LIGAND-RECEPTOR INTERACTIONS FOR SUPRAMOLECULAR DISASSEMBLY WITH APPLICATIONS IN SCREENING AND DRUG DELIVERY

Diego Amado Torres

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LIGAND-RECEPTOR INTERACTIONS FOR SUPRAMOLECULAR DISASSEMBLY WITH APPLICATIONS IN SCREENING AND DRUG DELIVERY

A Dissertation Presented

by

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To my mother, Adiela Torres Castillo.
ACKNOWLEDGMENTS

The people who have been directly involved with the achievement of my latest degree are many: family, faculty, lab mates, classmates, friends, and institutions. All of them are receivers of my eternal gratitude and admiration. However, in the world of chemistry it is necessary for me to acknowledge those who contributed most outstandingly in finishing my PhD degree. In an inverse chronological order of appearance in my life, those remarkable people are Professor Sankaran Thayumanavan, who formed part of the committee that brought me to UMass and then became my advisor during my PhD. Under his guidance, I learned and developed my taste for supramolecular chemistry. Professor Dr. Vladimir V. Kouznetsov, my mentor, advisor, and teacher during my Masters and Bachelor degrees in chemistry, back in my home country, Colombia. He taught me how organic chemistry can be frustrating most of the time, but always fascinating and many times beautiful.

I would have never gotten into chemistry if it was not due to the amazing teaching skills of Dr. Heriberto Acevedo, a remarkable science teacher during my years in high school. From him I learned that organic chemistry was the chemistry of life and a different world worthy of studying in detail. Of course, before all of this chemistry and science madness began, I had my mother to tell me to keep studying and learning throughout my childhood “for the best future belongs to those who study hard and excel at school.” Nothing of what I have achieved would have been achieved if it was not for her insisting on me to become a better student despite the fact that I was at the top of the class most years, and among those at the top the other times.
ABSTRACT

LIGAND-RECEPTOR INTERACTIONS FOR SUPRAMOLECULAR DISASSEMBLY WITH APPLICATIONS IN SCREENING AND DRUG DELIVERY

MAY 2014

DIEGO F. AMADO TORRES, B.S., UNIVERSIDAD INDUSTRIAL DE SANTANDER

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Proteins have the capacity to bind specific sets of compounds known as ligands, these are small molecules with a recurrent theme in their molecular design that is a characteristic exploited here to (i) identify particular affinities of small molecules for proteins with the aim of using them as ligands, inhibitors, or targeting moieties in more complex systems by means of a methodology that screens small molecules based on protein affinity; (ii) decorate a self-assembling supramolecular system at different positions, making it responsive to a complementary protein with the aim of exploring differences in disassembly and sensitivity of the release of encapsulated guest molecules, depending on the initial location of the ligand upon binding to a specific protein; (iii) decorate self-assembling and crosslinkable dendrons aiming to introduce a system incorporating multiple ligands, sequentially responsive to a reducing environment, and to specific proteins.
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2.1. Structure of the analyte molecules used in the assay and their literature-reported binding affinities
CHAPTER 1
INTRODUCTION

1.1 Protein Binding: from Self-assemblies to Ligands

Synthetic amphiphilic macromolecules that can spontaneously self-assemble in solution possess the capability of mimicking features of naturally occurring architectures such as cell membranes and endosomes, and macromolecules, such as proteins, RNA, and DNA.\(^1\) As a result, such systems are of great importance in supramolecular chemistry. Synthetic supramolecular systems often utilize the same kind of interactions that nature uses in its own designs, namely hydrophobic interactions, van der Walls forces, hydrogen bonds, electrostatic interactions, \(\pi-\pi\) interactions, and metal-ligand binding interactions.\(^2\) All of these non-covalent interactions are involved in the specific binding of ligands to proteins, which led to their inclusion by synthetic chemists in artificial systems in an attempt to emulate designs that evolution has refined over millions of years.\(^3\) Many of those artificial tailored systems include self-assembling molecules and macromolecules as scaffolds for nanostructures such as micelles, vesicles, lamellae, and aggregates along with less frequent nanostructures in other shapes.\(^4\) The main characteristics sought in self-assembling systems are often beneficial in encapsulation of guest molecules for drug delivery purposes and sensing applications.

Artificial self-assembling supramolecular systems are formed by a variety of molecules, including small molecule surfactants, block copolymers, random copolymers, and dendrons.\(^5,6\) The self-assembling capabilities of these molecules and
macromolecules in an aqueous environment is complemented by the encapsulation properties, usually of small molecules upon mixing them with the corresponding self-assembling motifs, i.e. small hydrophilic molecules trapped in the aqueous interior of vesicles, or hydrophobic molecules occupying hydrophobic pockets in a micelle, lamellae, or aggregate. As in naturally occurring proteins, which suffer conformational changes when bound to specific molecules, man-made self-assembling systems have been functionalized with particular features to induce conformational changes upon stimulation.\(^3,5\) A supramolecular system incorporating responsive features becomes more complex and interesting when a stimulus triggers either disassembly or a morphological transformation of the system.\(^7\) Such disturbances in assembly and morphology are usually followed by release of encapsulated small molecules, which are often a pharmacophore or a reporter chromophore. Nevertheless, disassembly and morphology changes are sought to be reversible in many cases, as a more accurate emulation of protein features.

The proteins’ capacity for binding to specific sets of compounds known as ligands, small molecules with a recurrent theme in their molecular design, is a characteristic exploited and described in this thesis for a system based on supramolecular analytical chemistry. It is desirable to identify particular affinities of small molecules for proteins, so they can be used as ligands, inhibitors, or targeting moieties in more complex systems.\(^8\) With that in mind, a methodology that screens small molecules based on protein affinity would be useful to identify hits that are synthetically more accessible, cheaper, and easier to modify than other known ligands.
Previously, the same protein-ligand binding capacity has been used to give specificity to self-assembling nanostructures, including soft and hard nanoparticles, towards a target protein. However, the importance of the ligand location on a nanoassembly has been disregarded, and nothing is known about how it affects either disassembly or the further release of encapsulated guest molecules.

Since this thesis is about the protein-ligand binding event and its influence on (i) discrimination of small molecules affinity in a screening assay, (ii) disassembly of dendrimeric aggregates and (iii) release efficiency, in this chapter, we will discuss some relevant aspects about determination of binding affinity and dendron responsiveness to diverse stimuli, what has been shown previously from our lab.

1.2 Protein-Ligand Binding Affinities, Importance and Discrimination

Protein-ligand interactions are known for their specificity and biological relevance, which is due to the myriad of physiological functions triggered upon a protein-ligand binding event. Mimicking the details and advantages of receptors’ binders has been an important task for pharmacological science; this problem has been approached by designing ligand-like small molecules with the purpose of either enhancing or reducing a physiological response after interaction with a specific protein. Discriminating protein-affinity of a new molecule when comparing it to a different molecule with already known binding affinity, i.e. how well the new molecule inhibits the action of a target protein by blocking its binding site, is a matter of great importance in the fields of drug discovery and bioresponsive materials.
Discriminating methods for rapid screening of small molecules is a crucial requirement in drug discovery and development. Even though there has been significant advancement in diversity-oriented organic synthesis to obtain a large library of small molecules, developing screening techniques with greater efficacy is still a challenging task for the pharmaceutical industry.\(^\text{11}\) Most of the current methods require either radio-labeling of the ligand molecule or labeling the receptor itself.\(^\text{12}\) Although there are a number of non-labeling methods, they involve expensive instrumentation and excessive time consumption. An efficient approach to address these issues would involve the development of versatile screening methods, utilizing simple analytical tools, thereby improving their broader applicability without compromising the speed of test.\(^\text{11}\)

In the past several decades, great efforts in medicinal chemistry and the advent of combinatorial chemistry have generated the possibility of synthesizing large libraries of organic molecules to target a number of diseases. However, only limited efforts have been made in finding new techniques for screening these huge numbers of molecular compounds.\(^\text{13}\)

For the screening of large numbers of molecules, the pharmaceutical industry requires simple and robust methods that could be easily transferred to automated drug screening. While high throughput screening (HTS) is currently available for such endeavors,\(^\text{14}\) these techniques require special instrumentation with highly complicated methodologies and expertise. Thus, it is pertinent to develop novel and more straightforward methods than the existing ones, using simple instrumentation
techniques to make the screening process and the identification of “hits” economical and time-saving.

The identification of “hit” molecules is important during the early stages of drug discovery. The following stages involve the transformation of the hit into a “lead” molecule, and then the optimization of those “lead” molecules. However, quantitatively assessing the binding strength of molecule-receptor interactions at an early stage plays a vital role in expediting the drug discovery process. Some of the most used methods for this purpose are described in the following sections.

1.2.1 X-Ray Crystallography

An increase in the speed and power of protein crystallography has had a significant impact on screening of small molecules through defining drug binding modes more rapidly and with greater certainty, which has significant importance on lead discovery.

In an X-ray approach to screening, protein crystals are exposed to either single molecules or molecule cocktails in a solvated environment. Since protein crystals contain extensive channels filled with solvent accounting for approximately 50% of the three-dimensional structure volume of the protein, ligand small molecules will generally diffuse rapidly into the crystals and interact as if they were in solution, as long as the crystal packing does not occlude the binding site. Once the initial protein crystals have been characterized, the small molecules can be visualized by collecting sets of X-ray data
on each soaked crystal under identical conditions. The aid of automatic procedures enables the rapid analysis of the protein–ligand complex solutions.\textsuperscript{16}

Figure 1.1. Fragment based approach to lead discovery; a) schematic representation of an active site with three pockets; b) two fragments binding two of the pockets; c) a scaffold linking three fragments binding the pockets; e) the real binding site defined by van der Waals surface; f) a fragment defined by X-ray crystallography.\textsuperscript{15}

However, the protein fragments are used at much higher concentrations than in conventional HTS approaches, assuming that the crystal is adequate for the study. Figure 1.1 shows a known binding site in a soaked protein crystal and illustrates how different small molecules are identified according to their fit within the protein.\textsuperscript{17} Although this method has several disadvantages including the required high concentrations and proper crystallization, high-throughput crystallography is now a viable screening method in the recognition of molecular fragments that bind protein targets and precise definition of the protein binding sites.\textsuperscript{18} It can subsequently be used as a rapid technique to guide lead optimization.
1.2.2 NMR Based Strategies

Several NMR based strategies have been developed with a particularly useful set for drug discovery.\textsuperscript{19} These include traditional chemical-shift mapping, ligand-based techniques that monitor changes in nuclear ligand spin relaxation properties upon binding, and diffusion measurements. However, some of these approaches are better suited to validate hits coming from HTS campaigns while others are better suited to guide hit optimization to lead compounds, mostly due to the high concentration of protein needed for the screening of large libraries of compounds.

One of the most often utilized for hit identification and validation by NMR is called chemical-shift mapping.\textsuperscript{20} This approach exploits the differences in chemical shift between free and bound target protein, in two-dimensional correlation spectra upon titration with a ligand or a mixture of ligands. This is a ligand-binding assay that can provide meaningful information on the binding site; it has advantages over other screening techniques, as compounds bound to the target protein can be found and characterized without need for an assay. When the structure of the target has been determined by NMR, it could be possible to derive ligand-protein distances. However, for target proteins over 30-40 kDa at least one amino acid needs to be labeled to reduce the spectral complexity.

The ligand-based techniques use a smaller amount of protein than the previous approach, and are based on the observation of perturbations induced by a substoichiometric amount of target protein on the NMR spectra of the ligand.\textsuperscript{21} Saturation transfer difference (STD) is one of these techniques in which a simple $^1$H
NMR is recorded for a ligand in the presence of a small amount of target i.e. ligand:protein 100:1, with and without selective irradiations of the protein resonances in regions that are not occupied by resonances of the small molecule ligand. This technique also exploits the relaxation differences between bound and free state of a ligand nuclei, even if the binding is transient in the presence of a substoichiometric amount of protein. This approach however, is less informative than chemical-shift mapping and is typically used for screening and validation.

Another interesting strategy uses both X-ray crystallography and NMR based methods for hit screening and validation along with in silico docking of compounds to predict those that should bind to a target protein. This is a very important aid used in the fragment-based drug design (FBDD) strategy, which combine the information acquired from either X-ray crystallography or NMR with computational simulations that help to save time and resources. FBDD is now an alternative to conventional HTS.\textsuperscript{15,19}

1.2.3 Mass Spectrometry

Mass spectrometry (MS) plays a role in nearly every step of the drug development process. However, it was not until recently that MS has emerged as an effective technique for identifying lead compounds based on the characterization of ligand-protein target interactions. One of the main advantages of this strategy is the small quantities of ligand and target protein required, and the capacity to study them without labeling them.\textsuperscript{22}
A MS approach stands apart in the identification of hits and lead compounds when is combined with electrospray ionization (ESI). ESI-MS reduces considerable constraints on the purity of both targets and ligands due to differentiations in molecular mass, allowing for detection of non-covalent complexes, which makes it useful in compound screening and structure-activity relationship (SAR) optimization.\textsuperscript{23}

A different method that can measure binding of small molecules to protein targets either directly or indirectly is affinity selection mass spectrometry (AS-MS). All varieties of AS-MS include a step of affinity selection, where the protein is equilibrated with at least one potential ligand; during this stage the protein forms a complex with any compound capable of binding. In the next step the resulting receptor-ligand complexes are separated from unbound components. In a final step, ligands are identified by MS or MS-MS.\textsuperscript{24}

AS-MS methods measure the binding event directly or indirectly. The direct methods separate protein-ligand complexes from unbound components within the mass spectrometer. Then, the mass of the non-covalent or covalent protein-ligand complex is measured in the gas phase. Indirect methods use a separation technique, usually chromatography, to isolate the protein-ligand complex formed from unbound components before MS. Both direct and indirect methods have been applied in screening of libraries of compounds, compound mixtures, and unpurified natural products extracts.
1.2.4 Microcalorimetry

Currently, isothermal scanning calorimetry, also known as isothermal titration calorimetry (ITC), permits direct determination of enthalpy values and gives complete thermodynamic profiles for binding events. The instruments for ITC most commonly use a power compensation design in which the temperature difference between a sample cell and a reference cell is monitored. While both cells are maintained at a constant temperature, a titration system delivers a reactant to the sample cell where any heat of reactant binding results in an imbalance between the reference and sample cells, which is compensated for by modulating the power applied to cell heaters.25

Figure 1.2 shows an example of ITC data of a ligand binding to a protein. The top panel shows the primary titration data, or raw data, in which power is displayed as a function of time. The programmed titration delivery steps are evident by the series of peaks that return to baseline. The area of each peak is the heat of reaction for each reactant addition and the magnitude decreases as the receptor binding site becomes saturated. The bottom panel shows the primary ITC data transformed into a binding isotherm displaying the characteristic sigmoidal shape. This binding isotherm is fit into a particular binding model to get the binding constant, binding enthalpy, and the stoichiometry of the binding event.
However, ITC is a laborious and time-consuming method. Even an experienced investigator can usually complete only a few experiments per day, which relegates calorimetry to the pharmaceutical industry for use as a secondary screening method for validation of other assays. High throughput calorimeters are still a work in progress with less than promising early results. Perhaps the most impressive results come from a hybrid technology between calorimetry and fluorimetry. ThermoFluor™ measures the fluorescence of dyes that sense protein unfolding; it is used to conduct miniaturized thermal shift assays in drug discovery.26

1.2.5 Fluorescence Based techniques

Fluorescence techniques can be easily implemented as a HTS.14 There are a number of methods involving fluorescence as screening techniques; although most of these methods are robust, many require specialized and costly instrumentation as well as the required knowledge to keep them working well.11,27 The most popular methods involve fluorescence-based displacement assays. Among them, fluorescence correlation spectroscopy (FCS) based assays are worth mention; these assays utilize the difference
in the diffusion time of bound and unbound ligands, and yet they require special instrumentation and expertise to correlate the data. Assays based on FRET partners are also of importance; although these assays require simpler fluorescent instruments the required labeling of the protein itself reduces their versatility.

Fluorescence-polarization activity-based protein profiling (fluopol-ABPP) technology, an interesting technique that has recently been developed, is compatible with HTS (Figure 1.3).

Fluorescence polarization, a measure of the apparent size of a fluorophore, is widely used to study molecular interactions. When excited with plane-polarized light, a fluorophore emits light parallel to the plane of excitation, unless it rotates in the excited state. The speed of molecular rotation and resulting extent of depolarization are inversely proportional to molecular volume. Typically, small fluorophores of less than 10 kDa rotate quickly and emit depolarized light that is registered as a low fluorescence polarization signal when free in solution, but, when they are bound to a protein, they rotate more slowly and emit highly polarized light that is registered as a high fluorescence polarization signal. The reaction between an activity-based probe and an enzyme results in a time-dependent increase in fluorescence polarization signal, enabling real time monitoring of enzymatic activity in a homogeneous assay format.
As a pictorial representation of this technique’s concept, in Figure 1.3, a protein is placed into a 384 well plate, followed by the addition of a different test compound into each well. The figure represents an inhibitor (a) and an inactive compound (b). A fluorescent ABPP probe is then introduced to each well and the plate incubated for some time. The reaction of the probe with unbound protein (but not with bound protein) will greatly increase the apparent mass of the probe, resulting in the maintenance of a strong fluorescence polarization signal.

1.3 Dendrimers, Mono-Dispersed Polymers

It was mentioned before that amphiphilic molecules are primarily interesting due to the self-assembling characteristics they exhibit, both in solution as micelles or vesicles and in aggregates. The driving force for the formation of these amphiphile assemblies is to maintain a favorable hydrophilic–lipophilic balance (HLB) between the hydrophilic and the lipophilic components. In solution, these molecules self-assemble only when they are above certain concentration, which is known as critical aggregation
concentration (CAC).\textsuperscript{30} Amphiphilic macromolecules exhibit CACs substantially lower than smaller surfactants. While small surfactants have CACs in the millimolar range, assemblies formed by macromolecules such as dendrimers, amphiphilic homopolymers, amphiphilic random copolymers or amphiphilic block copolymers have CACs lingering in the micro and nanomolar range.\textsuperscript{31,32,33} In a way, amphiphilic macromolecules could be seen as macro-surfactants. Although polymers form more stable self-assembling structures with lower CACs than the small molecule surfactants, the inherent polydispersity associated with their synthesis makes a challenge of the synthetic reproducibility, which could be a drawback for this kind of polymeric system in drug delivery, sensing, and in general in the study of nanoassemblies’ surface properties.\textsuperscript{34} In that sense, dendrimers have the advantage of synthetic reproducibility, monodispersity, and controlled molecular weight.

1.3.1 Structural Characteristics of Dendrimers

Among amphiphilic macromolecules, dendrimers are interesting due to their uniform structures, controlled functionalization, and defined molecular weight. Multivalency is another important feature that is provided by the highly branched dendrimer structures,\textsuperscript{35} which mean a high number of terminal functional groups are available for functionalization.

In fact, branching is the primary characteristics of dendrimers. For this reason, the macromolecules were initially called “arborols” by Newkome,\textsuperscript{36} and later on “dendrimers” by Tomalia, which became the term accepted in the literature.\textsuperscript{37} The
words dendrimer, and dendron, come from the Greek word δέντρον (read dendron), which means “tree”, and refers to the branched structures of the molecules. Usually, a dendrimer or a dendron contains a monomer or a single group called the core or the focal point. Typically, a dendrimer is symmetric around the core but a dendron branches out from the focal point in one hemisphere direction. The branches’ terminal groups are called the periphery in either case, as it can be seen from the cartoon depicting the backbone of dendrons in Figure 1.4.

**Figure 1.4.** Cartoon representation for the focal point, middle layer, and periphery of a dendron generation 1 (G1), and a dendron generation 2 (G2).

Amphiphilic dendrimers are formed using hydrophilic and hydrophobic components. As mentioned before, the non-covalent forces drive dendrimers to self-assemble above their CAC, when dispersed in aqueous media. Hydrophilic components, that often incorporate structures of carboxylates, phosphates or quaternary ammonium groups, render these dendrimers water soluble. However, the non-specific interactions of these charged species with biomolecules i.e. non-specific binding, often limit their use in biological applications. To address that issue, non-ionic and well known biocompatible hydrophilic molecules, such as poly(ethylene glycols), have been used as
the hydrophilic part. On the other hand, hydrophobic components dictate parameters such as stability and loading capacity.

There are also amphiphilic dendrimers that do not form assemblies. Instead, Fréchet-type dendrimers form unimolecular micelles in solution by displaying a hydrophilic periphery and a hydrophobic polyether core. Newkome, and Meijer have reported on similar kinds of dendrimers.

Dendrimers, as a type of monodispersed macromolecules, are synthesized in a controlled fashion. The appearance of dendrimers in specialized literature increased considerably in the last two decades, as well as their applications on a variety of fields. In our lab, a unique kind of biaryl amphiphilic self-assembling dendrons has been developed incorporating a range of different functionalities, with the goal of imparting the macromolecules with responsiveness towards different stimuli.

1.3.2 Divergent and Convergent Methods for Dendrimer Synthesis

Usually, a self-assembly is formed by amphiphilic macromolecules such as synthetic polymers, which show broad molecular weight distributions after synthesis; unlike natural amphiphilic polymers i.e. proteins, which are monodispersed despite their complexity. Thus, polymers are polydispersed regardless of the advances in polymerization techniques achieved during the last three decades. One way to access macromolecules with defined molecular weights is through iterative synthesis. Although this method is more tedious, time consuming and less efficient than step-by-step and radical polymerizations, it allows for successive and controlled attachment of
monomers. With this type of synthesis, it is possible to control the exact molecular weight and location of ligands and various functionalities into the macromolecule.

Dendrimer synthesis involves either divergent\textsuperscript{43,44} or convergent covalent attachment strategies,\textsuperscript{45} which generally require molecular scaffolds and synthetic pathways as outlined in Figure 1.5. Simply stated, the divergent strategy involves the controlled and iterative assembly of $AB_2$ type monomers, where the co-reactive nature of A and B allows for a controlled covalent attachment of the monomers. This might be visualized as a molecular tree growing from the stem towards the branches, and ending when it has reached the periphery of the branches or tree canopy. The convergent strategy also involves the controlled, iterative assembly of $AB_2$ type monomers, where the B and A components are co-reactive; in this case protection and deprotection...
protocols are used to build the dendrons, generation by generation. In essence, the molecular tree starts to grow from the periphery of the branches or tree canopy towards the inner branches, until reaching the stem (focal point), to produce a molecular tree, as shown in Figure 1.5. In general, dendrimers produced by the divergent strategy suffer an increase of reactive functionalities as the generation increases, which leads to a higher number of defects at higher generations. Dendrimers synthesized by the convergent strategy are nearly free of defects due to a fewer number of reactive functionalities that remain constant as the generation increases. However, steric hindrance produced by the growing bulky groups leads to reaction problems at higher generations.

An example of a single product generated using the convergent approach is shown in Figure 1.6.46 Briefly, a polyphenylazomethine-based dendrimer, which consist of a \( \pi \)-conjugated backbone synthesized up to the 4\(^{th} \) generation (G4) using the
convergent method, led to a single macromolecular structure, as it was observed by MALDI-TOF-MS. The product was monodispersed and showed a consistent molecular weight (m/z 5451.26 [M + H]+). Figure 1.7 shows an example of how the divergent approach affects the purity of the products by introducing defects that lead to byproducts of diverse molecular weights. Briefly, a poly(propylene imine) dendrimer synthesized via a divergent approach accumulated statistical defects as soon as the first synthetic step, which led to imperfections in the G1, G2, G3, and final products (Figure 1.7. with m/z 740.6, 1622.0, 3384.0, 6909.7 for increasing generations of this dendrimer).

1.3.3 Biomimetic Characteristics of Dendrimers

The synthesis of dendrons and dendrimers provides a precise and tunable strategy for the controlled construction of macromolecules. Formed by reproducible monomers, dendrimers are often compared to proteins due to their size and three-dimensional structure, characteristics imparted by the forming monomers. Each
architectural component bears a specific function that will define specific properties in
the nanostructures, generation by generation. Dendrimers’ monodispersity and three-
dimensional architecture resemble the homogeneity of the naturally occurring proteins,
and closely mimic the globular shape and scale of the bio-macromolecules. These
characteristics grant dendrimers and dendrons the epithet of artificial proteins.\textsuperscript{48,49}

The groups that are primarily exposed to the solvent and nanoenvironment
surrounding them lie at the surface of the nanostructure. These functionalities might
consist of either reactive or unreactive terminal groups that impart important properties
to the macromolecule such as solubility, charge surface, accessibility to the interior from
the outside and to the outside from the interior, surface functionalization, and
specificity towards a target or stimulus. Within the interior of the nanostructure lie the
group functionalities that are less exposed to the solvent; these functionalities define
the type of interior based on the groups’ composition and branching, the hydrophobic
properties of the macromolecules, and also the host-guest properties. The spherical 3D
nanostructure generates a nanoenvironment at the inner layers of the dendrimer that
allows for encapsulation of small molecules kindred to that nanoenvironment. The core,
or focal point, provides information about the kind of monomer forming the dendrimer;
the size, shape, and directionality are expressed by the connectivity of the core towards
the outer layers.\textsuperscript{4}

Such architectural components form the overall set of physicochemical
properties namely size, shape, flexibility, and reactivity of the dendrimers. In particular,
the encapsulation property of dendritic globular nanostructures and the possibility of
altering that property, as a response to an external stimulus, resemble protein’s characteristics, in which the active site is buried in hydrophobic pockets. Figure 1.8 shows a Fréchet type globular dendrimer which forms a unimolecular micelle, making it a system free of CAC. Such structure bears a hydrophilic periphery that might be formed by carboxylic acid functionalities (red beats in the figure) and a hydrophobic interior (red waving lines in the figure). Due to its globular shape and by means of the hydrophilic periphery of the macromolecule, the whole structure is water soluble regardless of its hydrophobic interior. Figure 1.8 clearly shows the voids in between hydrophobic branches (green), which, though unrealistic, are a pictoric representation of where the encapsulated hydrophobic molecules might remain trapped in a dendrimer (hydrophobic interior).

Amphiphilic dendrimers, such as the one represented in Figure 1.8, should adopt a globular shape in aqueous solution, displaying a hydrophobic core and hydrophobic inner layers (green color) that could be used as pockets to encapsulate small
hydrophobic molecules in between the branches of the unimolecular micelle inner layers. Encapsulation, one of the main characteristics of amphiphilic dendrons and dendrimers, will be discussed in the next section.

1.3.4 Encapsulation of Molecules within the Dendritic Architecture

Due to the interstitial space between branches in the dendritic architecture, the possibility of encapsulating hydrophobic small molecules has been explored thoroughly. In amphiphilic dendrimers, water solubility is given by the hydrophilic groups at the periphery, which wrap the hydrophobic inner layers screening them from the solvent. As a result, the dendritic structure is water soluble regardless of its encapsulated hydrophobicity. This phenomenon offers the possibility of using dendrimers and dendrons to interact with poorly soluble or non-water soluble molecules, such as drugs. The nature of guest non-covalent encapsulation could be simply physical entrapment or, could involve interactions with the dendritic structure depending on the branching functionalities.

Encapsulation of hydrophobic small molecules in the hydrophobic interior of dendrimers prevents them from precipitating out of an aqueous media. Given a hydrophobic drug molecule, encapsulation would enhance its bioavailability and circulation time in a biological system, improve its transit across biological barriers, and slow drug metabolism. Given the right functionalization, a dendrimer could act as a nanocarrier, taking the drug to specific types of cells i.e. active targeting rather than passive targeting through enhanced permeation and retention effect (EPR). Thus, a
A dendrimer with optimized characteristics would enhance drug bioavailability to diseased cells, and would reduce the risk of the drug reaching healthy cells, preventing the side effects of chemotherapies.\textsuperscript{52}

One of the first dendritic systems forming a unimolecular micelle, able to encapsulate guest molecules was described by Hawker and Fréchet.\textsuperscript{39} The macromolecule was a dendritic polyether synthesized by a convergent protocol and based on the electron rich 3,5-dihydroxybenzyl alcohol unit as the primary building block.

\textbf{Figure 1.9.} Unimolecular dendritic micelle for encapsulation of hydrophobic guests.
block. The final structure displayed carboxylate groups as terminal functionalities to
assess a hydrophilic periphery. Such a system had the capability of encapsulating
hydrophobic molecules like pyrene, anthracene, 1,4-diaminoanthraquinone, and
2,3,6,7-tetranitrofluorenone. The sequestration of these small molecules was tracked by
UV-VIS spectroscopy once they were solubilized by the dendrimer, which is shown in
Figure 1.9. For the encapsulated hydrophobic small molecules, the dendrimer showed a
high solubilizing power, which is also known as encapsulation efficiency. The reason for
this phenomenon was not only due to physical entrapment, but also to the formation of
stabilizing \( \pi-\pi \) interactions between the dendritic aromatic rings forming the
hydrophobic branches and the aromatic small molecules.
However, hydrophobic components in dendrimers could potentially cause precipitation of the entire system due to lack of solubility. Poly(ethylene glycol) (PEG) has been conjugated to dendrimers and dendrons with the purpose of increasing the hydrophilicity of the system and, in general, to translate the PEG properties to the dendrimers. That is, PEG is usually conjugated to the surface of a dendrimer or a dendron.

Figure 1.10. G2 dendritic unimolecular micelle functionalized with PEG.
dendron to provide a biocompatible hydrophilic shell, diminish non-specific interactions of the macromolecule in a biological media, and increase the circulation time of the conjugated system.\textsuperscript{53}

An example of a dendritic molecule capable of forming a unimolecular micelle with a hydrophobic core surrounded by a hydrophilic shell is shown in Figure 1.10. Briefly, the macromolecule was prepared by coupling a hydrophobic dendrimer with PEG. The monomer used to build the dendritic core was 4,4-bis(4'-hydroxyphenyl)pentanol, which provided flexibility and, consequently, contributed to the cavity size or container capacity of the dendritic architecture.\textsuperscript{53}

The G2 micelle shown in Figure 1.10 contains a hydrophobic alkyl-aryl core surrounded by a hydrophilic PEG shell. Such micelles were able to encapsulate hydrophobic small molecules like pyrene and indomethacin. In general, it was found that the encapsulation capacity for a unimolecular micelle depends on the generation and the PEG chain length that is grafted to the dendritic hydrophobic core.\textsuperscript{53}

Encapsulation of small molecules within a dendrimer becomes interesting and important from the pharmaceutical and sensing points of view, as long as there is a way of triggering or controlling their release. For that, it is necessary to design dendritic structures incorporating sensitive functionalities to render stimuli-responsive host-guest systems. The next section refers to a particular class of stimuli-responsive dendrons that self-assemble into aggregates able to encapsulate small hydrophobic molecules, rather than forming unimolecular micelles.
1.4 Stimuli-Responsive Supramolecular Aggregates formed by Biaryl Amphiphilic Dendrons

Biaryl amphiphilic dendrons present a particular design in which hydrophilic and hydrophobic moieties are displayed toward opposite directions in every layer of the dendron. Unlike Fréchet type dendrimers, biaryl amphiphilic dendrons do not form unimolecular micelles; instead these macromolecules self-assemble in water into aggregates of sizes around 100 nanometers containing hydrophobic pockets. Such pockets are product of the dendrons hydrophobic moieties collapsing towards the interior of the aggregate, and the PEG chains being exposed towards the aqueous solvent, shielding the nanoassembly from water. Surprisingly, when the same dendrons are in a non-polar media, such as toluene, the self-assembly process evolves towards an inverse micelle-like aggregate in which the hydrophobic moieties are exposed to the solvent and the hydrophilic branches collapse towards the interior of the aggregate, as shown in Figure 1.11.\textsuperscript{54,55}

Although, by definition, this type of nanoassembly has a CAC, which could be taken as a disadvantage when comparing them to dendritic unimolecular micelles; the
capability of these dendrons to self-assemble presents an opportunity to control the
disassembly and, if no cleavage of covalent bonds is involved, to make the disassembly
event reversible.56 Furthermore, adding the non-covalent encapsulation of guest
molecules, disassembly is the key to produce release of a structurally unaffected guest
molecule, since the guest is not covalently conjugated to the dendron. It has been
observed that disassembly could be triggered by diverse stimuli, depending on which
responsive unit has been incorporated into the molecular structure of the dendron.

1.4.1 Temperature Sensitive Dendrimeric Supramolecular Aggregates

This section refers to amphiphilic dendrons that undergo temperature-
dependent solubility changes once the lower critical solubility temperature (LCST), or
“cloud point”, is reached. There are many reports on temperature-sensitive hard and
soft nanoparticles.57,58,59,60 Such thermoresponsive materials could be utilized as drug
delivery vehicles for biomedical applications in thermotherapy (hyperthermia), which
involves exposure of the diseased cells or tissues to high temperature in order to
damage them. This therapy involves the use of clinically approved radiofrequency
ablation as a local heating system. LCST behavior has been observed and thoroughly
studied in poly(N-isopropylacrylamide) polymers (PNIPAM) and PEG based
macromolecules. It has been determined that the LCST depends greatly on the balance
between hydrophilicity and hydrophobicity of the system.61
Temperature-sensitive dendrimers could be prepared by conjugating them to thermoresponsive polymers, either at the periphery or at the focal point. There are amphiphilic dendrimers that showed the particularity of aggregating in micelle-like assemblies with thermosensitive properties (Figure 1.12.b), i.e. LCST behavior. Such dendrimers were formed using a hydrophobic oligo(p-phenylene vinylene) core and a hydrophilic periphery consisting of oligoethylene glycol. The temperature sensitivity was found to be dependent on the oligoethylene glycol moieties and on the dendrimer generation. Dendrimers with a backbone that displays hydrophobicity and hydrophilicity adequately balanced also act as temperature sensitive dendrimers. In fact, the first report on a thermoresponsive dendrimer including temperature sensitive functionalities as part of the backbone used oligoethylene glycol as a building block (Figure 1.12.a).
The concept of having hydrophilic and hydrophobic units in each building block is applied in biaryl amphiphilic dendrons, in which oligoethylene glycol acts as a hydrophilic moiety being present in every repeating unit of the molecular design along with hydrophobic decyl fragments. This confers the dendrons, and their assemblies, responsiveness toward changes in temperature in such a way that these systems exhibit a macroscopic transition, or LCST, in which the assembly separates from the aqueous phase at higher temperatures ($\geq 42 \, ^\circ\text{C}$ for G1, $\geq 32 \, ^\circ\text{C}$ for G2, and $\geq 31 \, ^\circ\text{C}$ for G3). At these temperatures the size of the supramolecular assemblies changes due to a decrease in

Figure 1.13. Structure of temperature sensitive G1, G2, and G3 dendrons.
the hydration of the oligoethylene glycol branches. Figure 1.13 shows the structure of G1, G2, and G3 biaryl amphiphilic dendrons.

It was not until recently that a sub-LCST transition was found in the assemblies formed by the biaryl G1 dendron; although this transition was not observed for the assemblies formed by either G2 or G3 dendrons. Therefore this transition, which occurs well below the LCST, was found to be dependent on the dendron generation. At this sub-LCST, the size of the dendron supramolecular assembly changes significantly in response to temperature variations due to an enhanced hydration of the oligoethylene glycol branches. A larger energetic penalty for reorganization could be the reason for a lack of sub-LCST transition in higher than G1 generation dendrons.

1.4.2 Photosensitive Dendrimeric Supramolecular Aggregates

Photoresponsive materials have found application in biomedical sciences, where they have been developed for photodynamic therapy in the treatments of superficial tumors and age related muscular degeneration. This technique involves the photochemical generation of a reactive single oxygen species (1^1O_2) directly on the diseased site to cause oxidative damage of cells. Although dendritic nanoparticles encapsulating or conjugated to photosensitizers have been developed, photodegradable dendrimers constitute a more interesting class of light-sensitive systems. The latter are called self-immolative dendrimers; after a photochemical reaction at the dendrimer core, the entire molecule is broken down into low molecular weight fragments,
ultimately resulting in the release of non-covalently encapsulated molecules or covalently conjugated peripheral groups, as depicted in Figure 1.14.\textsuperscript{50,65}

![Figure 1.14. Self-immolative photoresponsive dendron.](image)

In our lab, a class of photoresponsive biaryl amphiphilic dendrons was developed by incorporating a photosensitive linker in the hydrophobic face of the design.\textsuperscript{66} While PEG was used as the hydrophilic face, an \textit{o}-nitrobenzylester moiety was incorporated as a linker in the hydrophobic chains in such a way that after photocleavage, a residual

![Figure 1.15. Photosensitive biaryl amphiphilic dendron](image)
carboxylic acid functionality remains as part of the dendron backbone, terminating the amphiphilicity of the system and causing disassembly, as depicted in Figure 1.15. If small guest molecules are encapsulated in the hydrophobic core of the assembly, release can be observed after photocleavage due to a change of the core’s hydrophobicity into hydrophilicity, resulting in disassembly.

The same kind of previously mentioned micelle-like aggregates can be stabilized through photochemical crosslinking, if the photosensitive moieties incorporated at the hydrophobic face of the dendrons react with each other in a dimerization process after photo-stimulus. Since photodimerization of coumarin is well known, a coumarin derivative was used as terminal functional groups in the hydrophobic branches of the dendron. As expected, after photo-stimulation at 365 nm the aggregate stage in solution was locked. Since photodimerization of coumarin is reversible, the crosslinked micelle-like nanoassembly can be decrosslinked simply by using a 250 nm wavelength light, as it was inferred and it is shown in Figure 1.16.

![Figure 1.16. Crosslink of dendrons in the aggregate stage](image)

The crosslinking of dendritic aggregates increases the encapsulation stability of the system, since the equilibrium between monomeric stage and micelle-like aggregate...
stage is shifted then towards the aggregate stage, preventing the inherent leakage due to the equilibrium.

1.4.3 Protein-Sensitive Dendrimeric Supramolecular Aggregates

Bioresponsive materials are of great importance due to their potential to respond to pathologically relevant stimuli. Among the known bio-stimuli, some have greater relevance than others for they are directly related to diseases. Examples are imbalances in protein concentrations or enzymatic activities, which are known as primary imbalances of the human organism. Then, a system that disassembles and releases cargo in presence of proteins presents advantages in comparison to either thermoresponsive or photoresponsive systems.

The capability of a system to distinguish a target protein among a mixture by molecular recognition is of great importance. Such specificity, capability of encapsulation, nanometer size, and ability to prevent non-specific interactions are characteristics that have been described for biaryl amphiphilic dendrons. Particularly, the ability to respond to specific proteins can be embedded in these dendrons by incorporating specific protein substrates. Using this concept, a dendritic aggregate was

Figure 1.17. Enzymatic cleavage of hydrophobic moieties of a dendron. Change in HLB based on covalent modifications.
made responsive to an esterase, as shown in Figure 1.17.

In Figure 1.17 an esterase cleaves the ester functionalities linking the hydrophobic chains to the backbone of the dendron. As a result, the hydrophobic terminal groups of the dendron are transformed into carboxylic acid functionalities. Hence, by means of an enzymatic hydrolysis, the dendron loses its amphiphilicity and HLB, which results in the aggregate disassembly. Although disturbing the dendron's HLB by breaking covalent bonds and increasing its hydrophilicity is a successful approach, there are many non-enzymatic proteins that could be used as a target for drug delivery. For such targets, non-covalent interactions remain as an option to disrupt the HLB and trigger disassembly.

By tethering a ligand specific to the target protein, a dendron and its assembly acquire specificity towards that protein. After the binding event, the enhanced hydrophilicity of the protein disturbs the HLB of the dendritic aggregate, triggering disassembly, and, as depicted in Figure 1.18, the release of guest molecules.
1.5 Summary

The importance of ligand-protein interactions has been reviewed along with some of the most relevant methods for the screening of small molecules in the quest of the pharmaceutical industry for hits and lead molecules. Among the different screening methods, probably the most versatile and accessible are those based on fluorescence. Most important, although tethering with at least one fluorophore is required in most cases, fluorimetry based methods are rapidly adaptable as a HTS technique, which presents advantages for screening high numbers of compounds in less time.

Some interesting characteristics of classical amphiphilic dendrimers, which form unimolecular micelles, have been discussed in this chapter along with the recently developed biaryl amphiphilic dendrons, which self-assemble to form nanometer size aggregates able to non-covalently encapsulate hydrophobic small molecules. Unlike unimolecular micelles, aggregates formed by biaryl amphiphilic dendrons are not rigid, covalently bound nanostructures; on the contrary, when the biaryl dendrons are in water, they form micelle-like aggregates but when they are in a non-polar solvent, they form inverse micelle-like aggregates.

This chapter also explored the disassembly capability of the aggregates depending on the responsive features installed in the dendrons and when stimuli are applied. Once disassembly is triggered, the release of guest molecules is observed. We discussed relevant stimuli, such as proteins, which could cause disruption in the system’s HLB without breaking any covalent bond. For that method, the installation of a ligand, specific to the target protein is necessary. In the upcoming chapters of this
manuscript, we will discuss how the location of the ligand might affect disassembly of the dendritic nanoaggregates and the release of encapsulated guest molecules. From there, mechanisms to explain our results will be proposed.

1.6 References

Notes


70 Hayes, G. M.; Carrigan, P. E.; Miller, L. J. “Serine-arginine Protein Kinase 1 overexpression is associated with tumorigenic imbalance in Mitogen-Activated Protein Kinase pathways in breast, colonic, and pancreatic carcinomas”. Cancer Res. 2007, 67, 2072-2080.


2.1 Introduction

The ability of a small molecule to tightly bind to a target protein receptor is arguably the most important criterion in drug development. For this purpose, the development of reliable methods for rapidly screening small molecules against specific proteins is essential. Current methods for small molecule screening include X-ray crystallography,\textsuperscript{1} NMR,\textsuperscript{2} mass spectrometry,\textsuperscript{3,4,5} microcalorimetry,\textsuperscript{6} and fluorescence based techniques,\textsuperscript{7,8,9} as mentioned in detail in the previous chapter. These techniques are generally complementary to each other. While ITC gives the most valuable thermodynamic information, such as precise binding affinities, the difficulty of adapting it as a HTS technique makes it a more valuable method for validation those promising molecule candidates found using other techniques more easily adaptable to HTS, such as fluorescence and MS. On the other hand, techniques such as X-ray crystallography give valuable information about the binding site and ligand interactions but again, it is often only use as a validation method and less often for screening due to the amount of target protein required and the need for adequate crystals for the measurements.\textsuperscript{10} NMR is used as a validation method but also for screening, although the analysis of the acquired data requires great expertise. An extra inconvenience of NMR screening methods is the limitation in size for the target proteins, since samples larger than 30 kDa
require labeling of at least one amino acid close to the binding site in order to facilitate the interpretation of the data.

Among the different techniques, fluorescence stands apart as the premier tool due to its simplicity and speed in analysis along with the easy adaptability of it as a HTS method.\textsuperscript{11} However, most fluorescence-based approaches require labeling the target, drug candidate, or analyte with dyes.\textsuperscript{12,13} Tagging adds another step of planning and design to the screening process. To skip the labeling step, here we present a new label-free, fluorescence-based supramolecular platform to rapidly discriminate binding affinities of analytes against a target protein.

2.2 Approach and Molecular Design

The general scheme depicting the approach is shown in Figure 2.1. It is based on the observation that when a small molecule binds to a protein, nearby proteins, including enzymes, cannot bind to the small molecule, or at least not until it is dislodged from the initial protein.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2_1.png}
\caption{Label-free ligand screening. a) Probe characteristics; b) schematic illustration of the strategy.}
\end{figure}
In the strategy illustrated in Figure 2.1a, the probe is designed using a known ligand that is complementary to the protein and is connected to a pro-fluorophore through a linker. The key features of the molecular design are: (i) the linker is cleavable by an enzyme; (ii) enzymatic cleavage of the linker converts the non-fluorescent precursor to a water-soluble fluorophore; (iii) when the probe is bound to the target protein, the linker is sterically inaccessible for the enzyme, as depicted in Figure 2.1b. By designing a probe molecule that satisfies these requirements we envisaged the possibility of using this system to screen small molecule binders for the target protein. For this, the analyte small molecule with comparable or better binding affinity than the probe molecule would be able to displace it from the protein, depending on its relative concentration. This displacement would expose the enzyme-active linker, the cleavage of which should result in the formation of the fluorophore. The concentration-dependence of the fluorescence generation can then be used to evaluate the ability of the ligand candidates to bind the target protein.

To test this design hypothesis, we chose human carbonic anhydrase I (hCA1) as the target protein, an interesting and well characterized protein that has been

![Figure 2.2. Probe structure. Benzenesulfonamide as the ligand, an ester group as the cleavable linker, and umbelliferone as the reporter or fluorophore.](image)
implicated in a variety of pathophysiological processes.\textsuperscript{16,17} The molecular structure of our probe is shown in Figure 2.2. Arylsulfonamide is a ligand for hCA1,\textsuperscript{18,19,20,21} while the coumarin derivative, umbelliferone is an excellent fluorophore. The ligand and the fluorophore are linked through an aliphatic chain bearing an ester bond. Coumarin is attached to the ester through an acetal moiety, where the cleavage of the ester using porcine liver esterase (PLE) would release the fluorophore, umbelliferone.

Since the binding pocket in hCA1 is a 15 Å deep cleft,\textsuperscript{21} the linker length of the probe was designed to be modifiable in order to have two probes with different linker lengths as this would impact the enzyme’s accessibility to the ester moiety. In this way, we are making the linker length a critical component of our molecular design. As it will be shown in the synthesis of the probe, the attachment of the ligand is performed as a final step to allow for the use of a variety of ligands, depending on the chosen protein to target. This, along with the variations in the linker length renders the design modular.

\textbf{Chart 2.1.} Structure of the analyte molecules used in the assay and their literature-reported binding affinities.\textsuperscript{9}

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|c|c|c|c|}
\hline
Comp. & 1 & 2 & 3 & 4 & 5 & 6 & 7 \\
\hline
$K_D$ (M) & $9\times10^{-9}$ & $1.15\times10^{-6}$ & $1.67\times10^{-6}$ & 3.4x10$^{-6}$ & 90x10$^{-6}$ & N/A & N/A \\
\hline
\end{tabular}
\end{table}

The analytes to use, as represented in Figure 2.1b, must be a set of molecules with diverse affinities for hCA1, ranging from excellent ligands with nanomolar affinity to
ligands with middle and low affinity. These molecules are ethoxzolamide 1, acetazolamide 2, p-toluenesulfonamide 3, 4-carboxybenzensulfonamide 4, and 4-aminobenzenesulfonamide 5, which are shown in Chart 2.1. In this set, we have also included analyte molecules that have not been reported as ligands for hCA1 and that are unlikely to have any affinity for the protein, these molecules are p-iodoaniline 6 and p-nitrophenol 7. The molecules used as ligands and their binding constants ($K_D$) are shown in Chart 2.1. Analyte 8 was synthesized as a probe analogue; lacking the esterase substrate it will not be cleaved by PLE and, hence, will show no increase in fluorescence. This analogue should have similar binding affinity as the probe.

2.3 Results and Discussion

2.3.1 Synthetic Strategy

In order to have a modular design and a working probe for the conditions of our experiments, certain characteristics must be fulfilled. The most important of those requirements is that the reporter must be silent, if we are to track the binding of the analytes to the target protein; this means that the fluorescence of the fluorophore should be very low when it is linked to the benzenesulfonamide moiety. Also, it should be water soluble after enzymatic cleavage of the linker. For that purpose, 4-methylumbelliferone, a coumarin modified with a methyl group in position 4 and with a hydroxyl group in position 7 is an ideal molecule, for when it is O-alkylated at the position 7 its fluorescence decreases considerably and when it is not alkylated its solubility in water is better than an unmodified coumarin.
It was observed that when an ester bond is directly formed at the position 7 of 4-
methylumbelliferone, it is rapidly hydrolyzed in a buffer solution at pH 7.4. However,

when an acetal moiety is placed between the ester bond and the aromatic
umbelliferone the ester functionality remains stable during the timeframe of our
experiments. Also, by introducing a short spacer, as is the acetal, we prevent possible
hindrance from the 4-methylumbelliferone system on the PLE action over the ester
group.

The acetal and ester groups are formed in a two-step one-pot reaction between
4-methylumbelliferone 11, dibromomethane, and an alkyl carboxylic acid 9 in refluxing
acetone aided by the catalyst 18-crown-6, as shown in Scheme 2.1. The carboxylic acid is
the molecule that dictates the final length of the linker. As seen in Figure 2.2 for the two

Scheme 2.1. Synthesis of the probes, (a) and ester group cleavage by an esterase or a
nucleophile (b).
probes with \( n = 4 \) and \( n = 8 \), the carboxylic acids used were 6-bromohexanoic acid \( 9a \) and 10-bromodecanoic acid \( 9b \), respectively. To prevent nucleophilic attacks on the terminal carbon attached to the bromine, an planning on having a terminal primary amine the halogen was substituted by an azide \( 10a,b \) before esterification by reacting with sodium azide in \( N,N \)-dimethylformamide (DMF). Once the 4-methylumbelliferone has been O-substituted in the 7 position \( 12a,b \), the terminal azide is reduced to a primary amine \( 13a,b \) through a Staudinger reduction in tetrahydrofuran (THF).

This reduction is a critical step when \( n = 4 \), since the newly formed amine can act as a nucleophile and attack the carbonyl ester via an intramolecular cyclization to form a 7 membered \( \varepsilon \)-caprolactam, while recovering the 4-methylumbelliferone and releasing the formaldehyde byproduct (Scheme 2.1b). The yield for this step was low. Finally, the last step of the synthesis is the coupling of the ligand. Since the previously mentioned scaffold \( 13a,b \) holds a primary amine, it is convenient that the ligand moiety has a carboxylic acid functional group \( 14 \), in that way, the two fragments are tethered via amide coupling in DMF using 1,1′-carbonyldiimidazole (CDI) as the coupling agent.

![Scheme 2.2. Synthesis of analyte 8.](image-url)
The synthesis of the analyte 8 is faster and less troublesome to accomplish than the synthesis of the probes, since 8 lacks the ester and acetal functionalities that are prone to hydrolysis. So, as it is shown in Scheme 2.2, the analyte was synthesized starting from the O-alkylation of 4-methylumbelliferone 11 with the commercially available N-Boc protected 6-bromohexylamine 15, refluxing in acetone, using K$_2$CO$_3$ as the base and 18-crown-6 as a catalyst to get 16. Then, the primary amine is deprotected using trifluoroacetic acid in dichloromethane getting the molecule 17. Then, once again the terminal amine can be attached to the ligand 14 through amide coupling using CDI as the coupling agent to get the analyte 8.$^{22}$

2.3.2 Supramolecular Displacement$^{23}$

2.3.2.1 Hindering Enzymatic Action on the Probe

To first test our hypothesis, we chose the Probe A where the linker length is n=4 and exposed it to different concentrations of hCA1, as shown in Figure 2.3a. First, the probe itself does not hydrolyze in the buffer without the enzyme (green line). Secondly, while 5 µM concentration of Probe A with 50 nM PLE generated significant fluorescence within a few seconds (red line), this combination did not generate meaningful fluorescence in the presence of 20 µM hCA1 (blue line). At smaller concentrations of hCA1, such as 15 µM, there was intermediate fluorescence (purple line) in presence of PLE. Thirdly, we were surprised to find that the presence of hCA1 at a lower concentration (10 µM), enhanced the enzymatic cleavage of the probe (black line). This could be because the protein concentration is not enough to hinder enzymatic action on
the ester moiety of Probe A during the binding equilibrium but helps in improving the probe’s solubility, making it more available for the PLE.

When we tested 5 µM of Probe B, the probe itself also does not hydrolyze in the buffer without the enzyme (green line) and is stable in the sole presence of 20 µM hCA1 (orange line). Exposing Probe B to 50 nM PLE generated significant fluorescence within a few seconds (red line), similar to Probe A. However, under these conditions, a higher fluorescence in the presence of 20 µM hCA1 (blue line) is generated, unlike Probe A. This is due to the longer distance between the ligand moiety and the enzyme cleavable bond in Probe B, which makes it more difficult for hCA1 to hinder the action of PLE. Once again, the presence of hCA1 helped to improve the solubility of the probe and enhance enzymatic cleavage of the probe, as seen in Figure 2.3b. This could be because the protein does not sufficiently mask the ester moiety upon binding.

As shown in Scheme 2.1, both probes have a hydrophobic component that, to some extent, could compromise their water solubility, especially for Probe B where n =...
8. Then, hCA1 acts as a solubilizing agent for the probes, when it is at lower concentrations.

2.3.2.2 Pre-incubation Time

It is important for a HTS technique to be expedited in each of its steps. For this label-free supramolecular displacement assay to fulfill that requirement, the time for the binding event equilibration must be reasonable. Given the right concentration of hCA1 needed to efficiently hinder enzymatic action on the substrate, 20 µM in our experiments, the pre-incubation time with the probe was tested up to 18 hours.

As seen in Figure 2.4, the pre-incubation timeframe for Probe A-hCA1 was reduced to 15 minutes, after which PLE (50 nm) was added to the system. These experiments were accomplished without losing the steric masking provided to the probe by hCA1, since the difference in fluorescence intensity between just Probe A and PLE is meaningful.

Figure 2.4. Effective hCA1-Probe A pre-incubation time.
2.3.2.3 Competitive Displacement

With the identification of a combination of Probe A and the optimal relative Probe A:protein concentrations, along with their effective pre-incubation time, we were interested in testing the possibility of competitive displacement of the probe from the hCA1 binding pocket as an assay for the relative affinity of an analyte molecule. The analyte molecules used for this purpose were mentioned previously and are shown in Chart 2.1, along with their corresponding binding constants. Briefly, ligands 1-5 are known to be good inhibitors for hCA1, while molecules 6 and 7 are randomly chosen and are presumably not good ligands for hCA1. Analyte 8 should have a similar binding affinity as the probe.

![Figure 2.5. Displacement-mediated activity profiles generated by a) the analytes 1, 2, 4-7; and (b) 1, 3, 4, 8 with the slopes of the curve corresponding to the effective probe displacement by the analytes.](image)

When different amounts of these molecules were introduced into a solution containing a mixture of the Probe A:hCA1 (5:20 µM) and 50 nM PLE, the system showed an increase in fluorescence as a response to increasing concentrations of molecules 1-5. However, the response of the system to molecules 6 and 7 did not exhibit any
appreciable change in fluorescence with concentration. The fluorescence profiles for the set of molecules are shown in Figure 2.5a.

Three key features are evident from these plots: (i) analytes with strong to moderate binding affinities can displace Probe A and the displacement profile can be traced by the fluorescence evolution; (ii) since the relative concentration of Probe A vs. hCA1 dictates that there are some free proteins in the system, the fluorescence response remains flat in the initial part of the plot, which indicates that the ligands are first binding to the excess free proteins;24 (iii) the slope of the fluorescence change correlates with the analyte binding affinities, thus providing an opportunity to assess the relative binding affinities of the analyte molecules tested (Figure 2.5b). When there are no probe molecules remaining to be enzymatically hydrolyzed, the fluorescence stops increasing and the displacement profile reaches a final plateau.

The experiment is complete once the concentration of analyte has reached 100 µM, which is achieved in less than 45 minutes. During that time, Probe A must remain stable in the buffer media and PLE must be unable to hydrolyze the substrate. If so, the
increase in intensity accounts for the analyte binding affinity to the target protein and displacement of the probe. Figure 2.6a shows a control experiment with the highest fluorescence intensity of the reporter reached by enzymatic cleavage of Probe A (blue curve), and the probe in the buffer media (green line). The low fluorescence of the combination of PLE exposed to the pre-incubated probe:hCA1 (red line), accounts for the hCA1 esterase activity, which is much lower compared to the PLE activity on the probe. The conditions of this control experiment and the displacement assays are identical.

It is also important to note that the PLE activity is not inhibited by any of the analytes to which the enzyme is exposed in solution. To confirm this, 5 µM of the probe was exposed to 50 nM PLE in absence and presence of 40 µM of each analyte. As it is shown in Figure 2.6b, PLE activity is not inhibited to any extent by any of the analytes 1-8.

2.3.3 Correlation between Binding Affinity and Displacement

The analyte molecules 1-5 were chosen for this approach mainly because they

Figure 2.7. Correlation between binding constants and fluorescence profiles. Fitting equation: Log Slope = 0.2254 (-Log K_D) + 5.5209; R^2 = 0.9217
have been reported as effective inhibitors for hCA1. It is clear that the trend in the slopes obtained in our assays correlates well with the literature-reported $K_D$ values. Since the displacement rate of the probe is directly related to the affinity of the analyte for the binding pocket of the protein, which is reflected in the slopes of the fluorescence profiles, in Figure 2.5 we provide an example of the type of qualitative comparison that could be obtained with this method.

Molecules 3 and 4 have an identical functional group, benzene sulfonamide (Chart 2.1); however, while 3 has a methyl group in para- position to the sulfonamide, 4 has a carboxylic acid functional group, which provides different hydrophilic properties. This difference can be attributed to the higher affinity of 3, which is more hydrophobic than 4. Molecule 8 has a carboxamide functional group at the para- position that could be comparable to the carboxylic acid in 4, but this molecule is more hydrophobic than 3. To test whether the hydrophobicity indeed influences the binding affinity, we tested the relative binding affinity of 8 (Section 2.3.5). Our results suggest that 8 is indeed better than 3, but as seen in Figure 2.5b, it is not as good of an inhibitor as 1.

From Figure 2.5, and based on the analysis above, the displacement assay

\begin{table}[h]
\centering
\caption{Logarithmic correlation between binding constants and slopes from Figure 2.5a.}
\begin{tabular}{|c|c|c|c|c|}
\hline
Analyte & $K_D$ & Slope & -Log ($K_D$) & Log (Slope) \\
\hline
1 & 9.00E-09 & 2.16E+07 & 8.045757 & 7.334453751 \\
2 & 1.15E-06 & 9.33E+06 & 5.939302 & 6.969881644 \\
3$^\dagger$ & 1.67E-06 & 4.36E+06 & 5.777284 & 6.639486489 \\
4 & 3.40E-06 & 4.03E+06 & 5.468521 & 6.605305046 \\
5 & 9.00E-05 & 2.95E+06 & 4.045757 & 6.469822016 \\
8 & 1.22E-07 & 1.20E+07 & 6.913404 & 7.079181246 \\
\hline
\end{tabular}
\end{table}

$^\dagger$ From data in Figure 2.5b. Not included for the equation.
described here is useful to qualitatively discriminate binding affinities of small molecules for a target protein. Therefore, it is possible to evaluate the predictive capability of the assay developed here. Figure 2.7 shows a correlation between the literature-reported $K_D$ values and the slopes obtained from the linear regime of the plot in Figure 2.5a, where there is significant fluorescence change. In Figure 2.7 we provide a numeric correlation that could be used for predictive purposes as a calibration curve. In fact, using the equation from the calibration curve ($\text{Log Slope} = 0.2254 (\text{-Log } K_D) + 5.5209$), and the slope displayed in Figure 2.5b ($1.20 \times 10^{-7}$), the numeric value obtained for the binding constant of analyte 8 is $1.22 \times 10^{-7}$ M, which represents a better binding constant than analyte 3 but not as good as analyte 1, as was inferred qualitatively above. Table 2.1 shows the numeric values.

The reproducibility of the slopes obtained for the analytes and the experiment, in general, is an advantage for the assay, if it is to be adapted as a HTS method.

It has been reported that the coumaryl moiety could contribute to the binding affinity of a ligand. However, this could happen only if the sulfonamide ligand moiety is connected to the coumaryl through a short linker (two or three carbons) in such a way that when the ligand is completely bound to the binding pocket of the hCA1, the coumaryl is buried into the binding site, since it can interact only with the amino acid fragments buried deep in the binding cleft and not with the fragments close to the rim. Although the coumaryl moiety could be contributing to the improved affinity of 8, and consequently the affinity of the probe, this is not likely.
2.3.4 Adaptability as a HTS Method

The approach outlined here works well in providing an evaluation of relative affinities, but does not provide direct quantitative binding affinity measurements. However, the possibility of getting approximate binding constants through a calibration curve has been introduced as an option. Therefore, for such an approach to be useful, it is necessary that we can adapt this strategy for the rapid screening of analytes. For this purpose, we further tested the versatility of our approach in a 96-wells plate reader set-up. In such a setting, the volume of the components needed to generate a data point would be low and the screening can be done in a highly parallel fashion. The data generated from this experiment are shown in Figure 2.8 (compare with Figure 2.5a). The results indeed are consistent and reliably reproducible. As it is observed from Table 2.1, data for analyte 3 were incorporated from a similar experiment held at a different time and yet, its binding constant value remains between the values of analytes 2 and 3. This is an example of the methodology’s precision.
2.3.5 Inhibitory Concentrations

As it was mentioned before, during the analysis of Figure 2.5, our results suggest that analyte 8 is indeed better than analyte 3, but is not as good as 1. However, our attempts to independently obtain $K_D$ values for 8 through isothermal titration calorimetry (ITC) were unsuccessful due to precipitation issues in the concentration regimes needed for the experiment. Although ITC is the best validation method available, an alternative to compare the binding constants of Probe A, Probe B, and analyte 8 is to evaluate affinities through a comparative binding assay e.g. inhibitory concentration, or IC$_{50}$.\textsuperscript{27} It is possible to get such values via an hCA1 inhibition assay using 4-nitrophenyl acetate (NPA) as the substrate.\textsuperscript{28}

In an hCA1 inhibitory assay, it is the esterase activity of the protein that is evaluated. Since the active site is the same binding pocket targeted by the probes and analytes, a molecule that binds at that site could be potentially used as an inhibitor for hCA1. In the presence of hCA1, the ester bond in NPA is cleaved (Figure 2.9a); this event can be tracked by UV-Vis spectroscopy through observing the maximum absorbance of the $p$-nitrophenol ($\lambda_{abs} = 348$ nm), which is formed \textit{in situ}. So, hCA1 must be pre-incubated with the inhibitors and then exposed to the substrate, NPA. Figure 2.9 shows the inhibition profiles for Probe A, Probe B, and analyte 8.
The IC₅₀ is found based on the normalized maximum absorbance of the p-nitrophenol at a determined time of enzymatic action, in the presence of an inhibitor at different concentrations. Indeed, we found the IC₅₀ of 8 to be in the low micromolar region (0.10 μM), close to the IC₅₀ of Probe A (0.04 μM), although it was clearly a better hCA1 inhibitor than Probe B (1.37 μM). Figure 2.10 illustrates the plots of concentration of inhibitor against the percentage of hCA1 activity.
The analogues with shorter linkers than Probe B show a better IC$_{50}$. Although, for the analogue 8 and the Probe A the coumaryl moiety might be contributing to improve the affinity for hCA1, it could be that the less rigid nature of Probe B is diminishing its affinity for the target. The IC$_{50}$ values were measured in a Varian UV-Vis spectrophotometer CARY 100, following the procedures described in literature.

2.4 Summary

In summary, we have introduced a new approach to screen the binding of small molecules to proteins using a supramolecular displacement approach. Following are the noteworthy features of our approach: (i) a protein-specific ligand is attached to a fluorophore via an enzyme cleavable linker, which is chosen such that the cleavage results in the generation of a water-soluble, high quantum yield fluorophore,
umbelliferone; (ii) the linker length is chosen such that the enzyme-cleavable functional group is sterically masked from the enzyme, when bound to the protein; (iii) molecules with different binding affinities for the protein show different probe displacement profiles to expose the enzyme cleavable functionality and thus exhibit affinity-dependent fluorescence response; (iv) this approach can be rendered high throughput, as this is easily translated to a multi-well plate reader based fluorescence measurement; (v) a limitation of the approach is that one initial molecule with a reasonable binding affinity for the target protein must be known to successfully design the fluorescent probe and execute further ligand optimization. We envision that the design principles outlined here have the potential to be broadly adapted for rapid screening of small molecules against a protein target.

2.5 Experimental Section

2.5.1 Synthetic Procedures and Characterization of Compounds

All reagents and solvents were purchased from commercial sources and were used as received. $^1$H-NMR spectra were recorded on a 400 MHz Bruker spectrometer using residual proton resonance of the solvent as the internal standard. $^{13}$C-NMR spectra were recorded on a 100 MHz Bruker spectrometer. Fluorescence spectra were recorded using a JASCO FP-6500 spectrofluorimeter. FAB-MS spectra were measured on a JEOL JMS700. IR spectra were measured on a Bruker Alpha-P FT-IR.
2.5.1.1 General Synthesis of Azidoalkanoic Acids 10a,b

To a solution of bromo alkanoic acid 9a,b (1 equivalent) in DMF (15 mL) was added NaN\textsubscript{3} (3.0 equivalents). This reaction mixture was heated at 80 °C for 18 hours, stirring under argon atmosphere. After cooling to room temperature, most of the solvent was evaporated under vacuum. The crude material was mixed with water (50 mL) and extracted with ethyl acetate (3 x 50 mL). The organic layers were collected on Na\textsubscript{2}SO\textsubscript{4}. After filtration, the solvent was evaporated under vacuum to yield a yellowish oil that was used for the next step without further purification.

2.5.1.2 General Synthesis of Azidoalkanoic-Coupled 4-Methylumbelliferone 12a,b

In a two-necked round bottom flask, 4-methylumbelliferone 11 (1.2 equivalents) and dibromomethane (2.4 equivalents) were mixed and stirred in acetone (50 mL), with K\textsubscript{2}CO\textsubscript{3} (2.2 equivalents) and 18-crown-6 (0.2 equivalents) under argon atmosphere at reflux for 1 hour. Then, the azidoalkanoic acid 10a,b was added (1 equivalent) with extra dibromomethane (1.2 equivalents). The reaction mixture was refluxed for 18 hours. Then, the reaction crude was filtered and washed with acetone. The filtrates were collected and the solvent was evaporated. The crude was then poured into water and extracted with ethyl acetate (5 x 100 mL). The organic layers were dried over anhydrous Na\textsubscript{2}SO\textsubscript{4}, filtered, and concentrated in vacuo. The product was obtained after purification using Combiflash chromatography.
2.5.1.3 General Amide Coupling Reaction to get Probes a,b and Analyte 8

A mixture of 4-carboxybenzenesulfonylamine (1.5 equivalents) and carbonyldiimidazole (CDI) (1.8 equivalents) in anhydrous DMF was stirred for 20 minutes under argon atmosphere. Then, the resultant solution was added to a solution of 4-methylcoumarin-7-oxybenzene aminoalkanoate (1 equivalent) in anhydrous DMF stirring under argon atmosphere at 50°C. After 12 hours, the reaction was cooled to room temperature and the solvent was vacuum evaporated. Then, the crude was mixed with aqueous solution of sodium bicarbonate to be extracted with ethyl acetate with 1% methanol. The organic layers were dried over anhydrous Na$_2$SO$_4$, filtered and concentrated. The remaining crude solid was washed with acetone to obtain the product as the insoluble solid.

2.5.1.4 Synthesis of 4-Methylcoumarin-7-oxybenzene 6-Azidohexanoate 12a (DA-2-65)

According to the general procedure described above for coupling of umbelliferone with azidoalkanoic acids, the reaction of 4-methylumbelliferone 11 (4.71 g, 27.0 mmol), dibromomethane (13.94 g, 80.0 mmol), 6-azidohexanoic acid 10a (3.50 g, 22.3 mmol), K$_2$CO$_3$ (6.77 g, 50.0 mmol), and 18-crown-6 (1.18 g, 4.5 mmol) in acetone, yielded 1.98 g of a white solid 12a (35% yield). $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 7.56 (d, $J = 8.7$ Hz, 1H, b), 7.03 (d, $J = 2.1$ Hz, 1H, d), 6.99 (dd, $J = 8.7, 2.3$ Hz, 1H, c), 6.22 (s, 1H, a),
5.84 (s, 2H, e), 3.25 (t, \( J = 6.8 \text{ Hz} \), 2H, j), 2.44 (s, 3H, k), 2.42 (t, \( J = 7.5 \text{ Hz} \), 2H, f), 1.69 (tt, \( J = 7.4 \text{ Hz} \), 2H, i), 1.65-1.52 (m, 2H, g), 1.47-1.36 (m, 2H, h). \( ^{13} \text{C NMR (100 MHz, CDCl}_3 \) \( \delta \) 172.2, 161.1, 159.6, 155.2, 152.4, 125.9, 115.3, 113.3, 113.1, 103.5, 84.8, 51.3, 34.0, 28.6, 26.2, 24.3, 18.8. FAB-MS m/z calculated for \( \text{C}_{17}\text{H}_{19}\text{N}_3\text{O}_5 \) + H\(^+\): 346.3; found: 346.2.

### 2.5.1.5 Synthesis of 4-Methylcoumarin-7-oxymethyl 10-Azidodecanoate 12b

![Chemical Structure](image)

According to the general procedure described above for coupling of umbelliferone with azidoalkanoic acids, the reaction of 4-methylumbelliferone 11 (2.97 g, 16.9 mmol), dibromomethane (8.76 g, 50.4 mmol), 10-azidodecanoic acid 10b (3 g, 14.1 mmol), \( \text{K}_2\text{CO}_3 \) (4.29 g, 31.0 mmol), and 18-crown-6 (0.74 g, 2.8 mmol) in acetone, yielded 1.85 g of a white solid 12b (33% yield). \( ^{1} \text{H NMR (400 MHz, CDCl}_3 \) \( \delta \) 7.52 (d, \( J = 8.6 \text{ Hz} \), 1H, b), 6.99 (d, \( J = 2.3 \text{ Hz} \), 1H, d), 6.95 (dd, \( J = 8.6 \text{ Hz}, 2.4 \text{ Hz} \), 1H, c), 6.16 (s, 1H, a), 5.80 (s, 2H, e), 3.22 (t, \( J = 8.8 \text{ Hz} \), 2H, p), 2.39 (s, 3H, k), 2.38-2.32 (m, 2H, f), 1.65-1.54 (m, 4H, g-h), 1.45-1.15 (m, 10H, i-n). \( ^{13} \text{C NMR (100 MHz, CDCl}_3 \) \( \delta \) 171.4, 160.4, 154.0, 153.1, 152.1, 125.4, 118.1, 114.3, 110.3, 51.3, 34.1, 28.7 (5C), 26.4, 24.5, 18.6. IR-FT (cm\(^{-1}\)) 2917.2, 2849.1, 2085.0, 1707.6, 1262.1, 1128.1, 960.0, 842.5, 448.6.
2.5.1.6 Synthesis of 4-Methylcoumarin-7-oxyethyl 6-Aminohexanoate 13a (DA-2-121)

The 4-methylcoumarin-7-oxyethyl 6-azidohexanoate 12a (2.0 g, 5.8 mmol) was dissolved in anhydrous THF and then, triphenyl phosphine (1.82 g, 7.0 mmol) was added. The reaction was stirred under argon atmosphere for 5 hours at room temperature. Afterwards, water (1 mL) was added to the reaction and the temperature was increased to 50°C. After 5 hours, the reaction mixture was poured into water and extracted three times with dichloromethane. The organic layers were dried over anhydrous Na$_2$SO$_4$, filtered, and the solvent was evaporated. The product was purified by silica flash column on Combiflash to obtain 240 mg (15% yield) of a white solid 13a.

$^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 7.53 (d, $J = 8.7$ Hz, 1H, b), 6.99 (d, $J = 2.5$ Hz, 1H, d), 6.96 (dd, $J = 8.5$, 2.5 Hz, 1H, c), 6.18 (s, 1H, a), 5.99 (bs, 2H, q), 5.80 (s, 2H, e), 2.49-2.44 (m, 2H, j), 2.40 (s, 3H, k), 2.39-2.33 (m, 2H, f), 1.79-1.72 (m, 2H, g), 1.72-1.59 (m, 4H, h-i). $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 172.7, 162.1, 159.7, 155.0, 153.6, 126.0, 115.4, 113.1, 111.0, 103.5, 84.9, 43.2, 36.6, 34.1, 29.9, 23.4, 18.7. FAB-MS m/z calculated for C$_{17}$H$_{21}$NO$_5$ + H$: 320.3; found: 320.1.
2.5.1.7 Synthesis of 4-Methylcoumarin-7-oxymethyl 10-Aminodecanoate 13b (DA-2-50)

The 4-methylcoumarin-7-oxymethyl 10-azidodecanoate 12b (0.70 g, 1.75 mmol) was dissolved in ethanol (15 mL) and then, zinc (0.15 g, 2.27 mmol) was added to the mixture. An aqueous solution (5 mL) of ammonium chloride (0.22 g, 4.01 mmol) was added to the reaction mixture and then, it was refluxed for 30 minutes. The reaction was stopped and the ethanol was evaporated in vacuo. More water was added to the remaining mass and then extracted with ethyl acetate (3 x 20 mL). The organic layers were dried over anhydrous Na$_2$SO$_4$, filtered, concentrated, and purified by column chromatography. The product was recovered as 0.16 g of a white solid 13b (25% yield).

$^1$H NMR (400 MHz, MeOD) δ 7.75 (d, $J$ = 8.8 Hz, 1H, b), 7.09 (dd, $J$ = 8.8, 2.5 Hz, 1H, c), 7.05 (d, $J$ = 2.4 Hz, 1H, d), 6.24 (s, 1H, a), 5.89 (s, 2H, e), 2.90 (t, $J$ = 7.8 Hz, 2H, p), 2.47 (s, 3H, k), 2.39 (t, $J$ = 7.2 Hz, 2H, f), 1.64-1.58 (m, 4H, g,n), 1.40-1.27 (m, 10H, i-m), 1.24 (s, 2H). $^{13}$C NMR (100 MHz, MeOD) δ 175.3, 160.5, 159.0, 156.0, 154.1, 131.2, 115.1, 114.4, 109.1, 101.1, 85.6, 42.3, 35.3, 34.4, 30.1, 30.0, 29.9, 27.0, 19.6. FT-IR (cm$^{-1}$) 2917.2, 2849.1, 1724.1, 1614.8, 1262.1, 1128.1, 960.0, 842.5, 448.6. FAB-MS m/z calculated for C$_{21}$H$_{29}$NO$_5$ + H$: 375.5; found: 375.2.
2.5.1.8 Synthesis of 4-Methylcoumarin-7-oxy methyl 6-(4-Sulfamoyl benzamido)hex anoate, Probe A

According to the general procedure described above for the reaction of 4-methylcoumarin-7-oxy methyl 6-aminohex anoate 13a (0.16 g, 0.50 mmol) with 4-carboxybenzenesulfonamide 14 (0.15 g, 0.75 mmol) and CDI (0.15 g, 0.90 mmol) in DMF, 0.10 g of a white solid were obtained as the product Probe A (40%). $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 8.58 (t, $J = 5.4$ Hz, 1H, q), 7.96 (d, $J = 8.5$ Hz, 2H, x), 7.88 (d, $J = 8.3$ Hz, 2H, y), 7.73 (d, $J = 8.8$ Hz, 1H, b), 7.47 (s, 2H, z), 7.12 (d, $J = 2.4$ Hz, 1H, d), 7.06 (dd, $J = 8.7$, 2.5 Hz, 1H, c), 6.27 (s, 1H, a), 5.89 (s, 2H, e), 3.19 (dd, $J = 13.0$, 6.6 Hz, 2H, j), 2.40 (s, 3H, k), 2.39 (t, $J = 8.0$ Hz, 2H, f), 1.61-1.51 (m, 2H, g), 1.52-1.42 (m, 2H, i), 1.32-1.21 (m, 2H, h). $^{13}$C NMR (100 MHz, DMSO-$d_6$) $\delta$ 172.5, 165.7, 160.4, 159.6, 154.9, 153.9, 146.6, 138.1, 128.2, 127.4, 126.2, 115.1, 113.5, 112.6, 103.6, 85.0, 33.7, 29.2, 26.3, 24.5, 18.7. FAB-MS m/z calculated for C$_{24}$H$_{26}$N$_2$O$_8$S + H$^+$: 503.5; found: 503.2.

2.5.1.9 Synthesis of 4-Methylcoumarin-7-oxy methyl 10-(4-Sulfamoyl benzamido)decanoate, Probe B (DA-2-70)
According to the general procedure described above for the reaction of 4-methylcoumarin-7-oxymethyl 10-aminodecanoate 13b (0.16 g, 0.43 mmol) with 4-carboxybenzenesulfonylamine 14 (0.13 g, 0.65 mmol) and CDI (0.13 g, 0.78 mmol) in DMF, 0.09 g of a white solid were obtained as the product Probe B (38%). $^1$H NMR (400 MHz, DMSO-d6) δ 8.60 (t, $J = 5.0$ Hz, 1H, q), 7.97 (d, $J = 8.5$ Hz, 2H, x), 7.88 (d, $J = 8.2$ Hz, 2H, y), 7.74 (d, $J = 8.7$ Hz, 1H, b), 7.47 (s, 2H, z), 7.11 (d, $J = 2.4$ Hz, 1H, d), 7.07 (dd, $J = 8.7$, 2.5 Hz, 1H, c), 6.28 (d, $J = 1.1$ Hz, 1H, a), 5.89 (s, 2H, e), 3.23 (td, $J = 8.0$, 4.0 Hz, 2H, p), 2.40 (s, 3H, k), 2.36 (t, $J = 7.1$ Hz, 2H, f), 1.51-1.44 (m, 4H, g, n), 1.28-1.09 (m, 10H, h-m).

$^{13}$C NMR (100 MHz, DMSO-d6) δ 171.9, 165.0, 160.1, 158.9, 154.5, 153.4, 146.4, 137.5, 127.8, 126.81, 125.6, 114.8, 112.9, 112.2, 103.1, 84.3, 33.3, 28.6, 28.2, 26.6, 24.2, 18.1, 7.5. FAB-MS m/z calculated for C$_{28}$H$_{34}$N$_2$O$_8$S + H$^+$: 559.6; found: 559.3.

2.5.1.10 Synthesis of tert-butyl (6-((4-Methylcoumarin-7-yl)oxy)hexyl)carbamate 16 (DA-2-52)

The compound 16 was obtained from the O-alkylation of 4-methylumbelliferone 11 with the commercially available N-Boc protected 6-bromohexylamine 15 using K$_2$CO$_3$ (2 equivalents) as the base, and 18-crown-6 (0.2 equivalents) as the catalyst, refluxing in acetone for 13 hours (43% yield). C$_{21}$H$_{29}$NO$_5$, $^1$H NMR (400 MHz, CDCl$_3$) δ 7.48 (d, $J = 8.8$ Hz, 1H, b), 6.84 (dd, $J = 8.8$, 2.5 Hz, 1H, c), 6.79 (d, $J = 2.4$ Hz, 1H, d), 6.12 (sd, $J = 1.1$ Hz, 1H, a), 4.53 (bs, 1H, q), 4.00 (t, $J = 6.4$ Hz, 2H, e), 3.13 (dd, $J = 12.2$, 6.0 Hz, 2H, j), 2.39 (s,
3H, k), 1.90-1.74 (m, 2H, f), 1.59-1.33 (m, 15H, g-i, w). FAB-MS m/z calculated for 
C_{21}H_{29}NO_5 + H^+: 375.5; found: 375.2.

2.5.1.11 Synthesis of the Analyte 8 (DA-2-64)

After deprotection of the amine functionality in 16 with trifluoroacetic acid in 
dichloromethane at room temperature, the mixture was concentrated in vacuo and the 
remaining dry oil 17 was dissolved in anhydrous DMF and coupled with 4- 
carboxybezenesulfonamide 14 in presence of CDI following the procedure for the 
general synthesis of the probes (n=4, n=8). The product 8 was obtained as a white solid 
(7%). \(^1\)H NMR (400 MHz, MeOD) δ 7.96 (d, J = 8.0 Hz, 2H, x), 7.95 (d, J = 8.0 Hz, 2H, y), 
7.66 (d, J = 8.8 Hz, 1H, b), 6.93 (dd, J = 8.8, 2.4 Hz, 1H, c), 6.89 (d, J = 2.4 Hz, 1H, d), 6.16 
(d, J = 1.1 Hz, 1H, a), 4.09 (t, J = 6.3 Hz, 2H, e), 3.42 (t, 2H, j), 2.45 (s, 3H, k), 1.92-1.79 (m, 
2H, f), 1.74-1.64 (m, 2H, i), 1.64-1.54 (m, 2H, g), 1.54-1.44 (m, 2H, h). \(^{13}\)C NMR (100 MHz, 
DMSO-d6) δ 165.1, 162.0, 160.4, 154.9, 153.5, 146.2, 137.7, 128.0, 126.5, 125.7, 113.1, 
112.5, 111.1, 101.3, 68.4, 29.1, 28.5, 26.3, 25.3, 18.3. FAB-MS m/z calculated for 
C_{23}H_{26}N_2O_6S + H^+: 459.5; found: 459.2.
2.5.2. Protocols for Screening Experiments

2.5.2.1 Protocol for Screening of Analytes

A stock solution of the Probe (1.0 x 10^{-2} M) was prepared in DMSO and then 5 µL of this stock was dissolved in 10 mL of HEPES buffer (50 mM, pH 7.4). 1 mL of this solution was transferred to a cuvette. A solution of hCA1 (5.0 x 10^{-4} M) was prepared and 40 µL of it were mixed with the 1 mL Probe solution in the cuvette, leaving the final solution to stir gently for the pre-incubation time (15 min - 5 h). The concentrations of Probe and hCA1 in the cuvette are 5 and 20 µM respectively.

A solution of the esterase (PLE, 5.0 x 10^{-5} M) was prepared and 1 µL of this solution was transferred to the cuvette with the pre-incubated Probe and hCA1. A fluorescence spectrum at this time showed no increase in the intensity before and after exposure to the esterase. The concentration of PLE in the cuvette was 50 nM.

Stock solutions of the analytes (1 x 10^{-2} M) were prepared in DMSO. For a given analyte, 100 µL of its stock were diluted to 1 mL with the buffer and mixed well. Then, 5 µL of this analyte solution were transferred to the cuvette with the solution of Probe, hCA1, and PLE. After two minutes, 5 µL more of the analyte solution are added. The same volume of the analyte solution is added every two minutes until the end of the experiment. The evolution of the experiment was tracked by fluorimetry during 35 to 50 minutes, depending on the affinity of the analyte for the target protein. The increasing concentration of the analyte in the cuvette generates a fluorescence profile with time (sigmoidal shape), depending on the affinity of the analyte for the target protein.
2.5.2.2 Protocol for Screening of Analytes in a 96 Well Plate Reader

The protocol for the plate reader experiments follows the same principles applied in the cuvette based assay. From a stock solution of the Probe (1.0 x 10^{-2} M) in DMSO 5.0 µL were dissolved in 10 mL of HEPES buffer (50 mM, pH 7.4). To a different vial 6.0 mL of the Probe solution 5.0 µM were transferred and hCA1 was added until reaching the concentration of 20 µM. This solution was left stirring gently to pre-incubate (15 min to 1 h).

Solutions of 2.0 x 10^{-3} M of the analytes were prepared. 400 µL of the previous analyte solutions were diluted to 2.0 mL with buffer solution. A PLE solution (10 mL, 5 µM) was also prepared.

The wells in the plate are set with 200 µL of the pre-incubating solution. 2 µL of the PLE solution were added to the wells plate and their fluorescence were recorded. Then, 1 µL of the analyte solutions were added every two minutes and the evolution of the experiment was followed by fluorimetry.

2.5.2.3 Criteria Used for Calculating the Slope

Once the displacement-mediated activity profiles were generated, the data involved in the linear increment in fluorescence were selected to analyze the difference in binding affinity. Out of this selected data, only the data points with a higher value than the inflection point were selected to calculate the slope of the curve, trying to avoid any incidence of the excess hCA1 binding.
2.5.2.4 Finding Optimal Pre-incubation Time

A solution of Probe A (5 µM) in buffer (HEPES 50 mM, pH 7.4) was pre-incubated with hCA1 (20 µM) for different times (15 min, 1 h, and 5 h). Then, the solutions were exposed to PLE (50 nM) and the fluorescence spectra were registered. The evolution of the emission intensities with time were plotted in order to find the minimal time that is required for the pre-incubation of probe with hCA1 (Figure 2.4).

2.5.2.5 Enzymatic activity of PLE in Presence of the Analytes 1-8

To a cuvette, with 1 mL solution of Probe (5 µM) and analyte (1-8) (40 µM) were added 1 µL of PLE (50 µM) and the evolution of fluorescence (λ_{exc}: 365 nm, λ_{em}: 445 nm) was measured every minute for 7 minutes and after 20 to 50 minutes. The enzymatic activity of the PLE was not affected by any of the analytes as is shown in Figure 2.6b. A curve from a control experiment just exposing Probe A to PLE in the same concentrations as before is also shown.

2.6 References

Notes


27 Cheng, Y-C.; Prusoff, W. H. “Relationship between the inhibition constant (\(K_i\)) and the concentration of inhibitor which causes 50 per cent inhibition (\(I_{50}\)) of an enzymatic reaction”. Biochem. Pharmacol. 1973, 22, 3099-3108.


CHAPTER 3

PROTEIN-TRIGGERED SUPRAMOLECULAR DISASSEMBLY OF DENDRITIC AGGREGATES

BASED ON LIGAND LOCATION

3.1 Introduction

Host systems that can spontaneously self-assemble and stably encapsulate guest molecules under one set of conditions, but disassemble and release the guest molecules when external conditions change, have been of great interest in supramolecular chemistry due to implications in a variety of biomedical applications.\(^1\)\(^2\)\(^3\)\(^4\) While earlier studies have primarily focused on properties observed initially, i.e. self-assembly and binding, there has been a recent surge in interest in the latter features i.e. disassembly and release in response to an environmental change.

The responsiveness of the assemblies has primarily focused on physical or chemical changes. For example, there has been a significant interest in systems that disassemble in response to chemical stimuli such as pH\(^5\)\(^6\) or redox\(^7\)\(^8\) variations, as well as physical stimuli such as light,\(^9\)\(^10\) temperature,\(^11\)\(^12\)\(^13\) or a magnetic field.\(^14\)\(^15\) While systems incorporating features sensitive to light and magnetic field have been designed to respond to external triggers for biological applications, pH- and redox- responsive systems have been designed to respond to the inherent imbalances observed in certain diseased tissues. The anomalous pH or redox conditions in disease locations can be considered to be secondary imbalances, as the primary imbalances in biology are the result of aberrant protein concentrations or enzymatic activity.\(^16\)\(^17\)\(^18\) Therefore, there is
a growing interest in rendering supramolecular systems that respond to these primary factors in biology.\textsuperscript{19,20,21,22,23,24,25,26}

Protein-responsive systems can be broadly classified into two categories, \textit{viz.} covalent modification of the hosts to disable their capacity to hold the guest molecules and non-covalent modification of the host assemblies to produce the same effect.\textsuperscript{27,28,29} The former is often achieved by an enzyme-driven chemical reaction that modulates the host characteristics of the molecule; in the latter case, this is achieved due to a non-covalent binding interaction. While there have been several systems designed to be degradable or covalently modified by enzymes,\textsuperscript{30} supramolecular assemblies that lose their host capacities due to non-covalent binding with proteins have been limited.

Among the systems that undergo binding induced disassembly, polyelectrolyte assemblies that can non-covalently bind a complementarily charged surface to cause a disassembly have attracted some attention.\textsuperscript{31,32,33,34} Although non-specific in its interaction, the simplicity of these systems has proven useful in applications such as specific separations of peptides.\textsuperscript{35,36} It has also been clear that for a binding induced approach to be useful in applications such as delivery and sensing, where specificity is critical, strategies that use specific ligand-protein interactions are needed.\textsuperscript{37,38,39} Since dendritic macromolecules can be produced in high molecular weights, but with a great degree of control, these scaffolds have certain unique advantages for this strategy.\textsuperscript{40,41} For example, the critical aggregate concentrations of the dendrimer-based amphiphilic assemblies are low, an advantage that polymeric systems have over small molecule-based amphiphilic assemblies. Similarly, the control over functional group placements in
dendritic architectures captures the advantage that small molecules have over their polymeric counterparts.\textsuperscript{42}

The features mentioned above allow for structure-property relationship studies that unravel the factors that underlie the binding induced supramolecular disassembly process. In this chapter, we take advantage of this unique feature by incorporating specific locations within facially amphiphilic dendrons and interrogating the efficiency of supramolecular disassembly and molecular release in response to a specific protein binding event. We use both experiments and some of the molecular dynamics (MD) simulations developed by our collaborators to gain insights into the ligand-protein recognition based disassembly event.\textsuperscript{43}

3.2 Approach and Molecular Design

Facially amphiphilic dendrons, containing a biaryl-based internal repeat unit and an aryl peripheral unit, both consist of hydrophilic and hydrophobic functional groups as side chains in each of these repeat units.\textsuperscript{44,45} The amphiphilic functional groups are placed at opposite faces of the biaryl backbone of the dendron such that these molecules are endowed with the capability to form micelle-like assemblies in aqueous phase and form inverse micelle-like assemblies in apolar solvents.\textsuperscript{46,47} In preliminary findings from our lab, it has been shown that placing a ligand moiety at the hydrophilic face of the dendron can provide binding-induced disassembly of the micelle-like structure in the aqueous phase.\textsuperscript{37} In that work, the ligand moiety was placed at the focal point of the dendron, as this structure is synthetically most easily accessible. However,
the release efficiency of guests following to protein binding was relatively low reaching 43% for G1 and G2. In order to fully realize the potential of this process, it is critical that we understand the effect of the functional group placement, hence taking advantage of one of the most critical advantages of dendritic architectures in this supramolecular process. Thus, in this chapter, we investigate the effect of the placement of a protein-specific ligand moiety at specific positions of a dendron upon the accessibility of the complementary protein to the supramolecular assembly, and hence the effects on the concomitant guest release response as it has been represented in Figure 3.1.

The CAC of even the first generation of this kind of dendrons is substantially lower than the corresponding amphiphilic small molecule (µM compared to mM). Within each generation of dendrons however, the CAC gain has been relatively small, if
any, especially when one accounts for the difference in molecular weights. Therefore, here we focus on the G1 and G2 dendrons, which provide sufficient variations in the functional group placement. We targeted five different dendrons within these two generations, where the ligand placement is the key difference as it can be appreciated from the structures in Figure 3.1. Pentaethyleneglycol monomethylether (PEG) was chosen as the hydrophilic moiety not only for its ability to provide the macromolecules and the nanoassembly with the requisite solubility in the aqueous phase, but also for its propensity to present a charge neutral surface on the assembly that exhibits minimal non-specific interactions. Decyl moieties were incorporated as the hydrophobic components of the amphiphile. Biotin was chosen as the ligand, because of its well-established high affinity towards avidin.
As it is shown in Figure 3.2, a ligand can be incorporated on a G1 dendron either at the focal point or at the periphery. On a G2 dendron, a ligand can be incorporated on three different layers: (i) the focal point; (ii) the middle layer; and (iii) the periphery. To install a single ligand at a specific place, one of the PEG units was replaced by the ligand, attaching it to the dendron backbone via a hydrophilic linker. This placement allows the ligand to be exposed to the aqueous solution on the hydrophilic face of the assembly.

Note from Figure 3.2 that the focal point is synthetically the easiest place to attach a single functional group, since the focal point is a single and unique position in a dendron. However, to install a single functional group at the periphery, it is necessary to distinguish one of the peripheral monomers from otherwise identical positions within the dendron.

The G1 dendron has two such identical positions in the periphery, while the G2 dendron has four such locations. Similarly, the middle layer of the G2 dendron contains two similar positions that need to be distinguished for specifically placing a ligand moiety. Accordingly, the degree of difficulty in placing a unique functional group in the dendron increases as we move from the focal point to the periphery and as we increase the generation. To achieve these selective functionalization, we and others have developed a variety of synthetic strategies that afford multi-functionalized dendrons and dendrimers.42,51,52,53
3.3 Results and Discussion

3.3.1 Synthetic Strategy

In all our syntheses, we initially targeted dendrons containing an acetylene moiety, which will be used as the handle to “click” the biotin moiety in the last steps of the syntheses. Overall, we made the syntheses of the dendrons modular in order to assemble the targeted dendrons in a small number of steps. Accordingly, we first synthesized the biaryl protected AB₂ monomer 7 (Scheme 3.1), the propargyl-functionalized periphery unit 12, and the non-functionalized amphiphilic periphery unit 13 (Scheme 3.2) following the previously reported procedures.


Scheme 3.1 shows the synthesis of the protected AB₂ monomer 7. First, from commercially available 3,5-dimethoxybromobenzene 1 involving (i) deprotection of the phenolic hydroxyl groups from the methyl substituents, (ii) treatment of the phenolic
hydroxyls with MOM-Cl to introduce a protecting group easier to handle in 3 and, (iii) conversion of the bromo functionality to a stannane 5. The bottom aryl ring 6 was obtained from 4-bromo-3,5-dihydroxybromobenzoic acid 2. This involves, first, esterification of the acid followed by mono-O-alkylation of a phenolic hydroxyl by means of treatment with \( n \)-bromodecane in the presence of potassium carbonate and 18-crown-6 to get 4. Secondly, protection of the remaining hydroxyl by means of an O-acetylation to get 6. The stille coupling between the aryl stannane 5 and the arylbromide 6, followed by deprotection of the acetyl-protected hydroxyl and the reduction of the ester moiety, was accomplished in one step to generate the benzyl alcohol and biaryl AB\(_2\) monomer 7.

Scheme 3.2 shows the synthesis of periphery containing an alkyne moiety 10. First, mono O-alkylating 3,5-dihydroxybenzyl alcohol 8 using \( n \)-bromodecane in the presence of potassium carbonate and 18-crown-6 to get 9. The mono alkylated compound 9 could be further treated with propargyl bromide using the same conditions of O-alkylation than before to yield the propargyl bearing periphery 10. Compound 9 could also be treated with PEG-Ts in the same conditions to get amphiphilic periphery 11. The resultant hydroxymethyl compounds 10 and 11 were converted to the corresponding bromides 12 and 13 respectively, using phosphorus tribromide.
Scheme 3.2. Synthesis of periphery bearing either propargyl or PEG.

Scheme 3.3. Synthesis of biotin azide 18.

Scheme 3.4. AB₂ monomer.
In order to attach the biotin ligand to the dendrons through click chemistry, not only the installation of a propargyl unit on the dendrons structure is imperative, but also the functionalization of the ligand with a pendant azide. Scheme 3.3 shows such biotin-azide preparation. Briefly, it involves the amine-protection of 2-(2-aminoethoxy)ethanol 14 to get 15, which is followed by mesylation of the alcohol moiety and then, treatment with sodium azide to obtain the azide-compound 16. Next, the amine functionality of 16 was de-protected upon acidic treatment to get the amino-azide 17, which is coupled with biotin as a final step to the biotin azide 18.

To achieve the targeted dendrons G1 and G2 (see Figure 3.1 for structures), it is imperative to deprotect and further functionalize the AB₂ molecule 7. In Scheme 3.4 the functionalization of 7 is accomplished in two different ways, (i) using propargyl bromide and (ii) using PEG-Ts in the conditions for alkylation described above.

3.3.1.1 Synthesis of Final G1 Dendrons

To achieve G₁-P, the biaryl monomer 20 was first mono-alkylated with a periphery unit 12 to get the propargyl-functionalized scaffold 21 (Scheme 3.5) in 25% yield. The low isolated yield was because of the statistical distribution mono- and di-substituted products obtained in this reaction. It is worth to mention that although the di-substituted product has no further use in our synthetic design, the initially attempted mono-substitution of monomer 20 with periphery 13 generated also a di-substituted byproduct, which could have been used for the synthesis of G2 dendrons. However, the
isolation of such mono-alkylated product from the di-alkylated byproduct could not be accomplished due to their similar polarities and close retardation factors ($R_f$).

Molecule 21 was then treated with the amphiphilic peripheral monomer 13 under similar alkylation conditions to achieve the G1 dendron 22 containing the reactive propargyl moiety at its periphery. The G1 dendron 22 was then treated with the azide modified biotin 18 under click chemistry conditions\textsuperscript{54,55,56,57} in presence of copper(II) sulfate and sodium ascorbate to obtain the final G1 dendron with a single biotin moiety installed at the periphery G1-P in 70% yield.

\textbf{Scheme 3.5.} Synthesis of G1-P and installment of the ligand at the periphery.

Similarly, as shown in Scheme 3.6, the molecule G1-F was synthesized starting from the monomer 19, which was dialkylated with periphery units 13 to get the dendron 23 in a single step. Notice the difference in yields with the two steps required to get dendron 22. Then, by clicking the biotin azide 18 to the propargyl moiety present
in the biaryl repeat unit of 23, we got the G1 dendron with a single biotin moiety installed at the focal point G1-F.

![Scheme 3.6. Syntesis of G1-F.](image)

3.3.1.2 Synthesis of Final G2 Dendrons

Before the next steps towards the targeted G2 dendrons, the hydroxymethyl moieties of G1 dendrons 22 and 23 were transformed into the corresponding bromomethyl fragments 24 and 25 respectively, which are shown in Figure 3.3. These substitutions were accomplished using phosphorus tribromide in the same conditions that rendered the periphery amphiphiles 12 and 13 (Scheme 3.2). In the same way, the dendron obtained from dialkylation of the monomer 20 with two periphery units 13 is rendered into its bromomethyl derivative 26.
The syntheses of the targeted G2 dendrons were achieved using a similar set of synthetic strategies than for G1-P and G1-F, as shown in Scheme 3.7. To achieve the syntheses of these dendrons, the previously described amphiphilic G1 dendron 26 was treated with the biaryl monomer 20, where the mono-alkylated product 27 was separated from a statistical mixture in 24% yield. In this case, although the isolation of the mono-substituted product from the di-substituted byproduct was the bottle neck of the entire synthesis, the byproduct can be used as a control dendron for the disassembly and release experiments. Also using a brominated dendron 24, which is more difficult to obtain than 26, to mono-substitute 20 produces a di-substituted byproduct that does not have any further use in our designed synthesis. Mono-substituting 20 with brominated dendron 25, which takes the same number of synthetic steps than 26, also generates a byproduct that has no further use in our synthetic design.

Molecule 27 was then treated with the bromomethyl dendron 25 or 24 to obtain the G2 dendrons 28 or 29, containing the propargyl moiety at the middle layer or the periphery of the dendron respectively. Copper-catalyzed Huisgen reaction of 28 and 29 with the azide-functionalized ligand 18 afforded the dendrons with a single biotin

Figure 3.3. Brominated G1 dendrons.
moiety installed at the middle layer **G2-M**, and a single biotin moiety installed at the periphery **G2-P**, respectively (Scheme 3.7).

In a similar fashion than with dendron **G1-F**, to get a single biotin unit installed at the focal point of a G2 dendron, **G2-F**, we started from the monomer 19, which was di-alkylated with the brominated G1 dendron 26 to get the G2 dendron 30, bearing a propargyl fragment at the focal point in a single step. Then, by clicking the biotin azide

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**Scheme 3.7.** Synthesis of **G2-M** and **G2-P**.
to the propargyl moiety present in 26, we got the G2 dendron with a single biotin moiety installed at the focal point G2-F. This synthesis is shown in Scheme 3.8.

Scheme 3.8. Synthesis of G2-F.

3.3.2 Self-Assembly and Determination of CACs of Dendrons

The self-assembly properties of the target dendrons G1-F, G1-P, G2-F, G2-M, and G2-P in water were studied before trying to understand their disassembly. For this purpose, Nile red was used as the hydrophobic spectroscopic probe. After encapsulation, the CACs of the dendrons were determined using the fluorescence intensity of Nile red (λ_em = 615 nm), as shown in Figure 3.4. Since Nile red is a hydrophobic small molecule it is not water soluble and precipitates if it is exposed directly to an aqueous environment, and hence its emission spectrum cannot be detected (λ_ex = 550 nm). However, once the dye is encapsulated its emission spectrum
can be easily acquired. Then, Nile red is dispersed in the aqueous solution once it is encapsulated in the hydrophobic pockets of the dendritic containers.

Initially, when the dendron concentration is high, the intensity of the emission spectrum of the dye is high. However, when the concentration of the dendron in solution decreases the intensity of the emission of Nile red also decreases. Given the same amount of dye, when the dendron concentration goes down drastically, less dendron hold less dye molecules, and the non-encapsulated small molecules precipitate out of the solution. Finally, when the dendron reaches the CAC the emission intensity of Nile red decreases to a value that remains more or less stable, indicating that most of the hydrophobic pockets in the aggregate have been lost and only those hydrophobic moieties in the disassembled dendrons can hold a few molecules of dye. Such fluorescence intensity is close to cero, since the container properties of the assembly have been lost.

Figure 3.4. CAC plots based on nile red fluorescence for dendron assemblies a) G1-F, b) G1-P, c) G2-F, d) G2-M, e) G2-P.
Then, for each dendron encapsulating Nile red, the fluorescence intensities at 615 nm were recorded every time that the dendron concentration was reduced. The intensity values were plotted against the concentration of dendron to get a CAC curve, which should be sigmoidal ideally. From the curve, the point where the best fitted horizontal and vertical lines merge was taken as the CAC. Table 3.1 shows the CACs and the corresponding dendrons.

From Figure 3.4 is evident that the dendrons have CACs with close values, since the CAC curves look very similar, and as it is reflected in Table 3.1. As anticipated, the CACs of final G1 and G2 dendrons were determined to be in the low micromolar range.

### 3.3.3 Disassembly of the Dendritic Aggregates

With the installment of a single ligand at different layers of a dendron, and after knowing the lowest concentration at which they assemble into nanostructures, we investigated the response of the different dendritic aggregates in presence of the complementary protein extravidin and in presence of non-complementary proteins with diverse pl values and molecular weights, viz. α-chymotrypsin (Chy, pl = 8.1-8.6), pepsin (Pep, pl = 2.9), and myoglobin (Myo, pl = 7.2).

Prior to analyzing the interaction between the dendritic assemblies and the proteins, we analyzed the size of the assemblies in aqueous phase using dynamic light
scattering (DLS), with G1 dendron concentrations of 12.5 µM and G2 dendron concentrations of 10 µM; both concentrations are above the respective CACs. The sizes of the assemblies were found to be in a few tens of nanometers, ranging from ~30 nm to ~200 nm, 44 nm for G1-F, 220 nm for G1-P, 29 nm for G2-F, 92 nm for G2-M, and 51 nm for G2-P. These initial aggregate sizes are represented as red lines in Figure 3.5. The reason for the variations in size with the subtle change in the position of the ligand is not clear. However, note that the replacement of a hydrophilic PEG chain in a G1 or G2 dendron by a less hydrophilic pendant biotin decreases the PEG density on the assembly surface, decreasing hydrophilicity and increasing the chance of non-specific interactions with non-complementary proteins. Such decrease in PEG density seemed to be more relevant in G1, where substituting one of the three PEG chains in a dendron molecule could mean a reduction of up to 33% in hydrophilicity, while in a G2 it could mean a

Figure 3.5. DLS-size change of dendritic assemblies in aqueous phase upon interaction with different proteins. a) G1-F, b) G1-P, c) G2-F, d) G2-M, e) G2-P.
reduction of up to 14%. It is possible that these differences confer changes in the way the assemblies pack together in solution, which results in size variations among the biotin functionalized dendrimeric assemblies.

It is interesting however that the size of all these assemblies reduced to about ~13 nm in presence of extravidin at 2 µM. A particular difference in disassembly among the biotin-functionalized dendrons was observed in the case of the G2-P that disassembled into smaller aggregates of around ~7-8 nm in presence of extravidin, as seen in Figure 3.5e. G1-P also presented some deviations, showing larger aggregates around ~28 nm. However, these larger aggregates could also be formed by further aggregation of smaller assemblies.

Figure 3.6. DLS-size unchanged of control dendritic assemblies in aqueous phase upon interaction with different proteins. a) G1 dendron, b) G2 dendron, c) proteins. The decrease in the size of the assemblies was observed only in presence of the complementary protein, extravidin. In the presence of the non-complementary proteins, no disassembly was observed, although a tendency of the biotin functionalized dendrimeric assemblies to increase size, forming larger aggregates was noticed for G1-F and G1-P, as shown in Figure 3.5a,b. This aggregate enlargement did not occur when control dendrons G1 at a concentration of 12.5 µM, and G2 at a concentration of 10 µM,
both with PEG replacing the ligand moiety, were exposed to the same proteins. Figure 3.6a,b shows such control dendrons. This suggested that biotin functionalization of dendrons was the reason for such aggregate enlargement. Figure 3.6c shows what could be the size of the proteins at a concentration of 20 µM, since the same proteins at 2 µM were undetected by DLS.

Although DLS, in volume percentage, shows disassembly for the biotin-
functionalized dendritic aggregates when they are exposed to extravidin, the same data also shows that not all the aggregates suffer disassembly. This is shown in Figure 3.5 as a small and broad peak around 100 nm for all the dendritic aggregates when they are exposed to extravidin (red lines). To have a better appreciation of what particles with different size remain in solution after exposure to extravidin, we must refer to the percentage of intensity curves in DLS as shown in Figure 3.7.

Briefly, DLS measures Brownian motion and relates this to the size of the particles. It gives information about the size of the particles in three different distributions: (i) the number distribution or number percentage, depending on the number of particles of each size. (ii) The volume distribution, which shows the volume occupied by the detected particles according to the volume of a sphere \( ((4/3) \pi r^3) \), which means that by volume distribution a particle of 50 nm (area of the peak) is 1000 times larger than a particle of 5 nm (1:1000 ratio). (iii) The intensity distribution, which makes the area of the peak at 50 nm 1000000 times larger than the area for the 5 nm particle (1:1000000), which is due to large particles scattering much more light than small particles according to Rayleigh’s approximation \( I \sim d^6 \); where \( I \) is the intensity of light scattered and \( d \) is the diameter of the particle).

Then, from Figure 3.7 it is certain that non-specific proteins do not trigger disassembly of our dendritic aggregates since the size of the assemblies is not reduced; on the contrary, aggregation that helps increasing the size of the particles could be happening. On the other hand, when the dendritic aggregates were exposed to extravidin, it is clear how the distribution by number shows the formation of particles of
size around 10 nm in every case, similar to the distribution by volume. However, from
the intensity distribution it is clear that after exposure to extravidin, there are still some
large particles that either did not suffer disassembly or aggregated from smaller
particulates.

3.3.4 Release of Encapsulated Guest Molecules

Next, we investigated the host capabilities of the dendrons in the presence and
absence of complementary and non-complementary proteins. Specifically, we were
interested in assessing the effect of incorporating the ligand moieties at different
locations within the dendrons upon the disassembly-induced guest release from the
dendron host. To investigate these differences, Nile red was encapsulated in the
micelle-like nanoassemblies and its release was triggered as a consequence of binding
induced disassembly upon exposure to extravidin. Nile red is a hydrophobic molecule
that exhibits reduced fluorescence in water, unless it is sequestered in a hydrophobic
pocket. Therefore, the reduction in fluorescence is a good indicator of the binding
induced disassembly event.
As shown in Figures 3.8a and 3.8b, small differences in placement of the ligand in the dendron produced rather different responses. First, percentage of dye released was assessed after exposing 25 µM solutions of the dendrons to increasing concentrations of extravidin. We noticed that G1-P and G2-P responded to increasing concentrations of extravidin more than other dendrons studied. To further evaluate this behavior, we monitored the release profiles over time for all the dendritic assemblies upon exposing these assemblies to 14 µM of extravidin as shown in Figure 3.8b. During the first hour, the release in the G2-P assembly was as high as 65%, while the release of the G1-P assembly was around 22% increasing to 40% after 3 hours. Interestingly, the release from the G1-P assembly ultimately reached about 77%, which is comparable with the
81% observed for G2-P. In comparison, similar exposures to extravidin resulted in 35%, 25%, and 13% for G1-F, G2-F, and G2-M respectively. Interestingly, the release in G2-M is comparable to the release percentages observed due to non-specific interactions.

As a control experiment, the release of the Nile red from the dendritic assemblies was also monitored in the absence of any protein, as shown in Figure 3.8c. No discernible release (<10%) was seen in these dendritic assemblies. Similarly, Figure 3.8d shows that control dendrons lacking the biotin ligand also did not exhibit appreciable dye release in the presence of extravidin. These results show that the release profiles observed in Figure 3.8a and 3.8b are indeed due to the ligand-protein binding. Moreover, it is clear that among the second generation dendrons, G2-P assembly is the only one releasing efficiently the hydrophobic guests following to extravidin binding.
To test the selectivity in the systems towards the target protein further, the biotin-functionalized dendrons were exposed to increasing concentrations of non-complementary proteins, Chy, Pep, and Myo monitoring the change in Nile red fluorescence (Figure 3.9a-c). No significant change in the emission intensity was observed for any of the dendrons in the presence of Chy and Pep. However, while Myo did not exhibit any change in the fluorescence intensity in the G1-P, G2-M and G2-P based assemblies, there was a significant change in fluorescence in the G1-F and G2-F based assemblies, as it was further tested exposing the assemblies to constant concentrations of non-complementary proteins and it is shown in section 3.5.2.

![Graphs showing fluorescence intensity changes](image-url)

**Figure 3.9.** Exposure of dendritic assemblies to increasing concentrations of a) Chy, b) Pep, c) Myo, and d) absorption based percentage of release upon interaction of dendrons with 14 µM of Myo.
Interestingly, these latter dendrons also exhibited much smaller release in response to extravidin, as it was previously shown in Figure 3.9b. It is noteworthy that Myo is a metalloprotein and therefore the co-factors in metalloproteins could be simply quenching the fluorescence of the dye molecule without the need for releasing the contents from the amphiphilic assembly. In fact, such a phenomenon has been previously observed with polymer-surfactant co-assemblies.\textsuperscript{33}

To test this possibility, we investigated the change in absorption spectrum for Nile red in the presence of Myo. If it is simply a quenching phenomenon, no change should be present in the absorption spectrum since all dye molecules are still confined in the amphiphilic assembly without being released. Indeed, we noted that there was no change in the absorption spectrum over time, suggesting that the observed phenomenon is due to excited state quenching of the fluorescent dye molecules, as shown in Figure 3.9d.

### 3.3.4.1 A Particular Case of Quenching of the Cargo

In this part, we were interested in gaining insights into the observed fluorescence reduction in the presence of Myo, especially for G1-F and G2-F. There are two limiting mechanisms by which such quenching could occur: (i) inherently different encapsulation stabilities among the dendritic assemblies, causing the dye to leak out of G1-F and G2-F and bind to the hydrophobic pockets in the protein, where the proximity between the metalloprotein cofactor and the dye molecule causes fluorescence
quenching; or (ii) the possible non-specific interactions between assemblies formed by G1-F, and G2-F with Myo favour quenching of the cargo.

To test the possibility (i), we used a recently reported polymeric nanogel that has been well-established to have crosslink-density dependent encapsulation stabilities. Nile red-encapsulated nanogels with 0%, 20%, and 50% crosslink densities were exposed to Myo. If this mechanism was operative, there should be a crosslink density dependent emission dependent quenching. In all cases, we found that the extent of quenching was quite independent of the crosslink density (Figure 3.10). The quenching however was found to increase with increasing concentration of the Myo, as observed with G1-F and G2-F assemblies. These results are taken to suggest that Myo itself is not capable of binding the Nile red molecule, because the 0% crosslinked nanogel assembly has been found to be quite leaky. This rules out mechanism (i) and makes mechanism (ii) the most likely possibility.

**Figure 3.10.** Encapsulation stability and quenching. a) Quenching of Nile red (NR) fluorescence encapsulated in nanogels (NG, 1 mg/mL) at different crosslink densities and concentrations of Myo, b) quenching after a single exposure to Myo (5.5 mg/mL).
Within the realm of quenching, it is also important to know whether the quenching is due to dynamic collisions or non-specific binding (a static quenching event). Since dynamic and static quenching differ in their temperature dependence, we exposed the assemblies G1-F and G2-F encapsulating Nile red, to increasing concentrations of Myo at two different temperatures. The number of collisions between the dendritic assemblies and the protein was expected to increase at a higher temperature in the case of dynamic quenching, leading to an increase in quenching. On the other hand, in the case of static quenching, the weak dendritic assembly–protein complex would dissociate at a higher temperature, leading to a decrease in quenching. Figure 3.11 shows the Stern–Volmer plots for G1-F and G2-F respectively at 25 °C and 38 °C. The fact that the quenching increases with temperature supports the weakly bound complex hypothesis. Thus, it is interesting to note that G1-F and G2-F exhibit higher non-specific interaction and at the same time do not exhibit significant release of the guest molecules in response to the target extravidin. On the other hand, the dendrons G1-P and G2-P that exhibited the highest release in response to extravidin did not exhibit any non-specific quenching with Myo.
This evidence of a weakly bound complex between G1-F or G2-F and Myo, supports the idea that, even though PEG is coating these nanoassemblies, thethering of biotin to the focal point of the dendrons is generating assemblies where the PEG shell is not efficiently preventing non-specific interactions.

Additionally, the release of nile red observed by absorption from G1-F assembly, shown in Figure 3.9d, reached up to 20% which is high for mere non-specific interactions. Thus, it could be explained as due to the formation of a weak complex assembly-quencher, since formation of a ground-state complex that generates static quenching can result in different extinction coefficients for those fluorophores encapsulated but distant from the quencher metalloprotein, and for those closer to the metalloprotein. This results in perturbation of the fluorophore absorption spectrum and hence, in a false release profile that supports the static quenching in the system.

The idea of having two populations of fluorophores encapsulated in the same assembly, one of them not accessible to the quencher, is in turn supported by the downward curvature towards the x-axis displayed in the Stern–Volmer plots in Figure 3.11. Quenching of cargo. a) Stern-Volmer plot for G1-F + NR at 25 °C and 38 °C vs increasing concentration of Myo, d) Stern-Volmer plot for G2-F + NR at 25 °C and 38 °C vs increasing concentration of Myo.
3.11, a phenomenon seen in proteins having some triptophan residues on the surface and some buried.\textsuperscript{61}

\textbf{3.3.5 Making Sense of Disassembly and Release}

These latter data and those from the previous sections indicate that if the release of hydrophobic guests is triggered by the specific extravidin-biotin interaction, the selective binding with the complementary protein can occur more easily when a biotin ligand is grafted at the periphery than when it is grafted at the middle layer or at the focal point of the dendron, and that somehow this makes the release of hydrophobic guests faster for G2-P than for the other constructs, as observed in Figure 3.8a,b. If disassembly is controlled by the same interaction, since the biotin-extravidin binding is considered irreversible, when extravidin binds to a dendron in the assembly a reduction in the size of assembly will occur after some time, while the system reorganizes and equilibrates in smaller size assemblies as it was observed by DLS (Figure 3.5). Thus, although all systems reorganized into smaller size assemblies, the release was higher for the dendron systems with a ligand located at the periphery. The reason for this disparity was not entirely clear to us.
Based on the release kinetics, which was also faster for the dendritic systems with ligand at the periphery, we hypothesized that in these cases the assembly reorganization was drastic enough to produce a higher release. On the other hand, in the cases with a ligand at the middle layer and focal point the assemblies rearranged slower into smaller size structures, allowing for the encapsulated hydrophobic small molecules to still be accommodated in hydrophobic pockets. In fact, Figure 3.8b shows that even after 6 hours of G2-M (25 µM) exposure to a constant concentration of extravidin (14 µM), the release was as low as 13%. At this point, the interesting question of why is the release from G2-P so high compared to the release from G2-M and G2-F remain still open.

Nevertheless, based on our data and analysis at a molecular level, we propose a hypothesis involving the approach of a tetrameric extravidin to the biotin ligand surrounded by a different PEG nano-environment in the different locations, as represented in Figure 3.12. When biotin is grafted at the middle layer (Figure 3.12b) or

![Figure 3.12](image-url)
at the focal point (Figure 3.12c) it is surrounded by an environment rich in PEG. This makes the specific binding withextravidin, which implies deep penetration of the ligand inside one of the four binding pockets of tetrameric extravidin, very difficult compared to **G2-P** (Figure 3.12a). We have employed molecular dynamics (MD) simulations, carried out by our collaborators,\textsuperscript{43} to gain additional insights in the understanding of why the different positioning of a biotin ligand within the dendron scaffold has such a strong effect on the final properties.

First, it was important to understand how these molecules fold in solution. In fact, it is known that similar dendritic structures can undergo strong folding in solution,\textsuperscript{62,63} so that if the biotin ligand is backfolded and surrounded by PEG in the experimental conditions the specific binding with extravidin will be unlikely.

The entire simulation work was carried out with the AMBER 12 suite of programs.\textsuperscript{64} Molecular models were created with three different functionalization points for biotin, to understand how the individual dendrons arrange in solution. **G2-P**, **G2-M** and **G2-F** dendron models were created and parameterized according to similar studies on dendrons interacting with proteins.\textsuperscript{65,66,67} Starting configurations of the dendrons were then immersed in a simulation box (Figure 3.13a) containing explicit water molecules. All systems underwent 200 ns of MD in periodic boundary conditions at 25 °C (298 K) of temperature and 1 atm of pressure. During this time, all dendrons reached the equilibrium with good stability. The root mean square displacement (RMSD) and the radius of gyration ($R_g$) data extracted from the MD simulations were used to assess the system’s equilibration.
The size of G2 in water predicted by MD simulation does not change substantially depending on the tethering position of biotin, i.e. the radius of gyration ($R_g$) in the three cases is $9.4 \pm 10 \text{ Å}$ (Figure 3.13a). In general, the dendrons tend to compact the hydrophobic decyl chains at the core, and to surround them with hydrophilic PEG. On the other hand, in terms of distribution of the biotin ligand the situation is different. The plots in Figure 3.13b report the radial distribution function (RDF) $g(r)$ of the biotin ligand calculated with respect to the dendrons center and expressed as a function of the dendron radius ($R_g$) for the cases where biotin is grafted at the periphery ($G2$-$P$: red), the middle layer ($G2$-$M$: blue), or at the focal point ($G2$-$F$: black). In general, the $g(r)$ values give indication on the relative probability to find the biotin ligand at a certain distance from the dendron center, being the position of the $g(r)$ maximum peak the most probable one.

The biotin density, going from the center to the surface, is calculated at each simulation step, and the reported $g(r)$ data are averaged in time over the equilibrated phase MD trajectories (the last 100 ns). Thus, high and sharp peaks in $g(r)$ identify high
biotin density regions, but they also indicate high localization, confinement and backfolding (namely, atoms that cannot move are counted at each step in the same region of space).

On the contrary, flexible and fluctuating groups will have low and broad $g(r)$ peaks. Figure 3.13b shows that at the equilibrium biotin distribution is very different for \textbf{G2-P}, \textbf{G2-M} and \textbf{G2-F}. In particular, the biotin $g(r)$ maximum peak for \textbf{G2-P} (red curve) corresponds well with its $R_g$ indicating that, on average, the ligand availability on the surface is very good. On the contrary, the maximum peaks of black and blue $g(r)$ curves at a distance $r \approx 0.5$ $R_g$ suggest that biotin is considerably more backfolded in the case of \textbf{G2-F} and \textbf{G2-M}. These data give indication on how much the biotin ligand is available at the surface of the dendron, and thus also on the probability to have a specific binding with extravidin.

The experimental biotin-avidin energy of binding is known to be -20.4 kcal mol$^{-1}$\cite{1,68,69,70}, however the dendron-extravidin affinity will be worse for \textbf{G2-F} and \textbf{G2-M} dendrons since biotin is backfolded for them, unlike in \textbf{G2-P}. From the experimental biotin-avidin affinity and the biotin availability at the dendrons surface, it was possible to calculate the relative probability for the dendron-avidin specific binding (statistical weight). So, if we set the probability of having extravidin specifically bound to the biotin moiety in \textbf{G2-P} to 1, then the probability of having extravidin bound to the biotin moiety in \textbf{G2-F} and \textbf{G2-M} is reduced to $\sim 0.1$ due to reduced ligand availability. This means that the probability of extravidin specific binding to biotin ligands at the surface of \textbf{G2-P} aggregates is ten times higher than in the case of \textbf{G2-F} and \textbf{G2-M}.
3.3.5.1 Multivalent Binding of Extravidin

Given the differences of ligand availability for the dendrons, our results suggest that when extravidin gets in contact with a biotin at the surface of a G2-P aggregate, the formation of a specific binding will be an energetically favored event. In addition, it is worth noting that extravidin is a protein tetramer possessing four binding sites for biotin. Thus, after a first specific binding occurs between extravidin and one biotin at the G2-P aggregate surface, the protein can find also other biotin ligands available in the neighborhood to establish more specific bindings in a cooperative way. This will be an energetically favored thus highly probable event,\textsuperscript{71,72} according to the so-called multivalent effect, and to the fact that extravidin is energetically favored to bind four biotins at the same time.

The consequences of this behavior can be important. In fact, this tendency of extravidin to bind more biotin ligands at the same time, when available, can in principle speed up the disassembly process. Conceptually, since extravidin will tend to preserve its structure much more than the dendron aggregate, in case of cooperative binding to multiple ligands, it is reasonable to think that the dendrons from the aggregate will adapt over extravidin, rather than the unlikely opposite option: protein collapsing over the G2-P surface. This is consistent with a picture where G2-P aggregates are progressively degraded.
3.3.5.2. Proposed Mechanisms for Disassembly and Release

One key factor allowing for multivalent binding is biotin availability. Namely, biotin ligands must be accessible at the aggregate surface and free to complete specific interactions with extravidin. In fact, as we already discussed on Figure 3.13, the chance of having multivalent specific binding between the dendrons and extravidin will be extremely sensitive to biotin availability at the surface of the aggregates. As our results suggested, biotin availability is high for G2-P and low for G2-M and G2-F (Figure 3.13).

Figure 3.14. Proposed mechanism for disassembly and release from biaryl amphiphilic dendrons. a) In G2-P biotin is highly available for extravidin - exfoliation-like disassembly; b) in G2-M and G2-F biotin is less available for extravidin.
Thus, at the \textbf{G2-P} aggregates surface extravidin will find many accessible binding spots for completing specific interactions. Moreover, after a first specific binding is established, the same extravidin protein will be then energetically favored to bind more biotins from other \textbf{G2-P} dendrons. On the other hand, since the probability to have specific binding for \textbf{G2-M} and \textbf{G2-F} is reduced, even if a first specific interaction occurs between extravidin and one biotin ligand, it is reasonable to hypothesize that the chance of having multivalent extravidin binding at the surface of \textbf{G2-M} and \textbf{G2-F} aggregates will be lower.

In light of our overall results, we propose two different possible mechanisms for self-assembly schematized in Figure 3.14. (i) For the \textbf{G2-P} case, multivalent binding of extravidin leads to the rapid disassembly of the dendron aggregates in solution, in an exfoliation-like manner as in Figure 3.14a. (ii) For \textbf{G2-M} and \textbf{G2-F}, the aggregates would disassemble more slowly due to the destabilization induced by extravidin binding, as in Figure 3.14b. In principle, the first proposed mechanism would result in the rapid production of smaller aggregates limited by extravidin size, and in a higher level of hydrophobic guest release. Here, the velocity of the process and the small size of the aggregates would not allow for the structural rearrangement necessary to retain the guest molecules. The second proposed mechanism would most likely produce larger size aggregates and thus, lower levels of release.

To summarize this section, the high level of biotin availability of \textbf{G2-P} is compatible with its fast disassembly and hydrophobic guests in presence of extravidin, as shown by our DLS and fluorescence experiments. At the same time our data
demonstrates that G2-M and G2-F aggregates also disassemble in presence of extravidin (Figure 3.5), but more slowly. In addition, the final size of the disassembled aggregates is larger than that of G2-P (~14 vs ~7 nm), and no appreciable guest release is present during the disassembly of the G2-M and G2-F aggregates (Figure 3.8a,b).

3.4 Summary

Our research reveals that ligand placement on a supramolecular scaffold for binding induced disassembly greatly impacts disassembly and release of encapsulated guest molecules, as we have shown from the high release difference observed, for example, between G2-P and G2-M. The best place to attach a ligand, looking for a protein triggered release from a dendritic micelle-like nanostructure, is the periphery. MD simulations show backfolding of the ligand when attached to middle layer and focal point and a better availability for protein binding when the ligand is attached at the periphery. Incorporation of the biotin ligand in the dendrons gives the dendritic assemblies selectivity towards the target protein extravidin, regardless of the ligand position. Nonetheless, ligand positioning in the dendrons gives the assemblies sensitivity towards release upon binding of a target protein. In addition, multivalent binding of the same extravidin protein to other G2-P dendrons via specific biotin-extravidin interaction is an energetically favored event. These evidences allowed us to hypothesize two different mechanisms of disassembly induced by extravidin binding that depend on the ligand location in the scaffold. A fast one for G2-P based on high biotin availability at the aggregate surface, multivalent interactions and aggregates exfoliation. This mechanism
leads to fast formation of small disassembled aggregates and to a high release of hydrophobic guests. A second mechanism for \textbf{G2-M} and \textbf{G2-P} portraits a slower disassembly, based on extravidin binding induced aggregate destabilization, and the formation of larger and more ordered aggregates in solution that are still capable of retaining the guest molecules in their interior.

Substitution of a PEG unit in the dendron for a pendant biotin increased non-specific interactions of the assemblies with proteins, which was seen as the formation of larger aggregates in solution. This became more evident when the density of PEG chains on the hydrophilic face was low, as it is in a G1 dendron compared to a G2. This, in turn, facilitates the formation of a weak complex assembly-protein that was evidenced when a metalloprotein acted as a quencher, generating static quenching of the encapsulated fluorophore molecules. The research reported here gives a picture of how supramolecular disassembly and release might be largely affected by choosing a specific location for a trigger, rather than a random placement based on molecular architecture. Also, we have shown how controlled variations in PEG density could affect interactions of nanoparticles with proteins.

\textbf{3.5 Experimental Section}

\textbf{3.5.1 Synthetic Procedures and Characterization of Compounds}

All chemicals and solvents were purchased from commercial sources and were used as such, unless otherwise mentioned. $^1\text{H}$ NMR spectra were recorded on a 400 MHz Bruker NMR spectrometer using the residual proton resonance of the solvent as
the internal standard. Chemical shifts are reported in parts per million (ppm). When
peak multiplicities are given, the following abbreviations are used: s, singlet; bs, broad
singlet; d, doublet; t, triplet; m, multiplet. $^{13}$C NMR spectra were proton decoupled and
recorded on a 100 MHz Bruker spectrometer using the carbon signal of the deuterated
solvent as the internal standard. Fluorescence spectra were recorded using a JASCO FP-
6500 spectrofluorimeter. FAB-MS spectra were measured on a JEOL JMS700. MALDI-
TOF spectra were measured on a Bruker OmnifleX. IR spectra were measured on a
Bruker Alpha-P FT-IR.

3.5.1.1 General Procedure for the Synthesis of Dendritic Compounds

To a solution of the biaryl monomer AB$_2$ (1.0 equiv.) and the appropriate
bromobenzyl compound (1.0-3.0 equiv.) in anhydrous acetone, was added K$_2$CO$_3$ (3
equiv.) and 18-crown-6 (0.1 equiv.). The reaction mixture was refluxed under argon
atmosphere for 12-24 h (12 h for G1 and 24 h for G2). The progress of the reaction was
monitored by TLC. After completion of the reaction, acetone was evaporated and the
crude reaction mixture was partitioned between ethyl acetate and water. The aqueous
layer was extracted three times with ethyl acetate and the combined organic layer was
dried over Na$_2$SO$_4$. Afterwards, the solution was filtered and evaporated to dryness. The
crude product was purified by silica gel column chromatography, CombiFlash.
3.5.1.2 General Procedures for Incorporation of Biotin-azide to the Dendron using “Click” Chemistry

Procedure A: To a solution of dendritic acetylene compound (1.0 equiv.), and biotin-azide 18 (2 equiv.) in THF, was added the same volume of aqueous CuSO$_4$.5H$_2$O (0.2 equiv.) and sodium ascorbate (0.2 equiv.) in such a way that the final solution THF/H$_2$O was in a ratio 1:1. The reaction was heated at 50 °C for 24 h to 60 h, depending on dendron generation. After completion of the reaction, NH$_4$Cl solution was added to the reaction mass and then, extracted with ethyl acetate three times. The organic layers were collected and dried over anhydrous Na$_2$SO$_4$, filtered, concentrated, and the product purified by silica gel column chromatography, CombiFlash.

Procedure B: A mixture of the dendritic acetylene compound (1.0 equiv.), biotin azide 18 (3.0 equiv.), CuSO$_4$.5H$_2$O (1.0 equiv.) and sodium ascorbate (1.0 equiv.) in DMSO solvent was heated at 50 °C for 24-32 h (24h for G1 and 32h for G2). The progress of the reaction was monitored by TLC. After completion of the reaction, the reaction mixture was portioned between dichloromethane and saturated aqueous NH$_4$Cl solution. The aqueous layer was extracted twice with dichloromethane and the combined organic layer was dried over anhydrous Na$_2$SO$_4$ and evaporated to dryness. The crude product was isolated by silica gel column chromatography, CombiFlash.

3.5.1.3 Synthesis of compound 3 (DA-1-7)

![Chemical structure of compound 3 (DA-1-7)]
To a solution of 3,5-dihydroxybromobenzene (23 g, 121.6 mmol) in anhydrous THF (500 mL) in an ice bath was added Hunig’s base (74.2 mL, 425.8 mmol); the mixture was stirred at room temperature and under argon atmosphere for 15 min. At ice-bath temperature, methoxymethyl chloride (MOM-Cl) (32.3 mL, 425.8 mmol) was added slowly. The reaction mixture was allowed to stir overnight at room temperature. Then, it was poured into water to quench the remaining chloride and then, most of the THF was evaporated. The remaining aqueous solution was extracted three times with ethyl acetate. The combined organic layers were evaporated and subjected to silica gel column chromatography to yield 33 g (98%) of compound 3. For the characterization details of this compound please see reference 37.

3.5.1.4 Synthesis of compound 4 (DA-1-19)

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\includegraphics[width=1\textwidth]{diagram}
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The ethyl ester of 3,5-dihydroxy-5-bromobenzoic acid (30 g, 114.9 mmol) was mixed with 1-bromodecane (24.0 mL, 114 mmol), \( \text{K}_2\text{CO}_3 \) (47.6 g, 344.7 mmol) and 18-crown-6 (3.0 g, 11.5 mmol) in anhydrous acetone (500 mL) in Ar environment and refluxed overnight. Upon evaporation of the solvent, the mixture was dissolved in water and extracted three times with ethyl acetate. The combined extracts were dried over anhydrous \( \text{Na}_2\text{SO}_4 \). The crude product obtained upon evaporation of solvent was
purified by silica gel column chromatography to yield 15.2 g (33%) of compound 4. For the characterization details of this compound please see reference 73.73

3.5.1.5 Synthesis of compound 5 (DA-1-30)

To a solution of compound 3 (10 g, 36.1 mmol) in anhydrous THF (100 mL) was added n-BuLi (43.3 mL of a 2.5 M in hexane solution, 108.3 mmol) under argon atmosphere at -78 °C and stirred for 30 min. At the same temperature, SnBu₃Cl (29.4 mL, 108.3 mmol) was added and stirred from -78 to 25 °C, and then for 12 h. The reaction mixture was quenched with saturated aqueous NH₄Cl solution, the acetone was evaporated, and the remaining aqueous solution extracted three times with ethyl acetate. The organic layers were collected on anhydrous Na₂SO₄. The solvent was evaporated to afford the crude reaction mixture, which was purified by silica gel column chromatography to yield 14 g (75%) of product 5 as viscous oil. For the characterization details of this compound please see reference 37.

3.5.1.6 Synthesis of compound 6 (DA-1-24)
To a solution of mono-alkylated 4 (12 g, 30.0 mmol) in anhydrous dichloromethane (150 mL) was added triethylamine (12.0 mL 90.0 mmol) and cooled to 0 °C. Then, acetylchloride (6.4 mL, 90.0 mmol) was added dropwise and stirred at room temperature for 12 h. The reaction mixture was quenched with water and extracted three times with dichloromethane. The organic layers were collected on anhydrous Na₂SO₄. The solvent was evaporated to afford the crude reaction mixture, which was purified by silica gel column chromatography to afford 12.5 g (91%) of product 6 as viscous oil. For the characterization details of this compound please see reference 37.

3.5.1.7 Synthesis of compound 7 (DA-1-46)

![Chemical structure of compound 7]

The aryl stannane 5 (7 g, 14.3 mmol) and the bromo-ester 6 (6.35 g, 14.3 mmol) were dissolved in deoxygenated DMF (20 mL) under argon atmosphere. To this solution, 2.5 mol% of Pd(PPh₃)₂Cl₂ (0.25 g, 0.35 mmol) was added and the reaction mixture heated at 120-130 °C for 24 h. After cooling the reaction mixture to room temperature, the mixture was passed through a celite pad and washed with ethyl acetate. Finally the filtrate was washed with water and the organic layer was evaporated to dryness. The crude product was purified by silica gel chromatography to afford 4.4 g of biaryl coupled
compound (55% yield). For the characterization details of this compound please see reference 37.

Then, to a solution of the previous biaryl coupled compound (5 g, 8.9 mmol) in anhydrous THF (25 mL), was added LiAlH$_4$ (0.85 g, 22.3 mmol) in portions at 0 °C under argon atmosphere, stirring at room temperature for 12 h. The reaction mixture was quenched with ethyl acetate and then acidified using dilute HCl (1 N) solution. The resultant mixture was extracted three times with ethyl acetate and the combined organic extracts were dried over anhydrous Na$_2$SO$_4$. Upon evaporation of solvent, the crude was purified by silica gel chromatography to afford 3.3 g of compound 7 (85% yield). For the characterization details of this compound please see reference 37.

**3.5.1.8 Synthesis of compound 9 (DA-1-10)**

![Chemical Structure](image)

The mixture of 3,5-dihydroxybenzyl alcohol (10 g, 71.4 mmol), 1-bromodecane (13.4 mL, 64.2 mmol), K$_2$CO$_3$ (19.6 g, 142.8 mmol), sodium iodide (10.6 g, 71.4 mmol) and 18-crown-6 (1.8 g, 7.14 mmol) in anhydrous acetone (50 mL) was refluxed for 12 h. Upon evaporation of the solvent, the mixture was dissolved in water and extracted three times with ethyl acetate. The combined extracts were dried over anhydrous Na$_2$SO$_4$. The crude product obtained upon evaporation of solvent was purified by silica gel column chromatography to yield 8.2 g (41%) of compound 9. For the characterization details of this compound please see reference 73.
3.5.1.9 Synthesis of compound 10 (DA-1-16)

The mixture of compound 9 (2.8 g, 9.9 mmol), propargyl bromide (3.32 g mL from a 80% sln. in toluene, 29.8 mmol), K₂CO₃ (4.1 g, 29.8 mmol), and 18-crown-6 (0.26 g, 1.0 mmol) in anhydrous acetone (20 mL) was refluxed for 12 h. Upon evaporation of the solvent, the mixture was dissolved in water and extracted three times with ethyl acetate. The combined extracts were dried over anhydrous Na₂SO₄. The crude product obtained upon evaporation of solvent was purified by silica gel column chromatography to yield 3.0 g (95%) of compound 10. For the characterization details of this compound please see reference 37.

3.5.1.10 Synthesis of compound 11 (DA-1-12)

The mixture of compound 9 (5.1 g, 18.3 mmol), PEG-Ts (6.7 g, 16.5 mmol), K₂CO₃ (6.8 g, 49.4 mmol), and 18-crown-6 (0.4 g, 1.6 mmol) in anhydrous acetone (20 mL) was refluxed for 12 h. Upon evaporation of the solvent, the mixture was dissolved in water and extracted three times with ethyl acetate. The combined extracts were dried over anhydrous Na₂SO₄. The crude product obtained upon evaporation of solvent was
purified by silica gel column chromatography to yield 6.8 g (80%) of compound 11. For the characterization details of this compound please see reference 73.

3.5.1.11 Synthesis of compound 12 (DA-1-104)

To a stirring solution of 10 (1.8 g, 5.65 mmol) in dichloromethane (20 mL) was added PBr$_3$ (1 mL, 11.31 mmol) under argon atmosphere at room temperature. The reaction was monitored using TLC. After complete disappearance of the starting material the remaining PBr$_3$ was quenched by slow addition of a saturated NaHCO$_3$ solution. The resulting mixture was extracted with dichloromethane (3x50 mL). The combined organic layer was dried over anhydrous Na$_2$SO$_4$ and concentrated using vacuum. The crude compound was purified on silicagel column chromatography CombiFlash (1:4 Ethyl acetate, Hexanes) to afford 12 (1.65 g, 76%). $^1$H NMR (CDCl$_3$-400MHz) $\delta$ 6.57 (t, $J = 1.9$ Hz, 2H, a,b), 6.49 (t, $J = 2.2$ Hz, 1H, c), 4.67 (d, $J = 2.3$, 2H, d), 4.41 (s, 2H, f), 3.92 (t, $J = 6.5$ Hz, 2H, e), 2.53 (t, $J = 2.3$ Hz, 1H, g), 1.79-1.72 (m, 2H, h), 1.48-1.21 (m, 14 H, i), 0.87 (t, $J = 6.8$ Hz, 3H, j). $^{13}$C NMR (CDCl$_3$-100MHz) $\delta$ 162.4, 160.5, 158.8, 139.8, 108.6, 107.7, 101.9, 78.4, 75.9, 68.32, 56.0, 33.7, 32.0, 29.7, 29.5, 29.3, 26.1, 22.8, 14.3.
3.5.1.12 Synthesis of compound 13 (DA-1-35)

![Chemical Structure of Compound 13](image)

To a stirring solution of 11 (6.4 g, 12.4 mmol) in dichloromethane (20 mL) was added PBr$_3$ (16.1 mL from a 1M sln, 16.1 mmol) under argon atmosphere at room temperature. The reaction was monitored using TLC. After complete disappearance of the starting material the remaining PBr$_3$ was quenched by slow addition of a saturated NaHCO$_3$ solution. The resulting mixture was extracted with dichloromethane (3x50 mL). The combined organic layer was dried over anhydrous Na$_2$SO$_4$ and concentrated using vacuum. The crude compound was purified on silicagel column chromatography CombiFlash (1:4 Ethyl acetate, Hexanes) to afford 32 (5.0 g, 70%). For the characterization details of this compound please see reference 73.

3.5.1.13 Synthesis of compound 15 (DA-1-111)

![Chemical Structure of Compound 15](image)

To a solution of 2-(2-amino-ethoxy)-ethanol (10.0 mL, 100.0 mmol) in dichloromethane (50 mL) and an Ar atmosphere was added di-tert-butyl dicarbonate (32.8 g, 151.0 mmol) at ice bath temperature and stirred overnight. Water was added to the reaction mixture and extracted three times with dichloromethane. The combined organic extracts were dried over anhydrous Na$_2$SO$_4$. Afterwards, the solvent was
evaporated to dryness. The crude product **15** was taken for the next step without further purification.

**3.5.1.14 Synthesis of compound 16 (DA-1-112)**

![Structure of Compound 16]

To the solution of crude compound **15** (31.6 g, 100 mmol) in anhydrous toluene (100 mL) was added triethyl amine (13.3 mL, 100.0 mmol) and cooled to ice bath temperature. Then, methane sulfonyl chloride (7.7 mL, 100.0 mmol) was added dropwise at the same temperature. After 20 minutes stirring at room temperature (RT), tetrabutylammonium iodide (37 g, 100.2 mmol). An aqueous solution of sodium azide (40 g, 615.4 mmol) was added. Then, the reaction mass was heated at 70 °C for 6h. After cooling the reaction mixture to RT, water was added and extracted twice with ethyl acetate and then dried over anhydrous Na$_2$SO$_4$. After the evaporation of solvent, the crude product was purified by silica gel column chromatography to yield 18.0 g (78%) of compound **16**. For the characterization details of this compound please see reference 37.

**3.5.1.15 Synthesis of compound 17 (DA-1-128)**

![Structure of Compound 17]

To a solution of compound **16** (2 g, 8.2 mmol) in dichloromethane (40 mL) was added trifluoroacetic acid (0.80 mL, 10.4 mmol) at 0 °C. After stirring at RT for 8 hours,
the solvent was evaporated and dried under vacuum to obtain the Boc-deprotected compound 17 that was used as such for the next step.

3.5.1.16 Synthesis of compound 18 (DA-1-129)

Biotin (1.0 g, 4.1 mmol) was dissolved in DMF (25 mL) and heated to 50-60 °C for 45 minutes, and then allowed to cool to RT. To this solution was added carbonyldiimidazole (0.97 g, 6.0 mmol) previously dissolved in DMF (10 mL) and stirred at RT for 3 h. Then, the previous solution was added to a solution of compound 17 (1.1 g, 8.7 mmol) previously dissolved in DMF (5 mL) and stirred overnight at in Ar atmosphere. The solvent was removed under vacuum. The remaining solid was washed with water and then, with cold acetone under negative pressure in a fritted funnel. After a long drying the product was recover in 0.7 g (46%) of the product 18 as a white solid. For the characterization details of this compound please see reference 37.

3.5.1.17 Synthesis of compound 19 (DA-1-66)
To the propargylated product of the biaryl compound 7 (0.7 g, 1.3 mmol) dissolved in a mixture of methanol (20 mL), 1,4-dioxane (2 mL), and water (1 mL), 1.4 g of Dowex resin was added stirring under Ar atmosphere. The reaction mass was refluxed for 4 h. Then, it was filtered and the remaining solid was dissolved in water and extracted with ethyl acetate. The organic fractions were dried over Na₂SO₄. The solvent was evaporated and the product purified by silica gel chromatography getting 0.3 g of product 19 (58%). For the characterization details of this compound please see reference [1].

Compound 20 was obtained in exactly the same way as compound 19. The characterization details of this compound are in reference 37.

3.5.1.18 Synthesis of compound 21 (DA-1-107)

A mixture of compound 20 (0.25 g, 0.04 mmol), compound 12 (0.14 g, 0.04 mmol), K₂CO₃ (0.08 g, 0.06 mmol) and 18-crown-6 (0.01 g, 0.004 mmol) in acetone (15 mL) was refluxed for 12 h. Upon evaporation of the solvent, the mixture was dissolved in water and extracted three times with ethyl acetate. The combined extracts were dried over anhydrous Na₂SO₄. The crude product obtained upon evaporation of solvent
was purified by silica gel column chromatography to afford of compound 21 (0.079 g, 25%). $^1$H NMR (Acetone d$_6$-400MHz) $\delta$ 6.78-6.69 (m, 4 H, a,b), 6.51-6.50 (m, 3 H, c,d,e), 6.40 (d, $J = 2.36$, 1H, f), 5.01 (s, 2H, g), 4.78 (d, $J = 2.40$ Hz, 2 H, h), 4.63 (m, 2 H, i), 4.04 (t, $J = 4.68$ Hz, 2 H, j), 3.99 (t, $J = 6.48$ Hz, 2H, k), 3.91 (t, $J = 6.28$ Hz, 2 H, m), 3.65 (t, $J = 4.56$ Hz, 2 H, n) 5.53-3.52 (m, 11 H, o), 3.44-3.42 (m, 2H, p), 3.26 (s, 3H, q), 3.05 (t, $J = 2.36$, 1H, r), 2.84 (d, $J = 12.8$, 3H, u), 1.80-1.73 (m, 2H, v), 1.64-1.57 (m, 2H, w), 1.51-1.45 (m, 2H, x), 1.42-1.19 (m, 26H, y), 0.92-0.83 (m, 6H, z); $^{13}$C NMR (Acetone d$_6$-100MHz) $\delta$ 161.4, 159.9, 159.9, 158.2, 158.1, 157.9, 157.8, 144.3, 141.2, 137.3, 119.6, 112.4, 112.3, 109.9, 107.2, 106.8, 104.8, 104.6, 101.4, 79.7, 77.0, 72.6, 71.4, 71.4, 71.1, 71.1, 71.0, 70.2, 70.1, 69.5, 69.2, 68.6, 64.8, 64.7, 58.7, 56.3, 32.6, 32.6, 30.3, 30.3, 30.3, 30.1, 30.1, 26.8, 23.3, 23.3, 14.4, 14.3.

3.5.1.19 Synthesis of compound 22 (DA-1-127)

According to the general procedure for synthesis of dendritic compounds, the biaryl mono-G1-propargyl monomer 21 (0.13 g, 0.116 mmol) was reacted with the bromomethyl compound 13 (0.06 g, 0.127 mmol) to give compound 22 (0.12 g, 72%). $^1$H
NMR (Methanol d$_4$-400MHz) $\delta$ 6.69-6.67 (m, 2H, a), 6.65-6.51 (m, 7H, b), 6.46 (t, $J = 2.2$ Hz, 1H, f), 6.42 (t, $J = 2.2$ Hz, 1H, f'), 4.99-4.97 (m, 4 H, g, g'), 4.69 (d, $J = 2.36$ Hz, 2 H, h), 4.60 (s, 2H, i), 4.10-4.08 (m, 2H, j), 4.02-4.0 (m, 2H, j'), 3.94-3.79 (m, 10H, w, w'), 3.61-3.39 (m, 38H, q), 2.91 (t, $J = 2.40$ Hz, 1H, r), 1.78-1.70 (m, 4H, v, v'), 1.59-1.12 (m, 44H, y), 0.92-0.82 (m, 9H, z); $^{13}$C NMR (Acetone d$_6$-100MHz) $\delta$ 168.5, 167.4, 165.3, 159.4, 159.1, 156.6, 154.8, 150.7, 144.2, 140.8, 136.1, 133.6, 125.5, 124.4, 110.9, 110.4, 107.3, 106.3, 102.5, 100.5, 83.4, 83.0, 78.7, 77.1, 74.1, 71.6, 70.3, 70.2, 69.9, 67.7, 65.8, 58.0, 55.5, 34.7, 31.7, 31.5, 30.1, 25.9, 23.0, 22.4, 20.8, 13.4, 12.3. MALDI-ToF m/z expected for C$_{82}$H$_{130}$O$_{19}$: 1420.9; found 1442.6 for C$_{82}$H$_{130}$O$_{19}$+Na$^+$. 

**3.5.1.20 Synthesis of dendron G1-P (DA-1-164)**

According to the general procedure for click chemistry, the compound 22 (0.04 g, 0.0028 mmol) was reacted with biotin azide 18 (0.02 g, 0.0056) to give G1-P dendron (0.03 g, 70%). $^1$H NMR (Methanol d$_4$-400MHz); 8.22 (s, 1H, c), 6.81-6.72 (m, 3H, b), 6.70-6.63 (m, 5H, a), 6.62-6.53 (m, 2H, d) 6.47-6.43 (m, 1H, e), 5.25-5.15 (m, 2H, g) 5.10-4.95 (m, 4H, f, f'), 4.64 (s, 2H, i), 4.57 (t, $J = 4.8$ Hz, 2H, r), 4.52-4.40 (m, 1H, p), 4.29-4.22 (m,
1H, q), 4.13-4.11 (m, 3H, x), 4.04-4.03 (m, 2H, j'), 4.0-3.96 (4H, j), 3.93-3.87 (m, 3H, v'), 3.86-3.82 (m, 2H, w'), 3.81-3.78 (m, 3H, w), 3.69-3.41 (m, 41H, t), 3.35-3.29 (m, 2H), 3.27-3.26 (m, 6H), 3.13-3.08 (m, 1H), 2.98-2.82 (m, 2H), 2.72-2.62 (d, J = 12.4 Hz, 1H, m), 2.17-2.14 (t, J = 7.2 Hz, 2H, k), 1.82-1.71 (m, 4H, v, v'), 1.67-1.52 (m, 6H, h), 1.51-1.18 (m, 44H, y), 0.2-0.82 (m, 9H, z); $^{13}$C NMR (CDCl$_3$, 100 MHz) $\delta$ 160.4, 160.0, 158.9, 157.4, 157.2, 157.0, 142.0, 139.4, 135.9, 119.3, 112.1, 111.0, 106.1, 105.6, 104.4, 104.2, 102.1, 100.8, 94.6, 77.2, 71.9, 70.8, 70.6, 70.5, 69.9, 69.7, 69.6, 68.8, 68.0, 67.4, 65.4, 59.0, 55.9, 31.9, 30.9, 29.5, 29.4, 29.3, 29.1, 26.0, 25.9, 22.6, 14.1. MALDI-ToF m/z expected for C$_{96}$H$_{154}$N$_6$O$_{22}$S: 1777.34; found 1799.15 for C$_{96}$H$_{154}$N$_6$O$_{22}$S + Na$^+$, 1777.14 for C$_{96}$H$_{154}$N$_6$O$_{22}$S + H$^+$.  

**3.5.1.21 Synthesis of compound 23 (DA-1-187)**

According to the general procedure for synthesis of dendritic compounds, the biaryl monomer 19 (0.088 g, 0.2 mmol) was reacted with the bromomethyl compound 13 (0.30 g, 0.52 mmol) to give 0.20 g (yield: 70%) of compound 23. The characterization details of this compound are in reference 37.
3.5.1.22 Synthesis of dendron G1-F (DA-1-91)

According to the general procedure for click chemistry, the compound 23 (0.07 g, 0.05 mmol) was reacted with biotin azide 18 (0.02 g, 0.006 mmol) to give 0.04 g (83%) of G1-F dendron. The characterization details of this compound are in reference 37.

3.5.1.23 Synthesis of compound 24 (DA-2-78)

To a stirring solution of 22 (0.324 g, 0.228 mmol) in dichloromethane (10 mL) was added PBr₃ (0.22 mL, 2.28 mmol) under argon atmosphere at room temperature. The reaction was monitored using TLC. After complete disappearance of the starting material, the remaining PBr₃ was quenched by slow addition of saturated NaHCO₃ solution. The resulting mixture was extracted with dichloromethane (3x50 mL). The combined organic layer was dried over anhydrous Na₂SO₄ and concentrated using
vacuum. The crude compound was purified by column chromatography, CombiFlash (1:4 Ethyl acetate, Hexanes) to afford 24 (0.076 g, 22%). $^1$H NMR (Methanol d$_4$-400MHz) δ 6.73-6.41 (m, 11 H, a), 4.95 (s, 4H, b), 4.67 (d, J = 2.4 Hz, 2H, c), 4.54 (s, 2H, d), 4.12-4.06 (m, 2H, e), 4.05-3.97 (m, 2H, f), 3.96-3.40 (m, 32H, g), 2.91 (t, J = 2.3 Hz, 1H, h), 1.81-1.70 (m, 4H, i), 1.62-1.11 (m, 42H, j), 0.95-0.82 (m, 9H, k); $^{13}$C NMR (Methanol d$_4$-100MHz) δ 160.4, 160.1, 158.9, 157.5, 157.0, 156.7, 156.0, 157.9, 139.8, 130.0, 106.0, 105.6, 100.6, 100.4, 75.5, 71.5, 70.4, 70.3, 70.2, 70.1, 70.0, 69.9, 69.4, 69.2, 67.7, 67.2, 57.7, 55.3, 38.9, 31.7, 29.4, 29.3, 29.3, 29.1, 29.1, 25.9, 25.8, 22.4, 22.3, 18.7, 13.1, 13.1. MALDI-ToF m/z expected 1483.79 for C$_{82}$H$_{129}$BrO$_{18}$; found 1483.51 for C$_{82}$H$_{129}$BrO$_{18}$ + H$^+$.  

**3.5.1.24 Synthesis of compound 25 (DA-2-2)**

To a stirring solution of a G1 23 (0.53 g, 0.373 mmol) in dichloromethane (20 mL) was added PBr$_3$ (0.3 g, 1.12 mmol) under argon at room temperature. The reaction was monitored using TLC. After complete disappearance of the starting material the remaining PBr$_3$ was quenched by slow addition of saturated NaHCO$_3$ solution. The resulting mixture was extracted with dichloromethane (3x50 mL). The combined organic layer was dried over anhydrous Na$_2$SO$_4$, filtered and concentrated using vacuum. The
crude compound was purified by column chromatography using CombiFlash (1:4 Ethyl acetate, Hexanes) to afford 25 (0.432 g, 78%). $^1$H NMR (Methanol d$_4$ – 400 MHz); $\delta$ 6.89 (s, 1H, a), 6.76 (s, 1H, b), 6.58-6.53 (m, 7H, c), 6.42-6.40 (m, 2H, d), 4.96 (s, 4H, e), 4.57-4.55 (m, 4H, f, g), 4.47 (s, 1H, i), 4.08-4.06 (m, 4H, h), 3.94-3.78 (m, 11 H, i, j), 3.74-3.54 (m, 31 H, k), 3.53-3.46 (m, 5H, m), 2.91 (t, J = 2.3 Hz, 1H, n), 1.80-1.69 (m, 5H, p), 1.61-1.16 (m, 53H, q), 0.95-0.84 (m, 9H, z). MALDI-ToF m/z expected 1483.79 for C$_{82}$H$_{129}$BrO$_{18}$; found 1483.51 for C$_{82}$H$_{129}$BrO$_{18}$ + H$^+$. 

Compound 26 was synthesized in the same fashion.

3.5.1.25 Synthesis of compound 27

According to the general procedure for synthesis of dendritic compounds, the biaryl monomer 20 (0.072 g, 0.115 mmol) was reacted with the bromomethyl dendritic compound 26 (0.192 g, 0.115 mmol) to give the mono alkylated product 27 (0.061 g, 19%). $^1$H NMR (CDCl$_3$ – 400 MHz); $\delta$ 6.79-6.62 (m, 6H, a), 6.60-6.51 (m, 9H, b), 6.44-6.42
(m, 2H, c), 5.02 (s, 2H, d), 4.92 (s, 4H, e), 4.69 (s, 2H, f), 4.14-4.06 (m, 8H, g), 3.94-3.87 (m, 12H, k, n), 3.78-3.44 (m, 70H, p), 3.35 (s, 6H, h), 3.33 (s, 3H, i), 3.31 (s, 3H, j), 1.79-1.72 (m, 8H q), 1.69-1.58 (m, 4H, r), 1.46-1.38 (m, 4H, r), 1.37-1.15 (m, 48H, r), 0.91-0.79 (m, 12H, w); $^{13}$C NMR (CDCl$_3$-100MHz) $\delta$ 160.5, 160.1, 159.3, 159.1, 157.3, 157.2, 157.0, 141.9, 139.5, 138.4, 135.6, 119.8, 119.2, 110.3, 109.3, 106.3, 105.8, 105.2, 104.4, 103.6, 100.9, 72.0, 70.9, 70.7, 70.7, 70.5, 69.8, 68.9, 68.2, 67.5, 65.9, 65.6, 59.1, 32.0, 31.7, 29.7, 29.5, 29.4, 29.3, 26.2, 26.1, 22.8, 15.4, 14.2. MALDI-ToF m/z expected for C$_{124}$H$_{202}$O$_3$: 2221.91; found 2259.66 for C$_{124}$H$_{202}$O$_3$ + K$^+$, 2243.69 for C$_{124}$H$_{202}$O$_3$: + Na$^+$, 2221.73 for C$_{124}$H$_{202}$O$_3$ + H$^+$.

3.5.1.26 Synthesis of compound 28 (DA-2-12)

According to the general procedure for the synthesis of dendritic compounds, the dendron-mono-alkylated scaffold 27 (0.07 g, 0.031 mmol) was reacted with the bromo-dendron compound 25 (0.087, 0.059 mmol) to give compound 28 (0.091 g, 80%). $^1$H NMR (Acetone d$_6$ – 400 MHz); $\delta$ 6.92-6.86 (m, 2H, a), 6.78-6.59 (m, 21 H, a), 6.47-6.43
(m, 4H, a), 5.17-5.10 (m, 4H, b), 5.07-5.01 (m, 8H, c), 4.69 (d, J = 2.2 Hz, 2H, e), 4.67-4.63 (m, 2H, f), 4.22-4.20 (m, 1H), 4.15-4.04 (m, 13H, g), 4.02-3.91 (m, 14H, h), 3.84-3.77 (m, 9H, i), 3.71-3.40 (m, 97H, g, i), 3.29-3.23 (m, 18H, j), 2.96 (t, J = 2.2 Hz, 1H, k), 1.82-1.71 (m, 8H, h), 1.65-1.57 (m, 6H, h), 1.52-1.42 (10H, h), 1.42-1.17 (m, 88H, h), 0.92-0.82 (m, 21H, z); $^{13}$C NMR (Acetone d$_6$-100MHz) δ 161.4, 161.2, 160.0, 159.9, 157.9, 157.7, 154.6, 140.9, 140.8, 139.6, 119.3, 111.5, 111.3, 111.2, 106.8, 106.5, 105.8, 104.7, 101.4, 101.1, 80.0, 77.1, 72.6, 71.4, 71.3, 71.2, 71.0, 70.3, 70.1, 69.4, 69.3, 69.2, 68.6, 68.4, 58.8, 56.9, 32.7, 32.6, 26.9, 26.8, 23.3, 23.3, 14.4, 14.3. MALDI-ToF m/z expected for C$_{206}$H$_{330}$O$_{51}$: 3623.79; found 3645.49 for C$_{206}$H$_{330}$O$_{51}$ + Na$^+$. 

3.5.1.27 Synthesis of compound 29

According to the general procedure for synthesis of dendritic compounds, the dendron-mono-alkylated scaffold 27 (0.103 g, 0.046 mmol) was reacted with the bromo-dendron compound 24 (0.076 g, 0.051 mmol) to give compound 29 (0.12 g, 71%). $^1$H NMR (CDCl$_3$ – 400 MHz); δ 6.73-6.41 (m, 27H, a), 5.11-4.9 (m, 12 H, b, f), 4.73-4.65 (m,
4H, c), 4.16-4.02 (m, 10H, d, e), 3.97-3.81 (m, 20H, g, h), 3.77-3.44 (m, 104H, g), 3.38-3.32 (m, 18H, i), 2.52 (t, J = 2.2 Hz, 1H, k), 1.85-1.11 (m, 112H, g), 0.93-0.81 (m, 21H, j);

$^1$C NMR (CDCl$_3$-100MHz) δ 160.5, 160.1, 159.2, 159.2, 159.1, 158.9, 157.4, 157.3, 157.1, 157.1, 139.7, 139.4, 138.0, 136.3, 136.1, 119.9, 119.6, 110.3, 106.9, 106.3, 105.8, 105.3, 105.2, 104.9, 104.5, 101.2, 100.9, 72.0, 72.0, 70.9, 70.7, 70.7, 70.7, 70.6, 70.6, 70.5, 70.5, 69.8, 68.9, 68.2, 67.5, 65.4, 59.1, 59.1, 56.0, 32.0, 29.8, 29.7, 29.7, 29.5, 29.4, 29.4, 29.2, 26.2, 26.1, 22.8, 14.2.

3.5.1.28 Synthesis of dendron G2-M (DA-2-90)

According to general procedure for click chemistry, compound 28 (0.017 g, 0.005 mmol) was treated with biotin-azide 18 (0.005 g, 0.014 mmol) to give G2-M dendron (0.0074 g, 40%). $^1$H NMR (Methanol d$_4$ – 400 MHz); δ 7.71 (s, 1H, z), 6.82-6.41 (m, 27H, a), 5.65-5.58 (m, 4H, b, c), 4.68-4.51 (m, 4H, x, y, d), 4.40-4.32 (m, 5H, k), 4.31-4.23 (m, 5H, f), 4.18-3.97 (m, 9H, e), 3.89-3.72 (m, 15H, n, r), 3.69-3.63 (m, 12 H, k), 3.62-3.56 (m, 48 H, g, k, m, u, ), 3.55-3.48 (m, 35H, g, m, v), 3.32-3.25 (m, 3H, n, w), 2.86-2.72 (m, 4H),
2.41-2.30 (m, 8H, q), 2.12-1.95 (m, 16H, i, m), 1.85-1.62 (m, 33H, i, r), 1.60-1.48 (m, 17H, i, h), 1.45-1.20 (m, 80H, i, p), 1.01-0.85 (m, 21H, j). $^{13}$C NMR (Acetone d$_6$-100 MHz) δ 160.6, 160.3, 159.1, 157.2, 157.1, 157.0, 156.5, 144.0, 143.5, 140.0, 138.7, 136.5, 136.3, 119.4, 110.5, 106.0, 105.7, 105.1, 104.5, 104.0, 100.8, 100.7, 100.3, 70.3, 70.3, 70.2, 70.0, 69.6, 69.5, 69.3, 68.8, 68.7, 68.5, 68.4, 67.7, 67.5, 57.9, 31.8, 31.8, 29.6, 29.4, 29.2, 28.7, 28.5, 28.4, 26.1, 26.0, 25.4, 22.5, 22.5, 13.6, 13.6, 13.5. MALDI-ToF m/z expected for C$_{220}$H$_{354}$N$_6$O$_{54}$S: 3980.24; found 4023.08 for C$_{220}$H$_{354}$N$_6$O$_{54}$S + 2Na$^+$.  

3.5.1.29 Synthesis of dendron G2-P (DA-2-89)

According to the general procedure for click chemistry, compound 29 (0.022 g, 0.006 mmol) was treated with biotin-azide 18 (0.006 g, 0.018 mmol) to give (0.013 g, 17%) G2-P dendron. $^1$H NMR (CDCl$_3$ – 400 MHz); δ 7.78 (s, 1H, z), 6.82-6.40 (m, 27H, a), 6.24-6.22 (m, 1H, x), 5.57-5.56 (m, 1H, y), 5.32-5.17 (m, 2H, w), 5.11-4.89 (m, 12H, c), 4.75-4.66 (m, 2H, d), 4.60-4.37 (m, 4H, u, v), 4.36-4.21 (m, 2H, t), 4.19-3.32 (m, 155H, e,
f, i), 3.26-3.02 (m, 4H, r, s, q), 2.89-2.51 (m, 4H, r, q, k), 2.25-2.15 (m, 2H, h, k), 2.10-1.95 (m, 2H, g), 1.87-1.57 (m, 26H, j, l), 1.55-1.11 (m, 88H, i), 0.95-0.77 (m, 21H, ). $^{13}$C NMR (Acetone d$_6$-100 MHz) $\delta$ 160.9, 160.6, 159.8, 157.9, 157.6, 157.7, 156.5, 144.0, 143.5, 140.2, 138.8, 136.5, 136.3, 119.5, 110.6, 106.2, 105.9, 105.3, 104.7, 104.3, 100.9, 100.8, 100.4, 70.3, 70.2, 70.0, 69.6, 69.5, 69.3, 68.8, 68.7, 68.5, 68.4, 67.7, 67.5, 57.9, 35.3, 31.9, 31.8, 29.7, 29.5, 29.3, 29.2, 28.7, 28.5, 28.4, 26.1, 26.0, 25.4, 22.6, 22.5, 13.7, 13.6.

MALDI-ToF m/z expected for C$_{220}$H$_{354}$N$_6$O$_{54}$S: 3980.24; found 4025.46 for C$_{220}$H$_{354}$N$_6$O$_{54}$S + 2Na$^+$. 

3.5.1.30 Synthesis of compound 30

According to the general procedure for synthesis of dendritic compounds, the monomer 19 (0.03 g, 0.07 mmol) was reacted with the bromo-dendron compound 26 (0.30 g, 0.18 mmol) to give compound 30 (0.20 g, 70%). The characterization details of this compound are in reference 37.
3.5.1.31 Synthesis of dendron G2-F

According to the general procedure for click chemistry, compound 30 (0.050 g, 0.014 mmol) was treated with biotin-azide 18 (0.007 g, 0.020 mmol) to give (0.025 g, 46%) G2-F dendron. The characterization details of this compound are in reference 37.

3.5.2 Encapsulation, Disassembly, and Release Protocols

3.5.2.1 Encapsulation of Nile red

To encapsulate nile red in a dendron aggregate 2 mL 50 µM solutions of each dendron were prepared and stirred at 5 °C for 12 hours. Small amounts of Nile red were mixed with the dendron solutions and these heterogeneous mixtures sonicated for 4 hours. The solutions were stirred at room temperature until temperature equilibration and then stirred at 5 °C for 16 hours. Then, the samples were diluted to 25 µM and stirred at 5 °C for 12 hours more. After that, each sample was filtered through a syringe filter (0.22 µm). The 25 µM dendron solutions were thus ready for CAC measurement and dye release.
3.5.2.2 Determination of CACs

The CACs of the dendrons in aqueous solutions were determined using the fluorescence intensity of nile red ($\lambda_{em}= 615$ nm), as shown in Figure 3.4. 1 mL of 25 μM dendron solution as prepared above was transferred to a cuvette where its concentration was varied by replacing a measured volume of this solution with the same volume of water. An emission spectrum ($\lambda_{ex}= 550$ nm) was recorded for each concentration of the dendron and a decreasing fluorescence intensity was obtained from each spectrum. When the concentration of the dendron was below the CAC the change in fluorescence intensity became smaller each time. The intensity values were plotted against the concentration of dendron to get a curve (ideally sigmoidal). From the curve, the point where the best fitted horizontal and vertical lines merge was taken as the CAC.

3.5.2.3 Dye release upon exposure to an increasing concentration of protein

500 μL solution of dendron 25 μM encapsulating Nile red was exposed to protein 2 μM (extravidin, $\alpha$-chymotrypsin, pepsin, myoglobin) by adding 2 μL of protein solution from a stock 500 μM. After mixing well, the emission spectrum of Nile red was recorded ($\lambda_{em}= 615$ nm, $\lambda_{ex}= 550$ nm). Consecutive additions of 2 μL of protein 500 μM were made every two minutes, recording fluorescence after each time until completing 14 μM of protein in the 500 μL of dendron solution. Temperature was maintained at 25 °C. A control experiment to test stability of encapsulation over time was made by exposing the assemblies only to the buffer solution (HEPES 25 mM, pH 7.4) as shown in Figure
3.8c. A control experiment exposing 25 µM solutions of analog dendrimers G1 and G2, with PEG instead of ligand, to increasing concentration of extravidin was made to test the non-specificity of the assemblies lacking biotin towards the protein, as shown in Figure 3.8d.

3.5.2.4 DLS Measurements to monitor disassembly

The size of the dendrimeric assemblies and their change in size, when exposed to proteins, was measured by dynamic light scattering. For that, 12 µM solutions of the dendrons were prepared in water. Initial size of the assemblies was measured in 1 mL solution. Then, the assemblies were exposed to 2 µM protein and after mixing well, DLS was measured. The results for disassembly in presence of extravidin, interactions with α-chymotrypsin, pepsin, myoglobin, and control dendrons (PEG instead of ligand) are shown in Figure 3.5 and Figure 3.7. The proteins’ sizes are shown in Figure 3.6. All measurements were made in a Malvern Zeta-sizer.

3.5.2.5 Dye release upon a single exposure to 14 µM of protein

500 µL of dendron solution encapsulating Nile red was exposed to protein 14 µM (extravidin, α-chymotrypsin, pepsin, myoglobin) by adding 14 µL of protein solution from a stock 500 µM. After mixing well, the emission spectrum of Nile red was recorded ($\lambda_{\text{em}} = 615$ nm, $\lambda_{\text{ex}} = 550$ nm) every 15 minutes during the first hour, and then every hour during six hours. Release profiles over time for α-chymotrypsin, pepsin, and myoglobin are shown in Figure 3.15. Temperature was maintained at 25 °C.
3.5.2.6 Encapsulation stability test based on crosslink density:

Three different solutions of nanogels with 0%, 20%, and 50% crosslink densities encapsulating Nile red were prepared in a concentration of 1 mg/mL. The initial fluorescence was measured. Then, 1 mL of these solutions was exposed to 0.5 mg/mL myoglobin and the fluorescence recorded for 50 min. The concentration of myoglobin was increased to 1.0 mg/mL, 1.5 mg/mL, and 2.0 mg/mL measuring fluorescence for each change in myoglobin concentration for a few minutes. The final plots of time vs quenching percentage are shown in Figure S4a. When the nanogels were exposed to a high concentration of myoglobin, the change in quenching percentage was also high, Figure 3.10.

3.5.2.7 Stern-Volmer Plots for G1-F and G2-F

Two samples of dendron solution 25 µM in 500 µL encapsulating Nile red, at temperatures of 25 and 38 °C, were exposed to increasing concentrations of myoglobin. The value of the initial fluorescence of the dendrons (F₀) divided by the fluorescence of

Figure 3.15. Release profile of 25 µM dendron assemblies when exposed to 14 µM solution of a) α-chymotrypsin, b) pepsin, and c) myoglobin.
the same dendrons in presence of the myoglobin (F), in increasing concentration Vs the metalloprotein concentration was plotted in Figure 3.11.

3.6 References

Notes


17 Hayes, G. M.; Carrigan, P. E.; Miller, L. J. “Serine-arginine protein kinase 1 overexpression is associated with tumorigenic imbalance in mitogen-activated protein kinase pathways in breast, colonic, and pancreatic carcinomas”. Cancer Res. 2007, 67, 2072-2080.


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CHAPTER 4

SYNTHESIS OF A CROSSLINKABLE DENDRON FOR INTRACELLULAR TARGETING OF PROTEINS

4.1 Introduction

As discussed in Chapter 1, dendrimers possess high stability and the ability to form unimolecular micelles in aqueous solutions. On the other hand, as stated in Chapter 3, self-assembling dendrons can only form nanoassemblies in concentrations above their CAC, which inherently is a disadvantage if a self-assembling system is to be used as a drug delivery vehicle.¹

In the previous Chapter, we discussed the synthesis and structural advantages of dendrons, their characteristics, and their potential as scaffolds in drug delivery and sensing. As a matter of fact, we used biaryl amphiphilic self-assembling dendrons to study the stimulus-responsive disassembling properties of a supramolecular nanoassembly in the presence of a complementary protein, taking advantage of the CAC of the dendrons. The architecture of these dendrons was also used to discriminate the ligand location on the molecular structure that better led to a release of guest molecules upon protein binding.

A molecular system that requires being above a CAC to form nanoassemblies with some degree of stability, inevitably involves equilibrium between the nanoassembly stage and the molecular stage, as shown in Figure 4.1. During such equilibrium, the non-covalently encapsulated guest molecules are prone to leak out of
the assembly and into the media. In a biological system, this leakage can result in the carrier unloading the drug before it reaches the target site. Then, the leaked cargo could diffuse into normal tissue causing unwanted side effects.\(^2\) Also, a dendritic self-assembling system would have to overcome the dilution factor that implies translating from in vitro to in vivo conditions without going too close to or below its CAC.

![Figure 4.1. Inherent equilibrium of self-assembling dendrons.](image)

A solution to overcome this stability problem involves the incorporation of crosslinkers into the system, which can be adjusted to make the dendritic system responsive to environmental changes, such as a reducing environment.\(^3,4\) Crosslinking is a feature usually found in nanogels, although in a dendron, the crosslinkable units can be incorporated exactly in the desired locations due to the possibility of controlled functionalization during synthesis. Once the stability issue is corrected, a system could go through biological barriers, such as cell membranes, without losing cargo. Then, if the system is decorated with the right targeting moieties such as a ligand,\(^5\) it could reach the complementary protein in the cell.

Decoration of nanoparticles with ligands has tremendous implications in drug delivery and sensing due to the specificity that ligands confer to a system.\(^6\) In the
particular case of dendrons, as shown in the previous Chapter, protein binding through a ligand is the stimulus that triggers disassembly and release.$^{5,7,8}$ Targeting mechanisms for a delivery vehicle in cancer chemotherapy could be classified in two categories, passive and active targeting. Passive targeting is based on the propensity of nanoparticles of 10-200 nm in size to accumulate selectively in the tumor tissue. This is possible due to the increased permeability of the tumor vasculature and ineffective lymphatic drainage, also known as the enhanced permeation and retention (EPR) effect.$^9$ Active targeting is accomplished by decorating the nanoparticle with ligands that exhibit high affinity for receptors, which are usually overexpressed on the surface of cancer cells or as binding sites in overexpressed proteins inside the cell.$^{10}$

Here, we present a dendritic system designed to overcome encapsulation and stability problems resulting from an inherent CAC dependent equilibrium, with the potential capability to respond to different stimuli, as shown in Figure 4.2.

![Figure 4.2. Schematic of crosslinked dendritic aggregates.](image-url)
4.2 Approach and Molecular Design

Biaryl dendrons are facially amphiphilic in each layer of the design, the focal point and the periphery. This is accomplished by incorporating hydrophilic and hydrophobic side chains at opposite sides of the aryl moieties, which are oriented at opposite faces when the dendrons are in aqueous solution, due to the inherent twist of the biaryl.\textsuperscript{11} It has been shown in a recent work from our lab that dendrons can be crosslinked at the core of the assembly by using UV light, if the proper crosslinking moieties are incorporated in the hydrophobic face of the dendron, i.e. photodimerizable coumarin.\textsuperscript{12} In the same work, it was shown how the system could encapsulate hydrophobic guest molecules prior to crosslinking and preserve the crosslinking capacity. However, the responsiveness of the crosslink in that design was not applicable towards a biologically relevant stimulus, since light can only penetrate a few millimeters in organic tissue. To fully exploit the capability of dendrons to be adapted as crosslink nanoassemblies and stable nanocarriers, it is important to make the supramolecular aggregate responsive to biologically relevant stimuli by introducing, in this case, a redox responsive crosslinkable moiety and a ligand for protein targeting.
We targeted a single dendritic design, initially with the potential to be conjugated to any ligand able to perform a nucleophilic attack. The structure of the targeted dendron is shown in Figure 4.3. Pentaethyleneglycol monomethylether (PEG) was chosen as the hydrophilic moiety for the reasons mentioned in the previous chapter. Briefly, PEG confers water solubility and biocompatibility when it is installed as the outer shell in a nanoparticle.

Crosslinking in nanoparticles could be performed either at the surface or at the core. Our dendron has the crosslinking units as the hydrophobic moieties of the molecule, which means the hydrophobic chains will collapse towards the core of the self-assembly where they will crosslink upon a redox stimulus. For that, an alkyl chain with a pyridyl disulfide as a terminal group functions as both the crosslinking unit and the hydrophobic moiety. However, due to the reactivity of the pyridyl disulfide functionality, the hydrophobic fragments must be attached to the dendron in the last step of the synthesis via an orthogonal reaction. For this, the pyridyl disulfide bearing alkyl chain has an azide as the other terminal group. If the dendron backbone is functionalized with terminal alkyne functionalities, azide-alkyne click chemistry can be
performed to attach the crosslinkable hydrophobic moieties to the dendron in the last step of the synthesis.

It has been found that for this type of dendrons the gain in CAC from a G1 to a G2 is already small,\textsuperscript{13} while the gain from G0 to G1 is significant (mM to µM) this is a reason to limit the synthetic efforts to the synthesis of only a G1 type of crosslinkable dendrons. Also, the simplest crosslinkable dendron will have 3 of such functionalities, which corresponds to a G1 dendron with the redox responsive units at the hydrophobic face. A G2 dendron would have 7 of these functional groups, although after crosslinking, the formation of new covalent bonds between G1 dendrons in the assembly will render a crosslinked aggregate, not different from the crosslinked aggregate formed by a G2 dendron. Then, it is worthwhile to synthesize only a G1 dendron, which, in the end, after crosslink, does not present a meaningful difference with a G2.

A system that responds to a reducing environment is attractive from the drug delivery perspective, due to the high reducing environment in the cells.\textsuperscript{3} Although glutathione, the most abundant reducing agent in most cells, has an intracellular concentration of approximately 3 mM, its concentration in the blood is around 10 µM.\textsuperscript{14} Such difference in concentration is more than two orders of magnitude higher for the intracellular environment, which makes glutathione a highly desirable stimulus to target.

Given the possibility the aggregate remains preserved after it has been internalized by the cell and de-crosslinked by glutathione, it would be interesting to explore the possibility of triggering intracellular disassembly of the dendron self-
assembly. For that, once more, we take advantage of the control over the functionalization in the synthesis of a dendron to introduce a ligand as a second responsive functionality to target an overexpressed protein, which is to be found within a cancer cell.

Such target could be dihydrofolate reductase (DHFR), which is an enzyme that has a critical role in regulating the amount of tetrahydrofolate by reducing dihydrofolate acid to tetrahydrofolic acid in the cell.\textsuperscript{15}

Tetrahydrofolate, as well as its derivatives, is essential in the synthesis of purine and thymidylate, which in turn, are important for cell proliferation and cell growth.\textsuperscript{16,17} DHFR plays a central role in the synthesis of nucleic acid precursors, which makes it a target for anticancer drugs, along with the fact that folate is needed in rapidly dividing cells to make thymine.\textsuperscript{18} The deficiency of this enzyme causes deficiency in folate, which has been linked to health problems such as anemia. The overexpression of DHFR is a requirement for the rapid cell division shown by cancer cells. The highest concentrations of DHFR have been found in brain cancer cells.\textsuperscript{19}

Methotrexate (MTX), a folic acid derivative, is a ligand that has been found to bind efficiently to DHFR (nanomolar range).\textsuperscript{20,21,22} However, its poor solubility makes it difficult to functionalize, a requirement for its synthetic transformation.\textsuperscript{23} For that, we
functionalized this ligand with two hydrophilic fragments of amino-diethylene glycol in an attempt to increase the ligand solubility in different solvents. The targeted ligand is shown in Figure 4.4.

The functionalization of the dendron with the ligand can be accomplished at the benzylic position by simply transforming the benzylic hydroxyl into an excellent leaving group, trifluoromethanesulfonate (triflate, -OTf). Then, a hydroxyl from the ligand will attack the benzylic position displacing the triflate and forming the ligand-functionalized dendron. The reason to attach the ligand at the benzylic position of the focal point is related to the HLB of the dendron. Since the pyridildisulfide components are highly hydrophobic, a dendron in which a PEG chain has been replaced by a linker and ligand that are not as hydrophilic could simply precipitate out of solution. A way to overcome this problem, since we don’t want the ligand buried in the hydrophobic core, is to endow the ligand with a hydrophilic linker and then attach it to a position that does not involve substituting PEG in the molecule, i.e. the benzylic hydroxyl.
Scheme 4.1. Synthesis of the target G1 dendron.
4.3 Results and Discussion

4.3.1. Synthetic Strategy

In this synthesis, we target a dendron initially incorporating PEG chains as the hydrophilic components and propargyl functionalities in the positions, where we want the hydrophobic chains to be attached by click chemistry at a later step. Accordingly, and as shown in Scheme 4.1, we started with the synthesis by mono-protecting one phenolic hydroxyl in the ester of 4-bromo-3,5-dihydroxybromobenzoic acid 1 with tert-butyldimethylsilyl chloride (TBS-Cl), in presence of the base imidazole, to get the aryl scaffold 2. The remaining phenolic hydroxyl was then acetylated by reacting it with acetyl chloride using triethylamine as the base. From this reaction we obtained the bottom aromatic ring 3, which is protected by two different protective groups in such a way that it is possible to selectively deprotect the phenolic hydroxyls to make them react individually.

Once we got the bromoaryl 3, its reaction with the arylstannane 4 (compound 5 in Chapter 3) under Stille coupling conditions, generated the biaryl protected AB₂ monomer 5. Then, the acetyl group protecting one phenolic hydroxyl was hydrolyzed with lithium aluminum hydride (LiAlH₄) to deprotect the hydroxyl; simultaneously, the ester functionality was reduced to a methyl alcohol getting the mono-deprotected biaryl 6. The now available phenolic hydroxyl was O-monoalkylated using propargyl bromide in the presence of potassium carbonate and 18-crown-6 to get the alkyne bearing biaryl 7. The selective deprotection of the remaining phenolic hydroxyl in the bottom aromatic ring, using tetrabutylammonium fluoride (TBAF), led to the biaryl 8, which was O-
alkylated with PEG by reacting with PEG-Ts in the O-monoalkylation conditions already described. From the previous step, we got the MOM diprotected AB$_2$ compound 9.

Compound 9, which bears a PEG chain and a propargyl unit as well as the benzylic alcohol, was reacted with Dowex resin to deprotect the phenolic hydroxyls in the top ring to yield the AB$_2$ monomer 10. Utilizing a bromomethyl periphery unit 11 (compound 12 in Chapter 3) to O-alkylate the remaining phenolic hydroxyls, we got the G1 dendron 12 with the required three alkyne functionalities for click chemistry. Finally, the alkyne-azide click chemistry was possible between 12 and the hydrophobic molecule 13, which is functionalized with an azide on one end and with a pyridyldisulfide group (PDS) on the other end, to get the targeted crosslinkable G1 dendron 14 (Scheme 4.1).

However, before performing this previous reaction, the pyridyldisulfide moiety in 13 had not been reported to be stable under the click chemistry conditions used in the reaction with the dendron 12, as seen in Scheme 4.1, requiring certain synthetic efforts to achieve. For that reason, we run a test reaction to observe the behavior of the pyridyldisulfide moiety under the conditions of alkyne-azide click chemistry, as shown in Scheme 4.2. Briefly, 3-hydroxybenzyl alcohol 15 was O-alkylated with a propargyl substituent to get the product 16, which was set up to react with 13 in the click
chemistry conditions overnight. The desired product triazol-pyridyl disulfide 17 was obtained in acceptable yield and purified without major inconvenience.

![Chemical reaction diagram]

**Scheme 4.3.** Synthesis of the hydrophobic crosslinkable moiety.

The synthesis of the azide 13 is shown in Scheme 4.3 and was started from the commercially available 6-mercaptohexanol 18, which was functionalized and at the same time protected with 2,2'-dipyridyl disulfide 19, in acidic conditions,\(^2\) to get 6-(2-dithiopyridyl)hexanol 20. The terminal hydroxyl in 20 was functionalized into the mesylate 21 by reaction with mesyl chloride (MsCl). The mesyl group is a good leaving group and was substituted by an azide group to give the hydrophobic azide incorporating a crosslinkable moiety 13.

![Chemical reaction diagram]

**Scheme 4.4.** Modification of the ligand MTX.
The modifications made to the ligand MTX are shown in Scheme 4.4. After attempting to solubilize MTX in solvents such as $N,N$-dimethylformamide (DMF), dimethylsulfoxide (DMSO), methanol (MeOH) and mixtures of the solvents, MeOH/DMF, NMP/DMF, only $N$-methyl-2-pyrrolidone (NMP) was suitable to accomplish the reaction.\(^{23}\) For that, the carboxylic acid groups of MTX 22 were reacted in an amide coupling with the amino group of 2-(2-aminoethoxy)ethanol aided by the coupling agent HATU and Hüning's base ($N,N$-diisopropylethylamine, DIPEA)\(^{25}\) to get the more soluble modified ligand 23.

As mentioned before, to try to incorporate the ligand 23 to the structure of the dendron with the least possible variation in the HLB of the dendron, we decided to conjugate the ligand to the benzylic position of the dendron. For that, dendron 12 was reacted with triflyl chloride (TfCl) to afford 24,\(^{26}\) as shown in Scheme 4.5. Then, the

\[ \text{Scheme 4.5. Attaching the ligand to a G1 dendron at the benzylic position.} \]
ligand 23 substituted the benzylic position of the dendron aided by DBU, yielding dendron 25, which was clicked with the hydrophobic moieties 13 to afford the final target 26.

### 4.3.2 Self-Assembly and Crosslink

The self-assembly properties of dendron 14 in water were studied using DLS, with a dendron concentration of 25 µM, which is above the typical CAC found for these dendrons (2-11 µM). The size of the assemblies formed by this dendron is around 142 nm. The size of the initial aggregate is represented in Figure 4.5 as the curves in red color. The size discrimination by number of particles is consistent with the discriminations in volume and intensity, showing that the distribution of the nanoparticulates oscillates around a single size of dendritic assembly.

![Figure 4.5](image)

**Figure 4.5.** Size of the dendritic aggregates, before and after crosslink. a) distribution by number shows only one size of assembly, b) distribution by volume is consistent with the number %, c) distribution by intensity is also in the distribution range of number %.

To crosslink the assembly, we used a known reducing agent, dithiothreitol (DTT). In this experiment, DTT plays the same role that glutathione (GSH) would play in the intracellular environment, reducing the disulfide bonds and cleaving the pyridothione,27,28 which is a good leaving group (Scheme 4.6). The remaining
functionality, a thiol group, is attached to the backbone of the dendron by means of a triazol ring. The reactivity of the thiol groups to form disulfide bonds is the basis for the crosslinking reaction here.

The size of the crosslinked dendritic self-assembly distributes around 143 nm, which is 9 nm smaller than the size of the aggregate before crosslinking. The shrinking size of the nanoparticles is evidence that crosslink of the assembly indeed happened. More evidence that the nanoparticle could have crosslinked is the formation of the product from the reaction between DTT and the PDS moieties of the dendron, pyridothione. Pyridothione is a water soluble molecule that absorbs at 343 nm, making it possible to quantify its presence in the dendron solution upon reaction with DTT. Using UV-Vis spectroscopy, to monitor pyridothione absorbance, it was possible to quantify the degree of reaction of the PDS functionalities of the dendron as up to 63%, relative to the percentage of cleavage achieved when the dendron is exposed to a great excess of DTT during 48 hours, as shown in Figure 4.6.
To be able to quantify the PDS groups cleavage when the dendron and DTT were in solution in a molar ratio 1:3, which is equivalent to a ratio PDS:DTT 1:1 for the three PDS units per G1 dendron, the sample was exposed to an excess of DTT in such a way that the ratio PDS:DTT was increased to 1:20. After 48 hours of this reaction, the absorbance detected was taken as a relative 100% of the PDS reacted. Since the pyridothione molar absorptivity is known ($\varepsilon = 8.08 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$ at 343 nm), we can use it to calculate, theoretically, the 100% of 75 µM PDS that reacted (dendron at 25 µM x 3 PDS groups per dendron) simply by using the Beer-Lambert law. From that, it was found that only 74% of the PDS units were cleaved when the DTT excess was 20:1 respect the PDS groups. Based on this calculation, the percentage of crosslinking shown in Figure 4.6 is close to 50% for the ratio PDS to DTT 1:1.

**Figure 4.6.** Cleavage of pyridothione tracked by UV-Vis spectroscopy.
Next, to test if the equilibrium dendron-aggregate has been shifted towards the aggregate stage, the dendron self-assembly was exposed to a solution of water:methanol 50% and the change in size was observed through DLS. In general, methanol is a solvent in which these dendrons are solubilized, hence any aggregate would be expected to promptly dissociate in presence of this solvent. As shown in Figure 4.7, the size of the self-assembly increased from 142 to 332 nm for the non-crosslinked self-assembly, an increase in more than twice the initial size of the assembly that indicates swelling of the aggregate and a disruption in the non-covalent forces that held the assembly together. However, when the crosslinked dendritic assembly was exposed to the same conditions, water:methanol 1:1, the size increased only from 142 to 182 nm. The large difference in size between crosslinked dendritic assembly and G1 dendron in 50% methanol is also evidence of the crosslinking taken place and being effective in holding together the self-assembly.

**Figure 4.7.** DLS measurements of the stability of the assemblies in 50% methanol. a) distribution by number, b) distribution by volume, c) distribution by intensity.
The dendritic aggregates were also studied by transmission electron microscopy (TEM) before and after exposure to DTT. The TEM images of G1 and G1 crosslinked are shown in Figure 4.8. Before crosslinking, the images show more spherical shapes for the aggregates; after crosslinking, the shapes of the nanoparticulates became more oval in general. However, the size does not seem to change when going from non-crosslinked to crosslinked, as it was observed by DLS although the size does seem to be slightly larger in the images than what was observed by DLS.

Figure 4.8. TEM images of non-crosslinked dendritic aggregates. Panels (a) and (b); and crosslinked aggregates, panels (c) and (d). The panels come from different samples.
4.4 Summary

In summary, the functionalization of a dendron structure with hydrophobic moieties containing PDS can be easily performed at a later step of the synthesis through a reaction such as alkyne-azole click chemistry, which is orthogonal to the reactive functional groups in both the dendron and hydrophobic moiety.

We found that assemblies of PEG-PDS dendrons can be formed in aqueous solution. However, due to a higher inherent hydrophobicity, these dendrons cannot be deprived of any PEG chain for the attachment of a ligand on the hydrophilic face of the dendron. For that matter, the attachment of a ligand at the benzylic position of the backbone of the dendron was successfully achieved via a triflate leaving group. On the other hand, the solubility and hydrophilicity of the ligand MTX was accomplished by functionalization with a short aminodiethylene glycol chain.

Crosslinking of the PDS functionalities in the dendritic aggregates, compared to the non-crosslinked samples, gave the nanoparticulates more stability when they were exposed to a mixture 1:1 of water and methanol. Although only half of the PDS functionalities reacted, that was enough to confer the nanostructures more stability, potentially locking the inherent equilibrium of these amphiphilic dendrons in the self-assembly stage and preventing the leakage of encapsulated guest molecules.
4.5 Experimental Section

4.5.1 Synthetic Procedures and Characterization of Compounds

All chemicals and solvents were purchased from commercial sources and were used as such, unless otherwise mentioned. $^1$H NMR spectra were recorded on a 400 MHz Bruker NMR spectrometer using the residual proton resonance of the solvent as the internal standard. Chemical shifts are reported in parts per million (ppm). When peak multiplicities are given, the following abbreviations are used: s, singlet; bs, broad singlet; d, doublet; t, triplet; m, multiplet. $^{13}$C NMR spectra were proton decoupled and recorded on a 100 MHz Bruker spectrometer using the carbon signal of the deuterated solvent as the internal standard. Fluorescence spectra were recorded using a JASCO FP-6500 spectrofluorimeter. FAB-MS spectra were measured on a JEOL JMS700. MALDI-TOF spectra were measured on a Bruker Omniflex. IR spectra were measured on a Bruker Alpha-P FT-IR.

4.5.1.1 General Procedure for the Alkylation of Phenolic Hydroxyls

The phenolic hydroxyl group (1.0 equiv.) was solubilized in anhydrous acetone along with the benzyl bromide (1.0 equiv. for mono-alkylation and 2.20 equiv. for dialkylation). The base $\text{K}_2\text{CO}_3$ (2.0-3.0 equiv.), and 18-crown-6 (0.05-0.1 equiv.) were added. The reaction mixture was refluxed under argon atmosphere for 12-48 hours. The progress of the reaction was monitored by TLC. After completion of the reaction, the solvent was evaporated and the crude reaction mixture mixed with water and extracted with dichloromethane or ethyl acetate three times. The combined organic layer was
dried over anhydrous Na$_2$SO$_4$ and evaporated to dryness. The crude product was purified by column chromatography using silica gel as stationary phase.

4.5.1.2 General Procedures for “Click” Chemistry

To a solution of dendritic triacetylene compound (1.0 equiv.), and PDS-azide 13 (4 equiv.) in THF, was added the same volume of aqueous CuSO$_4$.5H$_2$O (1.0 equiv.) and sodium ascorbate (2.0 equiv.) in such a way that the final solution THF/H$_2$O was in a ratio 1:1. The reaction stirred at room temperature. After completion of the reaction, NH$_4$Cl solution was added to the reaction mass and then, extracted with ethyl acetate three times. The organic layers were collected and dried over anhydrous Na$_2$SO$_4$, filtered, concentrated, and the product purified by silica gel column chromatography.

4.5.1.3 Synthesis of compound 2 (DA-2-171)

The aromatic ester 1 (21 g, 80 mmol) was dissolved in 450 mL of dichloromethane along with the imidazole (5.50 g, 80 mmol). The temperature of the solution was reduced with an ice bath. The acetyl chloride was dissolved separately in 60 mL of dichloromethane and then added dropwise to the ester and base solution, stirring and under Ar atmosphere in the ice bath for 1 hour. After 12 hours stirring the reaction mass was washed two times with saturated NaHCO$_3$ solution. The organic layer was dried on Na$_2$SO$_4$, filtered, concentrated and purified by silica gel column
chromatography to get the product 2 (13.21 g, 44%). $^1$H NMR (CDCl$_3$-400MHz) $\delta$ 7.31 (s, 1H, a), 7.12 (s, 1H, b), 5.67 (bs, 1H, c), 4.35 (q, $J = 7.1$ Hz, 2H, d), 1.38 (t, $J = 7.1$ Hz, 3H, e), 1.05 (s, 9H, f), 0.28 (s, 6H, g).

4.5.1.4 Synthesis of compound 3 (DA-2-189)

The monoprotected ester 2 (11.4 g, 30.37 mmol) was dissolved in 125 mL of dichoromethane, after adding triethylamine (6.14 g, 60.75 mmol) the temperature of dropped by means of an ice bath and the acetyl chloride (4.8 g, 60.75 mmol) was added. The reaction was left stirring under Ar atmosphere for 18 h. Then, it washed two times with saturated NaHCO$_3$ solution. The aqueous layers were collected and extracted with 125 mL of dichloromethane, once. The organic layers were dried on Na$_2$SO$_4$, filtered, concentrated and purified by silica gel column chromatography to get the product 3 (10.95 g, 87%). $^1$H NMR (CDCl$_3$-400MHz) $\delta$ 7.42 (d, $J = 1.8$ Hz, 1H, a), 7.40 (d, $J = 1.9$ Hz, 1H, b), 4.35 (q, $J = 7.1$ Hz, 2H, d), 2.36 (s, 1H, c), 1.38 (t, $J = 7.1$ Hz, 3H, e), 1.05 (s, 9H, f), 0.29 (s, 6H, g). $^{13}$C NMR (CDCl$_3$-100MHz) $\delta$ 168.3, 165.1, 154.1, 149.5, 130.5, 117.8, 117.0, 61.4, 25.6, 20.8, 18.3, 14.2, 4.3. FAB-MS for C$_{17}$H$_{25}$BrO$_5$Si: m/z (r.i.) 417 (M$^+$, 39), 375 (40), 361 (83), 359 (80), 331 (28), 319 (25).
4.5.1.5 Synthesis of compound 5 (DA-3-43)

The solvent was degasified by bubbling Ar through it for 1 hour. The reagents 3 (6.91 g, 16.56 mmol) and 4 (7.32 g, 15.02 mmol) were placed in a 100 mL round-bottom-flask (RBF) that was also purged with Ar for 15 min. 50 mL of DMF were transferred with a syringe to the RBF with the reagents. The solution was heat to 110 °C. Then, the catalyst, bis(triphenylphosphine)palladium(II) dichloride (0.43 g, 0.61 mmol), was quickly added. The reaction was heat at 110-120 °C and stirred with Ar atmosphere for 24 hours. Then, the DMF was evaporated and the remaining mass was washed with 150 mL of ethyl acetate and filtered. The organic solution was washed with water (2 x 100 mL) and brine. The organic layer was dried on Na$_2$SO$_4$, filtered, concentrated and purified by silica gel column chromatography in CombiFlash to get the stille coupling product 5 (52.15 g, 27%). $^1$H NMR (CDCl$_3$-400MHz) δ 7.45 (d, $J = 1.6$ Hz, 1H, a), 7.42 (d, $J = 1.5$ Hz, 1H, b), 6.70 (t, $J = 2.3$ Hz, 1H, c), 6.61 (d, $J = 2.3$ Hz, 2H, h), 5.13 (s, 4H, i), 4.37 (q, $J = 7.1$ Hz, 2H, d), 3.46 (s, 6H, j), 2.04 (s, 3H, k), 1.39 (t, $J = 7.1$ Hz, 3H, e), 0.75 (s, 9H, f), 0.04 (s, 6H, g). FAB-MS for C$_{27}$H$_{38}$O$_8$Si: m/z (r.i.) 535 (M$^+$, 50), 503 (35), 477 (100), 447 (50), 373 (40), 345 (18).
4.5.1.6 Synthesis of compound 6 (DA-3-13)

Compound 5 (2.12 g, 3.97 mmol) was solubilized in 35mL of anhydrous. Stirring, under Ar atmosphere, and with an ice bath, the reducing agent LiAlH₄ (0.75 g, 19.85 mmol) was added in portions. After complete addition, the reaction was allowed to reach room temperature. After 24 hours stirring the reaction mass was quenched by adding 50 mL of ethyl acetate at 0 °C and stirring for a 10 min. The solvent was evaporated to again, add ethyl acetate and water, extracting the aqueous solution three times with ethyl acetate. The organic layers were collected and dried over Na₂SO₄. The solvent was evaporated and the product purified through column chromatography to get 6 (0.86 g, 48%). ¹H NMR (CDCl₃-400MHz) δ 6.74 (t, J = 2.3 Hz, 1H, c), 6.67 (d, J = 2.3 Hz, 2H, h), 6.63 (m, 1H, a), 6.49 (m, 1H, b), 5.16 (s, 4H, i), 4.61 (s, 2H, d), 3.46 (s, 6H, j), 0.74 (s, 9H, f), 0.07 (s, 1H, e), -0.01 (s, 6H, g). FAB-MS for C₂₃H₃₄O₇Si: m/z (r.i.) 451 (M⁺, 44), 433 (42), 393 (52), 331 (23).

4.5.1.7 Synthesis of compound 7 (DA-3-32)
Compound 6 (0.73 g, 1.62 mmol), propargyl bromide (0.39 g, 3.23 mmol), K$_2$CO$_3$ (0.45 g, 3.23 mmol), and 18-crown-6 (0.09 g, 0.32 mmol) were refluxed in 15 mL of acetone, as described in the general procedure for alkylations getting the product 7 after purification by chromatography (0.20 g, 25%). $^1$H NMR (CDCl$_3$-400MHz) $\delta$ 6.77 (d, $J$ = 1.2 Hz, 1H, a), 6.68 (m, 1H, c), 6,67 (d, $J$ = 2.0 Hz, 2H, h), 6.58 (m, 1H, b), 5.15 (s, 4H, i), 4.67 (d, $J$ = 5.4 Hz, 2H, d), 4.61 (d, $J$ = 2.4 Hz, 2H, n), 3.46 (s, 6H, j), 2.45 (t, $J$ = 2.4 Hz, 1H, k), 1.67 (t, $J$ = 5.87 Hz, 1H, e), 0.75 (s, 9H, f), 0.02 (s, 6H, g).

4.5.1.8 Synthesis of compound 8 (DA-3-35)

Compound 7 (0.2 g, 0.41 mmol) was dissolved in anhydrous THF, to which TBAF (0.43 g, 1.63 mmol) was added. The reaction was left stirring and in Ar environment at room temperature for 12 hours. THF was evaporated with air flow at room temperature. Then, 20 mL of NH$_4$Cl solution were added. The aqueous solution was extracted with ethyl acetate. The organic layers were collected and dried over Na$_2$SO$_4$. The solvent was evaporated and the product 8 was purified through column chromatography (0.15 g, 94%). $^1$H NMR (CDCl$_3$-400MHz) $\delta$ 6.78 (t, $J$ = 2.3 Hz, 1H, c), 6.71 (d, $J$ = 1.2 Hz, 1H, b), 6.70 (d, $J$ = 2.3 Hz, 2H, h), 6.68 (m, 1H, a), 5.17 (s, 4H, i), 4.68 (s, 2H,
d), 4.64 (d, \( J = 2.4 \) Hz, 2H, n), 3.48 (s, 6H, j), 2.46 (t, \( J = 2.4 \) Hz, 1H, k), 1.71 (bs, 1H, e).

FAB-MS for \( \text{C}_{20}\text{H}_{22}\text{O}_{7} \): m/z (r.i) 374(M⁺, 100), 329(53), 297(72), 267(50), 69(85).

4.5.1.9 Synthesis of compound 9 (DA-3-37)

According to the procedure for alkylation of phenolic hydroxyls, the biaryl 8 (0.14 g, 0.38 mmol) was mixed with the tosylate of pentaethylene glycol monomethyl ether PEG-Ts (0.16 g, 0.38 mmol), \( \text{K}_{2}\text{CO}_{3} \) (0.16 g, 1.15 mmol), and 18-crown-6 (0.02 g, 0.08 mmol) in anhydrous acetone and refluxed, stirring and under Ar atmosphere. After purification, 9 was obtained (0.16 g, 68%). \(^{1}\text{H} \) NMR (CDCl₃-400MHz) \( \delta \) 6.78 (s, 1H, b), 6.74 (s, 1H, a), 6.73 (d, \( J = 2.3 \) Hz, 2H, h), 6.68 (t, \( J = 2.3 \) Hz, 1H, c), 5.16 (s, 4H, i), 4.70 (s, 2H, d), 4.62 (d, \( J = 2.4 \) Hz, 2H, n), 4.08 (m, 2H, f), 3.71 (m, 2H, g), 3.61 (m, 11H, r), 3.53 (m, 5H, r), 3.48 (s, 6H, j), 3.37 (s, 3H, p), 0.07 (s, 1H, e). \(^{13}\text{C} \) NMR (CDCl₃-100MHz) \( \delta \) 157.5, 157.1, 155.6, 154.0, 142.1, 141.0, 135.5, 130.0, 119.6, 112.8, 111.7, 105.3, 104.9, 104.5, 103.7, 94.7, 78.8, 75.4, 71.9, 70.8, 70.7, 70.6, 70.5, 70.5, 69.6, 68.9, 68.8, 65.3, 59.0, 56.4, 56.0.
4.5.1.10 Synthesis of compound 10 (DA-3-39)

Compound 9 (0.16 g, 2.61 mmol) was dissolved in 10 mL of methanol, 1 mL of 1,4-dioxane, and 0.5 mL of water. Dowex resin was added (0.48 g) and the reaction was set to reflux, stirring and under Ar atmosphere. The mixture reacted for 4 hours. Upon completion of the reaction the solvents were evaporated and the remaining mass washed with acetone and filtered. The acetone was evaporated and 10 mL of water added to remaining mass, to extract with ethyl acetate (3 x 10 mL). The organic layers were dried over Na$_2$SO$_4$. The organic solution was filtrated and concentrated. After purification, product 10 was got (0.12 g, 90%). ESI-MS m/z calculated for C$_{27}$H$_{36}$O$_{10}$ + H$^+$: 520.57; found: 1061.0 for (2(C$_{27}$H$_{36}$O$_{10}$) + Na)$^+$, 543.2 for (C$_{27}$H$_{36}$O$_{10}$ + Na)$^+$.

Additional characterization details for compound 10 are found in reference 29.

4.5.1.11 Synthesis of compound 12 (DA-3-40)
According to the procedure for the alkylation of phenolic hydroxyls, compound 10 (0.12 g, 0.24 mmol), compound 11 (0.25 g, 0.52 mmol), K₂CO₃ (0.16 g, 1.18 mmol), and 18-crown-6 (0.03 g, 0.12 mmol) were mixed in anhydrous acetone and refluxed, stirring under Ar atmosphere. After purification, compound 12 was obtained (0.19 g, 62%). \(^1\)H NMR (CDCl₃-400MHz) δ 6.78 (s, 1H, b), 6.76 (s, 1H, a), 6.64 (d, \(J = 2.1\) Hz, 2H, c), 6.63 (d, \(J = 2.0\) Hz, 2H, d), 6.60 (d, \(J = 2.3\) Hz, 2H, i), 6.55 (d, \(J = 2.3\) Hz, 1H, z), 6.49 (t, \(J = 2.2\) Hz, 2H, x), 4.97 (s, 4H, e), 4.70 (s, 2H, g), 4.66 (d, \(J = 2.4\) Hz, 4H, w), 4.57 (d, \(J = 2.3\) Hz, 2H, f), 4.12-4.10 (m, 4H, h), 4.06-4.04 (m, 2H, i), 3.85-3.82 (m, 4H, j), 3.72-3.70 (m, 5H, k), 3.68-3.56 (m, 37H, k), 3.55-3.51 (m, 13H, k), 3.37 (s, 6H, u), 3.36 (s, 3H, y), 2.52 (d, \(J = 2.4\) Hz, 2H, v), 2.47 (d, \(J = 2.3\) Hz, 1H, n). \(^{13}\)C NMR (CDCl₃-100MHz) δ 160.1, 159.0, 158.8, 157.1, 155.6, 142.2, 139.7, 135.6, 120.0, 110.3, 106.7, 106.2, 105.5, 105.0, 101.3, 101.2, 78.9, 78.4, 77.2, 75.8, 75.6, 71.9, 71.9, 70.8, 70.6, 70.6, 70.5, 70.5, 70.5, 69.6, 67.5, 65.3, 59.0, 59.0, 56.4, 56.0, 53.4. ESI-MS m/z calculated for C₆₉H₉₆O₂₄ + H⁺: 1311.0; found: 1311.0 for (C₂₇H₃₆O₁₀ + H)⁺.
4.5.1.12 Synthesis of compound 14 (DA-3-41)

According to the procedure for click chemistry, compound 12 (185 mg, 0.145 mmol), compound 13 (124 mg, 0.463 mmol), CuSO$_4$.5H$_2$O (33 mg, 0.13 mmol), and sodium ascorbate (52 mg, 0.261 mmol) were mixed in 2 mL of THF and 2 mL of water. After purification of the reaction the product 14 was obtained (51 mg, 22%). $^1$H NMR (CDCl$_3$-400MHz) δ 8.44 (d, J = 4.7 Hz, 2H, x), 8.41 (d, J = 3.8 Hz, 1H, w), 7.70-7.58 (m, 7H, a, z), 7.19 (s, 1H, y), 7.08-7.03 (m, 3H, a), 6.77 (d, J = 5.8 Hz, 2H, a), 6.67-6.49 (m, 10H, a), 5.15 (s, 4H, b), 5.09 (s, 2H, c), 4.94 (s, 4H, d), 4.69 (s, 2H, e), 4.31 (t, J = 7.2 Hz, 4H, f), 4.18-4.04 (m, 8H, f, h), 3.83-3.81 (m, 4H, g), 3.70-3.48 (m, 57H, f, g), 3.36 (s, 6H, i), 3.34 (s, 3H, j), 2.76 (t, J = 7.2 Hz, 4H, k), 2.69 (t, J = 7.2 Hz, 2H, n), 1.91-1.84 (m, 6H, p), 1.76-1.56 (m, 12H, p), 1.45-0.89 (m, 19H, p). MALDI-ToF m/z expected for C$_{102}$H$_{144}$N$_{12}$O$_{24}$S$_{6}$: 2114.69; found 2152.66 for C$_{102}$H$_{144}$N$_{12}$O$_{24}$S$_{6}$ + K$^+$, 2136.69 for C$_{102}$H$_{144}$N$_{12}$O$_{24}$S$_{6}$ + Na$^+$, 2114.73 for C$_{102}$H$_{144}$N$_{12}$O$_{24}$S$_{6}$ + H$^+$. 

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4.5.1.13 Synthesis of compound 20 (DA-3-2)

The compounds 6-mercaptoethanol 18 (1 g, 7.45 mmol) and 2-mercaptoethanol 19 (2.46 g, 11.17 mmol) were dissolved in methanol (10 mL). Stirring, and under Ar atmosphere 250 µL of glacial acetic acid were added. The reaction was left 18 hours. The solvent was evaporated and NaHCO₃ solution was added, to be extracted with dichloromethane. After purification by column chromatography CombiFlash, the product 20 was obtained (1.53 g, 85%). ¹H NMR (CDCl₃-400MHz) δ 8.46 (ddd, J = 4.9, 1.8, 0.9 Hz, 1H, a), 7.74 (dt, J = 8.1, 1.0 Hz, 1H, d), 7.66 (ddd, J = 8.1, 7.4, 1.8 Hz, 1H, c), 7.09 (m, 1H, b), 3.62 (t, J = 6.5 Hz, 2H, e), 2.79 (t, J = 7.2 Hz, 2H, f), 1.71 (dd, J = 7.2, 7.1 Hz, 2H, g), 1.54 (dd, J = 5.0, 6.7 Hz, 2H, h), 1.38 (m, 4H, i, j). ¹³C NMR (CDCl₃-100MHz) δ 160.5, 149.3, 137.3, 120.6, 119.8, 62.8, 38.9, 32.5, 28.8, 28.2, 25.3. ESI-MS m/z calculated for C₁₁H₁₇NOS₂ + H⁺: 244.39; found: 266.07 for (C₁₁H₁₇NOS₂ + Na)⁺, 244.09 for (C₁₁H₁₇NOS₂ + H)⁺.

4.5.1.14 Synthesis of compound 21 (DA-3-4)

Compound 20 (4.67g, 19.20 mmol) and triethylamine (3.88 g, 38.39 mol) were solubilized in 120 mL of anhydrous THF. Under Ar atmosphere and stirring, the mesyl chloride (4.40 g, 38.39 mmol) was added. The reaction was left overnight, the THF was evaporated without heating the flask, water was added to the remaining mass, and then
extracted with dichloromethane. The organic layers were dried on Na₂SO₄, filtrated, concentrated, and purified through column chromatography to get product 21 (5.51 g, 90%). \(^1\)H NMR (CDCl₃-400MHz) δ 8.47 (ddd, J = 4.9, 1.7, 0.9 Hz, 1H, a), 7.74 (s, 1H, d), 7.72 (s, 1H, c), 7.67 (ddd, J = 7.7, 1.8 Hz, 1H, b), 4.20 (t, J = 6.5 Hz, 2H, f), 3.00 (s, 3H, k), 2.80 (t, J = 7.3 Hz, 2H, e), 1.77-168 (m, 4H, g, h), 1.46-1.38 (m, 4H, i, j). \(^{13}\)C NMR (CDCl₃-100MHz) δ 160.4, 149.3, 137.3, 121.0, 119.9, 69.8, 38.7, 37.4, 29.0, 28.6, 27.8, 25.0. ESI-MS m/z calculated for C₁₂H₁₉NO₃S₃ + H⁺: 322.48; found: 344.05 for (C₁₂H₁₉NO₃S₃ + Na)⁺, 322.06 for (C₁₂H₁₉NO₃S₃ + H)⁺.

4.5.1.15 Synthesis of compound 13 (DA-3-6)

![Image of compound 13]

Compound 21 (6.11 g, 19.00 mmol) was solubilized in 30 mL of anhydrous DMF mixing it with NaN₃ (1.85 g, 28.50 mmol). Stirring and under Ar atmosphere the reaction was left for 24 h. Then, more NaN₃ was added and left reacting 6 more hours. Afterwards, 250 mL of water were added to next extract with diethyl ether (3 x 100 mL). The organic layers were dried on Na₂SO₄, filtered and purified by column chromatography to get the product 13 (4.38 g, 86%). \(^1\)H NMR (CDCl₃-400MHz) δ 8.46 (ddd, J = 4.9, 1.7, 0.8 Hz, 1H, a), 7.72 (dt, J = 8.1, 0.9 Hz, 1H, d), 7.65 (m, 1H, c), 7.30 (ddd, J = 7.3, 4.9, 1.1 Hz, 1H, b), 3.24 (t, J = 6.9 Hz, 2H, f), 2.80 (m, 2H, e), 1.71 (dt, J = 14.7, 7.2 Hz, 2H, g), 1.58 (dt, J = 14.24, 7.0 Hz, 2H, h), 1.45-1.36 (m, 4H, i, j). \(^{13}\)C NMR
(CDCl$_3$-100MHz) $\delta$ 160.5, 149.4, 137.1, 120.6, 119.7, 51.3, 38.8, 28.7, 28.7, 27.9, 26.3.
FAB-MS m/z calculated for C$_{11}$H$_{16}$N$_4$S$_2$ + H$^+$: 269.2.

4.5.1.16 Synthesis of compound 23 (DA-3-132)

MTX Compound 22 (0.050 g, 0.11 mmol) was mixed with the 2-(2-aminoethoxy)ethanol (0.058 g, 0.55 mmol) in NMP (2 mL). Followed by the addition of DIPEA (0.071 g, 0.55 mmol). HATU (0.188 g, 0.495 mmol) was previously dissolved in NMP (2 mL) and this solution was poured into the MTX solution. Stirring and under Ar atmosphere, the reaction was left to complete for 5 days at room temperature. Diethyl ether was added to the reaction mass to precipitate a brown oil, which was washed once more with diethyl ether. The solution was separated from the oil, which was washed two more times with dichloromethane. The organic solution was separated from the oil. Then, the oil was mixed with acetone and sonicated. The product 25 (0.017 g, 25%) precipitated as a yellow solid that was vacuum filtered and dried. $^1$H NMR (DMSO-d$_6$-400MHz) $\delta$ 8.55 (d, $J = 5.1$, Hz, 1H, a), 7.73 (d, $J = 9.0$ Hz, 2H, b), 6.82 (d, $J = 8.9$ Hz, 2H, c), 6.61 (s, 2H, y), 4.78 (s, 2H, d), 4.59 (s, 2H, z), 4.31 (dd, $J = 13.6$, 8.4 Hz, 1H,
e), 3.60-3.35 (m, 16H, h), 3.21 (s, 3H, w), 2.17-2.11 (m, 2H, g), 2.10-2.08 (m, 2H, f). ESI-MS m/z calculated for $\text{C}_{28}\text{H}_{40}\text{N}_{10}\text{O}_{7} + \text{H}^+$: 329.68; found: 329.32 for ($\text{C}_{28}\text{H}_{40}\text{N}_{10}\text{O}_{7} + \text{H}$)⁺.

**4.5.1.17 Synthesis of compound 24 (DA-3-134)**

To a stirring mixture of dendron 12 (20 mg, 0.015 mmol) and DMAP (7.5 mg, 0.061 mmol) in anhydrous dichloromethane (1.2 mL), was added Tf-Cl (7.7 mg, 0.046 mmol) inside the previous solution. After 48 h the reaction was stopped, more dichloromethane (10 mL) and brine (10 mL) were added. It was extracted three times with dichloromethane. The organic extracts were dried over anhydrous Na₂SO₄. The solution was purified by flash chromatography to get the pure product 24 (7.47 mg, 34%). $^{19}\text{F}$ NMR (CDCl₃-300MHz) δ -78.26 (3F).
4.5.1.18 Synthesis of compound 25 (DA-3-136)

The dendron 24 (7.47 mg, 5.18 µmol) was dissolved in dichloromethane (1 mL), in a vial. The ligand 23 (3.26 mg, 5.18 µmol) was added followed by a drop of DBU (10 mg, 77.7 µmol). Stirring, the reaction was left in the capped vial. After 65 hours, water was added (2 mL), and then, extracted three times with dichloromethane. Then, the aqueous solution was saturated with NaCl to be extracted three more times with dichloromethane. The organic layers were dried over anhydrous Na₂SO₄. After purification by flash chromatography, the product 25 was obtained (3 mg, 30%). ¹H NMR (DMSO-d6-400MHz) δ 8.56 (d, J = 5.1 Hz, 1H, a) 7.73 (d, J = 8.9 Hz, 2H, b), 6.83-6.49 (m, 11H, c), 4.98 (s, 4H, d), 4.77 (s, 2H, e), 4.68-466 (m, 6H, f, g), 4.59-4.57 (m, 4H, h), 4.34-4.29 (m, 2H, i), 4.12-4.04 (m, 9H, j, k), 3.73 (m, 6H, j, k), 3.58-3.35 (m, 80H, j, k), 3.22-3.21 (m, 16H, n, p, j), 2.60-2.45 (m, 3H, x). MALDI-ToF m/z expected for C₉₇H₁₃₄N₁₀O₃₀: 1920.15; found 1943.70 for C₉₇H₁₃₄N₁₀O₃₀ + Na⁺.
4.5.1.19 Synthesis of compound 26 (DA-3-138)

According to the procedure for click chemistry, compounds 25 (3 mg, 1.56 µmol) and compound 13 (2.52 mg, 9.37 µmol) were mixed in presence of CuSO₄·5H₂O (0.58 mg, 2.34 µmol) and sodium ascorbate (0.58 mg, 2.34 V) to get the target dendron 26 (1.0 mg, 23%). ¹H NMR (DMSO-d6-400MHz) δ 8.55 (s, 1H, a), 8.43-8.41 (m, 3H, b), 7.82-7.61 (m, 9H, c), 7.22-7.19 (m, 4H, c), 6.86 (s, 1H, c), 6.71 (s, 1H, c), 6.67 (s, 2H, c), 6.61 (s, 2H, c), 6.57 (m, 2H), 6.53 (m, 3H, c), 5.10 (s, 4H, d), 5.06 (s, 2H, e), 4.94 (s, 4H, f), 4.78 (s, 2H, g), 4.50 (d, J = 5.6 Hz, 2H, h), 4.31 (t, J = 5.6 Hz, 4H, h), 4.33-4.30 (m, 3H, i), 4.06-3.90 (m, 5H, i, h), 3.71 (m, 5H, h), 3.57-3.35 (m, 65H, h), 3.20-3.18 (m, 11H, k), 2.80 (t, J = 7.1 Hz, 4H, n), 2.74 (t, 7.2 Hz, 2H, p), 1.78-1.03 (m, 34H, h, q).
4.5.2 Determination of Size of Self-Assembly and Crosslinked Nanoparticulates

4.5.2.1 Preparation of G1 Dendron Aggregates and DLS Measurements

The samples of dendrons were prepared in solutions of 25 \( \mu \text{M} \) and sonicated for 2 hours or until the solution looked clear. Next, the samples were stirred for 2 hours at room temperature before the DLS measurements were taken. The samples were not micro-filtered unless DLS revealed aggregate sizes higher than 1000 nm in diameter.

4.5.2.2 Preparation of Crosslinked Aggregates

A sample of dendron 25 \( \mu \text{M} \) in solution was taken a UV-Vis spectrum at 343 nm and then, since this dendron concentration makes a PDS concentration of 75 \( \mu \text{M} \), the sample was exposed to 75 \( \mu \text{M} \) of DTT by adding the corresponding microliters from a DTT stock solution 2 mM. After exposure to the crosslinking agent, the UV-Vis spectrum of the sample was taken. The absorbance at 343 nm was measured after 28 hours to verify cleavage of pyridothione groups.

With the objective of cleaving all the PDS functionalities in the dendron, a second sample with the same concentration of dendron as before was exposed to an excess concentration of DTT in such a way that the ratio PDS:DTT was at least 1:20. UV-Vis spectra were taken before and after exposure to DTT to check the highest absorbance for the sample.
4.5.2.3 DLS Measurements for Crosslinked Aggregates and Exposure to Organic Solvent

The samples from the crosslinking procedure were analyzed by DLS to make sure that the particulate size remains mono-dispersed. Micro-filtered was not necessary unless there were particulates with sizes larger than 1000 nm. However, the samples were micro-filtered before exposure to methanol. For that, 500 µL of the crosslinked dendron sample at 25 µM were diluted to 12.5 µM by adding 500 µL of methanol. The samples were allowed to equilibrate before DLS measurements.

4.6 References

Notes


5.1 Introduction

This thesis focused mainly on the importance of ligand availability for supramolecular interactions between macromolecules, such as proteins and small molecules or between proteins and synthetic macromolecules, such as dendrons. Using the knowledge on protein-ligand interactions that has been reported up to date, we chemically modified a few ligands with the aim of, upon binding to a specific protein, incorporating in our systems some of the characteristics shown by those proteins such as hydrophilic surface, globular shape, deep binding pockets, strong non-covalent interactions, etc.

An interesting and innovative system for screening of small molecules against a target protein was described in Chapter 2. There, we used the binding pocket of hCA1 to hinder, upon binding of a probe bearing an enzyme cleavable bond, the action of an enzyme on the probe. Such action would cleave and activate an otherwise silent fluorophore. The probe is displaced out of the protein binding pocket and made available for the enzyme, only in the presence of a small molecule (competitor) with affinity for the binding site. This concept could be used in the screening of small molecules looking for “hits”, and also to find relative binding constants for protein-ligand systems that are difficult to deal with using traditional methods.
In Chapter 3, we took advantage of the ligand-protein interaction and great binding affinity of the system biotin-extravidin, to study the best location to install a hydrophilic ligand on a dendron. Given the self-assembly property of our biotin-modified dendrons in aqueous solution, we encapsulated a fluorescent probe and studied dendron-disassembly and release efficiency upon protein binding. We found that installing a ligand at the periphery of these dendrons makes the ligand more available for the protein in solution, which gives better release profiles than when the ligand is tethered to the other locations.

Based on the knowledge gained from studying these supramolecular systems, in Chapter 4 we proposed the synthesis of a dendron modified with a particular ligand for targeting the intracellular enzyme DHFR, and endowed the dendron with redox-sensitive crosslinkable functionalities with the objective of de-crosslink in the intracellular reducing environment, and disassembly in the intracellular milieu upon protein binding.

Based on the research described in this thesis, there are some modifications that would be as interesting as pertinent to accomplish and that could give a deeper insight into the systems introduced by our group.

5.2 Supramolecular Displacement Conditions for Analytical Applications

As shown in Chapter 2, the length of the probe linker influenced the availability of the ligand for the enzyme. However, the shallowness or deepness of the binding pocket will have similar impact on such availability. To better understand this, and in
order to propose a better and more general probe design aimed to target a wider range of binding sites, it would be useful to study a few more systems.

An interesting target protein for this study is the protein β-lactoglobulin, which has a binding pocket that could bind either retinol at the rim of the binding site or a fatty acid at the bottom of the site. Results suggesting that bound retinol is more exposed to the environment make a retinol-based probe an interesting molecule to test. As shown in Figure 5.1, a retinol-based probe has a long linker that leaves the enzyme cleavable bond more exposed to the solvent than in the probes for hCA1, as shown by our preliminary results (Figure 5.2). Despite the good binding affinity of retinoic acid to β-lactoglobulin (39 nM), a probe synthesized with a linker between retinoic acid and 4-methylumbelliferone was not effectively protected by β-lactoglobulin from PLE cleavage. In our preliminary results, as shown in Figure 5.2, 125 µM of β-lactoglobulin was not enough to hinder esterase action on the probe linker. Compare to the 20 µM of hCA1 needed in Chapter 2 to protect Probe A, which means that further experiments

Figure 5.1. Retinoic acid- and fatty acids- based probe for β-lactoglobulin
are required to find the proper conditions to hinder enzymatic action on the β-lactoglobulin-probe complex.

![Graph](image)

**Figure 5.2.** Finding β-lactoglobulin (B-LG) concentration for esterase (PLE) activity inhibition. Probe at 5 μM, PLE at 50 nM.

However, palmitic acid has been found to coil in the β-lactoglobulin binding site.\(^3\)\(^4\) Hence, a probe based on the fatty acid could present a better inhibition profile than a retinol-based probe. Additionally, given the high propensity of hydrophobic small molecules to bind this target protein, it would be interesting to compare a hexanoic acid based probe to the previous two probes in terms of linker length and hindrance provided by β-lactoglobulin.\(^5\)

From a different perspective, but complementary to the interest of testing probes for a protein with a shallow binding site described above, it would be relevant to test this supramolecular displacement and activation of a silent fluorescence probe method against a disease relevant protein. To address this point, a good target would be the factor-inhibiting hypoxia-inducible factor (FIH), which is known to have a binding pocket 15 Å wide and to inhibit the hypoxic responses in the cell.\(^6\) Also, the screening
assays for FIH are scarce, and the preparation of the reagents requires high levels of expertise.⁷ A probe design could consist simply of 8-hydroxyquinoline as the ligand and 4-methylumbelliferone as the reporter, both linked through an ester bond. There is a set of ligands known to bind FIH, mostly quinoline derivatives such as clioquinol, which are commercially accessible ligands that would help the method to be easily transferred to HTS.⁸ This would be an excellent fire-proof test for our screening method.

5.3 Using Supramolecular Dendron Exchange to Improve Ligand Diversity

It was demonstrated recently that the dynamics of dendron exchange between dendritic aggregates involves only assemblies formed by G1 dendrons.⁹ This means that if we were to synthesize dendrons with the objective of exchanging them in solution to form aggregates with both dendrons, our synthetic efforts should be focused only on G1 dendrons. Given the synthetic challenge that implies functionalizing biaryl amphiphilic dendrons and the risk of precipitation involved in replacing more than one PEG chain in a G1 dendron, as mentioned in Chapter 3 and Chapter 4, the dynamic exchange could be used to achieve aggregates with two different kinds of ligands, avoiding design and synthetic problems.¹⁰
In Chapter 4, we showed a dendron design that incorporates a single ligand and potentially, could target an intracellular protein. The dendron was endowed with crosslinkable functionalities that also work as hydrophobic moieties. These functionalities could be de-crosslinked as response to a reducing environment, which is found in the intracellular media. However, a crosslinked dendritic aggregate would have more chance of reaching the intracellular milieu if it could go easily through the cell membrane.\textsuperscript{11}

It would be relevant to endow a system with three different responsive features towards three different targets (i) an overexpressed protein receptor in the cell membrane, (ii) a reducing intracellular environment, and (iii) an overexpressed protein in the intracellular milieu. Figure 5.3 depicts the characteristics of a dendritic couple that
merge their properties into a single assembly. Then, the action of a reducing agent such as DTT could lock those properties in the crosslinked nanoparticulate.

The dendron design in Chapter 4 already incorporates a redox responsive feature and a ligand for intracellular targeting, methotrexate (MTX). Since MTX shares structural features with folic acid, the incorporation of the latter into the dendron structure could be achieved utilizing similar conditions. The final structure of the dendrons will only differ in the ligand moieties.

5.4 Summary

In this final chapter a short summary for the previous chapters has been presented, highlighting the main features and properties of the systems described in this thesis. As a complement to that research, general ideas about molecular designs have been presented with the potential to take further what has been done and shown in Chapter 2 and Chapter 4.

We expect that the whole set of designs and ideas explored and proposed here, will help to improve the future molecular designs, and to understand better supramolecular systems involving proteins, dendrons, polymers, and small molecules.

5.5 References

Notes


APPENDICES
BIBLIOGRAPHY


