Characterization and Synthesis of Cyclodextrin Inclusion Complexes and their Applications as Fluorescent Probes for Sensing Biomacromolecules

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Abstract

Cyclodextrins (CDs) are macrocycles composed of several glucose units bound through α-1,4 glycosidic linkages. They can be chemically modified to display functional groups on their primary or secondary rim. CDs display these groups in defined geometries ideally suited to bind biomacromolecules. Moreover, CDs have a hydrophobic cavity that allows them to form stable host-guest complexes with lipophilic molecules. This combination of functionality and guest binding ability makes CDs important scaffolds for the design of functional supramolecular systems.

This thesis explored the interaction of heptakis-[6-deoxy-6-(2-aminoethylsulfanyl)]-β-cyclodextrin (1) with many hydrophobic guest molecules. The binding constants of CD host-guest interactions were measured using ITC and fluorometry-based approaches. These studies revealed 1 to form the highest affinity 1:1 cyclodextrin-guest complexes reported to date. This thesis then explored the use of CD inclusion complexes as biomacromolecular sensors.
CD 1 and its derivatives were used to develop self-assembling sensors. First, a library of polycationic CDs with differing charge distribution was synthesized. The sensing motif was synthesized by covalently linking a quinolinium fluorophore to lithocholic acid (LCA). The CD-based binding motifs and the LCA-based sensing motif self-assemble through host-guest interactions (i.e. 1 binding to LCA displays a $K_a = 5.52 \times 10^7$ M$^{-1}$). These inclusion complexes were then used as an array of self-assembling sensors capable of differentiating between pure and contaminated samples of heparin (anticoagulant).

To capitalize on the promise of CD 1 a new technique was explored to functionalize a single amine of 1. The technique relies on an S to N acyl transfer from a guest molecule to a CD host resulting in the mono-acylation of the host. The importance of the linker between the guest and the reactive acylating agent was fully explored. Furthermore, two CD probes are synthesized and are shown to display differential fluorescent responses with a small series of proteins.
Acknowledgments

This section allows me to thank the people that have been in my life this past five years. I need to thank them for the respect, patience and love that they have shown me. Without them I would not be the man that I am today.

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<th>Abbreviation</th>
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<tr>
<td>2, 6-ANS</td>
<td>6-anilino-2-naphthylsulfonic acid</td>
</tr>
<tr>
<td>A</td>
<td>Absorbance</td>
</tr>
<tr>
<td>Abs</td>
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<td>DIC</td>
<td>Diisopropyl carbodiimide</td>
</tr>
<tr>
<td>Abbreviation</td>
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</tr>
<tr>
<td>DMAP</td>
<td>4-(Dimethylamino)pyridine</td>
</tr>
<tr>
<td>DMF</td>
<td>N,N-dimethylformamide</td>
</tr>
<tr>
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</tr>
<tr>
<td>DS</td>
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h  hour

HS  Heparan sulfate

IC_{50}  Half maximal inhibitory concentration

ICD  induced circular dichroism

iPrOH  iso-Propanol

ITC  Isothermal Titration Calorimetry

K_a  association constant

k_{cat}  catalysis rate constant

k_{observed}  observed rate constant

k_{uncatalyzed}  uncatalyzed rate constant

\lambda_{max}  maximum absorbance wavelength

L  Liters

LCA  Lithocholic acid

LDA  Linear Discriminant Analysis

LMWH  Low Molecular Weight Heparin

M  Mass parent ion

M  Molar

m/z  Mass to Charge ratio

MALDI-MS  Matrix-Assisted Laser Desorption Ionization Mass Spectroscopy

mCPBA  meta-Chloroperbenzoic acid

xx
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDCK cells</td>
<td>Madin-Darby Canine Kidney Cells</td>
</tr>
<tr>
<td>MeI</td>
<td>Methyl iodide</td>
</tr>
<tr>
<td>MeOH</td>
<td>Methanol</td>
</tr>
<tr>
<td>MHz</td>
<td>Mega Hertz</td>
</tr>
<tr>
<td>mol</td>
<td>mole</td>
</tr>
<tr>
<td>MsCl</td>
<td>Methanesulfonyl chloride</td>
</tr>
<tr>
<td>MSNP</td>
<td>Mechanised Silica Nanoparticles</td>
</tr>
<tr>
<td>MSNT</td>
<td>1-(2-mesitylenesulfonyl)-3-nitro-1H-1,2,4-triazole</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular Weight</td>
</tr>
<tr>
<td>NaH</td>
<td>Sodium hydride</td>
</tr>
<tr>
<td>NaOH</td>
<td>Sodium hydroxide</td>
</tr>
<tr>
<td>NaN₃</td>
<td>Sodium azide</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
</tr>
<tr>
<td>NP</td>
<td>Nanoparticle</td>
</tr>
<tr>
<td>NTB²⁻</td>
<td>2-nitro-5-thiobenzoate</td>
</tr>
<tr>
<td>Nu</td>
<td>Nucleophile</td>
</tr>
<tr>
<td>OSCS</td>
<td>Oversulfated Chondroitin sulfate</td>
</tr>
<tr>
<td>ovt.</td>
<td>Overnight</td>
</tr>
<tr>
<td>PAA</td>
<td>Polyacrylic acid</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffer Saline</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet Derived Growth Factor</td>
</tr>
<tr>
<td>PDGFR</td>
<td>Platelet Derived Growth Factor Receptor</td>
</tr>
<tr>
<td>PGA</td>
<td>Polyglutamic acid</td>
</tr>
<tr>
<td>pH</td>
<td>decimal logarithm of hydrogen ion activity</td>
</tr>
<tr>
<td>Ph₃Cl</td>
<td>Trytil chloride</td>
</tr>
<tr>
<td>pI</td>
<td>Isoelectric point</td>
</tr>
<tr>
<td>pKₐ</td>
<td>decimal logarithm of the acid dissociation constant value of</td>
</tr>
<tr>
<td>PPI</td>
<td>Protein-Protein Interactions</td>
</tr>
<tr>
<td>Py</td>
<td>Pyridine</td>
</tr>
<tr>
<td>Rᵣ</td>
<td>Retention factor</td>
</tr>
<tr>
<td>ROE</td>
<td>Rotational Frame Nuclear Overhauser Effect</td>
</tr>
<tr>
<td>ROESY</td>
<td>Rotational Frame Nuclear Overhauser Effect Spectroscopy</td>
</tr>
<tr>
<td>RP-HPLC</td>
<td>Reverse Phase-High-Pressure Liquid Chromatography</td>
</tr>
<tr>
<td>r.t.</td>
<td>Room Temperature</td>
</tr>
<tr>
<td>S°</td>
<td>Entropy</td>
</tr>
<tr>
<td>SPPS</td>
<td>Solid Phase Peptide Synthesis</td>
</tr>
<tr>
<td>SPS</td>
<td>Solid phase synthesis</td>
</tr>
<tr>
<td>SS</td>
<td>Solid State</td>
</tr>
<tr>
<td>Succ</td>
<td>Succinimide</td>
</tr>
<tr>
<td>TBS</td>
<td>tert-butyldimethylsilyl</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin Layer Chromatography</td>
</tr>
<tr>
<td>TOCSY</td>
<td>Total Correlation Spectroscopy</td>
</tr>
<tr>
<td>TsCl</td>
<td>Tosyl chloride</td>
</tr>
<tr>
<td>UFH</td>
<td>Unfractionated Heparin</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra-Violet</td>
</tr>
<tr>
<td>UV/Vis</td>
<td>Ultra Violet/ Visible spectrum</td>
</tr>
<tr>
<td>V_{max}</td>
<td>Maximum rate</td>
</tr>
</tbody>
</table>
List of Appendices

Appendix A Binding isotherm equations

Appendix B Selected NMR spectra
1 A Brief Introduction to Cyclodextrins

Superficially it appears that bubble gum, deodorizers, drugs and tin cans do not share any similarities. However, closer inspection would show that the previous items all share the same additive: cyclodextrins (CDs). Cyclodextrins (CDs) are macrocyclic oligosaccharides linked through α-1,4-glycosidic bonds composed of 6, 7 or 8 glucose units referred to as α-, β- and γ-CDs respectively. CDs are hydrophilic compounds that have a hydrophobic cavity which allows them form inclusion complexes in water (Figure 1.1). Inclusion into the CD cavity alters the physicochemical properties of an included compound. This latter property has captivated the imagination of scientists for decades. Many uses of CDs have been developed by exploiting the hydrophobic nature of the cavity with the specific features of ad-hoc attached molecules. CDs and their inclusion complexes are commonly used in the food and drug industry, as well as in latex paint and other household items. Given the growing importance of CDs extensive reviews have been written on their industrial production, chemistry and uses. This chapter aims to give the reader a brief introduction of CD physicochemical properties, synthesis and some of its uses. For a more extensive review on CDs the reader is directed elsewhere.\(^1\) The work presented in this thesis deals exclusively with β-CDs, and as such from this point forwards the focus shall be placed on this CD.

\begin{figure}[h]
\centering
\includegraphics[width=0.7\textwidth]{CD_shape.png}
\caption{β-CD has a toroidal shape with a wide and a narrow rim, the interior of CD forms a hydrophobic cavity}
\end{figure}

1.1 Discovery and Production

Cavitands are macrocyclic organic compounds capable of acting as hosts, and include molecules such as cyclodextrins, calixarenes, resorcinarenes, cucurbiturils and criptands. The importance of cavitands was recognized in the 1987 Nobel Prize in Chemistry. The prize was awarded jointly
to Donald J. Cram, Jean-Marie Lehn and Charles J. Pedersen "for their development and use of molecules with structure-specific interactions of high selectivity". The concepts developed by these Nobel laureates and other pioneers garnered the modern age of supramolecular chemistry. CDs are one of the most important members of the supramolecular family and are used in the food, drug and home-products industry. This is because CDs are naturally occurring compounds that can be produced by bacterial fermentation in large scales. In contrast, other cavitands have to be synthesized, thus they are not cheaply available in large amounts. The other main advantage of CDs over other cavitands is that they are highly water soluble and non-toxic to humans.

CDs were discovered in 1891 by Villiers, who referred to them as cellulosine. Villiers reported obtaining a crystalline material from a starch digest of Bacillus amylobacter (obtained 3 g of this material per kg of starch). Shardinger later on realized that Villiers Bacillus amylobacter batches were contaminated with Bacillus macerans and that it was the latter bacterial species that were responsible for the synthesis of cellulosine. Shardinger then isolated B. macerans which produces two types of cellulosine; he referred to these isomorphs as crystalline dextrin-α and –β. These dextrins were assumed to be cyclical as they were non-reducing. However, the structures of α- and β-CD were not fully elucidated until 1965 using X-ray spectroscopy. β-CD has a donut or toroidal like structure. The CD cavity is 7.8 Å deep and the inside of the secondary rim has a diameter around 6.2 Å (Figure 1.1). CDs have a shallow truncated cone with a wide rim lined with 14 secondary hydroxyls (the C-2 and C-3 hydroxyls of the glucose units), and a narrow rim lined with 7 primary hydroxyls (C-6 hydroxyls). The interior of the cavity is lined with the hydrogen atoms from the C-3 and C-5 C-H bonds and the lone pairs of the glucose ring oxygens. The aforementioned elements impart a hydrophobic quality to the interior of the CD. Researchers have ascribed the hydrophobicity of the cavity to be similar to that of dioxane. However, it was not until 1954 that CDs ability to encapsulate organic compounds in water became widely accepted. The unusual properties of CD were first investigated by Schardinger, Pringsheim, Freudenburg, Cramer and French. At the time, the idea that CDs could encapsulate other molecules was met with skepticism. Cramer showed that in the presence of CDs a variety of chromophores exhibited changes in their UV absorption. He related these observations with the ability of CD to encapsulate these compounds in solution. Cramer continued to work in the
CD field and in the years leading up to 1966 he recorded the CDs ability to discriminate enantiomers and their ability to carry out catalytic conversions on included guests. The work by Cramer continues to fuel the imagination of researchers to the present day. Since the work of the aforementioned pioneers CD’s have been used as containers for organometallic, organic and inorganic compounds. These complexes are used for enantiomeric separations, the stabilization of drugs and to foster simplistic models of enzymatic reactions. All of these applications rely on the hydrophobic cavity of CDs.

Given the ubiquitous use of CDs as additives, many studies have been done to develop methods for the large scale preparation of CDs. CDs are synthesized from starch or starch derivatives using cyclodextrin glycosyl transferase (CGTase, EC 2.4.1.19). CGTases belong to the α-amylase family, or glycosyl hydrolase family 13, which rely on the α-retaining double displacement mechanism to hydrolyze or disproportionate oligoglucosides. CGTases are unique members of the glycosyl hydrolase family 13 in that they can also catalyze the cyclization of linear α(1→4)-linked glucans. The products of cyclization, an intramolecular transglycosylation reaction, are circular α(1→4)-linked oligoglucosides called cyclodextrins. CGTases obtained from different organisms produce a mixture of α-, β- and γ-CD in different ratios. CGTase is expressed by many microorganisms, however B. macerans have the largest market share of the commercially available CGTases. The types of CDs (α-, β- or γ-) produced by CGTases depends on the type of CGTase, raw material, reaction conditions and complexing agents used during the course of a reaction.

Industrially CDs are produced using one of two production processes: solvent process or non-solvent process. The raw material used in these two processes comes from potatoes. The starch is first liquefied; this process is normally achieved using jet-cooking. At this point the resulting starch solution is cooled down to the temperature at which the CGTase can operate. Afterwards, the “solvent” and “non-solvent” processes diverge. The “solvent” process utilizes complexing agents that influence the selectivity for the formation of a specific CD. Complexing agents form inclusion complexes with the CDs formed during the course of the enzymatic reaction. These inclusion complexes become insoluble and precipitate from solution. Thus, precipitating a specific type of CD (α, β or γ) resulting in a decrease of that type of CD in the reaction mixture. Once CD precipitates less product inhibition for that type of CD will occur.
Thereby resulting in an increased yield of that CD. Thus, the complexing agent reduces the CDs potential for further disproportionation and consequently a higher yield of that CD will be obtained. The judicious choice of complexing agent results in the selective formation of different types of CDs, for example: α-CD is preferentially formed by the use of 1-butanol, cyclohexane and various other C1-8-aliphatic alcohols; β-CD is preferentially formed using toluene and the addition of aromatic rings of similar size; γ-CD is produced by the use of bromotoluene and similarly sized aromatics. Once the reaction has gone to completion, the inclusion complex is removed by filtration or centrifugation. The product is then washed and distilled to remove the excess complexing agent. The CD products are then crystallized. Even though the complexing agent will select for one of the native CDs, the other two will be formed during the course of the reaction. In order to separate the unwanted CDs further purification techniques are employed. Of the three native CDs, β-CD is the easiest to purify from a mixture of CDs. β-CD is easy to separate because it has considerable lower water solubility than that of α- or γ-CD. This means that β-CD can be precipitated and purified in water easily. This fact is taken advantage of in in the non-solvent process which does not rely on complexing agents or organic solvents. As such, the β-CD obtained using the “non-solvent” process can be applied in the food industry without restrictions.

1.2 CD Binding Equilibria

The ability of CD to form inclusion complexes with guests has been thoroughly studied. Some general observations have been made. Complexation will not occur if the guest cannot fit properly into the CD cavity, as such steric factors are important contributors to the overall binding energy. If the guest fits in the CD cavity, the driving force for binding then depends on the thermodynamic interactions between the different components of the system: solvent, guest and CD. The first consideration is the solvent. In water, the solvent plays a key role in binding. This is because the water molecules inside the CD cavity, as well as the water molecules in close proximity to the cavity, are energetically different than the water molecules in the bulk solvent. This is due to the expanded hydrophobic sphere generated by the CD cavity, which forces the bulk solvent to rearrange around the CD cavity. This effect is comparable to a charged group creating an electric field which causes water molecules to form a “rigid” solution cage. Once a guest binds to the CD, water molecules previously trapped in and around the cavity become
“free” and are then able to interact with the bulk water. As such, the decisive role determining the overall complexation thermodynamics of inclusion complex formation in water arises due to the release of water molecules inside and around the CD cavity into the bulk solvent.\textsuperscript{18a,19} The second consideration for binding is the guest. Upon binding to the host, the highly organized water molecules surrounding the hydrophobic guest will be free to interact with the bulk solvent. Thus, inclusion formation in a polar solvent between a CD and a hydrophobic guest leads to an increase of entropy through the hydrophobic effect.

In conclusion, there are four main factors that contribute to energetically favorable binding interactions between CD and a guest.\textsuperscript{18c} One, displacement of polar water molecules by a guest from the apolar CD environment. Two, an increase of hydrogen bonds formed as trapped water molecules return to the bulk solvent. Three, reduction of non-favorable interactions between the bulk (polar) solvent and the apolar guest. Four, increase of Dipole-Dipole interactions between the guest and the CD cavity.

The stoichiometry of inclusion complexes of CD have been extensively studied using X-ray crystallography, NMR, circular dichroism and fluorescence spectroscopy.\textsuperscript{1,18b} The most commonly claimed ratio of CD inclusion complexes is 1:1 guest: host (stoichiometric ratios will be stated in order G: H). However, 2:1 and 2:2 ratios are observed, especially in the case of $\gamma$-CD. 1:1:1 component systems are even rarer; in these cases the third component is usually a solvent molecule or a small organic compound, such as a surfactant or small ion.\textsuperscript{20} It is assumed that complexes are formed through a bimolecular process; as such, complex formation can be expressed using this equilibrium:

$$H + G \rightleftharpoons H \cdot G$$

Where the association binding constant, denoted $K_a$, is expressed as follows:

$$K_a = \frac{[H \cdot G]}{[H][G]}$$

The Gibbs free energy change can be calculated from $K_a$, and the entropic and enthalpic contributions to binding can be obtained from measuring $K_a$ at different temperatures or by calorimetric studies.
Interestingly, the cavities of CDs (which are composed of D-glucose) are chiral. Therefore, CDs exhibit different binding energies for enantiomeric compounds. This quality has been exploited in chromatography for the separation of enantiomers.\textsuperscript{21} Furthermore, given the chiral quality of the CD cavity, it is possible to investigate and use it for sensing applications through circular dichroism or fluorescence spectroscopy. When a non-chiral guest binds to the chiral cavity of CDs, non-chiral chromophores will experience induced circular dichroism (ICD). In these cases the Cotton band that is observed upon complexation of the chromophore by CD will be positive if the excited state moment of the former aligns with the dipole axis in the CD cavity. Vice versa, the ICD Cotton band will be negative if it is perpendicular to that axis. Similarly, given the hydrophobic nature of the CD cavity, environmentally sensitive fluorophores will experience a shift in emission wavelength or increase in their excited lifetime upon entering the cavity.\textsuperscript{22}

1.3 Common Applications of CDs

CDs can change the physicochemical properties of a guest. When a guest compound is included in the CD cavity, the guest becomes surrounded by the atoms in the cavity of CD. Therefore, the hydrophobic groups of the guest that would be in contact with the solvent in the free-state interact with the atoms of the cavity of the CD instead. As a result, the solubility of the complex is dictated by the hydrophilic outer surface of the cyclodextrin. Thus, CDs can alter the physical properties of guests, such as increasing water solubility or reducing volatility (Table 1.1). The properties imparted to the guest due to inclusion formation with CDs have been ubiquitously applied in the drug, textile and home products industries.\textsuperscript{1} Below are some examples of compounds whose physicochemical properties have been altered by complexation with CDs.

To generate the desired inclusion complex CD is mixed with the guest of interest, using water as the solvent, until complexation is achieved. There are three commonly used industrial complexation methods: the slurry method, which uses 40-45% weight by weight of water to CD; the paste method, 20-30% weight by weight; and the dry mixing method. The amount of inclusion formation can be verified using techniques such as differential scanning calorimetry, organic solvent partitioning of the included guest, X-ray and NMR spectroscopy.\textsuperscript{3}
Table 1.1 Examples of CD applications

<table>
<thead>
<tr>
<th>Guest compound</th>
<th>Guest found in, used in or used for the treatment of</th>
<th>Effect of complex formation with CD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Limonin and Naringin</td>
<td>Citrus-based juice</td>
<td>Removes bitterness associated with the guest</td>
</tr>
<tr>
<td>Menthol</td>
<td>Chewing gum</td>
<td>Increases duration of flavor</td>
</tr>
<tr>
<td>Hexa- to Octadecanones</td>
<td>Food-can coatings</td>
<td>Removes stale flavor imparted by guest</td>
</tr>
<tr>
<td>Nicardipine hydrochloride</td>
<td>Angina/High blood pressure</td>
<td>Prolongs shelf-life of drug</td>
</tr>
<tr>
<td>Tolnaftate</td>
<td>Antifungal agent</td>
<td>Increases solubility of drug</td>
</tr>
<tr>
<td>Urushiols</td>
<td>Topical creams used to treat poison ivy rashes</td>
<td>Reduces rashes</td>
</tr>
<tr>
<td>Peroxyacetic acid</td>
<td>Bleaching products</td>
<td>Increases lifetime and thermal stability of products</td>
</tr>
</tbody>
</table>

1.3.1 Food Preparations

CDs are used in the food industry to disguise the taste of unpalatable compounds or to prolong their flavor lifetime. Limonin and Naringin, are abundantly found in citrus foods. These compounds impart an undesirable cloudiness and bitterness to the juice of canned orange slices. Adding β-CD to citrus products masks the bitter taste of limonin and naringin, thus resulting in more palatable citrus juices. Another common use for CD is in chewing gum. CDs can form inclusion complexes with flavor inducing compounds. It has been proven that the duration of flavor, as well as the flavor impact and sensation of coolness is higher with a CD-methoxypropane-1,2 complex. CDs are also ubiquitously found in can coatings. Can coatings can produce an off taste in the stored product. The stale flavor in can-stored foods arises from...
ketones containing 6-18 carbons; coatings made with adsorbed CDs are able to form inclusion complexes with the aforementioned ketones. Therefore, the ketones will bind to the CDs and remain attached to the can coating keeping the undesirable flavor constituents out of the stored food.

1.3.2 Drug Compositions

CDs are commonly used additives in drug preparation; they can improve the lifetime and solubility of a variety of drugs. The rate of photodegradation of Nicardipine hydrochloride, used for the treatment of angina and high blood pressure, was reduced tenfold through complexation with methylated β-CD. Inclusion complexes of various antifungal agents remain bioavailable by the action of CD. Tolnaftate is an anti-fungal agent used for the treatment of jock itch and athlete’s foot. The solubility of tolnaftate was improved drastically by adding β-CD. CDs are also used in cream treatments for poison ivy rashes. Urushiols are water insoluble oils responsible for the irritation of the skin after contact with poison ivy. Washing a poison ivy rash with water will just spread the oils causing larger irritation areas. It has been shown that CDs can bind to urushiols and increase their water solubility, thus making their removal by washing feasible.

1.3.3 Household Items

The boiling point of volatile guests can be increased by complexation with CDs. Upon complexation with β-CD, the CD-Menthol complex is non-odorous. This complex is taken advantage of in laundry sheets. During the drying process the CD-Menthol complex is transferred from the sheet to the fabrics being dried. At the beginning of the drying process, the environment is wet. In this milieu some of the complex will dissociate to give free CD and menthol, thus imparting dried laundry a fresh smell. However, most of the complex adsorbed on the users’ clothing will remain intact. When the fabric is worn again by the user, perspiration or other moisture in the environment will cause dissociation of the remaining complex, thus releasing further menthol and giving the user an impression of freshness. Similarly, complexed fragrant compounds can be released at a slower rate from CDs, thus giving perfumes a longer odorous lifetime. Moreover, CDs can also stabilize peroxyacetic acids used in bleaching products. The lifetimes and thermal stabilities of peroxides are greatly improved when they are
included in the CD cavity. Thus, bleach cleaning house-hold products that contain CD are more water soluble and user friendly.

The list of products containing CDs is immense. Other applications of CDs include their use in heat-curable silicone (masks catalyst for curing until heat is applied), diapers (odor control) and hair preparations (reduces volatility of malodorous mercaptans).³

1.3.4 Directing Chemical Reactivity

Through complexation CDs are able to mask certain portions of a molecule making them inaccessible during a reaction. This allows the chemist to use CDs to control the regioselectivity of a reaction. Thus, a reaction on a CD inclusion complex will affect only the exposed portions of a guest. Furthermore, the CD rim can be chemically modified to increase steric hindrance around the cavity for the prevention of a reaction or to impart greater regioselectivity. Similarly, in order to increase the rate of reaction of an included guest, catalytic groups can be added to the CD, in effect generating an enzyme mimic.

An example where CD controls the regioselectivity of a reaction comes from the synthesis of 2,6-naphthalenedicarboxylic acid. 2,6-Naphthalenedicarboxylic acid is used in high-performance polymers and is synthesized using β-CD in one step with 79% regioselectivity. Without the use of CD a shorter synthesis of 2,6-Naphthalenedicarboxylic acid is not feasible.³⁰ Another example of CD imparting regioselectivity during a reaction comes from the chlorination of anisole. In the presence of α-CD only para-substituted isomers are observed, whereas in the absence of α-CD both meta and para isomers are obtained (Figure 1.2).³¹

![Figure 1.2 Selective aromatic substitution using α-CD](image-url)
1.3.5 CD’s as Enzymatic Models

Enzymes achieve some of their catalytic efficiency by positioning their substrates into the appropriate geometric orientation for reaction. In this way enzymes can increase the local concentration of both the substrate and the catalytic group found in the enzyme’s cavity. Similarly, CD cavities have been employed to effect chemical transformations on included guests. For this reason CDs have been used as models for the study of enzymatic reactivity. There are many examples of CDs being used as artificial enzymes in the literature, here we present a few.

Bender compared the catalytic mechanism of α-chymotrypsin to that of CD. The similarities of these two catalysts for phenylester hydrolysis are as follows. First, both catalysts associate with their guests. Second, the bound ester substrates then react with the catalyst to form an acylated intermediate. Finally, hydrolysis of the acyl intermediate occurs in a slow step. Nevertheless, often times the rate accelerations observed with CDs do not reach the speeds as those observed with enzymes. An example of a comparable rate of reaction of a CD to an analogous enzymatic reaction was observed is the hydrolysis of a p-nitrophenyl ferrocinnamate. Breslow et al noted that by binding the acyl group into the cavity of the CD (instead of the leaving group) the entropy factors governing acylation of the CD’s secondary rim would be favorable. They set out to explore the reactivity of a variety of p-nitrophenyl esters (Figure 1.3).

\[
\begin{align*}
\text{R} & \quad \text{O} \quad \text{O} \quad \text{NO}_2 \\
\text{DMSO: 4mM phosphate buffer pH 6.8} & \quad \text{6} \quad : \quad 4 \\
\text{β-CyD} & \quad \text{R = OR} \\
\text{β-CD as an enzyme model of acylation} & \quad \text{HO} \quad \text{NO}_2 \quad + \quad \text{R} \quad \text{OH}
\end{align*}
\]

In order to increase the rate of acylation by CD, Breslow proposed to complex the acyl group in the CD cavity. He tested his hypothesis using a variety of substrates, including p-nitro
ferrocinnamate (Figure 1.3). Upon complex formation, the \( p \)-nitro ferrocinnamate positions its acetate leaving group next to the hydroxyl groups on the secondary rim of CD. The hydroxyls on the secondary rim then hydrolyze the ester (acetylation of secondary rim) and subsequently water hydrolyzes the newly formed acylated CD. Acetylation experiments were performed in 60% DMSO, 40% buffer solution. The buffer consisted of a 4 mM phosphate buffer at pH 6.8. \( \beta \)-CD concentrations were ranged from 0.2 mM to 20 mM and the concentration of the ester was kept constant at 0.1 mM. Under these conditions the authors observed a \( V_{\text{max}} \) of 0.18 s\(^{-1}\) proceeding 51,000 times faster than hydrolysis.\(^{32}\) In comparison, the \( V_{\text{max}} \) of \( \alpha \)-chymotrypsin for \( p \)-nitrophenyl acetate is 3.1 s\(^{-1}\). This example illustrates the rate increases that can be observed by optimizing the binding geometry between host and guest. The authors claimed that the rate acceleration was achieved by careful design of the guest. Through molecular modeling, it was determined that upon formation of the tetrahedral intermediate, the binding geometry of the \( p \)-nitrophenyl ferrocinnamate inside the CD cavity would remain unaffected. As such, the authors claim that the transition state’s binding energy to the CD is unaltered in comparison to the ground state complex.\(^{33}\) In comparison, the rate acceleration of \( m \)-\( \text{tert} \)-butylphenyl acetate by \( \alpha \)-CD is a mere 500 times faster than acylation by water. In this latter case, the substrates may be better bound than the transition state. The authors postulated that during the hydrolysis of \( m \)-\( \text{tert} \)-butylphenyl acetate, the geometry of the transition state forces the included guest to be pulled away from the cavity. Thus, they imply that the transition state is bound more weakly than the starting material thereby resulting in slower catalysis. Breslow’s analysis of these examples suggest that the catalytic rate increases in the presence of CDs will be larger when less binding energy is lost as the reaction proceeds.

An associated problem with CD catalysis is product inhibition. Product inhibition will be observed if the product is bound equally or more tightly than the starting material. As such, product inhibition is a drawback of CD catalytic systems. An example of a CD catalyst which avoids product inhibition was introduced by Breslow who used ditopic CD hosts and ditopic substrates (Figure 1.4).\(^{34}\) In these systems, after a reaction has taken place, the ditopic guest becomes hydrolyzed giving rise to two monotopic guests. The association constant of the monotopic guests is much weaker compared to that of the ditopic starting material guest, thereby rendering product inhibition less prevalent.
Large increases in the hydrolysis rates of a symmetrical phosphodiester capable of binding the two cavities of the CD dimer were observed. The experiments were performed in 1 mM of both La$^{3+}$ and di-$p$-nitrophenylphosphodiester, 3 mM HOOH and 0.2 mM CD dimer. The relative rate (rate observed over rate uncatalyzed) measured was $4.09 \times 10^7$. The hydrolysis of esters that bind into both cavities was also studied. The dimer CD with adamantyl ester at pH 7 exhibited a relative rate of $2.2 \times 10^5$ in comparison to the uncatalyzed hydrolysis under the following
experimental conditions: dimer $1 \times 10^{-4}$ M, $\text{Cu}^{2+} \ 2 \times 10^{-4}$ M. The relative rate of hydrolysis for the indole ester was $1.8 \times 10^{4}$ showing the guest dependent nature of hydrolysis.\textsuperscript{34}

Another example of enzymatic mimicry by cyclodextrins comes from the work of Bols et al.\textsuperscript{36} The authors synthesized a set of biomimetic oxidases capable of carrying out the same transformation as aldehyde oxidases. Aminophenol oxidase belongs to the family of aldehyde oxidases and catalyzes the oxidation of hydroxyanilines to quinone imines. Aminophenol oxidase displays a $k_{\text{cat}}$ of $10-20 \text{ s}^{-1}$ and a $K_M$ of $1-5 \text{ mM}$ for aminophenols. The same reaction can be achieved using a CD that has been modified to display an aldehyde on either its primary rim or secondary rim. In the presence of HOOH these CD biomimetic catalysts are able to effect the oxidation of aminophenol to a quinone imine. On average Bols’ CD aldehyde catalysts display a $k_{\text{cat}}$ of $5 \times 10^{-3} \text{ s}^{-1}$ and a $K_M$ of $14.6 \text{ mM}$.\textsuperscript{36} In order to study the effects on the rate of reaction, the authors synthesized a series of enzyme mimics which varied in the number of aldehyde groups the CD displayed, as well as in their position ($1^{\text{ary}}$ vs. $2^{\text{ary}}$, Figure 1.5).

![Figure 1.5](image)

**Figure 1.5** CD aldehydes as oxidase mimics. A) CD with varying amounts of aldehydes on their primary rim. B) CD with aldehyde on its secondary rim.

The relative rate of reaction ($k_{\text{observed}}$ over $k_{\text{uncatalyzed}}$) increased in correlation with the number of aldehydes displayed on CD. The increasing order of reactivity observed followed the following trend: 1 aldehyde $< 2$ aldehydes $< 3$ aldehydes. They conclude that this correlation is due to a higher probability of reaction between catalyst and bound substrate when more catalytic groups are available on the surface of CDs. They comment further stating that for this effect to take place, flexibility in the bound substrate must be present. Another observation made by the
authors for their systems is that tighter binding substrates (high $K_M$) tend to have lower $k_{cat}$ values. They comment that this may be due to the substrate being kept in a non-productive conformation (far away from catalyst and unable to explore proper orientation for catalysis due to tight binding). The authors comment further stating that when high $K_M$ are observed inclusion formation does not occur during reaction. This observation was tested using naphthalene 2-sulfonate which binds to the cavity with high affinity. Under these conditions, they observed no inhibition of aminophenol oxidation. This observation further indicates that catalysis by Bols’ systems does not necessarily involve the cavity. The role of CD in these cases might be to position the reacting groups in close proximity. However, if the orientation of both the substrate and CD’s catalytic group is not optimal, the reaction will not be sped up by binding CD.

The examples of enzymatic mimicry by CD have provided useful insight into the mechanisms of enzymes. Presently, molecular biology techniques allow physical organic chemists to alter specific amino acids at enzymatic catalytic sites. These perturbations provide catalytic detail of enzymes without the need to use mimics. Therefore, to the author’s knowledge, the investigation of CDs use as enzyme mimetics is not pursued as heavily as it once was. However, interest in CD chemistry has not diminished. CDs continue to be used ubiquitously in everyday life. The use of CD as drug carriers is also of current interest. Furthermore, given the large surface area that CD display on their primary and secondary rim, these compounds can be used to interact with large protein surfaces. As such, their use for probing protein-protein interactions is also of current interest. All of the aforementioned applications have led chemists to develop selective synthetic manipulations of CDs in order to produce a desired CD product.

1.4 Selective Functionalization of CDs

Cyclodextrin modifications have been used for different reasons, for example: increasing solubility of native CD, appending groups on their surface as enzymatic mimics, synthesis of CD protein conjugates and for making fluorescent probes. Modifying specific hydroxyl groups of CD is challenging as there are 21 hydroxyl groups on the surface of $\beta$-CDs, 7 of those are primary hydroxyl groups. The primary hydroxyl groups are more nucleophilic than their secondary hydroxyl counterparts, and as such can be selectively modified in the presence of the
latter. Many groups have taken advantage of this aspect of carbohydrate chemistry to selectively modify the primary rim. Examples of alkylation, esterification and further selective modifications of CDs have been reviewed elsewhere. This chapter focuses on common selective functionalization strategies used for derivatizing the primary rim of CDs (Figure 1.6).

Figure 1.6 CD derivatization. a) TsCl, NaOH, H₂O, 5h., 25% b) Nucleophile (Nu = NH₂R, N₃⁻, SR⁻, Im, RCOO⁻), solvent, heat. c)i) Ph₃CCl, pyridine, 80°C, 24h, 55%. ii) MeI, NaH, DMF 93%. d) BnCl, NaH, DMSO, 95% e) i) DIBAL-H (2eq.), toluene, 2h., 58% ii) MsCl, Et₃N, DCM, 12h. iii) NaN₃, DMF, 18h., 95%(two steps) iv) DIBAL-H (5eq.), toluene, 5d., 72%. 
The synthesis of CDs has been classified into three types of methodologies. One, “long”, involves multiple protection and deprotection steps. Two, “clever”, inclusion formation ability of CD is exploited to get the desired product efficiently. Third, “sledgehammer”, where indiscriminate reaction leads to statistical product mixtures and in order to get the product of interest complex separations are required. The following section focuses on the first and second type of strategies.

Monoderivatized CDs are often used as enzyme mimics, sensors and supramolecular delivery systems. The first step in the selective monoderivatization of CDs usually involves the formation of an electrophilic position on the primary rim of a CD. The activated hydroxyl group can then be displaced by nucleophilic substitution. The most commonly employed “clever” approach for generating a single electrophilic position on CD is tosylation. Monotosylation is a semi-selective process that gives rise to a mixture of mono- di- or tri-tosylated CDs. Furthermore, the mixture of products that results from monotosylation is complex. The complexity arises due to the generation of multiple di- and tri-tosylated CD regioisomers.

In order to increase the yield of monotosylated CD, tosylation is carried out using tosyl chloride in a 1:1 molar ratio to CD. The yield of the monotosylated CD can be further increased by judicious choice of bases and solvents employed during the reaction, for example: NaOH or CuSO₄ in water, or dry pyridine. Monotosylation is the most relied upon method to generate monofunctionalized CD, however, this method suffers from some limitations, such as low product yields and the requirement of multiple purification steps. The yield of the final product is reduced due to various reasons, such as chloride displacement of the tosylate, elimination to yield an alkene and 3,6-anhydride formation through intramolecular cyclization from a C-3 hydroxyl group. Purification of the final product requires multiple crystallization steps in water in order to recover the monotosylated compound. Final yields of monotosylated CDs vary in the literature between 30 and 60%. The monotosylated CD can then be functionalized using a nucleophile. The final monofunctionalized CDs using this technique are then used as enzyme mimics or sensors. These structures are not further functionalized and are limited to CDs that display a single functional group and multiple unfunctionalized hydroxyl groups.
In order to generate more complex CD structures a different strategy has to be used. “Clever”, “long” and “sledgehammer” approaches have been used to generate di-, tri-, tetra- and per-substituted CDs. Examples of “clever” approaches to generate specific CD regioisomers employ bis-electrophiles, whereas “long” approaches rely on selective de-benzylations of CDs. Finally, an example of a “sledgehammer” approach is tritylation of CD.

“Clever” disubstitution reactions on β-CD rely on the use of arenedisulfonyl chlorides. These compounds will form inclusion complexes with CD and then react with CD’s primary rim. Arenedisulfonyl chlorides are bis-electrophiles that generate two reactive positions on the CD rim. After displacement by two nucleophiles the CD will be functionalized with two equal functional groups. The regioselectivity of the final product is controlled by using different sizes of arenedisulfonyl chlorides (Figure 1.7). For example, AD isomers of β-CD are generated by using biphenyl-based disulfonates. AC isomers are generated by the use of benzophenone-based reagents. Finally, AB isomers can be formed using 1,3-benzenedisulfonyl chlorides.

“Long” functionalization approaches to generate more complex CDs rely on the selective debenzylation of perbenzylated CDs (CD(OBn)\textsubscript{21}). Perbenzylated CDs are generated by treating a native CD in DMSO with benzyl chloride and NaH. Mono-debenzylation and regioselective di-debenzylation of CD(OBn)\textsubscript{21} can be achieved by treating the starting material CD with a solution of diisobutylaluminum hydride (DIBAL-H) in toluene. The extent of debenzylation during a reaction is dependent on the temperature, the concentration of the DIBAL-H solution and the amount of DIBAL-H equivalents used. Mono-debenzylation is achieved by using 35
equivalents of DIBAL-H in a 0.1 M solution at room temperature. In order to achieve di-de-O-benzylation of CD(OBn)₂₁, 140 equivalents of a 0.5 M solution of DIBAL-H at 30 °C is used. Theoretically, 27 regioisomers could result from the di-de-O-benzylation of CD(OBn)₂₁ using DIBAL-H. Interestingly, the only isomer obtained using an excess of DIBAL is the AD regioisomer. These techniques were later used to incorporate two different functionalities on the primary rim of CDs, an amino- and carboxy-functional groups.

Two orthogonal functional groups can be installed on a perbenzylated CD primary rim using DIBAL-H. In order to increase the functionality on CDs further, a new protecting group was developed. This new protecting group strategy is capable of protecting two hydroxyls on CD simultaneously. In order to selectively protect the A and D positions of 6A,6D-dideoxy CD, these hydroxides can be bridged with 3-chloro-2-(chloromethyl)-1-propene. This methallyl bridge provides a protecting group for the hydroxyls in the A and D positions. The vinyl group present in the methallyl bridge can then be removed by Pd⁰-catalyzed double de-allylation, thus yielding two free hydroxides. Therefore, multiple steps have to be used in order to synthesize a per-functionalized CD. It is important to note, that the excessive amounts of DIBAL required for the selective debenzylaion of CDs generates a lot of waste. Relying on a similar methodology, selective desilylation can be used to generate 6A,6D unprotected CDs without using excessive DIBAL. DIBAL can be used to regiospecifically deprotect tert-butyldimethylsilyl (TBS) ethers on a per silylated CD’s primary rim. Mono de-silylated CD is prepared by treating CD(TBS)₂₁ with 3 equivalents of DIBAL-H at 0 °C for 3h in toluene. It was important to dilute the concentration of DIBAL-H to 0.15 M in order to increase the yield of the mono-de-silylated CD. The 6A,6D-de-O-silylated CD is obtained by increasing the concentration of DIBAL-H to 0.3M (6 equivalents) and the reaction time to 4h. De-silylation is an improved methodology, over the selective de-benzylation procedure, which requires a significantly lower amount of DIBAL-H in order to achieve the desired selective deprotection. Nevertheless, debenzylation and desilylation are both “long” methods requiring multiple synthetic and purification steps in order to per-functionalize CD.

An example of the “sledgehammer” approach to synthesize selectively protected CDs is tritylation. Selective tritylation of CDs relies on the chemoselectivity of primary alcohols and especially steric hindrance generated by the bulky trityl groups. The regioselectivity of tritylation
arises due to steric interactions. Once a trityl group is incorporated on the primary rim, the second trityl group will be incorporated as far away from that trityl group as possible. Regioselectivity of trityl group addition arises through the minimization of steric congestion in the primary rim of a per-tritylated CD. A considerable amount of work has been done to generate regioselectively functionalized tritylated CDs. To date 6-O-mono-, di-, tri-, tetra- and per-tritylated CDs have been prepared. The formation of regioisomers during tritylation is not specific. Therefore, arduous separation of regioisomers must be performed in order to obtain a specific regioisomer after a reaction. Separation and the low-selectivity during tritylation results in low yields of the final functionalized CD.

The approaches presented above now provide access to regioselectively multisubstituted CD hosts that will find use in the design of sensors, supramolecular assemblies and artificial enzymes. However, the aforementioned techniques rely on multiple manipulations of protecting groups in order to obtain the desired per-functionalized CD. This means that the CD chemist has to rely on methods that require extensive synthetic manipulations which lower the overall yield of the desired product and increase the difficulty and time required to obtain the desired final product.

1.5 Applications of Selectively Functionalized CDs

Supramolecular chemists rely on a repertoire of chemical tools to control the assembly of materials on surfaces or in solutions. These tools allow the chemist to tune different materials’ properties in order to develop monomolecular switches, electronics, polymers, protein probes, drug delivering nanoparticles and biological sensors. Supramolecular chemists commonly employ non-covalent self-assembly as one of these tools. Specifically, the non-covalent assembly of CDs with lipophilic guests has garnered a great deal of attention. This is not surprising given CDs intrinsic properties, such as water solubility, non-toxicity and their ability to form inclusion complexes with many molecules. What follows are three current examples of non-covalent chemistry employing CDs for the use for protein assembly, biomarker detection and mechanised nanoparticle drug delivery.

The ability to bring two biomacromolecules together, selectively, in a biological media allows researchers to investigate biological function within cells at a molecular level. However, protein-
protein interactions usually rely on non-covalent interactions in order to communicate with one another. In order to imitate this aspect of biology, supramolecular chemists have developed the use of non-covalent bio-orthogonal interactions to modulate protein-protein function. The applicability of these approaches in cellular environments was demonstrated by Luc Brunsveld et. al. by using the binding of CD to lithocholic acid in order to promote protein assembly.59a As a model protein system to study protein assembly through non-covalent association, enhanced yellow fluorescent protein (eYFP) and enhanced cyan fluorescent protein (eCFP) were chosen as the protein pairs. This system was used because the interaction of eYFP and eCFP can be easily measured using Förster resonance energy transfer (FRET). In FRET, eCFP acts as the donor and eYFP acts as the acceptor. eCFP has an excitation at 410 nm with an emission maximum at 474 nm. eYFP can accept the energy at that wavelength and subsequently emit at 527 nm. Therefore, when eYFP is within close proximity to eCFP, the emission maximum will be shifted from 474 nm to 527 nm. It is important to note that eCFP and eYFP will not inherently bind to each other. As such, by introducing a non-covalent interaction between these two proteins they can be brought together in solution. Brunsveld et al. covalently linked eCFP to lithocholic acid (LCA), and eYFP to CD (Figure 1.8). This modification ensures that the eYFP-CD construct will bind to the eCFP-lithocholic construct in solution. This interaction is brought about by inclusion complex formation between CD and lithocholic acid. The non-covalent interaction between these two protein constructs was then studied using FRET.
Interestingly, Brunsveld et al.’s construct exhibited a dimerization constant of \((1.6 \pm 0.2) \times 10^6\) M\(^{-1}\), similar to the reported association constant of CD and lithocholic acid.\(^{62}\) This system worked well at micromolar concentrations. The applicability of this approach for \textit{in vivo} studies was validated in Madin-Darby canine kidney (MDCK) cells. Brunsveld, et. al.’s approach worked well when the concentration of the interacting protein partners was above the association constant of CD and LCA. However, there are many protein partners whose concentrations in cellular environments exist in the sub-micromolar range. In order to study these proteins a CD-guest inclusion complex with a higher association constant would have to be developed.

The second example of the use of non-covalent assembly exemplifies the applicability of CD in order to bring together two partners orthogonally in a biological milieu. The work of Luc

\[\text{Figure 1.8 Luc Brunsveld’s protein FRET construct. eCFP is colored in blue, eYFP is colored in yellow.}\]
Brunsveld shows that host-guest complexing can be used as a bio-orthogonal method for coupling two biomolecules both in vitro and in vivo. Weissleder et al. used this tool in order to couple nanoparticles (NPs) to antibodies. The antibodies (Abs) were used to recognize a protein of interest, a.k.a. biomarker in a cell, whereas NPs were used for the detection of the aforementioned biomarkers. Weissleder et al. relied on the host-guest interactions between β-CD and adamantane (ADA) to bring their Abs and NPs together. In order to achieve this goal, NPs were linked to ADA and mono-thio-β-CD was anchored to maleimide-modified antibodies.

Biomarker labelling required the preparation of CD-Abs and ADA-NPs. CD-Abs were prepared by linking a mono-thio-CD through a Michael addition to a maleimide derivatized Ab. Maleimide modification of Abs is a non-selective process. Thus, the Michael addition of maleimide-Abs with mono-thio-β-CD results in multiple addition of CD to the surface of the Ab. Multiple addition of CD to an Ab coupled to the fact that NPs display multiple ADAs on their surface allows CD to interact with multiple ADAs. This results in a system that displays high avidity. Indeed, the authors observed a very high binding affinity (Kd = 5 ± 0.7 × 10^{-11} M) between the CD-Aba and ADA-NP constructs which is indicative of a multivalent binding interaction between host and guest. In comparison with other commonly utilized labelling techniques, this new β-CD-ADA system is far superior. The performance of other conventional methods: direct labelling with Aba-NP conjugate and two-step noncovalent labelling using avidin-biotin system was deemed less efficient. The success of the CD-ADA method was attributed to the high avidity and small size of the CD-ADA binding partners. These two reasons would promote the attachment of multiple NPs to a single biomarker, thus giving rise to a higher signal and more efficient labelling of biomarkers. Weissleder et al. successfully labelled biomarkers exposed on several human cancer cell lanes to show the applicability of their method.

The last example of the non-covalent use of CDs deals with the development of drug carriers. CDs have been used to increase the solubility of hydrophobic drugs in the past. Currently, there is an interest in using CDs for target specific delivery of drugs or sensors in humans. For example, Fraser Stoddart staunchly advocates the use of supramolecules to develop nanotechnology applications in medicine. A large amount of research has been dedicated to develop mechanised silica nanoparticles (MSNPs). MSNPs are silica nanoparticles that contain pores with an average diameter of around 2 nm; MSNPs have the ability to encapsulate a variety
of cargo molecules (guests) such as a variety of drugs, biomolecules and MRI contrast agents among others. Stoddart and others have mechanized MSNPs with nanovalve controls capable of stimuli-responsive release of cargo molecules. The nanovalves consist of a linker and a stopper. The linker is composed of a stimuli-responsive functional group. When the desired input is provided via a photochemical stimulus or a chemical stimulus, like a change in pH, the linker then causes the cap to dissociate from the MSNP. This release allows the cargo molecules of the MSNP to diffuse out into the MSNPs surroundings. The cap component is a supramolecule; below is an example where CDs have been used as the capping agent.

Nel et al. developed a pH responsive MSNP capable of delivering a payload inside lysosomes. Their method utilized an aromatic amine as the linker and underivatized β-CD as the cap. β-CD is capable of binding benzimidazole at the pH found in blood (pH = 7.4). However, if the benzimidazole guest becomes protonated in an acidic milieu, the β-CD-benzimidazole complex will dissociate. As such when an MSNP derivatized with benzimidazole-β-CD nanovalves gets incorporated into a lysosome, the MSNP’s cargo will be released (Figure 1.9).

**Figure 1.9** pH responsive MSNP. Cup is a pore in the MSNP and cubes are cargo.
NeI et al delivered doxorubicin, an anticancer agent, to the interior of KB-31 cells using the aforementioned pH responsive nanovalve MSNP delivery system. It remains to be seen if an MSNP capped via a non-covalent interaction will not leak in vivo.

1.6 Thesis Scope

The current applications of CDs commonly rely on underivatized CDs or monofunctionalized CD. This is because the synthetic protocols that have been developed to perfunctionalize CDs require multiple steps and have an overall low yield. As such, the most commonly functionalization on CD is monoderivatization. Monoderivatization is effected by monotosylation followed by nucleophilic displacement. There are countless examples of CD sensors and enzyme mimics generated using this method. However, these underivatized CDs suffer from some limitations (see above). For example, the dissociation constant of hydroxyl-based CDs are in the micromolar range; in order to study interactions of proteins present at submicromolar concentrations, a system with a lower dissociation constant is required. In Chapter 2, we present a CD system that exhibits a sub-micromolar association constant. Specifically, the high binding constant for lithocholic acid using heptakis-[6-deoxy-6-(2-aminoethylsulfanyl)]-β-CD (a per-amino CD) presented in Chapter 2 was used by Luc Brunsveld develop protein partners capable of operating in the submicromolar range. Furthermore, heptakis-[6-deoxy-6-(2-aminoethylsulfanyl)]-β-CD displays amino groups on its primary rim which provides a further advantage over per-hydroxylated CDs. The amino groups on heptakis-[6-deoxy-6-(2-aminoethylsulfanyl)]-β-CD can be easily acylated to generate a variety of CDs, whereas hydroxyl groups on the commonly employed monoderivatized CDs are not. The ease of functionalization of heptakis-[6-deoxy-6-(2-aminoethylsulfanyl)]-β-CD, combined with its submicromolar association constant with LCA, was used for making self-assembling sensors that were able to differentiate pure and contaminated heparin samples. This work is presented in Chapter 3. Moreover, the ease of functionalizing amino groups over hydroxyl groups can be taken advantage of to generate perfunctionalized CDs. The work presented in Chapter 4 provides a “clever” methodology to mono-acylate heptakis-[6-deoxy-6-(2-aminoethylsulfanyl)]-β-CD. This work goes on to show the differential fluorescent responses...
of two monofunctionalized CD per-aminino fluorescent probes towards a small series of proteins. Finally, Chapter 5 deals with some of the current work being undertaken in the laboratory, and provides some future perspectives on the work presented herein.
2 Remarkably Stable Inclusion Complexes with heptakis-[6-deoxy-6-(2-aminoethylsulfanyl)]-β-cyclodextrin

The synthesis of heptakis-[6-deoxy-6-(2-aminoethylsulfanyl)]-β-cyclodextrin was done by Dr. Jagt. Prof. Nitz performed the isothermal titration calorimetry experiments. This chapter has been reproduced with full permission from:


2.1 Introduction

Cyclodextrins (CDs) are readily available naturally occurring supramolecular hosts that are known to form inclusion complexes with many hydrophobic molecules.\textsuperscript{18a, b} Using CDs as hosts has facilitated the development of drug delivery agents, chiral receptors, chiral supports for chromatography, and the creation of many other innovative supramolecular structures.\textsuperscript{1} Remarkably, CD • guest complexes have even been used to mediate specific intracellular protein–protein interactions.\textsuperscript{59a, 68} The affinities of hundreds of guest molecules for cyclodextrins have been measured.\textsuperscript{2} For the majority of these complexes the association constant (K\textsubscript{a}) falls in the range of 10\textsuperscript{2} to 10\textsuperscript{5} M\textsuperscript{-1} and values in excess of 10\textsuperscript{6} M\textsuperscript{-1} are rarely reported.\textsuperscript{1} These affinities have proven to be sufficient for many uses but it would be desirable to have more stable complexes to expand the scope of CD application.

Heptakis-[6-deoxy-6-(2-aminoethylsulfanyl)]-β-CD (1, Figure 2.1) has been studied as a precursor to forming glycoclusters,\textsuperscript{69} as a camptothecin receptor,\textsuperscript{70} and as an inhibitor of anthrax lethal toxin.\textsuperscript{71} Despite the utility of this scaffold, few studies have analyzed the binding properties of 1 as a host for large hydrophobic guests. Recently, an insightful study examined the complexes of 1 with differentially charged tert-butylphenyl guests.\textsuperscript{72} It was noted that 1 had an exceptionally high affinity (K\textsubscript{a} = 4.0 × 10\textsuperscript{6} M\textsuperscript{-1}) for tert-butylbenzene sulfonic acid at pH 3.\textsuperscript{72} In this study Wenz \textit{et al.} convincingly establish that a large portion (12–15 kJ mol\textsuperscript{-1}) of the binding free energy is due to the electrostatic interaction of the cyclodextrin with the guest.
Here we report that host CD 1 forms complexes with association constants up to 2000 times greater than those of the parent β-CD with large hydrophobic guests (2–8, Figure 2.1). We were pleased to see that the affinity of lithocholic acid (4) for 1 reaches a $K_a$ as high as $5.5 \times 10^7$ M$^{-1}$.

**Figure 2.1** Structures of CD 1 and guest molecules 2-8.

To our knowledge this is the highest affinity interaction ever reported between a monomeric cyclodextrin and a small molecule. Detailed conformational analysis and binding studies of 1 show that the thiol ethers are situated over the hydrophobic cavity to allow for interaction with hydrophobic guest molecules. In addition, a conformational change of 1 at low pH was found to favour guest binding. The high affinity of the guests for 1 expands the possibilities for rapidly assembling new non-covalent structures for use in sensing and other biological applications.

### 2.2 Conformational Analysis of Host CD 1

#### 2.2.1 $^1$H-NMR Analysis of the Binding Orientation of Host-Guest Complexes of 1

The p$K_a$ values of the amines of 1 have been measured and the net charge of 1 at pH 7.5 is estimated to be $+5.5$. At pH 2.5 CD 1 should be fully protonated. Initial inspection of the $^1$H NMR spectra at pH 2.5 and 7.5 indicate that CD 1 has a significantly different rotamer population about C6 than is observed with native β-CD. In $^1$H NMR spectra of native β-CD, little
signal dispersion is present between the two diastereotopic protons at C6. The $J_{5-6}$ coupling constants of β-CD have been estimated using spin simulations and the values determined were consistent with C6 residing predominately in the gg conformer (Figure 2.2). In this conformation both C6 protons are oriented towards the hydrophobic cavity and the C6 hydroxyl groups point away from the cavity. In contrast, good signal dispersion is observed between the H6a and H6b protons of 1 (~0.2 ppm; Figure 2.4 and 2.5). To measure $J_{5-6}$ coupling constants of 1, 1D TOCSY NMR experiments were used to overcome the overlap with the ethyl protons. Values measured for H6b were consistently larger than those for H6a, falling between 6.4–8.6 Hz for H6b and below 2 Hz for H6a (Table 2.1). In glucosides it has been established that the tg rotamer is

![Diagram of predominant rotamers of 1 and β-CD.](image-url)
### Table 2.1 NMR data of $H_{6a}$ and $H_{6b}$ of free and complexed CD 1

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<th>Guest</th>
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<th>$J_{5-6a}/$Hz$^b$</th>
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$^a$ NMR experiments were carried out in imidazole (25 mM, pD 7.5) or maleic acid (25 mM, pD 2.5) buffer. $^b$ Coupling constants were obtained irradiating H4 using 1D TOCSY (90 ms mixing time) due to signal overlap with the protons a to the amine. $^c$ Coupling constants at pH 2.5 could not be measured due to low solubility of the guest at this pH.

sparsely populated due to the interaction with the hydroxyl group at C4, leaving an approximate 60 : 40 mixture of $gt$ and $gg$ rotamers. Thus, given the $tg$ isomer is disfavored, the measured coupling constants indicate that the predominant rotamer of 1 at C6 is the $gt$ species (Figure 2.2). This C6 conformer is consistent with observed chemical shifts of $H_{6a}$ and $H_{6b}$ which follow the same trend as the ring protons that resonate downfield ($H_3$ and $H_5$) when oriented towards the hydrophobic cavity of the CD. At pH 2.5 the coupling constant of $H_{6b}$ decreases as does the dispersion between $H_{6a}$ and $H_{6b}$. This is consistent with a small change in the rotamer population towards the $gg$ rotamer likely due to increased electrostatic repulsion at pH 2.5. An observed decrease in signal dispersion between the ring protons at pH 2.5 in comparison to the spectra at pH 7.5 was also observed suggesting the overall CD conformation may change between the two pH values (Figure 2.4 and 2.5).
The $g_l$ rotamer of host CD 1 would position the polarizable sulfur atoms over the hydrophobic cavity, allowing them to interact with a hydrophobic guest. Due to the increased size and polarizability of the sulfur atom, the thiol ethers of 1 are significantly more hydrophobic than the primary hydroxyl groups of β-CD. In binding interactions of cucurbiturils with alkyl thioethers it was found that thioethers bind with higher affinity to the hydrophobic cavity of the cucurbituril than the corresponding methylene derivatives. Thus, the orientation and hydrophobicity of the thioethers will enlarge the hydrophobic cavity of 1 over β-CD, favoring the binding of large hydrophobic guests.

### 2.2.2 NMR Analysis of the Binding Orientation of Host-Guest Complexes of CD 1

In order to determine if the thioethers dramatically influenced the binding orientation of common CD guests, ROESY NMR studies were carried out with 1 : 1 complexes of CD 1 and guests 2–4 at pH 7.5. To study the effect of the protonation state of 1 on binding, guest 3 was also studied at pH 2.5. Adamantanes are considered to be nearly ideal in size for binding at the secondary face of β-CD. Complexes of 1 and 1-adamantanamine (2) or adamantane-1-carboxylic acid (3) displayed intermolecular ROE contacts between the adamantane protons and the H3 and H5 protons situated in the CD cavity (Figure 2.7, 2.8, 2.10, 2.11, 2.13 and 2.14). However, despite the enlarged hydrophobic cavity provided by the thioethers and a favorable electrostatic interaction between 3 and 1, no ROE contacts could be observed between the adamantanes and either of the H6 protons of 1. In the two adamantane complexes the rotamer populations about C6 were similar to those observed with uncomplexed 1 (Table 2.1). From these results it can be concluded that at both pH 2.5 and 7.5, binding occurs in the same orientation as is known for the native β-CD: at the secondary face with the polar functional groups directed towards the solvent.

Similarly, we confirmed that the binding orientation for lithocholic acid (4) towards 1 is analogous to that proposed for the native β-CD complex. Intermolecular contacts were observed between methyl substituents at positions 18 and 21 of 4 and the H6 protons of 1. Consistent with the rotamer populations discussed above, H6$_a$ was observed to have a stronger interaction with 4 than H6$_b$ (Figure 2.15 and 2.16). This binding orientation is the same as that proposed by
Breslow and Yang for β-CD\textsuperscript{62} with the carboxylic acid positioned through the primary face of the CD ring.

2.3 Affinities of Hydrophobic Guests for Host CD 1 Measured by Fluorescence Spectroscopy

Fluorescence spectroscopy was used to evaluate the affinity of guests for 1 (Table 2.2). Due to the large increase in fluorescence emission observed upon complexation with 1, the affinity of the dyes 2-anilino-6-naphthalenesulfonate (5, 2,6-ANS) and \(N,N\)-4-diethylaminocoumarin-3-carboxylic acid (6, DEAC) could be measured directly (Figure 2.18 and 2.19). Upon complexation with native β-CD, a change in fluorescence spectra was observed only with guest 5. Competitive fluorescence experiments were carried out with the non-fluorescent guests and either guest 5 or 6 as the fluorescent probe with 1 and β-CD. Due to the large difference in affinity of ANS (5) and the LCA derivatives (4, 7, 8) for native β-CD, the association constants of the LCA derivatives for native β-CD could not be accurately measured by a competitive titration. All of the titration isotherms fit well to a 1 : 1 binding model.
Table 2.2 Association constants of host CD 1 with guests 2-8 measured by fluorescence experiments vs. literature values for β-CD.

<table>
<thead>
<tr>
<th>Guest</th>
<th>pH</th>
<th>( \log K_a ) β-CD (literature value)</th>
<th>( \log K_a ) 1&lt;sup&gt;c,d&lt;/sup&gt;</th>
<th>( \Delta \Delta G^\circ ) 1 vs. β-CD (kJ mol(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>7.5</td>
<td>3.83&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.49&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-3.4</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>(3.9)&lt;sup&gt;1,2,f&lt;/sup&gt;</td>
<td>5.23&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-7.5</td>
</tr>
<tr>
<td>3</td>
<td>7.5</td>
<td>4.46&lt;sup&gt;b&lt;/sup&gt;, (4.5)&lt;sup&gt;1,2&lt;/sup&gt;</td>
<td>5.64&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-6.5</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>&gt; 5</td>
<td>6.67&lt;sup&gt;b&lt;/sup&gt;</td>
<td>—</td>
</tr>
<tr>
<td>4&lt;sup&gt;e&lt;/sup&gt;</td>
<td>7.5</td>
<td>(6.0)&lt;sup&gt;78&lt;/sup&gt;</td>
<td>7.74&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-9.9</td>
</tr>
<tr>
<td>5</td>
<td>7.5</td>
<td>(3.32)&lt;sup&gt;a&lt;/sup&gt;, (3.4)&lt;sup&gt;79,80&lt;/sup&gt;</td>
<td>5.61&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-12.6</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>3.31&lt;sup&gt;a&lt;/sup&gt;, (3.3)&lt;sup&gt;79&lt;/sup&gt;</td>
<td>6.64&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-19</td>
</tr>
<tr>
<td>6</td>
<td>7.5</td>
<td>(3.7)&lt;sup&gt;68&lt;/sup&gt;</td>
<td>6.43&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-15.6</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>—</td>
<td>6.17&lt;sup&gt;a&lt;/sup&gt;</td>
<td>—</td>
</tr>
<tr>
<td>7</td>
<td>7.5</td>
<td>—</td>
<td>7.27&lt;sup&gt;b&lt;/sup&gt;</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>—</td>
<td>7.20&lt;sup&gt;b&lt;/sup&gt;</td>
<td>—</td>
</tr>
<tr>
<td>7</td>
<td>7.5</td>
<td>—</td>
<td>6.72</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>—</td>
<td>6.48</td>
<td>—</td>
</tr>
</tbody>
</table>

Association constants were determined in phosphate buffers (0.05 M) at the designated pH by:  
<sup>a</sup> direct fluorescence titration or <sup>b</sup> competitive fluorescence titration using DEAC (6) or ANS (5).  
<sup>c</sup> Combined sources of errors in the titrations are estimated to be <5% for direct and <10% for the competitive titrations.  
<sup>d</sup> Titration data can be found in the supplementary information.  
<sup>e</sup>
Determined only at pH 7.5 due to the low solubility of 4 at pH 2.5. f2 formed an insoluble complex with ANS and b-CD at pH 2.5.

2.3.1 Stability of Complexes with Adamantanes

The adamantanes (2 and 3) bind to native β-CD with moderate affinity at pH 7.5. Interestingly, the affinity of adamantan-1-carboxylic acid (3) increases at pH 2.5 and adamantanamine (2) binds with similar affinity at both pH 7.5 and 2.5 (Table 2.2). One explanation for this observation is that 3 is more readily desolvated upon binding in its neutral form.

The adamantanes (2 and 3) bind to 1 with increased affinity over the native β-CD at pH 7.5 (Table 2.2). Since it was shown that the adamantanes do not contact the thioethers (see above), and that the effect is present for both positively and negatively charged adamantanes, this increase may reflect a difference in conformation of the hydrophobic cavity of 1 when compared to native β-CD. Interestingly, at pH 2.5 the affinity of both adamantanes 2 and 3 for 1 increased in comparison to pH 7.5. This increase in affinity for adamantane 3 was expected as an increase is also observed for native β-CD. The change in affinity for 2 between pH 2.5 and pH 7.5 suggests that 1 displays a difference in conformation or binding geometry at low pH that favours guest binding. This change may be the result of greater electrostatic repulsion between the ammonium ions of 1 at low pH altering its conformation.

2.3.2 Stability of Complexes with Aromatic Compounds

Due to their ability to span the enlarged hydrophobic cavity and position their anionic functional group for favorable electrostatic interactions, the two dyes 2,6-ANS (5) and DEAC (6) were expected to bind with increased affinity to 1 compared to β-CD. Calculated Coulombic energies for complexes with 1 have been previously shown to correlate well with observed changes in free energy upon binding differentially charged guests. Using these calculations, at pH 7.5, electrostatic interaction between a monoanionic guest and the partially protonated amines on the primary face of 1 (with a net charge of +5.5) would stabilize the complex by approximately $-12 \text{ kJ mol}^{-1}$. At pH 2.5, when 1 is fully protonated and thus has a net charge of +7, this value would change to approximately $-15 \text{ kJ mol}^{-1}$. 


2,6-ANS (5) is known to form complexes with β-CD and it has been suggested that its linear geometry allows full encapsulation of the dye with the sulfonate directed towards the primary face of the CD. The observed increase in binding affinity with 1 compared to the native β-CD at pH 7.5 agrees well with the calculated −12 kJ mol⁻¹ of Coulombic stabilization energy. Binding of the guest to 1 at pH 2.5 was significantly higher in affinity than at pH 7.5 (ΔΔG = −5.9 kJ mol⁻¹). Only roughly half of this increase in binding energy can be attributed to the increase in Coulombic stabilization at pH 2.5. This observation is consistent with the observed increase in affinity for the adamantane derivatives at pH 2.5 and may similarly reflect a change in conformation of 1 at low pH which favors guest binding.

In the case of DEAC (6), at pH 7.5, the increased affinity for 1 compared to β-CD was slightly higher than would be expected based on Coulombic interaction energies alone. A possible explanation for this may be that 6 preferentially interacts with the extended hydrophobic cavity. At low pH—in contrast to compounds 2, 3 and 5—a slight decrease in binding affinity is observed. However, considering the fact that the dye is neutral at this pH (pKₐ ~ 4.6) and no electrostatic stabilization upon binding would be expected, the binding affinity is higher than would be predicted. A change in the desolvation penalty upon binding of the coumarin when in its neutral form may be partially responsible for the larger than expected affinity at pH 2.5.

2.3.3 Stability of Complexes with Lithocholic Acid

Finally, we analyzed the stability of the complexes of 1 with lithocholic acid (4). It is known that 4 spans the hydrophobic cavity of native β-CD and associates with exceptional affinity (Kₐ = 1 × 10⁶ M⁻¹). We were particularly interested in the affinity of this guest for 1 as it can be readily functionalized at both termini and, for this reason, makes an ideal building block for supramolecular structures. At pH 7.5 the association constant for binding of 4 to 1 (Kₐ = 5.5 × 10⁷ M⁻¹) increases by more than 1.5 orders of magnitude compared to the native β-CD (Kₐ = 1 × 10⁶ M⁻¹).

---

1To ensure that 6 binds in its neutral form at pH 2.5, the absorbance spectra of both the complex and the free dye were measured at both pH 2.5 and pH 7.5. Although at pH 2.5 the absorbance maxima are blue shifted upon binding to 1, the spectra are significantly different than that of the complex at pH 7.5 suggesting DEAC is binding in its neutral state (6 pH 7.5 λₘₐₓ = 412 nm, 6·1 pH 7.5 λₘₐₓ = 412 nm, 6 pH 2.5 λₘₐₓ = 437 nm, 6·1 pH 7.5 DEAC λₘₐₓ = 429 nm).
The relative increase in affinity is smaller than that observed for 5 and 6. This may be due to the carboxylate of 4 being positioned farther away from the positively charged amines of 1 resulting in a reduced Coulombic stabilization. In order to assess the contribution of electrostatic interactions on the observed binding, lithocholic acid amides 7 and 8 were studied. In these derivatives the favorable electrostatic interaction of the carboxylate is removed and a repulsive ammonium ion is placed close to (8), and far from (7), the host – guest interface. Compared to 4, guest 7 shows a relatively small reduction in affinity towards 1 (ΔΔG = 2.7 kJ mol⁻¹). Guest 8 shows a more dramatic decrease in binding affinity. These relative reductions in affinity observed are likely due to the distance of the ammonium ion from the positively charged CD. The binding affinity of guest 8 is also more sensitive to the increasing charge on the CD at low pH. If the assumption is made that the electrostatic repulsion between 7 and 1 is negligible, due to the distance between the charges of these molecules, the electrostatics can be estimated to be responsible for less than a third of the observed increase in affinity of 4 for 1 compared to the native β-CD. The remainder of the increase in free energy of binding for guests 4 and 7 can likely be attributed to the increased hydrophobic cavity of 1. Unfortunately, 4 was not sufficiently soluble at pH 2.5 to obtain accurate complexation data. Analysis of compound 7 at pH 2.5 gave a similar affinity to that observed at pH 7.5. This large hydrophobic guest may bind to 1 with different constraints than the smaller guests (2, 3, 5, and 6) and any change in conformation of the CD at low pH does not alter the overall binding energy.

The affinity of the lithocholic acid derivatives 4, 7 and 8 could not be measured for native β-CD using a competitive titration with guest 5 due to a large difference in affinity between these compounds towards β-CD.

2.4 Investigation of the Increased Affinity at Low pH by Isothermal Titration Microcalorimetry

To gain further insight into the increased affinity that some of the guest compounds (i.e. 2, 3, 5 and 6) have displayed for 1 at low pH, we analyzed the binding of 5 to 1 by isothermal titration microcalorimetry (ITC). These titrations were complicated by the observation of a second binding event to 1 at the high guest concentrations required in these experiments. In ITC experiments, the CD and guest concentrations are typically well above the dissociation constants
of the complex and thus weaker binding sites can be observed. However, using a sequential two site binding model, and holding the first association constant at the value determined by fluorescence titration, the thermodynamic parameters of the binding events could be fitted to the calorimetry data (Table 2.3). As is common in the majority of binding events in aqueous solution, entropy–enthalpy compensation is observed between the two pH values investigated for the binding of 5 to 1. Examination of the differences in entropy ($T\Delta S^\circ = +17 \text{ kJ mol}^{-1}$) and enthalpy ($\Delta\Delta H^\circ = +10 \text{ kJ mol}^{-1}$) of the first binding event at the two pH values indicates that the

<table>
<thead>
<tr>
<th>Guest</th>
<th>pH</th>
<th>$\Delta G^\circ / \text{kJ mol}^{-1}$</th>
<th>$\Delta H^\circ / \text{kJ mol}^{-1}$</th>
<th>$T\Delta S^\circ / \text{kJ mol}^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>7.5</td>
<td>32 ± 3</td>
<td>-59 ± 5</td>
<td>-28 ± 4</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>38 ± 4</td>
<td>-48 ± 4</td>
<td>-11 ± 2</td>
</tr>
</tbody>
</table>

Isothermal titration microcalorimetry was performed in phosphate buffers (50 mM) at 25°C. Full experimental details and curve fits can be found in the experimental section (Heading 2.4). Errors represent combined uncertainties in the entire experiment.

larger difference in the entropy of binding is responsible for the overall increased stability of the complex at low pH. Given that the increased stability of the complexes at low pH is observed for a series of guests investigated (2, 3, 5, 6), and that with 5 little dependence on pH was observed upon complexing with native β-CD, it is likely that the changes in binding free energy are a result of a difference in conformation or solvation of 1 at the two pH values. One possible explanation, consistent with our results, is a decrease in the cone angle of the cyclodextrin due to the strong electrostatic repulsion between the cysteinaminyl functionalities resulting in a conformation that deepens the hydrophobic cavity and favours the binding of adamantane and aromatic guests.

Due to the lack of solubility of the lithocholic acid derivatives (4, 7, 8), the binding of these compounds to CDs could not be analyzed by ITC. Lithocholic acids are known to form colloidally stable supramolecular aggregates with defined structures in aqueous solution. These structures are in a slow equilibrium with very small amounts of free lithocholic acid. It was
observed when 1 is added to a colloidally stable suspension of the lithocholic acid (4) aggregates, the CD inclusion complex slowly forms over a 4–5 hour period (Figure 2.3). This slow equilibrium is not suitable for investigation with ITC.

**Figure 2.3** Time course of binding between lithocholic acid guest 4 to 1. CD 1 (1.8 μM) was dissolved in phosphate buffer (50 mM, pH 7.5) and fluorescent guest 6 (10 μM) was added. The competitive guest 4 (1.8 μM) was added either as a solution in DMSO (■) (1.8% final DMSO concentration in solution) or as a colloidally stable suspension in phosphate buffer (50 mM pH 7.5) (•) at time 0.

### 2.5 Conclusions

We have demonstrated that through the simple substitution of the hydroxyl groups at the primary face of β-CD for cysteinaminyl functionalities, the affinity for large hydrophobic guests improved dramatically. At pH 7.5, the enlarged hydrophobic cavity of 1 and favorable Coulombic interactions with the protonated amines on the primary face are identifiable reasons for the observed increase in affinity. When the pH of the solution is lowered to 2.5, a further increase in binding affinity is observed for adamantane guests (2 and 3) as well as 2,6-ANS (5) beyond that which would be expected by electrostatic effects alone. We hypothesize that this is due to a change in conformation of the host 1 at low pH which favors binding of these guests.

To the authors’ knowledge, the complex between 1 and lithocholic acid (4) is the most stable monomeric CD host complex measured to date. The affinity of the interaction does not match the
most stable synthetic complex ever generated in water between cucurbit[7]uril and bis(trimethylammonium)ferrocene, but the asymmetry and ease of modification of both the lithocholic acid and CD 1 suggest they can be readily elaborated to form a wide variety of useful, exceptionally stable host–guest complexes.

2.6 Experimental Section

2.6.1 Instrumentation

$^1$H NMR spectra were recorded on a Varian 400 spectrometer at T = 297 K. ROESY NMR experiments were conducted on a Varian 500 spectrometer at T = 297 K. Absorption spectra were measured on a Schimadzu UV-2401PC spectrophotometer, whereas fluorescence spectroscopy was performed on a Fluorolog-3 spectrofluorometer. Isothermal Titration Calorimetry was performed on a Microcal calorimeter.

2.6.2 ROESY NMR Experiments

ROESY NMR experiments were performed with 4 mM solutions of the 1:1 complexes of cyclodextrin (CD) 1 and the respective guests (G). A maleic acid buffer (25 mM) and an imidazole buffer (25 mM) were used to adjust the pH of the samples to pH 2.5 and 7.5 respectively. 1H NMR spectra of the studied complexes and free 1 are supplied as reference material.
Figure 2.4 $^1$H NMR CD 1 at pH 7.5

Figure 2.5 $^1$H NMR CD 1 at pH 2.5
Figure 2.6 $^1$H NMR Complex 1•2 in D$_2$O pD 7.5
Figure 2.7 ROESY Complex 1•2 in D$_2$O pD 7.5
Figure 2.8 Expansion of ROESY Complex 1•2 in D$_2$O pD 7.5
Figure 2.9 $^1$H NMR Complex 1•3 in D$_2$O pD 7.5
Figure 2.10 ROESY Complex 1•3 in D$_2$O pD 7.5
Figure 2.11 Expansion of ROESY Complex 1•3 in D$_2$O pD 7.5
Figure 2.12 $^1$H NMR Complex 1•3 in D$_2$O pD 2.5
Figure 2.13 ROESY Complex 1•3 in D_{2}O pD 2.5
Figure 2.14 Expansion of ROESY Complex 1•3 in D₂O pD 2.5
Figure 2.15 $^1$H NMR Complex 1•4 in D$_2$O pD 7.5
Figure 2.16 ROESY Complex 1•4 in D$_2$O pD 7.5
Figure 2.17 Expansion of ROESY Complex 1•4 in D$_2$O pD 7.5
2.6.3 Determination of Association Constants for Host-Guest Complexes Using Fluorescence Spectroscopy

2.6.3.1 Direct Titration of 1 or β-CD to Guests 5 or 6

Due to the large increase in fluorescence emission observed upon complexation, the affinity of the fluorescent dyes 5 and 6 could be measured directly. Phosphate buffers (50 mM) were used to adjust the pH of the samples to pH 2.5 and 7.5. Plotting fluorescence versus the cyclodextrin concentration, the association constants (Kₐ) could be determined by fitting the data to equation 1 in Appendix A derived below. Association constants were calculated from the average of 3 independent titrations. The included graphs are a representative fit of the data.

**Figure 2.18** Fluorescence (λex = 319 nm) of 0.27 μM 2,6-anilinonaphthalene sulfate (5, 2,6-ANS) in phosphate buffer 50 mM at pH 2.5 (left panel) and 7.5 (right panel) in the absence (solid line) and presence (dotted line) of 6 μM CD 1.
Figure 2.19 Fluorescence ($\lambda_{\text{ex}} = 420$ nm) of 10 $\mu$M 7-diethylaminocoumarin-3-carboxylic acid (6, DEAC) in phosphate buffer 50 mM at pH 2.5 (left panel) and 7.5 (right panel in the absence (solid line) and presence (dotted line) of 10 $\mu$M CD 1.

Figure 2.20 Fluorescence titration of 1 with guest 5 pH 2.5
**Figure 2.21** Fluorescence titration of 1 with guest 5 pH 7.5

**Figure 2.22** Fluorescence titration of 1 with guest 6 pH 2.5

**Figure 2.23** Fluorescence titration of 1 with guest 6 pH 7.5
2.6.3.2 Competitive Fluorescence Experiments of 1 or β-CD with Guests 2-4, 7 or 8

Competitive fluorescence experiments were carried out with the nonfluorescent guests (2, 3, 4 and 7) using N,N-diethylaminocoumarin-3-carboxylic acid (6) as the fluorescent probe. Plotting fluorescence versus the guest concentration, the association constants (Kₐ) could be determined by fitting the data into equation 2 in Appendix A derived below. Association constants were calculated from the average of 4 independent titrations.
Figure 2.26 Competitive fluorescence titration of 1 and 6 with guest 2 pH 2.5

Guest (G): 2  
\[ \text{pH} = 2.5 \]  
\[ [\text{CD}] = 1.8 \, \mu\text{M} \]  
\[ [6] = 10 \, \mu\text{M} \]  
\[ \lambda_{\text{ex}} = 415 \, \text{nm} \]  
\[ \lambda_{\text{em}} = 456 \, \text{nm} \]  
\[ K_a = 1.69 \times 10^5 \, \text{M}^{-1} \]  
\[ \pm 0.074 \times 10^5 \, \text{M}^{-1} \]  
\[ \log(K_a) = 5.23 \]

Figure 2.27 Competitive fluorescence titration of 1 and 6 with guest 2 pH 7.5

Guest (G): 2  
\[ \text{pH} = 7.5 \]  
\[ [\text{CD}] = 1.8 \, \mu\text{M} \]  
\[ [6] = 10 \, \mu\text{M} \]  
\[ \lambda_{\text{ex}} = 415 \, \text{nm} \]  
\[ \lambda_{\text{em}} = 456 \, \text{nm} \]  
\[ K_a = 3.11 \times 10^4 \, \text{M}^{-1} \]  
\[ \pm 0.21 \times 10^4 \, \text{M}^{-1} \]  
\[ \log(K_a) = 4.49 \]

Figure 2.28 Competitive fluorescence titration of 1 and 6 with guest 3 pH 2.5

Guest (G): 3  
\[ \text{pH} = 2.5 \]  
\[ [\text{CD}] = 1.8 \, \mu\text{M} \]  
\[ [6] = 10 \, \mu\text{M} \]  
\[ \lambda_{\text{ex}} = 415 \, \text{nm} \]  
\[ \lambda_{\text{em}} = 456 \, \text{nm} \]  
\[ K_a = 4.65 \times 10^5 \, \text{M}^{-1} \]  
\[ \pm 0.21 \times 10^5 \, \text{M}^{-1} \]  
\[ \log(K_a) = 6.67 \]
Figure 2.29 Competitive fluorescence titration of 1 and 6 with guest 3 pH 7.5

Guest (G): 3
pH = 7.5
[CD] = 1.8 µM
[6] = 10 µM
λ<sub>ex</sub> = 415 nm
λ<sub>em</sub> = 456 nm

K<sub>a</sub> = 4.35 x 10<sup>9</sup> M<sup>-1</sup>
± 0.13 x 10<sup>7</sup> M<sup>-1</sup>

log(K<sub>a</sub>) = 5.64

Figure 2.30 Competitive fluorescence titration of 1 and 6 with guest 4 pH 7.5

Guest (G): 4
pH = 7.5
[CD] = 1.8 µM
[6] = 10 µM
λ<sub>ex</sub> = 415 nm
λ<sub>em</sub> = 456 nm

K<sub>a</sub> = 5.52 x 10<sup>9</sup> M<sup>-1</sup>
± 0.29 x 10<sup>7</sup> M<sup>-1</sup>

log(K<sub>a</sub>) = 7.74

Figure 2.31 Competitive fluorescence titration of 1 and 6 with guest 7 pH 2.5

Guest (G): 7
pH = 2.5
[CD] = 1.8 µM
[6] = 10 µM
λ<sub>ex</sub> = 415 nm
λ<sub>em</sub> = 456 nm

K<sub>a</sub> = 1.57 x 10<sup>7</sup> M<sup>-1</sup>
± 0.11 x 10<sup>7</sup> M<sup>-1</sup>

log(K<sub>a</sub>) = 7.20
Figure 2.32 Competitive fluorescence titration of 1 and 6 with guest 7 pH 7.5

Figure 2.33 Competitive fluorescence titration of 1 and 6 with guest 8 pH 2.5

Figure 2.34 Competitive fluorescence titration of 1 and 6 with guest 8 pH 7.5
2.6.4 Isothermal Titration Microcalorimetry

A solution of 5 was titrated into a solution of 1 at 25° C with 300 rpm stirring. Both solutions were made in phosphate buffer (pH 2.5 and 7.5, 50 mM) and dialyzed overnight using 100 MWCO dialysis tubing (spectrapore). The final concentration of 5 was determined using absorbance spectrometry ($\varepsilon = 26,000$ cm$^{-1}$ M$^{-1}$, $\lambda_{\text{max}}$ 319 nM). Heats of dilution were determined in separate experiments. The data was fit to a two sequential site binding model. During the curve fitting the association constant of the first binding event was held at the value determined by fluorescence titration. Representative curve fits from titration data are found below. Data reported are the average of two titrations.
Figure 2.37 ITC of 5 into 1 at pH 7.5.

ITC of 5 into 1 at pH 7.5.

[1] = 0.050 mM (Cell)
[5] = 1.1 mM (Titrant)
5 was titrated in 8 µL injections, with the exception of the first injection of 3 µL

K1 3.5 \times 10^5 \text{ M}^{-1}
\Delta H_1 -1.431 \times 10^4
\pm 0.018 \times 10^4 \text{ cal/mol}
\Delta S_1 -22.55 \text{ cal/mol}
K2 9293 \pm 239 \text{ M}^{-1}
\Delta H_2 -956.0
\pm 553.3 \text{ cal/mol}
\Delta S_2 14.95 \text{ cal/mol}

Figure 2.38 ITC of 5 into 1 at pH 2.5.

ITC of 5 into 1 at pH 2.5.

[1] = 0.060 mM (Cell)
[5] = 1.4 mM (Titrant)
5 was titrated in 9 µL injections, with the exception of the first injection of 3 µL

K1 4.2 \times 10^5 \text{ M}^{-1}
\Delta H_1 -1.157 \times 10^4
\pm 0.010 \times 10^4 \text{ cal/mol}
\Delta S_1 -8.486 \text{ cal/mol}
K2 5.326 \times 10^4
\Delta H_2 -5151
\pm 307.3 \text{ cal/mol}
\Delta S_2 4.347 \text{ cal/mol}
3 Pattern-Based Recognition of Heparin Contaminants by an Array of Self-Assembling Fluorescent Receptors

The idea of using pattern-based recognition to assess the purity of heparin samples was conceived by Dr. Jagt. My involvement in this work stems from the measurement of association constants of CD 1 with lithocholic acid. I synthesized cyclodextrins 9 and 10. I also carried out fluorescence measurements to characterize which sensors gave us the strongest responses and the concentrations of the host and guests required for maximal fluorescence responses. This sensor and the H:G concentrations were then used by Dr. Jagt for the linear discriminant analysis experiments. This chapter has been reproduced with full permission from: Jagt, R.B., Gómez-Biagi, R.F., and Nitz, M. (2009) Pattern-based recognition of heparin contaminants by an array of self-assembling fluorescent receptors. Angewandte Chemie International Edition English. 48: 1995-1997

3.1 Introduction

During late 2007 and early 2008, 81 patients in the USA and Germany died and hundreds were seriously injured after being administered contaminated unfractionated heparin sulfate (UFH) while undergoing anticoagulation therapy. Multiple orthogonal analytical techniques, including extensive high-field NMR spectroscopy, HPLC, and capillary electrophoresis, were needed to identify the contaminant as the semisynthetic glycosaminoglycan, oversulfated chondroitin sulfate (OSCS). Even though some batches of heparin were found to contain up to a third of this non-natural form of chondroitin sulfate, its presence was masked in standard quality-control assays owing to the inherent anticoagulant activity of OSCS. The development of quick and reliable tests for heparin contaminants is currently of great interest. Herein we report the design and evaluation of a fluorescent receptor array that is able to assess the quality of a heparin sample by quickly differentiating UFH from OSCS and other commonly encountered negatively charged polymers.

The design of selective receptors for biological macromolecules, such as UFH, poses a significant challenge. As an alternative, chemists have turned towards differential arrays which do not rely on receptors that are specific for a particular molecule, but on a unique diagnostic
pattern that is derived from an array of receptors with broad specificity. Many of the most successful examples of solution-phase differential receptor arrays are based on indicator-displacement assays (IDAs). The power of IDAs lies in their modular nature, which enables many unique receptors to be constructed rapidly with a minimum number of synthetic steps. Herein we describe an alternative modular receptor array that does not rely on dye displacement, but on the binding of an environmentally sensitive fluorophore proximal to the analyte-recognition site (Figure 3.1).

![Figure 3.1 Putative Binding Model for Self-assembled fluorescent probe](image)

A wide variety of different colorimetric and fluorescent heparin indicators have been reported with mechanisms of heparin sensing based on boronic acids, heparin-specific peptides, labeled heparin-binding proteins, changes in polymer conformation, and fluorophore aggregation. However, none of these strategies provide a modular design that enables the facile generation, and optimization, of a receptor array capable of differentiating between negatively charged polymers.

To design a modular receptor with a high propensity for binding negatively charged biopolymers, we employed the cycloextrin (CD) 1 as a scaffold. The primary amine groups of 1 were functionalized to provide the amide and guanidino derivatives 9-12 as a small collection of polycationic receptors (Figure 3.2). The different modes of electrostatic recognition...
of CDs 1, 9-12 towards the polyanionic biopolymers provides the diversity that is needed to generate specific response patterns in the receptor array for each analyte.

![Chemical structures](image)

**Figure 3.2** Modular self-assembling fluorescent receptors for polyanionic biopolymers. The receptors consist of a polycationic cyclodextrin host (1, 9-12) and a fluorescent reporter tethered to a lithocholic acid guest (13-15).

Conveniently, 1 also forms a remarkably stable inclusion complex ($K_d=18$ nm) with lithocholic acid (LCA). The stability of LCA–β-cyclodextrin complexes has previously enabled the innovative application of this complex in directing protein–protein interactions. According to previous NMR spectroscopic studies, LCA binds to 1 with the carboxylic acid oriented through the primary rim of the CD. This binding orientation provides a simple and modular way to position a fluorophore near the positively charged analyte-binding site of CDs 1, 9-12 (Figure 3.3).
Figure 3.3 Model of CD 1 (stick model) bound to the LCA–quinolinium conjugate 13 (ball-and-stick model). The model shows the relative geometry and scale of the inclusion complex but is not the global-minimum-energy structure. Positively charged ammonium groups of the putative UFH-binding site are emphasized with enlarged spheres.

The quinolinium fluorophore employed is an efficient fluorescent reporter of heparin-binding events driven by electrostatic interactions in aqueous solution.\textsuperscript{92a} Thus, the synthesis of a quinolinium fluorophore tethered through a variable spacer to LCA (to give 13-15), followed by complexation with CDs 1, 9-12, rapidly generated fifteen potential fluorescent receptors for the desired analytes.

3.2 Optimization of the Linker Region for the LCA-Quinolinium Sensing Motif

An initial evaluation of complexes 1 · 13-15 was carried out with UFH as the analyte. The optimum conditions with respect to both fluorescence response and binding affinity for UFH were a 1:1 ratio of 1 to the LCA–fluorophore conjugate 13 in phosphate-buffered saline (PBS; NaPO₄ 12 mm, NaCl 140 mm, pH 7.4). At a concentration of 7 μM, all three complexes 1 · 13-15 displayed a linear increase in fluorescence upon titration with UFH (1–13 μg mL\textsuperscript{-1}; Figure 3.4). The relative increase in fluorescence ($F/F_0$) was greater with shorter
Figure 3.4 Fluorescence increase for $1 \cdot 13$ ($\Delta$), $1 \cdot 14$ ($\circ$), and $1 \cdot 15$ ($\Box$) at a concentration of 7 μm in PBS when titrated with UFH ($\lambda_{ex} = 320$ nm, $\lambda_{em} = 430$ nm). Error bars indicate ± one standard deviation.

LCA/quinolinium tethers X (Figure 3.2), and the largest increase in fluorescence (six-fold) was observed for the $1 \cdot 13$ complex. Presumably, the shorter tethers bring the fluorophore closer to the polycationic binding site and thus maximize the change in environment experienced by the quinolinium ion upon analyte binding. Although control experiments with 13-15 in the absence of a CD showed a fluorescence increase (2.5-fold) for 13 when titrated with UFH, the presence of 1 is essential for both the magnitude and linearity of the observed response (Figure 3.5).
Figure 3.5 Solutions of 13-15 (7 μM) in phosphate buffer saline (PBS) at pH 7.4 were titrated with unfractionated heparin (UFH) in the presence (■) and absence (▲) of an equimolar amount of polycationic cyclodextrin 1 (λ ex = 320 nm, λ em = 430 nm).
3.3 Pattern-Based Discrimination of Analytes using Self-Assembling Probes

3.3.1 Mapping the Fluorescence Responses of the Self-Assembling Probes

Assays were conducted with CDs 1, 9-12 and the LCA–fluorophore conjugate 13 in 96-well plates to demonstrate the potential of the corresponding self-assembled fluorescent receptor complexes in pattern-based recognition. The following analytes were chosen to evaluate the discriminatory power of the array: unfractionated and low-molecular-weight heparin (UFH and LMWH), heparan sulfate (HS), dermatan sulfate (DS), chondroitin sulfate A (CS), oversulfated chondroitin sulfate (OSCS), polyglutamic acid (PGA), and polyacrylic acid (PAA). Eight individual measurements of $F/F_0$ were made for each fluorescent-receptor/analyte combination at a concentration of 5 μM for the receptor complex and an analyte concentration of 6 μg mL$^{-1}$. The responses for each analyte are shown in Figure 3.6. Most notably, the signal profile for OSCS was dramatically different from that observed for any of the other polyanions. The use of 11 ⋅ 13

![Figure 3.6](image_url)  
**Figure 3.6** Distinctive diagnostic patterns for the analytes tested. The data represent the averages of eight individual measurements with a standard deviation ≤5%. All measurements were made in PBS.
produced a large response with OSCS, whereas little or no binding was observed with the other analytes and this receptor complex. As expected, the two forms of heparin (UFH and LMWH) gave nearly identical response patterns. The difference in overall magnitude of the signals with UFH and LMWH may be caused by the smaller number of high-affinity binding sites in LMWH than in UFH. The less highly charged analytes HS, DS, and PGA gave similar signal profiles. The most similar response to that of heparin with receptors 1, 9-12 ⋅ 13 was observed for PAA; however, the highly charged receptor complex 1 ⋅ 13 was able to distinguish these analytes.

3.3.2 Analytical Power of Array Tested Using Linear Discriminant Analysis

The statistics program SYSTATII was used to perform a linear discriminant analysis (LDA)97 of the fluorescence array data. A graphical representation of this analysis in the form of a two-dimensional score plot is given in Figure 3.7. The dispersion between the groups of analytes

II SYSTAT, version 12.0, Systat Software Inc., Richmond, CA, USA.
Figure 3.7 Two-dimensional LDA score plot for the analysis of UFH (a), LMWH (b), HS (c), DS (d), CS (e), OSCS (f), PGA (g), and PAA (h). Eight separate measurements were made for each analyte.

in the score plot is indicative of the analytical power of the receptor array in discriminating between analytes. “Jackknifed” classification matrices \textsuperscript{III} were taken to evaluate the LDA results, and all analytes were discriminated with 100 \% accuracy. \textsuperscript{IV} In a second set of experiments, in which UFH, DS, and OSCS were investigated at concentrations of 2, 4, 6, and 8 \(\mu\text{g mL}^{-1}\), both the analytes and their concentrations could be distinguished readily (Figure 3.8).

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\textsuperscript{III} One measurement at a time was treated as an unknown, and the remainder were used as the training set.

\textsuperscript{IV} The predictive ability of the receptor array was determined by a cross-validation routine, in which 50 \% of the dataset was taken out randomly and used as a training set for the LDA, and the omitted remainder was reclassified.
Figure 3.8 Average F/F0 values of eight independent fluorescence measurements of the complexes 1, 9-12 • 13 (5 μM) with DS, UFH, and OSCS (6 μg/mL) and mixtures thereof in PBS at pH 7.4 (Standard deviation ≤5%)

LDA of the data obtained in Figure 3.8 was then performed and is displayed in Figure 3.9.

Figure 3.9 LDA score plot of the data in Figure 3.8 with DS (red), UFH (green), and OSCS (blue) at 2 μg mL⁻¹ (circle), 4 μg mL⁻¹ (cross), 6 μg mL⁻¹ (square), and 8 μg mL⁻¹ (triangle).
3.3.3 Assessment of Heparin Sample Purity Using Self-Assembled Probes

We were interested in whether our receptor array would also be able to accurately distinguish a pure sample of UFH from one containing a natural contaminant, such as DS, or the potentially deadly contaminant OSCS. Mixtures of UFH containing 10, 25, 50, and 75% OSCS and DS were submitted to our receptor array at a total concentration of 6 μg mL⁻¹. Furthermore, a 1:1 mixture of OSCS and DS and a 1:1:1 mixture of all three analytes was tested (Figure 3.10).

**Figure 3.10** Average F/F₀ values of eight independent fluorescence measurements of the complexes **1, 9-12 • 13** (5 μM) with DS, UFH and OSCS (6 μg mL⁻¹) and mixtures thereof in PBS at pH 7.4 (standard deviation ≤ 5%)

Remarkably, the resultant LDA score plot of the data in Figure 3.10 enables the identification of both the ratio of the mixture and the identity of its components (Figure 3.11). Discrimination with 100% accuracy was possible for samples with as little as 10% contamination; thus, this type of differential array has great potential for the quality control of heparin.
Figure 3.11 Two-dimensional LDA score plot for the analysis of UFH (a) and the contaminants DS (b) and OSCS (c). Mixtures of UFH with 10, 25, 50, and 75% DS (d–g) or OSCS (h–k) were analyzed, as well as 1:1 DS/OSCS (l) and 1:1:1 DS/OSCS/UFH (m). Eight separate measurements were made for each analyte and mixture.

3.4 Conclusions

In summary, we have described a novel strategy for the rapid assembly of an array of supramolecular fluorescent receptors based on a modified cyclodextrin host–guest complex. The analytical power of these differential arrays was demonstrated by accurate discrimination between heparin and common potential contaminants of this important drug. As both 1 and LCA are relatively easy to functionalize, other self-assembling fluorescent receptors based on this scaffold can be envisioned for a much broader variety of analytes.
3.5 Experimental Section

3.5.1 Instrumentation

All chemicals were purchased from Sigma Aldrich with the exception of heparan and unfractionated heparin sulfate which were purchased from Celsus Laboratories. Oversulfated chondroitin sulfate was prepared according to a known literature procedure. All reactions were performed under a nitrogen-atmosphere and at room temperature, unless stated otherwise. 1H- and 13C-NMR spectra were recorded on a Varian 400 spectrometer at $T = 297$ K. Absorption spectra were measured on a Schimadzu UV-2401PC spectrophotometer, whereas fluorescence spectroscopy was performed on a Fluorolog-3 spectrofluorometer. Fluorescence measurements for the differential receptor array were carried out on a Tecan Safire2 plate reader using Corning black polystyrene flat bottom 96-well plates. Solutions were prepared with distilled water purified via a milliQ system. Linear discriminant analysis (LDA) was performed using SYSTAT, version 12.0, Systat Software Inc., Richmond, CA (USA).

3.5.2 Synthetic Procedures

![Diagram of synthetic procedures](image)

Figure 3.12 Synthesis of 1
Cyclodextrin (CD) 1 was prepared according to the reaction sequence in Figure 3.8. Per-(6-deoxy-6-bromo)-β-CD (17) was prepared from β-cyclodextrin according to a literature procedure.98,V

2,2,2-Trifluoro-N-(2-mercaptoethyl)acetamide (16) A mixture of cysteinamine•HCl (2 g, 17.6 mmol), ethyl 2,2,2-trifluoroacetate (2.1 mL, 17.6 mmol), and Et₃N (4.88 mL, 35.2 mmol) in MeOH (50 mL) was stirred for 12 h. Subsequently, the mixture was neutralized with AcOH (conc.) and the solvent removed under diminished pressure. The residue was redissolved in EtOAc (100 mL) and washed with water (3 x 50 mL). The organic layer was dried on MgSO₄, filtered, and evaporated to give 16 as a clear oil. Yield: 82% (2.4 g, 14.4 mmol). ¹H-NMR (CD₃OD, 400 MHz): δ = 3.61 (t, 1H, J = 6.8 Hz), 2.90 (t, 1H, J = 6.8 Hz); ¹³C-NMR (CD₃OD, 100 MHz): δ = 159.2 (q, JCF = 37.1 Hz), 117.5 (q, JCF = 286.6 Hz), 39.9, 37.5.

Heptakis-[6-(2-aminoethylsulfanyl)-6-deoxy]-β–CD Heptahydrochloride salt (1). NaH (60% in mineral oil, 500 mg, 12.5 mmol) was added in small portions to a solution of freshly prepared 16 in dry DMF (10 mL). After stirring for 1 h, 17 (1 g, 0.69 mmol) in dry DMF (5 mL) was added dropwise at 0 °C. The mixture was stirred for 24 h at r.t. and quenched with water (10 mL). The volume of the mixture was reduced to 5 mL under diminished pressure and 96% EtOH (5 mL) and Et₂O (80 mL) were added. The resulting suspension was centrifuged (4000 rpm, 30 min) and the pellet resuspended in water (50 mL) and centrifugated (4000 rpm, 30 min). Analysis of the product by MALDI-TOF mass spectrometry at this point showed partial deprotection of the trifluoroacetetyl groups. Further deprotection with neat ethylene diamine (5 mL, 12 h) rendered the product water soluble. 96% EtOH (5 mL) and Et₂O (80 mL) were added, the resulting suspension centrifuged (4000 rpm, 30 min), and the pellet dissolved in NH₄OH aq. (50 mL) for full deprotection (48 h). The volume of the reaction mixture was reduced to 5 mL under diminished pressure and freeze-dried. The solid residue was dissolved in water (5 mL) and the remaining solution was adjusted to pH 4 using dilute HCl and freeze-dried. In order to remove traces of ethylene diamine, EtOH, and Et₂O retained by the cyclodextrin cavity, the residue was dissolved in a minimum amount of water, precipitated using 96% ethanol, and

V It was conveniently found removal of any impurities inherent to the preparation of 17 could be removed by recrystalization from DMSO/Methanol = 2/9 at 0 °C.
centrifuged (4000 rpm, 30 min). The pellet was redissolved in water (5 mL), extracted with CH$_3$Cl (4 x 10 mL), and freeze dried. The product was obtained as a white powder. Yield: 86% (1.07 g, 0.59 mmol). $^1$H-NMR (D$_2$O, 400 MHz): δ = 5.18 (d, 1H, $J = 3.7$ Hz), 4.08 (ddd, 1H, $J = 2.2, 6.8, 9.3$ Hz), 3.97 (m, 1H), 3.69 (m, 2H), 3.31 (t, 2H, $J = 6.7$ Hz), 3.22 (dd, 1H, $J = S 4 2.1, 14.4$ Hz), 3.04 (m, 3H); $^{13}$C-NMR (D$_2$O, 100 MHz) δ = 101.7, 83.7, 72.8, 71.7, 38.8, 32.8, 30.1; ESI-MS (positive mode) calcd for ([C$_{56}$H$_{105}$O$_{28}$N$_7$S$_7$ + 2H$^+$]/2) = 774.8. Found: $m/z$ 774.8; Exact mass (ESI-MS) calcd: ([M + 2H$^+$]/2) = 774.7599. Found: $m/z$ 774.7643.

![Figure 3.13 Synthesis of 9](image)

**Heptakis-[6-(2-(2,6-diguanidinohexanamido)-ethylsulfanyl)-6-deoxy-]-β–CD**

**Heptahydrochloride salt (9).** Potassium carbonate (0.125 g, 0.9 mmol) was added to a solution of 1a (50 mg, 0.027 mmol) in water, followed by the addition of 1-$H$-pyrazole-1-carboxamidine hydrochloric acid (0.130 g, 0.9 mmol). The resulting mixture was stirred at 60 °C for 2 days, after which a fresh portion of the aforementioned guanidylating reagent was added (0.125g, 0.9 mmol). The reaction was stirred for an additional 2 days at 60° C. The solution was diluted and the product purified by heparin affinity preparative HPLC. The collected fractions were dialyzed for 5 days using 500 MWCO dialysis tubing and subsequently freeze-dried. Yield: 10% (5.2 mg). $^1$H-NMR (D$_2$O, 400 MHz): δ = 5.15 (d, 1H, $J = 3.0$ Hz), 3.97 (dd, 2H, $J = 10.0, 19.8$ Hz), 3.71 (dd, 1H, $J = 3.1, 9.9$ Hz), 3.60 (t, 1H, $J = 9.2$ Hz), 3.49 (t, 2H, $J = 6.4$ Hz), 3.32 (d, 1H, $J =13.3$ Hz), 2.96 (m, 3H); $^{13}$C-NMR (D$_2$O, 100 MHz): δ = 157.0, 101.9, 84.7, 72.8, 72.1 (2C), 40.9, 32.9, 31.7; ESI-MS (positive mode) calcd for ([C$_{63}$H$_{119}$N$_{21}$O$_{28}$S$_7$ + 4H$^+$]/4) = 461.4. Found: $m/z$ 461.4; Exact mass (ESI-MS) calcd: ([M + 4H$^+$]/4) = 461.4217. Found: $m/z$ 461.4205.
General procedure for synthesis of trifluoracetylprotected amino acids succinimidyl esters (18-20). The corresponding amino acid (40 mmol) was dissolved in a solution of Na (80 mmol) in MeOH (100 mL). For the synthesis of 20 the natural L-enantiomer of lysine was used. After addition of ethyl 2,2,2-trifluoroacetate (44 mmol) the reaction mixture was stirred for 12 h. The mixture was neutralized with AcOH (conc.) and the solvent removed under diminished pressure. The resulting solid was redissolved in EtOAc (200 mL) and washed with 2M HCl (3 x 100 mL) and water (2 x 100 mL). The organic layer was dried on MgSO₄, filtered, and evaporated. The products were thus obtained as white solids in near quantitative yield. To a solution of the protected amino acids (20 mmol) in EtOAc (200 mL) N-hydroxy succinamide (21 mmol) and N,N'-dicyclohexyl-carbodiimide (21 mmol) are added. After stirring for 12 h, the reaction mixture is filtered and washed with sat. NaHCO₃ (3 x 100 mL). Removal of the solvent under diminished pressure affords the products (18-20) as white solids.
2-(2,2,2-trifluoroacetamido)acetic acid. $^1$H-NMR (D$_2$O, 400 MHz): $\delta = 4.09$ (s, 2H); $^{13}$C-NMR (D$_2$O, 100 MHz): $\delta = 171.9, 159.4$ (q, $J_{CF} = 27.9$ Hz), 115.8 (q, 1H, $J_{CF} = 285.6$ Hz), 41.1; ESI-MS (negative mode) calcd for (C$_4$H$_4$F$_3$NO$_3$ - H$^+$) = 170.0. Found: m/z 170.0; Exact mass (ESI-MS) calcd: (M - H$^+$) = 170.0070. Found: m/z 170.0070.

6-(2,2,2-trifluoroacetamido)hexanoic acid. $^1$H-NMR (CD$_3$OH, 400 MHz): $\delta = 4.28$ (t, 2H, $J = 7.1$ Hz), 2.30 (t, 2H, $J = 7.4$ Hz), 1.61 (m, 4H), 1.37 (m, 2H); $^{13}$C-NMR (CD$_3$OH, 100 MHz): $\delta = 177.5, 159.0$ (q, $J_{CF} = 36.7$ Hz), 117.6 (q, $J_{CF} = 286.5$ Hz), 40.6, 34.7, 29.5, 25.6, 27.3; ESI-MS (negative mode) calcd for (C$_8$H$_{12}$F$_3$NO$_3$ - H$^+$) = 226.1. Found: m/z 226.1; Exact mass (ESI-MS) calcd: (M - H$^+$) = 226.0696. Found: m/z 226.0695.

2,6-bis(2,2,2-trifluoroacetamido)hexanoic acid. $^1$H-NMR (CD$_3$OD, 400 MHz): $\delta = 4.46$ (m, 1H), 3.33 (t, 2H, $J = 7.1$ Hz), 2.03 (m, 1H, 1.85 (m, 1H), 1.64 (m, 2H), 1.46 (m, 2H); $^{13}$C-NMR (CD$_3$OD, 100 MHz): $\delta = 173.8, 159.1$ (dq, $J_{CF} = 10.2, 37.0$ Hz), 117.5 (dq, $J_{CF} = 12.8, 286.7$ Hz), 54.0, 40.4, 31.5, 29.3, 24.3; ESI-MS (negative mode) calcd for (C$_{10}$H$_{12}$F$_6$N$_2$O$_4$ - H$^+$) = 337.1. Found: m/z 337.1; Exact mass (ESI-MS) calcd: (M - H$^+$) = 337.0628. Found: m/z 337.0622.

2-(2,2,2-trifluoroacetamido)acetic acid, succinimidyl ester (18). Washed with pentane. Yield: 95% (5.1 g, 19 mmol). $^1$H-NMR ((CD$_3$)$_2$CO, 400 MHz): $\delta = 9.10$ (bs, 1H), 4.56 (d, $J = 6.1$ Hz, 2H), 2.89 (s, 4H); $^{13}$C-NMR ((CD$_3$)$_2$CO, 100 MHz): $\delta = 207.2, 171.0, 166.7, 159.2$ (q, $J_{CF} = 37.3$ Hz), 117.9 (q, $J_{CF} = 287.0$ Hz), 40.5, 27.3; ESI-MS (positive mode) calcd for (C$_8$H$_7$F$_3$N$_2$O$_5$ + Na$^+$) = 291.0. Found: m/z 291.0; Exact mass (ESI-MS) calcd: (M + Na$^+$) = 291.0199. Found: m/z 291.0186.

6-(2,2,2-trifluoroacetamido)hexanoic acid succinimidyl ester (19). Recrystallized (CH$_2$Cl$_2$/Pentane = 1/1). Yield: 85% (5.5 g, 17 mmol). $^1$H-NMR (CDCl$_3$, 400 MHz): $\delta = 6.84$ (bs, 1H), 3.35 (q, 2H, $J = 6.6$ Hz), 2.82 (s, 4H), 2.61 (t, 2H, $J = 7.1$ Hz), 1.78 (td, $J = 7.2, 15.1$ Hz, 2H), 1.62 (td, $J = 7.2, 14.4$ Hz, 2H), 1.46 (m, 2H); $^{13}$C-NMR (CDCl$_3$, 100 MHz): $\delta = 169.3, 168.4, 157.3$ (q, 1H, $J_{CF} = 36.8$ Hz), 115.8 (q, $J_{CF} = 287.8$ Hz), 39.4, 30.7, 28.0, 25.5, 25.4, 24.0; ESI-MS (positive mode) calcd for (C$_{12}$H$_{13}$F$_3$N$_2$O$_5$ + Na$^+$) = 234.1. Found: m/z 234.1; Exact mass (ESI-MS) calcd: (M + Na$^+$) = 237.0825. Found: m/z 237.0829.
2,6-bis(2,2,2-trifluoroacetamido)hexanoic acid, succinimidyl ester (20). Recrystallized (EtOAc/Pentane = 1/1). Yield: 76% (6.6 g, 15.2 mmol). $^1$H-NMR ((CD$_3$)$_2$CO, 400 MHz): $\delta =$ 8.42 (d, 1H, $J = 7.1$ Hz), 7.84 (bs, 1H), 4.29 (dd, 1H, $J = 8.3$, 13.6 Hz), 2.76 (dd, 2H, $J = 6.0$, 12.2 Hz), 2.28 (s, 4H), 1.54 (m, 1H), 1.42 (m, 1H), 1.07 (m, 4H); $^{13}$C-NMR ((CD$_3$)$_2$CO, 100 MHz): $\delta =$ 207.3, 171.1, 168.8, 158.8 (dq, $J_{CF} = 4.8$, 156.9 Hz), 118.0, (dq, $J_{CF} = 32.7$, 287.2 Hz), 52.9, 40.9, 32.1, 29.8, 27.3, 24.3; ESI-MS (positive mode) calcd for (C$_{14}$H$_{15}$F$_6$N$_3$O$_6$ + Na$^+$) = 458.1. Found: m/z 458.1; Exact mass (ESI-MS) calcd: (M + Na$^+$) = 458.0757. Found: m/z 458.0744.

General procedure for the synthesis of 10-12. To a solution of 18 or 19 or 20 (4 mmol), Bu$_4$NCl (400 mg, 1.4 mmol), and 2,6- lutidine (1 mmol, 116 μL) in dry THF (5 mL) a solution of 1 (130 mg, 0.072 mmol) and Bu$_4$NCl (800 mg, 2.8 mmol) in dry THF (12 mL) was added over the course of 2 h using a syringe pump. After stirring for 12 h, the reaction was quenched with MeOH (10 mL) and stirred for another 6 h. Subsequently, the solvent was reduced to 2 mL under diminished pressure and Et$_2$O (50 mL) was added. The resulting suspension was centrifuged (4000 rpm, 30 min) and the pellet resuspended in water (50 mL), followed by centrifugation (4000 rpm, 30 min). Analysis of the products by MALDI-TOF mass spectrometry at this point showed partial deprotection of the trifluoroacetyl groups. Further deprotection with neat ethylene diamine (5 mL, 12 h) rendered the product water-soluble. 96% EtOH (5 mL) and Et$_2$O (80 mL) were added, the resulting suspension centrifuged (4000 rpm, 30 min), and the pellet dissolved in NH$_4$OH aq. (50 mL) for full deprotection (48 h). The volume of the reaction mixture was reduced to 5 mL under diminished pressure and freeze-dried. The solid residue was dissolved in water (5 mL) and the remaining solution was adjusted to pH 4 using dilute HCl and freeze-dried. In order to remove traces of ethylene diamine, EtOH, and Et$_2$O retained by the cyclodextrin cavity, the residue was dissolved in a minimum amount of water, precipitated using 96% ethanol, and centrifuged (4000 rpm, 30 min). The pellet was redissolved in water (5 mL), extracted with CH$_3$Cl (4 x 10 mL), and freeze dried.

Heptakis-[6-(2-(2-aminoacetamido)-ethylsulfanyl)-6-deoxy-]-$\beta$–CD Heptahydrochloride salt (10). Yield: 82% (130 mg, 0.059 mmol). $^1$H-NMR (D$_2$O, 400 MHz): $\delta =$ 5.22 (d, 1H, $J =$ 3.1 Hz), 3.96 (dt, 2H, $J =$ 4.1, 9.1 Hz), 3.86 (s, 2H), 3.68 (dd, 1H, $J =$ 3.4, 10.2 Hz), 3.61 (t, 1H, $J =$ 9.6 Hz), 3.54 (t, 2H, $J =$ 6.7 Hz), 3.26 (d, 1H, $J =$ 12.0 Hz), 2.95 (dd, 1H, $J =$ 8.6, 13.7 Hz), 2.87 (t,
2H, J = 6.7 Hz); $^{13}$C-NMR (D$_2$O, 100 MHz): $\delta = 167.0, 100.2, 82.7, 72.9, 71.8, 71.6, 40.6, 39.1, 33.4, 31.9; $ ESI-MS (positive mode) calcd for ([C$_{70}$H$_{126}$N$_{14}$O$_{35}$S$_7$ + 2H$^+$]/2) = 974.3. Found: m/z 974.3; Exact mass (ESI-MS) calcd: ([M + 2H$^+$]/2) = 974.3350. Found: m/z 974.3384.

**Heptakis-[6-(2-(6-aminohexanamido)-ethylsulfanyl)-6-deoxy-]-β–CD Heptahydrochloride salt (11).** Yield: 66% (122 mg, 0.047 mmol). $^1$H-NMR (D$_2$O, 400 MHz): $\delta = 5.15$ (d, 1H, $J = 3.4$ Hz), 3.86 (m, 2H) 3.72 (dd, 1H, $J = 3.5, 9.9$ Hz), 3.59 (t, 1H, $J = 9.2$ Hz), 3.45 (m, 2H), 3.33 (d, 1H, $J = 12.7$ Hz), 3.02 (dd, 2H, $J = 6.9, 8.7$ Hz), 2.79-2.98 (m, 3H), 2.28 (t, 2H, $J = 7.5$ Hz), 1.69 (m, 4H), 1.42 (m, 2H); $^{13}$CNMR (D$_2$O, 100 MHz): $\delta = 176.3, 101.7, 84.6, 73.2, 72.0, 39.4, 39.1, 35.7, 32.9, 31.9, 26.7, 25.5, 25.1; $ ESI-MS (positive mode) calcd for ([C$_{98}$H$_{182}$N$_{14}$O$_{35}$S$_7$ + 3H$^+$]/3) = 780.7. Found: m/z 780.7; Exact mass (ESI-MS) calcd: ([M + 3H$^+$]/3) = 780.7051. Found: m/z 780.7062.

**Heptakis-[6-(2-(2,6-diaminohexanamido)-ethylsulfanyl)-6-deoxy-]-β–CD Heptahydrochloride salt (12).** Yield: 42% (82 mg, 0.038 mmol). $^1$H-NMR (D$_2$O, 400 MHz): $\delta = 5.20$ (bs, 1H), 4.06 (t, 1H, $J = 6.6$ Hz), 3.97 (m, 2H), 3.67 (m, 2H), 3.55 (t, 2H, $J = 5.2$ Hz), 3.24 (d, 1H, $J 12.2$ Hz), 3.05 (dd, 1H, $J = 6.9, 8.7$ Hz), 2.80-3.02 (m, 3H), 1.97 (m, 2H), 1.77 (m, 2H), 1.53 (m, 2H); $^{13}$C-NMR (D$_2$O, 100 MHz): $\delta = 169.8, 101.4, 84.2, 72.9, 71.9, 53.3, 39.2, 33.5, 31.9, 30.8, 26.575, 21.6; $ ESI-MS (positive mode) calcd for ([C$_{98}$H$_{189}$N$_{21}$O$_{35}$S$_7$ + 3H$^+$]/3) = 815.7. Found: m/z 815.7; Exact mass (ESI-MS) calcd: ([M + 3H$^+$]/3) = 815.7306. Found: m/z 815.7370.
Compounds 13-15 were prepared according to the reaction sequence in Figure 3.15. Compounds 25-27 were prepared according to a literature procedure and crystallized (CH₂Cl₂/pentane = 1/1).

**tert-Butyl 2-(quinoli-6-yloxy)acetate (21).** NaH (60% in mineral oil, 332 mg, 8.3 mmol) was added to a solution of 6-hydroxyquinoline (1 g, 6.9 mmol) in dry DMF (30 mL) and the mixture was stirred for 30 min. Subsequently, tert-butyl bromoacetate (1.2 mL, 6.9 mmol) was added and the reaction was stirred for 12 h. After removal of the solvent under diminished pressure, the
residual oil was taken up in CH$_3$Cl (150 mL) and washed with water (3 x 50 mL). The organic layer was dried on MgSO$_4$, filtered, and evaporated. The resulting residue was purified by column chromatography (EtOAc/pentane = 1/1, Rf = 0.45) and the product was obtained as a red oil. Yield: 96% (1.7 g, 6.6 mmol). $^1$H-NMR (CDCl$_3$, 400 MHz): $\delta = 8.78$ (d, 1H, $J = 3.1$ Hz), 8.01 (dd, 1H, $J = 1.3, 8.1$ Hz), 8.03 (d, 1H, $J = 9.2$ Hz), 7.44 (dd, 1H, $J = 2.8, 9.2$ Hz), 7.34 (dd, 1H, $J = 4.2, 8.3$ Hz), 7.0 (d, 1H, $J = 2.8$ Hz), 4.63 (s, 2H), 1.49 (s, 9H); $^{13}$C-NMR (CDCl$_3$, 100 MHz): $\delta = 167.5, 155.9, 148.3, 144.6, 134.8, 131.1, 128.9, 122.0, 121.4, 106.4, 82.5, 65.8, 28.0$; ESI-MS (positive mode) calcd for (C$_{15}$H$_{17}$NO$_3$ + H$^+$) = 260.1. Found: $m/z$ 260.1; Exact mass (ESI-MS) calcd: (M + H$^+$) = 260.1281. Found: $m/z$ 260.1272.

*tert*-Butyl 2-((N-methyl quinolinium)-6-yloxy)acetate iodide (22). MeI (0.92 mL, 14.8 mmol) was added to a solution of compound 21 (0.75 g, 3.7 mmol) in 10 mL of dry THF. The reaction was stirred for 12 hours, during which the product precipitated. Filtration afforded the product as a bright yellow solid. Yield: 78% (1.2 g, 2.9 mmol). $^1$H-NMR (CD$_3$OD, 400 MHz): $\delta = 9.21$ (d, 1H, $J = 5.7$ Hz), 9.08 (d, 1H, $J = 8.5$ Hz), 8.47 (d, 1H, $J = 9.7$ Hz), 8.02 (dd, 1H, $J = 5.8, 8.5$ Hz), 7.95 (dd, 1H, $J = 2.9, 9.7$ Hz), 7.76 (d, 1H, $J = 2.8$ Hz), 4.92 (s, 2H), 4.68 (s, 3H), 1.51 (s, 9H); $^{13}$C-NMR (CD$_3$OD, 100 MHz): $\delta = 168.9, 160.0, 148.5, 147.1, 136.3, 133.1, 129.4, 123.4, 121.7, 110.3, 84.1, 67.0, 46.494, 28.341$; ESI-MS (positive mode) calcd for (C$_{16}$H$_{20}$NO$_3$ +) = 274.1. Found: $m/z$ 274.1; Exact mass (ESI-MS) calcd: (M+) = 274.1437. Found: $m/z$ 274.1444.

2-((N-methyl quinolinium)-6-yloxy)acetic acid chloride (23). Compound 22 (1.1 g, 2.7 mmol) was dissolved in conc. HCl (20 mL) and left standing for 12 h. After removal of the solvent under diminished pressure, the residue was recrystallized from EtOH, filtered, and washed with Et$_2$O. The product was obtained as a pale yellow solid. Yield: 89% (610 mg, 2.4 mmol). $^1$H-NMR (D$_2$O, 400 MHz): $\delta = 9.04$ (d, 1H, $J = 5.3$ Hz), 8.93 (d, 1H, $J = 8.5$ Hz), 8.34 (d, 1H, $J = 9.7$ Hz), 7.95 (dd, 1H, $J = 5.8$ Hz), 7.90 (m, 1H), 7.63 (d, 1H, $J = 2.9$ Hz), 4.94 (s, 2H), 4.62 (s, 3H); $^{13}$C-NMR (D$_2$O, 100 MHz): $\delta = 172.7, 157.6, 147.0, 146.0, 135.0, 131.5, 127.8, 122.2, 120.4, 109.1, 65.5, 45.5$; ESI-MS (positive mode) calcd for (C$_{12}$H$_{12}$NO$_3$ +) = 218.1. Found: $m/z$ 218.1; Exact mass (ESI-MS) calcd: (M$^+$) = 218.0811. Found: $m/z$ 218.0817.

**General procedure for the synthesis of 25-27.** Lithocholic acid succinimidyl ester (24, 800 mg, 1.7 mmol) in dry DMF (5 mL) was slowly added to the corresponding diamine (67.2 mmol) in
dry DMF (5 mL) in the course of 1 h using a syringe pump, after which the mixture was stirred for 12 h. After removal of the solvent under diminished pressure the residue was redissolved in CH₂Cl₂ (200 mL) and extracted with water (3 x 100 mL). The organic layer was dried on MgSO₄, filtered, and evaporated. The resulting residue was suspended in water (50 mL) and separated by centrifugation (4000 rpm, 30 min). The product was obtained as a white powder.

\textbf{\textit{N-8-Lithocholylamino-ethylamine (25).}} Yield: 87\% (619 mg, 1.48 mmol). \textsuperscript{1}H-NMR (CD₃OD, 400 MHz): δ = 3.54 (m, 1H), 3.26 (t, J = 6.3 Hz, 2H), 2.76 (t, J = 6.3 Hz, 2H); 0.90- 2.29 (m, 34H), 0.69 (s, 3H); \textsuperscript{13}C-NMR (CD₃OD, 100 MHz): δ = 177.3, 72.5, 58.0, 57.5, 44.0, 43.6, 42.3, 41.9, 41.6, 37.3, 36.9, 36.5, 35.7, 34.2, 33.3, 31.3, 29.3, 28.4, 27.7, 25.3, 24.0, 22.0, 18.9, 12.6; ESI-MS (positive mode) calcd for (C₂₆H₄₇N₂O₂ +) = 419.4. Found: m/z 419.4; Exact mass (ESIMS) calcd: (M+) = 419.3632. Found: m/z 419.3639.

\textbf{\textit{N-8-Lithocholylamino-butylamine (26).}} Yield: 79\% (600 mg, 1.34 mmol). \textsuperscript{1}H-NMR (CD₃OD, 400 MHz): δ = 3.54 (m, 1H), 3.17 (t, J = 6.4 Hz, 2H), 2.69 (t, J = 6.4 Hz, 3H); 0.90- 2.29 (m, 38H), 0.69 (s, 3H); \textsuperscript{13}C-NMR (CD₃OD, 100 MHz): 176.8, 72.5, 58.0, 57.5, 44.0, 43.6, 41.9, 41.6, 40.1, 37.3, 37.2, 36.9, 36.5, 35.7, 34.2, 33.4, 31.3, 30.4, 29.3, 28.4, 27.8, 27.7, 25.3, 24.0, 22.0, 18.9, 12.6; ESI-MS (positive mode) calcd for (C₂₈H₅₁N₂O₂ +) = 447.4. Found: m/z 447.4; Exact mass (ESI-MS) calcd: (M+) = 447.3945. Found: m/z 447.3963.

\textbf{\textit{N-8-Lithocholylamino-3,6-dioxaoctylamine (27).}} Yield: 83\% (706 mg, 1.4 mmol). \textsuperscript{1}H-NMR (CD₃OD, 400 MHz): 3.63 (m, 4H), 3.55 (m, 5H), 3.35 (t, J = 5.4 Hz, 2H), 2.83 (m, 2H), 0.9-2.30 (m, 34H), 0.68 (s, 3H); \textsuperscript{13}C-NMR (CD₃OD, 100 MHz): δ = 176.9, 72.6, 71.4, 71.3, 70.7, 58.0, 57.5, 44.0, 43.6, 41.9, 41.6, 40.4, 37.3, 37.2, 36.9, 36.5, 35.7, 34.1, 33.3, 31.3, 29.3, 28.4, 27.7, 25.3, 24.0, 22.0, 18.9, 12.6; ESI-MS (positive mode) calcd for (C₃₀H₅₄N₂O₄ + H⁺) = 507.4. Found: m/z 507.4; Exact mass (ESI-MS) calcd: (M + H⁺) = 507.4156. Found: m/z 507.4153.

\textbf{General procedure for the synthesis of 13-15.} Compound 23 (200 mg, 0.78 mmol) and KPF₆ (72.6 mg, 0.79 mmol) were dissolved in dry DMF and stirred at 50 °C for 10 min. Subsequently, \textit{N}-hydroxysuccinimide (94 mg, 0.82 mmol) and \textit{N},\textit{N}-diisopropylcarbodiimide (128 μL, 0.82 mmol) were added. After stirring for 30 min at 50 °C, the reaction was allowed to cool down to r.t. Subsequently, compound 25, 26, or 27 (0.78 mmol) was added. After stirring for 12 h, the solvent was removed under diminished pressure and the residue extracted with CH₂Cl₂ (100
mL). The organic layer was washed with water (3 x 50 mL), dried on MgSO₄, filtered, and reduced to 2 mL under diminished pressure. The product was subsequently precipitated with pentane and recrystallized from iPrOH.

*N*-2-Lithocholylamino-ethyl-((*N*-methylquinolinium)-6-yloxy) acetamide (13). Yield: 45% (229 mg, 0.35 mmol). ¹H-NMR (CD₃OD, 400 MHz): δ = 9.18 (d, J = 5.7 Hz, 1H), 9.09 (d, J = 8.4 Hz, 1H), 8.49 (d, J = 9.7 Hz, 1H), 8.06 (dd, J = 2.8, 9.6 Hz, 1H), 7.82 (d, J = 2.8 Hz, 1H), 4.79 (s, 2H), 4.68 (s, 3H), 3.54 (m, 1H), 3.39 (m, 4H), 0.90-2.29 (m, 34H), 0.65 (s, 1H); ¹³C-NMR (CD₃OD, 100 MHz): δ = 177.5, 170.0, 159.6, 148.6, 147.3, 136.5, 133.2, 129.7, 123.5, 121.7, 110.4, 72.4, 68.6, 58.0, 57.5, 46.3, 43.9, 43.5, 41.9, 41.6, 40.7, 39.7, 37.2, 36.9, 36.5, 35.7, 34.2, 33.3, 31.2, 29.3, 28.4, 27.7, 25.3, 24.0, 22.0, 18.9, 12.5; ESI-MS (positive mode) calcd for (C₃₈H₅₆N₃O₄⁺) = 618.4. Found: m/z 618.4; Exact mass (ESI-MS) calcd: (M⁺) = 618.4265. Found: m/z 618.4273.

*N*-4-Lithocholylamino-butyl-((*N*-methyl quinolinium)-6-yloxy) acetamide (14). Yield: 26% (136 mg, 0.20 mmol). ¹H-NMR (CD₃OD, 400 MHz): δ = 9.17 (d, J = 5.7 Hz, 1H), 9.06 (d, J = 8.5 Hz, 1H), 8.48 (d, J = 9.7 Hz, 1H), 8.02 (m, 2H), 7.79 (d, J = 2.8 Hz, 1H), 4.79 (s, 2H), 4.67 (s, 3H), 3.53 (m, 1H), 3.32 (t, J = 6.7 Hz, 2H), 3.16 (t, J = 6.7 Hz, 2H), 0.90-2.29 (m, 38H), 0.67 (s, 3H); ¹³C-NMR (CD₃OD, 100 MHz): δ = 176.8, 169.6, 159.7, 148.6, 147.2, 136.5, 133.1, 129.6, 123.5, 121.7, 110.3, 72.4, 68.6, 58.0, 57.5, 46.3, 44.0, 43.6, 41.9, 41.6, 40.0, 37.3, 37.2, 36.9, 36.5, 35.7, 34.2, 33.4, 31.2, 29.3, 28.4, 27.8, 27.7, 25.3, 24.0, 22.0, 18.9, 12.5; ESI-MS (positive mode) calcd for (C₄₀H₆₀N₃O₄⁺) = 646.5. Found: m/z 646.5; Exact mass (ESI-MS) calcd: (M⁺) = 646.4578. Found: m/z 646.4574.

*N*-8-Lithocholylamino-3,6-dioxaoctyl-((*N*-methylquinolinium)-6-yloxy)acetamide (15). Yield: 34% (187 mg, 0.27 mmol). ¹H-NMR (CD₃OD, 400 MHz): δ = 9.17 (d, 1H, J 5.8 Hz), 9.07 (d, 1H, J 8.4 Hz), 8.48 (d, 1H, J 9.7 Hz), 8.02 (m, 2H), 7.80 (d, 1H, J 2.8 Hz), 4.83 (s, 1H), 4.67 (s, 1H), 3.62 (m, 5H), 3.53 (m, 4H), 3.34 (d, 2H, J = 6.1 Hz), 0.90-2.29 (m, 36H), 0.67 (s, 3H); ¹³C-NMR (CD₃OD, 100 MHz): δ = 176.9, 169.8, 159.7, 148.6, 147.3, 136.5, 133.1, 129.6, 123.5, 121.7, 110.5, 72.4, 71.4, 71.3, 70.7, 70.5, 68.7, 56.0, 57.5, 46.3, 44.0, 43.6, 41.9, 41.6, 40.4, 40.1, 37.3, 37.2, 36.9, 36.5, 35.7, 34.1, 33.3, 31.2, 29.3, 28.4, 27.7, 25.3, 24.0, 22.0, 18.9,
12.5; ESI-MS (positive mode) calcd for \((C_{42}H_{64}N_{3}O_{6})^{+}\) = 706.5. Found: \(m/z\) 706.5; Exact mass (ESI-MS) calcd: \((M^{+}) = 706.4800\). Found: \(m/z\) 706.4786.

### 3.5.3 Discriminant Receptor Array

Solutions of the polycationic CD binding motifs 1, 9-12 (10 μM) and LCA-fluorophore conjugate 13 (10 μM) were prepared in 2 x phosphate buffer saline (2 x PBS, pH 7.4). The fluorescent receptor complex solutions (40 μL) were combined with each analyte or analyte mixture in water (40 μL) in a 96 well-plate. Eight replicates were prepared for each fluorescent receptor/analyte combination. The increase in fluorescence intensity after adding the analytes to the self-assembled fluorescent receptors was measured with a fluorescence plate reader. For each fluorescent receptor/analyte combination the relative fluorescence increase \(F/F_0\) was calculated. To confirm the reproducibility of the data, for the analytes DS, UFH, and OSCS, the same experiment was repeated with new stock solutions. The deviation for each recognition pattern \((F/F_0)\) was below 5%. In order to evaluate if the resultant recognition patterns can be applied for discrimination and identification, the SYSTAT statistical software package was used to perform a linear discriminant analysis (LDA). Jackknifed classification matrices were used to evaluate discrimination results. Furthermore, the predictive ability of the receptor array was determined by a cross-validation routine, in which randomly 50% of the dataset was omitted. The randomly selected data was used as the training set for the LDA, and the omitted remainder was classified.

### 3.5.4 Linear Discriminant Analysis (LDA) and Jackknifed Classification Matrixes of Fluorescent Probes

**Table 3.1** LDA for the discrimination of 8 different negatively charged macromolecules (see Figure 3.6).

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Table 3.2 Summary of the Jacknifed classification matrix for Table 3.1

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Table 3.3 Cumulative proportion of total dispersion for Table 3.1

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The data from Figure 3.6 was used but this time 50% of the data was taken out randomly and reclassified.
**Table 3.4** LDA (cross-validation) for the discrimination of 8 different negatively charged macromolecules

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**Table 3.5** Summary of the Jacknifed classification matrix for Table 3.4

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Table 3.6 Cumulative proportion of total dispersion for Table 3.4

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Table 3.7 LDA for mixtures of DS, UFH, and OSCS (Figure 3.8)

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Table 3.8 Summary of the Jacknifed classification matrix for Table 3.7

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</table>

**Table 3.9** Cumulative proportion of total dispersion for Table 3.7

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<td>0.698</td>
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<td>0.997</td>
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</table>

The data from Figure 3.8 was used but this time 50% of the data was taken out randomly and reclassified.

**Table 3.10** LDA (cross-validation) for the discrimination of 8 different negatively charged macromolecules

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Table 3.11 Summary of the jacknifed classification matrix for Table 3.10

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<th>Analyte (6 μg/mL)</th>
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<td>DS:UFH (1:9)</td>
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<tr>
<td>UFH</td>
<td>100</td>
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<tr>
<td>UFH:OSCS (9:1)</td>
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Table 3.12 Cumulative proportion of total dispersion for Table 3.10

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Table 3.13 LDA for different concentrations of DS, UFH, and OSCS (Figure 3.10)

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<td>9 • 13</td>
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<tr>
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<td>12 • 13</td>
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Table 3.14 Summary of the Jacknifed classification matrix for Table 3.13

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<th>Analyte (6 μg/mL)</th>
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<td>DS (4 μg/mL)</td>
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<td>Variable</td>
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<td>-------------------</td>
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<tr>
<td>DS (6 μg/mL)</td>
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<td>DS (8 μg/mL)</td>
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Table 3.15 Cumulative proportion of total dispersion for Table 3.13

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<th>0.997</th>
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</table>

The data from Figure 3.10 was used but this time 50% of the data was taken out randomly and reclassified.

Table 3.16 LDA (cross-validation) for different concentrations of DS, UFH, and OSCS

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<th>F-to-remove</th>
<th>Tolerance</th>
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<table>
<thead>
<tr>
<th>Analyte (6 μg/mL)</th>
<th>% correct</th>
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</thead>
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<tr>
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<tr>
<td>DS (4 μg/mL)</td>
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<td>DS (6 μg/mL)</td>
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<td>DS (8 μg/mL)</td>
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<td>-------</td>
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<td>OSCS (8 µg/mL)</td>
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<tr>
<td>Total</td>
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</table>

**Table 3.18** Cumulative proportion of total dispersion for Table 3.16

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<tbody>
<tr>
<td>0.807</td>
<td>0.966</td>
<td>0.997</td>
<td>1.000</td>
<td>1.000</td>
</tr>
</tbody>
</table>
4 Mono-acylation of a polyamine-β-cyclodextrin based on guest mediated acyl migration

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4.1 Introduction

In comparison with native β-CDs, mono-derivatized β-CDs have a broader range of applications due to their increased functionality.13 The majority of mono-derivatized β-CDs that have been produced rely on the mono-tosylation of a primary alcohol on CD's primary rim.42a, 100 The toluenesulfonyl ester then serves as a leaving group allowing for the introduction of a variety of nucleophiles yielding the desired mono-functionalized CD.13 Other innovative methods to functionalize CDs include selective benzyl deprotections,39, 47, 49, 101 tritylations,54 and the introduction of biselectrophiles.102 Starting from unfunctionalized CDs, the yields of these typical mono-functionalizations can be good with the use of the correct protecting groups and chromatographic separations. To complement the available methods,13 here we report a new method to mono-functionalize heptakis-[6-deoxy-6-(2-aminoethylsulfanyl)]-β-cyclodextrin (1) in a single step via a guest mediated S → N acyl delivery.

Amine functionalized CD 1 has served as a versatile skeleton for sensor design and binds a variety of guests with exceptional affinity.103 Further elaboration of 1 will allow the development of more complex supramolecular sensors and other heterofunctionalized CD derivatives.

4.2 Enabling Mono-acylation of 1

CD 1 forms a stable host-guest complex with 7-diethylaminocoumarin-3-carboxylic acid (\(K_a = 2.7 \times 10^6\) M).67 The shape complementarity and electrostatic interactions in the complex orient the coumarin's carboxylate towards the primary amines of 1. Thus, it was hypothesized that functionalization of the coumarin as an activated acyl donor would allow acylation of 1 upon formation of a host–guest complex in a covalent capture strategy.103b The coumarin could then remain bound in the cavity of the cyclodextrin to prevent further acylation.
The proposed mono-acylation reaction would be analogous to transesterification reactions between activated esters and the secondary rim of β-cyclodextrins. The mechanism and rates of these acylations have been studied extensively. However, the transesterification reactions have not been optimized to allow high yields of mono-acylated cyclodextrins to be isolated.

Thioesters were chosen as the mild acylating agents to functionalize the coumarin; thioesters are slow to hydrolyze but will undergo direct aminolysis under ambient conditions. The thioglycolic acid esters also provided increased aqueous solubility to the coumarin acylating agents. The two thioester guests (28 and 29) were synthesized to explore the effect of the spacing between the hydrophobic guest and the thioester on acylation (Figure 4.1). It was found that the introduction of an alkyl spacer to DEAC did not dramatically alter its binding affinity to 1 ($K_a = (1.5 \pm 0.1) \times 10^6$ M, Figure 4.7).
4.3 Investigation of Aminolysis Rate Acceleration by 1

The aminolysis of the thioester guests 28 and 29 was evaluated for the reaction rate and the selectivity of the reaction for mono-acylation of 1. The S-N acyl migration for the aminolysis reactions of 28 and 29 was monitored using UV/Vis spectroscopy. Coumarin thioester 28 undergoes a blue shift upon aminolysis (λ_{max} = 450 to 431 nm). The reaction of thioester 29 was followed by release of thiol, which could be quantified with Ellman’s reagent. The reaction rate of thiols with excess Ellman’s reagent is six orders of magnitude greater than the observed rates of aminolysis by 1 and ethylenediamine (EDA). EDA was chosen to benchmark the rate of aminolysis of the thioesters in the absence of complex formation as the second pKa of EDA.
(7.63) is comparable to the least basic amine of 1 (pKa 7.37). EDA was used at an equivalent concentration to the amines of CD 1. The reaction progress of thioester aminolysis with 1 and EDA is shown in Figure 4.2 and the initial rates are tabulated (Table 4.1). The relative rate of

**Figure 4.2** Aminolysis of thioester guests 28 and 29 overtime at 296 K borate buffer (100 mM, pH 8.5). Reagents and conditions (■) EDA (6.8 × 10⁻³ M) and 28 (9.7 × 10⁻⁶ M). (▼) EDA (6.8 × 10⁻³ M), 29 (9.7 × 10⁻⁶ M), and Ellmans reagent (5.1 × 10⁻² M), (●) 1 (9 × 10⁻⁴ M) and 28 (9.7 × 10⁻⁶ M) (▲) 1 (9 × 10⁻⁴ M), 29 (9.7 × 10⁻⁶ M), and Ellmans reagent (5.1 × 10⁻² M).

**Table 4.1** Initial rates of thioester aminolysis

<table>
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<tr>
<th>Amine</th>
<th>Thioester</th>
<th>Initial rates / M min⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDA</td>
<td>28</td>
<td>(9.6 ± 1.3) × 10⁻⁹</td>
</tr>
<tr>
<td>EDA</td>
<td>29</td>
<td>(38 ± 6) × 10⁻⁹</td>
</tr>
<tr>
<td>1</td>
<td>28</td>
<td>(370 ± 25) × 10⁻⁹</td>
</tr>
<tr>
<td>1</td>
<td>29</td>
<td>(1300 ± 1.5) × 10⁻⁹</td>
</tr>
</tbody>
</table>
aminolysis with EDA of 28 was found to be 38 times slower than aminolysis by 1. Interestingly, a similar difference in initial rates was observed with 29 undergoing aminolysis 34 times faster with 1 than with EDA. The acceleration of aminolysis observed with 1 is below the accelerations observed for the transesterification reactions with β-CD. However, the aminolysis experiments clearly show that the non-covalent inclusion complex formation accelerates the reaction.

4.4 Analysis of Acylation Using Reverse Phase HPLC

The extent of acylation of 1, resulting from the aminolysis of 28 and 29, was evaluated with reverse phase HPLC (RP-HPLC) (Figure 4.3) and MALDI-MS (Figure 4.11).

![Figure 4.3 RP-HPLC traces of the aminolysis of 28 and 29](image)

**Figure 4.3** RP-HPLC traces of the aminolysis of 28 and 29 (Borate buffer 100 mM, pH 8.5 at 298 K, 16 h). The z-axis (not displayed) is the normalized absorption at 430 nm. A) 28 (1 × 10^{-5} M). B) 5 (1 × 10^{-5} M). C) Reaction of 28 (1 × 10^{-5} M) and 1 (1 × 10^{-5} M). D) Reaction of 28 (1 × 10^{-5} M) and 1 (2 × 10^{-5} M). E) Reaction of 29 (1 × 10^{-5} M) and 1 (1 × 10^{-5} M). F) Reaction of 29 (1 × 10^{-3} M) and 1 (1 × 10^{-3} M).

The mono-acylated cyclodextrin product 30 elutes at 23.7 min. In aminolysis reactions with equimolar ratios of 28 and cyclodextrin 1 the monoacylated CD 30 was not the sole product; di-acylated and tri-acylated derivatives of 1 were identified in the compounds eluting between 25 and 40 min (Figure 4.3). The integration of the peak at 23.7 min revealed a yield of 44% corresponding to the desired mono-acylated product. When the mole ratio of 1 to thioester 28 was doubled (2 : 1), solely mono-acylated CD 30 was produced along with unreacted CD 1.
(Figure 4.3 (D)). Under these reaction conditions purified product was obtained with a 45% yield (based on 1). The reaction between 1 and 29 at an equimolar concentration resulted exclusively in mono-acylation (Figure 4.3 (E)), and a final isolated yield of 78% (based on 1). Even in a saturated reaction solution of 29 and 1 (1 × 10⁻³ M) the reaction proceeded with exquisite selectivity, yielding only the mono-functionalized product (Figure 4.3 (F)). Further experiments carried out with a 2 fold excess of guest to cyclodextrin 1 also showed excellent selectivity with thioester 29 and little selectivity with thioester 28 (Figure 4.4 (1–2)).

![HPLC traces](image_url)

**Figure 4.4** HPLC traces for the reaction of 1 with two equivalents of 28 or 29. 1. (A) 29 (2×10⁻⁵ M). (B) Reaction of 29 (2×10⁻⁵ M) and 1 (1×10⁻⁵ M) in 6 mL borate buffer (10 mM, pH 8.5) overnight at room temperature. 2. (A) 28 (2×10⁻⁵ M). (B) Reaction of 28 (2×10⁻⁵ M) and 1 (1×10⁻⁵ M) in 6 mL borate buffer (10mM, pH 8.5) overnight at room temperature.

4.5 Evaluating the Selectivity of Acylation Using NMR Spectroscopy

To probe the source of the differing selectivity between the two thioesters, NMR studies were carried out to determine if both 30 and 31 form intramolecular inclusion complexes. Due to spectral overlap in the desymmetrized CDs 30 and 31, 2D TOCSY experiments were necessary to identify the products’ spin systems and gCOSY experiments performed to assign the identity resonances (Table 4.4). Similarly, the coumarin resonances were identified by gCOSY and ROESY experiments.
ROE cross-peaks between the $^1$H resonances of the coumarin and the H-3’s of the glucose rings of 30 and 31 are diagnostic of inclusion complexes (Figure 4.5).

**Figure 4.5** Key ROESY cross-peaks for the structural elucidation of 30 and 31 in D$_2$O. Spectra were acquired in a 500 MHz at 298 K with a mixing time of 300 ms. A) 30 ($1.9 \pm 0.5 \times 10^{-3}$ M). B) 30 ($4.3 \pm 0.5 \times 10^{-3}$ M). C) 31 ($9.5 \pm 0.5 \times 10^{-3}$ M).

Contacts were not observed between coumarin’s methyl resonances and 30’s cavity ($^1$H-3 or $^1$H-5) at a concentration of $1.9 \pm 0.5 \times 10^{-3}$ M, whereas at higher concentrations ($6.3 \pm 0.5 \times 10^{-3}$ M) two sets of coumarin methyl signals appeared at 1.25 and 1.40 ppm (Figure 4.5). Strong ROE cross-peaks between glucose $^1$H-3 and the downfield shifted coumarin methyl signal were observed. The concentration dependent behaviour of the NMR signals suggests that intermolecular complexes form with CD 30 and self-inclusion of the coumarin is not favoured in CD 30 (Figure 4.6). In CD 31, a strong ROE cross-peak between the coumarin methyl resonance and the glucose $^1$H-3’s was observed across all concentrations investigated.
Figure 4.6 $^1$HNMR Array of different concentrations of 30. (A) 6.3±0.5 $\times$ 10$^{-3}$ M. (B) 4.3±0.5 $\times$ 10$^{-3}$ M. (C) 1.9±0.5 $\times$ 10$^{-3}$ M.

From the observed ROE signals for cyclodextrin 31 and the appended coumarin, it is apparent that 31 adopts a self-included conformation. This intramolecular inclusion may explain the selectivity of the acylation reactions observed. After acylation of 1 with 29, the coumarin of 31 occupies the CD’s cavity, preventing further acylations with free thioester 29 in solution, whereas with CD 30, the coumarin does not self-include as efficiently, leaving the cavity open to facilitate subsequent acylations.

4.6 Establishing the Potential of Mono-Acylated Polyamine CD’s as Fluorescent Probes

As a simple test of the potential of CD’s 30 and 31 in a fluorescent probe design, the fluorescent responses of these supramolecules were evaluated upon binding negatively charged biomolecules. The DEAC fluorophore is environmentally sensitive and thus any changes to the
local environment upon interaction of the CD with a negatively charged binding partner should translate into a change in the fluorescence signal. The fluorescence responses of CDs 30 and 31 were evaluated with differentially charged proteins lysozyme (pI ≈ 11) and glucose oxidase (pI ≈ 4.5) as well as two negatively charged polysaccharides (Figure 4.7).

**Figure 4.7** Fluorescence response of 30 (grey bar) and 31 (black bar) at a host concentration of $1.65 \times 10^{-5}$ M in phosphate buffer saline pH 7.4. The analyte concentration in solution was 1 mg/mL. Standard deviation is below 1%. A) lysozyme. B) glucose oxidase. C) chondroitin sulphate A. D) heparin.

Of the two proteins the negatively charged protein, glucose oxidase, gave the larger change in the fluorescence signal upon addition to CD 30 or 31. Titrations with the polysaccharides chondroitin sulfate and heparin also lead to differential responses. Chondroitin, which is weakly negatively charged, gave a limited response. The highly negatively charged heparin gave a strong increase in the fluorescence signal (Figure 4.5). Although this is a preliminary result it clearly illustrates that binding interactions between biomolecules and CDs 30 and 31 will differentially affect the fluorescence emission. The varied fluorescence responses observed are promising for the future generation of differential sensor arrays from these scaffolds, as a multivalent presentation of potential recognition sites for biomolecules could be generated by elaborating the primary amines of 30 and 31.

### 4.7 Conclusions

In conclusion, an effective covalent-capture strategy has been developed to mono-acylate 1 using hydrophobic thioesters without the use of protecting groups. Thioester aminolysis experiments
indicate that inclusion complex formation of the thioester guest with 1 leads to significant acylation rate acceleration. To control the extent of acylation of 1 the thioester guest must form a self-inclusion complex after acylation. The potential of the coumarin functionalized CDs in a fluorescent sensor design has been established.

4.8 Experimental

4.8.1 Instrumentation and Chemicals

Unfractionated heparin was purchased from Celsius laboratories isolated from pig intestinal mucosa and was obtained as the sodium salt. Egg white lysozyme was purchased from BioShop Canada Inc. All other chemicals were purchased from Sigma Aldrich. Solvents were purchased from Acros chemicals. All reactions were performed under a nitrogen-atmosphere and at room temperature, unless stated otherwise. $^1$H- and $^{13}$C-NMR spectra were recorded on a Varian 400, 500 or Bruker 400 MHz spectrometers at T = 297 K. Absorption spectra were measured on a Schimadzu UV-2401PC spectrophotometer at T = 296 K. Fluorescence studies were performed using a Perkin-Elmer LS-50BLuminescence Spectrophotometer. HPLC separations and analysis were performed on a Waters 1525 Binary HPLC pump and 2487 dual λ absorption detector using a Waters XBridgeTM Prep BEH130 C18 5 μm (10 × 250 mm) reverse phase analytical column. Silica chromatography was performed with SiliCycle Silica-P Flash Silica Gel. High resolution mass spectra were obtained from an ABI/Sciex QStar mass spectrometer with an ESI source. The MALDI spectra were taken in a Waters® Micromass® MALDI micro MX™ (matrix-assisted laser desorption/ionization time-of-flight mass spectrometer [MALDI-ToF MS]).
4.8.2 Synthetic procedures

Compounds 32 and 33 were prepared according to a literature procedure.\textsuperscript{107}

2-((7-(diethylamino)-2-oxo-2H-chromene-3-carbonyl)thio)acetic acid (28). 32 (0.2 g, 0.52 mmol) was added to a solution of thioglycolic acid (0.054 mL, 0.78 mmol) and triethylamine (0.22 mL, 1.6 mmol) in DCM (15 mL). The reaction was stirred for approximately 16 h. After removal of the solvent under diminished pressure, the residue was taken up in a minimum amount of methanol (3 mL) and diluted with water (25 mL). The solution was cooled to 0°C in an ice bath, and then acidified using 1M HCl. The solution was allowed to stand for 1h at 0oC, the resulting solid was collected by filtration, and washed with water. The product was obtained as a dark yellow powder (TLC MeOH/DCM; 1/10, Rf = 0.4). Yield = 85\% (0.146 g, 0.43 mmol).\textsuperscript{107}

\textsuperscript{1}H NMR δ H (400 MHz, d6-DMSO) 8.55 (s, J = 7.4 Hz, 1H), 7.70 (d, J = 9.1 Hz, 1H), 6.83 (dd, J = 9.1 Hz, 2.4 Hz, 1H), 6.61 (d, J = 2.2 Hz, 1H), 3.73 (s, 2H), 3.52 (q, J = 7.1 Hz, 4H), 1.16 (t, J = 7.0, 6H).\textsuperscript{13}C-NMR δ C (100 MHz, d6-DMSO) 186.52, 170.89, 159.97, 158.70, 154.17,
2-((4-(7-(diethylamino)-2-oxo-2H-chromene-3-carboxamido)butanoyl)thio)acetic acid (29). (i) 34 (0.57 g, 1.6 mmol) and \(N\)-hydroxysuccinimide (0.38 g, 3.3 mmol) were dissolved in a minimum amount of THF (15 mL). The solution was cooled in an ice bath. DCC (0.37 g, 1.8 mmol) was dissolved separately in 1 mL THF and subsequently added to the aforementioned solution. The reaction was allowed to warm to room temperature overnight. The reaction was filtered through a plug of silica, washed with EtOAc, and the filtrate was evaporated under diminished pressure. The residue was dissolved in DCM, washed with a sodium bicarbonate solution (1 M), brine, dried over MgSO\(_4\), filtered, and evaporated. The residue was brought up in a minimum amount of DCM, precipitated using pentane, and a canary yellow powder was collected by filtration (TLC conditions: EtOAc, Rf = 0.4). Yield = 69% (0.49 g, 1.1 mmol). This compound was used without further purification in the next step (ii). \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 8.89 (t, \(J = 5.9\) Hz, 1H), 8.68 (s, 1H), 7.41 (d, \(J = 9.0\) Hz, 1H), 6.63 (dd, \(J = 9.0\) Hz, 2.5 Hz, 1H), 6.48 (d, \(J = 2.3\) Hz, 1H), 3.54 (dd, \(J = 13.0\) Hz, 6.8 Hz, 2H), 3.44 (q, \(J = 7.1\) Hz, 4H), 2.83 (br s, 4H), 2.76 – 2.64 (m, 2H), 2.06 (dt, \(J = 17.8\) Hz, 7.1 Hz, 2H), 1.22 (t, \(J = 7.1\) Hz, 6H). \(^{13}\)C NMR (100 MHz, CDCl\(_3\)) \(\delta\) 169.23, 168.44, 163.66, 162.96, 157.87, 152.78, 148.38, 131.36, 110.33, 110.17, 108.58, 96.80, 45.29, 38.61, 28.84, 25.80, 25.08, 12.64. HRMS-ESI m/z calcd for C\(_{22}\)H\(_{26}\)N\(_3\)O\(_7\) [M+H]\(^+\) 444.1765, found 444.1777. (ii) The succinimidyl ester of 34 (0.47 g, 1.05 mmol) was dissolved in a solution of DCM (20 mL) and triethylamine (0.44 mL, 3.2 mmol). Thioglycolic acid (0.12 mL, 1.7 mmol) was added to the aforementioned solution. The reaction was stirred for approximately 16 h. The solvent was removed under diminished pressure. The residue was taken up in a minimum amount of methanol (3 mL) and diluted with water (25 mL). After cooling the solution to 0°C using an ice bath, the product was precipitated by acidification using 1 M HCl. The solution was allowed to stand for 1 h at 0°C, filtered, and the solid washed with water. The product was obtained as a canary yellow powder (TLC conditions: MeOH/DCM; 1/9, Rf = 0.2). Yield = 56% (0.23 g, 0.55 mmol). \(^1\)H NMR (400 MHz, d6-DMSO) \(\delta\) 12.89 – 12.65 (br s, 1H), 8.64 (t, \(J = 5.9\) Hz, 1H), 8.61 (s, \(J = 16.3\) Hz, 1H), 7.64 (d, \(J = 9.0\) Hz, 1H), 6.77 (dd, \(J = 9.0\), 2.4 Hz, 1H), 6.58 (d, \(J = 2.2\) Hz, 1H), 3.66 (s, 2H), 3.45 (q, \(J = 7.0\) Hz, 4H), 3.30 (dd, \(J = 13.0\), 6.7 Hz, 2H), 2.65 (t, \(J = 7.5\) Hz, 2H), 1.79 (p, \(J = 7.2\) Hz,
2H), 1.12 (q, \(J = 7.1\) Hz, 6H). \(^{13}\)C NMR (100 MHz, dmso) \(\delta\) 198.06, 170.23, 163.00, 162.34, 157.87, 153.06, 148.31, 132.22, 110.76, 110.15, 108.32, 96.53, 45.00, 41.23, 38.71, 31.84, 25.84, 12.99. HRMS-ESI m/z calcd for C20H25N2O6S [M+H]\(^+\) 421.1427, found 421.1421.

Compound 1 was prepared according to a literature procedure.\(^{103a}\) The synthesis of 30 and 31 is described below.

**Mono-[6-deoxy-6-(7-(diethylamino)-N-(2-mercaptoethyl)-2-oxo-2H-chromene-3-carboxamide]-hexakis-[6-deoxy-6-(2-aminoethylsulfanyl)]-\(\beta\)-cyclodextrin (30).** 1 (0.02 g, 12.8\(\times\)10\(^{-3}\) mmol) and 28 (0.002 g, 6.4\(\times\)10\(^{-3}\) mol) in 640 mL of borate buffer pH 8.5 (0.01 M) was stirred overnight. The solvent was removed under diminished pressure. The residue was taken up in a minimum amount of water and purified by RP-C18-HPLC. The collected fractions were freeze dried. Yield (based on 1) = 45\% (0.0051 g, 2.9\(\times\)10\(^{-3}\) mmol). \(^1\)H NMR (500 MHz, D\(_2\)O) \(\delta\) 8.53 (s, 1H), 7.61 – 7.56 (m, 1H), 7.12 – 6.99 (m, 1H), 6.66 – 6.54 (m, 1H), 5.26 – 5.22 (m, 1H), 5.22 – 5.20 (m, 1H), 5.14 (m, 2H), 5.03 (s, 2H), 4.93 – 4.90 (m, 1H), 4.64 – 4.58 (m, 1H), 4.55 – 4.49 (m, 1H), 4.36 – 4.30 (m, 1H), 4.24 (s, 2H), 4.04 – 2.68 (m, 98H), 1.39 (m, 1H), 1.28 (m, 9H). HRMS-ESI m/z calcd for C70H120N8O31S7 [M+2H]\(^{2+}\) 896.3047, found 896.3046.

**Mono-[6-deoxy-6-(7-(diethylamino)-N-(4-((2-mercaptoethyl)amino)-4-oxobutyl)-2-oxo-2H-chromene-3-carboxamide]-hexakis-[6-deoxy-6-(2-aminoethylsulfanyl)]-\(\beta\)-cyclodextrin (31).** 1 (0.01 g, 6.4\(\times\)10\(^{-3}\) mmol) and 29 (0.003g, 6.4\(\times\)10\(^{-3}\) mmol) in 640 mL of borate buffer pH 8.5 (0.01 M) was stirred overnight. The solvent was removed under diminished pressure. The residue was taken up in a minimum amount of water and purified by RP-C18-HPLC. The collected fractions were freeze dried. Yield (based on 1) = 78\% (0.0095 g, 5.0\(\times\)10\(^{-3}\) mmol). \(^1\)H NMR (500 MHz, D2O) \(\delta\) 8.72 (s, 1H), 7.74 (d, \(J = 8.9\) Hz, 1H), 6.74 (d, \(J = 8.7\) Hz, 1H), 6.41 (s, 1H), 5.22 (s, 2H), 5.14 (s, 2H), 5.06 (s, 2H), 5.02 (s, 1H), 4.13 (t, \(J = 8.3\) Hz, 1H), 4.08 (br s, 1H), 4.03 – 3.86 (m, 4H), 3.84 – 2.57 (m, 75H), 2.46 – 2.29 (m, 2H), 2.13 (br s, 1H), 1.96 (br s, 1H), 1.39 (t, \(J = 6.9\) Hz, 6H). HRMS-ESI m/z calcd for C74H127N9O32S7 [M+2H]\(^{2+}\) 938.8310, found 938.8334.
4.8.3 Fluorescence Studies

Due to the large increase in fluorescence emission observed upon complexation, the affinity of the coumarin 34 could be measured directly. Borate buffer 100mM pH 8.5 was used. Plotting fluorescence versus the cyclodextrin concentration, the association constants (K_a) was determined to be K_a = (1.5 ± 0.08) x 10^6 M by fitting the data to a 1:1 binding isotherm. Association constants were calculated from the average of 3 independent titrations. The included graphs are a representative fit of the data (Figure 4.7).

The biomacromolecule stocks (heparin, chondroitin sulfate A, lysozyme, and glucose oxidase) were prepared by dissolving 50 mg of the analyte into 10 mL of phosphate buffer saline (PBS) pH 7.4 buffer. For these experiments the concentration of 30 or 31 was kept constant at 1.65 x 10^{-5} M. Each fluorescence spectra was taken from a separately prepared solution where the concentration of the biomacromolecule in question was increased for a total of eight spectra. The final concentrations of the biomacromolecules in question were 0 mg/mL, 0.05 mg/mL, 0.1 mg/mL, 0.15 mg/mL, 0.2 mg/mL, 0.33 mg/mL, 0.67 mg/mL and 1 mg/mL (Figure 4.10).

![Fluorescence spectra](image)

**Figure 4.9** Fluorescence spectra of 1 and 34, and titration. (A) Fluorescence titration of 1 with guest 34 (4×10^{-9}M). (B) Fluorescence (λex = 368 nm) of 4×10^{-9}M 34 in the absence (blue line) and presence (red line) of 4.6×10^{-6}M 1.
Figure 4.10 Fluorescence spectra of 30 and 31 in the presence of biomacromolecules
(A) 30 and lysozyme. (B) 31 and lysozyme. (C) 30 and glucose oxidase. (D) 31 and glucose oxidase. (E) 30 and heparin. (F) 31 and heparin. (G) 30 and chondroitin sulfate A. (H) 31 and chondroitin sulfate A.

4.8.4 UV/Vis Reaction Studies

The S→N acyl transfer was followed by UV/Vis spectroscopy. During the aminolysis by 1 or ethylenediamine of 28 a blue shift (450 to 431 nm) in absorbance was observed. This change in absorbance was correlated to the disappearance of 28 as a function of time. The decrease in concentration of 28 was followed by observing the change in absorbance at a 455 nm. The observation of an isosbestic point indicates that no long lived intermediates were formed in the reaction. (Figure 4.9 (A) and (B)). Thus the concentration of 28 could be calculation Beer’s law and linear regression analysis using the following formula:

\[
\frac{A - \varepsilon_a c_t^0}{\varepsilon_t - \varepsilon_a} = c_t
\]

Where A is the absorbance at 455 nm, \(\varepsilon_a\) is the molar absorptivity of the amide product at 455 nm (13 700 L mol\(^{-1}\) cm\(^{-1}\)), \(\varepsilon_t\) (54 600 and 48 400 L mol\(^{-1}\) cm\(^{-1}\), outside and inside 1’s cavity respectively) is the molar absorptivity of the thioester starting material at 455 nm, \(c_t^0\) is the initial concentration of the thioester, and \(c_t\) is the concentration of the thioester at a given time.

When 29 reacted with 1, there was no change in the absorption spectrum. The formation of 31 was followed by adding Ellman’s reagent to the reaction solution. As thioglycolic acid was formed during the reaction, it reduced Ellman’s reagent to NTB\(^{-2}\), which has an absorption maxima at 412 nm. The concentration of NTB\(^{-2}\) produced as the reaction proceeded was determined from a calibration curve using 34 (final concentration of \(9\times10^{-4}\)M), and titrating in a known amount of thioglycolic acid. The aminolysis reaction using 29 was followed by observing the change in absorbance units starting at \(t=0\) min. at a wavelength of 427 nm. The concentration of the product (31) was correlated to the formation of NTB\(^{-2}\) using the calibration curve shown in Figure 4.11(F).

Stocks were prepared as follows: 1 in borate buffer (100 mM, pH 8.5) and EDA in DMSO were prepared in \(7.4\times10^{-3}\)M and 3.4 M concentrations respectively. The thioester solutions of 28 and
29 in DMSO were prepared in $1.6 \times 10^{-2}$ M and $5.9 \times 10^{-2}$ M concentrations respectively. Ellmans reagent ($5.1 \times 10^{-2}$ M) was prepared in DMSO. For each of the reactions these stocks were diluted into a total volume of 1 mL borate buffer (100 mM, pH 8.5) at room temperature in a quartz cuvette. The final concentration of the components was; 1 $9 \times 10^{-4}$ M, EDA $6.8 \times 10^{-3}$ M, 28 and 29 $9.7 \times 10^{-6}$ M, and DTNB $9 \times 10^{-5}$ M. All of the reactions were carried out at 296 K.

Control experiments for the hydrolysis of 28 and 29 were performed at a concentration of $9.7 \times 10^{-6}$ M in borate buffer (100 mM, pH 8.5). No appreciable hydrolysis was observed after 24 h at room temperature (data not shown).
Figure 4.11 Reaction spectra of aminolysis and calibration curve. (A) EDA (6.8×10^{-3}M) and 28 (9.7×10^{-6}M) in 1 mL borate buffer (100mM, pH 8.5), spectra were taken at 7.2 min intervals for a total of 100 spectra. (B) 1 (9×10^{-4}M) and 28 (9.7×10^{-6}M) in 1 mL borate buffer (100 mM, pH 8.5), spectra were collected at 5 min intervals. 100 spectra were recorded. (C) 1 (9×10^{-4}M) and 29 (9.7×10^{-6}M) in 1 mL borate buffer (100 mM, pH 8.5), 85 spectra were recorded at 3 min intervals. (D) Calibration curve for thioglycolic acid concentrations.
intervals. (D) \(1 \times 10^{-4} \text{M}, \ 29 \ (9.7 \times 10^{-6} \text{M}), \) and Ellmans reagent \((5.1 \times 10^{-2} \text{M})\) in 1 mL borate buffer \((100 \text{ mM, pH 8.5})\), 85 spectra were collected at 3 min intervals. (E) \(1 \times 10^{-4} \text{M}, \ 34 \ (9.7 \times 10^{-6} \text{M}), \) and Ellmans reagent \((5.1 \times 10^{-2} \text{M})\) in 1 mL borate buffer \((100 \text{ mM, pH 8.5})\). After the first spectra subsequent spectra represents a titration of 2 μL of thioglycolic acid \((7.9 \times 10^{-4} \text{M})\) in 1 mL borate buffer \((100 \text{ mM, pH 8.5})\) for a total of 8 additions. (F) Calibration curve constructed from E at 427 nm.

### 4.8.5 HPLC Evaluation of Acylation

HPLC elution method is displayed in Table 4.2 below.

<table>
<thead>
<tr>
<th>Table 4.2 Ramping method for RP-HPLC</th>
</tr>
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<td>Min.</td>
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<tr>
<td>15</td>
</tr>
<tr>
<td>30</td>
</tr>
<tr>
<td>70</td>
</tr>
</tbody>
</table>

When 1 was reacted with 28 or 29, with a 2 : 1 ratio of guest vs. host, better selectivity of monoacylation is observed with 29. This can be seen in Figure 4.10. Aliquots of the reaction between 1 and 28 with a 1 : 1 ratio and 2 : 1 ratio of guest vs. host were collected between 25 and 35 min. The mixture was analyzed by MALDI-TOF.
4.8.6 MALDI-TOF Evaluation of acylation

![Chemical structures of compounds 30 and 31]

**Table 4.3 Calculated MW of n-acylated 30 and 31**

<table>
<thead>
<tr>
<th>Compound</th>
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<th>MW (g/mol) [M+Na]^+</th>
</tr>
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Figure 4.12 MALDI-TOF MS spectra of HPLC traces. (A) Spectrum taken of compounds eluting between 25-40 min collected from the HPLC of the reaction between 1 and 28 at $1 \times 10^{-5}$.
M (Figure 4.3-C). (B) Spectrum taken of the compounds eluted between 25-40 min collected from the HPLC of the reaction between 1 at $1 \times 10^{-5}$M and 28 at $2 \times 10^{-5}$M (Figure 4.10-2B). (C) Spectrum of 30. (D) Spectrum of 31.

4.8.7 NMR Tables and Spectra

4.8.7.1 2D TOCSY of 31 and 30

Table 4.4 Chemical shifts of $^1$H-1 scalar couplings of 31 and 30.

<table>
<thead>
<tr>
<th>Compound (Concentration)</th>
<th>30 (1.9±0.5 × 10$^{-3}$M)</th>
<th>30 (4.3±0.5 × 10$^{-3}$M)</th>
<th>31 (9.5±0.5 × 10$^{-3}$M)</th>
</tr>
</thead>
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<td>Position</td>
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<td>$\delta$ (ppm)</td>
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<tr>
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### 4.8.7.2  gCOSY of 3 and 2

**Table 4.5** Assignments of the scalar couplings of the ring sugar $^1$H’s of 31 and 30.

<table>
<thead>
<tr>
<th>Compound (Concentration)</th>
<th>30 ($1.9\pm0.5 \times 10^{-3}$M)</th>
<th>30 ($4.3\pm0.5 \times 10^{-3}$M)</th>
<th>31 ($9.5\pm0.5 \times 10^{-3}$M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Position</td>
<td>δ (ppm)</td>
<td>COSY</td>
<td>δ (ppm)</td>
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<td>$^1$H-2</td>
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</tr>
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<td>5.03 &amp; 5.04</td>
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<sup>a</sup> overlapped
### 4.8.7.3 ROESY of 31 and 30

**Table 4.6** ROESY cross-peaks in the aromatic region of 31 and 30.

<table>
<thead>
<tr>
<th>ROESY assignments of the aromatic region of 3 and 2.</th>
<th>30 (1.9±0.5 × 10^-3M)</th>
<th>30 (4.3±0.5 × 10^-3M)</th>
<th>31 (9.5±0.5 × 10^-3M)</th>
</tr>
</thead>
<tbody>
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<td>ROE δ (ppm)</td>
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<td>1H-B</td>
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</table>
5 Current Work and Future Perspectives

The work outlined in Chapters 3 and 4 clearly demonstrates that CDs can interact with biomacromolecules through electrostatic and hydrophobic interactions. CD-protein interactions can be used for making discriminant arrays to determine the identity of the biomacromolecules being probed. However, multiple synthetic steps are required to generate these compounds. In principle solid phase peptide chemistry (SPPS) on CD scaffolds could be used to quickly generate a library of derivatized CDs. This Chapter outlines two steps that have been achieved towards attaching CDs on solid phase synthesis (SPS) beads. First, CDs have been mono-acylated with a functionalized linker. Second, after monoderivatization, the linker can be used to generate an orthogonal functional group to the amines on the CD. Future work entails using the functional group from the linker to attach CDs onto SPS beads. Finally, the strategies required to generate unsymmetrically derivatized CDs will be discussed.

5.1 Supramolecular Protein-Protein Interaction Inhibitors

Protein-protein interactions (PPIs) control numerous cellular processes; many diseases are caused by miss-regulated protein-protein interactions.\textsuperscript{108} PPIs often bury large interfacial areas between interacting protein partners. The interface is composed of a complementary surface between protein partners composed of small peptide secondary structural elements. The surface exposed residues regulate the interaction between protein partners. Therefore, developing compounds capable of inhibiting PPIs entails mimicking the projections of residues displayed by a complementary protein surface using synthetic analogues. Small molecules do not have large surface areas and as such are not suitable to mimic protein surfaces. However, large molecules, such as cavitands, possess advantageous properties as PPI inhibitors: they possess scaffolds capable of encompassing a large surface area to mimic the interaction region of a PPI interface and the functionalization of their upper rim allows geometric control over the residues to be projected. In effect, cavitand-based PPI interface mimetics can present clusters of hydrophobic groups, electrostatic groups and metal coordinating groups to selectively bind a protein surface.\textsuperscript{109} Below are two examples of modified cavitands used to disrupt PPIs: platelet-derived growth factor (PDGF) binding to its partner platelet-derived growth factor receptor (PDGFR) and citrate synthase (CSase) dimerization.
The first example of a cavitand mimic to inhibit PPI is a calixarene binding to PDGF. PDGF is one of many growth factors involved in the regulation of cell growth and division. Its major role is in blood vessel formation (angiogenesis) stemming from pre-existing blood vessel tissue. PDGF elicits its biological activity by binding with PDGFR. The dimerization of these protein partners eventually leads to the activation of signal transduction pathways. In cancer, PDGF is often overexpressed resulting in the generation of blood vessels that will feed tumor growth. Hamilton et al. developed a series of calixarene-based PPI inhibitors; these compounds inhibited the interaction of PDGF with PDGFR. Hamilton’s calixarenes exhibited anti-angiogenesis activity and as such have the potential to become anti-cancer agents. They designed a scaffold capable of binding to the large PPI interface between PDGF and PDGFR; calix[4]arene was a perfect candidate. Calix[4]arenes possess four positions on their upper rim that were used to attach the recognition domain capable of interacting with PDGF. This generated a complimentary surface mimicking that of PDGFR. The best calix[4]arene inhibitor that displayed potent anti-angiogenic properties against human tumors in mice is displayed in Figure 5.1. This cavitand was derivatized with four G-D-G-Y cyclic peptides and displayed an IC50 value of 250 nM (compound 84).
This example demonstrates how cavitands can be successfully applied in order to inhibit a PPI. However, the synthesizing these heavily modified calix[4]arenes requires multiple synthetic steps, as such this approach cannot be applied to quickly generate a library of compounds for screening cavitands as PPI inhibitors. Moreover, it is unclear whether PPI inhibition in this case is due to a complementary surface display by the symmetrical calix[4]arene or whether it is an effect of multivalency. There are four G-D-G-Y ligands being exposed on the surface of the calix[4]arene, if one of this groups binds to a pocket in PDGF it is likely that the high association constant of this system for PDGF arises through avidity. In order to truly synthesize a PPI inhibitor that relies on binding to a PPI interface, an unsymmetrically derivatized cavitand would have to be synthesized.

The second example of a cavitand mimic to inhibit a PPI is a CD binding to CSase. CSase is involved in the Krebs cycle, it catalyzes a Claisen condensation of acetyl-Coenzyme A and oxaloacetate to form citrate. This enzyme is only active as the dimer; dimerization of the enzymes involves the interaction of hydrophobic patches on citrate synthase. Breslow et al.
proposed that a CD dimer capable of binding to the hydrophobic patches of citrate synthase would be able to inhibit the dimerization of the enzyme, thus leading to its inhibition.\textsuperscript{114} They synthesized a variety of CD constructs in order to inhibit CSase and other protein dimers and tetramers. CD dimers form tight complexes with hydrophobic residues of peptides. As such, CD dimers should block protein dimerization by binding to the hydrophobic residues in the interfacial region between CSase dimers.\textsuperscript{115} Interestingly, only CD dimers that were linked through their secondary rim were able to inhibit CSase dimerization. One of the most effective CSase dimerization inhibitors was a CD dimer that contained a pyridine-2,6 diamide linker linked to two mono-C-3aminodeoxyCDs (Figure 1.2).

\textbf{Figure 5.2} CD dimer capable of binding CSase

This CD dimer was able to inhibit the catalytic activity of CSase with an IC\textsubscript{50} of 140 μM. It was proven that PPI inhibition using CD dimers was due to binding to CSase and not by scavenging of the substrate or the cofactor of CSase. They propose that inhibition reflects the dissociation of the enzyme to the inactive monomer through inhibition of dimerization. However, these dimers interact non-specifically with hydrophobic residues found in PPI interfaces. Furthermore, the mode of PPI disruption is not clear. It might be that the CD dimer inhibition arises through non-specific binding to the surface of a CSase monomer. A CD with a complimentary surface for PPI inhibition would have to display different residues on its surface.

Traditionally large organic molecules (MW > 750 Da) are not considered as viable drug candidates (based on Lipinski’s rules).\textsuperscript{116} Nevertheless, large compounds such as cavitands exhibit advantages over small molecules for PPI inhibition. For example, “epitope transfer” can be generated on cavitands whereas small molecules do not have the large surface area required for mimicking a PPI epitope. Encouragingly some large molecules have shown utility as
pharmaceuticals. An example of a CD-based drug being used in the clinic is Sugammadex. Sugammadex is used for the reversal of neuromuscular blockade during anaesthesia induced by the agent rocuronium. Therefore, large macromolecules could have applications as drugs. Molecules like antibodies and peptides, polymers and nanoparticles that modulate PPIs have proved to be promising as drug candidates. However, synthesizing epitopes on cavitands remains a challenging synthetic feat; finding the correct display of epitopes will likely require the generation of a library of cavitand-based PPI inhibitors. A solution to this problem may be found in the use of solid phase synthesis (SPS) to generate large libraries of potential cavitand-based PPI inhibitors. CDs are ideally suited for this task given their size and relative ease of rim modification. Moreover, the ability of per-amino CDs to bind protein surfaces has already been demonstrated in the work presented in this thesis (Chapters 3 and 4). Described below is the current work aimed at developing a method to attach per-amino CDs on beads to perform solid phase peptide synthesis (SPPS) on per-amino CDs and develop a library of compounds that could be used for inhibiting PPIs or for the development of protein sensing cavitands.

5.2 Enabling Perfunctionalization of CD

As reviewed in previous chapters, there are a variety of methods capable of regioselectively modifying CDs. The most commonly employed method of generating mono-substituted CD is through nucleophilic displacement carried out on mono-tosylated CD. Indeed the majority of monoderivatized CD in the literature are used without further elaborating the rest of the primary hydroxyls on the CD’s primary rim. Other methods used to increase the functionality on the primary rim of CDs usually yield the desired per-derivatized CD with protected hydroxyl groups. The hydroxyl groups of these structures could be de-protected to yield the free hydroxides and then be subsequently modified. This latter step would require further synthetic manipulations adding to the already complex organic manipulations required to achieve a multi-functional CD system.

Solid phase synthesis (SPS) could be a very useful tool to solve the difficulties in purification that arise during the functionalization of hetero-functionalized CDs. This idea has been validated recently; a method was published that utilizes an SPS approach to get a variety of C-6 mono-functionalized CDs. The SPS strategy relied on linking CDs covalently through a
phosphodiester bridge to different guests immobilized on SPS beads. The beads were loaded with 4-hydroxy-3-nitrophenylacetic acid subsequently functionalized with a set of phosphoramidite derivatives. At this point, the phosphite linker was oxidized to yield a phosphate triester. Deprotection using 2-cyanoethyl phosphate protecting group afforded the phosphodiester activated solid support (SS). Finally, coupling of CD is performed in presence of 1-(2-mesitylenesulfonyl)-3-nitro-1H-1,2,4-triazole (MSNT) and final cleavage from the resin is effected using ammonium hydroxide. The yields of mono-derivatized CD phosphodiesters are moderate (40%). However, this method does not selectively mono-acylate CD and this method yields only mono-functionalized CDs with hydroxyl groups on their primary rim (Figure 5.3).
In order to functionalize CDs further, a SPS approach could be used to append per-amino CD’s to SPS beads. An immobilized CD could then be subjected to solid phase peptide synthesis (SPPS) to append a variety of different peptides on the CD primary rim (Figure 5.4).
The method used in Chapter 4 was altered in order to generate a CD capable of binding to a SS. In Chapter 4 a method was used to selectively monoacylate a per-amino CD. Amine groups, in the presence of hydroxyls, can be selectively acetylated to yield chemically robust amides; there are a multitude of functional groups that could be incorporated easily onto the CD amines at this point. A multi-functional CD could be built by mono-acylating a per-amino CD and subsequently per-acylating that structure. Unfortunately, the mono-acylation method has some limitations. In principle only the specific acylating DEAC guest was able to mono-acylate CD as
described in Chapter 4. In order to increase the range of functional groups that can be incorporated through mono-acylation a reactive group on the linker connecting the guest and the monoderivatized CDs was incorporated. The linker of the monoacylated CD will contain a disulphide bond which can later be reduced. Reduction of the linker will result in the formation of two thiols. One thiol will be linked to the now non-covalently bound guest, whereas the second thiol will be linked to the CD primary rim. The thiol on the primary rim can then be reacted selectively in the presence of the amines on the CD (Figure 5.5). There exists a wide range of chemical reactions that thiols can undergo in the presence of amines.\textsuperscript{119} By utilizing this strategy after mono-acylation of a per-amino CD and reduction of the linker, many other functional groups could be incorporated on the CD primary rim.

\textbf{Figure 5.5} Selective functionalization of amino CD. The polygon, square and stars represent different functional groups.
5.3 Incorporation of a Functional Linker onto CD 1

The new mono-acylating guest compound is made up of three components: the guest, the acylating agent and the linker. DEAC was used once again as the guest of choice. *p*-Chlorophenol ester was chosen as a mild acylating agent to mono-acylate 1. Finally, the linker connecting the guest and the acylating agent is a disulfide; disulfides can be easily reduced to yield a free thiol which, in the presence of amines, can be selectively functionalized. A disulfide linker (35) was synthesized to validate the applicability of this approach (Figure 5.6).

The synthesis of 36 began with cystamine hydrochloride. The first step is to generate an unsymmetrical dithiol linker. First, cystamine was mono-oxidized using *m*-CPBA to yield a sulfinothiolate.\(^\text{120}\) Second, the sulfoxide on cystamine was displaced using the 3-mercaptopropanoic acid to generate 38. At this point, compound 35 is esterified with DCC and DMAP in DCM in the presence of *p*-chlorophenol. 35 was then explored as a mono-acylation reagent for CD 1. The mono-acylation reaction with CD 1 was carried out in borate buffer (10 mM, pH 8) at r.t. in the presence of 35 (1 eq.). After stirring overnight, another equivalent of 35 was added to the reaction solution, which was stirred for another day. The final mono-acylated CDs can be purified using RP-HPLC (Figure 5.6).
**Figure 5.6** Synthesis of 36. Equimolar amounts of 1 and 35 (1 mM total conc.) stirred in borate buffer pH 8 (10 mM)

The yield of the mono-acylation reaction of 1 with 35 to yield 36 was low (10% final yield, 50% max. conversion by MALDI, Figure 5.7).
Figure 5.7 Equimolar amounts of 1 and 35 (1 mM total conc.) stirred in borate buffer pH 8 (10mM) for 2 d. MW of 1 is 1568.7, MW of 35 is 1999.4.

This experiment shows the potential to selectively functionalize per-amino CD without the need for tedious protecting group manipulations. In the future, we could link a mono-thio-hexa-amino CD to a SS and attach amino acids to the free amines on CD. There are some limitations to mono-acylating CD with 35. One such limitation is the low yields of reaction. In its current form the reaction does not go to completion, but only mono-acetylated CDs are observed in the crude reaction mixture. Perhaps more equivalents of 35 or longer acylation reaction is required. Alternatively, a different approach could be used to attach CD to a SS. Drawing inspiration from the diphosphate reaction of CD it could be feasible to mono-acylate the CD onto a SS directly. The rest of the amines on the mono-acyl CD would be free. At this point using commonly employed SPPS techniques would allow the generation of a library of CD displaying amino acids.
to be easily synthesized. These compounds could be tested for their efficacy as PPI inhibitors. Another use of the free thiol generated from the disulphide linker is that a variety of fluorophores could be attached to the per-amino CD. These compounds could be used as biomolecular sensors similar to those presented in Chapters 3 and 4.

The work in this thesis has illustrated the potential for CDs as biomacromolecular sensors. In the future these compounds could be used to inhibit PPIs and give rise to PPI inhibition drugs. Alternatively, CDs could be used for sensing applications. Much work remains to be done in these areas.

5.4 Experimental Section

![Chemical结构](image)

**Figure 5.8** Synthesis of 35. i) mCPBA (75%) 1.2 eq, MeOH/DCM; 1/5, stir ovt r.t.. ii) 3-mercaptopropanoic acid 1.5 eq., MeOH 4h, r.t. iii) 33X 1.6 eq., DMF, DIPEA 2eq., rt. stir ovt. iv) p-Cl-phenol 1eq, DMAP (cat.), DCC 0.95 eq., DCM

**Synthesis of 3-((2-aminoethyl)disulfanyl)propanoic acid (37).** Cystamine hydrochloride (4 g, 18 mmol) was dissolved in MeOH (250 mL), the resulting solution was cooled to 0 °C in ice. A solution of mCPBA (75%, 3.7 g, 21 mmol) dissolved in DCM (50 mL) was added dropwise. The reaction was stirred overnight at r.t. The reaction solution was evaporated to give a white residue.
The solid was triturated with ether. After decanting the white solid it was suspended in MeOH (150 mL) and to this heterogeneous solution was added 3-mercaptopropionic acid (3.1 mL, 36 mmol). The resulting solution was stirred for 1 h at r.t. The solvent was then removed by evaporation, and the resulting solid was titurated with ether. The resulting off-white solid was purified by dry loading on a silica plug (50 g). The final compound was collected after washing the plug sequentially with DCM (400 mL), EtOAc (400 mL), 5% MeOH in EtOAc (500 mL), 10% MeOH in EtOAc (500 mL), 25% MeOH in EtOAc (500 mL). Off-white solid. Yield = 62% (2 g, 11 mmol).

Synthesis of 3-((2-(7-(diethylamino)-2-oxo-2H-chromene-3-carboxamido)ethyl)disulfanyl) propanoic acid (38). The succinimidyl ester of diethylaminocoumarin 33 VI (1 g, 2.8 mmol) was dissolved in a solution of DMF (100 mL) and DIPEA (1.5 mL, 9 mmol). 37 (0.12 mL, 1.7 mmol) was added to the aforementioned solution. The reaction was stirred for approximately 16 h. The solvent was removed under diminished pressure. The residue was taken up in DCM (150 mL) and washed with 1M HCl (3 x 100 mL). The organic layer was dried over MgSO4, filtered, and evaporated to give a yellow residue. The final compounds 38 was purified using silica chromatography. Yellow needles (TLC conditions: DCM/MeOH; 9/1, Rf = 0.5). Yield = 50% (0.6 g, 1.4 mmol). 1H NMR (399 MHz, cdcl3) δ 9.14 (t, J = 5.4 Hz, 1H), 8.70 (s, 1H), 7.49 – 7.37 (m, 1H), 6.64 (dd, J = 9.0, 2.4 Hz, 1H), 6.48 (d, J = 2.4 Hz, 1H), 3.75 (dd, J = 12.8, 6.4 Hz, 2H), 3.44 (q, J = 7.1 Hz, 4H), 2.95 (dt, J = 13.4, 7.0 Hz, 4H), 2.80 (t, J = 6.9 Hz, 2H), 1.23 (t, J = 7.1 Hz, 6H). 13C NMR (100 MHz, cdcl3) δ 185.10, 184.98, 162.92, 157.89, 152.96, 148.72, 131.54, 110.29, 109.82, 108.57, 96.72, 45.28, 39.07, 37.96, 34.41, 33.68, 12.66. HRMS-ESI m/z calcd for C19H25N2O5S2 [M+H]+ 425.1214, found 425.1205.

Synthesis of 4-chlorophenyl 3-((2-(7-(diethylamino)-2-oxo-2H-chromene-3-carboxamido)ethyl)- disulfanyl)propanoate (35). 38 (0.05 g, 0.12 mmol), p-chlorophenol (0.011 mL, 0.12 mmol) and DMAP (0.001 g, 8.2 × 10^-3 mmol) were dissolved in a minimum amount of DCM (20 mL). The solution was cooled in an ice bath. DCC (0.022 g, 0.11 mmol) was dissolved separately in 1 mL DCM and subsequently added to the reaction solution. The

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VI Refer to reference 107 for synthesis of this compound (33)
reaction was allowed to warm to room temperature overnight. The reaction was filtered through a plug of celite, washed with EtOAc, and the filtrate was evaporated under diminished pressure. The residue was dissolved in DCM, washed with a sodium bicarbonate solution (1 M), brine, dried over MgSO₄, filtered, and evaporated. The residue was dry loaded on silica. The product was obtained after silica chromatography using pentane/EtOAc; 1/2 as the eluent. The product was a yellow powder (TLC conditions: pentane/EtOAc; 1/3, Rf = 0.55). Yield = 55% (0.035 g, 0.066 mmol). ¹H NMR (399 MHz, cdcl₃) δ 9.08 (t, J = 5.6 Hz, 1H), 8.67 (s, 1H), 7.40 (d, J = 9.0 Hz, 1H), 7.34 – 7.28 (m, 2H), 7.07 – 7.01 (m, 2H), 6.64 (dd, J = 9.0, 2.4 Hz, 1H), 6.47 (d, J = 2.4 Hz, 1H), 3.77 (q, J = 6.4 Hz, 2H), 3.45 (q, J = 7.1 Hz, 4H), 3.07 – 3.00 (m, 4H), 2.99 – 2.93 (m, 2H), 1.24 (t, J = 7.1 Hz, 6H). ¹³C NMR (100 MHz, cdcl₃) δ 170.31, 163.61, 162.96, 157.90, 152.84, 149.24, 148.36, 131.43, 131.38, 129.64, 123.15, 110.20, 108.57, 97.02, 96.76, 45.31, 38.82, 38.26, 34.44, 33.10, 12.65. HRMS-ESI m/z calcd for C25H27ClN2O5S2 [M+H]⁺ 535.1111, found 535.1122.

**Synthesis of mono-[4-chlorophenyl 3-((2-(7-(diethylamino)-2-oxo-2H-chromene-3-carboxamido)ethyl)- disulfanyl)propanoate]-hexakis-[6-deoxy-6-(2-aminoethylsulfanyl)]-β-cyclodextrin (36).** 1 (0.01 g, 3.65 × 10⁻³ mmol) and 35 (0.002 g, 3.65 × 10⁻³ mmol) were dissolved in 3.6 mL (final conc. 1 mM) borate buffer pH 8.5 (0.01 M) was stirred overnight. The solvent was removed under diminished pressure. The residue was taken up in a minimum amount of water and purified by RP-C18-HPLC. The collected fractions were freeze dried. Yield (based on 1) = 15% (0.001 g, 5.5×10⁻⁴ mmol). ¹H NMR (500 MHz, d₂o) δ 8.75 (s, 1H), 7.74 (t, J = 7.5 Hz, 1H), 6.76 (d, J = 9.1 Hz, 1H), 6.44 (s, 1H), 5.22 – 5.01 (m, 9H), 4.15 – 2.62 (m, 124H), 2.34 (d, J = 15.6 Hz, 3H), 2.11 – 1.97 (m, 3H), 1.39 (t, J = 7.1 Hz, 7H), 1.20 (dt, J = 14.6, 7.2 Hz, 3H). HRMS-ESI m/z calcd for C75H130N9O32S9 [M+H]³⁺ 652.2166, found 652.2097.
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Appendix A

Titration Isotherm Equations
**Equation A)** Direct titrations

\[ CD \rightleftharpoons CD \cdot G \]

Where CD is cyclodextrin 1 and G is a fluorescent guest molecule.

\[ K_G = \frac{[CD \cdot G]}{[CD]_f [G]_f} \]  \hspace{1cm} (1)

Where \([CD]_f\) is the concentration of free 1, \([G]_f\) is the concentration of free fluorescent guest, and \(K_G\) is the association constant of the guest and 1.

\[ \text{% bound fluorophore} = \frac{(F - F_{\text{min}})}{(F_{\text{max}} - F_{\text{min}})} \]  \hspace{1cm} (2)

Where \(F\) is the fluorescence emission detected and \(F_{\text{min}}\) and \(F_{\text{max}}\) are the minimum and maximum fluorescence signal that was detected (i.e. when \([CD] = 0\) and \([CD] \gg [G]\), respectively. Eqn (3) can then be defined as follows:

\[ \frac{[CD \cdot G]}{[G]_f} = \frac{(F - F_{\text{min}})}{(F_{\text{max}} - F_{\text{min}})} \]  \hspace{1cm} (3)

And

\[ [G]_f = [G]_i - [CD \cdot G] \]  \hspace{1cm} (4)

\[ [CD]_f = [CD]_i - [CD \cdot G] \]  \hspace{1cm} (5)

Where \([G]_f\) is the total concentration of the guest molecule and \([CD]_f\) is the total concentration of 1. Eqn. (6) was obtained by substituting (4) and (5) into (1):
\[ K_g = \frac{[CD \cdot G]}{[CD, I]G_r - (CD, I CD \cdot G + G_r CD \cdot G + CD \cdot G)^2} \]  \hfill (6)

And solved for \([CD \cdot G]\) as a quadratic function where the variable \(x = [CD_r]\):

\[ [CD \cdot G] = \frac{(K_g x + K_g[G_r] + 1) \pm \sqrt{(-K_g x - K_g[G_r] - 1)^2 - 4K_g^2 x[G_r]}}{2K_g} \]  \hfill (7)

We now define \(q\) as:

\[ q = \frac{(K_g x + K_g[G_r] + 1) \pm \sqrt{(-K_g x - K_g[G_r] - 1)^2 - 4K_g^2 x[G_r]}}{2K_g} \]

By inserting (7) into (3) and solving for \(F\), the final expression was obtained for the direct titration of a fluorescent guest and 1,

\[ F = \frac{q(F_{max} - F_{min}) + [G_r]F_{min}}{[G_r]} \]  \hfill (8)

Plotting fluorescence versus \([CD_r]\), \(K_s\) of the fluorophore was determined by fitting the data to (8).

**Equation B) Competitive titrations**

CD \cdot G \rightleftharpoons CD \rightleftharpoons CD \cdot DEAC

Competitive titration of coumarin 6 (DEAC) versus non-fluorescent guest (G) with 1 were fit using the following equation:

\[ [G_r] = [G_r] - [CD \cdot G] \]  \hfill (9)
Where \([G_r]\) is the concentration of free guest and \([G_t]\) is the total concentration of guest.

\[
K_c = \frac{[CD \cdot G]}{[CD] [G_t]}, \tag{10}
\]

And,

\[
[CD \cdot G] = [CD_r] - [CD \cdot DEAC] - [CD_g] \tag{11}
\]

Where \([CD_r]\) is the concentration of free 1 and \([CD_t]\) is the total concentration of 1, and \(K_c\) is the association constant of the guest and 1. Assuming that \([CD_r]\) remains very small throughout the titration, the \([CD_g]\) term in equation (11) can be ignored. Expression (12) was obtained by substituting (9) into (10) and subsequently substituting (11) into their ensuing equation.

\[
K_0 = \frac{([CD_r] - [CD \cdot DEAC])}{[CD_r][G_t] - [CD] [CD \cdot DEAC]} \tag{12}
\]

Rearranging (12) and (1) to equal \([CD_r]\) (and therefore to each other), by rearranging the resulting (13) was obtained:

\[
0 = [CD \cdot DEAC] [K_c - K_0] + [CD \cdot DEAC] [K_c - K_0] [CD] [CD \cdot DEAC] [K_c - K_0] + [CD] [K_c] [DEAC] \tag{13}
\]

Solving (13) as a quadratic function, where \(y = [CD \cdot DEAC]\), and \(x = [G_r]\), \(q_2\) was defined as follows:
Inserting $q_2$ into (3) (where $G = \text{DEAC}$) and solving for $F$, the final expression for the competitive titration of 6 versus guest and 1 is given,

$$F = \frac{q_1(F_{\text{max}} - F_{\text{shift}}) + [\text{DEAC}_1]F_{\text{min}}}{[\text{DEAC}_1]}$$  \hspace{1cm} (14)
Appendix B

Selected NMR Spectra
