Synthesis of Mono-Functionalized Cucurbit[n]urils and Exploration of their Applications

by

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Doctor of Philosophy in Chemistry

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To my dear Grandma
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Abstract

The present doctoral thesis describes the synthetic procedure of clickable mono-functionalized cucurbit[n]urils ($n = 6, 7$) and mainly on the exploration of new applications based on the mono-functionalized CB$n$. In principle, with the functionalized clickable group, various functionalized CB$n$ derivatives can be achieved. One explored application is based on the functionalization of CB7 on the surface of nano-/macro-particles, thus can be applied to quantify the surface coverage densities of particles. The other one is based on a chromophore attached to CB7 which makes the host molecule fluorescent and enables it to form a host-guest FRET pair with a corresponding fluorescent guest, which can be applied to DNA sensing. Besides these, the binding constants between CB$n$ and inorganic cations were systematically studied.

The first part of the thesis focuses on the synthesis and characterizations of mono-functionalized CB6 and CB7, including mono-hydroxylated CB6 or CB7, propargyl attached CB6 or CB7, and fluorophore attached CB6 or CB7, and related compounds.

The second part of the thesis reports a host-guest FRET pair based on the macrocyclic host CB7 labelled with carboxyfluorescein as acceptor and the nucleic stain DAPI as donor and guest. This supramolecular FRET pair is to be used for quantitative sensing of DNA with an excellent linear dependence of the ratiometric fluorescence intensities. Such approach can be applied to quantify DNA accurately and potentially be used in real-time PCR.

The third part of the thesis demonstrates a strategic supramolecular application to precisely control the coverage densities on the surface of nano-/macro-particles. The key is to functionalize CB7 on the surface of particles. After that, incubation of CB7-functionalized particles with two high-affinity guests, resulted in a simple linear relationship between surface coverage densities of one fluorescent guest and the mole fraction of this guest in the incubation mixture. This suggests a highly modular supramolecular strategy for the stable immobilization of application-relevant molecules on particle surfaces and a precise control of their surface coverage densities.

In the last part, I summarize the main projects during my PhD and give the outlook about exploring more applications based on mono-functionalized CB7.
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I would also like to thank my former and present colleagues I worked with during my four-year study in the Nau group for providing a very harmonious and knowledgeable environment. In particular, I am thankful to Ms. Yan-cen Liu for helpful discussions about fundamental knowledge of supramolecular chemistry and all the fun during our many trips in Germany. I thank Dr. Khaleel I. Assaf for answering my questions all the time with patience and a smile, for his guidance on NMR analysis and his encouragement from time to time. I thank Dr. Chusen Huang for his guidance with cell culture experiments and fruitful suggestions in one of my research projects. I thank Dr. Maik Jacob for his help to calculate the Förster distance. I thank Dr. Andrea Barba-Bon for the help in running the first column in my life and the beautiful memory of our Berlin trip. I thank Dr. Suhang He, Mr. Mohammad Ata Alnajjar, Mr. Nilam Mohamed, and Ms. Yao Chen for sharing with me a pleasant time in the lab. Also, I thank Dipl.-Chem. Thomas Schwarzlose for his encouragement and help. I thank Dr. Frank Biedermann, Dr. Haibo Zhang, and Dr. Xiaojuan Wang for the shared projects.

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List of Publications


2. Huang, Haihong, Baosheng Ge, Shuai Zhang, Jiqiang Li, Chenghao Sun, Tongtao Yue, and Fang Huang. “Using Fluorescence Quenching Titration to Determine the Orientation of a Model Transmembrane Protein in Mimic Membranes.” *Materials*, 2019, 12, 349.


**Manuscript in Preparation**

Participation in Scientific Conferences

1. 02/2019 SupraChem 2019, Würzburg, Germany. Poster entitled: “Ratiometric DNA sensing with a host–guest FRET pair”.

2. 06/2016 5th International Conference on Cucurbiturils (ICCB), Brno, Czech Republic. Poster entitled “Cucurbit[7]uril-Functionalized Polymer Microparticles”.
Chapter 1. Introduction
Chapter 1 Introduction

1.1 Supramolecular Chemistry

Supramolecular chemistry, which is one of the today’s most interested research fields in chemistry, was introduced by Jean-Marie Lehn in 1978 for the definition, consolidation and generalization of the domain of crown ether chemistry, the chemistry of molecular recognition, and host-guest chemistry. In 1967, Charles Pederson had investigated the selective binding of crown ethers to specific metals and cation transfer in biphasic solvent.\(^1\) This accidental discovery paved the way for Donald J. Cram and Jean-Marie Lehn to express their creativity in unique ways and wholly develop the field of supramolecular chemistry.\(^2-4\) They were jointly awarded the 1987 Nobel Prize in chemistry for a recognition of crown ether, host-guest and molecular recognition chemistries.

Supramolecular chemistry has been described as “chemistry beyond the molecule”. Over the past 50 years, this field has grown into a major branch of chemistry and has promoted innumerable developments: from fundamental knowledge to practical applications, from noncovalent interactions to drug delivery, and from polymer materials to solid-state engineering. As a result, it has led to the appearance and establishment of supramolecular science and technology, as a wide range of multidisciplinary and interdisciplinary field that provides highly fertile soil for scientists’ creativity in all disciplines.

The importance of supramolecular chemistry was again established by the 2016 Nobel Prize for Chemistry, which was awarded to Jean-Pierre Sauvage, Sir J. Fraser Stoddart, and Bernard L. Feringa in recognition of their work in molecular machines, in which supramolecular chemistry was basic in showing the noncovalent bond into visibility. Particularly, they used supramolecular chemistry as a kit to create mechanical power, promoting molecules that can operate as machines, including molecular knots,\(^5, 6\) molecular elevator,\(^7, 8\) molecular motor\(^9, 10\) and nanocar.\(^11\)

Until now, numerous families of supramolecular host molecules (natural and synthetic) have been reported and investigated. Cucurbit[\(n\)]urils (CB\(n\)) as one of most young macrocycles is very much related to my research during my 4-year PhD study. Therefore, in Chapter 1 I will focus on the CB\(n\) properties, functionalizations and applications, as well as the mechanisms of Cu(I)–catalyzed azide-alkyne cycloaddition related to CB\(n\) functionalizations and the principle of förster resonance energy transfer associated with CB\(n\) applications.
1.2 Supramolecular Chemistry of Cucurbit[n]urils

Cucurbit[n]urils (CBn, n = 5–8, 10, 13–15) are cyclic oligomers composed of different glycoluril units connected by methylene groups (Figure 1.1), which represent as a remarkable water soluble molecular hosts. CB6 was the first one to be synthesized in 1905 by Behrend, but its structure was not shown until 1981. Shortly after 2000 CBn family was expanded to have more species, such as CB5, CB7, CB8 and CB10, by Kim, Day and Isaacs. Recently, CB13, CB14 and CB15 which show twisted crystal structures (Figure 1.2) have been discovered by Tao. In the recent two decades, although applications of large CBn are very limited, CBn (n = 5–8) have been well explored in fundamental and applied science, including formation of supramolecular hydrogels, supramolecular tandem enzyme assays, catalysis, biological molecules recognition, and materials science.

Figure 1.1 Chemical structures of CB5, CB6, CB7 and CB8.

Figure 1.2 X-ray crystal structure of twisted CB14, hydrogen atoms are hidden for clarity.
1.2.1 Basic Properties of Cucurbit[n]urils

CBn are barrel-shaped macrocycles with highly symmetric structure, negatively charged carbonyl portal and hydrophobic cavity. Like the cyclodextrins, the CBn homologues have the same depth (9.1 Å), but their portal diameter, inner cavity diameter and cavity volume vary systematically with the number of united glycoluril units (Figure 1.3 and 1.4). The CBn structures are rather rigid, so the suitable guests for CBn to form host-guest complex are size selective. As shown in Table 1.1, the thermal stability of CBn is high enough for CBn to keep complete structure under 370 °C. The solubility of CBn varies on sizes, CB5 and CB7 show modest solubility in water, but CB6 and CB8 are slightly soluble in water. Therefore, the poor solubility of CB6 and CB8 is one of the limitations of the CBn family to be explored for some applications in aqueous solution.

![Model of the strict CBn macrocycles.](image)

**Figure 1.3** Model of the strict CBn macrocycles.

<table>
<thead>
<tr>
<th>CBn</th>
<th>(p) (Å)</th>
<th>(d) (Å)</th>
<th>(h) (Å)</th>
<th>(V) (Å³)</th>
<th>(s) (mM)</th>
<th>Stability (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CB5</td>
<td>2.4</td>
<td>4.4</td>
<td>9.1</td>
<td>68</td>
<td>20-30</td>
<td>&gt; 420</td>
</tr>
<tr>
<td>CB6</td>
<td>3.9</td>
<td>5.8</td>
<td>9.1</td>
<td>142</td>
<td>0.018(^{42})</td>
<td>425(^{43})</td>
</tr>
<tr>
<td>CB7</td>
<td>5.4</td>
<td>7.3</td>
<td>9.1</td>
<td>242</td>
<td>20-30</td>
<td>370</td>
</tr>
<tr>
<td>CB8</td>
<td>6.9</td>
<td>8.8</td>
<td>9.1</td>
<td>367</td>
<td>&lt; 0.01</td>
<td>&gt; 420</td>
</tr>
</tbody>
</table>

\(p\): portal diameter; \(d\): inner cavity diameter; \(h\): the height; \(V\): cavity volume; \(s\): water solubility; Values are taken from ref. 15, 42, 43.

Not only the size of portal decides what kind of guest fits with CBn, but also the packing coefficient (PC). PC representing the ratio of the guest size and the host cavity volume, as one of testing standards to estimate the fit goodness of host-guest complex, was introduced by Rebek and Mecozzi in 1998.\(^{44}\) A value of ca. 0.55 was reported to get the best binding between host and guest molecules, while larger or smaller PC value accompanied by lower affinities. PC value has been proven useful for a couple of macrocyclic hosts, as well as CBn, which was reported by Nau group to be applied for predicting the stability of CBn complex successfully.\(^{45}\)
The CBₙ host-guest chemistry is obviously and intuitively related with dimensions of CBₙ structures, also with other driving forces, such as electrostatic effect and hydrophobic effect. Electrostatic effect plays an important role in biochemical molecular recognitions, as well as in supramolecular chemistry. Figure 1.5 shows the calculated electrostatic potential of CBₙ (n = 5-8), which spotlights the preference of the positive charge associated and the reluctance of the anions with the portals of CBₙ.

Indeed, the electrostatic potential has a significant influence for the recognition behavior of CBₙ. The first reports of CBₙ complex was formed with metal cations being as a lid bound to the portals of CBₙ. The common cations (inclusive of alkali and alkaline earth metal cations, transition metal cations, lanthanides and actinides, as well as ammonium ions) have been proven to bind with CBₙ with varying affinities and increase the CBₙ solubility significantly.
Figure 1.5 Calculated electrostatic potential (EP) at the B3LYP/6-31G* level of theory for (a) CB5, (b) CB6, (c) CB7, and (d) CB8 in the $\sigma_h$ plane (left) and in the $\sigma_v$ plane (right). Reprinted by permission from ref. 61.

Figure 1.6 Schematic illustration of the release of high-energy water molecules from the CB7 cavity upon binding of a hydrophobic guest. Reprinted by permission from ref. 46.

The advantage of applying CB$n$ into molecular recognition is that the formation of CB$n$ based complex can be conducted in aqueous solution with high binding affinity. Generally, compared with organic solvents the presence of water decreases the host-guest binding affinity
as water molecules compete strongly to form hydrogen bond. However, this is not the case for CBn, which are able to solve this problem by encapsulating high-energy water (Figure 1.6) in the cavity.\textsuperscript{62} And the release of high-energy water accounts for a large proportion of the overall hydrophobic effect for the complex formation. The number of high-energy water molecules accommodated in the cavity is depending on CBn cavity volume. The molecular dynamics simulations conducted by Biedermann shows that high host-guest binding affinity can be gained by the removal of all water molecules. CB7 was found to have the highest energy gain compared with other homologues, as it encapsulates more high energy water molecules than CB5 and CB6 (more than twice), and has more suitable cavity size to host higher energy water molecules than CB8.\textsuperscript{63}

### 1.2.2 Specific Host–Guest Binding of Cucurbit[n]urils

CB5 is considered to be the smallest macrocycle in CBn family until now. In respect to its small cavity volume (68 Å\textsuperscript{3}), CB5 has been limited to use in the formation of numerous guest molecules compared to the larger family members. Even with such small cavity and portal sizes, CB5 and its derivative have found to be available for the formation of host-guest complex with alkali and alkaline earth cations\textsuperscript{64} and ammonium cations\textsuperscript{64}, as well as divalent transition metal cations.\textsuperscript{55} Instead of these small cations, CB5 and derivatives are reported to encapsulate small gas molecules, such as O\textsubscript{2}, N\textsubscript{2}, Ar, CO, N\textsubscript{2}O, CO\textsubscript{2}, He, H\textsubscript{2}, Ne, Kr, Ar, Xe, Rn and CH\textsubscript{4}.\textsuperscript{65,66} In particular the very recent study by the Nau group on CB5 binding affinities with noble gases by replacement of ethane or methane as NMR probes in water is attracting attention.\textsuperscript{66} In this study, the contributions to host–guest binding was identified and a conclusion was drawn that the binding process is driven by differential cavitation energies rather than dispersion interactions. Their discovery show that the cavitation energy drives the noble gas into CB5 cavity, which supplies have an impact on the improvement of gas storage materials and the understanding of biological receptors.

CB6 is the most abundant homologue from the synthesis of CBn, with a moderate volume of 142 Å\textsuperscript{3}, which is well known to complex with aliphatic amines. Alkylammonium and alkylidiammonium ions were firstly studied to bind with CB6 by Mock in aqueous formic acid, with the determination of their binding constants.\textsuperscript{67} It can be well explained by the strong interactions between the negative carbonyl rim and the positively charged protonated amines. With further investigations Mock and coworkers found that aliphatic amines bind with CB6 has structure selectivity.\textsuperscript{68,69} Consequently, CB6 shows a chain length dependent selectivity with
strongest binding affinities towards pentano- and hexano-bridged diammonium ions. Moreover, the binding affinity of CB6 with imidazolium-based ions have also been studied broadly, and applied to make monofunctionalized-CB6 as well as to separate the CBn homologues. In addition, the small cavity of CB6 limits to include fluorescent moiety into the cavity, while CB6 is successfully used for fluorescence sensor designs with alkyldiammonium-dye as smartly synthesized guest to explore applications for indicator displacement assays.

Compared with CB6, CB7 with the advantages of relative large cavity volume and better water solubility, has reported to be the most popular host in CBn family. Compared with other host, CB7 can recognize and host with a wider scope of guests with high binding affinities. On account of the special properties of CB7, a couple of fluorescent dyes such as acridine orange, berberine chloride and DNA stain dye DAPI, are shown to form stable complex with CB7 with moderate binding constants of ca. 10⁶, which makes CB7 favoured in many indicator displacement strategies. What is more, CB7 is reported to bind with diamantane diammonium with a binding constant up to 10¹⁷ M⁻¹, which goes over the biotin-(strept)avidin pair as the strongest non-covalent interaction found in nature. More three dimensional compounds like other adamantane derivatives and ferrocene also form very stable complex with CB7 (10¹² - 10¹⁵ M⁻¹). The ultra-high binding of CB7-guest pair implicates that this system can be used in some applications where biotin-(strept)avidin pair plays a role, which makes the transition from fundamental research into practical applications.

CB8 has a cavity volume of 367 Å³ which is 1.5 times larger than that of CB7, while CB8 has the similar binding properties with smaller CBn homologues. CB8 also shows relatively strong binding with adamantane and ferrocene derivatives. With a large cavity volume, CB8 can also bind with large guests, for example cyclen and cyclam, to form “a macrocycle inside macrocycle” complex. As well as fullerene which can bind with CB8 portals, with each portal sitting one fullerene molecule. Very interestingly, alkylammonium ions armed with long aliphatic chains are reported to form U-shape within CB8 cavity. Moreover, nitroxide derivatives have also been proven to form complex with CB8. One of the unique properties of CB8 is the ability to encapsulate two guests, and the two guests can be the same or two different molecules. This unique trait makes CB8 applicable in many fields such as sensing, catalysis, and supramolecular polymeric materials.
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1.3 Functionalization of CBn and Their Applications

Acting as cyclodextrin macrocycles, the functionalization expanded their applications in numerous varying areas, the development and prosperity of CBs family also demand functionalization. The initial motivation to modify CBs may be ascribed to the poor solubility of CB6 and CB8 in common solvents, which limited this host family to explore more practical applications. The first synthesized functionalized CBs was $\text{Me}_10\text{CB}_5$ derived from dimethylglycouril by Stoddart and co-workers in 1992.\(^{103}\) Since then a wide range of fully or partially alkyl-modified CBs derivatives are reported, and the derivatives are coming from various sources, including dimethylglycoluril,\(^{104, 105}\) cyclohexanoglycoluril,\(^{106, 107}\) diphenylglycoluril,\(^{108}\) and cyclopentanoglycoluril.\(^{109, 110}\) In 2011, Isaacs and coworkers reported the $p$-xylylenediammonium ion as a template to synthesize methylene-bridged glycoluril hexamer.\(^{111}\) This hexamer is available to further react with substituted phthalaldehydes to achieve monofunctionalized CB6 derivatives (Figure 1.7). In addition, they specially designed a “clickable” group onto the skeleton of CB6 derivative which provides a convenient way for introducing further functionality.\(^{112}\) Furthermore, modified CB6 with a fluorophore (naphthalene group) were applied by Isaacs to detect cancer-associated nitrosamines and basic amino acids as supramolecular sensors.\(^{113, 114}\) This principle can also be used to obtain CB7 derivatives by reacting the hexamer with modified glycoluril. With the advantage of the special properties of CB7, Isaacs and co-workers used functionalized CB7 for targeting delivery drug molecule to cancer cells,\(^{115}\) forming vesicle-type assemblies,\(^{116}\) endowing biopharmaceuticals with PEGylation,\(^{117}\) releasing drug molecules,\(^{118}\) as well as sensing and imaging the cells.\(^{119}\)
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Figure 1.7 Functionalized CB6 and CB7 derivatives from Isaacs’s group.

Generally the alkyl-modified CBs derivatives can improve the solubility of host molecules in common solvents, but they are synthesized indirectly and difficult to be functionalized further. Therefore, direct functionalization of CBs would be appealing. Kim and co-workers achieved a breakthrough in this field to obtain fully-hydroxylated CBN (n = 5-8) by direct oxidation of CBs in the presence of K$_2$S$_2$O$_8$ in 2003. Through this method CB5 and CB6 derivatives can be reacted very efficiently with yields of 42% and 45%, however, for CB7 and CB8, the yields are lower than 5%. This functionalization creates a platform for further modifications and countless potential applications, including CBs-based supramolecular polymers, fluorescent capsules, drug delivery, enrichment of proteins, and biocompatible supramolecular hydrogels. In 2012, Scherman and co-workers separated monohydroxylated CB6 through the oxidation of CB6 in the presence of bisimidazolium salts, and further functionalization with reactive alkenyl and alkynyl was also reported. In 2013, Kim and co-workers improved their previous method and separated monohydroxylated CB7 and they successfully applied monohydroxylated CB7 to form supramolecular velcro for reversible underwater adhesion. Two years later, Kim group conjugated a fluorophore Cy3 with CB7 (CB7-Cy3) from monohydroxylated CB7. The conjugation was proven to form fluorescence resonance energy transfer (FRET) pair with adamantylamine-Cy5, and this high affinity host-guest FRET pair was used for single vesicle content mixing assay. The ultra-high affinity between CB7-Cy3 and adamantane or ferrocene derivatives makes this dye conjugated CB7 applicable for protein imaging in cell culture. Very recently, CB7 functionalized with
alkenyl\textsuperscript{133} and alkynyl\textsuperscript{134} were reported by following Kim’s method, applying to form supramolecular assembly and for drug encapsulation, respectively.

![Synthesis of monohydroxylated-CBn (n = 5-8).](image)

**Figure 1.8** Synthesis of monohydroxylated-CBn (n = 5-8).

In 2015, Bardelang and Ouari described a photochemical method to functionalize CB\textsubscript{n} (n = 5-8) with single alcohol in high conversions by using H\textsubscript{2}O\textsubscript{2} and UV light (Figure 1.8).\textsuperscript{135} This method was considered to be the milestone of functionalized CB\textsubscript{n} with respect to easy operation and excellent yields. Following this procedure, Nau and Hennig synthesized one CB7 derivative with propargyl conjugated which is clickable for further reactions. They functionalized CB7 on the surface of macro- and nanoparticles to achieve precise control of surface coverage densities on polymers.\textsuperscript{41} They also attached a fluorescent dye with CB7 (CB7-CF) and a FRET pair was made between CB7-CF and DNA stain dye DAPI. The special properties of host-guest interactions motivated them to apply this system into sensing DNA and explore potential application in real time PCR.\textsuperscript{36}

### 1.4 Cu (I)-Catalyzed Azide-Alkyne Cycloaddition

The Cu (I)-catalyzed azide-alkyne [3+2] cycloaddition (CuAAC) reaction, often referred to as click chemistry, was firstly reported by Tornøe and Meldal for the synthesis of solid state peptide in 2001.\textsuperscript{136} Subsequently, the CuAAC effect was described by the groups of Meldal and Sharpless with two independent publications in 2002.\textsuperscript{137,138} Since then this reaction has received considerable attention among researchers from different disciplines in the fact that the reaction is insensitive to harsh conditions and quantitative to couple azido and ethynyl molecules through the formation of chemical stable 1,2,3-triazole. Additionally, the triazole formed is chemically inert to usual reactive conditions, including reduction, oxidation and hydrolysis.\textsuperscript{139} As a result, CuAAC reaction is proved to be one of the most straightforward ways to connect
two molecules and has been applied in numerous research areas, including biochemistry, materials science and medical science.\textsuperscript{140}

\textbf{Figure 1.9} Examples of efficient \textit{N}-donor ligands for CuAAC reactions. Donor atoms for copper bonding are in red.

In principle, a CuAAC reaction only needs three components, a terminal azide, a terminal alkyne and a Cu catalyst, and it is worth noting that any source of copper can be accepted as a precatalyst for versatile CuAAC reaction. All other reagents (such as ligands, solvents, and base) and reactions conditions (temperature, \textit{N}_2 atmosphere) are elective. Among these, ligands (Figure 1.9) which can coordinated with Cu have been proven to accelerate the reaction rate and protect the Cu catalyst from oxidation.\textsuperscript{140, 141} Polydentate \textit{N}-donor chelators are popular ligands used in CuAAC reactions, since the auxiliary nitrogen donors has strong electron donating effect which make it easier for Cu to coordinate thus to be more reactive than those with oxygen or sulfur donors.
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Since CuAAC reaction is used currently in numerous fields, to understand the intrinsic mechanism of this reaction becomes more and more urgent. Tedious work has been performed to demonstrate the mechanism of CuAAC reaction, not only experimentally\textsuperscript{142-146} but also theoretically.\textsuperscript{147-151} Sharpless and co-workers firstly proposed a mononuclear mechanism which started with ethynyl copper followed by the subsequent formation of three intermediates (Figure 1.10 a). Following experiments found that the mononuclear mechanism questionable and more than one copper ion involved in this reaction, which was further proved by density functional theory (DFT) investigations. One remarkable experiment was conducted by Fokin and co-workers applying isotopic labelling in CuAAC reaction, through this method bis-copper-intermediates (binuclear) were observed by electrospray ionization mass spectrometric technique.\textsuperscript{145} Plus the outstanding work from other groups, the dinuclear mechanism of CuAAC reaction is increasingly accepted.\textsuperscript{143, 146, 152} In the dinuclear mechanism, the second copper was involved to form the metallacycle structure which could alleviate the ring strain and lower the activation barrier (Figure 1.10 b).

1.5 Förster Resonance Energy Transfer (FRET)

Förster resonance energy transfer, also known as fluorescence resonance energy transfer or resonance energy transfer, is a mechanism proposed by Theodor Förster in 1948 to describe the energy transfer between two chromophores (referred to as a donor and an acceptor).\textsuperscript{153} FRET is an electrodynamic happening that non-radiative energy can be transferred through long range dipole-dipole interactions between the two chromophores in the absence of photon, therefore this energy transfer is distance dependent. This process occurs between a donor chromophore
in excited state and an acceptor in ground state. The prerequisite of FRET to occur is the fluorescence spectrum of donor molecules overlaps with the absorbance spectrum of acceptor molecules (Figure 1.11).

![Spectral overlap between the absorbance spectrum of CB6-carboxyfluorescein (CB6-CF, donor) and the fluorescence spectrum of rhodamine-spermine (R-S, acceptor).](image)

**Figure 1.11** Spectral overlap between the absorbance spectrum of CB6-carboxyfluorescein (CB6-CF, donor) and the fluorescence spectrum of rhodamine-spermine (R-S, acceptor).

The theory of FRET is relatively complicated, while it is well explained by taking into account one donor and one acceptor separated by a distance.15 With this assumption, the rate of FRET is given by equation 1.1. $Q_D$ represents the quantum yield of the donor in the absence of acceptor. The term $\kappa^2$ represents the relative orientation of the donor and acceptor transition dipoles, which is usually assumed to be $2/3$. $N$ represents Avogadro’s number. $\tau_D$ is the donor lifetime, $n$ is the refractive index of the solution (typically assumed to be 1.4 for biomolecules in aqueous solution), and $r$ is the distance between donor and acceptor. In equation 1.1 the transfer rate $k_T(r)$ is described as a function of $r$ to highlight its distance dependence.

$$k_T(r) = \frac{Q_D \kappa^2}{\tau_D r^6} \left( \frac{9000 \ln 10}{128 \pi^5 N n^4} \right) \int_0^\infty F_D(\lambda) E_A(\lambda) \lambda^4 d\lambda$$

(1.1)

Generally, the rate of FRET from a donor to an acceptor is given by equation 1.2. $R_0$ is known as Förster distance, a distance between the donor and acceptor at which the FRET efficiency is 50%. Typically, $R_0$ is in the range of 20 to 90 Å, which are comparable to the size of biological molecules. $r$ is the distance between the donor and acceptor. It is obvious that the rate of FRET is proportional to $r^{-6}$ with a significant dependence on the distance.

$$k_T(r) = \frac{1}{\tau_D} \left( \frac{R_0}{r} \right)^6$$

(1.2)
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\( J(\lambda) \) is the spectral overlap integral, which represents the degree of spectral overlap between the emission spectrum of donor and the absorbance spectrum of acceptor. Where \( F_D(\lambda) \) is the donor emission spectrum normalized to an area of 1, \( \varepsilon_A(\lambda) \) represents the molar extinction coefficient of the acceptor which can be obtained from acceptor absorption spectrum. As the name suggests, \( \lambda \) is the wavelength.

\[
J(\lambda) = \int_0^\infty F_D(\lambda) \varepsilon_A(\lambda) \lambda^4 d\lambda
\] (1.3)

From equation 1.1 and 1.2, equation 1.4 can be deduced as followed. This equation expresses that the Förster distance can be calculated from donor emission spectrum and acceptor absorption spectrum plus the quantum yield of the donor.

\[
R_0^6 = \frac{9000(\ln 10)Q_D\kappa^2}{128\pi^5N^4} \int_0^\infty F_D(\lambda) \varepsilon_A(\lambda) \lambda^4 d\lambda
\] (1.4)

Once the value of Förster distance \( (R_0) \) is known, equation 1.1 can be easily transferred to equation 1.4. The FRET efficiency \( (E) \) is given by equation 1.5. With the substitution of equation 1.2 into 1.5, equation 1.6 can be easily derived. From this equation, one can predict the FRET efficiency with an estimated or calculated \( r \) value.

\[
E = \frac{k_T(r)}{\tau_D^{-1} + k_T(r)}
\] (1.5)

\[
E = \frac{R_0^6}{R_0^6 + r^6}
\] (1.6)

The FRET efficiency can be measured by both steady state fluorescence spectroscopy and time-resolved fluorescence spectroscopy. The calculation is given by equation 1.7, where \( F_{DA} \) and \( F_D \) are the fluorescence intensity of the donor in the presence and absence of acceptor, \( \tau_{DA} \) and \( \tau_D \) are the fluorescence lifetime of the donor in the presence and absence of acceptor.

\[
E = 1 - \frac{F_{DA}}{F_D} = 1 - \frac{\tau_{DA}}{\tau_D}
\] (1.7)

With the experimental proof of FRET theory in 1967, this theory has been well explored in countless applications, particularly in biochemistry, such as protein folding, medical diagnostics, optical imaging, membrane fusion, DNA analysis and so on.

1.6 References


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Chapter 2

Synthesis of Mono-functionalized CB6 and CB7
In the past decade, the supramolecular chemistry of the CB\textit{n} family of molecular containers has rapidly developed due to the exceptionally high affinity and selectivity in aqueous solution.\textsuperscript{1-7} Accordingly, unfunctionalized CB\textit{n} have been used to create functional supramolecular systems including molecular machines, materials for capture and release of volatile compounds, supramolecular polymers, solubilizing agents for insoluble drugs, supramolecular catalysts, and chemical sensing ensembles. In order to further extend the supramolecular chemistry of CB\textit{n} it is necessary to develop efficient synthetic methods to prepare functionalized CB\textit{n} derivatives.

### 2.1 Introduction

A big step in this direction is accomplished by Kim group who performed the direct perhydroxylation of cucurbituril using K\textsubscript{2}S\textsubscript{2}O\textsubscript{8} as an oxidant to yield a series of hydroxy-substituted (OH)\textsubscript{2n}CB\textsubscript{n},\textsuperscript{8} which has served as a trigger for the synthesis of many other functionalized cucurbituril.\textsuperscript{9-14} These reactions represent the first direct covalent derivatizations of CB\textit{n}. These functionalized products show various potential applications, including the synthesis of chromatographic materials,\textsuperscript{15} formation of ion channels, construction of sensors as well as their utility in ion separation and capture applications.\textsuperscript{16} They also show promise in molecular devices,\textsuperscript{17} as antibacterial agents that are stable against enzymatic degradation,\textsuperscript{18} as functional CB\textit{n}-based polymers,\textsuperscript{19-23} and for the formation of CB\textit{n}-based supramolecular polymers.\textsuperscript{24} Further areas include application in drug delivery,\textsuperscript{25, 26} as fluorescent capsules,\textsuperscript{27} and as supramolecular biocompatible hydrogels.\textsuperscript{28}

Scherman and coworkers isolated monohydroxylated CB6 by harnessing the host-guest chemistry of bisimidazolium salts with CB6 with a much high solubility.\textsuperscript{9} Afterwards, Kim group improved their method and obtained monohydroxylated CB7 in one step with a weak point of low quantitative reaction conversion.\textsuperscript{29} Recently, Bardelang and Ouari developed a convenient photochemical method to synthesize monohydroxylated CB\textit{n} directly with relatively high yield by using hydrogen peroxide and UV light,\textsuperscript{30} and what we are following is this direct synthesis method.
2.2 Synthesis and Separation of CBn

Figure 2.1 The reaction between glycoluril and formaldehyde.

60 mL 9 M sulfuric acid is mixed with 20 mL formaldehyde (37% aqueous solution) at room temperature and then cooled down the temperature to 2-5 °C using an ice bath. Then glycoluril is then added in small portions with vigorous stirring. The temperature is increased to 95 °C in an oil bath and the mixture is refluxed for 72 h.

200 mL distilled water and 800 mL acetone are added into the reaction solution with vigorous stirring, in order to participate all CBn homologues. The suspension is settled down, filtered and washed with 250 mL mixed solution of acetone and water (v/v: 4:1). The filtrate is collected and decanted subsequently with small portion of acetone/water solution in order to remove the concentrated acid. The precipitate is dissolved in 400 mL distilled water. CB5 and CB7 would dissolve but not CB6 and CB8 whose solubilities in water are very low. By this step, the mixture of CB6 and CB8 is the solid in the filter and mixture of CB5 and CB7 is in the filtrate.

To get CB6, 10 g of the mixture of CB6 and CB8 (dry solid) is dissolved in 100 mL 3 M HCl. Then the solution is filtrated, CB6 stays in the solution and little CB6 and CB8 are left in the filter. The solution is evaporated to get the solid, 200 mL methanol is added to disperse the solid again, and then the solution is filtrated following by 3 times water (3 × 50 mL) washing and 3 times methanol (3 × 30 mL) washing.

To get CB7, a volume of 300 mL acetone is added to the filtrate, which would precipitate mainly the CB7. Then the solid is washed a couple of times with acetone and dried under vacuum, relatively pure CB7 can be obtained.
2.3 Synthesis of CB6-OH and CB7-OH

![Synthesis of CB6-OH and CB7-OH](image)

**Figure 2.2** Synthesis of CB6-OH and CB7-OH.

Take CB7 as an example, CB6 follows the same procedure. 1 g (0.86 mmol) CB7 was dissolved in 125 mL of a mixture of Millipore water and 12 M HCl (3:2 v/v) and introduced in a 250 mL quartz glass round bottom flask under nitrogen. 65 µL (0.62 mmol) 30% hydrogen peroxide in H₂O was added and the solution was vigorously stirred during irradiation of UV light (254 nm) for 48 h. The reaction was monitored by ¹H NMR by taking aliquots of the reaction mixture.

The solvent was then evaporated under reduced pressure affording a white solid. The crude product containing a mixture of CB7-(OH)ₙ (with n = 0, 1, 2, 3) was separated by column chromatography. Therefore, the mixture was dissolved in 950 µL H₂O/HCOOH 1:1 and loaded onto silica gel 60 (0.04-0.063 mm) and the column was eluted with H₂O/AcOH/HCOOH 10:10:1.5. The eluent was collected in fractions of 2 mL (>250 fractions) and the fractions containing pure CB7-OH were combined. Evaporation of the solvent gave 150 mg CB7-OH as a white solid. The ¹H NMR was in accordance with the reported spectrum and the identity and purity of the obtained material was additionally confirmed by mass spectrometry (Figure 2.3 and 2.4). The ESI-MS spectra were obtained in the presence of cystamine, as cystamine can dramatically increase the water solubility of CBₙ.
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Figure 2.3 Mass spectrum of CB7-OH with 1 mM cystamine in Millipore water. Traces of CB6 were presumably enriched during column chromatography.

Figure 2.4 Mass spectrum of CB6-OH with 1 mM cystamine in Millipore water.
2.4 Synthesis of CB6-OPr and CB7-OPr

![Chemical Structures]

Figure 2.5 Synthesis of CB6-OPr and CB7-OPr.

Take CB7-OPr for example. 20 mg (17 µmol) CB7-OH was dissolved in 1.5 mL anhydrous DMSO. 10 mg (0.4 mmol) NaH (95% purity as solid) was added, and the mixture was stirred at room temperature for 3 h. Subsequently, the mixture was cooled to 0 °C, 0.5 mL (4.4 mmol) propargyl bromide was added, and the reaction mixture was stirred at room temperature for 12 h. 50 mL diethyl ether was added, and the resulting precipitate was three times triturated with 25 mL MeOH. Drying under high vacuum afforded a pale yellow solid, which was subjected a second time to the same reaction conditions. This gave the desired CB7-OPr quantitatively as confirmed by mass spectrometry (Figure 2.7), 1H NMR (Figure 2.8), MS (ESI, +ve): 685.3, [CB7-OPr+Cys+2H]^{2+}.

Noteworthy, the functionalization of propargyl group with CB6 and CB7 need to be reacted twice to achieve a high yield. This process can be well followed by MS spectra. As can be seen from Figure 2.6 and 2.7, after one time reaction free mono-hydroxylated CB6 and CB7 is still left in a large amount. However, with one more time reaction under the same conditions, nearly 100% yield is obtained finally. In addition, this functionalization lowers the solubility of CB6-OH and CB7-OH. Consequently, for both CB6-OPr and CB7-OPr, NMR spectra can only be shown in the presence of guest molecules, such as p-xylylenediamine with CB7-OPr.
Figure 2.6 Mass spectra of product started with CB6-OH with 1 mM cystamine in Millipore water.
Figure 2.7 Mass spectrum of product started with CB6-OH with 1 mM cystamine in Millipore water.
Figure 2.8 $^1$H NMR spectra of CB7-OPr in 1% DCl in D$_2$O in absence (top) and presence of substoichiometric amounts (middle) or excess (bottom) of the cavity binder p-xylidendiamine.

2.5 Synthesis of CB6-Carboxyfluorescein (CB6-CF)

Figure 2.9 Synthesis of CB6-CF.
21 mg (20 μmol) CB6-OPr was dissolved in DMSO/H$_2$O (3.4 mL/0.9 mL, 4.3 mL in total). Subsequently, 6-Carboxyfluorescein-Azide (40 μmol), CuSO$_4$ (40 μmol), sodium ascorbate (40 μmol) and TBTA (20 μmol) were added. The solution is stirred at room temperature for 24 h. 50 mL diethyl ether was added, and the resulting precipitate was washed three times with 25 mL MeOH. Drying under high vacuum afforded a dark solid. The crude product containing unreacted CB6-OPr, 6-FAM-Azide, and CB6-CF, was purified by column chromatography. In detail, the mixture was dissolved in 600 μL H$_2$O/HCOOH 1:1 and loaded onto silica gel 60 (0.04-0.063 mm) and the column was eluted with H$_2$O/AcOH/HCOOH 10:10:1.5. The eluent was collected in fractions of 2 mL (ca. 50 fractions) and the fractions containing pure CB6-CF were combined. Evaporation of the solvent gave 8 mg (7.6 μmol, 38% yield) CB6-CF as a brown solid. The product identity was confirmed by mass spectrometry (Figure 2.10), [CB6-CF+Cys+2H$^+$$]^{2+}$ is calculated at 832 and found at 832.

Figure 2.10 The mass spectrum of CB6-CF.

2.6 Designed FRET Pair Based on CB6-CF and R-S

Our initial aim is to design a FRET pair based on host-guest interactions and try to explore its potential applications. Therefore, after the successful synthesis of CB6-CF, we designed a guest molecule rhodamine-spermine (R-S) which was afforded by Prof. Pischel (structures are shown in Figure 2.11 a, b). Figure 2.11 c illustrates a FRET pair based on supramolecular host-guest interaction between CB6-CF and R-S. In this FRET pair, CB6-CF and R-S are considered as the donor and the acceptor respectively. As a result of the simulated calculation based on the complex of CB6-CF and R-S (as shown in Figure 2.12), the maximum distance between acceptor and donor depends on the molecules orientation, which is calculated to be 0.9 nm and 1.4 nm in two possible structures. This calculation demonstrates the designed FRET system possible to occur theoretically.
Figure 2.11 Illustration of host-guest FRET pair based on CB6-CF and R-S.

Figure 2.12 DFT calculation of the structure in different possible orientations for the CB6-CF/R-S complex in gas phase.
2.6.1 Spectral Characterization of the Designed FRET pair

Figure 2.13 Normalized absorption (dashed) and emission (solid) spectra of CB6-CF (black) and R-S (green) in 10 mM (NH₄)₂HPO₄, pH 7.5. \( \lambda_{\text{exc,CB6-CF}} = 490 \text{ nm} \) and \( \lambda_{\text{exc,R-S}} = 560 \text{ nm} \).

Figure 2.13 shows the spectroscopic properties of CB6-CF and R-S. The spectral overlap between the CB6-CF emission spectrum (black solid line in Figure 2.13) and the R-S absorption spectrum (green dashed line) demonstrates this FRET pair works in principle. The results from control experiments show that CB6-CF could be selectively excited around 450 nm, where the absorbance of R-S is insignificant.

2.6.2 Spectral Properties of the Designed FRET Pair

Steady state fluorescence spectroscopy is used to characterize the spectral properties of this FRET pair. Titrations of 50 nM CB6-CF with increasing amounts of R-S were carried out to investigate the performance of the FRET system (Figure 2.14 a, b). Overall, the spectral changes during the titrations can be grouped into three different concentration regions of R-S. At low concentrations of R-S (0-0.12 \( \mu \text{M} \)), the fluorescence of CB6-CF steeply decreases by a factor of two and R-S is not fluorescent at all (Figure 2.14 a). We presume that this is due to formation of the host-guest complex leading to fluorescence quenching of the fluorescein dye due to the close spatial proximity of the rhodamine dye. In fact, analysis of the fitting curve of this concentration region suggested a nearly quantitative binding affording a lower limit of the binding constant of \( K_a > 6 \times 10^8 \text{ M}^{-1} \). This is accordance with the very strong binding reported for spermine to CB6 \( (K_a \text{ ca. } 10^{12} \text{ M}^{-1}) \). With increasing concentrations of R-S (0.36-0.76 \( \mu \text{M} \)), the fluorescence intensity of CB6-CF starts to slightly increase. We believe that this is due to a residual fluorescence emission from excess R-S. The solution now contains ca. 10 times more
R-S than CB6-CF. This is supported by Figure 2.14 d (only R-S), where a slight increase in fluorescence around 521 nm becomes visible in this concentration range.

**Figure 2.14** Fluorescence titrations ($\lambda_{exc} = 450$ nm, slits: 10/10) and control experiments with increasing amounts of R-S (0 to 4.36 $\mu$M) in presence of a) 50 nM CB6-CF and 0-0.12 $\mu$M R-S, b) 50 nM CB6-CF and 0.12-4.36 $\mu$M R-S, c) 50 nM 6-FAM azide, and d) in absence of fluorescein. Experiments were performed in 10 mM (NH$_4$)$_2$HPO$_4$, pH 7.5.

Addition of even larger amounts of R-S (>0.76 $\mu$M, >15 fold excess) then leads to a continuous decrease in the fluorescence intensity of fluorescein. At these high concentrations, R-S shows significant absorbance in the spectral region of fluorescein emission ($\varepsilon = 110000$ M$^{-1}$cm$^{-1}$, $A > 0.08$). This may lead to reabsorption of the emitted photons and thus a decrease in fluorescence. This is supported by a similar decrease in fluorescence upon addition of R-S to a solution containing 6-FAM-azide lacking the CB6 receptor (Figures 2.14 c).

In summary, it becomes apparent that a host-guest complex between CB6-CF and R-S is formed. The spectral changes of the titration and the control experiments are in clear accordance with this interpretation. However, a fluorescence increase in the R-S emission at low concentrations of R-S (Figure 2.14 a) was not observed, which currently suggests that FRET is not the quenching mechanism.
2.6.3 Quenching Mechanism

As noted above, the absence of a fluorescence increase in the region of rhodamine emission during the titration of CB6-CF with R-S suggests that FRET is not the (only) operative fluorescence quenching mechanism. We thus considered that static quenching, either of fluorescein by rhodamine or of rhodamine by fluorescein after FRET, may cause the lack of fluorescence increase.

Figure 2.15 a) Titration of R-S (110 nM) with CB6-CF (From 0 to 1.33 µM) in 10 mM (NH₄)₂HPO₄, pH 7.5 using direct excitation of R-S (λₑₓ = 550 nm). b) CB6-CF concentration dependence of the fluorescence intensity of R-S at 580 nm.

Figure 2.16 a) Titration of 0.5 µM R-S with CB6 (0 to 30 µM) in 10 mM (NH₄)₂HPO₄, pH 7.5. (λₑₓc = 560 nm). b) CB6 concentration dependence of the fluorescence intensity of R-S at 580 nm.

To test whether there is static quenching of rhodamine by fluorescein (after FRET) in this system or not, a control titration experiment was conducted. We used a constant concentration of R-S and added increasing amounts of CB6-CF (Figure 2.15). The excitation wavelength was 550 nm, such that rhodamine is directly excited without exciting CB6-CF.
Under these conditions, any change in rhodamine fluorescence must occur because of the formed complex. In fact, we observed a slight decrease of R-S fluorescence intensity, which was, however, not strong enough to fully explain the absence of rhodamine emission upon excitation of CB6-CF.

In another control experiment, parent unmodified CB6 was added to a solution containing 0.5 µM R-S (shown in Figure 2.16). At a lower concentration of CB6, from 0 to 0.3 µM, the intensity somehow decreased. When the concentration was higher than 0.6 µM, the intensity started to increase. Also here, the fluorescence changes are small and do not explain the absence of the expected increase in rhodamine fluorescence after FRET.

Based on the proposed analysis of the spectral properties, the designed FRET pair based on CB6-CF and R-S is not suitable for further study. We need to select another FRET pair with proper spectral properties. A new FRET pair based on CB7 host-guest interaction is later achieved and this content will be described in detail in Chapter 3.

### 2.7 Further Synthesis of Mono-Functionalized CB7

![Examples of mono-functionalized CB7 derivatives.](image)

**Figure 2.17** Examples of mono-functionalized CB7 derivatives.
Figure 2.18 MALDI-TOF spectra of CB7-TAMRA and CB7-Cy3.
Besides CB6-CF, we further synthesized four mono-functionalized CB7 derivatives, one will be explained in the next Chapter and the other three includes two fluorescent dyes in different emission range and one functional molecule with –COOH at the end (Figure 2.17 and 2.18). CB7-TAMRA and CB7-Cy3 are reacted by CB7-OPr with commercial 5-TAMRA azide and Cyanine 3 azide. CB7-O2O is reacted with synthesized azido molecule with functional groups from Prof. Dr. Leif Schroeder. The synthesis procedures to get CB7-TAMRA and CB7-Cy3 are the same with CB6-CF procedure. The procedure to get CB7-O2O is quite different which takes tedious work to optimize the reaction conditions.

![Figure 2.19 The synthesis route of CB7-Nle and CB7-O2O.](image)

From our collaborator Prof. Dr. Leif Schroeder, we received two chemicals (1 and 2, Figure 2.19) designed to react with CB7-OPr. The first try was following the proved successful procedure of CB6-CF, unfortunately no product was acquired by characterizations with TLC and MS. However, click reaction is well known as efficient reaction and it should work in principle between azide and alkyne. Therefore, twelve different reaction conditions were tried for the reaction by varying the Cu source, ligand and solution composition (Table 2.1). The stock solutions of chemicals used in table 2.1: CB7-OPr: 30 mM in DMSO; NaHCO3: 1 M in H2O; NaAsc: 1 M in H2O; TBTA: 376 mM in DMSO; BPDS: 388 mM in H2O; CuSO4: 1 M in H2O; CuI: 0.5 M in DMSO; CuBr: 0.5 M in DMSO; DIPEA: 0.742 g/mL in H2O. For each of the reaction it combines 3 steps. Firstly it was reacted for 24 h in room temperature. Without new spot observed on TLC temperature was increased to 50 °C for another 24 h reaction time.
Chapter 2 Synthesis of Mono-Functionalized CB6 and CB7

If still no new spot shown on TLC, the temperature then increased to 90°C. The first and second step were characterized by TCL, as well as the third step, no new spot appeared by all of them. Finally we chose MALDI-TOF to detect products after the third step reaction due to the very low solubility of mono-functionalized CB7 derivatives.

**Table 2.1 Different conditions for the reactions between CB7-OPr and 1 or 2.**

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<th>No.</th>
<th>Azide</th>
<th>CB7-OPr</th>
<th>Cu source</th>
<th>Ligand</th>
<th>Azide</th>
<th>NaAsc/Base</th>
<th>Solvent</th>
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<td>1.5 µL = Cu²⁺</td>
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<td>BPDS</td>
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<td>15 µL</td>
<td>1 µmol</td>
<td>CuSO₄</td>
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<tr>
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<td>CuI</td>
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<td>CuBr</td>
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<td>0.7 µL</td>
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<td>2 µmol</td>
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As shown in Figure 2.20, MS of Samples from condition 11 and 12 show the peak 1574 m/z, which comes from the complex of CB7-O2O with Na+, demonstrating that CB7-O2O can
be successfully synthesized by using condition 11 and 12. The peak is very tinny because of the low solubility of the aimed product. The explanation of no new spot shown on TLC plate is still missing. In conclusion, the difficulty of the click reactions based on CB7-OPr is depend on the azido molecules. To successfully synthesize the target product, optimization of reaction conditions plays a crucial role.

2.8 References


Chapter 3. Ratiometric DNA Sensing with a Host-Guest FRET Pair
Chapter 3 Ratiometric DNA Sensing with a Host-Guest FRET Pair
This chapter is derived from the following publication:

In this chapter, a supramolecular host-guest FRET pair based on a carboxyfluorescein-labelled cucurbit[7]uril (CB7-CF, as acceptor) and the fluorescent dye 4',6-diamidino-2-phenylindole (DAPI, as donor) is developed for sensing of DNA. In comparison to the commercial DNA staining dye SYBR Green I, the new chemosensing ensemble offers dual-emission signals, which allows a linear ratiometric response over a wide concentration range.

3.1 Introduction

Fluorescent chemosensors are receiving increasing attention, especially for biological analytes.1-2 For example, numerous commercially available fluorescent dyes, such as SYBR-Green I, bind to the minor groove of double-stranded DNA (dsDNA), which can be exploited for sensing in medical diagnostics, e.g., for real-time PCR.3-6 Most of these dye-intercalation techniques are based on single-wavelength monitoring, whereas dual emitting probes, which allow ratiometric sensing for more precise quantification, are mostly based on the recognition of DNA hybridization. This has been achieved by using pyrene-functionalized molecular beacons (with either oligonucleotide7 or peptide8 backbones), locked nucleic acids,9 and perylene-based probes,10-12 but these probes only respond to complementary DNA sequences.

An alternative sensing strategy to direct binding, such as intercalation, is indicator displacement. Macrocycles such as cyclodextrins,13-15 calixarenes,16-18 crown ethers,19-21 and cucurbit[n]urils22-23 (Cbn) have been abundantly used in combination with fluorescent dyes to quantify analytes by the latter strategy, including the cited examples of the most desirable ratiometric sensing. With the aim to monitor analyte formation or depletion and to measure the kinetics of enzymatic reactions, macrocycle/dye reporter pairs have also been implemented in supramolecular tandem assays,24-31 including the enzymatic conversion of nucleotides,22,32-41 but they have not yet been employed for DNA sensing.

Cbn macrocycles, and in particular the medium-sized homologue CB7, stand out as recognition motifs due to their high analyte affinities and selectivities.42-44 The known assays rely on a direct affinity between analyte and CBn, which results in a competitive displacement of the fluorescent indicator dye. However, although indirect interactions between DNA or nucleotides and CBn, mediated by other guests, have been described,45-52 applications to DNA sensing are elusive. Herein, we establish a nonconventional approach to DNA sensing, which relies neither on a simple intercalation of a fluorescent dye with the analyte (DNA) nor on a direct binding of DNA to a recognition site, but rather on a competitive binding of a fluorescent
dye to an acceptor-labelled CB7 and DNA; this allows a ratiometric DNA sensor based on fluorescence resonance energy transfer (FRET) between the two chromophores to be set up according to Figure 3.1. The new application complements recent work on fluorescent dye-conjugated CBn derivatives with cyanine-3 and tetramethylrhodamine for the detection of vesicle fusion\textsuperscript{53-55} and cellular imaging\textsuperscript{56}.

![Figure 3.1](image_url) \textbf{Figure 3.1} (a) Molecular structures of CB7-CF and DAPI. (b) Schematic illustration of a DNA chemosensing ensemble based on FRET between DAPI (donor) and CB7-CF (acceptor).

\section*{3.2 Synthesis of CB7-CF}

The synthesis of CB7-carboxyfluorescein (CB7-CF) involved labelling through click reaction of the azide derivative of carboxyfluorescein (CF, using commercial 6-FAM-azide), with monofunctionalised propargyl-CB7 (CB7-OPr)\textsuperscript{57-58} (see Figure 3.2). Since the CF chromophore is equatorially attached to the outer wall of CB7, its hydrophobic cavity remains accessible for guest binding. Intramolecular or intermolecular complexation of CF in the cavity, which presents a common obstacle when using monofunctionalized CBn derivatives,\textsuperscript{59} does not interfere, because of the anionic nature of CF, which is incompatible with the cation-receptor propensity of CBn homologues.\textsuperscript{60}
3.3 Optical Characterizations of FRET Pair

![Diagram of CB7-Pr and CB7-CF synthesis](image)

**Figure 3.2** Synthesis of CB7-CF.

**Figure 3.3** (a) Normalized absorption (solid) and emission (dashed) spectra of DAPI (blue) and CB7-CF (green). (b) Emission spectra ($\lambda_{\text{ex}} = 360$ nm) of 2 µM DAPI (blue), 1 µM CB7-CF (green), and a mixture of 2 µM DAPI and 1 µM CB7-CF (orange).

The encapsulation of the fluorescent DNA stain 4',6-diamidino-2-phenylindole (DAPI, as chloride salt), by CB7-CF allows efficient FRET to occur. The spectral characterization of the FRET pair was performed by UV-Vis absorption and fluorescence spectroscopy (Figure 3.3). The emission spectrum of DAPI has a significant overlap with the absorption spectrum of CB7-CF, which fulfills the principal requirement of FRET. The distance between DAPI and CB7-CF was modelled to be between 5.9 Å (minimum) and 10.8 Å (maximum), which falls
sufficiently far below the calculated critical Förster radius of the system \((R_0 = 33.3 \, \text{Å}, \text{Figure 3.4})\) to allow quantitative FRET (>99%).

**Figure 3.4** DFT-optimized structures (B3LYP/3-21G*) of different possible co-conformations for the CB7-CF/DAPI complex (in gas phase). The distance \(d\) between the center of mass of the CF and DAPI is given in Å. The structure shown in (a) was found to be more stable than that in (b) by 4.9 kcal/mol.

Optical titration experiments were limited by the reduced water solubility of CB7-CF (ca. 5 µM in pure water and ca. 30 µM in the presence of 0.5% DMSO), but the fluorescence intensities were sufficiently high to allow an analysis also in this solubility range. The fluorescence titration of the donor (DAPI, \(\lambda_{ex} = 360\) nm) upon addition of acceptor (CB7-CF) was performed first in the presence of 0.5% DMSO (Figure 3.5 a). As expected, there was a strong rise in fluorescence of CB7-CF, which afforded, after correction for direct excitation of CF at the excitation wavelength of DAPI, a binding constant of \((1.4 \pm 0.1) \times 10^6\) M\(^{-1}\). Noteworthy, the fluorescence of the donor did not decrease, as expected for quantitative FRET, but showed a slight increase. This phenomenon is well known in FRET experiments with commercial labelling agents (6-FAM-azide) and can be traced back to a nonquantitative labelling with acceptor.\(^6\) The comparison of the fluorescence intensity of DAPI alone and that extrapolated to high concentrations of CB7-CF, along with the expected fluorescence enhancement factor in an unlabelled CB7 cavity (ca. 12, see Figure 3.6) allowed us to estimate the labelling degree with CF as 90%.

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\(^6\) The comparison of the fluorescence intensity of DAPI alone and that extrapolated to high concentrations of CB7-CF, along with the expected fluorescence enhancement factor in an unlabelled CB7 cavity (ca. 12, see Figure 3.6) allowed us to estimate the labelling degree with CF as 90%.
Figure 3.5 (a) Fluorescence spectral changes ($\lambda_{\text{ex}} = 360$ nm) with increasing amounts of CB7-CF (0 to 15 µM) in the presence of 1 µM DAPI; the inset shows the fluorescence titration ($\lambda_{\text{em}} = 520$ nm) with increasing concentration of CB7-CF ($R^2 = 0.998$). Corrected for direct excitation of CF. (b) Fluorescence spectral changes ($\lambda_{\text{ex}} = 360$ nm) with increasing amounts of DAPI (0 to 7.4 µM) in the presence of 1 µM CB7-CF; the inset shows the fluorescence titration ($\lambda_{\text{em}} = 450$ nm) with increasing concentration of DAPI in the presence of only DAPI (black curve, $R^2 = 0.998$), 1 µM CB7-CF (red curve, $R^2 = 0.999$), and 1 µM CB7 (blue curve, $R^2 = 0.998$). Fitting curves were obtained by a 1:1 host-guest binding isotherm.

Figure 3.6 Variation of the fluorescence spectrum ($\lambda_{\text{ex}} = 360$ nm) of 1 µM DAPI in 10 mM (NH$_4$)$_2$HPO$_4$, pH 7.2, upon addition of CB7. Inset: CB7 concentration dependence of the fluorescence intensity at 470 nm, $R^2 = 0.998$. The curve was fitted by 1:1 binding stoichiometry. The reverse titration allowed us to demonstrate operation of the FRET system in neat water (10 mM ammonium phosphate buffer, pH 7.2, Figure 3.5 b), where the solubility of CB7-
CF was limited to ca. 5 μM. Upon addition of increasing amounts of donor (DAPI) to a CB7-CF solution (1 μM) its fluorescence (λ<sub>ex</sub> = 360 nm, λ<sub>obs</sub> = 450 nm) increased. However, the fluorescence increase (red line) fell far below the 12-fold increase observed upon addition to a corresponding CB7 solution (1 μM, blue line). This is due to the fact that the DAPI fluorescence in the CB7-CF cavity is effectively quenched by FRET (also reflected in an increased CF fluorescence) and not enhanced as in unlabelled CB7, where a microenvironmental effect enhances the fluorescence.<sup>62</sup> Comparison of the fluorescence intensity of DAPI in the presence of CB7-CF with that in the absence of any host further indicates a slight increase (compare red and black lines), which can be attributed to the nonquantitative acceptor labelling already exposed in the titration with acceptor (see above, Figure 3.5 a). Fitting of the fluorescence titrations by taking into account these boundary conditions afforded a binding constant between DAPI and CB7-CF of (5.2 ± 0.5) × 10<sup>6</sup> M<sup>-1</sup>, comparable to that between DAPI and CB7 in the same ammonium phosphate buffer ((2.8 ± 0.2) × 10<sup>6</sup> M<sup>-1</sup>), but below that in neat water (11 × 10<sup>6</sup> M<sup>-1</sup>),<sup>62</sup> due to the competition of ammonium cation binding to CB7 in the buffer.<sup>63</sup>

![Image](http://example.com/image.png)

**Figure 3.7** (a) Fluorescence titrations (λ<sub>ex</sub> = 360 nm) with increasing amounts of DAPI (0 to 7.4 μM) in the presence of 1 μM CB7-CF as well as 20 μM AMADA. (b) Fluorescence spectra (λ<sub>ex</sub> = 360 nm) of 1 μM CB7-CF and 7.4 μM DAPI without (black line) and with (red line) 32 μM AMADA in 10 mM (NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub>, pH 7.2. The addition of AMADA leads to a strong fluorescence decrease of CB7-CF.

Note that the value in the aqueous buffer lies slightly above that determined in the presence of 0.5% DMSO (1.4 × 10<sup>6</sup> M<sup>-1</sup>, see above), a co-solvent which is expected to slightly reduce binding as a consequence of a reduced hydrophobic driving force.<sup>64</sup> The reversibility of the FRET process and the analyte responsiveness of the system was established through control experiments with 1-aminomethyladamantane (AMADA), a known competitive binder of CB7 (see Figure 3.7).
Figure 3.8 Time-resolved fluorescence decay traces of 5 µM DAPI alone (blue) and in the presence of 10 µM CB7-CF (black) and CB7 (red); instrument response function is shown in magenta. $\lambda_{\text{exc}} = 373$ nm, $\lambda_{\text{obs}} = 450$ nm.

Table 3.1 Fitting results of lifetime decays of DAPI with and without CB7 or CB7-CF in 10 mM (NH$_4$)$_2$HPO$_4$, pH 7.2 with 0.5% DMSO.

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<tr>
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<th>$B$</th>
<th>Rel%</th>
<th>$\chi^2$</th>
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<td>DAPI$^a$</td>
<td>$\tau_1 = 0.29$</td>
<td>0.056</td>
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<tr>
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<td>0.026</td>
<td>100</td>
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<tr>
<td>DAPI+CB7-CF</td>
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<td>$\tau_2 = 3.05$</td>
<td>0.010</td>
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</table>

$^a$ Without DMSO.

The FRET pair was also characterized by nanosecond time-resolved fluorescence spectroscopy (Figure 3.8 and Table 3.1). DAPI has a long fluorescence lifetime component (2.75 ns). Upon complexation by CB7, the lifetime is reduced to ca. 1.6 ns, which presents an unusual but previously documented response; generally, an increase in fluorescence intensity upon CB7 complexation is accompanied by an increased fluorescence lifetime. This unusual behavior points to an increase of the radiative lifetime ($\tau$) of DAPI upon CB7 complexation, which is likely related to a conformational realignment of the chromophore. From the Förster radius of the FRET pair, a quantitative (>99%) fluorescence quenching of DAPI was expected in the DAPI-CB7-CF complex. Unfortunately, due to the interference of nonquantitative (90%) acceptor labelling, a residual long-lived component attributable to DAPI remained even in the
presence of an excess of CB7-CF. Noteworthy, the fluorescence lifetime of the CF chromophore in CB7-CF (3.6 ns) remained virtually the same as that in free CF,\textsuperscript{67} which demonstrates that the CF chromophore in CB7-CF and also in the DAPI-CB7-CF complex is not quenched.

### 3.4 DNA Sensing by FRET Pair

![Figure 3.9](image)

**Figure 3.9** (a) Fluorescence spectra (\(\lambda_{ex} = 360\) nm) of 0.5 \(\mu\)M DAPI and 1 \(\mu\)M CB7-CF with increasing concentration of salmon sperm DNA (0 to 20 \(\mu\)g/mL). The inset shows the linear relationship between the fluorescence intensity ratio (\(I_{450}/I_{520}\)) and the concentration of salmon sperm DNA (0 to 20 \(\mu\)g/mL). (b) Temperature-dependent fluorescence intensity at 450 nm and 520 nm (\(\lambda_{ex} = 360\) nm) from 20 to 90 °C. The inset shows the reversible changes in \(I_{450}/I_{520}\) ratios between 20 and 90 °C.

DAPI is well known to intercalate strongly into the minor groove of DNA (binding affinity of 10\(^7\) M\(^{-1}\) per nucleotide, or 10\(^{10}\) M\(^{-1}\) for a typical DNA with ca. 1000 base pairs),\textsuperscript{68-69} but binds with micromolar affinity to CB7,\textsuperscript{62} which should allow for an efficient relocation of the dye from CB7 upon addition or formation of DNA if the concentrations are favourably selected.\textsuperscript{70} Accordingly, we conducted a fluorescence titration of the pre-assembled FRET pair (0.5 \(\mu\)M DAPI and 1 \(\mu\)M CB7-CF) with Type III salmon sperm DNA (Figure 3.9 a). With increasing concentration of DNA, DAPI is relocated from CB7-CF, where it no longer serves as FRET donor and, thereby, reduced the CF fluorescence at 520 nm, into DNA, where its own fluorescence at 450 nm becomes enhanced. The ratio of the fluorescence intensity values (\(I_{450}/I_{520}\)) increased linearly in the picomolar range (up to 20 \(\mu\)g/mL), which sets up an excellent DNA chemosensing ensemble with an LOD value (limit of detection) of ca. 60 ng/mL (inset in Figure 3.9 a). A plateau value was reached at DNA concentrations above 0.1 mg/mL (Figure 3.10 a), indicating that DAPI relocation from CB7 to DNA is quantitative.
Figure 3.10 (a) Fluorescence spectra ($\lambda_{ex} = 360$ nm) of 0.5 $\mu$M DAPI and 1 $\mu$M CB7-CF with increasing concentration of salmon sperm DNA (0 to 0.5 mg/mL). The inset shows the corresponding change in the fluorescence intensity ratio at 450 nm and 520 nm. (b) (c) Variation of the fluorescence spectrum of 0.5 $\mu$M DAPI ($\lambda_{ex} = 360$ nm) and of 0.5 $\mu$M SYBR ($\lambda_{ex} = 497$ nm) upon addition of salmon sperm DNA. The inset shows the dependence of the fluorescence intensity at 450 nm on DNA concentration and at 520 nm on DNA concentration. (d) Normalized fluorescence intensity of DAPI, SYBR-Green, and DAPI/CB7-CF with same concentration of fluorescence dye in dependence on DNA concentration. Note that for DAPI/CB7-CF the ratiometric response is shown.

The performance of the dual-wavelength monitoring system was directly contrasted (Figure 3 b-d) to DAPI alone and also to commercial SYBR-Green I, a gold standard for the detection of DNA in PCR technologies. Although the conventional intercalators excelled with respect to the absolute fluorescence enhancement factor upon DNA addition, the DAPI/CB7-CF system showed a much larger linear-response range, which is advantageous for absolute quantification. With identical instrumental settings, SYBR-Green I showed an approximately linear response up to 3 $\mu$g/mL, while the range of the FRET system extends up to 20 $\mu$g/mL (Figure 3.9 a). Consequently, we propose that the DAPI/CB7-CF FRET pair can be used as a
complementary and non-proprietary indicator dye for nucleotide sensing technologies, including real-time PCR. As a proof of principle, we carried out fluorescence-based DNA melting experiments, which demonstrated that the FRET system responds reversibly to the melting of DNA in dependence on temperature (Figure 3.9b); interestingly, the heating and cooling curves displayed a large hysteresis, presumably as a consequence of the more complex competitive equilibria involved when compared to a simple dye-intercalation approach.

### 3.5 Conclusions

In conclusion, we have introduced a host-guest FRET pair based on the macrocyclic host CB7 labelled with carboxyfluorescein as the acceptor and the nucleic stain DAPI as donor and guest. We demonstrated that the resulting host-guest FRET ensemble can be used for quantitative sensing of DNA with an excellent linear dependence of the ratiometric fluorescence intensities ($I_{450}/I_{520}$). The approach opens a new avenue to highly accurate DNA quantification such as real-time PCR, for which very few alternatives exist to the presently employed proprietary dyes. Moreover, this approach provides potential strategy for new genetic method to convert single-wavelength sensor into ratiometric probes.

### 3.6 References

(50) X. J. Zhang, Y. M. Zhang, Z. Wang, Y. Chen and Y. Liu, ChemistrySelect, 2016, 1, 685-690.


Chapter 4. Precise Supramolecular Control of Surface Coverage Densities on Polymer Micro- and Nanoparticles
Chapter 4 Precise Supramolecular Control of Surface Coverage Densities on Polymer Micro- and Nanoparticles

This chapter is derived from the content of the following publication:
Shuai Zhang, Zoe Domínguez, Khaleel I. Assaf, Mohamed Nilam, Thomas Thiele, Uwe Pischel, Uwe Schedler, Werner M. Nau, and Andreas Hennig, Precise Supramolecular Control of Surface Coverage Densities on Polymer Micro- and Nanoparticles, Chem. Sci., 2018, 9, 8575-8581.

In this chapter we report the controlled surface functionalization of micro- and nanoparticles by supramolecular host-guest interactions. Our idea is to exploit the competition of two high affinity guests for binding to the surface-bound supramolecular host cucurbit[7]uril (CB7). To establish our strategy, surface azide groups were introduced to hard-sphere (poly)methylmethacrylate particles with a grafted layer of poly(acrylic acid), and a propargyl derivative of CB7 was coupled to the surface by click chemistry. The amount of surface-bound CB7 was quantified with the high-affinity guest aminomethyladamantane (AMADA), which revealed CB7 surface coverage densities around 0.3 nmol/cm² indicative of a 3D layer of CB7 binding sites on the surface. The potential for surface functionalization was demonstrated with an aminoadamantane-labeled rhodamine (Ada-Rho) as a second high-affinity guest. Simultaneous incubation of CB7-functionalized particles with both high-affinity guests, AMADA and Ada-Rho, revealed a simple linear relationship between the resulting surface coverage densities of the model fluorescent dye and the mole fraction of Ada-Rho in the incubation mixture. This suggests a highly modular supramolecular strategy for the stable immobilization of application-relevant molecules on particle surfaces and a precise control of their surface coverage densities.

4.1 Introduction

The possibility to precisely control the attachment of application-relevant molecules to the surfaces of micro- and nanoparticles creates a powerful platform technology with a large number of conceivable applications. A vast number of different methods have therefore been explored, but the performance of these materials is still limited by the shortcomings of existing surface functionalization methods.\textsuperscript{1-3} For example, the highly specific and strong ($K_a \sim 10^{15}$ M$^{-1}$) binding interaction between the small-molecule ligand biotin with the proteins avidin or streptavidin is popular for surface attachment in initial proof-of-principle studies with nanoparticles,\textsuperscript{1} but it has also been noted that the tetrameric structure of the proteins and their large size (i) may induce crosslinking, (ii) prevent a precise control of conjugate stoichiometry, and (iii) limit the maximum
achievable surface coverage densities.\textsuperscript{4-6} Alternative and refined strategies to reliably functionalize particle surfaces with molecular components are thus highly desired to achieve the transition from proof-of-principle to real applications of nanobioconjugate materials.\textsuperscript{1-3}

Numerous supramolecular host-guest systems have been previously investigated with the goal to equip nanoparticles with molecular recognition capabilities, and this has enabled a large variety of potential applications.\textsuperscript{7-22} The typically \( \mu \text{M} \) to mM affinities of most synthetic host-guest systems ensure reversible binding, which is highly desirable, e.g., in nanoparticle polymer composites, to control nanoparticle assembly, or for drug delivery. However, for surface functionalization aiming towards bioanalytical applications, host-guest complexes are required, which are sufficiently stable at much lower concentrations. To account for the low binding affinity, multivalent systems have been explored, but this strategy sacrifices – similar to the (strept)avidin/avidin system – control over the binding stoichiometry and induces particle crosslinking.\textsuperscript{11, 14, 23, 24}

Cucurbit[n]urils (CB\textit{n}, \( n = 5–8, \ 10, \ \text{and} \ 14 \)) composed of \( n \) glycoluril units comprise a class of biocompatible macrocycles, which stand out from all other supramolecular host molecules by remarkably high binding affinities (\( K_a > 10^{17} \text{ M}^{-1} \))\textsuperscript{25-30} towards certain guest molecules. This exceeds the affinity of biotin with (strept)avidin and clearly suggests CB\textit{n} hosts as a complementary tool in bioconjugation.\textsuperscript{4, 31} Moreover, CBs are highly biocompatible as shown in various applications, e.g., in enzyme and membrane transport assays,\textsuperscript{32-36} for immobilization of proteins and cells on planar surfaces,\textsuperscript{37-39} for enrichment and isolation of proteins by affinity-beads,\textsuperscript{40-42} for supramolecular PEGylation of biopharmaceuticals,\textsuperscript{43} for multi-stimuli-responsive release,\textsuperscript{44} and for protein imaging.\textsuperscript{45} CB\textit{n} hosts were also adsorptively attached to metal surfaces such as planar\textsuperscript{46, 47} and spherical\textsuperscript{48-50} gold surfaces, and to iron oxide nanoparticles through multidentate binding of their carbonyl-fringed portals.\textsuperscript{51, 52} However, this adsorptive surface functionalization affects the host-guest recognition properties of the cavity since one of the portals is involved in surface binding. Moreover, the presence of two carbonyl-fringed portals may lead to particle aggregation.\textsuperscript{46, 51-53}

Herein, we present for the first time the covalent surface modification of small, hard-sphere core-shell particles with cucurbit[7]uril (CB7). To demonstrate this, we use polymer microparticles, also known as beads or microspheres, as well as nanoparticles. These particles play important roles, e.g., in optical tweezers,\textsuperscript{54} drug delivery,\textsuperscript{55} medical imaging,\textsuperscript{56, 57} and in diagnostic, multiplexing bead-based assays,\textsuperscript{58-60} as well as lateral flow immunoassays.\textsuperscript{61, 62} Our
approach affords, similar to the previously established planar surfaces\textsuperscript{37-39} and porous resins of large (>40 µm) sepharose beads,\textsuperscript{40-42} a suitable supramolecular strategy to subsequently immobilize application-relevant molecules. Moreover, we demonstrate herein, that host-guest chemistry allows an unprecedented control of surface functionalization of particles in an easily quantifiable manner.

### 4.2 Particle Synthesis

![Figure 4.1 a) Synthesis of propargyl-CB7 (CB7-OPr). b) Synthesis of CB7-functionalized PMMA microparticles. c) Optical microscopy image (at 40-fold magnification) of 5 mg/ml CB7-functionalized PMMA particles in 10 mM (NH\textsubscript{4})\textsubscript{2}HPO\textsubscript{4}, pH 7.2.](image)

For the synthesis of CB7-functionalized particles, we decided to use copper-catalyzed azide-alkyne click chemistry (Figure 4.1). To obtain the required propargyl-CB7 (CB7-OPr), a different procedure than the recently reported method by Zhang and coworkers was established.\textsuperscript{63} First, monohydroxylated-CB7 was synthesized according to the
method of Bardelang and coworkers. Quantitative conversion into CB7-OPr was then achieved by repeated cycles of crude product resubmission to propargyl bromide and sodium hydride in DMSO, and the identity and purity of CB7-OPr was confirmed by comparison with the reported data (see Electronic Supplementary Information, ESI†). As particles, we used microparticles (mean diameter of 2.55 µm) composed of a compact, hard-sphere poly(methylmethacrylate) (PMMA) core and a grafted layer (111 µmol/g) of poly(acrylic acid) (PAA). These particles were chosen for their ease of handling, optical transparency, and the possibility for microscopic observation, and they were previously characterized by us in detail. In addition, commercially available nanoparticles (mean diameter of 110 nm) with a hard-sphere polystyrene core and surface carboxylic acids were also tested.

Azide groups were introduced by reaction of surface COOH groups with 11-azido-3,6,9-troxaundecan-1-amine (ATA) using standard amide coupling protocols, and CB7 was finally covalently bound to the surface by Cu-catalyzed click chemistry (Figure 4.1 a, b). The resulting particles were washed into 10 mM (NH₄)₂HPO₄, pH 7.2, which was also used for all subsequent experiments. Inspection by optical microscopy indicated no increased tendency for aggregation compared to the PAA- and ATA-functionalized particles, and by IR spectroscopy, which was in accordance with surface-immobilized CB7.

4.3 Quantification of Surface-Bound CB7

The most compelling evidence for surface functionalization with CB7 was obtained by successful extraction of aminomethyladamantane (AMADA) from 10 mM (NH₄)₂HPO₄, pH 7.2 using CB7-functionalized particles (Figure 4.2). The ultra-strong affinity of AMADA to CB7 (Kₐ ca. 10¹⁵ M⁻¹) was previously exploited by us to evaluate various surface quantification methods. Therein, AMADA-putrescine was used as a chemical labelling agent for surface COOH groups by amide formation and the number of surface-bound AMADA was determined by extraction of CB7 and subsequent quantification of remaining CB7 by the fluorescent dye acridine orange (AO). Similarly, remaining AMADA is now quantified by addition of a known concentration of CB7 and AO to the supernatant.
Figure 4.2 Quantification of CB7 on particle surfaces. a) Incubation of CB7-functionalized particles with AMADA and subsequent centrifugation gives a supernatant, which can be analyzed to afford the concentration of remaining AMADA by addition of the fluorescein dye acridine orange (AO) and CB7. b) Dependence of fluorescence spectral changes ($\lambda_{\text{exc}} = 450 \text{ nm}, \lambda_{\text{obs}} = 510 \text{ nm}$) of the supernatant on the volume of added CB7-functionalized particles stock solution (10 mg/mL) during incubation with 25 µM AMADA. And the bottom figure compares CB7-functionalized (filled circles) and ATA-functionalized particles as control (open circles) in 10 mM (NH$_4$)$_2$HPO$_4$, pH 7.2.

In accordance with our expectations, an increase of the fluorescence with increasing volume of the particle stock solution immediately indicated the particle-dependent extraction of AMADA and thus successful immobilization of CB7 on the particle surface (Figure 4.2b). The clearly linear dependence with no indications of the typical curvature of a reversible binding isotherm is consistent with quantitative binding between surface-bound CB7 and AMADA, as well as with quantitative binding in the supernatant analysis (Figure 4.3a). As a consequence, we can determine the average (bulk) CB7 surface coverage densities from the intersection between the linear increase and the plateau region in the inset of Figure 4.2, which indicates the amount of particles required to extract all AMADA from solution.$^{65, 66}$
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and Nanoparticles

Figure 4.3 a) Competitive fluorescence titration ($\lambda_{\text{exc}} = 450$ nm) of 2 µM AO and 1.1 µM CB7 in 10 mM (NH$_4$)$_2$HPO$_4$, pH 7.2. The inset shows the corresponding fluorescence titration ($\lambda_{\text{em}} = 510$ nm) plot normalized to the initial fluorescence intensity and demonstrates quantitative 1:1 binding between AMADA and CB7 in solution. b) Dependence of fluorescence spectral changes ($\lambda_{\text{exc}} = 450$ nm, $\lambda_{\text{obs}} = 510$ nm) of the supernatant on the volume of added CB7-functionalized polymer particles stock solution (10 mg/mL) during incubation with (dimethylaminomethyl)ferrocene (surface coverage density = 5.5 µmol/g). The inset compares CB7-functionalized (filled circles) and ATA-functionalized particles as control (open circles).

This simple method to determine the resulting CB7 loading capacities and surface coverage densities was highly reproducible (Table 4.1, coefficient of variation of ca. 2% for $n = 7$) and enabled us to evaluate various reaction conditions and their reproducibility during surface functionalization in a straightforward manner (Table 4.2). For example, we could show that washing the particles after click reaction with buffer containing EDTA or not had no influence on the resulting surface coverage, which suggests that no copper ions remained on the particle surface (cf. entry #1 and #2 in Table 4.2). Furthermore, we could establish that the click reaction is considerably reproducible. The resulting loading capacities of three different reaction batches varied by less than 1% (Table 4.2, entry #1).
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**Table 4.1** Reproducibility measurements for the quantification of surface-bound CB7.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>loading capacity (µmol/g)</th>
<th>coupling yield (%)</th>
<th>CB7 surface density (nmol/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.66</td>
<td>5.10</td>
<td>0.286</td>
</tr>
<tr>
<td>2</td>
<td>5.81</td>
<td>5.23</td>
<td>0.294</td>
</tr>
<tr>
<td>3</td>
<td>5.52</td>
<td>4.97</td>
<td>0.279</td>
</tr>
<tr>
<td>4</td>
<td>5.88</td>
<td>5.30</td>
<td>0.297</td>
</tr>
<tr>
<td>5</td>
<td>5.63</td>
<td>5.07</td>
<td>0.285</td>
</tr>
<tr>
<td>6</td>
<td>5.74</td>
<td>5.17</td>
<td>0.290</td>
</tr>
<tr>
<td>7</td>
<td>5.80</td>
<td>5.23</td>
<td>0.293</td>
</tr>
<tr>
<td>average</td>
<td>5.72</td>
<td>5.15</td>
<td>0.289</td>
</tr>
<tr>
<td>standard deviation</td>
<td>0.11</td>
<td>0.10</td>
<td>0.006</td>
</tr>
<tr>
<td>coefficient of variation</td>
<td>ca. 2%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 4.2** Reaction results for CB7 surface functionalization.

<table>
<thead>
<tr>
<th>entry #</th>
<th>reaction conditions&lt;sup&gt;b&lt;/sup&gt;</th>
<th>loading capacity (µmol/g)</th>
<th>coupling yield (%)&lt;sup&gt;c&lt;/sup&gt;</th>
<th>CB7 surface density (nmol/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1) 3 h at pH 5.0, 2) 24 h (with EDTA)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>5.69 ± 0.04</td>
<td>5.13 ± 0.03</td>
<td>0.288 ± 0.002</td>
</tr>
<tr>
<td>2</td>
<td>1) 3 h at pH 5.0, 2) 24 h (without EDTA)</td>
<td>5.67</td>
<td>5.11</td>
<td>0.287</td>
</tr>
<tr>
<td>3</td>
<td>1) 3 h at pH 5.0, 2) 48 h</td>
<td>6.2</td>
<td>5.6</td>
<td>0.32</td>
</tr>
<tr>
<td>4</td>
<td>1) 3 h at pH 7.2, 2) 24 h</td>
<td>4.4</td>
<td>4.0</td>
<td>0.22</td>
</tr>
<tr>
<td>5</td>
<td>1) 6 h at pH 7.2, 2) 24 h</td>
<td>4.8</td>
<td>4.3</td>
<td>0.24</td>
</tr>
</tbody>
</table>

<sup>a</sup> Values were determined with the AMADA assay (Section 5.1). <sup>b</sup> Functionalization of COOH surface groups with 1) azide groups was performed in pH 5.0 or pH 7.2 buffer for 3 h or 6 h and then with 2) CB7-OPr in a click reaction for 24 h or 48 h (see Section 4.1 and 4.2 for details). <sup>c</sup> With respect to 111 µmol/g surface COOH. <sup>d</sup> From three replicates.
The resulting CB7 surface coverage densities (ca. 0.3 nmol/cm², i.e. 1.8 CB7 molecules/nm² or a loading capacity of ca. 5.7 µmol/g) were significantly higher than the value for a CB7 monolayer on planar gold surfaces (ca. 0.08 nmol/cm²),\textsuperscript{38} which is in accordance with a grafted 3D layer of PAA on the particle surface and thus a 3D layer of surface-bound CB7. Interestingly, the overall coupling yields in our two-step functionalization protocol were in very good agreement with typical coupling yields for amide formation only (ca. 5% with respect to COOH groups of surface PAA),\textsuperscript{6,66} which suggests that the second step, the click reaction to attach CB7 onto the surface, is nearly quantitative. As controls, azide-functionalized particles lacking CB7 gave no change in fluorescence intensity (inset of Figure 4.2b), which excluded unspecific binding of AMADA to the particle surface. As additional controls, identical values for the CB7 surface coverage density were determined by extraction of (dimethylaminomethyl)ferrocene (Figure 4.3b), which further excludes any unspecific binding. Moreover, poly(styrene) nanoparticles with surface carboxylic acid groups could be similarly surface-functionalized and analyzed, which afforded CB7 surface coverage densities of ca. 0.1 nmol/cm² (Figure 4.4).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure4.png}
\caption{a) Dependence of fluorescence spectral changes ($\lambda_{\text{exc}} = 450$ nm, $\lambda_{\text{obs}} = 510$ nm) of the supernatant on the volume of added CB7-functionalized (poly)styrene nanoparticles stock solution (10 mg/mL) during incubation with 4.56 µM AMADA. b) Respective titration plot.}
\end{figure}

\section*{4.4 Supramolecular Surface Functionalization}

The possibility to reliably immobilize application-relevant molecules on the surface of CB7-functionalized particles was demonstrated with an aminoadamantyl-labeled rhodamine (Ada-Rho, from Prof. Uwe Pischel) as a model fluorescent dye (Figure 4.5). Immobilization was simply achieved by addition of CB7-functionalized particles to a
buffered aqueous solution containing Ada-Rho and washing, which gave a bright red fluorescence from surface-bound Ada-Rho in fluorescence microscopy, whereas the size of the particles remained same as judged by comparison of the bright-field images of particles with and without Ada-Rho. The absorbance as well as the fluorescence of the supernatant decreased linearly with increasing amounts of CB7-functionalized particles indicating quantitative binding (Figure 4.5). As controls, when the CB7 cavity was blocked by pre-incubation with the stronger binder AMADA, the particles were unable to extract Ada-Rho. Similarly, no Ada-Rho extraction was observed with ATA-functionalized particles (Figure 4.6).

Figure 4.5 a) Surface functionalization of CB7 particles with Ada-Rho. b) Fluorescence microscopy images ($\lambda_{\text{exc}} = 546 \text{ nm}$) of CB7-functionalized particles (5 mg/mL) with surface-bound Ada-Rho.
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and Nanoparticles

Figure 4.6 Variation of a) fluorescence intensity ($\lambda_{\text{exc}} = 520 \text{ nm}$, $\lambda_{\text{obs}} = 580 \text{ nm}$) and b) absorbance ($\lambda_{\text{obs}} = 570 \text{ nm}$) of the supernatant (150 µL) of a mixture of 10 µM Ada-Rho and varying amounts of CB7 particles (10 mg/mL) after centrifugation and dilution to 2000 µL in 10 mM (NH$_4$)$_2$HPO$_4$, pH 7.2.

Figure 4.7 Variation of a) fluorescence intensity ($\lambda_{\text{exc}} = 520 \text{ nm}$, $\lambda_{\text{obs}} = 580 \text{ nm}$) and b) absorbance ($\lambda_{\text{obs}} = 570 \text{ nm}$) of the supernatant (220 µL) of a mixture containing 1.7 µM Ada-Rho and varying amounts of ATA-functionalized particles (10 mg/mL) after centrifugation and dilution to 2000 µL in 10 mM (NH$_4$)$_2$HPO$_4$, pH 7.2.

As in the case of AMADA (see above), the loading capacity of Ada-Rho could be determined from the intersection between the linear decrease and the plateau region of the titration plots, which revealed that the amount of Ada-Rho on the surface was significantly lower (3.0 µmol/g) than the amount of CB7 (5.7 µmol/g). Such a dependence of the surface coverage density on the size of the immobilized molecule is common, and may be due to steric repulsion between molecules at adjacent binding sites, a size-dependent diffusion through the grafted 3D PAA network, or a conformational
rearrangement of the PAA chains in response to the presence of the hydrophobic dye, which may block otherwise available binding sites.

Figure 4.8 Quantification of remaining CB7 binding sites on Ada-Rho-functionalized particle surfaces. a) Fluorescence spectra of different samples. b) Dependence of fluorescence spectral changes ($\lambda_{\text{exc}} = 450$ nm, $\lambda_{\text{obs}} = 510$ nm) of the supernatant on the volume of added functionalized polymer particles stock solution (10 mg/mL) during incubation with 25 µM AMADA in 10 mM (NH$_4$)$_2$HPO$_4$, pH 7.2.

Figure 4.9 Extraction with CB7-functionalized beads using solutions of 0.44 µM Ada-Rho (solid circles) or a mixture of 0.44 µM Ada-Rho and 0.22 µM AMADA (open circles). The intersections refer to final Ada-Rho surface loadings of 3.0 µmol/g and 1.8 µmol/g, respectively.

The latter explanation can be ruled out, because particles, in which all binding sites were saturated with Ada-Rho could still extract additional 2.8 µmol/g AMADA from solution (Figure 4.8) and the sum of Ada-Rho (3.0 µmol/g) and AMADA (2.8 µmol/g) was in excellent agreement with the CB7 surface coverage density determined with AMADA only (5.7 µmol/g). In other words, approximately 50% of all available
CB7 binding sites were occupied when the particles were incubated with Ada-Rho first, and then, the remaining binding sites could be occupied in a second incubation step with AMADA.\textsuperscript{70} A different result was, however, obtained, when the CB7-functionalized particles were incubated with a mixture of both, Ada-Rho and AMADA. This reduced the amount of Ada-Rho that can be extracted with a specific amount of particles (Figure 4.9), which is consistent with a competitive occupation of the CB7 binding sites by AMADA, and suggests an elegant method to control the surface coverage density of CB7-functionalized particles.

4.5 Supramolecular Control of Surface Coverage Densities

Figure 4.10 Variation of absorbance ($\lambda_{\text{obs}} = 570$ nm) of the supernatant (370 µL) containing a mixture of 2 nmol AMADA and Ada-Rho (molar fraction of Ada-Rho: a) 1.0, b) 0.75, c) 0.5, and d) 0.25) incubated varying amounts of CB7 functionalized particles after centrifugation and dilution to 2000 µL in 10 mM (NH$_4$)$_2$HPO$_4$, pH 7.2. The insets show the dependence of absorbance at $\lambda_{\text{obs}} = 570$ nm on the volume of added particle stock solution (7 mg/mL).
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In order to investigate in detail how the presence of AMADA during incubation with Ada-Rho influences the resulting surface coverage densities, varying amounts of CB7 particles were incubated with mixtures containing different mole fractions of AMADA and Ada-Rho. This indicated that the amount of particles, which are required to extract Ada-Rho from solution, remained approximately the same despite varying total Ada-Rho concentrations in the incubation solution (Figure 4.10). Consequently, the surface coverage densities of Ada-Rho as determined by our extraction-based surface quantification method depended linearly on the molar fraction of AMADA and Ada-Rho over its entire range (Figure 4.11).

Figure 4.11 Dependence of resulting Ada-Rho surface coverage densities on the mole fraction of the competitor AMADA in mixtures of AMADA and Ada-Rho (6 µM total concentration).

It is noteworthy that such a simple linear relationship came as a surprise, because first, the number of available binding sites is different for AMADA and Ada-Rho (see above), and second, the resulting surface concentrations of two competitors should, in a thermodynamically equilibrated mixture, also depend on their binding affinities.30,70 We conclude that the occupation of the CB7 binding cavities is diffusion-limited, which leads to a kinetically controlled competitive occupation of the CB7 binding sites with AMADA and Ada-Rho. In addition to the mechanistic insights, our results clearly demonstrate that the surface coverage densities of application-relevant functional molecules on CB7-functionalized particles can be precisely adjusted by using two competitive cavity binders. This allowed us to prepare a series of particles with exactly known Ada-Rho surface coverage densities in a straightforward manner, which could then be analyzed by fluorescence spectroscopy and microscopy (Figure 4.12 and 4.13).
Therein, a linear increase in the fluorescence intensity was observed at low surface coverage densities (up to ca. 1 µmol/g), whereas higher surface fluorophore coverage densities did not lead to a further increase in fluorescence intensity.

**Figure 4.12** a) Steady-state fluorescence spectra ($\lambda_{exc} = 520$ nm) of Ada-Rho-labeled particles (0.2 mg/mL) in 10 mM (NH$_4$)$_2$HPO$_4$, pH 7.2 with increasing surface coverage densities of Ada-Rho (adjusted by using mixtures of Ada-Rho and AMADA, see Figure 4.10). The inset shows the dependence of the normalized fluorescence intensity ($\lambda_{em} = 585$ nm) on the surface coverage densities. b) Dependence of the mean fluorescence intensity within the regions of interest (ROIs) in fluorescence microscopy images (see Figure 4.12). Error bars represent the standard deviation ($n = 3$).

This result is consistent with our previous observations with covalently bound surface fluorophores and originates most likely from self-quenching due to an increased probability of non-fluorescent aggregate formation at high surface coverage densities.\(^6\)\(^6\) It is important to note that uncertainties arising from the covalent surface modification protocol needed to be previously eliminated by control measurements with absolute fluorometry involving an integrating sphere set-up,\(^6\) whereas in this report, we exploit supramolecular host-guest chemistry to unambiguously determine and control the fluorophore surface coverage densities. Another interesting consideration is that our competition-based surface functionalization protocol applies an excess of two competitors for a limited number of accessible binding cavities, which could lead to a more homogeneous distribution of surface coverage densities within a particle population than methods relying on substoichiometric amounts of reagent. The latter require a very efficient mixing to prevent a local depletion during reagent addition to a reaction mixture.
Figure 4.13 Fluorescence microscopy images (546 nm bandpass filter) of Ada-Rho-labeled particles (5 mg/mL) with increasing surface coverage densities of Ada-Rho (expressed as loading capacities, LC). The white circles represent the automatically assigned ROIs by the software ImageJ.

Figure 4.14 Time-dependent dissociation of Ada-Rho from CB7-functionalized particles in presence of the competitor AMADA. Ada-Rho functionalized particles (2 mg/mL) were incubated with 50 mM (open circles) or 100 mM (filled circles) AMADA in 10 mM (NH₄)₂HPO₄, pH 7.2 and the fluorescence of the supernatant (λ<sub>exc</sub> = 520 nm, λ<sub>obs</sub> = 585 nm) was measured after certain time intervals. As control, the fluorescence of the supernatant did not show any release of surface-bound Ada-Rho in absence of the competitor AMADA (filled squares).

In contrast, when the particles were first surface-functionalized with Ada-Rho and subsequently incubated with high concentrations of AMADA for a longer period, a
slow dissociation of Ada-Rho from the surface was observed (Figure 4.14). This clearly demonstrates the principal reversibility of the host-guest interaction despite the strong affinity. Furthermore, it enables the determination of exchange kinetics on particles surfaces and presents a complementary conceptual framework for kinetic studies of host-guest systems.\textsuperscript{71, 72}

### 4.6 Conclusions

In conclusion, we have introduced polymer particles surface-functionalized with the supramolecular host molecule CB7. Therefore, we synthesized monofunctionalized CB7 bearing a propargyl group, which can be covalently bound to azide-functionalized surfaces of polymer particles by click chemistry. The successful reaction and the resulting number of CB7 molecules on the particle surface was reliably quantified and the ease of subsequently introducing other molecular components was demonstrated with the fluorescent dye Ada-Rho. Compared with covalent conjugation strategies, simple mixing of the two components in water suffices and other additives such as coupling reagents are not required. Overall, this provides a reliable host-guest-based surface functionalization method in water with wide-ranging perspectives. We have shown that it allows to precisely control surface coverage densities, which unfolds numerous perspectives in varying areas. For example, it allows the investigation of fluorescence quenching mechanisms on surfaces and validation of absolute fluorometry,\textsuperscript{6, 69} the systematic testing of hitherto unverified, theoretical quantification models for spherical substrates in x-ray photoelectron spectroscopy (XPS),\textsuperscript{68} as well as straightforward construction of multimodal probes for medical imaging,\textsuperscript{73} potentially for clinical applications, which require regulatory clearance and thus metrological traceability.\textsuperscript{67} Varying surface coverage densities can have a significant impact on the efficiency of nanoparticle-based diagnostics and therapy.\textsuperscript{1-3, 74-77} We also demonstrated the controllable release of surface-bound Ada-Rho by a stronger cavity binder. This suggests the use of CB7-functionalized particles for the construction of stimuli-responsive release systems.

### 4.7 References

Chapter 4 Precise Supramolecular Control of Surface Coverage Densities on Polymer Micro- and Nanoparticles


(18) A. Wei, *Chem. Commun.*, 2006, 0, 1581-1591.


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(70) The reported binding constants for the cationic ammonium forms of 1-aminoadamantane and 1-aminomethyladamantane are \(1.7 \times 10^{14} \text{ M}^{-1}\) and \(7.7 \times 10^{14} \text{ M}^{-1}\), respectively (ref. 27). As a consequence of this strong affinity, the resulting host-guest complex with CB7 has also an appreciable kinetic stability. Assuming a maximally diffusion-controlled association reaction (\(k_{\text{ass}} = 7.4 \times 10^{9} \text{ M}^{-1} \text{ s}^{-1}\)), the dissociation half-life times are \(>4.4 \text{ h}\) and \(>20 \text{ h}\) for 1-aminoadamantane and 1-aminomethyladamantane.


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Summary and Outlook

The thesis describes the synthetic procedure of clickable mono-functionalized cucurbit[$n$]urils ($n = 6, 7$) and mainly on the exploration of new applications based on the mono-functionalized CB$n$. we explore two applications based on mono-functionalized CB7.

One is to attach fluorescein on the outer rim of CB7, thus a host-guest FRET pair based on the macrocyclic host CB7 labelled with carboxyfluorescein as acceptor and the nucleic stain DAPI as donor and guest was designed. This supramolecular FRET pair is used for quantitative sensing of DNA with an excellent linear dependence of the ratiometric fluorescence intensities. Compared with commercial DNA staining dyes, the FRET pair with dual-wavelength can offer more precise results. Such approach can be applied to quantify DNA accurately and potentially be used in real-time PCR.

For the second application, mono-functionalized CB7 is introduced on the surface of nano-/macro-particles. After that, incubation of CB7-functionalized particles with two high-affinity guests, resulted in a simple linear relationship between surface coverage densities of one fluorescent guest and the mole fraction of this guest in the incubation mixture. This suggests a highly modular supramolecular strategy for the stable immobilization of application-relevant molecules on particle surfaces and a precise control of their surface coverage densities.

For the future plan based on mono-functionalized CB7, I would like to suggest new members in our group to synthesize CB7 derivatives attathed with organelle recognition functional groups and apply this into cell culture. The other promising project is to connect two CB7 toghther to dimerize guests and biomolecules, for example to form proteins dimer through host-guest interactions.
Summary and Outlook
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Supporting Information for Chapter 3

Materials and Methods

CB7 and CB7-OPr were synthesized according to literature procedures.\(^1\) DAPI (4’,6-diamidino-2’-phenylindole dihydrochloride) and Type III salmon sperm DNA (ca. 2000 base pair)\(^2\) were commercial samples (Sigma Aldrich) and used as received without further purification. 6-FAM-azide was purchased from Baseclick and used as received. Reagents for synthesis were from Fluka, Carl Roth, and Sigma-Aldrich. TLC was performed on SIL G/UV254 (Macherey-Nagel). Buffers and salts were of the highest purity available from Fluka and Sigma-Aldrich and used as received.

UV-Vis absorption measurements were performed with a Varian Cary 4000 spectrophotometer and the fluorescence spectra were recorded on a Varian Cary Eclipse spectrofluorometer. All measurements were performed at ambient temperature, except for the fluorescence-based DNA melting experiment, which was recorded in a temperature range from 20 to 90 °C with rectangular quartz cuvettes with 1-cm optical path length. The fluorescence lifetimes were measured by time-correlated single-photon counting (FLS920, Edinburgh Instruments Ltd.). For the lifetime measurements, DAPI and CB7-CF were excited at 373 nm by using a diode laser (PicoQuant, LDH-P-C 375, fwhm ca. 50 ps) and the fluorescence was followed at 470 nm. \(^1\)H NMR spectra were recorded on a Jeol ECS400 MHz and chemical shifts (\(\delta\)) are reported in ppm relative to TMS (\(\delta = 0\) ppm). Mass spectra were recorded on a Bruker MALDI TOF spectrometer; HCCA (\(\alpha\)-cyano-4-hydroxycinnamic acid) was used as a matrix.

Synthesis and Characterization of CB7-CF

8.7 mg (7.2 μmol) CB7-OPr was dissolved in 0.7 mL anhydrous DMSO and 6 mg (13 μmol) 6-FAM-azide was added. Then, 10 mg (50 μmol) sodium L-ascorbate was added into 2.8 mL 55% DMSO aqueous solution containing 4.47 mg (28 μmol) CuSO\(_4\) and 14.86 mg (28 μmol) tris(benzyltriazolylmethyl)amine (TBTA). These two solutions were mixed and stirred at room temperature for 24 h. 50 mL diethyl ether was added, and the resulting precipitate was washed three times with 25 mL MeOH. Drying under high vacuum afforded a dark solid. The crude product containing unreacted CB7-OPr, 6-FAM-Azide, and CB7-CF, was purified by column chromatography. In detail, the mixture was dissolved in 600 μL H\(_2\)O/HCOOH 1:1 and loaded onto silica gel 60 (0.04-0.063 mm) and the column was eluted with H\(_2\)O/AcOH/HCOOH 10:10:1.5. The eluent was collected in fractions of 2 mL (50 fractions) and the fractions containing pure CB7-CF were combined. Evaporation of the solvent gave 4 mg (2.4 μmol, 33%
yields) CB7-CF as a brown solid. The product identity was confirmed by mass spectrometry (Figure S2) and NMR (Figure S3). $^1$H NMR (400 MHz, D$_2$O with excess $p$-xylene diamine$^2$), δ (ppm) = 8.55 (H1), 8.44 (H2), 7.96 (H7), 7.75 (H6), 7.48 (H4), 7.46 (H3), 7.37 (H5, H10, Hb$^{\text{free}}$), 6.58 (Hb$^{\text{bound}}$), 5.73 (Hd), 5.52 (He), 4.79 (H11, HOD), 4.24 (H14, Hc), 4.00 (H13), 3.92 (Ha$^{\text{free}}$), 3.49 (Ha$^{\text{bound}}$), 1.90 (H12). MALDI-TOF MS calculated for [CB7-CF] 1675.43, found 1675.67; calculated for [CB7-CF–H$^+$+Na$^+$] 1697.41, found 1697.63.

Fig. S1 Synthesis of CB7-CF.

Fig. S2 MALDI-TOF mass spectra of CB7-CF (HCCA matrix, positive mode).
Fig. S3 $^1$H NMR spectra of CB7-CF in D$_2$O with excess $p$-xylene diamine (see ref. 2) to aid in solubilization (magenta peak assignments).

(a)

(b)

d = 5.9 Å

d = 10.8 Å

Fig. S4 DFT-optimized structures (B3LYP/3-21G*) of different possible co-conformations for the CB7-CF/DAPI complex (in gas phase). The distance (d) between the center of mass of the CF and DAPI is given in Å. The structure shown in (a) was found to be more stable than that in (b) by 4.9 kcal/mol.
**Fig. S5** Fluorescence titrations ($\lambda_{ex} = 360$ nm) with increasing amounts of DAPI (0 to 7.4 µM) in the presence of (a) 1 µM CB7, (b) 1 µM CB7-CF as well as 20 µM AMADA, and (c) only DAPI. All experiments were performed in 10 mM (NH$_4$)$_2$HPO$_4$, pH 7.2.

**Fig. S6** (a) Excitation spectrum ($\lambda_{em} = 520$ nm) of 1 µM CB7-CF in 10 mM (NH$_4$)$_2$HPO$_4$, pH 7.2. (b) Emission spectra of 1 µM CB7-CF at varying excitation wavelengths.

**Data Analysis**

Binding constants were calculated from the fluorescence titrations (Fig. 3b, inset) by assuming a 1:1 complex stoichiometry and performing a nonlinear fitting according to eq. S5. [G] and [HG] are the concentrations of the uncomplexed guest and the host-guest complex, and [G]$_0$
and \([H]_0\) are the total concentrations of guest and host. \(K_a\) is the association constant of the guest with the host. \(a\) and \(b\) are constants depending on instrumental settings of the fluorometer.

At a suitable low concentration, the fluorescence intensity of a fluorophore has a linear relationship with fluorophore concentration (eq. S1).

\[
I = a[G] + b[HG]
\] (S1)

Conservation of mass requires that:

\[
[G]_0 = [G] + [HG]
\] (S2)

From eq. 1 and eq. S2 one obtains:

\[
I = (a - b) \cdot [G] + b \cdot [G]_0
\] (S3)

According to the law of mass action, the concentration of host-guest complex under equilibrium conditions is:

\[
[G] = \frac{[G]_0 - [H]_0 - \frac{1}{K_a}}{2} + \sqrt{\frac{([H]_0 + [G]_0 + \frac{1}{K_a})^2}{4} - [H]_0[G]_0}
\] (S4)

Substitution of eq. S4 into eq. S3 affords eq. S5, which can be implemented into fitting programs and which was used in the fitting in Figure 3b, inset, in the main text.

\[
I = (a - b) \cdot \frac{[G]_0 - [H]_0 - \frac{1}{K_a}}{2} + \sqrt{\frac{([H]_0 + [G]_0 + \frac{1}{K_a})^2}{4} - [H]_0[G]_0 + b \cdot [G]_0}
\] (S5)

**Fig. S7** Variation of the fluorescence spectrum (\(\lambda_{ex} = 360\) nm) of 1 \(\mu\)M DAPI in 10 mM \((NH_4)_2HPO_4\), pH 7.2, upon addition of CB7. Inset: CB7 concentration dependence of the fluorescence intensity at 470 nm, \(R^2 = 0.998\). The curve was fitted by 1:1 binding stoichiometry.
Fig. S8 Fluorescence spectra ($\lambda_{ex} = 360$ nm) of 1 μM CB7-CF and 7.4 μM DAPI without (black line) and with (red line) 32 μM AMADA in 10 mM (NH$_4$)$_2$HPO$_4$, pH 7.2. The addition of AMADA leads to a strong fluorescence decrease of CB7-CF.

Fig. S9 Time-resolved fluorescence decay traces of 5 μM DAPI alone (blue) and in the presence of 10 μM CB7-CF (black) and CB7 (red); instrument response function is shown in magenta. $\lambda_{exc} = 373$ nm, $\lambda_{obs} = 450$ nm.

Table S1. Fitting results of lifetime decays of DAPI with and without CB7 or CB7-CF in 10 mM (NH$_4$)$_2$HPO$_4$, pH 7.2 with 0.5% DMSO.

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<th>Rel%</th>
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<td>$\tau_2 = 3.05$</td>
<td>0.010</td>
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</table>

$^a$ Without DMSO.
Fig. S10 Fluorescence spectra ($\lambda_{ex} = 360$ nm) of 0.5 μM DAPI and 1 μM CB7-CF with increasing concentration of salmon sperm DNA (0 to 0.5 mg/mL). The inset shows the corresponding change in the fluorescence intensity ratio at 450 nm and 520 nm.

Fig. S11 Variation of the fluorescence spectrum ($\lambda_{ex} = 360$ nm) of 0.5 μM DAPI in 10 mM (NH₄)₂HPO₄, pH 7.2 upon addition of salmon sperm DNA. Inset: Dependence of the fluorescence intensity at 450 nm on DNA concentration.

Fig. S12 Variation of the fluorescence spectrum ($\lambda_{ex} = 497$ nm) of 0.5 μM SYBR Green in 10 mM (NH₄)₂HPO₄, pH 7.2 upon addition of salmon sperm DNA. Inset: Dependence of the fluorescence intensity at 520 nm on DNA concentration.
Fig. S13 Normalized fluorescence intensity of DAPI, SYBR-Green, and DAPI/CB7-CF in dependence on DNA concentration. Note that for DAPI/CB7-CF the ratiometric response is shown (compare Figures S10-S12).

**CF Labelling Degree Calculation**

As the FRET efficiency is >99%, the DAPI fluorescence in Fig. 3a should not increase upon addition of CB7-CF. However, there is a slight fluorescence increase by ca. 30% at 460 nm of DAPI, attributed to complexation by unlabelled CB7. For comparison, complexation of DAPI by unlabelled (parent) CB7 affords a fluorescence increase by a factor of 12 (Fig. S8, at 460 nm). The ratio of these two factors (1.3 divided by 12) is 11%, which represents the degree of unlabelled CB7 corresponding to ca. 90% labelling efficiency.

**Limit of Detection**

The limit of detection (LOD) of the FRET probe was determined according to eq. S6 from the slope of the calibration curve, $b$, and the standard deviation of the y-intercept (inset in Fig. 4a) of the calibration curve, $S_a$ (with $n \geq 7$). The molecular weight of salmon sperm DNA was taken from the literature as $1.3 \times 10^6$ g/mol.$^2$

$$\text{LOD} = \frac{3S_a}{b}$$  \hspace{1cm} (S6)

**References**

Materials and Methods

Reagents for synthesis were from Fluka, Sigma-Aldrich, or Acros Organics NV, Belgium. Azobisisobutyronitrile (AIBN) was from Molekula GmbH, Germany. Analytical thin layer chromatography (TLC) was performed on SIL G/UV_{254} (Macherey-Nagel). Buffers and salts were of the highest purity available from Fluka, Sigma-Aldrich and used as received. Methyl methacrylate was destabilized with aluminum oxide (neutral for chromatography 50-200 µm, 60A) prior to use. Poly(styrene) nanoparticles (average diameter 110 nm) with surface carboxylic acid groups were from Kisker Biotech GmbH & Co. KG (Steinfurt, Germany). Cucurbit[7]uril (CB7) was synthesized according to established literature methods. Functionalization of microspheres was carried out in standard Eppendorf plastic tubes. Concentrations of fluorescent dye stock solutions were determined using an extinction coefficient of 90800 M^{-1} cm^{-1} in acetonitrile/phosphate buffer for Ada-Rho. The photoreactions were carried out in a Luzchem LZC-4V photoreactor with 14 G8T5 lamps from SANKYO DENKI (six lamps from top and four lamps from each side) in a 250-mL quartz glass round bottom flask. IR spectra were recorded on a Bruker Equinox 55 equipped with an IRScope and ATR unit and are reported as wavenumbers in cm^{-1} with band intensities indicated as s (strong), m (medium), w (weak), and br (broad). ^{1}H spectra were recorded on a Jeol ECS400 MHz and chemical shifts (δ) are reported in ppm relative to TMS (δ = 0 ppm). ESI-MS was performed on a Bruker HCT ultra and mass spectra are reported as mass-per-charge ratio m/z (intensity in %, [assignment]). Absorbance measurements were performed with a Varian Cary 4000 spectrophotometer. Fluorescence was measured with a Varian Cary Eclipse spectrofluorometer equipped with a temperature controller. All spectroscopic measurements were performed in 3.5 mL polymethacrylate fluorimeter cuvettes (Sigma-Aldrich) or 3.5-mL quartz glass cuvettes (Hellma Analytics, Müllheim, Germany). Fluorescence microscopy images were captured by an Axiovert 200 (Zeiss) with a filter (BP 546/12, LP 590) through an Evolution QEi Media Cybernetics camera by using a 40× objective, and processed with the software ImageJ 1.48 V (https://imagej.nih.gov/ij/index.html).
Abbreviations
AIBN: azobisisobutyronitrile; AMADA: 1-aminomethyladamantane; ATA: 11-azido-3,6,9-trioxaundecan-1-amine; CB7: cucurbit[7]uril; EDC: 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride salt; DMSO: dimethyl sulfoxide; EDTA: ethylenediaminetetraacetic acid; ESI-MS: electrospray ionization mass spectrometry; FT-IR: Fourier transform infrared spectroscopy; HR-MS: high resolution mass spectrometry; MES: 2-(N-morpholino)ethanesulfonic acid; m.p.: melting point; NMR: nuclear magnetic resonance spectroscopy; IR: infrared; PAA: poly(acrylic acid); PMMA: poly(methyl methacrylate); rcf: relative centrifugal force; r.t.: room temperature; SDS: sodium dodecyl sulfate; TBTA: tris(benzyltriazolylmethyl)amine; TLC: thin layer chromatography.
Synthesis

Synthesis of CB-OH

1 g (0.86 mmol) CB7, synthesized as previously reported, was dissolved in 125 mL of a mixture of Millipore water and 12 M HCl (3:2 v/v) and introduced in a 250-mL quartz glass round bottom flask under nitrogen. 65 µL (0.62 mmol) 30% hydrogen peroxide in H2O was added and the solution was vigorously stirred during irradiation of UV light (254 nm) for 48 h. The reaction was monitored by 1H NMR by taking aliquots of the reaction mixture. The solvent was then evaporated under reduced pressure affording a white solid. The crude product containing a mixture of CB7-(OH)n (with n = 0, 1, 2, 3) was purified by column chromatography. Therefore, the mixture was dissolved in 950 µL H2O/HCOOH 1:1 and loaded onto silica gel 60 (0.04-0.063 mm) and the column was eluted with H2O/AcOH/HCOOH 10:10:1.5. The eluent was collected in fractions of 2 mL (>250 fractions) and the fractions containing pure CB7-OH (as confirmed by TLC, see Fig. S2) were combined. Evaporation of the solvent gave 150 mg CB7-OH as a white solid. The 1H NMR was in accordance with the reported spectrum, and the identity and purity of the obtained material was additionally confirmed by mass spectrometry (Fig. S1) and TLC (Fig. S2).
Fig. S1 Mass spectrum of CB7-OH with 1 mM cystamine in Millipore water. Traces of CB6 were presumably enriched during column chromatography (cf. Fig. S2).

Fig. S2 TLC of CBn mixture and CB7 derivatives.
Synthesis of CB7-OPr

20 mg (17 µmol) CB7-OH was dissolved in 1.5 mL anhydrous DMSO. 10 mg (0.4 mmol) NaH (95% purity as solid) was added, and the mixture was stirred at room temperature for 3 h. Subsequently, the mixture was cooled to 0 °C, 0.5 mL (4.4 mmol) propargyl bromide was added, and the reaction mixture was stirred at room temperature for 12 h. 50 mL diethyl ether was added, and the resulting precipitate was three times triturated with 25 mL MeOH. Drying under high vacuum afforded a pale yellow solid, which was subjected a second time to the same reaction conditions. This gave the desired CB7-OPr quantitatively as confirmed by mass spectrometry (Fig. S3), $^1$H NMR (Fig. S4), and IR spectroscopy: MS (ESI, +ve): 685.3 (100, [CB7-OPr+Cys+2H]$^{2+}$). IR (KBr) cm$^{-1}$ 806 (s), 968 (s), 1234 (s), 1322 (s), 1376 (s), 1473 (s), 1733 (s), 2120 (w), 2933 (m), 2998 (w), 3432 (s).

Fig. S3 Mass spectrum of CB7-OPr with 1 mM cystamine in Millipore water.
Fig. S4 $^1$H NMR spectra of CB7-OPr in 1% DCl in D$_2$O in absence (top) and presence of substoichiometric amounts (middle) or excess (bottom) of the cavity binder p-xylene diamine (pXD).
Synthesis of Ada-Rho

A mixture of 1-bromoadamantane (0.5 g, 2.32 mmol) and 1,4-diaminobutane (1.89 g, 11.62 mmol) was heated in a sealed tube at 190 °C for 20 h. Then, 2 M HCl (60 mL) and diethylether (60 mL) were added, and the aqueous layer was separated and made alkaline with 50% aq. NaOH (60 mL). The product was extracted with diethylether, the organic layer was dried over anhydrous MgSO₄, and after removal of the solvent, a pale yellow semi-solid was obtained (0.26 g, 1.17 mmol, 50% yield). The product was used without further purification in the next step. ¹H NMR (400 MHz, CDCl₃) δ (ppm): 2.70 (t, J = 6.6 Hz, 2H), 2.58 (t, J = 6.6 Hz, 2H), 2.06 (s, 3H), 1.71-1.53 (m, 12H), 1.51-1.44 (m, 4H).

N-Adamantanyl-1,4-diaminobutane (15 mg, 0.067 mmol) and triethylamine (15 mg, 0.14 mmol) were dissolved in 1 mL dry tetrahydrofuran and the solution was stirred for 5 minutes. Afterwards, lissamine rhodamine B sulfonyl chloride (38.9 mg, 0.067 mmol), dissolved in 5 mL dry tetrahydrofuran, was added and the mixture was heated at 70 °C in a sealed tube for 72 hours. After that time, the solvent was removed and the crude was subjected to purification by silica gel column chromatography, using dichloromethane/methanol (9/1) as eluent. This procedure yielded the final product Ada-Rho as purple solid (18 mg, 0.024 mmol, 35% yield). ¹H NMR (400 MHz, (CD₃)₂SO) δ (ppm): 8.52 (m, 1H, NH-adamantyl), 8.43 (d, J = 1.9 Hz, 1H, CH-phenylsulfonate), 8.05 (t, J = 6.0 Hz, 1H, SO₂NH), 7.96 (dd, J = 8.0 and 1.9 Hz, 1H, CH-phenylsulfonate), 7.49 (d, J = 8.0 Hz, 1H, CH-phenylsulfonate), 7.11-6.92 (m, 6H, 6 × CH-xanthylum), 3.64 (q, J = 7.0 Hz, 8H, 4 × CH₃CH₂N), 2.92 (dt, J = 6.0 Hz, 2H, SO₂NHCH₂CH₂CH₂CH₂NH), 2.88-2.78 (m, 2H, SO₂NHCH₂CH₂CH₂CH₃H₂NH), 2.09 (s, 3H, 3
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$\times$ CH-adamantyl), 1.84 (s, 6H, $3 \times$ CH$_2$-adamantyl, SO$_2$NHCH$_2$CH$_2$CH$_2$NH), 1.21 (t, $J = 7.0$ Hz, 12H, $4 \times$ CH$_3$CH$_2$N) ppm; $^{13}$C NMR (101 MHz, (CD$_3$)$_2$SO) $\delta$ 157.3$^a$, 157.1$^a$, 155.0$^a$, 147.9$^a$, 141.6$^a$, 133.0$^a$, 132.6$^b$, 130.7$^c$, 126.6$^c$, 125.7$^c$, 113.7$^a$, 113.4$^b$, 95.4$^b$, 56.1 (quart. C-adamantyl), 45.3 ($4 \times$ CH$_3$CH$_2$N), 42.0 (SO$_2$NHCH$_2$CH$_2$CH$_2$NH), 38.5 (SO$_2$NHCH$_2$CH$_2$CH$_2$NH), 37.5 ($3 \times$ CH$_2$-adamantyl), 35.2 ($3 \times$ CH$_2$-adamantyl), 28.4 ($3 \times$ CH$_2$-adamantyl), 26.3 (SO$_2$NHCH$_2$CH$_2$CH$_2$NH), 23.5 (SO$_2$NHCH$_2$CH$_2$CH$_2$NH), 12.5 ($4 \times$ CH$_3$CH$_2$N) ppm.

$^a$ quaternary C corresponding to xanthylum or phenylsulfonate skeleton; $^b$ CH corresponding to xanthylum skeleton; $^c$ CH corresponding to phenylsulfonate skeleton

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**Fig. S5** $^1$H NMR spectrum of Ada-Rho in (CD$_3$)$_2$SO.
Preparation and Characterization of CB7-Functionalized Particles

Synthesis of PMMA/PAA Microparticles

**PMMA microparticles:** Poly(methylmethacrylate) (PMMA) polymer microbeads were prepared by dispersion polymerization. In order to obtain a narrow size distribution, a custom-made modified reaction chamber was employed to control the polymerization temperature and the stirring speed of the sealed polymerization flasks. The bead size can be controlled by parameters like monomer, stabilizer, and radical initiator concentration as well as reaction temperature. A typical procedure for bead preparation is given in the following: 7 g poly(vinylpyrrolidine) K90 (average molecular weight 1.300,000) and 600 mg (1.35 mmol) sodium bis(2-ethylhexyl) sulfosuccinate (aerosol-OT) were dissolved in 170 ml methanol. The mixture was transferred to the reaction flask containing 15 ml (14.1 g, 141 mmol), destabilized methyl methacrylate, and 200 mg (1.2 mmol) azobisisobutyronitrile (AIBN). The sealed flask was placed in the reaction chamber, where the polymerization was performed at a stirring speed of 20 rpm at a temperature of 55 °C for 21 hours. After cooling down to room temperature, the resulting bead suspension was poured into 600 ml water to precipitate the PMMA beads. After decantation of the water, the beads were washed several times with water and removed from...
the solution by centrifugation in 50 ml Falcon tubes. These washing-centrifugation cycles were repeated until no more methyl methacrylate could be detected in the supernatant.

**Bead functionalization:** A 300 ml of a 0.17 % (w/v) suspension of 2.55 µm PMMA particles were mixed with 9 g (125 mmol) acrylic acid, 150 mg (0.52 mmol) sodium dodecylsulfate (SDS), and 1.50 ml of 0.15 M benzophenone as photoinitiator in methanol. After 5 min equilibration time, the suspension was exposed for 8 min to UV light with 20 mW/cm² intensity, while the suspension was vigorously stirred. After irradiation, the suspension was centrifuged and washed several times with distilled water to remove unreacted compounds, additives, and homopolymer. Absence of PAA in solution was confirmed by conductometry (conductance <10 µS/cm).

**Azide-Functionalized Particles**

**ATA-functionalized PMMA microparticles:** 10 mg PMMA microparticles (111 µmol/g COOH) were washed into 660 µL reaction buffer (0.1 M MES, pH 5.0 or 10 mM (NH₄)₂HPO₄, pH 7.2) by repeated centrifugation, supernatant removal and resuspension cycles. Subsequently, 60 µL of 40 mM 11-azido-3,6,9-trioxaundecan-1-amine (ATA) in reaction buffer were added. The reaction was started by adding 80 µL of 100 mg/mL (0.52 M) EDC hydrochloride freshly dissolved in 4 °C cold water. Total reaction volume was 800 µL, final conditions were 12.5 mg/mL microparticles (corresponding to 1.11 µmol COOH groups), 2.4 µmol ATA, and 42 µmol EDC. After 3 h or 6 h reaction time, the particles were washed into 1 mL 10 mM (NH₄)₂HPO₄, pH 7.2 to afford a 10 mg/mL stock solution of ATA-functionalized particles.

**ATA-functionalized PS nanoparticles:** 10 mg commercially available PS nanoparticles were washed into 660 µL reaction buffer (0.1 M MES, pH 5.0) and 18 µL of 400 mM 11-azido-3,6,9-trioxaundecan-1-amine (ATA) in reaction buffer were added. The reaction was started by adding 74 µL of 1 g/mL (5.2 M) EDC hydrochloride freshly dissolved in 4 °C cold water. Total reaction volume was 800 µL, final conditions were 12.5 mg/mL nanoparticles, 7.2 µmol ATA, and 389 µmol EDC. After 3 h reaction time, the particles were washed into 1 mL 10 mM (NH₄)₂HPO₄, pH 7.2 (by 10x centrifugation at 28600 rcf for 40 min) to afford a 10 mg/mL stock solution of ATA-functionalized particles.
Fig. S7 Optical microscopy images (at 40fold magnification) of a) PMMA-PAA and b) ATA-functionalized microparticles.
CB7-Functionalized Particles

32 µL of a 3 mM CB7-OPr stock solution in DMSO was added to 400 µL ATA-functionalized particles (10 mg/mL) in 10 mM (NH₄)₂HPO₄, pH 7.2 buffer, and 10 µL of a freshly prepared 20 mM sodium ascorbate solution in 10 mM (NH₄)₂HPO₄, pH 7.2 was mixed with 20 µL of a 10 mM Cu²⁺/tris(benzyltriazolylmethyl)amine (TBTA) in 55% DMSO stock solution. Both solutions were combined and the resulting reaction mixture was shaken for 24 h. The solution was then centrifuged (3.5 min, 16000 rcf) and the supernatant was discarded after centrifugation. The particles were thoroughly washed (20 times) with 10 mM (NH₄)₂HPO₄, pH 7.2 or 10 mM (NH₄)₂HPO₄, 1 mM EDTA, pH 7.2 (which gave identical results), and the total buffer volume was finally adjusted to afford a particle concentration of 10 mg/mL. Nanoparticles were prepared in the same way except for centrifugation at 28600 rcf for 40 min.

Fig. S8 (a) IR spectra (KBr pellet) of ATA-functionalized particles before (black) and after (red) click reaction with CB7-OPr, and a mixture of ATA-functionalized particles and CB7-OPr (blue). (b) IR spectra of ATA-functionalized particles before (black) and after (red) click reaction with CB7-OPr in a narrow range.
Surface Quantification Methods
Quantification of Surface-Bound CB7 with AMADA

For quantification of surface-bound CB7, aliquots from the as prepared CB7-functionalized particle stock solution (10 mg/mL) were diluted with 10 mM (NH₄)₂HPO₄, pH 7.2 to afford a final volume of 475 µL, to which 25 µL 60 µM AMADA was added (final AMADA concentration 3 µM). The mixture was briefly vortexed, sonicated, and then shaken for 5 min. After centrifugation for 10 min at 16000 rcf, 400 µL of the supernatant was transferred to a new Eppendorf tube and centrifugation was repeated. 350 µL of the final supernatant were transferred into a 3-mL poly(methylmethacrylate) cuvette containing 1290 µL 10 mM (NH₄)₂HPO₄, pH 7.2, and 160 µL 10 µM CB7 and 200 µL 10 µM acridine orange were added. The final volume was 2000 µL and final concentrations were 0.8 µM CB7, 1 µM AO, and 0-0.525 µM AMADA (depending on the amount of AMADA extracted).

Then, a fluorescence spectrum was recorded (λ_ex = 450 nm) and the fluorescence intensities at λ_em = 520 nm were plotted against the volume of particle stock solution and normalized to the fluorescence intensity in absence of particles (V = 0 µL). Linear fitting of the initial linear increase of the titration plot gave the slope of the fitted line, a, and the y-intercept, b (see, for example, inset of Fig. 2b in the main manuscript).

Assuming quantitative binding between AMADA and CB7 on the particle surface, the loading capacity of the particles, i.e. the amount of CB7 per particle mass, can be obtained from the intersection of the fitted line and the final plateau value in the titration plot, y∞, indicating that all extraction of the molecule by the CB7-functionalized particles is complete. The volume of particle stock solution needed to completely extract the molecule, x, is thus:

\[ x = \frac{y_{\infty} - b}{a} \]  

[EQ1]

The mass of particles needed to completely extract the molecule, m, is then obtained by the mass concentration of particle stock solution, ρ_{Particle}:

\[ m = \frac{\rho_{Particle} (y_{\infty} - b)}{a} \]  

[EQ2]

This gives the loading capacity of the particles as the mass of particles needed to extract a specific amount of molecules, n = c·V, as

\[ \text{Loading capacity} \left( \text{in } \frac{\mu\text{mol}}{g} \text{ of particles} \right) = \frac{a·c·V}{\rho_{Particle} (y_{\infty} - b)} \]  

[EQ3]

where a is slope and b the y-intercept of the fitted line, y∞ is the final plateau value in the titration plot, c·V is the amount of the molecule to be extracted (e.g., 25 µL of 60 µM AMADA stock solution or 500 µL of 3 µM during incubation), and ρ_{Particle} is the mass concentration of CB7.
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Particle stock solution (here: 10 mg/mL). The reproducibility of the method was evaluated by repeated measurements with a randomly selected batch of CB7-functionalized particles (Table S1).

**Quantification of Surface-Bound Ada-Rho**

Varying volumes of the CB7-functionalized particles (10 mg/mL) were transferred into 1.5 mL Eppendorf tubes and 10 mM \((\text{NH}_4)_2\text{HPO}_4\), pH 7.2 was added to achieve a total volume of 80 µL. Then, 100 µL of 20 µM Ada-Rho was added and the mixture was incubated for 17 min. After addition of 20 µL Triton X-100 to prevent unspecific absorption of Ada-Rho (sodium dodecylsulfate performed equally well), the mixture was briefly vortexed and then centrifuged for 27 min at 16000 rcf. Afterwards, 150 µL of the supernatant was diluted into 1850 µL 10 mM \((\text{NH}_4)_2\text{HPO}_4\), pH 7.2 and absorption and fluorescence spectra were recorded.

The fluorescence intensities at \(\lambda_{em} = 580\) nm and the absorbance values at \(\lambda = 570\) nm were plotted against the volume of particle stock solution (Fig. S10), and linear fitting of the initial linear decrease of the titration plot gave the slope of the fitted line, \(a\), and the y-intercept, \(b\).

The loading capacity of the particles, i.e. the mass of particles needed to extract a specific amount of Ada-Rho, was calculated similarly as above (see EQ3) by additionally considering the amount of unspecifically absorbed Ada-Rho, \(n_{\text{unspecific}}\):

\[
\text{Loading capacity (in } \frac{\mu\text{mol}}{g} \text{ of particles)} = \frac{a\cdot(c\cdot V - n_{\text{unspecific}})}{\rho_{\text{Particle}}(y_{\infty} - b)} \quad [\text{EQ4}]
\]

As controls, AMADA-blocked CB7-functionalized particles were prepared by incubation of 100 µL 10 mg/mL CB7-functionalized particles and 1 mL 10 µM AMADA in 10 mM \((\text{NH}_4)_2\text{HPO}_4\), pH 7.2 for 17 min and subsequent centrifugation. The pellet was resuspended in 10 mM \((\text{NH}_4)_2\text{HPO}_4\), pH 7.2 and then subjected to the procedure above to test whether Ada-Rho could still bind to the particles (Fig. S12).

Unoccupied binding sites on the Ada-Rho and CB7-functionalized were quantified by the AMADA-based method (Fig. S13, see Section 5.1 for experimental procedure).
Supramolecular Control of Surface Coverage Densities

In order to prepare particles with different surface coverage densities of Ada-Rho, varying volumes of CB7 functionalized particles (7 mg/mL) were transferred into 1.5 mL Eppendorf tubes and 10 mM (NH₄)₂HPO₄, pH 7.2 was added to achieve a total volume of 140 µL. Then, 200 µL of a solution containing Ada-Rho and AMADA (10 µM total concentration) was added and the mixture was incubated for 17 min. After addition of 100 µL 1% SDS, the mixture was briefly vortexed and then centrifuged for 27 min at 16000 rcf. Afterwards, 370 µL of the supernatant was diluted into 1630 µL 10 mM (NH₄)₂HPO₄, pH 7.2 and absorption spectra were recorded.

The absorbance values at λ = 570 nm were plotted against the volume of particle stock solution (Fig. S15), and linear fitting of the initial linear decrease of the titration plot gave the slope of the fitted line, \( a \), and the y-intercept, \( b \). The surface coverage density with Ada-Rho was then calculated using EQ4, in which \( c \) is the concentration of Ada-Rho only. The dependence of the resulting surface coverage density on the molar fraction of AMADA is shown in Fig. 4 in the main text.

Particles for fluorescence microscopy were then prepared by incubating 40 µL of 10 mg/mL CB7 functionalized particles with 200 µL of a solution containing Ada-Rho and AMADA (10 µM total concentration) for 17 min, addition of 100 µL 1% SDS and centrifugation for 27 min. The particles were finally washed with 10 mM (NH₄)₂HPO₄, pH 7.2 and the particle concentration was adjusted to 5 mg/mL. The fluorescence of the particle suspensions was determined by fluorescence spectroscopy (Fig. 5a in the main text) and by fluorescence microscopy (Fig. 5b and Fig. S16). For the latter, 5 µL of the particle suspension were deposited in the center of a Thermo SCIENTIFIC 76×26 mm clear-white glass slide and a 22×22 mm ROTH #1 cover glass was placed on the particle suspension to ensure that particles distribute homogeneously between glass slide and cover glass. The images were captured with a Zeiss Axiovert 200 and an Evolution QEi Media Cybernetics imaging camera through a 40× objective using a BP 546/12, LP 590 filter set. The dependence of the fluorescence intensities within the field of view on the surface coverage densities with Ada-Rho (Fig. 5b) was obtained by automatic assignment of the regions of interest (ROIs) and averaging the intensity within all ROIs with the software ImageJ 1.48 V (Fig. S16). The brightness and contrast of the image in the main manuscript (Fig. 3b) was enhanced. The images in the SI (Fig. S16) are unedited.
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Supporting Figures and Tables

Supporting Tables S1 and S2

Table S1. Reproducibility measurements for the quantification of surface-bound CB7.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>loading capacity (µmol/g)</th>
<th>coupling yield (%)</th>
<th>CB7 surface density (nmol/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.66</td>
<td>5.10</td>
<td>0.286</td>
</tr>
<tr>
<td>2</td>
<td>5.81</td>
<td>5.23</td>
<td>0.294</td>
</tr>
<tr>
<td>3</td>
<td>5.52</td>
<td>4.97</td>
<td>0.279</td>
</tr>
<tr>
<td>4</td>
<td>5.88</td>
<td>5.30</td>
<td>0.297</td>
</tr>
<tr>
<td>5</td>
<td>5.63</td>
<td>5.07</td>
<td>0.285</td>
</tr>
<tr>
<td>6</td>
<td>5.74</td>
<td>5.17</td>
<td>0.290</td>
</tr>
<tr>
<td>7</td>
<td>5.80</td>
<td>5.23</td>
<td>0.293</td>
</tr>
<tr>
<td>average</td>
<td>5.72</td>
<td>5.15</td>
<td>0.289</td>
</tr>
<tr>
<td>standard deviation</td>
<td>0.11</td>
<td>0.10</td>
<td>0.006</td>
</tr>
<tr>
<td>coefficient of variation</td>
<td>ca. 2%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table S2. Reaction results for CB7 surface functionalization. a

<table>
<thead>
<tr>
<th>entry #</th>
<th>reaction conditionsb</th>
<th>loading capacity (µmol/g)</th>
<th>coupling yield (%)c</th>
<th>CB7 surface density (nmol/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1) 3 h at pH 5.0, 2) 24 h (with EDTA)d</td>
<td>5.69 ± 0.04</td>
<td>5.13 ± 0.03</td>
<td>0.288 ± 0.002</td>
</tr>
<tr>
<td>2</td>
<td>1) 3 h at pH 5.0, 2) 24 h (without EDTA)</td>
<td>5.67</td>
<td>5.11</td>
<td>0.287</td>
</tr>
<tr>
<td>3</td>
<td>1) 3 h at pH 5.0, 2) 48 h</td>
<td>6.2</td>
<td>5.6</td>
<td>0.32</td>
</tr>
<tr>
<td>4</td>
<td>1) 3 h at pH 7.2, 2) 24 h</td>
<td>4.4</td>
<td>4.0</td>
<td>0.22</td>
</tr>
<tr>
<td>5</td>
<td>1) 6 h at pH 7.2, 2) 24 h</td>
<td>4.8</td>
<td>4.3</td>
<td>0.24</td>
</tr>
</tbody>
</table>

a Values were determined with the AMADA assay (Section 5.1). b Functionalization of COOH surface groups with 1) azide groups was performed in pH 5.0 or pH 7.2 buffer for 3 h or 6 h and then with 2) CB7-OPr in a click reaction for 24 h or 48 h (see Section 4.1 and 4.2 for details). c With respect to 111 µmol/g surface COOH. d From three replicates.
Supporting Figures S9 to S19

**Fig. S9** Competitive fluorescence titration ($\lambda_{\text{exc}} = 450 \text{ nm}$) of 2 µM AO and 1.1 µM CB7 in 10 mM (NH$_4$)$_2$HPO$_4$, pH 7.2. The inset shows the corresponding fluorescence titration ($\lambda_{\text{em}} = 510 \text{ nm}$) plot normalized to the initial fluorescence intensity and demonstrates quantitative 1:1 binding between AMADA and CB7 in solution.

**Fig. S10** Dependence of fluorescence spectral changes ($\lambda_{\text{exc}} = 450 \text{ nm}$, $\lambda_{\text{obs}} = 510 \text{ nm}$) of the supernatant on the volume of added CB7-functionalized polymer particles stock solution (10 mg/mL) during incubation with (dimethylaminomethyl)ferrocene (surface coverage density = 5.5 µmol/g). The inset compares CB7-functionalized (filled circles) and ATA-functionalized particles as control (open circles).
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Fig. S11 a) Dependence of fluorescence spectral changes ($\lambda_{\text{exc}} = 450$ nm, $\lambda_{\text{obs}} = 510$ nm) of the supernatant on the volume of added CB7-functionalized (poly)styrene nanoparticles stock solution (10 mg/mL) during incubation with 4.56 µM AMADA. b) Respective titration plot.

Fig. S12 Variation of a) fluorescence intensity ($\lambda_{\text{exc}} = 520$ nm, $\lambda_{\text{obs}} = 580$ nm) and b) absorbance ($\lambda_{\text{obs}} = 570$ nm) of the supernatant (150 µL) of a mixture of 10 µM Ada-Rho and varying amounts of CB7 particles (10 mg/mL) after centrifugation and dilution to 2000 µL in 10 mM (NH$_4$)$_2$HPO$_4$, pH 7.2.

Fig. S13 Variation of a) fluorescence intensity ($\lambda_{\text{exc}} = 520$ nm, $\lambda_{\text{obs}} = 580$ nm) and b) absorbance ($\lambda_{\text{obs}} = 570$ nm) of the supernatant (220 µL) of a mixture containing 1.7 µM Ada-Rho and varying amounts of ATA-functionalized particles (10 mg/mL) after centrifugation and dilution to 2000 µL in 10 mM (NH$_4$)$_2$HPO$_4$, pH 7.2.
**Fig. S14** Incubation of Ada-Rho with AMADA-blocked CB7-functionalized particles. a) Fluorescence spectra of different samples. b) Dependence of fluorescence spectral changes ($\lambda_{exc} = 520$ nm, $\lambda_{obs} = 570$ nm) of the supernatant on the volume of added AMADA-blocked CB7 functionalized polymer particles stock solution (10 mg/mL) during incubation with 2.5 µM Ada-Rho in 10 mM (NH$_4$)$_2$HPO$_4$, pH 7.2.

**Fig. S15** Quantification of remaining CB7 binding sites on Ada-Rho-functionalized particle surfaces. a) Fluorescence spectra of different samples. b) Dependence of fluorescence spectral changes ($\lambda_{exc} = 450$ nm, $\lambda_{obs} = 510$ nm) of the supernatant on the volume of added functionalized polymer particles stock solution (10 mg/mL) during incubation with 25 µM AMADA in 10 mM (NH$_4$)$_2$HPO$_4$, pH 7.2.

**Fig. S16** Extraction with CB7-functionalized beads using solutions of 0.44 µM Ada-Rho (solid circles) or a mixture of 0.44 µM Ada-Rho and 0.22 µM AMADA (open circles). The intersections refer to final Ada-Rho surface loadings of 3.0 µmol/g and 1.8 µmol/g, respectively.
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Fig. S17 Variation of absorbance ($\lambda_{\text{obs}} = 570$ nm) of the supernatant (370 µL) containing a mixture of 2 nmol AMADA and Ada-Rho (molar fraction of Ada-Rho: a) 1.0, b) 0.75, c) 0.5, and d) 0.25) incubated varying amounts of CB7 functionalized particles after centrifugation and dilution to 2000 µL in 10 mM (NH$_4$)$_2$HPO$_4$, pH 7.2. The insets show the dependence of absorbance at $\lambda_{\text{obs}} = 570$ nm on the volume of added particle stock solution (7 mg/mL).
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**Fig. S18** Fluorescence microscopy images (546 nm bandpass filter) of Ada-Rho-labeled particles (5 mg/mL) with increasing surface coverage densities of Ada-Rho (expressed as loading capacities, LC). The white circles represent the automatically assigned ROIs by the software ImageJ.

**Fig. S19** Time-dependent dissociation of Ada-Rho from CB7-functionalized particles in presence of the competitor AMADA. Ada-Rho functionalized particles (2 mg/mL) were incubated with 50 mM (open circles) or 100 mM (filled circles) AMADA in 10 mM (NH₄)₂HPO₄, pH 7.2 and the fluorescence of the supernatant ($\lambda_{\text{exc}} = 520$ nm, $\lambda_{\text{obs}} = 585$ nm) was measured after certain time intervals. As control, the fluorescence of the supernatant did not show any release of surface-bound Ada-Rho in absence of the competitor AMADA (filled squares).

**References**

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Chem. Comm., 2019, 55, 671-674  
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Materials, 2019, 12, 349  
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