q water. The concentration of CB7 and amantadine hydrochloride stock solution were determined by fluorescence titration against a known concentration of MDAP dye and CB7⊃MDAP receptor complex, respectively, by exciting the sample at 339 nm and collecting the emission intensity at 454 nm in Milli-Q water. The concentration of the 1-adamantanol stock solution was determined by ITC measurements. The stock solution concentrations of chemosensors 1 and 2 were determined by fluorescence titration with a known concentration of amantadine hydrochloride by exciting the sample at 350 nm and detecting the emission intensity at 540 nm in Milli-Q water. The stock solution of polyamine (cadaverine, spermine and spermidine) were prepared to the aqueous solution with a certain concentration directly by weighing. In general, chemosensor solutions should be stirred for 30 minutes in the desired medium to ensure full equilibration along with a stable baseline for spectroscopical measurements.

4.4.1.2. Nuclear Magnetic Resonance (NMR) Spectroscopy.

¹H and ¹³C NMR spectra were recorded either in deuterium oxide, Chloroform-d₃ or DMSO-d₆ on a Bruker Avance 500 spectrometer at 25°C. The ¹H and ¹³C NMR chemical shifts (δ) are given in ppm and refer to residual protons on the corresponding deuterated solvent.

4.4.1.3. High-Performance Liquid Chromatography (HPLC).

Analytical HPLC experiments were performed on an LC-2000Plus HPLC system equipped with a UV-2075 UV-Vis detector and a Kromasil 100 C18 5 μM LC column (250 × 4.6 mm, Agela) at a flow rate of 0.8 mL/min. Preparatory HPLC was performed on the same system but equipped with a Kromasil 100 C18 5 μM LC precolumn (50 × 20 mm, Agela) and a Kromasil 100 C18 5 μM LC preparative column (250 × 50 mm, Agela) for purification of CB7-HEG-BC (1) and CB7-TEG-BC (2) at a flow rate of 12 mL/min. All crude samples were dissolved in a mixture of water and ACN (v/v = 3/1) to get a final concentration of 1.5 mg/mL with one drop of TFA to improve the solubility.

4.4.1.4. Electrospray Ionization Ion Mobility-Mass Spectrometry (ESI-IM-MS).

This part of the work was done in cooperation with Dr. Ananya Baksi.

All samples (only for chemosensors 1 and 2) were dissolved in Milli-Q water to reach a final concentration of 500 µM. The stock solutions were diluted 20 times with Milli-Q water and then diluted with ACN (v/v = 1/2). 20 µL formic acid was added to acidify 2 mL diluted samples, which were directly infused into a Waters’ Synapt G2S HDMS mass spectrometer with an ESI source coupled with an ion mobility cell. All data were collected in positive ion mode in the mass range of 200-2000 m/z. Optimized parameters are as follows: flow rate: 5 µL/min, capillary voltage: 1 kV, cone voltage: 20 V, source offset: 30 V, source temperature: 80°C, desolvation temperature: 200°C, cone gas flow: 0 L/min, desolvation gas flow: 400 L/min, nebulizer: 2.5 bar. Guest inclusion was confirmed by IMS, where additional optimized parameters used are as follows: He flow: 180 mL/min, IMS gas flow: 90 mL/min, trap: 2 mL/min, IMS wave velocity: 850 m/S, wave height: 40 V. Collision cross-sections were calibrated using polyalanine
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(2+ ions). The mass resolution was 35000, and the IMS resolution was 25-30. About 300 spectra acquired over 5 min were averaged for each measurement.

4.4.1.5. Electrospray Ionization Mass Spectrometry (ESI-MS).

The mass spectrum was performed on a microOTOF-Q (208-320 Vac, 50/60 Hz, 1800VA), Bruker. A small amount of samples were dissolved into 1 mL MeOH and then treated in an ultrasound bath for 30 seconds. All the data were collected in the positive mode in the mass range m/z 100-2000. Optimized parameters are as follows: dry temperature: 100°C, dry gas: 3.0 L/min, ion energy: 5.0 eV, collision energy: 10.0 eV, collision RF: 800 Vpp, transfer time: 120 μs, prepuls storage: 10 μs.

4.4.1.6. Absorbance Spectra.

Absorbance spectra were measured in Milli-Q water on a Jasco V-730 double-beam UV-Vis spectrophotometer at 25°C. PMMA cuvettes with a light path of 10 mm and dimensions of 10 × 10 mm from Brand with a spectroscopic cut-off at 220 nm were utilized for UV-Vis absorption experiments. The samples were equilibrated using a water thermostatic cell holder STR-812, while the cuvettes were equipped with a stirrer allowing rapid mixing.

4.4.1.7. Fluorescence Spectra.

Steady-state emission spectra and time-resolved emission profiles were recorded on a Jasco FP-8300 fluorescence spectrometer equipped with a 450 W Xenon arc lamp, double-grating excitation and emission monochromators. Standard correction curves corrected the emission spectra for source intensity (lamp and grating) and the emission spectral response (detector and grating). All titration and kinetic experiments were carried out at 25°C using a water thermostatic cell holder STR-812, while the cuvettes were equipped with a stirrer allowing rapid mixing. PMMA cuvettes with a light path of 10 mm and dimensions of 10 × 10 mm from Brand with a spectroscopic cut-off at 300 nm were utilized for fluorescence-based titration experiments.

4.4.1.8. Amantadine determination by a chemosensor in urine and saliva

In order to simulate urine from Parkinson's patients receiving amantadine treatment, urine samples from three healthy donors were each spiked with 10 μM amantadine and subsequently diluted with water to reach a final concentration of 2 μM amantadine following the biorelevant concentration. The urine was used for measurements without any processing steps. It was used within 3 days after excretion and stored in the fridge. Morning and daily urine samples were used to probe different urine compositions. However, the higher concentration of ingredients in the morning urine did not affect the sensors. The fitting curves were obtained through a linear fit function using the software Origin.

In order to simulate saliva from Parkinson's patients receiving amantadine treatment, saliva samples from three healthy donors were each spiked with 2 μM amantadine following the biological relevant concentration. The saliva was used after centrifuging at 8000 rpm for 10 minutes to get rid of the insoluble component. It was used within 3 days after excretion and stored in the fridge. The fitting curves were obtained through a linear fit function using the software Origin.
4.4.2. Synthesis and characterization

4.4.2.1. Synthesis of glycoluril

According to the procedures in the literature, glyoxal (50 g, 861 mmol, 1.0 eq, 125 ml of a 40 wt. % solution in H₂O), urea (129.4 g, 2.154 mol, 2.5 eq), and 250 mL of H₂O were added into a 1 L round-bottom flask equipped with a stirrer. The reaction mixture was heated to 90°C and was stirred for 5 min. Concentrated H₂SO₄ (10 mL) was added dropwise over 5 min, and a white solid formed 10 min after adding the acid. The reaction mixture was stirred for an additional 12 h. The reaction mixture was cooled to room temperature, and 50% NaOH aqueous solution was added until the pH of the reaction was adjusted to 14. The stirring was stopped, and the reaction mixture was cooled to 0°C. The suspension was isolated by vacuum filtration on a Büchner funnel, and the solid was washed with cold water (2 x 1000 mL). The solid was left to dry on the Büchner funnel under suction for 3 h, was transferred to a 700 mL beaker, and was dried for 16 h at 80°C to yield 45.0 g (304 mmol, 92% yield) of glycoluril as an off-white powder.

¹H NMR (500 MHz, DMSO-d₆) δ 7.17 (s, 4H), 5.24 (s, 2H).

Figure 4.22 ¹H-NMR of glycoluril in DMSO-d₆
4.4.2.2. Synthesis and purification of CB7

According to the procedures in the literature, formaldehyde (14 mL of a 37% aqueous solution, 182 mM) was mixed with sulfuric acid (60 mL of a 9 M aqueous solution), and the magnetically stirred mixture was cooled down to 5°C. Glycoluril (11.4 g, 80 mM) was added, which dissolved slowly to produce a thick transparent gel. After 30 min, the gel became too viscous to allow further stirring, and at this point, the temperature was elevated to 100°C, resulting in the gel dissolved again. The reaction mixture was heated for 72 h and then poured into 200 mL of distilled water. Upon adding 800 mL of acetone, all CBn oligo- and polymers were precipitated from the solution. The solid mixture was filtered and subsequently washed with 1.5 L of cold acetone/water (v/v = 8:2) to remove the acid and produce a mixture of CBn as a white powder. 400 mL of distilled water was added, and all water-soluble CBn was dissolved except for CB6 and CB8. When the filtrate was treated with 300 mL of acetone, a new precipitate mainly containing CB7 appeared. This precipitate was dissolved into 100 mL of distilled water again, and 20 mL of acetone was added, which caused the precipitation of CB7 with other unidentified homologues. The further addition of acetone (100 mL) into filtrate led to the precipitation of the major fraction of CB7. The product was washed with acetone and methanol and subsequently dried under vacuum at 80°C for 24 h.

\[ ^1H \text{NMR (500 MHz, D}_2\text{O) } \delta 5.78 \text{ (d, } J = 15.4 \text{ Hz, 14H), 5.54 \text{ (s, 14H), 4.24 \text{ (d, } J = 15.4 \text{ Hz, 14H).} \]

Figure 4.23 \[ ^1H \text{NMR of CB7 in D}_2\text{O} \]

An additional consistent step for CB7 isolation was carried out with imidazolium salt sometimes because a small amount of other CBn residual exists in the product by following the above procedure. In contrast to conventional isolation of CBn homologues based on the solubility differences in water or acid,
the imidazolium salt is capable of complexing with one CBn resulting in significant solubility changes and readily tunable by the exchange of counter ion, e.g., C₄mimBr for CB5 and CB7 isolation; NpmimBr for CB6 and CB8. The CB7 isolation from crude products involving the imidazolium salt was briefly discussed. According to the procedure in the literature, the crude CB7 product (5 g, ≤ 4 mmol) was dissolved into water (220 mL). After the ultrasound and fully stirring for a half-hour, the insoluble solid was removed by filtration. C₄mimBr (4.5 mmol, 1 g) was added into filtrate to form the complex with CB7. Then, NH₄PF₆ salt (4.5 mmol, 734 mg) was added to exchange the Br ion and resulting in the precipitation of the CB7⊃C₄mimPF₆ complex, which was collected by centrifugation. The solid product of the complex was dissolved again into water (200 mL) in the presence of C₄mimBr (5 mmol, 1.1 g) and heated to 80°C until most of the solid dissolved. After the counter ion was exchanged from PF₆⁻ to Br⁻, the solution of CB7⊃C₄mimBr complex was concentrated at 50 mL and precipitated upon the addition of methanol (150 mL). A solid-state metathesis (SSM) was employed to remove the C₄mimBr from the CB7 cavity. Briefly, the CB7⊃C₄mimBr complex was suspended into 50 mL DCM, and then NH₄PF₆ salt (5 mmol, 815 mg) was added and refluxed for several days. ¹H NMR was employed to monitor the decomposition of CB7⊃C₄mimBr complex. The final product was obtained by centrifugation and washed with methanol.

4.4.2.3. Synthesis and separation of mono-hydroxylated CB7 (CB7-(OH)₁)

![Chemical Structure]

The mono-hydroxylation of CB7 was based on Kaifer’s procedure. Briefly, CB7 (500 mg, 0.43 mmol), (NH₄)₂S₂O₈ (147.2 mg, 0.645 mmol) and NaHSO₃ (67.1 mg, 0.645 mmol) were placed into a 100 mL round-bottomed flask. Afterwards, 60 mL of purified water was added and kept at 65°C for 12 h. After finishing the reaction, the flask was connected to a rotary evaporator in a 70°C water bath to concentrate the reaction mixture further. When the volume of the reaction mixture was reduced to about 15 mL, the evaporation was stopped, and the resulting solution in the flask was cooled to about 30°C and ready for chromatography.

The concentrated solution was loaded onto the CHP20P resin column. The column was then eluted with purified water (15 mL per fraction). The fractions were monitored by ESI-MS, for which species was mixed with an excess of C₈mimBr₂. The CB7 peak is at m/z 719²⁺ (CB7⊃C₈mim), CB7-OH is at m/z 727²⁺ (CB7-OH⊃C₈mim) and CB7-(OH)₂ is at m/z 735²⁺ (CB7-(OH)₂⊃C₈mim). The fractions in which CB7-OH was the dominant portion were collected. After evaporation, the product was obtained as a white powder (89.7 mg, 0.076 mmol, 17.7%).

¹H NMR (500 MHz, D₂O) δ 5.77 (d, J = 15.4 Hz, 2H), 5.67 (d, J = 15.5 Hz, 10H), 5.45 (m, 14H), 5.21 (s, 1H), 4.42 (d, J = 15.6 Hz, 2H), 4.16 (m, 12H). ESI-MS: [M⊃C₈mim]²⁺ Calcd. for: 727.2844; found: 727.2900.
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4.4.2.4. Synthesis of mono-propargylated CB7 (CB7-(OPr)_1)

According to the procedures in the literature,\textsuperscript{166} CB7-OH (50 mg, 42.4 μmol, 1 eq) was dissolved in 5 mL anhydrous DMSO. NaH (24.9 mg, 1.04 mmol, 24.5 eq) was added under N\textsubscript{2} at 0°C, and the mixture was stirred at room temperature for 2 h. Subsequently, the mixture was cooled to 0°C, propargyl bromide (1.04 mmol, 24.5 eq, 144 μL) was added under N\textsubscript{2}, and the reaction mixture was stirred at room temperature for 24 h. 30 mL mixture of diethyl ether and MeOH (v/v = 1:1) was added, and the resulting precipitate was three times triturated with 25 mL MeOH and dried in the oven at 80°C. The product was obtained as a brown powder (36.2 mg, 0.030 mmol, 70%).
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4.4.2.5. Synthesis of berberrubine (BC-OH)

According to literature procedures,\textsuperscript{289} berberine hydrochloride (740 mg, 2.0 mmol) was dissolved in 4 mL DMF. After heating and stirring at 160°C for 2 h, the solvent was removed under reduced pressure to yield a dark red solid. The crude product was purified by column chromatography (silica, CH\textsubscript{2}Cl\textsubscript{2}/methanol (v/v = 10/1)) to give berberrubine as red solid (497 mg, 1.6 mmol, 70 %).

\textsuperscript{1}H NMR (500 MHz, DMSO-\textit{d}\textsubscript{6}) \(\delta\) 9.07 (s, 1H), 7.98 (s, 1H), 7.61 (s, 1H), 7.21 (d, \(J = 7.8\) Hz, 1H), 6.96 (s, 1H), 6.35 (d, \(J = 7.8\) Hz, 1H), 6.09 (s, 2H), 4.48 (t, \(J = 6.1\) Hz, 2H), 3.72 (s, 3H), 3.04 (t, \(J = 6.1\) Hz, 2H) ppm. \textsuperscript{13}C NMR (126 MHz, DMSO-\textit{d}\textsubscript{6}) \(\delta\) 167.5, 150.1, 148.8, 147.7, 146.2, 133.7, 132.5, 129.7, 122.3, 121.7, 120.4, 117.6, 108.7, 105.2, 102.0, 101.3, 56.1, 52.8, 28.0 ppm. ESI-MS: Calcd. for [M-Cl]\textsuperscript{+}: 322.1074; found: 322.1341.

Figure 4.26 ESI-MS of CB7-(OPr)\textsubscript{1} with C\textsubscript{8}mimBr\textsubscript{2} in the mixture of water and methanol (v/v = 1:1).

Figure 4.27 \textsuperscript{1}H NMR spectrum (500 MHz) of BC-OH in DMSO-\textit{d}\textsubscript{6}. 
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**Figure 4.28** $^{13}$C NMR spectrum (126 MHz) of BC-OH in DMSO-$d_6$.

**Figure 4.29** ESI-MS of BC-OH in the mixture of water and methanol (v/v = 1:1).

Additional information on details is available via the Chemotion repository: https://dx.doi.org/10.14272/reaction/SA-FUHFF-UHFFADPSC-GYFSYEVKFO-UHFFADPSC-NUHFF-NUHFF-NUHFF-ZZZ

4.4.2.6. **Synthesis of Dibromo-hexaethylene glycol (Br-HEG-Br)**

According to literature procedures,$^6$ 100 mL CH$_2$Cl$_2$ were cooled down to $-78^\circ$C using a cooling mixture of dry ice and acetone. NBS (8.72 g, 49.0 mmol) and triphenylphosphine (12.84 g, 49.0 mmol) were added into a round bottom flask under stirring. After 10 min, hexaethylene glycol (4.23 g, 15.0 mmol) was added to the solution, and the reaction mixture was stirred overnight. CH$_2$Cl$_2$ was removed under reduced pressure, and the crude product was purified on a silica column, eluted with hexane/ethyl acetate (v/v = 2/1). The product was obtained as a colorless oil (3.98 g, 9.8 mmol, 65 %).
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$\text{^1H NMR (500 MHz, CDCl}_3\text{)} \delta 3.81 (t, J = 3.2 \text{ Hz, 4H}), 3.66 (m, 16H), 3.47 (t, J = 3.2 \text{ Hz, 4H}) \text{ ppm}.$ $\text{^13C NMR (126 MHz, CDCl}_3\text{)} \delta 71.36, 70.83, 70.75, 70.69, 30.47 \text{ ppm}.$

Figure 4.30 $\text{^1H NMR spectrum (500 MHz) of Br-HEG-Br in CDCl}_3.$

Figure 4.31 $\text{^13C NMR spectrum (126 MHz) of Br-HEG-Br in CDCl}_3.$

Additional information on details is available via the Chemotion repository: https://dx.doi.org/10.14272/reaction/SA-FUHFF-UHFFFADPSC-LACSKPODWQ-UHFFFADPSC-NUHFF-NUHFF-NUHFF-ZZZ

4.4.2.7. Synthesis of berberine-hexaethylene glycol-bromide (BC-HEG-Br)

A solution of BC-OH (50.0 mg, 140 µmol), K$_2$CO$_3$ (58.0 mg, 420 µmol) and Br-HEG-Br (285 mg, 700 µmol, 180 µL) in 2 mL DMF was heated to 80°C under N$_2$ for 2 h. Excess of Br-HEG-Br was used to obtain preferentially the mono-substituted product. Afterwards, the mixture was cooled to room
temperature and poured in 40 mL Et₂O. The crude product precipitated immediately. The crude product was dissolved in DCM and subjected to an isocratic silica column, eluted with DCM/methanol (v/v = 10/1), and therefore purified to give a yellow powder of BC-HEG-Br (73.8 mg, 110 µmol, 81 %). ¹H NMR (500 MHz, DMSO-d₆) δ 9.77 (s, 1H), 8.95 (s, 1H), 8.20 (d, J = 9.1 Hz, 1H), 8.01 (d, J = 9.1 Hz, 1H), 7.81 (s, 1H), 7.10 (s, 1H), 6.18 (s, 2H), 4.93 (t, J = 6.2 Hz, 2H), 4.42 (t, J = 4.6 Hz, 2H), 4.07 (s, 3H), 3.80 (t, J = 4.6 Hz, 2H), 3.68 (t, J = 5.8 Hz, 2H), 3.60 - 3.43 (m, 18H), 3.21 (t, J = 6.3 Hz, 2H) ppm. ¹³C NMR (126 MHz, DMSO-d₆) δ 150.6, 149.9, 147.7, 145.5, 142.5, 137.5, 132.9, 130.7, 126.6, 123.6, 122.0, 120.5, 120.2, 108.5, 105.4, 102.1, 73.1, 70.3, 69.8, 69.7, 69.7, 69.5, 69.5, 69.4, 69.3, 57.0, 55.5, 39.5, 32.2, 26.4 ppm. ESI-MS: Calcd. for [M]+: 648.1083; found: 648.1792.

Figure 4.32 ¹H NMR spectrum (500 MHz) of BC-HEG-Br in DMSO-d₆.

Figure 4.33 ¹³C NMR spectrum (126 MHz) of BC-HEG-Br in DMSO-d₆.
Figure 4.34 ESI-MS of BC-HEG-Br in the mixture of water and methanol (v/v = 1:1).

Additional information on details is available via the Chemotion repository:
https://dx.doi.org/10.14272/reaction/SA-FUHFF-UHFFADPSC-ZGCIIPCWPNL-UHFFADPSC-NUHFF-MUHFF-NUHFF-ZZZ

4.4.2.8. Synthesis of berberine-hexaethylene glycol-azide (BC-HEG-N₃)

To a solution of BC-HEG-Br (73.8 mg, 100 µmol) in 1 mL DMF, NaN₃ (32.9 mg, 500 µmol) was added at room temperature. After stirring at 60°C for 6 h, the mixture was cooled to room temperature. 50 mL CH₂Cl₂ were added and 3 x 50 mL NaCl saturated solution was added in extraction cycles. The combined organic extracts were dried over anhydrous Na₂SO₄. The crude solution was concentrated and purified by an isocratic silica column eluted with CH₂Cl₂/methanol (v/v = 10/1) to give BC-TEG-N₃ as yellow powder (66.6 mg, 95.0 µmol, 95 %). ¹H NMR (500 MHz, D₂O) δ 9.60 (s, 1H), 8.29 (s, 1H), 7.94 (d, J = 8.7 Hz, 1H), 7.82 (s, J = 8.9 Hz, 1H), 7.30 (s, 1H), 6.90 (s, 1H), 6.06 (s, 2H), 4.84 (t, J = 6.0 Hz, 2H), 4.49 (t, J = 3.1 Hz, 2H), 4.02 (s, 3H), 3.92 (t, J = 3.1 Hz, 2H), 3.72 (t, J = 3.5 Hz, 2H), 3.65 – 3.58 (m, 16H), 3.41 (t, J = 4.3 Hz, 2H), 3.20 (t, J = 6.0 Hz, 2H) ppm. ¹³C NMR (126 MHz, D₂O) δ 151.8, 150.6, 150.1, 147.7, 144.3, 141.9, 137.6, 133.2, 130.2, 126.3, 123.9, 121.9, 119.9, 108.4, 104.9, 102.3, 72.9, 69.8, 69.6, 69.5, 69.5, 69.5, 69.4, 69.3, 69.2, 56.6, 56.0, 50.1, 26.7 ppm. ESI-MS: Calcd. for [M⁺]: 611.2712; found: 611.2696.
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Figure 4.35 $^1$H NMR spectrum (500 MHz) of BC-HEG-N$_3$ in D$_2$O.

Figure 4.36 $^{13}$C NMR spectrum (126 MHz) of BC-HEG-N$_3$ in D$_2$O.

Figure 4.37 ESI-MS of BC-HEG-Br in the mixture of water and methanol (v/v = 1:1).

Additional information on details is available via the Chemotion repository:
https://dx.doi.org/10.14272/reaction/SA-FUHFF-UHFFADPSC-FIXMWOGOKF-UHFFADPSC-NUHFF-MUHFF-NUHFF-ZZZ

CB7-(Opr)₄ (8.7 mg, 7.2 μmol) and BC-HEG-N₃ (9.0 mg, 13.0 μmol) were dispersed in 0.7 mL DMSO. Then, sodium L-ascorbate (10.0 mg, 50.0 μmol) was added into 2.8 mL 55% DMSO aqueous solution containing CuSO₄ (4.5 mg, 28.0 μmol). These two solutions were mixed and stirred at room temperature for 24 h. Afterwards, 50 mL diethyl ether was added, and the resulting precipitate was washed three times with 25 mL methanol. Drying under a high vacuum afforded a dark solid. The product was purified by HPLC (C18, v (acetonitrile) / v (0.1% TFA aqueous) = 1/3) and obtained as yellow solid (3.6 mg, 1.9 μmol, 27%). ESI-MS: [M + Na]⁺ Calcd. for: 925.3076; found: 925.3130. Due to the complex structure, a precise characterization by ¹H and ¹³C NMR was not possible. However, as described in the previous section, the formation and purity of the compound were characterized by several methods.

Figure 4.38 ¹H NMR spectrum (500 MHz) of chemosensor 1 in D₂O.
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Figure 4.39 Analytical HPLC trace at 350 nm of chemosensor 1 using a 25 % acetonitrile/75 % 0.1 % TFA aqueous as eluents.

Additional information on details is available via the Chemotion repository:
https://dx.doi.org/10.14272/reaction/SA-FUHFF-UHFFADPSC-PLPZWDLHCM-UHFFADPSC-NUHFF-MUHFF-NUHFF-ZZZ

4.4.2.10. Synthesis of Dibromo-tetraethylene glycol (Br-TEG-Br)

According to literature procedures, 100 mL CH₂Cl₂ was cooled down to −78°C using a cooling mixture of dry ice and acetone. NBS (4.36 g, 24.5 mmol) and triphenylphosphine (6.42 g, 24.5 mmol) were added to the reaction mixture under stirring. After 10 min, tetraethylene glycol (2.13 g, 11.0 mmol) was added, and the solution was stirred overnight. The solvent was removed under reduced pressure, and the crude product was purified by an isocratic silica chromatography with hexane/ethyl acetate (v/v = 4/1) as eluents. The product was obtained as a colorless oil (3.15 g, 9.9 mmol, 90 %). ¹H NMR (500 MHz, CDCl₃) δ 3.82 (t, J = 3.2 Hz, 4H), 3.68 (s, 8H), 3.48 (t, J = 3.2 Hz, 4H) ppm. ¹³C NMR (126 MHz, CDCl₃) δ 71.2, 70.7, 70.6, 30.4 ppm.
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Figure 4.40 $^1$H NMR spectrum (500 MHz) of Br-TEG-Br in CDCl$_3$.

Figure 4.41 $^{13}$C NMR spectrum (126 MHz) of Br-TEG-Br in CDCl$_3$.

Additional information on details is available via the Chemotion repository:
https://dx.doi.org/10.14272/reaction/SA-FUHFF-UHFFFFADPSC-IJFMWHXEB-UHFFFFADPSC-NUHFF-NUHFF-NUHFF-ZZZ

4.4.2.11. Synthesis of berberine-tetraethylene glycol-bromide (BC-TEG-Br)

A solution of BC-OH (122 mg, 300 µmol), K$_2$CO$_3$ (141 mg, 1.0 mmol) and Br-TEG-Br (546 mg, 1.7 mmol, 1.1 mL) in 2 mL anhydrous DMF was heated to 80°C under nitrogen atmosphere for 2 h. Excess of Br-TEG-Br was used to obtain preferentially the mono-substituted product. Afterwards, the mixture was cooled to room temperature and poured in 40 mL Et$_2$O. The crude product precipitated immediately. The crude product was dissolved in DCM and purified by an isocratic silica column.
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chromatography with CH₂Cl₂/methanol (v/v = 10/1) as eluents. After removal of the solvents, BC-TEG-Br was obtained as a yellow powder (69 mg, 100 µmol, 35 %). ¹H NMR (500 MHz, DMSO-d₆) δ 9.78 (s, 1H), 8.95 (s, 1H), 8.20 (d, J = 9.2 Hz, 1H), 8.01 (d, J = 9.1 Hz, 1H), 7.81 (s, 1H), 7.10 (s, 1H), 6.18 (s, 2H), 4.93 (t, J = 6.2 Hz, 2H), 4.42 (t, J = 4.4 Hz, 2H), 4.06 (s, 3H), 3.81 (t, J = 4.4 Hz, 2H), 3.65 (t, J = 5.8 Hz, 2H), 3.60 – 3.46 (m, 10H), 3.22 (t, J = 3.1 Hz, 2H) ppm. ¹³C NMR (126 MHz, DMSO-d₆) δ 151.0, 150.3, 148.2, 145.9, 143.0, 137.9, 133.4, 131.1, 127.0, 124.1, 122.9, 120.9, 120.7, 108.9, 105.9, 102.6, 73.5, 70.8, 70.2, 70.1, 70.0, 69.9, 69.8, 57.5, 55.9, 32.7, 26.9 ppm. ESI-MS: Calcd. for [M]⁺: 560.1218; found: 560.1365.

Figure 4.42 ¹H NMR spectrum (500 MHz) of BC-TEG-Br in DMSO-d₆.

Figure 4.43 ¹³C NMR spectrum (126 MHz) of BC-TEG-Br in DMSO-d₆.
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Figure 4.44 ESI-MS of BC-TEG-Br in the mixture of water and methanol (v/v = 1:1).

Additional information on details is available via the Chemotion repository: https://dx.doi.org/10.14272/reaction/SA-FUHFF-UHFFADPSC-BTUUNLJUIAC-UHFFADPSC-NUHFF-MUHFF-NUHFF-ZZZ

4.4.2.12. Synthesis of berberine-tetraethylene glycol-azide (BC-TEG-N₃)

To a solution of BC-TEG-Br (55 mg, 8.6 μmol) in 1 mL DMF, NaN₃ (56 mg, 900 μmol) was added at room temperature. After stirring at 60°C for 6 h the mixture was cooled down to room temperature. 50 mL CH₂Cl₂ were added and washed by 3 x 50 mL NaCl saturated solution. The combined organic extracts were dried by anhydrous Na₂SO₄. The crude solution was concentrated to 2 mL and purified by an isocratic silica column with CH₂Cl₂/MeOH (v/v = 10/1) as eluents to obtain BC-TEG-N₃ after solvent removal as yellow powder (50 mg, 8.3 μmol, 96 %). ¹H NMR (500 MHz, DMSO-d₆) δ 9.78 (s, 1H), 8.95 (s, 1H), 8.20 (d, J = 9.2 Hz, 1H), 8.01 (d, J = 9.1 Hz, 1H), 7.81 (s, 1H), 7.10 (s, 1H), 6.18 (s, 2H), 4.93 (t, J = 6.2 Hz, 2H), 4.42 (t, J = 4.4 Hz, 2H), 4.06 (s, 3H), 3.81 (t, J = 4.4 Hz, 2H), 3.60 – 3.48 (m, 12H), 3.22 (t, J = 3.1 Hz, 2H) ppm. ¹³C NMR (126 MHz, DMSO-d₆) δ 150.6, 149.9, 147.7, 145.5, 142.6, 137.5, 133.0, 130.7, 126.6, 123.6, 122.0, 120.3, 120.3, 108.5, 105.5, 102.1, 73.1, 69.8, 69.6, 69.6, 69.5, 69.4, 69.2, 57.0, 55.5, 50.0, 26.4 ppm. ESI-MS: Calcd. for [M]+: 523.2187; found: 523.2245.
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Figure 4.45 $^1$H NMR spectrum (500 MHz) of BC-TEG-N$_3$ in DMSO-$d_6$.

Figure 4.46 $^{13}$C NMR spectrum (126 MHz) of BC-TEG-N$_3$ in DMSO-$d_6$.

Figure 4.47 ESI-MS of BC-TEG-N$_3$ in the mixture of water and methanol (v/v = 1:1).

Additional information on details is available via the Chemotion repository:
https://dx.doi.org/10.14272/reaction/SA-FUHFF-UHFFADPSC-RJLCCSUQZU-UHFFADPSC-NUHFF-MUHFFNUHFF-ZZZ

CB7-(Opr)$_1$ (8.7 mg, 7.2 μmol) and BC-TEG-N$_3$ (7.9 mg, 13.0 μmol) were dissolved in 700 μL DMSO. In a second flask, sodium L-ascorbate (10.0 mg, 50.0 μmol) was added to 2.8 mL 55% DMSO aqueous solution containing CuSO$_4$ (4.5 mg, 28.0 μmol). The two solutions were mixed and stirred at room temperature for 24 h. Afterwards, 50 mL diethyl ether was added, and the resulting precipitate was washed three times with 25 mL MeOH. Drying under a high vacuum afforded a dark solid. The product was purified by HPLC (C18, v (acetonitrile) / v (0.1% TFA aq.) = 1/3) and obtained as yellow solid (1.2 mg, 690 nmol, 10 %). ESI-MS: [M+Na]$^2+$ Calcd. for: 881.2814; found: 881.2889. Due to a precise characterization of the complex structure by $^1$H and $^{13}$C NMR was not possible.

Figure 4.48 $^1$H NMR spectrum (500 MHz) of chemosensor 2 in D$_2$O.
Figure 4.49 Analytical HPLC trace at 350 nm of chemosensor 2 using a mixture of 25 % acetonitrile/75 % 0.1 % TFA aqueous for elution.

Additional information on details is available via the Chemotion repository: https://dx.doi.org/10.14272/reaction/SA-FUHFF-UHFFFADPSC-SCCUVBVHKK-UHFFFADPSC-NUHFF-MUHFF-NUHFF-ZZZ

4.4.2.14. Synthesis of N-adamantyl-4-methylpyridine (Ad-MePy)

The synthesis of DASAP and of the starting materials were carried out by Alicja Siennicka.

According to the literature procedures, 1-bromoadamantane (2.15 g, 10.0 mmol), 4-(dimethylamino) pyridine (60.0 mg, 500 μM) and picoline (4.67 g, 50.2 mmol, 4.82 mL) were mixed under nitrogen atmosphere. After adding 180 μL distilled water (180 mg, 10.0 mmol), the mixture was refluxed for 60 h. Afterwards, the mixture was cooled down to room temperature and slowly poured into chloroform. A white precipitate was formed immediately and washed several times with chloroform. The product was dried and obtained as a white solid. (2.30 g, 7.49 mmol, 75 %). 

\[^1^H\text{NMR}\ (500\ MHz,\ D_2O)\ \delta\ 8.89\ (d,\ J = 6.9\ Hz,\ 2H),\ 7.86\ (d,\ J = 6.6\ Hz,\ 2H),\ 2.63\ (s,\ 3H),\ 2.36\ (s,\ 3H),\ 2.29\ (d,\ J = 2.6\ Hz,\ 6H),\ 1.81\ (dd,\ J = 28.3,\ 12.5\ Hz,\ 6H)\ \text{ppm}.\]

\[^{13}\text{C\ NMR}\ (126\ MHz,\ D_2O)\ \delta\ 159.2,\ 139.8,\ 128.2,\ 68.6,\ 41.7,\ 34.6,\ 29.8,\ 20.9\ \text{ppm}.\]

ESI-MS: Calcd. for [M]: 228.1447; found: 228.1739.
Figure 4.50 $^1$H NMR spectrum (500 MHz) of Ad-MePy in D$_2$O.

Figure 4.51 $^{13}$C NMR spectrum (126 MHz) of Ad-MePy in D$_2$O.

Figure 4.52 ESI-MS of Ad-MePy in the mixture of water and methanol (v/v = 1:1).

Additional information on details is available via the Chemotion repository: https://dx.doi.org/10.14272/reaction/SA-FUHFF-UHFFADPSC-JROBPRZYLG-UHFFADPSC-NUHFF-MUHFF-NUHFF-ZZZ
4.4.2.15. Synthesis of 4-[4-(Dimethylamino)styryl]-1-adamantylpyridinium (DASAP)

![Synthesis reaction diagram]

Ad-MePy (250 mg, 814 μM) was dissolved in MeOH (3 mL). To this solution, 4-dimethylamino benzaldehyde (121 mg, 814 μM) and 2-3 drops of piperidine were added, and the reaction mixture was heated to 85°C and kept stirring overnight under a nitrogen atmosphere. Afterwards, the mixture was cooled down to room temperature, and 10 mL of ethyl acetate was added. After storage at 5°C overnight, the precipitate was collected and washed three times with ethyl acetate, one time with hexane and one time with diethyl ether. The residue was then recrystallized from a mixture of dichloromethane and ethyl acetate (v/v =1/2) to give a red-purple solid (108 mg, 247 μmol, 30 %). In analogy to the commercial dye 4-[4-(Dimethylamino)styryl]-1-methylpyridinium iodide that is commonly abbreviated as DASP, the abbreviation DASAP for the herein introduced dye 4-[4-(Dimethylamino)styryl]-1-adamantylpyridinium bromide is suggested. ¹H NMR (500 MHz, MeOH-d₃) δ 8.85 (s, 2H), 7.98 (d, J = 6.3 Hz, 2H), 7.85 (d, J = 15.8 Hz, 1H), 7.62 (d, J = 8.1 Hz, 2H), 7.10 (d, J = 15.9 Hz, 1H), 6.79 (d, J = 7.4 Hz, 2H), 3.07 (s, 6H), 2.42 – 2.22 (m, 9H), 1.87 (s, 6H) ppm. ¹³C NMR (126 MHz, MeOH-d₃) δ 155.9, 154.0, 144.4, 141.0, 131.6, 124.2, 123.6, 117.6, 113.1, 68.7, 43.0, 40.2, 36.2, 31.6 ppm. ESI-MS: Calcd. for [M]⁺: 359.2482; found: 359.2506.

Figure 4.53 ¹H NMR spectrum (500 MHz) of DASAP in MeOH-d₃.
Figure 4.54 $^{13}$C NMR spectrum (126 MHz) of DASAP in MeOH-$d_3$.

Figure 4.55 ESI-MS of BC-TEG-N$_3$ in the mixture of water and methanol (v/v = 1:1).

Additional information on details is available via the Chemotion repository:
https://dx.doi.org/10.14272/reaction/SA-FUHFF-UHFFADPSC-ULNWEZYJTN-UHFFADPSC-NUHFF-MUHFF-NUHFF-ZZZ
4.5. Supplementary data


Figure 4.56 Emission spectra of (a) 5 μM berberine, (b) 5 μM BC-HEG-N₃, and (c) 5 μM BC-TEG-N₃ upon addition of CB7 in water, λₑₓ = 350 nm. Interval time between each titration step: 50 seconds.

4.5.2. Fluorescence titration of guests to chemosensors

4.5.2.1. Amantadine

Figure 4.57 Fitting plots of normalized emission intensity at 540 nm of 1 μM 1 in (a) 1X PBS and (b) surine at 25°C, upon addition of amantadine. Intervals between titration steps: 200 seconds. Normalized fluorescence-based kinetic traces at varied concentrations of amantadine and 1 μM 1 in 1X PBS at 25°C. Concentration of amantadine for each curve: 0, 0.25, 0.50, 1 and 2 μM, λₑₓ = 350 nm.

Figure 4.58 Fitting plots of normalized emission intensity at 540 nm of 1 μM 2 in (a) 1X PBS and (b) surine at 25°C, upon addition of amantadine. Intervals between titration steps: 200 seconds. Normalized fluorescence-based kinetic traces at varied concentrations of amantadine and 1 μM 2 in 1X PBS at 25°C. Concentration of amantadine for each curve: 0, 0.25, 0.50, 1 and 2 μM, λₑₓ = 350 nm.
4.5.2.2. Cadaverine

**Figure 4.59** Fitting plot of normalized emission intensity at 540 nm of 1 μM 1 in water (a), 1X PBS (b) and plot of normalized emission intensity at 540 nm of 1 μM 1 in 10X PBS (c) at 25°C, upon addition of cadaverine. Interval time between titration steps: 200 seconds in water, 20 seconds in 1X PBS and 10X PBS. (d) Normalized fluorescence-based kinetic traces at varied concentrations of cadaverine and 1 μM 1 in 1X PBS at 25°C. The concentration of cadaverine for each curve: 0, 50, 100, 250 and 500 μM, $\lambda_{ex} = 350$ nm.

**Figure 4.60** Fitting plot of normalized emission intensity at 540 nm of 1 μM 2 in water (a), 1X PBS (b) and plot of normalized emission intensity at 540 nm of 1 μM 2 in 10X PBS (c) at 25°C, upon addition of cadaverine. Interval time between titration steps: 200 seconds in water, 20 seconds in 1X PBS and 10X PBS. (d) Normalized fluorescence-based kinetic traces at varied concentrations of cadaverine and 1 μM 2 in 1X PBS at 25°C. The concentration of cadaverine for each curve: 0, 50, 100, 250 and 500 μM, $\lambda_{ex} = 350$ nm.
4.5.2.3. Spermine

**Figure 4.61** Fitting plot of normalized emission intensity at 540 nm of 1 μM 1 in water (a), 1X PBS (b) and plot of normalized emission intensity at 540 nm of 1 μM 1 in 10X PBS (c) at 25°C, upon addition of spermine. Interval time between titration steps: 200 seconds in water, 20 seconds in 1X PBS and 10X PBS. (d) Normalized fluorescence-based kinetic traces at varied concentrations of spermine and 1 μM 1 in 1X PBS at 25°C. The spermine concentration for each curve: 0, 5, 25, 50, and 100 μM, λ_{ex} = 350 nm.

**Figure 4.62** Fitting plot of normalized emission intensity at 540 nm of 1 μM 2 in water (a), 1X PBS (b) and plot of normalized emission intensity at 540 nm of 1 μM 2 in 10X PBS (c) at 25°C, upon addition of spermine. Interval time between titration steps: 200 seconds in water, 20 seconds in 1X PBS and 10X PBS. (d) Normalized fluorescence-based kinetic traces at varied concentrations of spermine and 1 μM 2 in 1X PBS at 25°C. Concentration of spermine for each curve: 0, 5, 25, 50 and 75 μM, λ_{ex} = 350 nm.
4.5.2.4. Spermidine

Figure 4.63 Fitting plot of normalized emission intensity at 540 nm of 1 μM 1 in water (a) and plot of normalized emission intensity at 540 nm of 1 μM 1 in 10X PBS (b) at 25°C, upon addition of spermidine. Interval time between titration steps: 200 seconds in water and 20 seconds in 10X PBS; (c) Normalized fluorescence-based kinetic traces at varied concentrations of spermidine and 1 μM 1 in 1X PBS at 25°C. The spermidine concentration for each curve: 0, 50, 100, and 250 μM, λ<sub>ex</sub> = 350 nm.

Figure 4.64 Fitting plot of normalized emission intensity at 540 nm of 1 μM 2 in water (a) and plot of normalized emission intensity at 540 nm of 1 μM 2 in 10X PBS (b) at 25°C, upon addition of spermidine. Interval time between titration steps: 200 seconds in water and 20 seconds in 10X PBS. (c) Normalized fluorescence-based kinetic traces at varied concentrations of spermidine and 1 μM 2 in 1X PBS at 25°C. Concentration of spermidine for each curve: 0, 50, 100, 250 and 480 μM, λ<sub>ex</sub> = 350 nm.
4.5.2.5. 1-Adamantanol

**Figure 4.65** Fitting plot of normalized emission intensity at 540 nm of 1 μM (a) 1 and (b) 2 in 10X PBS at 25°C, upon addition of 1-adamantanol. Interval time between titration steps: 3000 seconds in 10X PBS. (c) Normalized fluorescence-based kinetic traces at varied concentrations of 1-adamantanol and 1 μM 1 in 1X PBS at 25°C. Concentration of 1-adamantanol for each curve: 0.0, 5.5, 11.0, 21.0 and 43.5 μM. (d) Normalized fluorescence-based kinetic traces at varied concentrations of 1-adamantanol to 1 μM 2 in 1X PBS at 25°C. Concentration of 1-adamantanol for each curve: 0.0, 2.5, 6.7, 22.3 and 44.2 μM, $\lambda_{ex} = 350$ nm.

4.5.2.6. Nandrolone

**Figure 4.66** Fitting plot of normalized emission intensity at 540 nm of 1 μM (a) 1 and (b) 2 in 1X PBS at 25°C, upon addition of nortestosterone. Interval time between titration steps: 500 seconds in 1X PBS.
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Normalized fluorescence-based kinetic traces at varied concentrations of nortestosterone and 1 μM (c) 1 and (d) 2 in 1X PBS at 25°C. Concentration of nortestosterone for each curve: 0.0, 5.4, 10.8, 21.6 and 43.2 μM, \( \lambda_{ex} = 350 \text{ nm} \).

4.5.3. Overlay of kinetic traces on the addition of amantadine to 1 and 2

![Graph showing overlay of kinetic traces](image)

**Figure 4.67** Overlay of normalized fluorescence-based kinetic traces for the addition of 1.5 μM amantadine to 1 μM 1 (red line) and 1 μM 2 (black line) in water (a) and 1X PBS (b) at 25°C, \( \lambda_{ex} = 350 \text{ nm} \). (Normalization method [0,1] in Origin software package was used).

4.5.4. Detection limit of chemosensor 2 for amantadine

![Graph showing detection limit](image)

**Figure 4.68** Fitting plot of emission intensity monitored at 540 nm of 1 μM 2 in 1X PBS at 25°C, upon addition of amantadine, \( \lambda_{ex} = 350 \text{ nm} \). The emission intensity of 2 without any amantadine was measured for five individual samples in 1X PBS, and based on these measurements, the standard deviation (σ) was determined. The limit of detection (LOD) was then calculated with the equation \( 3\sigma/S \). S is the slope between intensity versus amantadine concentration (ΔF is the ratio of fluorescence intensity change, and C is the concentration of amantadine in μM).
4.5.5. 100 nM amantadine detection by chemosensor 2 in surine

**Figure 4.69** Fitting plot of the normalized emission intensity at 540 nm *versus* concentration of 2 in surine spiked with 100 nM amantadine at 25°C, $\lambda_{ex} = 350$ nm. Interval time between titration steps: 200 seconds.
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5. Distinguishing biorelevant analytes by salt-responsive CB7-based chemosensor in biofluids

This chapter is derived from the work that has been submitted to the Journal of the American Chemical Society (after final revision): Hu, C.; Jochmann, T.; Chakraborty, P.; Neumaier, M.; Levkin, P. A.; Kappes, M. M.; Biedermann, F. The synthesis, analysis, and data organization were carried out by me under the supervision of Dr. Frank Biedermann. The PCA and pairwise pattern analysis was carried out in cooperation with Thomas Jochmann. The part of mass spectrometry, ion mobilogram experiments and DFT calculation were collaborated with Papri Chabraborty, Dr. Marco Neumaier and Prof. Dr. Manfred M. Kappes. The manuscript was written and revised through the contributions of all authors above.

The dissociation caused by the salt competitive binding to supramolecular receptors is one of the major constraints for most host-guest chemosensors in biofluids or similar environments. Many relevant metabolites share a similar affinity with artificial binders, so it is difficult to distinguish them using established emission-based chemosensing methods such as indicator displacement assay (IDA). In this chapter, a novel cucurbit[7]uril-dye conjugate was applied, namely CB7-NBD, which can respond to salt-induced adaptation on analytes distinction via the facile approach with different salt types and concentrations. The new parameters provide additional information about the system at a low synthetic effort. In addition, an intuitive data-driven way of translating human-visible differences in curves into pairwise differences is presented. Combining ion mobility experiments with density functional theory (DFT) calculations yielded new insight into the binding mechanism and revealed an unprecedented ternary complex geometry for CB7. This chapter presents the non-selectively binding but salt-adaptive cucurbit[n]uril system for sensing applications in biofluids such as urine, saliva, and blood serum.

5.1. Introduction

Artificial receptors that utilize non-covalent recognition motifs are crucial elements in the design of fast-response, facile, low-cost, and parallelizable sensing technology, e.g., for monitoring biochemical processes and molecular diagnostics. Over the years, supramolecular research has focused on a complex with a static structure, taking advantage of preorganization and template effects to prepare them. Recently, there has been a growing interest in studying and exploiting labile interactions between molecular components in supramolecular systems. So far, mainly dynamic covalent bonding motifs were built into molecular frameworks to adapt the constitution to internal or external factors by reorganizing and exchanging building blocks. Similarly, synthetic receptors were developed with these concepts. However, most receptor designs have been based on "static" macrocyclic systems with no obvious way to incorporate dynamic covalent bonds into the framework.

Most synthetic receptors that display high binding affinities for small bioactive molecules, e.g., metabolites, neurotransmitters, and drugs, have been constructed from macrocyclic hosts with a deep binding cavity, taking advantage of multivalent binding, preorganization and of the non-classical hydrophobic effect, i.e., high-energy water release, gain of differential cavitation energies or the chaotropic effect. Representative examples are cryptands, calix[n]arenes, cavitands,
naphthotubes,\textsuperscript{348-350} and cucurbit[$n$]urils.\textsuperscript{117, 215} Unfortunately, these macrocyclic systems are structurally symmetric and consequently rather unselective receptors. Besides, they do not mimic the asymmetric and selective binding pocket of proteins.

As the introduction mentioned above, though the propensity of CB$n$ to strongly bind a wide range of biorelevant analytes\textsuperscript{241, 351, 279} has spurred the development of assays for bioprocess monitoring, the applications of CB$n$-based chemosensor in biofluids remain scarce yet\textsuperscript{56, 352} because of the relatively low selectivity and the subject to competitive binding by salts occurring in biofluids.

To confront these issues, in the previous chapter, an indicator-CB7 conjugated strategy for designing a responsive supramolecular system was provided to detect ultra-high binding affinity drug amantadine in biofluid. Nevertheless, this unimolecular chemosensor cannot sense analytes with less binding affinity. According to the established systems, it always required the preparation of a series or library of differentially selective chemosensors combined with multivariant data analysis.\textsuperscript{343, 353, 354}

This line of thought should have led us to prepare a library of CB$n$-based chemosensors to achieve the differential array sensing of less binding analytes. However, established CB$n$ analogues with particular structures (e.g., chiral CB$n$, acyclic CB$n$, and nor-seco-CB$n$) have not brought the desired achievements.

Given the above situation, a potential strategy that could convert the ‘enemy factors’ into ‘assistant tools’ for the differential sensing of analytes was figured out. Instead of the library of chemosensors, the type and concentration of salt were set as the variable parameters to modulate the binding properties between CB$n$ and analytes. According to the distinctive and distinguishable responsiveness of each host-guest combination to the different salt types and concentrations, a tailored signal output can be obtained with individual features, namely the salt-adaptive CB$n$ chemosensor.

5.2. Results

5.2.1. Design and preparation of CB7-based chemosensor

To satisfy the chemosensor with tunable features responded by metal-cation addition, the idea of following the chapter 4 that covalently tethered reporter dye onto CB$n$, which endows the chemosensor with the resistance of dilution and competitive salt binding, was adopted here firstly. However, the inevitable enhancement of binding strength \textit{via} covalently tethered the strong binding dye resulted in the limited detection of ultra-high analytes rather than other weakly ones by chemosensors, such as CB7-BC conjugates. On the other hand, positively charged berberine moiety will electrostatically repel the metal ions and prevent their binding to CB$n$ portals. This feature would only ensure the resistance of cation and stability in a high salinity medium but no salt-responsiveness. Thereby, both relatively weakly binding affinity and non-charge of the dye were prerequisites for designing a salt-responsive unimolecular CB$n$-based chemosensor, which could be regulated by salt while considering the displacement by the guest with weak affinity.

After screening the dye with a suitable size for the encapsulation by CB7 and the feature of environment-responsive non-charged fluorescent, a highly solvent-polarity responsive fluorescent dye, namely nitrobenzoxadiazole (NBD),\textsuperscript{355} was identified as a promising potential dye for establishing the
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chemosensor. Firstly, the binding performance of NBD to CB7 was investigated via photophysical characterization. In principle, the significant emission changes for this polarity-sensitive dye should be observed after encapsulated into the hydrophobic cavity of CB7 if the binary complex is formed. As expected, any hardly detectable spectral change of NBD upon addition of CB7 can be detected for the emission spectra in aqueous media by excitation at 475 nm (max absorption for NBD). Notably, the NBD was conjugated with a short flexible tetraethylene-glycol-azide (NBD-TEG) as a control compound for CB7-NBD. The resulting data barely indicated an intermolecular interaction between NBD-TEG and CB7 in an aqueous solution.

![Absorption (dashed line) and emission (solid line) spectra of NBD-TEG (5 μM, black line) upon addition of excess CB7 (red line), \( \lambda_{\text{ex}} = 475 \text{ nm} \).](image)

**Figure 5.1** Absorption (dashed line) and emission (solid line) spectra of NBD-TEG (5 μM, black line) upon addition of excess CB7 (red line), \( \lambda_{\text{ex}} = 475 \text{ nm} \).

Likewise, via an azide-alkyne Huisgen cycloaddition reaction, NBD-TEG was covalently tethered to the propargyl-functionalized monosubstituted CB7 to obtain the CB7-NBD conjugate, as shown in Figure 5.2. The detailed synthesis and characterization information is given in the experimental section.

![Preparation of CB7-NBD conjugate via an azide-alkyne Huisgen cycloaddition.](image)

**Figure 5.2** Preparation of CB7-NBD conjugate via an azide-alkyne Huisgen cycloaddition.

The chemical structure and conformation of obtained CB7-NBD in aqueous media were investigated by \(^1\text{H} \) NMR experiments. Due to the limited solubility of this conjugate in water, the sample dissolution
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was assisted with NaCl addition. This phenomenon proved the interaction between CB7-NBD and salt intuitively and will further analyze this phenomenon from the perspective of spectroscopy. From the proton NMR spectra of NBD-TEG and CB7-NBD, it was observed that the significant upfield shift of peaks belonging to NBD moiety after tethering to CB7 (Figure 5.3 (a) and (b)). This phenomenon provided solid evidence that NBD was encapsulated into the cavity of CB7 in a folded, unimolecular conformation.

Figure 5.3 Overlay of $^1$H NMR (500 MHz, D$_2$O) spectra of (a) NBD-TEG (black), (b) CB7-NBD (red), (c) CB7-NBD with an excess of cadaverine (blue), and (d) CB7-NBD with an excess of amantadine (pink). The appearance of the singlet peak at 8.04 ppm (marked with a green square) confirmed the triazole formation via click reaction.

5.2.2. Photophysical features of CB7-NBD in aqueous solution

In contrast with the result of NBD-TEG shown in Figure 5.1, the absorption and emission spectra of CB7-NBD in the aqueous phase were characterized that the maximum absorbance peak and emission wavelength were at 475 nm and 540 nm around, respectively, and displayed highly emissive intensity than NBD-TEG at the same concentration (Figure 5.4).

Figure 5.4 Absorption (dashed line) and emission (solid line) spectra of CB7-NBD (5 μM, black line) and NBD-TEG (5 μM, red line), $\lambda_{ex} = 475$ nm.
It is noteworthy that the significant emission enhancement of CB7-NBD solution occurred upon the addition of salts from the emission spectra (Figure 5.5 (a) and (b)). In the aspect of the absorption spectra, the maxima shifts were also observed and attributed to a strengthening of the unimolecular self-inclusion complex through cation co-binding.

**Figure 5.5** (a) Schematic illustration of emission enhancement of CB7-NBD induced upon addition of NaCl and photographic images of 200 μM CB7-NBD in MiliQ water in the absence (left cuvette) and presence (right cuvette) of 20 mM NaCl. Images were recorded at ‘day light’ (left image) and under UV irradiation (right image). (b) Absorption (dashed line) and emission (solid line) spectra of CB7-NBD in the absence (black line) and presence (red line) of excess NaCl, λ_{ex} = 475 nm.

In order to demonstrate the stability of CB7-NBD in high salinity solution, two examples of traditional non-covalent binary CBn⊃dye chemosensors (CB7⊃BC and CB7⊃MDAP) and another unimolecular conjugated chemosensor (CB7-BC) were involved into the NaCl titration experiments as well. The decreasing emission intensity of chemosensor solution with the increasing NaCl concentration revealed the gradual expulsion of dye from the cavity of CB7 upon salt addition (Figure 5.6).

**Figure 5.6** Plot of emission intensity of CB7-NBD conjugate (black) (λ_{ex} = 475 nm, λ_{em} = 550 nm), CB7-BC conjugate (purple) (λ_{ex} = 350 nm, λ_{em} = 540 nm), CB7⊃BC complex (red) (λ_{ex} = 350 nm, λ_{em} = 540 nm) and CB7⊃MDAP complex (blue) (λ_{ex} = 339 nm, λ_{em} = 454 nm) in MiliQ water as a function of increasing concentration of NaCl.
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5.2.3. Interaction of CB7-NBD with biorelevant analytes

Two typical CB7 binding guests, cadaverine and amantadine, were utilized to investigate the binding performances between CB7-NBD and competitive guests. As shown in Figures 5.3 (c) and (d), the displacement of NBD from the CB7 cavity can be concluded from the characteristic \(^1\)H NMR shifts. Addition of excess analytes into a solution of CB7-NBD results in the NBD protons (marked with red and blue squares) undergoing significant downfield shifts (\(\Delta \delta = 0.5 \) and 0.3 ppm, respectively). Furthermore, the interpretation of NBD displacement was supported by the significant intensity decrease (Figure 5.7 (a)) and slight bathochromic shift (Figure 5.7 (b)) in the prospective of the emission signal of CB7-NBD, which gave the evidence of the dye displaced from the cavity and exposure into the polar solvent environment upon the addition of guests.

![Emission spectra](image)

**Figure 5.7** (a) Emission spectra (\(\lambda_{ex} = 475 \) nm) of CB7-NBD (1.0 \( \mu \)M) upon addition of cadaverine. (b) Absorbance spectra in the water of 5 \( \mu \)M CB7-NBD upon addition of 66.8 \( \mu \)M cadaverine.

Afterwards, the binding affinity of CB7-NBD with typical CB7-binding organic guests was investigated by the emission-based titration experiment in water and saline buffered media. As shown in Table 5.1, it was observed that only modestly reduced binding affinity to CB7-NBD with guests compared to the parent CB7 alone, such as \( \log K_a = 7.0 \) versus 7.5 for PheGly\(^{161} \) or 6.7 versus 7.4 for methyl viologen (\( \text{M}_2\text{V} \))\(^{283} \) complexation in deionized water. It demonstrated that the low energetic cost was consumed on the expulsion of NBD from the CB7 cavity rather than the strong affinity reducing of CB7-BC with corresponding guest displacement. On top of that, the data in the table also provided an intuitive comparison of binding affinity between deionized water and 1X PBS as medium. While an attenuated affinity of CB7-NBD with guests was observed in the presence of salt, the residual binding strength is still appreciable high in contrast to other reported synthetic receptors that become disfunctional in the presence of salts. In conclusion, this novel chemosensor may possess the capability for analyte detection in buffered biologically media.
Table 5.1 Binding affinities of CB7-NBD with (bio)organic analytes from fluorescence titration experiments in aqueous media*.

<table>
<thead>
<tr>
<th>analytes</th>
<th>log $K_a$ (M$^{-1}$)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H$_2$O</td>
<td>1X PBS</td>
<td></td>
</tr>
<tr>
<td>putrescine (Put)</td>
<td>5.2</td>
<td>$\leq 3.0^a$</td>
<td></td>
</tr>
<tr>
<td>cadaverine (Cad)</td>
<td>7.0</td>
<td>4.0</td>
<td></td>
</tr>
<tr>
<td>spermidine (Spd)</td>
<td>6.3</td>
<td>3.3</td>
<td></td>
</tr>
<tr>
<td>spermine (Spm)</td>
<td>7.8</td>
<td>3.9</td>
<td></td>
</tr>
<tr>
<td>agmatine (Agm)</td>
<td>5.5</td>
<td>$\leq 3.0^a$</td>
<td></td>
</tr>
<tr>
<td>tyramine (Tyr)</td>
<td>5.8</td>
<td>3.8</td>
<td></td>
</tr>
<tr>
<td>amantadine (AdNH$_2$)</td>
<td>$\geq 8.0^b$</td>
<td>$\geq 8.0^b$</td>
<td></td>
</tr>
<tr>
<td>L-phenylalanyl glycine (PheGly)</td>
<td>7.0</td>
<td>4.5</td>
<td></td>
</tr>
<tr>
<td>nandrolone (Nan)</td>
<td>$\geq 8.0^b$</td>
<td>4.2</td>
<td></td>
</tr>
<tr>
<td>methyl viologen (M$_2$V)</td>
<td>6.7</td>
<td>4.1</td>
<td></td>
</tr>
<tr>
<td>1-adamantanol (AdOH)</td>
<td>$\geq 8.0^b$</td>
<td>$\geq 8.0^b$</td>
<td></td>
</tr>
<tr>
<td>pinacol</td>
<td>5.2</td>
<td>4.1</td>
<td></td>
</tr>
<tr>
<td>4-fluorophenethylamine (4F-PEA)</td>
<td>6.4</td>
<td>4.1</td>
<td></td>
</tr>
<tr>
<td>1,8-octanediyl-bis(methylimidazolium) (C$_8$mim)</td>
<td>$\geq 8.0^b$</td>
<td>5.9</td>
<td></td>
</tr>
</tbody>
</table>

$^a$, $^b$ Binding curves too flat or too steep, respectively, thus $K_a$ determination was not attempted.

$^c$ $K_a$ determination not attempted due to slow equilibration. *10 µM NaCl was added to CB7-NBD (1 µM) for solubility reasons. The estimated error in log $K_a$ is 0.2. 1X PBS consists of 137 mM NaCl, 2.7 mM Na$_2$HPO$_4$, and 1.8 mM KH$_2$PO$_4$.

5.2.4. Response of CB7-NBD$⊃$guest complexes to salts

Although the universal trend of attenuated affinity in the presence of high salt in the medium can be found among the different analytes, it was also observed that the degree of affinity weakening is not quite the same between different analytes. For instance, log$K_a$ of spermine dropped from 7.8 in water to 3.9 in 1X PBS, but log$K_a$ of pinacol was only reduced from 5.2 in water to 4.1 in 1X PBS. I concluded that the sensitivity of each CB7-NBD$⊃$guest to salt concentration varies significantly between the different analytes, which provides an unprecedented opportunity to create a salt-tunable supramolecular differential sensing system for analytes distinction.
Afterwards, a fluorescent emission-based titration of NaCl into CB7-NBD⊃guest complex was carried out, as shown in Figure 5.8. A unique intensity restoring behavior of CB7-NBD⊃cadaverine complexes was observed upon the addition of NaCl into their aqueous solution, which demonstrated the repulsion of protonated cadaverine from CB7 cavity and the re-binding of NBD moiety occurred in the presence of sodium cations.

![Figure 5.8](image)

**Figure 5.8** Representative emission-based titration of the CB7-NBD⊃cadaverine complex with NaCl$_{aq}$ ($\lambda_{ex} = 475$ nm).

The corresponding absorbance spectra of CB7-NBD with other typical analytes upon the salt addition were investigated, as shown in Figure 5.9. Conversely, for the ultra-high binding and part of non-charged guests, the spectral reversal caused by the addition of NaCl is not very pronounced, which indicates that the high-affinity guests remain buried in the cavity while the NBD fluorophore is located mostly outside. Especially, CB7-NBD inclusion complexes with ultra-high affinity guests (*e.g.*, amantadine and 1-adamantan) were almost irresponsible to the addition of NaCl.

![Figure 5.9](image)

**Figure 5.9** Absorbance spectra in water of 5 μM CB7-NBD upon addition of (a) 1-adamantan, (b) amantadine, (c) pinacol, (d) nandrolone, (e) cadaverine, (f) spermidine, and subsequent addition of NaCl.
After all, adding salt into the CB7-NBD complex with different guests can lead to the competitive displacement of guests and induce the adaptation of different supramolecular conformations, as shown in Figure 5.10, which is dependent on the type of guests and salts, and salt concentration. The variation in fluorescent signal is determined by a combination of factors. Therefore, the change in the fluorescence signal is characteristic for different analytes.

**Figure 5.10** Schematic representation of salt-induced analyte distinction by CB7-NBD. Upon titrating with salts, some analyte types are expelled from the chemosensor while the dye-moiety is rebound. Other CB7-NBD analyte complexes may adopt a different binding geometry but remain intact in the presence of salts. One or more equivalents of metal cations can be co-complexed as a function of salt concentration and type. These processes provide analyte-indicative information and enable their distinction.

An assay that enables differential sensing of analytes has been proposed and preliminarily validated. The distinction of analytes can be achieved via the differential emission responses of CB7-NBD complexes with analytes to the addition of different types and concentrations of salts.

### 5.2.5. Analytes distinction by microplate assay

The array-based analytes distinction assay via the salt addition was carried out in a fluorescence plate reader, satisfying the emission responses of different CB7-NBD analytes complex versus various increasing salts concentration in parallel experiments.

Firstly, a series of typical CB7-binding biorelevant guests with various affinities and features (e.g., charge, size, etc.) was chosen in Figure 5.11. In the assay preparation stage, a sub-stoichiometric concentration of CB7-NBD was added to the aqueous solution of the analytes to ensure that a high degree of complexation with the chemosensors is relatively independent of the binding affinity. Then, the aqueous stock solution of salt was titrated stepwise into the mixture solution, and the emission was recorded after a certain interval between each titration. The intensity was plotted against the salt concentration in the final mixture in the microplate wells.
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**Figure 5.11** Chemical structures of the tested analytes. All compounds are shown in their native charge state in water, pH 7.

Taking NaCl as a titration example, the normalized plot curves of emission intensity versus concentration of NaCl was demonstrated in Figure 5.12 (a). Compared to the spectral information, these curves were more intuitive in contrasting the differences between each analyte with CB7-NBD in response to salt. To demonstrate this unique feature belonging to CB7-NBD, two traditional non-covalent IDA chemosensors (CB7▷BC and CB7▷MDAP) and one unimolecular conjugated chemosensor (CB7-BC) were carried out in the same experiments as the control (Figure 5.12 (b)). Much more steeply increasing curves for CB7-NBD can be observed for polyamines and dicationic guests. In contrast, the non-charged guests, nandrolone (Nan), phenylalanine glycol (PheGly), and pinacol, caused only modest emission responses as their complexes with CB7-NBD (Figure 5.12 (a)). Meanwhile, flat signal response curves were found for amantadine (AdNH₂) and 1-adamantanol (AdOH), whose host-guest complexes with CB7-NBD are mostly inert to salt addition. This subgroup of ultra-high analytes can be directly identified among the numerous analytes by this strategy. It is noteworthy that the emission intensity of one analyte with CB7-NBD at different salt concentrations could also offer specific information compared to other analytes. For instance, before 500 mM NaCl in the mixture, the plot traces of C₃mim and pinacol were mostly identical, but the obviously accelerated differentiation appeared after 600 mM of NaCl addition. The intensity of C₃mim continued to rise while pinacol leveled off. In contrast, the traditional non-covalent chemosensors (Figure 5.12 (b) and (c)) or cationic unimolecular chemosensors (Figure 5.12 (d)) were not able to distinguish the analytes in this way.
Figure 5.12 Normalized plot of the emission intensity as a function of NaCl concentration. The assay was initiated by preparing a mixture of chemosensor ((a) 1.0 μM CB7-NBD, (b) 1.0 μM CB7 + 1.2 μM BC, (c) 1 μM CB7 and 1.2 μM MDAP, and (d) 1 μM CB7-BC) and 200 μM of the analyte in 10 mM phosphate buffer, pH 7.45, followed by the subsequent titration with stock solutions of inorganic salts. Three replicas of the emission responses for each analyte-salt combination are shown.

In addition, titrating with other inorganic salts can harvest additional and complementary information to distinguish analytes. For instance, when titrating LiCl\textsubscript{aq} into the mixture of CB7-NBD analytes complex in solution, an obviously different emission intensity plot for spermidine can be observed among the numerous curves when NaCl\textsubscript{aq} or KCl\textsubscript{aq} titrating.

Figure 5.13 Plot of the emission intensity at 550 nm of 1 μM CB7-NBD in the presence of 200 μM analytes as a function of the concentration of (a) LiCl and (b) KCl in 10 mM PB (λ\textsubscript{ex} = 475 nm).
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5.2.6. Data analysis of analyte salt-response differences

The PCA and pairwise pattern analysis was carried out in cooperation with Thomas Jochmann.

In order to integrate the multi-dimensional information from various concentrations of salt and analytes into a more visible and intuitional presentation way, the principal component analysis (PCA) was introduced into differential sensing studies for analyzing the above results. For instance, PCA was performed solely on the single-salt NaCl-addition data with all analytes processed together and visualized the first two principal components of the emission curves, as shown in Figure 5.14. For most analytes, distinct data clusters were observed and substantiated by non-overlapping 95% confidence ellipses computed from each analyte's replica measurements. Except for ultra-high binding affinity analytes, other analytes can be fully separated from each other without serious overlapping areas. Conversely, the PCA data resulting from differential sensing assay by the traditional non-covalent chemosensor CB7⊃BC cannot supply a clear differential performance as CB7-NBD, where the large overlap among the confidence ellipses of each analyte was observed basically.

Figure 5.14 PCA plot with 95% confidence ellipses resulting from the emission intensity at 550 nm (λex = 475 nm) as a function of the concentration of NaCl with (a) 1 μM CB7-NBD, (b) 1 μM CB7 + 1.2 μM BC, (c) 1 μM CB7 + 1.2 μM MDAP and (d) 1 μM CB7-BC in 10 mM PB spiked with 200 μM analytes. The black loading vectors reflect the influence of each concentration on the first two principal components. Orthogonal vectors indicate uncorrelated information between the corresponding measurements, whereas parallel vectors indicate redundant information.
Besides NaCl, other alkaline salts were also capable of demonstrating great performance for analyte distinction in the perspective of PCA results. The slight but clear distinction between AdOH and AdNH$_2$ was achieved by the addition of RbCl, CsCl, or CsF. Although the results obtained by titration of alkaline earth metal salts were not as ideal as alkaline salt (probably due to the multivalent cations or precipitates forming with phosphate ions), they also enriched dimensionally the differential sensing information. See the data from experimental sections.

Meanwhile, the analytes distinction capability can be enhanced by jointly processing the data from all salt-addition data into one, namely, combining the dependence of salt types with salt concentration. The two-dimensional PCA was utilized again for this analysis and is shown in Figure 5.15.

![Figure 5.15 PCA plot with 95% confidence ellipses resulting from processing all above data input.](image)

The main superiority of the system is to harvest additional data by salt addition with one chemosensor instead of the traditional receptor-library-based approaches that faces additional synthetic efforts. Furthermore, it is not required to employ redundant types of salt at various concentrations, but one or two types of salts added at four different concentrations were already enough to reach the goal of analyte distinction.

In order to present the differences between the salt-responses of any pairs of specimens in a single and intuitive quantity, the root mean squared difference of the salt-induced emission response $I_{em}$ was calculated at each salt concentration (offset-corrected ($I_{em}(c) = I_{em}(c) - I_{em}(c = 0)$) and normalized ($I_{em}(c) = I_{em}(c)/\max(|I_{em}(all \ concentrations \ and \ analytes)|)$):

$$\Delta(I_a, I_b) = \sqrt{\frac{\sum_c (I_a(c) - I_b(c))^2}{N_{\text{conc}}}}, \quad (5.1)$$

Where $c$ stands for the salt concentration and, again, $N_{\text{conc}}$ for the number of different salt concentrations used in the assay.

The corresponding plots that depict the pairwise distance $\Delta (I_a, I_b)$ between any two specimens (replicas/samples) are shown in Figure 5.12. When NaCl was the titrant, except CB7-NBD complexes with ultra-high affinity guests (AdNH$_2$ and AdOH) were unresponsive, it is generally feasible to pairwise distinguish any of the two analytes from each other in this colorimetric pattern. Likewise, the standard
non-covalent chemosensors (CB7⊃BC and CB7⊃MDAP) did not allow for a clear distinction of the analytes from their corresponding emission response patterns due to the full dissociation in the presence high concentration of salt. In addition, strong unimolecular chemosensor (CB7-BC) can only distinct a few high-affinity guests, e.g., Nan, AdNH₂ or C₈mim, rather than other low-affinity analytes.

Figure 5.16 Pairwise pattern of difference between emission responses of the three individual replicas for each analyte with (a) 1 μM CB7-NBD, (b) 1 μM CB7 + 1.2 μM BC, (c) 1 μM CB7 + 1.2 μM MDAP and (d) 1 μM CB7-BC in 10 mM PB spiked with 200 μM analytes. The three replicas per analyte are depicted inside the blocks as smaller squares. The blocks correspond to different analytes and are separated by thick white lines. Yellow color marks small distances (curves are very similar); violet marks larger distances (curves are more different).

To further demonstrate the different salt-responses, differential sensing of the analytes mixture was carried out following the same salt-addition procedure. For example, the mixture of spermine and nandrolone was investigated and found to be distinguishable through titration with NaCl, as shown in Figure 5.16.
Figure 5.17 (a) Plot of the emission intensity, (b) PCA plot with 95% confidence ellipses resulting from the emission intensity at 550 nm of 1 µM CB7-NBD in the presence of 200 µM nandrolone (Nan) and spermine (Spm) mixture as a function of the concentration of NaCl in 10 mM PB (λ_ex = 475 nm). (c) Pairwise pattern of differences between emission responses of the three individual replicas for each analyte.

5.2.7. Differentiation of biorelevant amines by salt-addition assays

I also investigated the salt-addition responses of CB7-NBD for a series of biorelevant amines. Six universal and important biogenic amines were chosen here, i.e., putrescine, cadaverine, agmatine, tyramine, spermidine, and spermine. Some are important biomarkers for disease diagnosis or important metabolites for health quantification. Besides, their similar chemical structure and binding affinity to CB7 is a challenge to test the system and assay for analytes distinction. The array-based emission data for salt-addition (alkali chlorides) to the solution containing the mixture of amines (500 µM, for fully complexing the CB7 at most) and CB7-NBD (1 µM) in 10 mM phosphate buffer at pH 7.45, was carried out. Fortunately, the obvious distinction between each pair of analytes by salt-addition assay can be observed in both the PCA and pairwise pattern plots, as the example of LiCl addition shows in Figure 5.18. In addition, similar experiments were carried out with the concentration of amines at 200 µM, and a great distinction was also obtained that revealed the assay is not disturbed by concentration differences if the analyte is more excess than the chemosensor.
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Figure 5.18 (a) Plot of the emission intensity, (b) PCA plot with 95% confidence ellipses resulting from the emission intensity at 550 nm of 1 μM CB7-NBD in the presence of 500 μM amines as a function of the concentration of LiCl in 10 mM PB (λ<sub>ex</sub> = 475 nm) and (c) pairwise pattern of difference between emission responses of the three individual replicas for each amine.

5.2.8. Differential sensing of analytes by salt-addition assay in biofluids

Encouraged by the successful application for analytes distinction in 1X PBS by salt-addition assay, I moved forward to transform the assays in biofluid, where it is always a challenge for standard non-covalent CBn-based chemosensors for sensing applications due to their disintegration or unselective binding properties. Firstly, the salt-addition assay with CB7-NBD in analyte-spiked human urine and deproteinized human serum were tested. As shown in Figure 5.19, all spiked specimens can be distinguished from each other with the CB7-NBD by salt-addition assay, analogous to the aforementioned results in water and 1X PBS. Furthermore, the salt-addition assay was also successfully applied in artificial saliva and artificial synthetic urine (surine) for analytes distinction.
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Figure 5.19 PCA plots with 95% confidence ellipses resulting from the emission intensity at 550 nm ($\lambda_{ex}$ = 475 nm) as a function of the concentration of NaCl with 1 μM CB7-NBD in (a) human urine spiked with 200 μM analytes and (b) diluted human deproteinized serum spiked with 200 μM analytes. The corresponding colorimetric plots for differences in emission response between pairwise replicas/samples were presented on the right side.

5.2.9. Binding geometries by ion mobility experiments and DFT calculations

The part of mass spectrometry, ion mobilogram experiments and DFT calculations in this section were collaborated with Papri Chabraborty, Dr. Marco Neumaier and Prof. Dr. Manfred M. Kappes.

After exploring and applying CB7-NBD for analytes differential sensing by salt-addition assay, I was interested to study the geometry of its binding modes by ion mobility experiments in combination with DFT calculations to unravel the conformation of analyte-bound or unbound chemosensor. I also expected to identify differences in the complex geometries of literature-known CBn-guest complexes. 291, 356 Firstly, Na⁺·CB7-NBD complex was geometry-optimized by dispersion-corrected DFT calculations and displayed as a representative energetically lowest structure out of ten investigated conformers in Figure 5.20 (a). It showed an expected inclusion structure in agreement with the aforementioned schematic illustration and NMR results. Meanwhile, the bonding interaction of Na⁺ with -CO- groups of CB7 and the -NO₂ moiety of NBD with Na-O distances of 2.34 Å and 2.23 Å, respectively, was found from the DFT calculation. Herein, the structure and Mulliken charge distributions were then used to calculate Collision Cross Sections (CCSs) based on the trajectory-method (TM). 357 Based on this predicted result,
the direct experimental support for identifying the structure was obtained from ion mobility experiments, and only one conformer of the Na⁺·CB7-NBD complex was observed in the gas phase, as shown in Figure 5.20 (b). Moreover, a great match was found between the experimentally determined CCSs (336 Å) and calculated CCSs (359 Å) with only a 6.9% deviation. In comparison with the inclusion conformation of Na⁺·CB7-NBD, the other conformation with the unbound NBD moiety exhibited the higher DFT energies (+1.64 eV for folded and +3.84 eV for unfolded) and much larger CCSs values (388 Å for folded and 489 Å for unfolded) than the experimental values.

Like wise, the DFT calculation and ion mobility experiments were carried out to characterize three representative CB7-NBD complexes, AdNH₂, AdOH, and Cad, respectively. The results from calculation and experiments for [CB7-NBD·Na⊃adamantanol]⁺ and the [CB7-NBD·Na⊃amantadine·H]²⁺ complex demonstrated that an exclusion type complex exhibited the lowest DFT energy with the NBD moiety inside of the CB7 cavity. However, the inclusion type complex showed larger DFT energy and calculated CCSs than the experimental value. This was a converse situation compared to an aqueous solution, where the AdOH and AdNH₂ were bound inside the CB7 cavity while NBD was displaced and exposed to the solution environment corresponding to the results from NMR data. It can be interpreted that molecules are in an isolated state in the gas phase, and there is less existing the conventional driving force between host and guest. This might cause the different topologies in the gas phase in contrast to the solution phase. There are two protonated cadaverine states for the chemosensor complex with cadaverine in solution to investigate separately. Similar to the previous two analytes, the monoprotonated [CB7-NBD⊃cadaverine·H]⁺ complex was also in an exclusion-type conformation as the energetically lowest candidate than others, which was calculated by DFT and in agreement with the ion mobility experiment. All of the corresponding computational and experimental CCSs values were summarized in Table 5.2.

Figure 5.20 (a) Structures of [CB7-NBD·Na]⁺ obtained at the DFT level (BP86/disp3-bj/def2-SV(P)). The NBD moiety inside the cavity is depicted with van der Waals spheres. Hydrogen atoms are not shown for clarity. The CB7 unit is shown in light grey. Atoms in blue, red and grey refer to nitrogen, oxygen and carbon atoms, respectively. The sodium cation is shown in purple. (b) Mass spectra and ion mobilograms of [CB7-NBD·Na]⁺.
Table 5.2 Experimental and theoretical data relevant to CB7-NBD/guest system with experimental collision cross-sections ($^{\text{TIMS}}$CCS$_{N2}$), calculated collision cross-sections ($^{\text{calc}}$CCS$_{N2}$), and relative DFT energies $\Delta$E$_{\text{DFT}}$ (0 eV refers to the structure with the highest stability). Bold structures best agree with the experimental CCSs and exhibit the highest stability.

<table>
<thead>
<tr>
<th>Compound</th>
<th>m/z</th>
<th>$^{\text{TIMS}}$CCS$_{N2}$ (Å$^2$)</th>
<th>$^{\text{calc}}$CCS$_{N2}$ (Å$^2$)</th>
<th>$\Delta$E$_{\text{DFT}}$ (eV)</th>
<th>Topology</th>
<th>CCSs deviation exp. vs calc. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[CB6-Na]$^+$</td>
<td>1019</td>
<td>270</td>
<td>285.3</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>[CB7-H]$^+$</td>
<td>1163</td>
<td>305</td>
<td>322.7</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>[CB7-NBD-H]$^+$</td>
<td>1598</td>
<td>337</td>
<td>366.9</td>
<td>0.00</td>
<td>inclusion</td>
<td></td>
</tr>
<tr>
<td>[CB7-NBD-Na]$^+$</td>
<td>1620</td>
<td>336</td>
<td>359.3</td>
<td>0.00</td>
<td>inclusion</td>
<td></td>
</tr>
<tr>
<td>[CB7-NBD•cadaverine•2H]$^{2+}$</td>
<td>851</td>
<td>375</td>
<td>387.7</td>
<td>0.00</td>
<td>dual inclusion</td>
<td></td>
</tr>
<tr>
<td>[CB7-NBD•cadaverine•H]$^+$</td>
<td>1700</td>
<td>349</td>
<td>389.5</td>
<td>0.16</td>
<td>exclusion</td>
<td></td>
</tr>
<tr>
<td>[CB7-NBD•AdOH]$^+$</td>
<td>1772</td>
<td>352</td>
<td>381.0</td>
<td>0.00</td>
<td>exclusion</td>
<td></td>
</tr>
<tr>
<td>[CB7-NBD•AdNH$_2$•H]$^{2+}$</td>
<td>886</td>
<td>386</td>
<td>386.7</td>
<td>0.00</td>
<td>exclusion</td>
<td></td>
</tr>
<tr>
<td></td>
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</tbody>
</table>

Next, for the double protonated [CB7-NBD•cadaverine•2H]$^{2+}$ complex, DFT calculated suggested a very unusual dual CB7-inclusion complex that the lowest energetically conformation is simultaneous inclusion of NBD moiety and cadaverine•2H$^+$ inside the cavity as shown in Figure 5.21. Indeed, the CCSs
value was in agreement with the measured one as well. The other inclusion structure that NBD dangling over the CB7 portal is out of consideration due to its high DFT value and large deviation from the experimental value.

**Figure 5.21** (a) Structures of \([\text{CB7-NBD} \supset \text{cadaverine} \cdot 2\text{H}]^{2+}\) obtained at the DFT level (BP86/disp3-bj/def2-SV(P)). The guest molecules and the NBD unit inside the cavity are depicted with van der Waals spheres. Hydrogen atoms are not shown for clarity, except for the two protonated amino groups of cadaverine. The CB7 unit is shown in light grey. Atoms in blue, red and grey refer to nitrogen, oxygen and carbon atoms, respectively. The sodium cation is shown in purple. (b) Mass spectra and ion mobilities of \([\text{CB7-NBD} \supset \text{cadaverine} \cdot 2\text{H}]^{2+}\).

### 5.3. Conclusion

In this chapter, a unimolecular cucurbit[7]uril-dye conjugate system (CB7-NBD) was established and introduced for differential sensing of analyte by its unique salt-adaptive behaviors in buffered media and biofluids. Compared to the dissociation of traditional IDA-based non-covalent supramolecular systems in high salinity media, CB7-NBD exhibited the required dilution stability and salt resistance and maintained the binding affinity to a broad scope of analytes accompanied by fluorescent responses. Importantly, the binding behavior with analytes can be modulated through the addition of salts. The characterization of analytes binding with CB7-NBD was investigated by NMR, photophysics, ion mobility experiments, and DFT calculations. A salt-addition assay for analytes differential sensing strategy was developed to take advantage of the salt-adaptive responses. Instead of needing to synthesize a series of “differentially selective” receptors, merely the concentration and type of salts sufficed as parameters for creating high dimensional information for analytes differential sensing. After processing by data analysis, the results were visualized by PCA plots. Moreover, another intuitive colorimetric depiction was utilized to quantify the differences between each analyte (including replica). The presented CB7-NBD chemosensor combined with the salt-addition assay enables the sensing of biorelevant analytes in biofluids, such as urine, saliva, and serum.
Distinguishing biorelevant analytes by salt-responsive CB7-based chemosensor in biofluids

5.4. Experimental part

5.4.1. Material and methods

All solvents and purchased chemicals were used as received from suppliers without any further purification. The stock buffer solutions of phosphate-buffered saline (1X PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, and 1.8 mM KH₂PO₄) at pH 7.45 were prepared from Gibco™ PBS tablets by dissolving a tablet in 500 mL of distilled water. The stock solution of 10 mM phosphate buffer (PB) was prepared with sodium dihydrogen phosphate (0.6 g) in 500 mL of distilled water, and the pH was adjusted by 0.1 M NaOH to 7.45. Artificial saliva was purchased from Pickering Laboratories and used as received. Artificial synthetic urine (surine) was purchased from Cerilliant and was used as received. Healthy coworkers voluntarily donated their own urine. See also the section “ethical approval and informed consent” below. Human serum was purchased from Sigma and used in a deproteinized form. The deproteinization was performed according to the literature procedure. Specifically, 500 µL of 4 M perchloric acid was added to 5 mL of cold human serum, mixed by shaking and centrifuged for 5 min at 10000 rpm at 4 °C. The supernatant was removed from the precipitated protein, and the pH was adjusted to 7.0 by dropwise addition of KOH (4 M). KClO₄ that precipitated was removed by centrifugation, and the deproteinized serum was diluted with 70% 1X PBS buffer for further measurements. The stock solution concentrations of cucurbit[7]uril-tetraethylene glycol-nitrobenzoxadiazole (CB7-NBD) were determined by fluorescence titration upon the addition of known concentration of amantadine hydrochloride by exciting the sample at 475 nm and collecting the emission intensity at 550 nm in Milli-Q water. The amantadine hydrochloride and 1-adamantanol stock solution concentration were determined by fluorescence titration against a known CB7⊃berberine receptor complex by exciting the sample at 350 nm and collecting the emission intensity at 540 nm in Milli-Q water. The concentrations of stock solutions of berberine chloride (BC), L-phenylalanyl glycine (PheGly), methyl viologen (M₂V), and nortestosterone (nandrolone) were determined by using their molar absorption coefficients (berberine chloride: 22300 M⁻¹ cm⁻¹ at 344 nm, L-phenylalanyl glycine: 195 M⁻¹ cm⁻¹ at 257.6 nm, methyl viologen: 20700 M⁻¹ cm⁻¹ at 257 nm, and 19-nortestosterone: 15320 M⁻¹ cm⁻¹ at 248 nm) by UV-Vis absorption titration measurements in Milli-Q water. The stock solutions of other analytes were prepared as the aqueous solution with a certain concentration directly by weighing. In general, chemosensor solutions should be stirred for 30 minutes in the desired medium to ensure full equilibration along with a stable baseline for spectroscopical measurements.

5.4.1.1. Nuclear Magnetic Resonance (NMR) Spectroscopy.

¹H and ¹³C NMR spectra were recorded either in deuterium oxide or chloroform-d₅ on a Bruker Avance 500 spectrometer at 25 °C. The ¹H and ¹³C NMR chemical shifts (δ) are given in ppm and refer to residual protons on the corresponding deuterated solvent.

5.4.1.2. High-Performance Liquid Chromatography (HPLC).

Preparative HPLC was performed on an LC-2000Plus HPLC system equipped with a UV-2075 UV-Vis detector, a Kromasil 100 C18 5 μM LC precolumn (50 × 20 mm, Agela), and a Kromasil 100 C18 5
μM LC preparative column (250 × 50 mm, Agela) for purification of CB7-NBD at a flow rate of 12 mL/min. All crude samples were dissolved in a mixture of water and ACN (v/v = 65/35) to get a final concentration of 1.5 mg/mL with one drop of TFA to improve the solubility.

5.4.1.3. Absorbance Spectra.

Absorbance spectra were measured at 25°C in Milli-Q water or PBS buffer on a JASCO V-730 double-beam UV-Vis spectrophotometer. PMMA cuvettes with a light path of 10 mm and dimensions of 10 × 10 mm from Brand with a spectroscopic cut-off at 220 nm were utilized for UV-Vis absorption experiments. The samples were equilibrated using a water thermostatic cell holder STR-812, while the cuvettes were equipped with a stirrer allowing rapid mixing.

5.4.1.4. Fluorescence Spectra.

Steady-state emission spectra and time-resolved emission profiles were recorded on a JASCO FP-8300 fluorescence spectrometer equipped with a 450 W Xenon arc lamp, double-grating excitation, and emission monochromators. Emission spectra were corrected for source intensity (lamp and grating) and the emission spectral response (detector and grating) by standard correction curves. All of the titration and kinetic experiments were carried out at 25°C by using a water thermostatic cell holder STR-812, while the cuvettes were equipped with a stirrer allowing rapid mixing. PMMA cuvettes with a light path of 10 mm and dimensions of 10 × 10 mm from Brand with a spectroscopic cut-off at 220 nm were utilized for fluorescence-based titration experiments.

5.4.1.5. 96-well Plate Reader.

Salt-adaptive analytes distinction assay was performed in a CLARIOstar Plus fluorescence plate reader (BMG Labtech). The total volume of solution before titration was 200 μL in each microplate well. All of the titration and kinetic experiments were carried out at 25°C with a temperature control system. For detection settings, the excitation was in the wavelength range from 450 nm to 490 nm, and emission was collected from 530 nm to 570 nm with a dichroic mirror at 511.2 nm. The pump speed of the injector was selected as 430 μL/s for the titration steps. Shaking of the microplate was carried out at 300 rpm frequency for 60 seconds before each cycle.

5.4.1.6. Ethical Approval and Informed Consent.

All procedures in studies involving human participants were in accordance with the formal statement of ethical principles published by the World Medical Association in the declaration of Helsinki in 1964 and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants included in the study.

5.4.1.7. Trapped Ion Mobility Spectrometry (TIMS).

This part was carried out by Papri Chabraborty.

Ion mobility spectrometry (IMS) is a technique that can be coupled to mass spectrometry (MS). IMS provides collision cross sections (CCSs) of ions and thus insights into their gas-phase structures. All IMS measurements were performed with a Bruker TIMS TOF instrument. TIMS is a type of the various IMS methods which can determine the ion mobilities at a very high resolution (~200). In TIMS, ions are
accumulated, and an electric field gradient (EFG) traps the ions against a constant flow of buffer gas (N₂). The difference in pressure (Δp) between the entry and exit of the TIMS tunnel is the source of this flow. Lowering the EFG results in ion elution according to their mobilities. In my experiments, Δp of 1.7 mbar, inverse mobility gap of 0.1 Vs/cm², ramp time of 500 ms, and accumulation time of 10 ms were used. Source conditions for electrospraying the ions in TIMS TOF were capillary voltage 5 kV, nebulizer 0.3 bar, dry gas 3.5 L/min, and dry temperature 200°C.

For the determination of the collision cross-section of the ions from TIMS, a calibration is necessary. Commercially available Agilent Tune Mix and the drift tube CCSs values (in N₂) published by Stow et al.³⁶² were as used for calibration. TIMS records inverse mobilities (1/k) of ions which were then converted to TIMSCCS_N₂.

5.4.1.8. DFT Method and Calculation of Collision Cross Sections.

This part was carried out by Papri Chabraborty and Dr. Marco Neumaier.

The quantum chemical calculations were performed with the TURBOMOLE package³⁶³ with the BP86-functional²⁶⁴, ³⁶⁵ and def2-SV(P)³⁶⁶ basis set. For dispersion correction, the DISP3³⁶⁷ method with Becke and Johnson damping (BJ-damping) was used.³⁶⁸ Prior density-functional theory (DFT) calculations structures were generated manually in Avogadro³⁶⁹ (version 1.2.0) and preoptimized with the Merck molecular force field MMFF94.³⁷⁰-³⁷⁵

We used the Mulliken population analysis as implemented in TURBOMOLE to assign partial charges on each atom. The atomic coordinates of the optimized DFT structures and the partial charges were used as input data for the IMoS 1.09³⁷⁶ package in order to calculate Collision Cross Sections (CCSs) based on the trajectory method (TM). In IMoS, the interaction of the cucurbituril ions with the collision gas nitrogen is modeled with a Lennard-Jones (LJ)-type interaction plus an ion-induced dipole potential. Since nitrogen is anisotropic, the ion–quadrupole³⁷⁷ interaction can also be taken into account. Based on the cucurbituril-nitrogen interaction potential, the scattering angle (χ) was calculated by a series of trajectory calculations, and the momentum transfer cross section (calcCCSN₂) was obtained by numerical integration of χ.³⁷⁸

5.4.1.9. Data Analysis Details

For each of the considered analytes, the emission intensities from CB7-NBD as a function of the concentration of the salt was plotted. The overall distinguishability of different analytes was assessed and visualized: First, the curve distance between any two replicas based on the root mean squared difference of the emission intensities at the measured concentrations was quantified. Second, these distances into colored maps of pair-wise distance were translated separately for each salt.
5.4.2. Synthesis and characterization

5.4.2.1. Chemical structures of non-covalent reporter dyes for CB7

![Chemical structures of reporter dyes for CB7](image)

**Figure 5.22** Chemical structures of reporter dyes for CB7.

5.4.2.2. Synthesis of NBD-tetraethylene glycol-azide (NBD-TEG)

![Synthesis of NBD-TEG](image)

11-azido-3,6,9-trioxaundecan-1-amine (20.0 mg, 91.6 μmol, 18.2 μL) was added to a suspension of K₂CO₃ (28.5 mg, 206.2 μmol) in 2 mL dry DCM under nitrogen and stirred for 10 min. Subsequently, the solution of NBD-chloride (27.4 mg, 137.5 μmol) in 1 mL dry DCM was added and keep the reaction stirring at room temperature for 3 h under nitrogen. The solvent was removed by rotary evaporation and the crude product was purified by a column chromatography (gradient 0-50 % ethyl acetate in hexane) to give a brown viscous liquid of NBD-TEG (25.0 mg, 65.6 μmol, 71.6 %). ¹H NMR (500 MHz, CDCl₃) δ 8.52 (d, J = 8.6 Hz, 1H), 7.03 (br, 1H), 6.21 (d, J = 8.6 Hz, 1H), 3.93 – 3.88 (t, J = 4.8 Hz, 2H), 3.74 (s, 12H), 3.44 – 3.39 (t, J = 4.8 Hz, 2H). ¹³C NMR (126 MHz, CDCl₃) δ 144.5, 144.3, 144.1, 136.6, 124.2, 98.8, 70.9, 70.9, 70.8, 70.7, 70.2, 68.3, 50.8, 43.9 ppm. ESI-MS: Calcd. For [M+Na]⁺: 404.1289; found: 404.1337.

![NMR spectrum of NBD-TEG](image)

**Figure 5.23** ¹H NMR spectrum (500 MHz) of NBD-TEG in CDCl₃.
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Figure 5.24 $^{13}$C NMR spectrum (126 MHz) of NBD-TEG in CDCl$_3$.

Additional information on details is available via the Chemotion repository:
https://dx.doi.org/10.14272/reaction/SA-FUHFF-UHFFADPSC-ZTJNWVOHDV-UHFFADPSC-NUHFF-NUHFF-NUHFF-ZZ

5.4.2.3. Synthesis of cucurbit[7]uril-TEG-NBD conjugate (CB7-NBD)

CB7-(Opr)$_1$ (20.0 mg, 16.4 μmol) and NBD-TEG (9.4 mg, 24.7 μmol) were dispersed in 3 mL DMSO. Then, sodium L-ascorbate (3.3 mg, 16.4 μmol) was added into 55% DMSO aqueous solution (2 mL) containing CuSO$_4$ (2.6 mg, 16.4 μmol). Both solutions were mixed, and the reaction was kept stirring at room temperature for 24 h. Afterwards, 10 mL MeOH was added to the mixture, and the resulting precipitate was washed with MeOH (3 x 35 mL). Drying under 80°C in the oven and afforded a dark
solid. The product was purified by HPLC (C18, v (acetonitrile) / v (0.1% TFA aqueous) = 35%/65%) and obtained the product as yellow flappy solid after freeze-drying (3.6 mg, 1.9 μmol, 27 %). ESI-MS: [M + Na]^+ Calcd. for: 1620.486; found: 1620.483. Due to the complex structure, a precise peak assignment by ^1^H and ^13^C NMR was not possible.

Figure 5.25 ^1^H NMR spectrum (500 MHz) of CB7-NBD in D$_2$O.

Figure 5.26 Analytical HPLC trace of CB7-NBD recorded at 475 nm using a mixture of 35 % acetonitrile/65 % 0.1 % TFA aqueous as eluent.

Additional information on details is available via the Chemotion repository:
https://dx.doi.org/10.14272/reaction/SA-FUHFF-UHFFADPSC-ICDIQORYLK-UHFFADPSC-NUHFF-NUHFF-NUHFF-ZZZ
5.5. Supplementary data

5.5.1. Binding affinities for analytes with CB7-NBD in water and 1X PBS

Figure 5.27 Fitting plot of normalized emission intensity at 550 nm ($\lambda_{ex} = 475$ nm) of $1 \mu$M CB7-NBD in water at $25^\circ$C, upon addition of analytes (except nandrolone).
Figure 5.28 Fitting plot of normalized emission intensity at 550 nm ($\lambda_{ex} = 475$ nm) of 1 µM CB7-NBD in 1X PBS at 25°C, upon addition of aliquots of the analyte.
5.5.2. Emission-based titration of salts to CB7-NBD with analytes

**Figure 5.29** Plot of the emission intensity at 550 nm of 1 μM CB7-NBD in the presence of 200 μM analytes as a function of the concentration of different salts in 10 mM PB (λ<sub>ex</sub> = 475 nm).
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5.5.3. PCA plots for analytes resulting from salt-addition assays

![PCA plots for analytes resulting from salt-addition assays](image)

**Figure 5.30** PCA plot with 95% confidence ellipses resulting from the emission intensity at 550 nm ($\lambda_{ex}$ = 475 nm) as a function of the concentration of salt with 1 $\mu$M CB7-NBD in 10 mM PB spiked with 200 $\mu$M analytes.
5.5.4. Pairwise plot of the difference between analytes by using salt-addition assay

Figure 5.31 Pairwise pattern of difference between emission responses at 550 nm of the three individual replicas for each analyte (200 μM) in the presence of 1 μM CB7-NBD with the titration of salt in 10 mM PB (λ_{ex} = 475 nm).
5.5.5. **Emission-based titration of salts to CB7-NBD with biorelevant amines**

![Graph showing emission intensity at 550 nm of 1 μM CB7-NBD in the presence of 500 μM amines as a function of the concentration of different salts in 10 mM PB (λ_ex = 475 nm).]

**Figure 5.32** Plot of the emission intensity at 550 nm of 1 μM CB7-NBD in the presence of 500 μM amines as a function of the concentration of different salts in 10 mM PB (λ_ex = 475 nm).

5.5.6. **PCA plots for biorelevant amines resulting from salt-addition assays**

![PCA plots with 95% confidence ellipses resulting from the emission intensity at 550 nm (λ_ex = 475 nm) as a function of the salt concentration with 1 μM CB7-NBD in 10 mM PB spiked with 500 μM amines.]

**Figure 5.33** PCA plot with 95% confidence ellipses resulting from the emission intensity at 550 nm (λ_ex = 475 nm) as a function of the salt concentration with 1 μM CB7-NBD in 10 mM PB spiked with 500 μM amines.
5.5.7. **Pairwise plot of the difference between amines by using salt-addition assay**

![Pairwise pattern of difference between emission responses at 550 nm of the three individual replicas for each amine (500 μM) in the presence of 1 μM CB7-NBD with the titration of salt in 10 mM PB (λ_ex = 475 nm).](image)

**Figure 5.34** Pairwise pattern of difference between emission responses at 550 nm of the three individual replicas for each amine (500 μM) in the presence of 1 μM CB7-NBD with the titration of salt in 10 mM PB (λ_ex = 475 nm).

5.5.8. **Emission-based titration of NaCl to CB7-NBD with analytes in biofluids**

![Emission-based titration of NaCl to CB7-NBD with analytes in biofluids](image)

**Figure 5.35** (a) Plot of the emission intensity, (b) PCA plot with 95% confidence ellipses resulting from the emission intensity at 550 nm of 1 μM CB7-NBD in the presence of 200 μM analytes as a function of the concentration of NaCl in surine (λ_ex = 475 nm) and (c) pairwise pattern of difference between emission responses of the three individual replicas for each analyte.
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Figure 5.36 (a) Plot of the emission intensity, (b) PCA plot with 95% confidence ellipses resulting from the emission intensity at 550 nm of 1 μM CB7-NBD in the presence of 200 μM analytes as a function of the concentration of NaCl in artificial saliva ($\lambda_{ex} = 475$ nm) and (c) pairwise pattern of difference between emission responses of the three individual replicas for each analyte.
5.5.9. Geometry-optimized molecular structures and corresponding DFT calculations

The part was collaborated with Dr. Marco Neumaier.

The DFT calculations were performed without and with dispersion correction and generally found more compact structures in the latter case when considering the same topology. This difference is least pronounced for the pure cucurbit[n]uril ions [CB6·Na]⁺ and [CB7·H]⁺ with differences in the collision cross sections (CCSs) of below 0.5%. For the studied CB7-NBD structures and for the intermolecular complexes CB7-NBD⊃cadaverine and CB7-NBD⊃1-adamantanol, the CCSs of the dispersion corrected structures (considering the same topology) can be smaller by up to ~6%. Similar to other studies, it was also found a different DFT stability order in some cases when accounting for dispersion (e.g., for doubly and singly protonated CB7-NBD⊃cadaverine) and inclusion complexes are typically more favored compared to exclusion complexes. Studying the influence of dispersion is not the scope of this work and will possibly be addressed in a future publication. Therefore, I focus on the dispersion corrected structures, which also show better agreement with experimentally determined CCSs from the ion mobility measurements. Note that due to the flexibility and size of the molecule, it is not readily possible to sample the whole potential energy surface. Therefore, I focus more on the differences in topologies (e.g., folded versus open/unfolded structures) with respect to CCSs.

IMoS TM calculations in which the ion–quadrupole interaction was explicitly taken into account led to CCSs being consistently smaller (by ~6-8 %) than the pure LJ (plus ion-induced dipole) potential based CCSs, also showing overall a better agreement with the experimental CCSs of the gas-phase structures. Therefore, the results based on the additional inclusion of the ion–quadrupole interaction will be shown only.

5.5.9.1. [CB6·Na]⁺ and [CB7·H]⁺

For [CB6·Na]⁺ (Figure 5.37 (a)) and [CB7·H]⁺ (Figure 5.37 (b)), which were chosen as test systems, a good agreement of ~5-6% between the experimentally determined N₂ collision cross sections (TIMS\text{CCS}_{N₂}) (Figure 5.38) and the calculated CCSs (\text{calc\text{CCS}}_{N₂}) was found with \text{calc\text{CCS}}_{N₂} being somewhat larger (Table S2).
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Figure 5.37 Side views (left) and top views (right) of (a) [CB6·Na]$^+$ and (b) [CB7·H]$^+$

Figure 5.38 Mass spectra and ion mobilogram of [CB6·Na]$^+$ and [CB7·H]$^+$.

5.5.9.2. [CB7-NBD·Na]$^+$ and [CB7-NBD·H]$^+$

The gas-phase structures of [CB7-NBD·Na]$^+$ and [CB7-NBD·H]$^+$ unfolded (Figure 5.39) and folded (Figure 5.40) structures can be ruled out based on the high DFT energies and based on the large $^{calc}$CCS$_{N2}$ of ~490Å$^2$ and ~385Å$^2$ which is ~45% and ~15% larger than the corresponding experimental values (Table S2). The best agreement with $^{TIMS}$CCS$_{N2}$ was found for an inclusion type complex (Figure 5.41) which also exhibits the lowest DFT energy. Note that all structures were fully geometry optimized, and structures of [CB7-NBD·Na]$^+$ and [CB7-NBD·H]$^+$ of the same topology cannot readily be compared, i.e., they can differ in relative DFT energy and to some extent in topology and consequently CCSs as well.
Distinguishing biorelevant analytes by salt-responsive CB7-based chemosensor in biofluids

Figure 5.39 Side views (left) and top views (right) of the unfolded complexes a) [CB7-NBD·Na]^+ (3.84 eV) and b) [CB7-NBD·H]^+ (3.22 eV).

Figure 5.40 Side views (left) and top views (right) of the folded complexes (a) [CB7-NBD·Na]^+ (1.64 eV) and (b) [CB7-NBD·H]^+ (0.86 eV).
Distinguishing biorelevant analytes by salt-responsive CB7-based chemosensor in biofluids

Figure 5.41 Side views (left) and top views (right) of the inclusion complexes (a) [CB7-NBD·Na]$^+$ (0 eV) and (b) [CB7-NBD·H]$^+$ (0 eV).

Figure 5.42 Mass spectra and ion mobilogram of [CB7-NBD·H]$^+$.

5.5.9.3. [CB7-NBD·cadaverine·2H]$^{2+}$

For [CB7-NBD·cadaverine·2H]$^{2+}$, two structures were found that have almost the same $\text{calc} \text{CCS}_{N_2}$ showing very good agreement with the experiment (~3%) and that are close in energy (Figure 5.43 (a) and (b)). As the CCSs resolution of the timsTOF mass spectrometer is not sufficient to distinguish between these two structures, a mixture of both or only one of the structures might be present in the gas phase. An inclusion complex, as shown in Figure 5.43 (c), can be ruled out based on $\text{calc} \text{CCS}_{N_2}$ and $\Delta \text{E}_{\text{DFT}}$. 
Distinguishing biorelevant analytes by salt-responsive CB7-based chemosensor in biofluids

**Figure 5.43** Side views (left) and top views (right) of the a) dual inclusion (0 eV), b) exclusion (0.16 eV) and c) inclusion complex (0.67 eV) of [CB7-NBD⊃cadaverine·2H]²⁺.

5.5.9.4. [CB7-NBD⊃cadaverine·H]⁺

For [CB7-NBD⊃cadaverine·H]⁺ an exclusion type complex, as shown in Figure 5.44 (b), was found to be most stable, and its calculated CCSs of 381.0 Å² show a reasonably good agreement with the experimental value (348.9 Å²). A dual inclusion complex (Figure 5.44 (a)) exhibits higher DFT energy (0.50 eV) but shows a similarly good agreement with the experimental CCSs. See Figure 5.45 for ion mobility spectra and experimental CCS. An inclusion complex where the NBD moiety is replaced by [cadaverine·H]⁺ (Figure 5.44 (c)) can clearly be ruled out as a gas-phase structure as it shows a much larger calculated CCS (413.1 Å²) and a considerably higher relative DFT energy of 1.22 eV.
Distinguishing biorelevant analytes by salt-responsive CB7-based chemosensor in biofluids

Figure 5.44 Side views (left) and top views (right) of the a) dual inclusion (0.50 eV), b) exclusion (0 eV) and c) inclusion complex (1.22 eV) of [CB7-NBD⊃cadaverine·H]+.

Figure 5.45 Mass spectra and ion mobilogram of [CB7-NBD⊃cadaverine·H]+.
5.5.9.5. [CB7-NBD·Na⊃AdOH]⁺

For [CB7-NBD·Na⊃AdOH]⁺, the best possible agreement in terms of CCSs was found for an exclusion complex, as shown in Figure 5.46 (a). This structure also exhibits higher stability compared to the inclusion complex (Figure 5.46 (b)).

![Figure 5.46 Side views (left) and top views (right) of the a) exclusion (0 eV) and b) inclusion complex (0.71 eV) of [CB7-NBD·Na⊃AdOH]⁺.](image)

**Figure 5.46** Side views (left) and top views (right) of the a) exclusion (0 eV) and b) inclusion complex (0.71 eV) of [CB7-NBD·Na⊃AdOH]⁺.

![Figure 5.47 Mass spectra and ion mobilogram of [CB7-NBD·Na⊃AdOH]⁺.](image)

**Figure 5.47** Mass spectra and ion mobilogram of [CB7-NBD·Na⊃AdOH]⁺.
5.5.9.6. \([\text{CB7-NBD-Na} \supseteq \text{AdNH}_2 \cdot \text{H}]^{2+}\)

For \([\text{CB7-NBD-Na} \supseteq \text{amandatine} \cdot \text{H}]^{2+}\), best agreement with the experimental collision cross-section (\(\text{TIMS}^{\text{CCS}}_{\text{N}_2} = 386.1 \ \text{Å}^2\)) is found within \(\sim 4\%\) for exclusion complexes, whereas the structure shown in Figure 5.48 (a) also exhibits the lowest DFT energy. The inclusion type complex (Figure 5.48 (c)) has a larger \(\text{calc}^{\text{CCS}}_{\text{N}_2}\) of 419.4 \(\text{Å}^2\) which is \(\sim 9\%\) larger compared to the experimental value and also exhibits higher DFT energy (0.63 eV).

\[
\begin{array}{c}
\Delta E_{\text{DFT}} = 0 \text{ eV} \\
\text{calc}^{\text{CCS}}_{\text{N}_2} = 403.0 \text{ Å}^2 \\
\end{array}
\]

\[
\begin{array}{c}
\Delta E_{\text{DFT}} = 0.47 \text{ eV} \\
\text{calc}^{\text{CCS}}_{\text{N}_2} = 399.5 \text{ Å}^2 \\
\end{array}
\]

\[
\begin{array}{c}
\Delta E_{\text{DFT}} = 0.63 \text{ eV} \\
\text{calc}^{\text{CCS}}_{\text{N}_2} = 419.4 \text{ Å}^2 \\
\end{array}
\]

**Figure 5.48** Side views (left) and top views (right) of the two exclusion complexes a) (0 eV) and b) (0.47 eV) and c) inclusion complex (0.63 eV) of \([\text{CB7-NBD-Na} \supseteq \text{AdNH}_2 \cdot \text{H}]^{2+}\).
Figure 5.49 Mass spectra and ion mobilogram of [CB7-NBD·Na⊕AdNH₂·H]²⁺.
Distinguishing biorelevant analytes by salt-responsive CB7-based chemosensor in biofluids
6. Conclusion and outlook

Cucurbit[n]urils represent one of the most attractive macrocyclic receptors that can be used for molecular sensing and imaging applications due to its specific binding affinity to a broad range of guests and excellent physical and chemical properties. The well-established IDA-based chemosensor consisting of CBn and reporter dyes is a powerful tool for fluorescence-based sensing of biologically relevant small molecules. However, a major drawback of CBn-based chemosensors is the inevitable dissociation of their host-guest complexes in the presence of cations, e.g., in buffers or biofluids. My thesis aims to address this problem by designing and characterizing novel CBn-based chemosensors that have improved salt-resistance and are thus able to overcome current limiting obstacles that exist for the use of this class of macrocycles as future chemosensors.

In chapter 4, unimolecular covalent CB7-dye conjugates with both lengths of the tethered linker (TEG and HEG) were synthesized and characterized. Furthermore, their potential and advantages for sensing application in saline media and biofluids are presented. Berberine was chosen as the reporter dye because of the strong emission enhancement observed upon CB7-binding and the possibility for its chemical modification, which is required for covalent etherification to yield CB7-dye conjugates. The macrocyclic CB7-berberine conjugate was successfully prepared via copper-catalyzed cycloaddition between the CB7-alkyne and the azide-terminated berberine-linker, which allowed covalently tethering of the dye at the periphery of CB7. The conformation of the resulting CB7-BC conjugates was characterized as a unimolecular self-capsulated structure by ion mobility experiments in the gas phase and by photophysical measurements in the solution phase. Regarding their stability in high-salt media, both conjugates showed good resistance to cation- and dilution-induced disassembly, which is typically observed in previously reported CB7⊃BC or CB7⊃MDAP pairs. Importantly, the CB7-dye conjugate can be used to detect high-affinity analytes, e.g., nortestosterone (nandrolone) and adamantyl derivatives, as confirmed by NMR and photophysical technology.

Furthermore, the binding kinetics of CB7-BC to the two structurally similar analytes (amantadine and adamantanol) was investigated based on their emission changes as a function of time and compared with the dysfunction of the non-covalent CB7 dye. In biologically relevant buffered saline and biofluid (human real urine and saliva), the detection and quantification of the Parkinson’s drug amantadine were achieved at the medically relevant concentration range and a unique chemosensor addition method was utilized herein to circumvent the matrix influences between different samples. In the end, an estimated S$_N$2-type guest exchange mechanism was suggested to interpret the prominent properties of unimolecular conjugated CB7-dye in contrast with the traditional non-covalent pairs.

Inspired by the findings on how unimolecular CBn-based chemosensors perform in a high salinity medium, it was realized that salt plays a delicate role in this type of supramolecular system and that salt effects could be explored to tune the chemosensor performance to the point where even differential sensing is possible. In chapter 5, in order to extend the sensing capability for weak binding affinity analytes and make salt involved as a tunable factor for mediating the supramolecular binding between
host and guest, a novel unimolecular CB7 conjugate was equipped with a neutral and highly emissive dye (nitrobenzoxadiazole, NBD) with a modest affinity to CB7 than berberine. Indeed, CB7-NBD can be used in 1X PBS to detect analytes with weak affinity, which was not possible with CB7-BC and non-covalent reporter pairs in such an environment. Meanwhile, the conformation of obtained CB7-NBD complexes with different analytes was investigated by NMR, ion mobility experiments, and DFT calculations. In particular, emission-based titration experiments were performed to evaluate the binding affinity of CB7-NBD with dozens of biorelevant analytes, and the emission recovery observed upon subsequent addition of salts was characterized. According to the different responses upon salt addition into different CB7-NBD analyte complexes, a ‘salt-addition assay’ strategy for differential analyte sensing was developed. The PCA plot and the other data-driven approach to convert the data differences into an intuitive pairwise colorimetric pattern were utilized to evaluate the distinction of analytes by this assay. Meanwhile, the type of salt was also a new parameter to provide more dimensional information for analytes differential sensing. This salt-adaptive chemosensor system was also exploited for analytes sensing capability in real biofluids in analogy with the previous chapter.

Overall, the unimolecular CBn-based conjugate for sensing application in this work opens a range of multiple possibilities for the design and preparation of more attractive chemosensors with specific and smart features combined with their current existing strengths in the future. Concerning the choice of receptors, other CBn homologues (mainly for CB6 and CB8) and other artificial could be utilized to satisfy the requirements for different classes of analytes sensing. Especially for CB8, the larger cavity can provide more potential binding conformation between analytes and conjugate, e.g., in the ABA model, to create more novel sensing mechanisms and target a wider scope of specific analytes with high but flexible affinity. As an outlook, one strategy for expanding the potential applications of chemosensors, which is equipping the conjugate with a ‘controllable button,’ can be envisioned. For example, a light-responsive linker connecting the host and the indicator could be installed in the chemosensor design in the future to release the tethered indicator when exposed to light, resulting in chemosensors with spatiotemporally controlled on/off function. This concept also applies to other stimulus-dependent groups typically used in dynamic covalent chemistry, such as -S-S bonds (redox responsive) or Schiff bonds (pH-responsive). For indicators, the readout signal is not only limited to the fluorescent emission but also other detection forms, such as absorbance, chemical shifts in NMR (\(^1\)H, \(^{13}\)C, \(^{15}\)N, or \(^{19}\)F), circular dichroism (CD), and so on. For instance, using the fluoroalkane or fluoro aromatic compound as the indicator and monitoring the process of guest displacement by \(^{19}\)F NMR or MRI technique for imaging application is one of the idea frameworks. For the prospect of applications, the objects for sensing or analyzing may not be limited to the small molecules, and it is likely used as the chemosensor for macromolecular detection, e.g., peptides, antibodies, and proteins. The abundant structural features on these macromolecules can provide more multi-dimensional interactions and responses to conjugated chemosensors and potentially harvest the identical fingerprint information for each analyte. Meanwhile, the conjugated chemosensors could also be applied in cellular imaging or real-time monitoring for drug
metabolism taking advantage of its resistance to salt and the lack of background from the free dye in contrast to the non-covalent reporter pairs.
Conclusion and outlook
7. List of abbreviations

Å Angstrom
ABA Associative binding assays
ACN Acetonitrile
aq. Aqueous
BC Berberine (chloride)
°C Degree Celsius
CB14 Cucurbit[14]urils
CB5 Cucurbit[5]urils
CB6 Cucurbit[6]urils
CB7 Cucurbit[7]urils
CB8 Cucurbit[8]urils
CBn Cucurbit[n]urils
CCSs Collision Cross Sections
CD Circular dichroism
CuSO₄ Copper(II) sulfate
D₂O Deuterium oxide
DASAP trans-4-[4-(Dimethylamino)styryl]-1-adamantylpyridinium
DCM Dichloromethane
de novo New
DFT Density functional calculation
DMF Dimethylformamide
DMSO Dimethyl sulfoxide
e.g., exemplari gratia, for example
eq. Equation
Et₂O Diethyl ether
etc. et cetera, and so forth
eV Electron volts
FRET Fluorescence resonance energy transfer
GDA Guests displacement assays
h Hour
H₂O₂ Hydrogen peroxide
HEG Hexaethylene glycol
HPLC High-Performance Liquid Chromatography
i.e., id est, that is to say
IDA Indicator displacement assay
ITC Isothermal titration calorimetry
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>K₂S₂O₈</td>
<td>Potassium persulfate</td>
</tr>
<tr>
<td>Kₐ</td>
<td>Association constant</td>
</tr>
<tr>
<td>kᵢn</td>
<td>Complexation rates</td>
</tr>
<tr>
<td>kₒₗₐₜ</td>
<td>Decomplexation rates</td>
</tr>
<tr>
<td>L</td>
<td>Liter</td>
</tr>
<tr>
<td>LOD</td>
<td>Limit of detection</td>
</tr>
<tr>
<td>M</td>
<td>Mole</td>
</tr>
<tr>
<td>MDAP</td>
<td>2,7-Dimethyldiazapyrenium dication</td>
</tr>
<tr>
<td>MeOH</td>
<td>Methanol</td>
</tr>
<tr>
<td>mg</td>
<td>Milligram</td>
</tr>
<tr>
<td>mL</td>
<td>Millilitre</td>
</tr>
<tr>
<td>mM</td>
<td>Millimolar</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrum</td>
</tr>
<tr>
<td>ms</td>
<td>Milliseconds</td>
</tr>
<tr>
<td>MTS</td>
<td>3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)(-2)-(4-sulfophenyl)-2H-tetrazolium</td>
</tr>
<tr>
<td>NBD</td>
<td>Nitrobenzoxadiazole</td>
</tr>
<tr>
<td>NBS</td>
<td>N-Bromosuccinimide</td>
</tr>
<tr>
<td>nm</td>
<td>Nanometer</td>
</tr>
<tr>
<td>nM</td>
<td>Nanomolar</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>PB</td>
<td>Phosphate buffer</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PC</td>
<td>Packing coefficient</td>
</tr>
<tr>
<td>PCA</td>
<td>Principal component analysts</td>
</tr>
<tr>
<td>PDT</td>
<td>Photodynamic therapy</td>
</tr>
<tr>
<td>PLA</td>
<td>Polylactic Acid</td>
</tr>
<tr>
<td>PLGA</td>
<td>Poly(lactic-co-glycolic acid)</td>
</tr>
<tr>
<td>PMMA</td>
<td>Poly(methyl methacrylate)</td>
</tr>
<tr>
<td>SDA-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SN₂</td>
<td>Substitution nucleophilic (bi-molecular)</td>
</tr>
<tr>
<td>SSM</td>
<td>Solid-state metathesis</td>
</tr>
<tr>
<td>TEG</td>
<td>Tetraethylene glycol</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic acid</td>
</tr>
<tr>
<td>TIMS</td>
<td>Trapped Ion Mobility Spectrometry</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>UV-Vis</td>
<td>Ultraviolet-visible</td>
</tr>
<tr>
<td>β-CD</td>
<td>β-cyclodextrin</td>
</tr>
<tr>
<td>λ&lt;sub&gt;ex&lt;/sub&gt;</td>
<td>Excitation wavelength</td>
</tr>
<tr>
<td>μM</td>
<td>Micromolar</td>
</tr>
<tr>
<td>σ</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>Abs</td>
<td>Absorption of a photon</td>
</tr>
<tr>
<td>conc.</td>
<td>Concentration</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>ESI</td>
<td>Electrospray ionization</td>
</tr>
<tr>
<td>g</td>
<td>Gram</td>
</tr>
<tr>
<td>I&lt;sub&gt;abs&lt;/sub&gt;</td>
<td>Intensity of transmitted light</td>
</tr>
<tr>
<td>I&lt;sub&gt;rel&lt;/sub&gt;</td>
<td>Normalized intensity</td>
</tr>
<tr>
<td>μmol</td>
<td>Micromole</td>
</tr>
</tbody>
</table>
8. Appendices

8.1. Curriculum Vitae

Name Changming Hu
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Education
Since 10/2018 PhD candidate, Chemistry, Institute of Nanotechnology (INT) of the Karlsruhe Institute of Technology (KIT)
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Supervised by Prof. Dr. Hong Chen and Prof. Dr. Qian Yu
Research Direction: Fabrication of biofunctionalized surface based on host-guest interaction

09/2011-06/2015 Bachelor, Material science and Engineering, College of Chemistry, Chemical Engineering and Materials Science of Soochow University

Scholarships and Honors
09/2018-09/2022 Funded by Chinese Scholarship Council (CSC) for PhD student (48 months)
2018 1st class scholarship
2017 3rd class scholarship
2016 1st class scholarship
2014 2nd class scholarship

Skills
- Professional skills: Organic synthesis and characterization, Polymer synthesis and characterization, Spectroscopic instrumental analysis, biomolecules relevant detection, cell/bacterial culture and operation, interface analysis and characterization, etc.
- Software: Microsoft office, Origin Lab, ChemOffice, Endnote, Citavi, Illustrator, Photoshop, MestReNova, etc.
- Instrument Operation: NMR, ESI-MS, LC-MS, HPLC, UV-vis spectrometer, Fluorescent spectrometer, Circular dichroism, Dynamic light scattering, SEC (GPC), FT-IR, microscope, TEM, SEM, etc.
8.2. Publications


*This publication was featured as front cover in Chemical Science.*


*This publication was featured with the front cover in Chemical Science.*


*This publication was featured with the inside front cover in the Journal of Materials Chemistry B.*


8.3. Acknowledgments

In this section, I would like to express my sincere appreciation to all of the people who accompanied and help with me during this long but brief period of my PhD life.

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I would like to appreciate my former and present colleagues during this four-year life in the Biedermann group. I am thankful to Dr. Stephan Sinn for teaching me about photophysical related knowledge and training the fundamental operations for all instruments when I first came, and I am thankful to Dr. Pierre Picchetti for sharing me with lots of experiences and suggestions on organic synthesis work and chatting with me about many interesting scientific ideas and career perspective in my final year. I thank Dr. Laura Grimm for plenty of detailed instrument training (HPLC, titrator, freeze dryer, etc.) and help with data analysis (constant binding fitting). I thank Philipp Avon and Alicja Siennicka for introducing all of the regulations in labs and institute and offering me assistance with the synthesis of cucurbit[n]uril and other compounds when I had problems. I thank Amrutha Prabodh, Rui Kang and Joana Krämer for their help and skills in photophysical related works in analysis labs, i.e., absorption, fluorescent, circular dichroism spectrometry and platereader use. I thank Dr. Pronay Biswas, Dr. Yanan Luo, Yichuan Wang, Chunting Zhong, Manoj Nilima, Wenjing Wang, and Patrick Gruhs for sharing a pleasant time in the lab.

I would also like to gratitude the cooperated colleagues in charge of supporting the additional techniques, which is out of my major but greatly improved the scientific level of my projects. I thank Prof. Dr. Manfred Kappes, Dr. Macro Neumaier, Dr. Ananya Baks, and Papri Chakraborty for your important contribution to mass spectrometry, ion mobility experiments, and DFT calculations. Your professional works provided strong support for your research and publication. I thank Thomas Jochmann for data analysis by the machine learning approach in my analysis differential sensing section.

I would like to thank the China Scholarship Council (CSC) for funding my whole PhD period and offering this great opportunity to study in Germany.

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grandfather for your encouragement and support when I was obstructed by troubles in deep distress. Thanks to my friends for your sharing their experiences and life to enrich my view and keep in contact with the world outside of academics. Finally and importantly, I would like to thank my dearest girlfriend for your everlasting love and endless support in the past nearly nine years. From the inseparable life during our college and master periods to our separated life by time and space in these four years, you never hesitate to company with me and contribute the efforts for our future. Like the mirror and soul of life, I appreciate the patient listening and the most useful advice from you.
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