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DOTTORATO DI RICERCA IN
SCIENZA E TECNOLOGIA DEI MATERIALI

CICLO XXXVII

Recognition of Bacterial Metabolites, Gram-negative Bacteria and their Cell Wall by Calix[4]arene-based Receptors

Coordinatore:

Chiar.mo Prof. Enrico Dalcanale

Tutore:

Chiar.mo Prof. Alessandro Casnati

Dottoranda: Eloisa Tosi

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Abstract

This thesis deals with the study of calix[4]arenes receptors, in particular their synthesis and first interaction studies with bacterial metabolites, Gram-negative bacteria and their artificial models of cell wall.

The first chapter of this thesis is a general introduction of the topic of bacteria recognition and how this can be made with supramolecular receptors, such as calix[4]arenes, or other multivalent ligands. Among the techniques that can be used to study this interaction, a focus is set on the quartz crystal microbalance, a mechanical system that is able to assess the presence of an interaction by detecting a variation in the mass that is set on the sensor surface.

Chapter 2 is about the interaction of two negatively charged calix[4]arenes with four biogenic polyamines (1,4-diaminobutane, 1,6-diaminohexane, spermidine and spermine), and the study of this interaction has been carried out via ^1H NMR titrations, resulting in a complex system with multiple equilibria occurring. To clarify the system fluorescence titrations were performed, allowing the determination of the association constants of the complexes formed.

The third chapter is about the synthesis of derivatives of two calix[4]arenes already studied in this research group, bearing four methyl-L-proline and four methyl-L-phenylalanine at the upper rim respectively. These receptors have proven to be selective ligands for Gram-negative bacteria, opening the possibility to be exploited for multiple applications. Two derivatives that have been synthesized possess alkyl chains at the lower rim, that will allow the study of the ligand properties of the macrocycle changed upon alkylation of the phenolic groups. The other two derivatives possess alkyl chains that terminate with an azide, allowing the attachment of the calixarene to the surface. The synthetic strategy employed has not led to the products, and for time reasons I was not able to find a suitable strategy. Moreover, two models of the calixarenes with a monovalent amino acid moiety have been synthesized, with an azide on the other end to allow the linkage to the surface and study another type of multivalent surface.

Chapter 4 concerns the work carried out in a six-months research period in University of Twente, the Netherlands, where quartz crystal microbalance was used to study the multivalent interactions between zwitterionic surfaces and Gram-negative bacterial membrane models. In the first model the supported lipid bilayers were used as models for the Gram-negative bacteria cell wall on the surface and solutions of methyl-L-proline and methyl-L-phenylalanine calix[4]arenes were flowed: the interaction was not detected with this system. Given that only the two monovalent calixarene models were successfully obtained, these were the sole molecules immobilized on a surface in the second approach. By subsequently introducing a vesicle solution over the modified surface, the interaction between the vesicles and the calixarene-functionalized surface was examined.

Chapter 5 is a preliminary study on a new project, the SAMBA project, where the aim is to obtain nano-objects made of polymers with different shapes, bearing units of calix[4]arenes with properties of Gram-negative bacteria ligands. This work represents a preliminary investigation into the feasibility of conducting RAFT polymerization by anchoring the RAFT agent to the lower rim of calix[4]arene. After synthesizing calixarenes functionalized with between one and four RAFT agent units, polymerization was carried out using the calixarene with two RAFT agents, yielding the polymers subsequently analyzed.

Chapter 1

Introduction

1.1 Antimicrobial resistance

Antimicrobial resistance (AMR) arises when microorganisms, such as bacteria, fungi, viruses, and parasites, develop the ability to withstand treatments that were once effective against them. This growing challenge poses a critical threat to global health, affecting countries worldwide. As infections become harder to treat with existing medicines, the future of healthcare appears increasingly uncertain. Costs implicated in AMR are currently rising in all countries, because resistant infections lead to more severe illnesses, extended hospital stays and increased medical expenses. In Europe, for example, the economic toll of AMR exceeds nine billion euros annually, and in the United States, the Centers for Disease Control and Prevention (CDC) reports that AMR contributes an additional \$20 billion in healthcare costs, alongside \$35 billion in lost productivity each year.¹

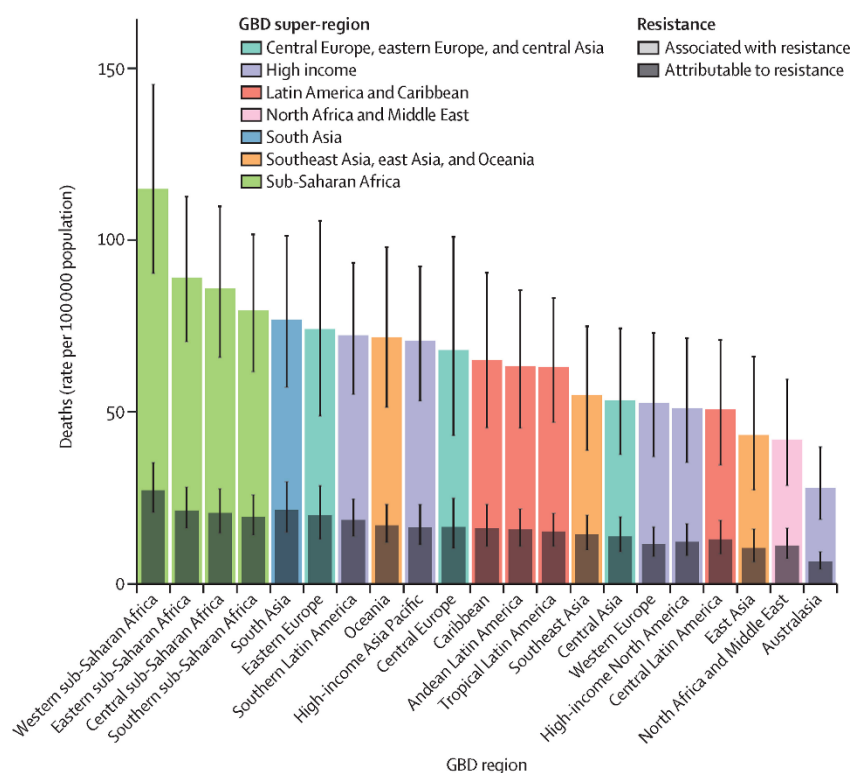


Figure 1. All-age rate of deaths attributable to and associated with bacterial antimicrobial resistance by GBD region, 2019. Estimates were aggregated across drugs, accounting for the co-occurrence of resistance to multiple drugs. Error bars show 95% uncertainty intervals. GBD=Global Burden of Diseases, Injuries, and Risk Factors Study. Figure adopted from reference

2.

A key concern within AMR is bacterial resistance to antibiotics. Over two million people in the U.S. are infected with drug-resistant bacteria annually, resulting in at least 23,000 fatalities. This resistance not only weakens the immune system capacity to fight off infections but also complicates recovery for patients undergoing treatments like dialysis, chemotherapy, and surgery.¹ Those with chronic illnesses, such as diabetes, asthma, and arthritis, are especially vulnerable as antibiotics become less effective.

Among the most notorious cases of AMR is methicillin-resistant *Staphylococcus aureus* (MRSA), a bacterium that causes numerous deaths annually, and also multidrug-resistant Gram-negative bacteria (MDR-GNB) have made treating conditions such as pneumonia and urinary infections more difficult. Resistance to medications used for tuberculosis, gonorrhea, and typhoid fever is also increasing

globally, particularly in countries like India, Russia, the Philippines, and South Africa, where cases of multidrug-resistant tuberculosis (MDR-TB) are expected to rise sharply by 2040.³

The World Health Organization (WHO) has long acknowledged the importance of a coordinated international response to combat antimicrobial resistance (AMR). In 2001, the WHO introduced its Global Strategy for Containment of Antimicrobial Resistance, offering a set of guidelines to slow the development and minimize the spread of resistant organisms.⁴

The BacHound project,⁵ in which this thesis is involved, aims at helping in overcoming this global problem by developing molecular systems that could identify in rapid, efficient and relatively cheap way pathogenic bacteria. The creation of smart chemical agents can be a useful tool to enable the rapid identification of bacterial species *in vitro*, by the selective molecular recognition of the entire bacterial cells and/or small molecules (such as bacterial metabolites) produced under normal or pathological conditions. In fact, pathogenic bacteria in the human gut can disrupt the balance of gut microbiota, undermining its crucial role in maintaining digestive health, immunity, and overall homeostasis.

1.2 Gut microbiota

Mammals host a diverse and active microbial community, known as the microbiota, with the most active and numerous populations residing in the colon, containing over 10^{13} bacterial cells.⁶ The host has evolved mechanisms to coordinate and integrate conserved pathways involved in metabolic signaling, microbial detection, and immune responses to thrive in a microbe-rich environment. This ongoing communication between the host and its microbiota is essential for maintaining homeostasis. When this interaction is disrupted, either due to alterations in the microbiota or host, it is called dysbiosis. This disruption can be linked to various human diseases, both within and beyond the gastrointestinal system. The gut microbiota composition, along with their metabolic activity to a lesser degree, has been associated with various diseases, including those related to obesity, chronic inflammation, cardiovascular conditions, cancer, stress, and neurodegenerative illnesses (Figure 2).⁷

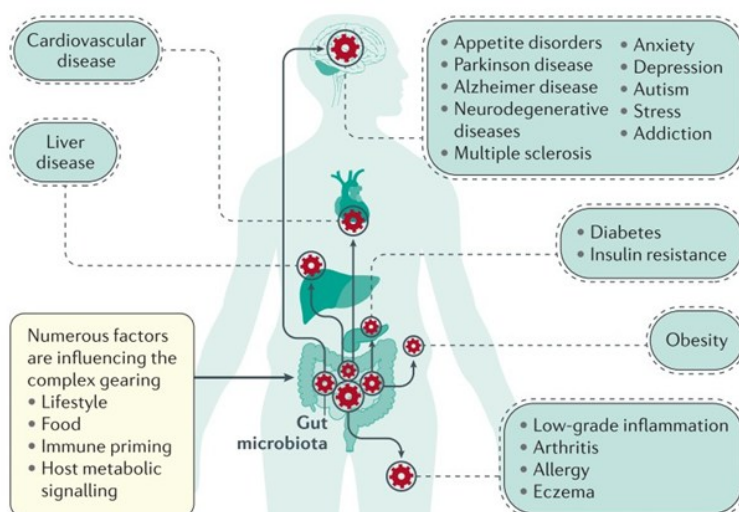


Figure 2. The composition and, consequently, the health of the gut microbiota can be associated with several diseases.

Figure adopted from reference 7.

Recent advances in the characterization of microbial species and communities have revealed the crucial role of microbial metabolism in the host immune system. A healthy microbiota provides vital molecular signals, conveyed via microbial surface antigens and metabolites, that are indispensable for

the development of immune tissues and the regulation of immune responses.⁶ The composition of the gut microbiota is shaped by a multitude of environmental and physiological factors within the gastrointestinal tract. The various regions of the tract exhibit considerable variation in terms of transit time, pH, oxygen exposure, nutrient availability, host secretions, mucosal surfaces, and immune system interactions, all of which influence microbial colonization.⁸

Bacteria present in the human gut microbiota can be divided into three families: Gram-positive, Gram-negative, and Mycobacteria. This classification is based on the molecular composition of their outer cell wall. The primary differentiating factor between Gram-negative and Gram-positive bacteria is the presence of an outer membrane in the former, which is absent in the latter. Also, mycobacteria lack an outer membrane and can be distinguished by a relatively thick cell wall. Gram-positive bacteria (Figure 3b) are characterized by a single lipid membrane that is surrounded by a thick layer of peptidoglycans and lipoteichoic acid. The latter anchored to the cell wall via diacylglycerol. In contrast, the cell wall of Gram-negative bacteria (Figure 3a) is composed of a thin layer of peptidoglycans situated within the periplasmic space between the outer and inner bilayer membranes. In mycobacteria (Figure 3c), the cell wall is composed of a layer of peptidoglycans and arabinogalactan, along with mycolic acid, which imparts lipophilic properties to the system.⁹

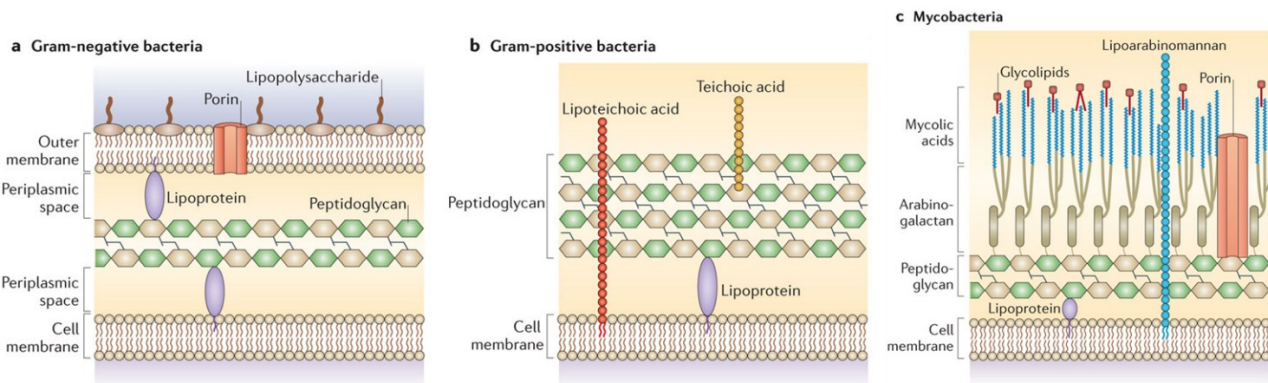


Figure 3. Cell wall structure of a) *Gram-negative bacteria*, b) *Gram-positive bacteria* and c) *Mycobacteria*. Figure adopted by reference 9.

Figure 4 illustrates the distinctive molecular patterns characteristic of each cell wall type. Teichoic acid represents a significant proportion, approximately 60% in Gram-positive bacterial cell walls. It is synthesized via polymerization of glycerol or ribitol, and characterized by a high phosphate diester content. Lipopolysaccharides (LPS) and lipooligosaccharides (LOS) constitute 75% of the Gram-negative bacterial cell wall. They expose the polysaccharide chain (Figure 4b), while the core, known as Lipid A, consists of two saccharide residues, each of which contains a phosphate group in monoester form, carrying a negative charge. In mycobacteria, the molecular pattern is predominantly hydrophobic, due to the presence of mycolic acid chains, which belong to the homologous series of long-chain C60-C90 α -alkyl- β -hydroxy fatty acids (Figure 4c).

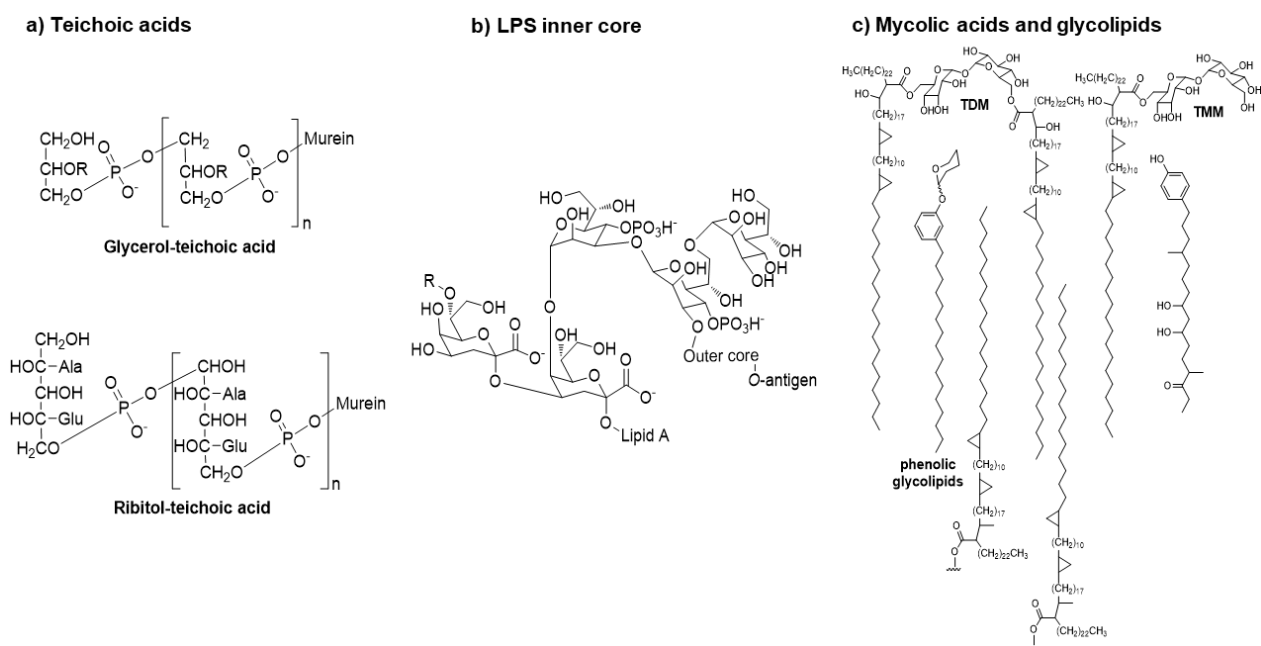


Figure 4. Molecular patterns characteristic of cell walls of Gram-positive bacteria (a), Gram-negative bacteria (b) and Mycobacteria (c). Figure adopted from reference 5.

1.3 Bacteria sensing

Identifying bacteria, particularly when differentiating between various species in a mixed bacterial sample, is vital for guiding the treatment of bacterial infections.¹⁰ The current methods for bacterial identification, such as culture-based techniques or molecular technologies like polymerase chain reaction (PCR), are time-consuming or require sophisticated equipment, which makes them unsuitable for rapid, high-throughput clinical applications.¹¹ Optical and electrochemical methods offer more straightforward alternatives.

Recently, novel approaches have been developed that employ biosensors where proteins, enzymes, nucleic acids or aptamers are linked to the sensor surface and also used for bacteria detection.¹² Although these biosensors are often too specific, lack sensitivity, can easily degrade and the need to build up sensors for each analyte makes them impractical when dealing with multiple analytes in complex bacterial mixtures.¹⁴ To address these limitations, array-based biosensors have been developed. These systems, which draw inspiration from animal olfactory systems, can detect subtle differences between bacterial species by converting their chemical or physical properties into useful analytical data. In an array-based biosensor, multiple sensing elements are present, which interact with the target in a specific manner, transducing the molecular event into a detectable signal.¹⁵ In comparison to a conventional single-component biosensor, an array-based biosensor is capable of distinguishing a multitude of targets without the need for external intervention. Furthermore, an array-based biosensor is able to discern even the slightest variations in the chemical composition or physical characteristics of the objects under examination.¹⁶

The use of array-based biosensors for bacterial detection provides valuable tools for the assessment of key characteristics of each bacterial class, utilizing multiple sensor elements. The differentiation of bacteria can be achieved by examining their surface physicochemical properties and metabolites, as previously illustrated, which serve as useful targets. Furthermore, the interactions between the sensing elements and bacterial cells, including both electrostatic and hydrophobic/hydrophilic interactions,

whether specific or non-specific, play a role in distinguishing between different bacterial species.¹⁷ Besides all these advantages, biosensors suffer of some important weakness: i) their biological components can degrade over time, losing their sensitivity and specificity thus limiting the lifetime; ii) they are often sensitive to environmental conditions such as temperature, pH, and humidity; iii) their preparation is expensive, especially on a large scale, due to the complexity of the manipulation of their biological elements; iv) reproducibility of the data across different batches is affected by quality issues in their fabrication. For all these reasons, the development of more stable and versatile sensors based on small molecular systems exploiting the principle of supramolecular chemistry is highly desirable.

1.4 Supramolecular sensors

In recent decades, the observation of specific host-guest interactions in nature has contributed to the growing interest of supramolecular chemistry in biomedical applications. As a result, supramolecular chemists are now trying to replicate and harness these interactions to design and develop synthetic receptors and sensors. Generally, in supramolecular chemistry sensor binding of the analyte (the guest) to the receptor (the host) is the event that triggers the change of a detectable signal, like for example a change in the color or fluorescence, in the redox potentials or, more generally, in some physical properties.¹⁸ In Figure 5 are illustrated the most common non covalent intermolecular interactions that can be established between the receptor and the analyte, that comprise electrostatic interactions, hydrogen bonds, π - π stacking, cation- π and anion- π , van der Waals interactions, or hydrophobic effects.

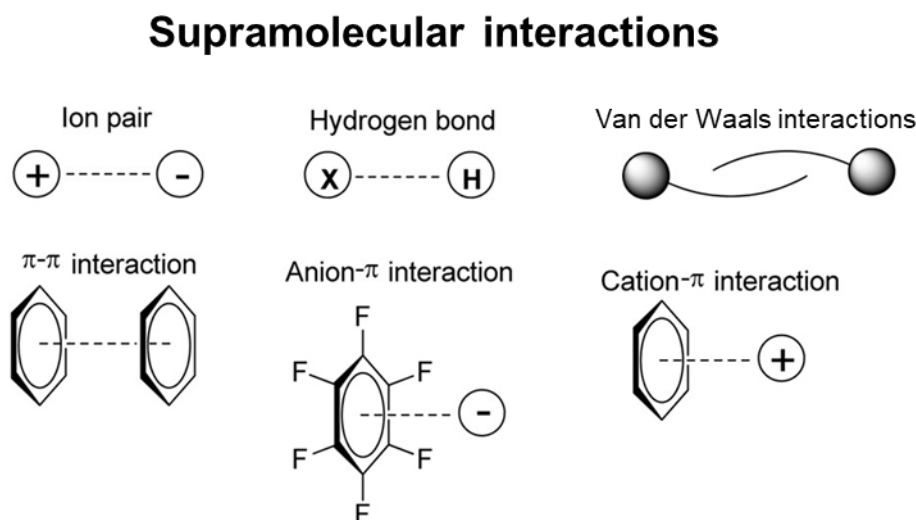


Figure 5. Most common supramolecular interactions. Figure adopted from reference 18.

The most classic approach, distinguished by high sensitivity, is the receptor-spacer-reporter method. This technique is based on photoluminescence detection, in which a fluorophore is covalently linked in proximity to the recognition site of the sensor element (Figure 6), thereby enabling modulation of fluorescence upon binding that can be detected. This approach draws inspiration from the "lock and key principle", which is exemplified by enzymes, whose active site is complementary to a specific substrate.

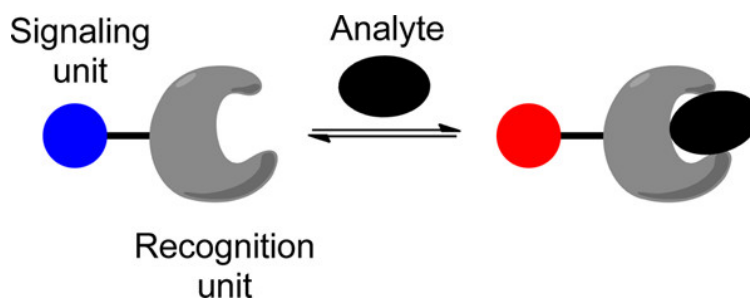


Figure 6. Illustration of the classical approach, receptor-spacer-reporter method. Figure adopted from reference.18.

A more innovative competitive strategy, referred to as the indicator displacement assay (IDA), involves the displacement of a non-covalently bound fluorescent indicator from the receptor following interaction with the target analyte (Figure 7). This method is particularly valuable, as modifications to the indicator environment have led to changes in its photophysical properties. As the fluorescence of the indicator can be either enhanced or quenched upon analyte binding, the sensor is referred to as “fluorescence-on” sensor or “fluorescence-off sensor”, respectively, the former being more sensitive than the latter.

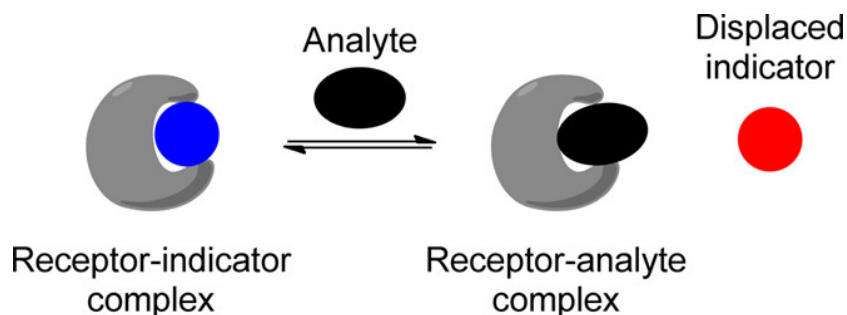


Figure 7. Illustration of the IDA approach, indicator displacement assay. Figure adopted from reference 18.

An illustrative example of this approach (Figure 8) in the detection of bacteria documented by Rotello *et al.*,¹⁹ using an array of gold nanoparticles (NPs) functionalized with different quaternary ammonium amphiphiles. The positively charged NPs strongly interact with an anionic fluorescent polymer, poly(paraphenylene ethynylene) (PPE), whose fluorescence is quenched. However, in the presence of bacteria, the fluorophore is released, resulting in the restoration of fluorescence.

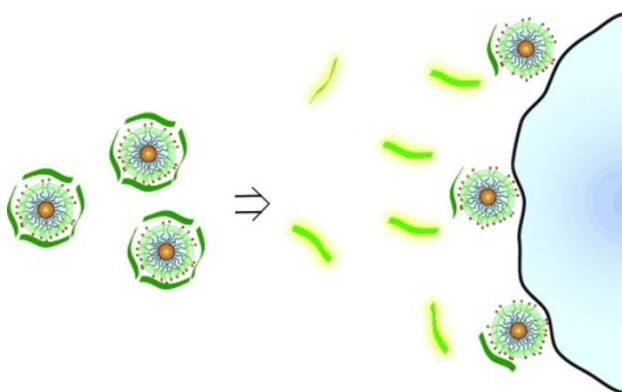


Figure 8. Process of displacement of the anionic PPE from the nanoparticles upon interaction of the NPs with the bacteria. Figure adopted by reference 19.

1.5 Molecular target used for the detection of bacteria by supramolecular sensors

Certain biomarkers found on bacterial surfaces, such as polysaccharides, proteins, peptides, and other membrane structures, can serve as recognition molecules for the rapid and direct detection of bacteria. Trying to target these particular membrane structures, several recognition elements (ligands) can potentially be envisaged.

Boronic acids

Boronic acid interacts specifically with cis-diol groups to form cyclic esters, a feature that is commonly utilized for the detection and quantification of bacteria and other cis-diol-containing biomolecules, including carbohydrates and glycoproteins.^{20–22} Boronic acid derivatives, such as 4-mercaptophenylboronic acid (MPBA, Figure 9a), are particularly effective in recognizing saccharides and glycosylated molecules that are present on bacterial surfaces, making them useful in the detection of bacteria. For instance, Zhang *et al.*²³ introduced a colorimetric detection method for Gram-negative bacteria using silver nanoparticles (AgNPs) functionalized with MPBA. MPBA reacts with the diol group of the saccharides present on the bacterial cell surface, allowing the attachment of the NPs to the bacteria and thus preventing the NPs from clumping. This interaction inhibits a change of color and its intensity, associated with the concentration of bacteria cells. The color change can be detected either by UV-vis spectroscopy or by visual inspection.

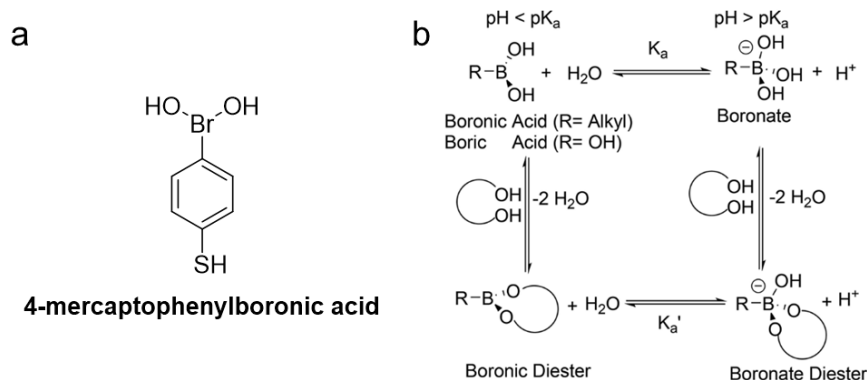


Figure 9. a) Structure of MPBA. b) Equilibria of boronic acids and diols in aqueous solutions. Figure adopted from reference 24.

The interaction between boronic acid and cis-diol can be regulated by modulating the pH. In an alkaline environment, the complex is formed, whereas in an acidic environment, it is dissociated.²⁵ Wu *et al.*²⁶ developed a sensor system for the detection of bacteria utilizing MPBA and mercaptoethylamine (MA)-functionalized AgNPs. A Wulff-type boronate, which includes amines in proximity to the boronic acid, was employed to enhance binding with cis-diol groups at neutral pH through B-N coordination. The pH-sensitive interaction resulted in the separation of nanoparticles, enabling the accurate identification and classification of 17 bacterial species based on color changes, with a 100% success rate.

Glycopeptide Antibiotics

Glycopeptide antibiotics, like vancomycin and polymyxin, exhibit a strong affinity for the D-Ala-D-Ala dipeptide on bacterial cell walls, allowing them to bind effectively to both Gram-positive and Gram-negative bacteria.²⁷ These antibiotics can be used as recognition molecules for detecting bacteria. One

example is a fluorescence sensor array using carbon dots functionalized with vancomycin, boronic acid, and polymyxin to distinguish various bacterial strains. The resulting fluorescence response pattern enable the classification of *D. desulfuricans*, *P. aeruginosa*, *E. coli*, *S. sciuri*, *S. aureus*, and *L. monocytogenes*.²⁸

A similar approach, introduced by Wang *et al.*,²⁹ involves multidimensional sensing arrays. The sensor array consisted of negatively charged vancomycin-templated gold nanoparticles (AuNPs) and positively charged cetyltrimethylammonium bromide (CTAB)-templated gold nanoclusters (AuNCs), and it was able to successfully identify Gram-negative bacteria, including both resistant and non-resistant *E. coli* strains and other species of Gram-negative bacteria.

1.6 Calixarenes

As synthetic supramolecular elements for discrimination among bacteria metabolites or bacteria, we opt to use macrocycles, and particularly calixarenes. Calixarenes are among the most extensively studied macrocycles of the past century, along with cyclodextrins and crown ethers. The term "calix[n]arene" was first introduced by Gutsche, who observed that the tetramer assumes a cup-like shape in the solid state due to hydrogen bonding between hydroxyl groups (the cone shape in Figure 10),³⁰ and they are the product of the condensation in basic conditions of phenols and formaldehyde.

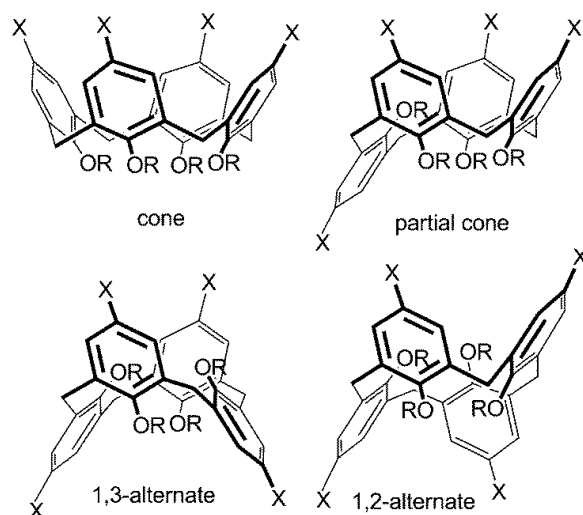


Figure 10. Limiting conformation of a calixarene. When R groups are bulkier than ethyl the interconversion between the conformations is blocked. Figure adopted from reference 31.

Macrocycles with an even number of phenolic units are synthesized with high yields, typically in the range of 60-85%. In contrast, those with an odd number of units are produced with significantly lower yields (Figure 11).

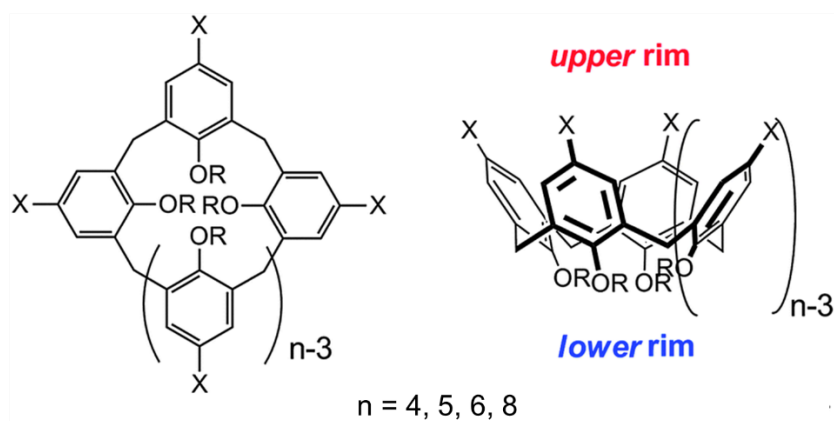


Figure 11. On the left: planar representation of calix[n]arene. On the right: representation of calix[n]arene where upper and lower rim are indicated. Figure adopted from reference 31.

The cone-shaped conformation can be achieved through the introduction of four converging groups with a steric hindrance equal to or greater than propyl. Even though the conformational interconversion between these limiting conformations is prevented with bulky R groups, also the cone conformation still retains a certain degree of conformational flexibility, which can be attributed to the so-called "breathing" movements of the macrocycle, that interconverts between the two C_{2v} structures called flattened cones (Figure 12a). In contrast, the cone structure of tetrahydroxy calix[4]arenes is more rigid, as it is stabilized by hydrogen bonds between hydroxyl groups on the lower rim, strengthened by the deprotonation of one hydroxyl group ($pK_{a1} = 3$) that occurs at physiological pH (Figure 12b).

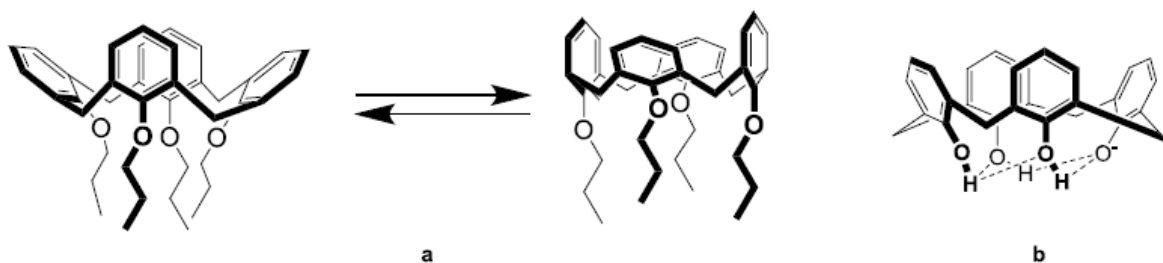


Figure 12. a) Interconversion of tetrapropoxy calix[4]arene between the two flattened cone conformations. b) Hydrogen bonds between the free hydroxyl groups of calix[4]arene at physiological pH.

1.7 Calixarenes as multivalent ligands for biomacromolecules

Properly functionalized calixarenes can be considered as multivalent objects due to the presence of repeating phenolic units. The synthetic processes developed in the last forty years allow the decoration of the upper or lower rim of calixarenes with different ligating groups, from simple monofunctional ones to more complex amino acids, peptides or sugars.³¹ Modulation of the distance between the groups on the calixarene scaffold is feasible through the utilization of spacers, thereby facilitating an enhanced adaptation of the ligand to the target. Also, the size of the macrocycle and the valency play a key role in the binding process. Typically, calix[8]arenes and calix[6]arenes exhibit a larger cavity in comparison to calix[4]arenes, coupled with enhanced conformational mobility. But, as a consequence, higher flexibility and lower preorganization.

The modification of one of the rims of the calixarene with highly polar groups allows the establishment of typically polar interaction, such as electrostatic and hydrogen bonds, while the cavity maintains a lipophilic character. The coexistence of groups with such varying polarities within a single structure, which is sometimes referred to as "hybrid character", is analogous to the environment found within protein and enzyme binding pockets. In these pockets, the aromatic rings of phenylalanine and tryptophan residues are in close proximity to the NH–CO peptide groups of the protein backbone, which act as hydrogen bond donors and acceptors, or with the polar side chains of amino acids. The proximity of different binding units serves to enhance their interaction potential.³¹

Two examples of cooperation between the calixarene lipophilic cavity and the polar groups attached at the upper rim in the binding of biomolecules are shown in Figure 13. Compound **4** (on the left) presents two *N*-linked L-Ala that are close to a diethylene triamine, to form a bridge between two distal phenolic units of the calixarene. The structure of this macrobicyclic ligands mimic the binding region of the antibiotic vancomycin, whose antimicrobial activity is due to its ability to bind the peptidoglycan sequence L-Lys-D-Ala-D-Ala of the peptidoglycan of Gram-positive bacteria. Compound **3a** is a para-tetrasulfonate calix[4]arene, functionalized at the upper rim with four sulfonate anions. It can bind the lateral chain of Lys (Figure 13 on the right) and Arg, accommodating the alkyl chain in the lipophilic cavity, while the anionic upper rim sulfonates establish hydrogen bonds and electrostatic interactions with the ammonium cations. These kinds of complexes are well studied in both solution and solid state.

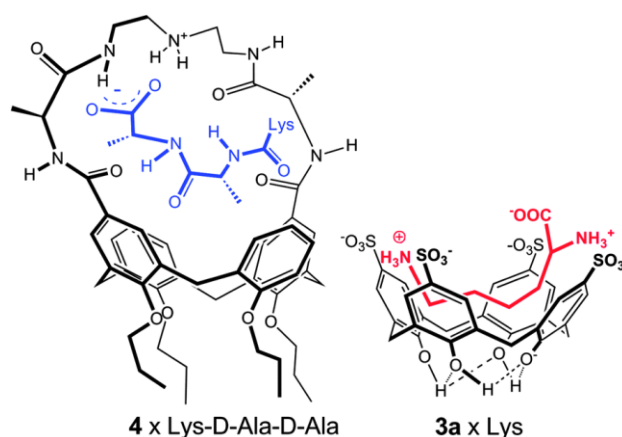


Figure 13. Structures of the complexes between macrobicyclic ligand **4** and L-Lys-D-Ala-D-Ala on the left, and on the right p-sulfonato calix[4]arene **3a** with L-Lys. Figure adopted from reference 31.

1.8 Multivalency

Multivalency is a common phenomenon that can be observed in both biological and synthetic systems. "Multivalency" is defined as the ability of a system having multiple ligating units to engage multiple noncovalent interactions with another systems having multiple receptor units.³² In Figure 14 is exemplified a multivalent interaction between a virus and a cell surface.

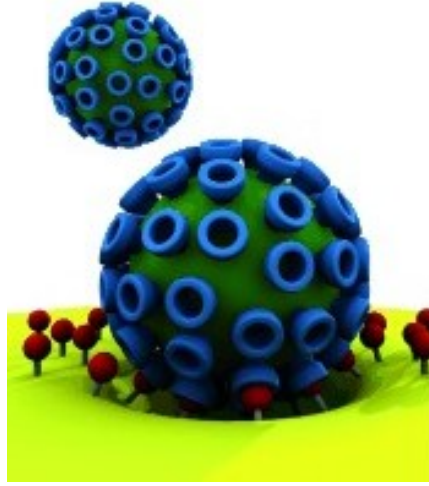


Figure 14. Example of multivalent binding: a virus on a cell surface. Figure adopted from reference 32.

For a positive multivalent effect, the strength of the multivalent interaction exceeds the sum of the single monovalent interactions. Multivalency is due to the favorable entropy contribution.

Multivalency is typically defined by a favorable increase in avidity entropy, which grows as the valency of the complex rises, thereby encouraging binding and offsetting the loss of conformational entropy that happens during the creation of a multivalent complex.

From a thermodynamic viewpoint, the following formula can be applied to describe the multivalent binding:

$$\Delta G_{multi}^0 = n \Delta G_{mono}^0 + \Delta G_{interaction}^0$$

Where ΔG_{multi}^0 represents the standard free energy linked to multivalent binding, while ΔG_{mono}^0 refers to the standard free energy for a monovalent ligand binding to its receptor. The term $\Delta G_{interaction}^0$ is included to reflect the additional free energy that compensates for the favorable and unfavorable influences of tethering in the multivalent system.

To quantify the multivalent effect, Whitesides³³ introduced a parameter, enhancement factor (β), defined as the ratio of the binding constant for a multivalent ligand interacting with a multivalent receptor (K_{multi}) to the binding constant for the monovalent ligand binding to a single multivalent receptor (K_{mono}):

$$\beta = K_{multi}/K_{mono}$$

This ratio is useful when the precise number of active binding interactions is unknown. A positive multivalent effect is characterized by $\beta \gg 1$.

If the valency of the cluster is determined, the enhancement factor can be normalized by dividing it by the valency, yielding the parameters β/n . This normalized value allows for the comparison of multivalent systems with varying valency, structure, and linker design and is a measure of the increase of the binding of each single multivalent interaction. Systems with high β/n values (much greater than 1) are more effective than monovalent systems, while those with lower β/n values (much less than 1) exhibit lower binding strength and show a negative multivalent effect.

1.9 Quartz crystal microbalance (QCM-D)

Among the techniques that consent to study the interaction between macrocycles or other multivalent objects, the quartz crystal microbalance (QCM) can be a useful tool for its ability to quantify low concentrations of analytes and its ability to perform real-time monitoring.³⁴

The quartz crystal microbalance (QCM) is a nanogram-sensitive technique that employs acoustic waves generated by an oscillating crystal of quartz to measure differences of mass taking place on the surface exposed to the analyte solution. The detection mechanism is based on the piezoelectric properties of quartz (“piezo” derives from the Greek word *πιέζειν* that means “to press”). In a piezoelectric material, the pressure applied to the material generates an accumulation of electric charges.³⁴ Consequently, when alternating electric fields are applied to a quartz crystal, it undergoes repeated expansion and contraction of its lattice structure. Indeed, modern QCM instruments comprise a QCM chip, wherein a circular quartz crystal is located between two metal electrodes. When an electric field is applied to the quartz disk, a mechanical oscillation ensues, contingent on the disk design. This oscillation is influenced by fundamental parameters such as the material thickness, mass, shape, and chemical structure.³⁵ In Figure 15 a schematic illustration of the QCM chip is represented.

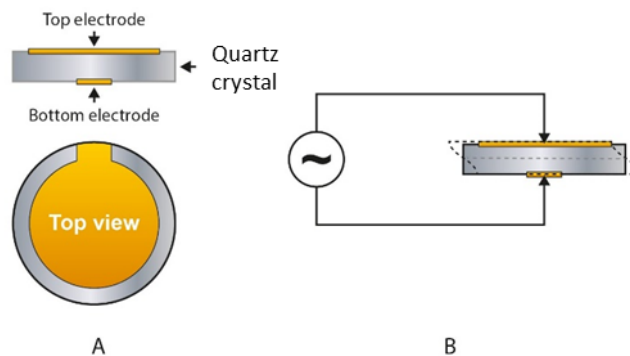


Figure 15. A) sketch of a QCM chip, where the piezoelectric material (quartz, in this case) is placed between two electrodes, made of gold in this case. B) mechanical oscillation of the crystal induced by an alternating voltage.

Resonant oscillation is obtained by incorporating the crystal into an oscillating circuit, where both the electrical and mechanical oscillations are close to the crystal fundamental frequency.³⁵ Resonance is triggered when an alternating current is applied at a frequency near the crystal’s resonance frequency (f_0). The QCM reaches resonance when the standing wave formed by the alternating expansion and contraction is an odd multiple of the quartz plate thickness. Resonant frequencies in QCMs typically fall within the MHz range, and there is a tradeoff between frequency (related to sensitivity) and crystal thickness: the higher the frequency, the thinner the quartz. A common resonant frequency of 5 MHz corresponds to a crystal thickness of about 330 μm .³⁴

Sauerbrey's demonstration³⁶ of the theoretical and experimental link between the change of frequency and the mass adsorbed to its surface paved the way for the extensive utilization of QCM as a mass sensor. This linear relationship between the frequency shift (Δf) and the adsorbed mass (Δm) is described by the equation:

$$\Delta m = \frac{C}{n} \Delta f$$

where n is the harmonic number, and C is a constant given by:

$$C = \frac{(t_q \rho_q)}{f_0}$$

with t_q being the quartz thickness and ρ_q the quartz density ($\sim 17.7 \text{ Hz ng/cm}^2$ for a 5 MHz resonating crystal).

The Sauerbrey equation is only applicable when the adsorbed mass is negligible in comparison to the quartz crystal mass and exhibits rigid attachment coupled with the oscillation of the quartz material. Consequently, if the driving voltage is turned off, a long time is required for the system to decay. In contrast, a flexible material takes a significantly shorter time to dampen the crystal oscillation.³⁷ Furthermore, the distribution of the adsorbed substance must be uniform across the surface of the crystal.³⁴ Initially the application of this device exclusively concerned studies in the gas phase, monitoring volatile analyte adsorption in vacuum systems, etching studies, and aerospace contamination studies.^{35,38} A significant advancement occurred around 1980 when QCMs were successfully adapted for use in liquid phases, thereby greatly increasing their potential applications. These liquid-based QCM applications became especially relevant in the field of biotechnology, particularly in biosensing. Nevertheless, it became apparent that in numerous studies, the adsorbed or deposited films did not satisfy the assumptions of the Sauerbrey equation. The viscous and elastic properties of certain polymers, the presence of hydration water, and the existence of trapped water between adsorbed molecules frequently result in alterations to the frequency that do not align with the Sauerbrey assumption that the adsorbed mass is rigid. This led to the development of new theoretical frameworks which also consider the viscoelastic effects and account for changes in not only resonance frequency but also in the dissipative loss of energy (D).³⁸

In a quartz crystal microbalance with dissipation monitoring the dissipation value represents the mechanical properties of the surface-adhering layer and, when considered alongside Δf , can be used to quantify the mass, thickness, and viscoelastic properties of soft layers. This level of detail cannot be obtained by examining the Δf response alone. The combined insights provided by Δf and ΔD in **QCM-D** (Quartz Crystal Microbalance with Dissipation Monitoring) make it especially well-suited for analyzing and characterizing hydrated systems, which are often viscoelastic in nature.³⁹

1.9.1 Quartz Crystal Microbalance with Dissipation Monitoring

One of the most explored fields of application of QCM-D is biology for the sensing of small analytes, large biomacromolecules or even microorganisms. The sensing of bacteria with QCM-D, for instance, has often been proposed because it is label free and allows real-time monitoring. Several examples are present in literature of biosensors for monitoring microorganisms: among different bacteria, probably the most commonly proposed are *Chlamydia*,⁴⁰ *Salmonella*,⁴¹ *Bacillus*,⁴² *Pseudomonas*⁴³ and *E. coli*.^{44–46} In this specific context, the monitoring of dissipation is a particularly valuable tool due to the inherent non-rigid properties of cells. The core principle of QCM-D technology relies on the fact that "soft" masses, like bacterial cells, adhering to the crystal surface do not completely couple with the crystal oscillations, leading to a dampening effect. Consequently, the mass absorbed on the crystal surface tends to be underestimated when applying the traditional Sauerbrey equation. To address this, a rapid method was introduced for obtaining simultaneous measurements of both the frequency (f) and the dissipation factor (D), defined as:

$$D = \frac{E_{dissipated}}{2\pi E_{stored}}$$

$E_{dissipated}$ refers to the energy lost during a single oscillation cycle, while E_{stored} represents the energy retained within the oscillating system. D reflects the total contribution of all processes that cause energy loss within the oscillating system.³⁸

Figure 16 is a clear example of how rigid or soft materials have a corresponding response in the dissipation values in a QCM-D graph.

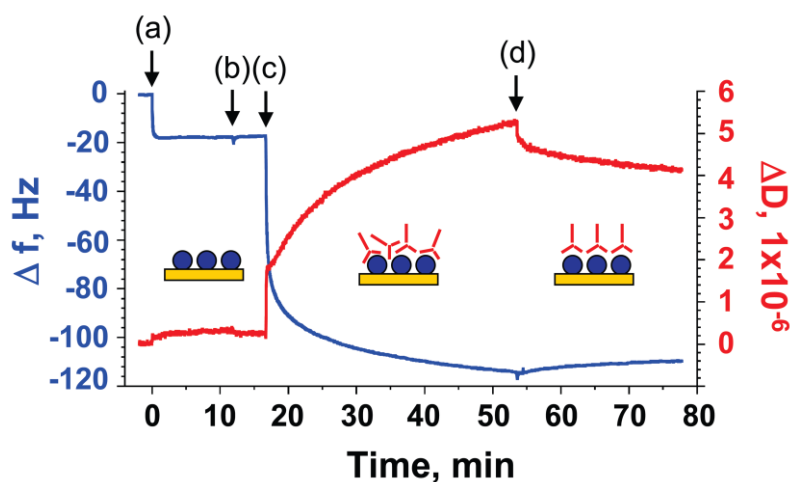


Figure 16. The frequency (Δf) is shown in blue, on the left axis, while on the right axis is reported the dissipation (ΔD) in red. a) illustrates the adsorption of human serum albumin and (c) of an antibody specific to human serum albumin (c). Steps (b) and (d) represent buffer rinses. Figure adopted from reference 34.

In step (a), the human serum albumin absorption generates a frequency shift without a corresponding change in dissipation. This indicates that the human serum albumin film is of a rigid nature as it adsorbs onto the QCM surface. In contrast, the adsorption of the antibody in step (c) results in notable alterations in both frequency and dissipation, indicating not only the mass adsorption on QCM but also an enhancement in viscoelastic properties due to the incorporation of water. Furthermore, the rinsing step at (d) demonstrates a discernible conformational alteration in the antibody distribution as some of the aspecifically physisorbed molecules are removed.³⁴

Another characteristic of viscoelastic materials is the presence of not overlapping multiple overtones. In QCM-D, overtones play an essential role in characterizing viscoelastic properties. Overtones are higher harmonics of the crystal's fundamental resonance frequency, with each one representing a frequency that is a multiple of the fundamental (commonly the 3rd, 5th, or 7th harmonics). By studying multiple overtones, researchers acquire depth-resolved insights into both the viscoelastic and mass properties of thin films deposited on the sensor surface.

For a thin, rigid layer, shifts in frequency (Δf) and dissipation (ΔD) across the overtones typically coincide, indicating a negligible viscoelastic response. However, in thicker layers, variations in Δf and ΔD appear across the overtones due to the increased dampening effect within the viscous material, which reveals layer structure and mechanical properties. This “spreading” of the overtones is particularly informative for analyzing viscous films since each overtone responds differently depending on layer thickness and softness. This characteristic informs modeling adjustments, where viscoelastic models are necessary to interpret the non-linear behavior of Δf and ΔD shifts accurately.

The unique sensitivity of overtones derives from their distinct penetration depths: as the overtone frequency increases, the acoustic wave's penetration depth decreases. Consequently, higher overtones provide enhanced sensitivity to material properties closer to the sensor surface, while lower overtones

offer insights into bulk material characteristics. By examining changes in both frequency and dissipation across overtones, QCM-D enables a precise analysis of complex viscoelastic behaviors, particularly in soft or hydrated films, thereby delivering a detailed mechanical profile of the material.⁴⁷

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Biogenic Polyamine Binding to Anionic Calix[4]arenes

2.1 Introduction

One of the aims of the BacHound research project is to develop selective receptors for the detection of microbial metabolites. The deviation of microbial metabolite concentrations from their physiological values has been related to the onset of certain pathologies¹ in organs or functions (from liver, to bones and central nervous system). The intestinal epithelial cells of human beings are, in fact, at the interface between the intestinal flora and the host, and their interaction influences the immune response and the risk of disease.² Therefore, there is an urgent need to develop new tools for the selective detection of bacterial strains and of their metabolites.

The following table presents a list of diseases and clinical conditions that have been linked to an imbalance of the microbial metabolites present within the human body.

Table 1. Microbial metabolites or components that are implicated in disease.³

Human disease and preclinical models	Microbial metabolites or components
Allergic and immune disorders	
Ashtma	SCFAs
Inflammatory bowel disease	SCFAs, B vitamins
Cancer	
Colorectal cancer	SCFAs, B vitamins
	N ¹ ,N ¹² -diacetylspermine
Gynaecological and reproductive disorders	
Bacterial vaginosis and other sexually transmitted infections	Polyamines, HBP
Preterm labor	SCFAs
Metabolic disorders	
Cardiovascular disease	TMAO
Kidney disease	SCFAs, p-Cresol
Obesity and metabolic syndrome	TMAO
Type 2 diabetes	TMAO
Neurological disorders	
Autism spectrum disorder	4-EPS
Central nervous system dysfunction	SCFAs
Other gastrointestinal disorders	
Infectious colitis (<i>Clostridium difficile</i>)	Bile acids

4-EPS, 4-ethyl phenol sulfate; HBP, D-glycero-β-D-manno-heptose-1,7-biphosphate; SCFAs, short-chain fatty acids; TMAO, trimethylamine N-oxide.

This chapter is dedicated to the detection of biogenic polyamines, a distinctive and intriguing class of microbial metabolites. The discussion is centered on putrescine (1,4-diaminobutane), hexamethylenediamine (1,6-diaminohexane), spermidine, and spermine, shown in Figure 1, as well as affinity studies conducted with calix[4]arene-based negatively charged receptors.

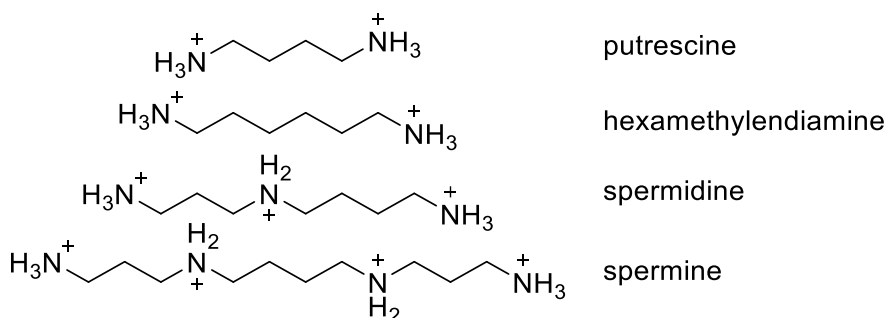


Figure 1. Polyamine studied in this chapter.

2.1.1 Biogenic Polyamines

Biogenic polyamines are polycationic molecules distinguished by the presence of an alkyl chain of varying length containing a number of ammonium ions that can range from two to four. These molecules are found in almost all living cells and are crucial for cellular growth across a wide range of organisms, from bacteria to mammalian cells. High levels of polyamines have been related to the onset of pathological conditions, including cancer.³ Polyamines play a complex role in managing oxidative stress. At normal levels, they protect cells from oxidative damage by acting as antioxidants and supporting cellular repair mechanisms. However, an excessive polyamine metabolism can contribute to oxidative stress and related diseases.² Moreover, several bacterial pathogens, including *Helicobacter pylori*, *Salmonella Typhimurium*, *Shigella* species, *Staphylococcus aureus*, *Streptococcus pneumoniae* and *Vibrio cholerae*, have been demonstrated to rely on polyamines for their virulence and survival within a host. Polyamines are also crucial for normal host cell functions and imbalanced levels of their concentrations can lead to problems: low concentrations of polyamines and ornithine decarboxylase (a crucial enzyme for the biosynthesis of putrescine in both mammalian and bacteria cells) have been associated with cell growth defects, whereas high levels have been demonstrated to be toxic and contribute to carcinogenesis.¹ For this reason, the regulation of polyamine levels within cells is a complex and fundamental process, involving tightly controlled processes of biosynthesis, degradation, uptake, and excretion.³

Moreover, polyamines also stabilize different biomolecules inside cells. They are, in fact, capable of interacting with a number of biological macromolecules, including nucleic acids (particularly RNA), ATP, certain proteins, and phospholipids, thanks to their full protonation state under physiological conditions.^{4,5,6} The types of interaction involved in these complexes are not limited to electrostatic interactions: in fact, hydrogen bonds and hydrophobic interactions with the hydrocarbon chains also play a significant role.

The interaction between nucleic acids and polyamines occurs primarily at the phosphate group of the pentose phosphate backbone. Several studies have shown that the presence of polyamines within a cell is crucial for maintaining chromatin conformation. By binding to DNA, the polyamines can interact with the polyphosphate backbone and the N7 of purine and O2 of thymine.⁷ Moreover, the inherent flexibility of alkyl chains allows for multiple interactions with a vast array of subsites, including those of proteins. The interaction with proteins has been more extensively studied with enzymes that are involved in the synthesis and metabolism of polyamines themselves, or that bind negatively charged moieties, thereby locally masking the charge. For instance, carboxylates can constitute sites of binding, including those of polyamines that act as bridges between two different proteins. This phenomenon leads to conformational changes and protein aggregation.⁷

2.1.2 Negatively charged calix[4]arenes

Calixarene-based ligands have shown remarkable potential in interacting with proteins and other biomolecules, demonstrating high efficiency and selectivity in recognition processes. This is due to their multivalent nature, the different functionalities that can be attached to their structure at different distances from the aromatic units, and the different geometric arrangements they offer for presenting binding units.⁸ The synthesis of water-soluble derivatives of these macrocycles has allowed their use as ligands for biological macromolecules, including enzymes, proteins, nucleic acids and polysaccharides.⁹ Calixarene derivatives have therefore the potential to be utilized in the development of a number of different applications, including anti-tumor agents, immune response modulators, gene

or drug delivery systems, and agents able to inhibit the adhesion of viruses, bacteria, and bacterial toxins to host cells. This section presents a description of the most commonly occurring negatively charged calixarenes, along with an analysis of their applications in the field of recognizing small molecules and biomacromolecules of biological interest.

2.1.2.1 *para*-Sulfonato-calix[4]arene

The *para*-sulfonato-calix[4]arene (Figure 2, left) has been shown to be an exemplary receptor for a wide range of biomolecules. Amongst other types of macrocycles, the calix shape and the presence of the lipophilic cavity play a pivotal role in facilitating additional interactions, besides the main electrostatic ones. In numerous instances, the cavity is employed for the accommodation of alkyl chains or aromatic nuclei, due to hydrophobic effects of more specific CH- π interactions.^{10,11}

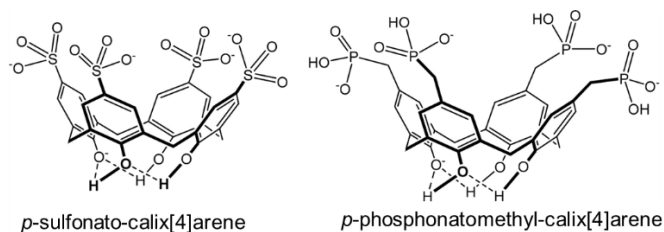


Figure 2. The structures of *p*-sulfonato and *p*-methylphosphonato-calix[4]arenes. The presence of strong H-bonds between the phenolic hydroxyl groups, one of which is deprotonated at physiological pH,¹² stabilizes the cone conformation for these derivatives.

Among proteins, it has been demonstrated to facilitate protein crystallization of cationic proteins like cytochrome c and lysozyme. This is achieved by binding the ammonium head-groups of the side chain of positively charged amino acids, such as lysine and arginine, and establishing CH- π interactions with the alkyl chains of the aforementioned amino acids within the hydrophobic cavity.¹³ Figure 3 illustrates the crystal structure of the asymmetric unit of *p*-sulfonato-calix[4]arene and cytochrome c, which comprises three calixarene units and two cytochrome c units.

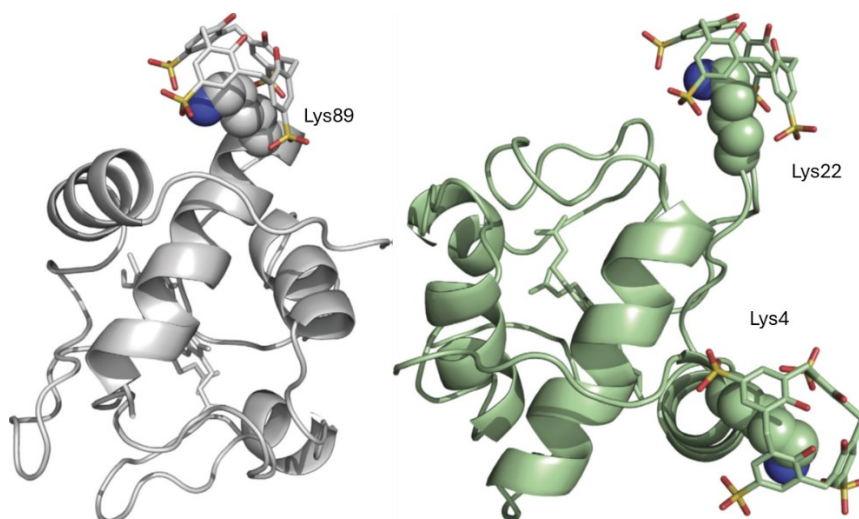


Figure 3. Representation of the interaction between *p*-sulfonato-calix[4]arene and the side chain of the cytochrome c lysine residue. The calixarene is binding the side chains of Lys89, Lys4 and Lys22.¹⁴

Perret *et al.*¹⁵ have conducted several complexation studies in solution with *p*-sulfonato-calix[4]arene, which revealed that the strongest binding constants are with cationic amino acids like lysine, arginine, and histidine. This suggests that electrostatic binding is a crucial factor in the interactions between *p*-

sulfonato-calix[n]arenes and amino acids or proteins. *p*-sulfonato-calix[4]arene has also demonstrated complexation properties with bioactive molecules, as evidenced by the crystalline structures obtained through X-ray diffraction with guests such as aminobenzimidazole, thiamine, spermine, and streptidine.¹⁶ Of particular interest is the complex with spermine, which is one of the polyamines involved in this work, and whose asymmetric unit is shown in Figure 4.

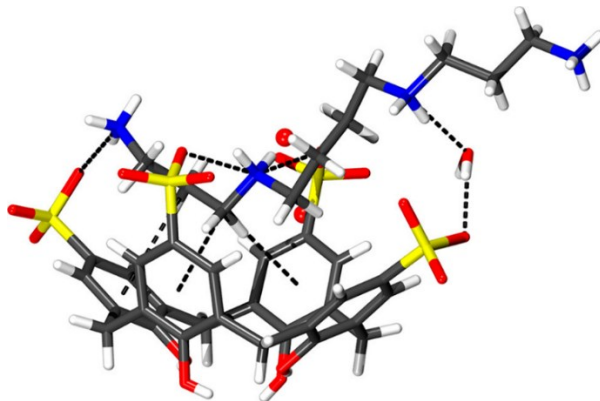


Figure 4. The asymmetric unit of the crystal structure of the complex of *p*-sulfonato-calix[4]arene with spermine. Figure adapted from reference 16.

It is noteworthy that the spermine cation is included within the calix[4]arene cavity, where interactions such as CH- π with the aromatic moieties occur. Furthermore, hydrogen bonds are observed between the ammonium ions (also acting as H-bond donors) and the oxygens of the sulfonate groups (acting as H-bond acceptors), or with a water molecule bridging NH and SO groups. The portion of the spermine that is included within the calix[4]arene cavity is stabilized by a number of host-guest interactions, whereas the portion that is situated outside is more disordered.¹⁶

2.1.2.2 *para*-Sulfonatomethyl-calix[4]arene

Another negatively charged calixarene, the *para*-sulfonatomethyl-calix[4]arene (Figure 5), has been previously synthesized and studied for its interaction with biomolecules.^{17,18}

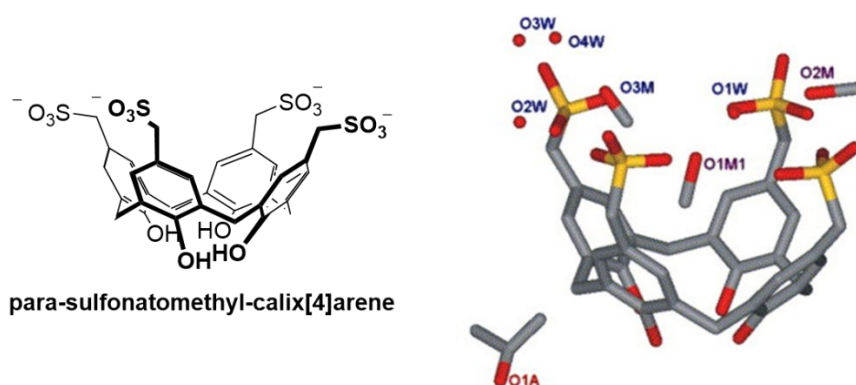


Figure 5. Structure of *para*-sulfonatomethyl-calix[4]arene and its X-ray crystal structure. Figure adapted from reference 18.

Perret *et al.*¹⁸ have addressed the need to increase the flexibility of the sulfonate group with respect to *p*-sulfonato-calix[4]arene at the upper rim of the calixarene and to extend the depth of the hydrophobic cavity. This objective was achieved by the insertion of methylene groups between the aromatic core and the ionizable functions, resulting in the formation of *p*-sulfonatomethyl-calix[4]arene. Moreover, they carried out the first crystallographic investigation of this novel water-soluble calixarene.

In our group, with the collaboration of Professor Crowley group at the National University of Galway, studies have been made to test its ability to act as molecular glue for the crystallization of cationic proteins, such as cytochrome c. The use of *p*-sulfonatomethyl-calix[4]arene results in a significantly more effective and easy crystallization accompanied by a higher quality of the crystals compared to *p*-sulfonato calix[4]arene.¹⁹ Crowley and his research team successfully determined the X-ray structure (Figure 6) of one of these crystals.

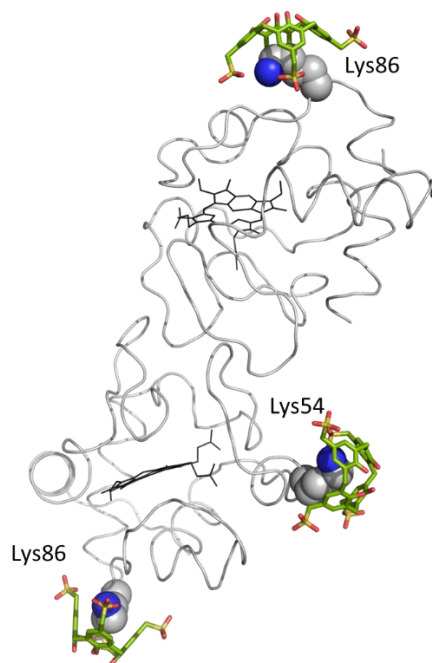


Figure 6. X-ray structure of the complex of *p*-sulfonatomethyl-calix[4]arene and cytochrome c.

The unit cell is constituted of two proteins and three calix[4]arenes, and each calixarene interacts with a single lysine residue, specifically incorporating Lys54 and Lys86 on protein unit A, while only one residue of Lys86 is included on unit B. For all the three calixarene ligands, only one of the sulfonate groups is pointing towards the calixarene cavity and establishing an ion pair with the ammonium group of the lateral chain of the amino acids. The other three sulfonato head-groups are pointing outward from the macrocycle. The incorporation of lysine side chains closely resembles the resolved structures of *para*-sulfonato-calix[4]arene (described in the previous paragraph) and *para*-phosphonomethyl-calix[4]arene (described in the following paragraph), where cation- π and CH- π interactions with the lysine ε -CH₂ groups are observed.

2.1.2.3 *para*-Phosphonomethyl-calix[4]arene

Calixarenes bearing phosphonate groups at the upper rim have also been studied for this same purpose of protein binding. One of these ligands, the *para*-phosphonomethyl-calix[4]arene,²⁰ also presents a methylene bridge between the ionic group and the calixarene scaffold. Studies were carried out to test its properties as a molecular glue for the crystallization of cytochrome c.¹³ The results demonstrated that it exhibits more favorable properties and a higher selectivity in the binding compared to the *p*-sulfonato-calix[4]arene. The structure of the obtained crystal is illustrated in Figure 7A. The phosphonated ligand was observed to exhibit a higher degree of specificity than the *para*-sulfonato-calix[4]arene ligand, with the former one displaying only two binding sites (Lys54 and Lys86, as illustrated in Figure 7B) instead of three.

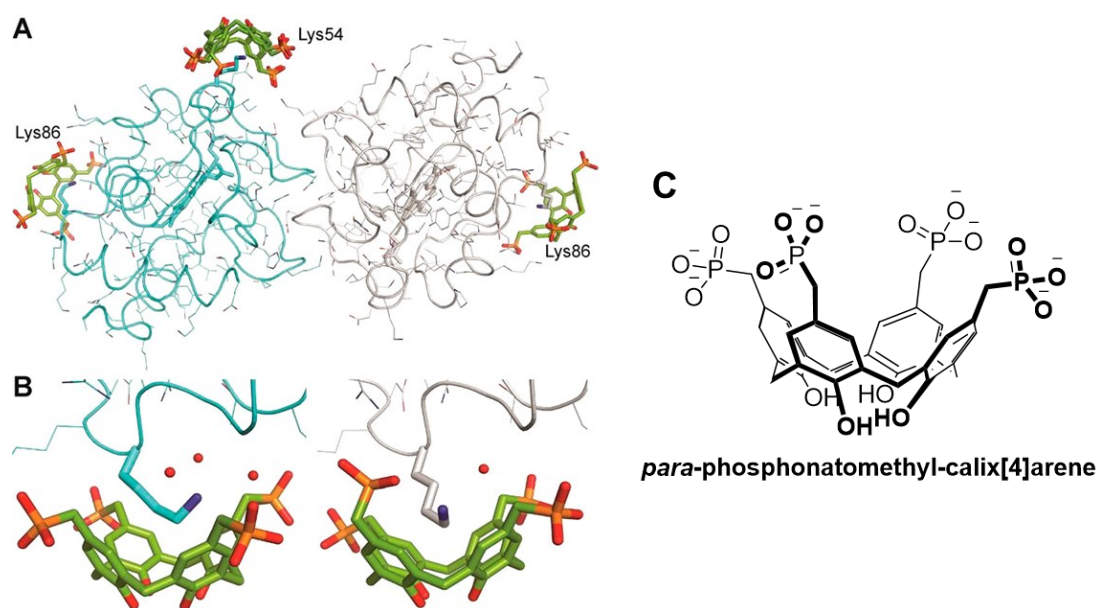
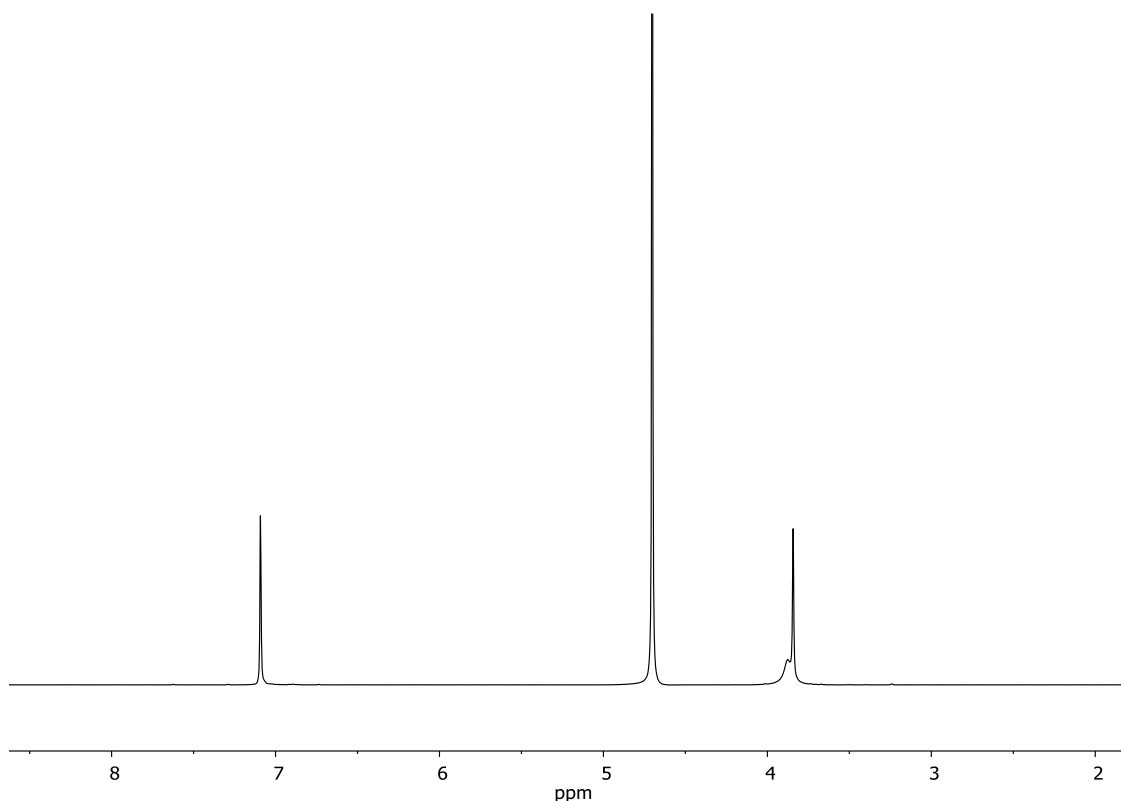


Figure 7. A) Asymmetric unit of the complex between *p*-phosphonatomethyl-calix[4]arene and cytochrome *c* and that involves two proteins and three calixarenes. B) The two different binding modes: the calixarene in figure B (on the left) lacks direct salt bridges between the phosphonate groups and the ammonium head-groups, while the ligand in figure B (on the right) show one phosphonate group establishing a salt bridge with the ammonium of the Lys. C) Molecular formula of the *p*-phosphonatomethyl-calix[4]arene.¹³

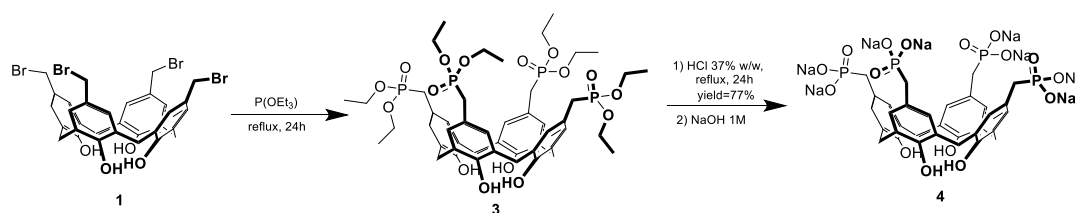
The crystal structures of the two phosphonato calixarenes exhibit similarities, including the inclusion of the lysine side chain within the cavity and the formation of cation- π and CH- π interactions. As for *p*-phosphonatomethyl-calix[4]arene, only one of the calixarene presents a phosphonate group pointing inwards the cavity and establishing a salt bridge with the ϵ -ammonium groups of a Lys.

2.1.3 Aim of this work

The aim of this work is to study the possibility to use the two negatively charged calixarenes, *p*-sulfonatomethyl- (**2**) and *p*-phosphonatomethyl- (**4**) calix[4]arene for the detection of the biogenic polyamines of interest. In the following section, the results of the study on the interaction between these two anionic receptors and four different polyamines will be reported. All complexation studies were carried out in aqueous solutions. Initially, we run ^1H NMR titration experiments in order to characterize the host-guest complexes and their stability. Complexes were also studied, in collaboration with the group of Prof. Marco Montalti at the University of Bologna, by using fluorescence titrations.

Figure 9. ^1H NMR of pure product **2** recorded in D_2O (400 MHz, 298K).

The synthesis of the methyl phosphonate **4** was carried out similarly to that described in literature,²⁰ but also in this case using the bromomethyl derivative as starting compound instead of the chloromethyl derivative (Figure 10). The following step to obtain **3** is a Michaelis-Arbuzov reaction, where the triethylphosphite acts as a nucleophile to obtain the final diethylphosphonate **3**. The isolation of the latter compound turned out to be particularly difficult due to the high boiling point of the residual triethylphosphite which always remains in traces.

Figure 10. Synthesis of product **4**.

However, it was decided to proceed with the subsequent hydrolysis step of the phosphonic esters, by reaction with 37% w/w HCl at reflux. The solid obtained was then titrated till neutrality by using an aqueous solution of NaOH 0.1 M, and the octasodium salt **4** was then isolated as a dark purple solid after removal of the water by freeze drying. Figure 11 shows the ^1H NMR spectrum of compound **4**.

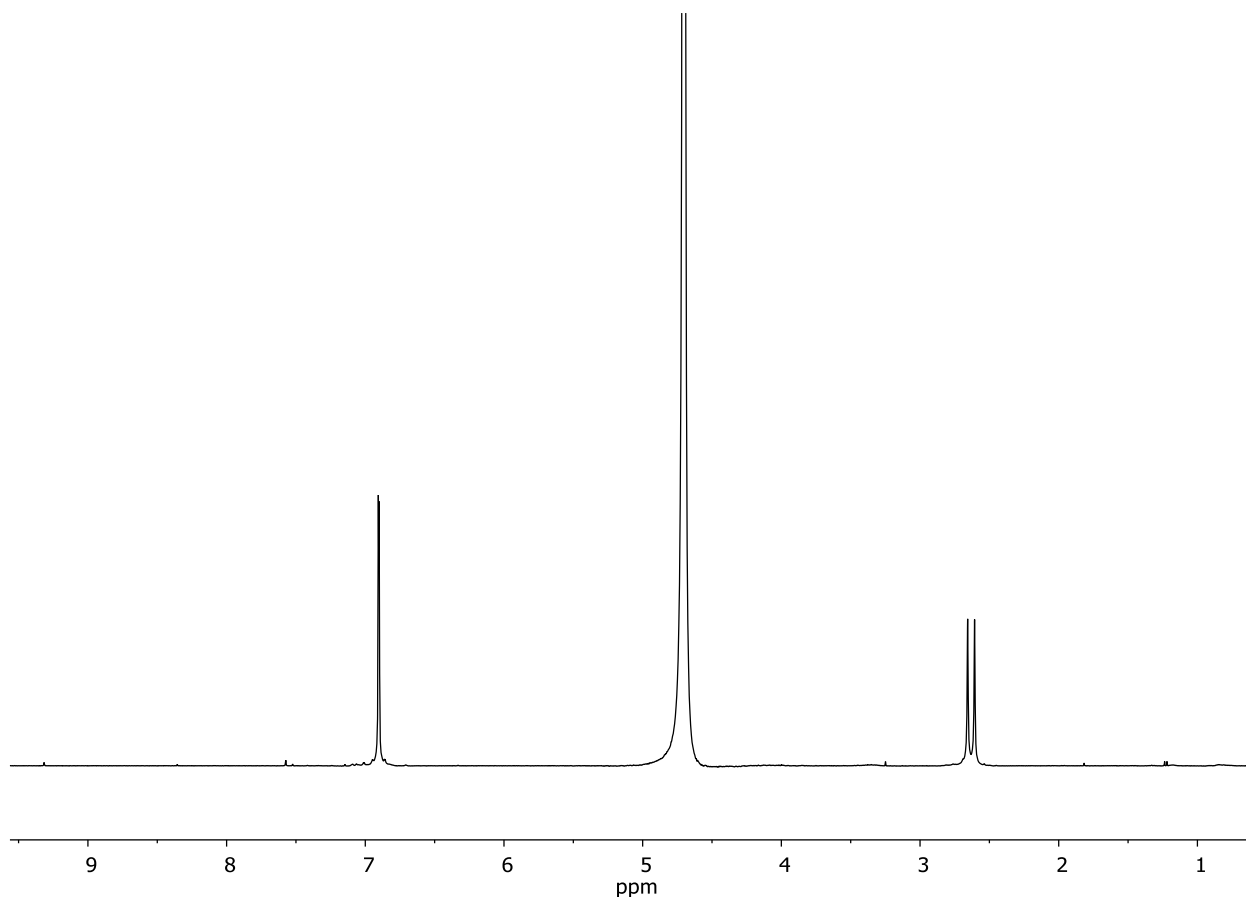


Figure 11. ^1H NMR of pure product **4** recorded in D_2O (400 MHz, 298K).

The diagnostic signals are due to the coupling of the phosphorus with the protons of the methylene groups (ArCH_2P) at the upper rim, which in fact give rise to a doublet at 2.95 ppm with a J_1 of 20.0 Hz. The aromatic protons at 7.10 ppm also appears as a doublet due to the H-P coupling, J_4 of 2.4 Hz, while the signals of the ArCH_2Ar protons are very broad, therefore not visible, in the range from 4.5 to 3.00 ppm.

2.2.2 ^1H NMR complexation studies with polyamines

The complexation properties of negatively charged calixarenes **2** and **4** towards different polyamines were investigated by ^1H NMR titration. The concentration of the calixarene, the host (H), was kept constant at 0.002 M, and then subsequent aliquots of the guest (G) stock solution were added, gradually increasing the G concentration and consequently the G/H ratio. The polyamine stock solution used for host **2** was 0.033 M, whereas the solution used for host **4** was 0.05 M. The titrations were conducted in deuterated phosphate buffer at pD 7.3 to mimic the physiological conditions, where all the polyamines studied should predominantly be present in their fully protonated forms (see pK_a values of Figure 12). The use of the buffer is also necessary to avoid an increase of the pH upon the addition of the polyamine, and variations of the protonation state of the guest during the titration.

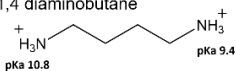
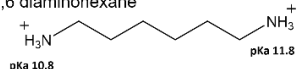
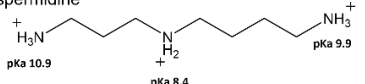
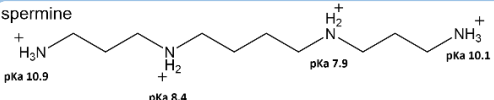
		pH 7.3	
		Protonated	Deprotonated
1,4 diaminobutane		99.2% PA-H ₂ ²⁺ (PA)	0.8% PA-H ⁺ (PAd)
1,6 diaminohexane		99.97% PA-H ₂ ²⁺ (PA)	0.03% PA-H ⁺ (PAd)
spermidine		92% PA-H ₃ ³⁺ (PA)	8% PA-H ₂ ²⁺ (PAd)
spermine		75% PA-H ₄ ⁴⁺ (PA)	25% PA-H ₃ ³⁺ (PAd)

Figure 12. pKa of the polyamines investigated in this chapter.

2.2.2.1 ¹H NMR titrations of calixarene **2** with polyamines at 25 °C

The first titration reported, with calixarene **2** as host and 1,4-diaminobutane as guest, was carried out at 298 K. The observation of the ¹H NMR spectra (Figure 13 and 14) reveals that the signals of the 1,4-diaminobutane are in a fast exchange regime, with significant shifts of the guest signals from high to low fields upon increasing of the guest concentrations (traces 2 → 8).

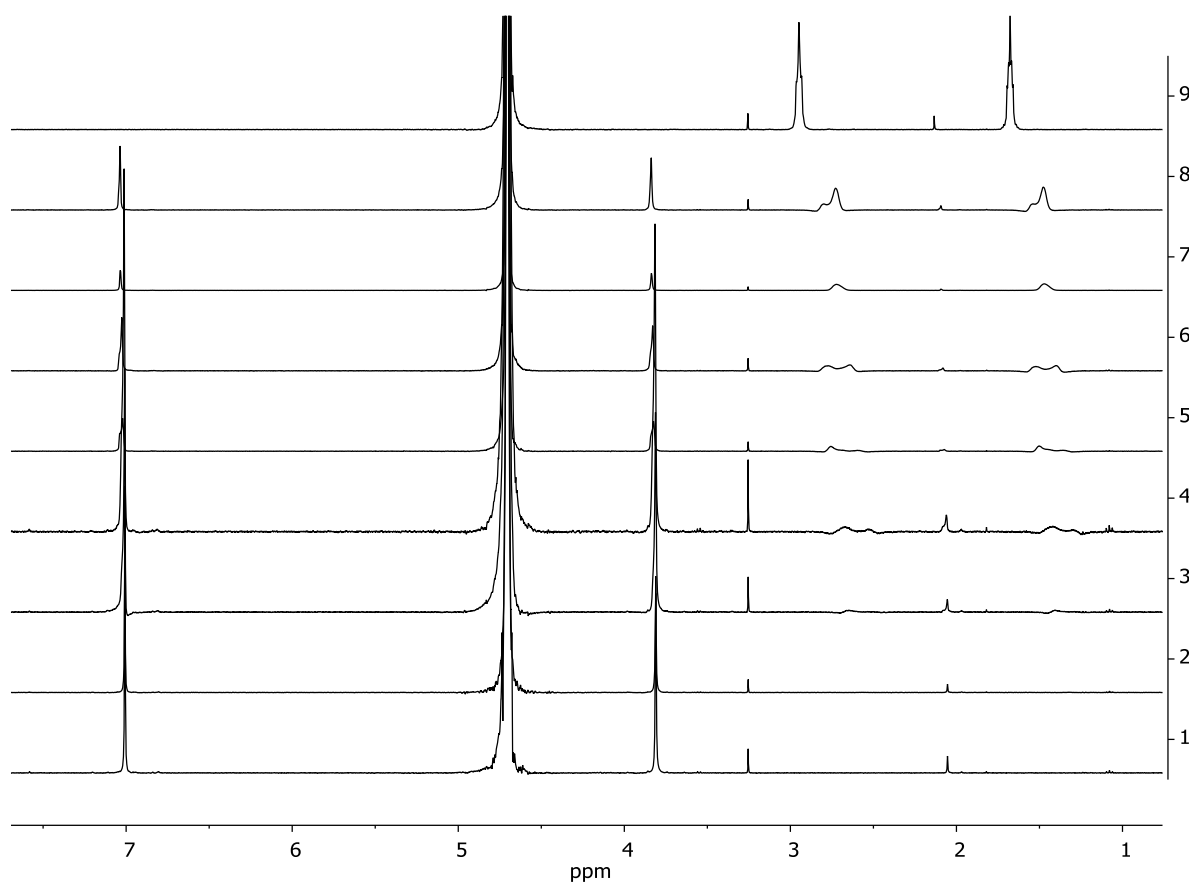


Figure 13. ¹H NMR spectrum of (from the bottom to the top): the host **2** (0.002 M, trace 1), then increasing the concentration of the guest (1,4-diaminobutane); trace 2) +0.4 eq; 3) +0.8 eq; 4) +1.2 eq; 5) +2 eq; 6) +2.8 eq; 7) +3.6 eq; 8) +4.8 eq. Trace 9 corresponds to the spectrum of a pure of 1,4-diaminobutane (0.006 M, the same concentration as at the end of the titration, trace 8). All the spectra were recorded in deuterated phosphate buffer 0.1 M pD 7.3 (400 MHz, 298 K).

This shift is compatible with a dispersion of the positive charges on the guest due to the interaction with the negatively charged sulfonate groups of the host and/or with a partial inclusion of the methylene protons in the calixarene cavity. Regarding the methylene (ArCH_2SO_3) and the aromatic signals of the host, only extremely small, although unidirectional, shifts from high to low fields, accompanied by a slight broadening, are observed from trace 5 to trace 7 (from 2 eq to 3.6 eq of G). Trace 8 presents a very small shift back to higher fields and sharpening of the peak (Figure 14a). As far as the signals of the ArCH_2Ar protons are concerned, the singlet that is superimposed to that of the ArCH_2S group at 3.82 ppm, broadens and nearly immediately disappears. This fact is thought to be originated by the progressive rigidification of the cone calixarene conformation that changes this signal from a singlet (fast exchange between axial and equatorial protons in the free ligand) to two broad signals given by the complex, where the exchange between the two protons (axial and equatorial) is nearly at the coalescence. This observation is common to all the following examples of complex formation between calixarene **2** or **4** with all the polyamine used and will be no longer discussed in detail.

The guest signals present a more complex picture. Each of the two CH_2 peaks at 2.95 and 1.58 ppm of the free guest split into two broad signals (Figure 14b). Looking at them in detail, in traces 2 to 4 the peaks are very broad and significantly shifted to higher fields with respect to the free guest (track 9). In track 5 (2 equivalents of G) two signals of the guest appear, and they grow in intensity upon addition of more equivalents of guest, indicating the possible formations of more species of the complexes (trace 6). At the end of the titration (trace 7) these two peaks merge and in traces 8 (almost 5 eq of G) one species dominates the other. It is likely that, being the host a tetraanion and the guest a dication, once the 1:1 dianionic complex is formed, it still shows affinity for another dicationic guest, so that at high G/H ratios (traces 5-8) also the 1:2 H/G complex forms. The simultaneous presence of multiple equilibria, and the broad and often superimposed signals, did not allow to determine reliable binding constants even when a model of 1:1 and 1:2 complex formation was considered.

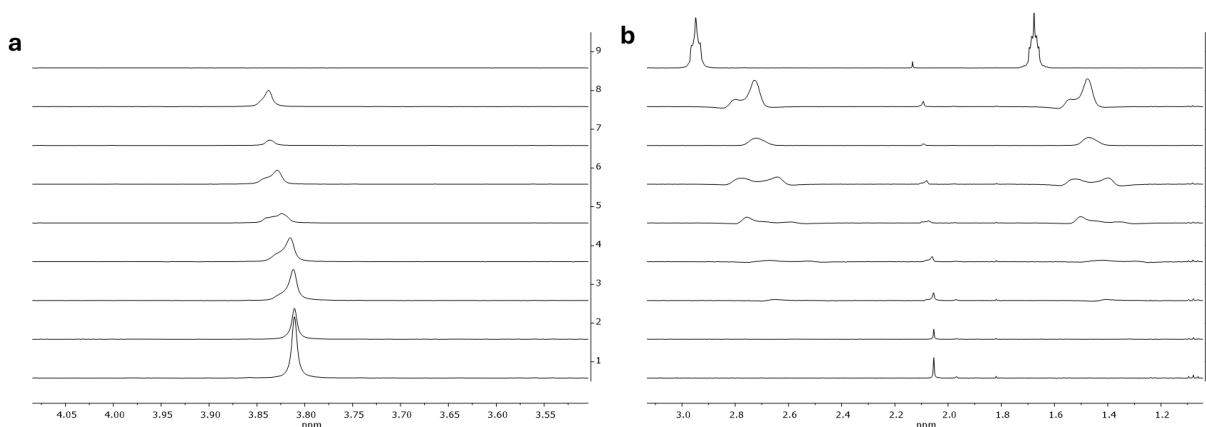


Figure 14. magnification of a) the host methylene bridge signal and b) the guest signals in the titration in Figure 13.

The titration of **2** with 1,6-diaminohexane (298 K, Figure 15 and 16) shows an evolution of the NMR spectra very similar to that of the previous titration, also due to the similarity of the two guests.

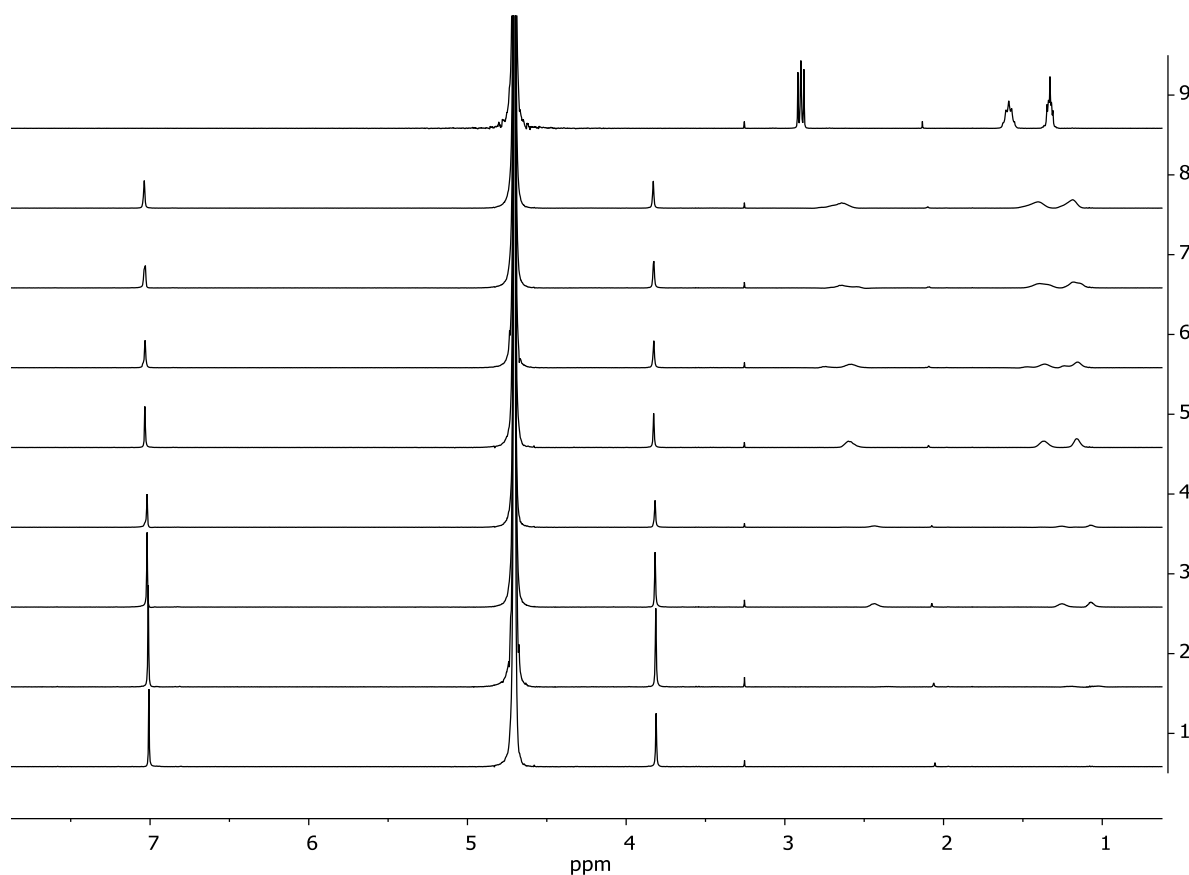


Figure 15. ^1H NMR spectrum of (from the bottom to the top): host **2** (0.002 M, track 1), then increasing the concentration of the guest (1,6-diaminohexane), track 2) +0.4 eq; 3) +0.8 eq; 4) +1.2 eq; 5) +2 eq; 6) +2.8 eq; 7) +3.6 eq; 8) +4.8 eq. Track 9 is the spectrum of pure 1,6-diaminohexane (0.006 M, the same concentration as at the end of the titration, trace 8). Spectra were recorded in deuterated phosphate buffer 0.1 M pD 7.3 (400 MHz, 298 K).

Looking at the host signals, the equilibrium between the complex and the free species appears to be in a fast exchange regime, with a shift to lower field of both the methylene $\text{ArCH}_2\text{SO}_3^-$ (Figure 16a) and the aromatic signal (Figure 16), during the titration.

Regarding the guest signals (Figure 16b), the situation looks very similar to that of the previous titration with 1,4-diaminobutane: the signals of the proton in α position to the ammonium head group (NCH_2) first move to lower fields (traces 2-5) and then split into two broad peaks starting from track 6 (2.8 eq of G), merging again in a single signals at track 8 (4.8 eq of G). All signals resonated at higher field with respect to the free guest (track 9), again because of charge neutralization upon binding and/or inclusion in the calixarene cavity. Also in this case, besides the 1:1 complex, the presence of a 1:2 complex is likely.

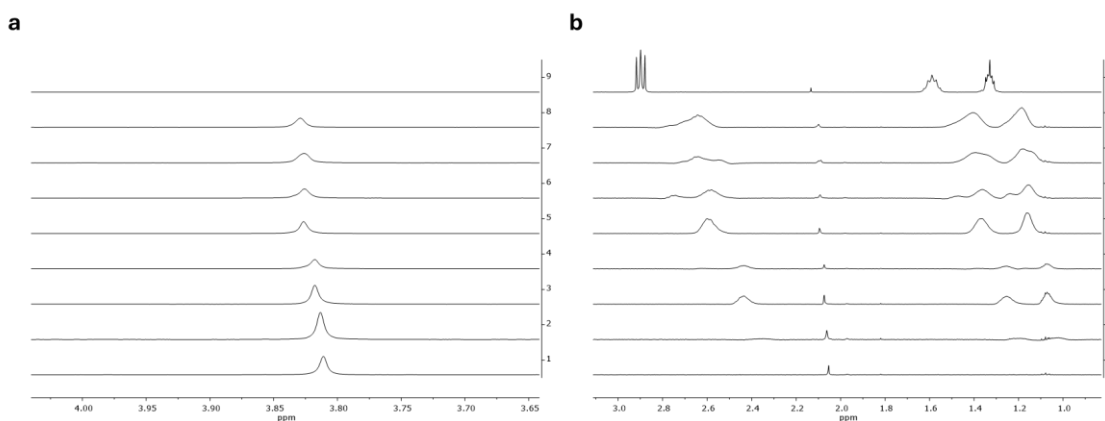


Figure 16. Magnification of a) the host methylene bridge signal and b) one of the guest signals in the titration in Figure 15.

The titration of calixarene **2** with spermidine, a tricationic polyamine, is reported in Figure 17. The pK_a of the ammonium ions of spermidine are respectively 10.9, 9.9 and 8.4. Spermidine is therefore present mainly as trication at pH 7.3 although a small amount of triamine is also present as dicationic species (approximately 8%). However, since upon binding of the triamine to the tetraanionic sulfonatocalix[4]arene the positive charges on the guest are shielded, it might also be possible that the pK_{a3} of the bound guest increases significantly so that it also results 100% protonated. The possible presence of two forms of the guest, even though at rather different concentrations, might lead to the presence of different complexes. From this point of view, the situation might be even more complicated than in the previous titrations. Moreover, also in this case, there is not a precise match between the charges of the host and of the guest, so that the 1:1 H/G complex results still to be monoanionic and therefore potentially able to bind another triammonium ions (Guest).

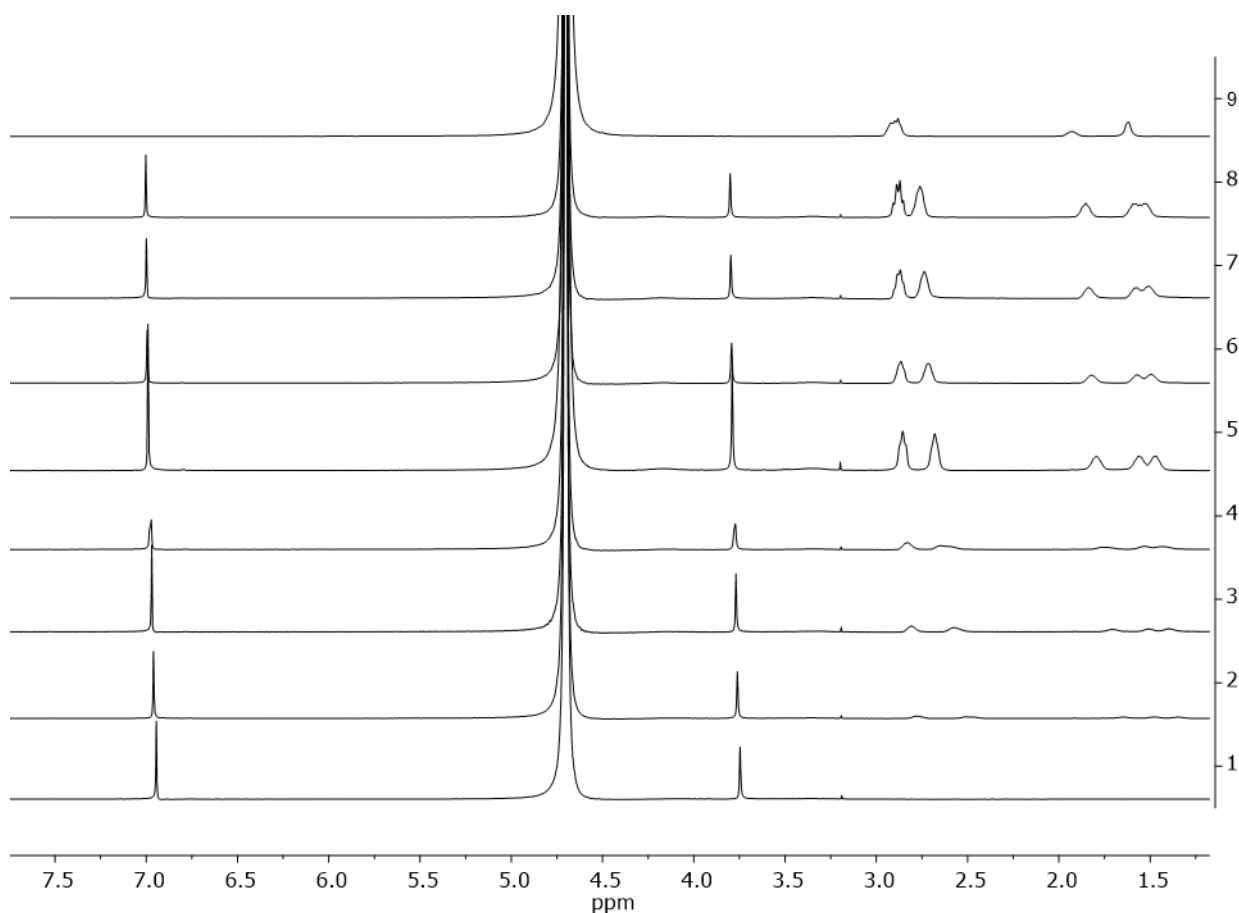


Figure 17. ^1H NMR spectrum of (from bottom to top) host **2** (0.002 M, track 1), then increasing the concentration of the guest (spermidine); track 2) +0.4 eq; 3) +0.8 eq; 4) +1.2 eq; 5) +2 eq; 6) +2.8 eq; 7) +3.6 eq; 8) +4.8 eq. Track 9 corresponds to the spectrum of pure spermidine (0.006 M, like at the end of the titration, trace 8). Spectra were recorded in deuterated phosphate buffer 0.1 M pD 7.3 (400 MHz, 298 K).

Indeed, as can be seen in Figure 18a, it is likely that in this case there are complexes characterized by different exchange regimes with the free species. In tracks 2 and 3 (G/H ratio < 1), the host signal undergoes a downfield shift, suggesting a fast exchange between the host and the 1:1 complex on the NMR timescale. Upon the addition of 1.2 equivalent of G (track 4) a shoulder starts to rise beside the 1:1 complex signal, indicating the presence of different stoichiometry complexes. In track 5 (2 equivalents of G) the equilibrium is shifted to a possible 1:2 complex, due to the presence of only the complex signal, that starts to move slightly to lower fields from track 6 until the end of the titration. This second complex formed (from track 5 on) is probably the 1:2 complex and this appears to be in a fast exchange regime as the complex peak in track 4 moves from 3.789 ppm to 3.800 ppm (track 8).

Much more relevant are the changes connected with the signals of the guest (Figure 18b). At low G/H ratios, the signals appear broad, showing a split into two distinct peaks (observed in trace 3 at 1.50 and 1.39 ppm). Starting from trace 5, the splitted peaks coalesce, indicative of a fast exchange regime. The interpretation of these spectra and the identification of the species present is however far to be clarified.

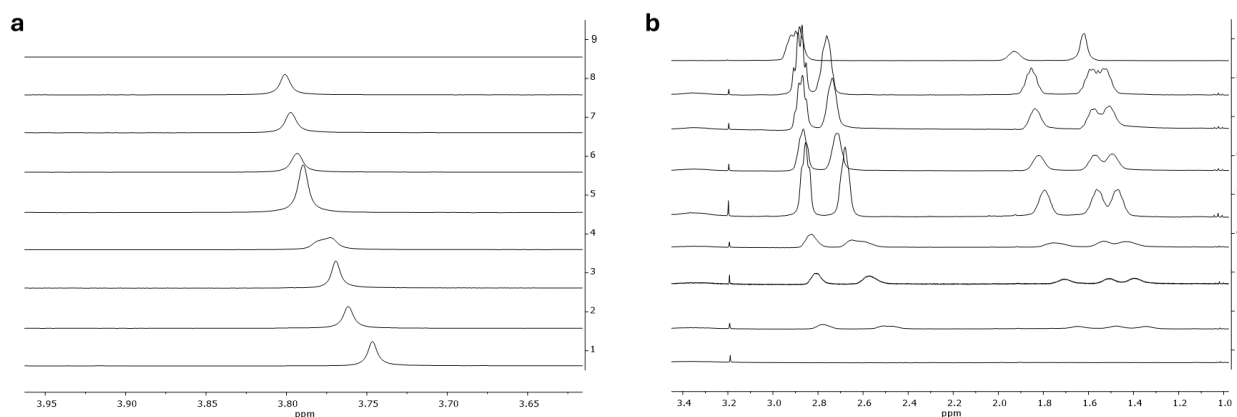


Figure 18. Magnification of a) the host methylene bridge signal of the host and b) magnification of the guest signals in the titration in Figure 17.

Finally, in Figure 20 the titration of **2** with spermine is shown. Spermine is a tetra cationic polyamine and, similarly to spermidine, the range of pK_a of the ammonium ions is quite wide. The lowest pK_a is that of one of the secondary ammonium ions, resulting in 7.9. Therefore, at pH 7.3 there is 25% of a tricationic polyamine but, again, the protonation state can be also influenced by the present of the charged host. As in the previous titration, the presence of two protonation state of the guest can have great influence in the shape of both the host and guest signals along the titration.

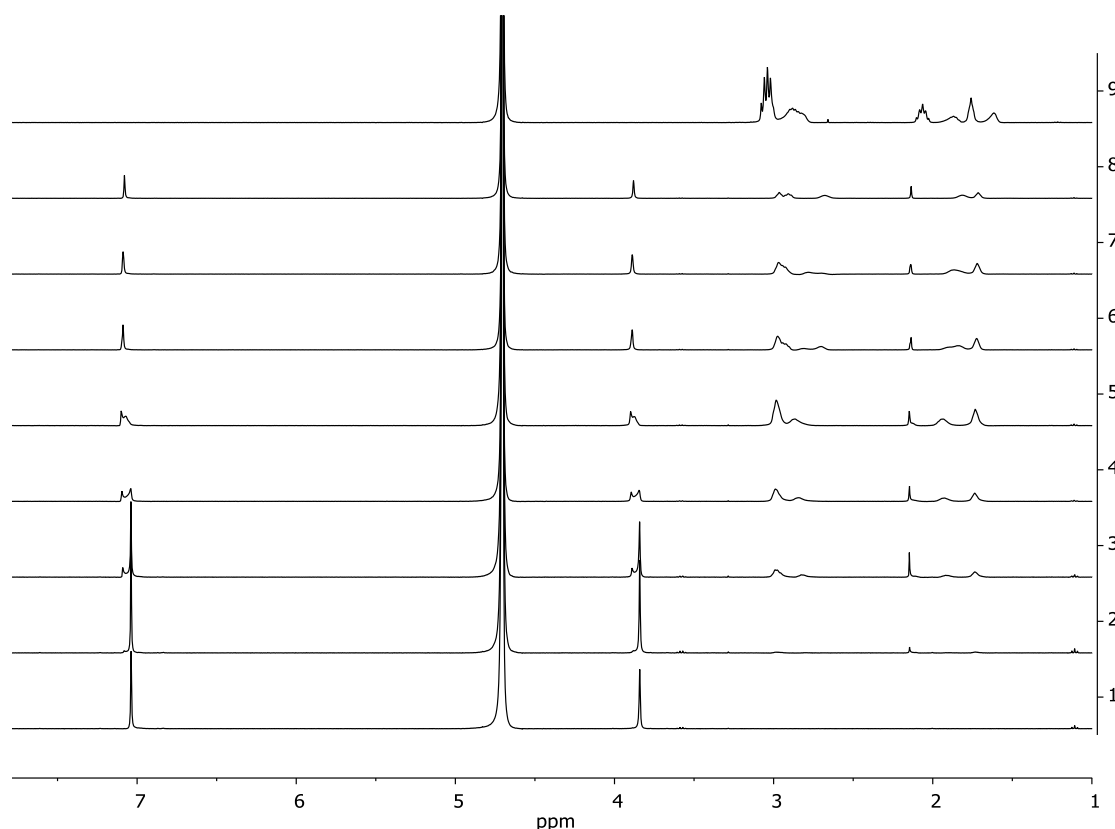


Figure 20. The ^1H NMR spectra of the host **2** (0.002 M, track 1) and, from the bottom to the top, an increase of the concentration of the guest (spermine); track 2) +0.4 eq; 3) +0.8 eq; 4) +1.2 eq; 5) +2 eq; 6) +2.8 eq; 7) +3.6 eq; 8) +4.8 eq. Track 9 corresponds to only to the spermine spectrum (0.006 M like at the end of the titration, track 8). Spectra were recorded in deuterated phosphate buffer 0.1 M pD 7.3 (400 MHz, 298 K).

Both the aromatic and ArCH₂S signals of the host (Figure 21a) indicate the presence of different species. Looking at tracks 2 to 5, it appears that the 1:1 host-guest complex is in a slow exchange regime with the free host, due to the appearance and increase of intensity of a singlet for the ArCH₂S at 3.90 ppm, accompanied by the decrease of the intensity of the free host signal at 3.84 ppm. At track 5, when in the solution there are 2 equivalents of guest for each equivalent of the host and, more markedly from track 6 (2.8 eq of G), this signal of the ArCH₂S of the 1:1 complex moves slightly to lower fields, to end at 3.88 ppm, probably due to a fast exchange regime formation of a 1:2 host-guest complex.

The magnification of the guest signals is shown in Figure 21b. From the beginning to the end of the titration the guest signals show small shifts, while more relevant are the changes in their shapes, generally becoming broader. The multiplet at 3.05, for instance, disappears already in traces 2-3 giving rise to signal at higher field and indicating that its methylene protons (⁺H₃NCH₂) are shielded. Moreover, the four signals from 1.55 ppm to 2.10 ppm (belonging to the NCH₂CH₂ protons) in the free guest (track 9), merge into two even broader signals. Also in this case, however, it is possible to observe a breakpoint in the evolutions of the signals between traces 5 (G/H = 2) and 6 (G/H = 2.8) indicating the presence of different complexes possibly having different stoichiometries.

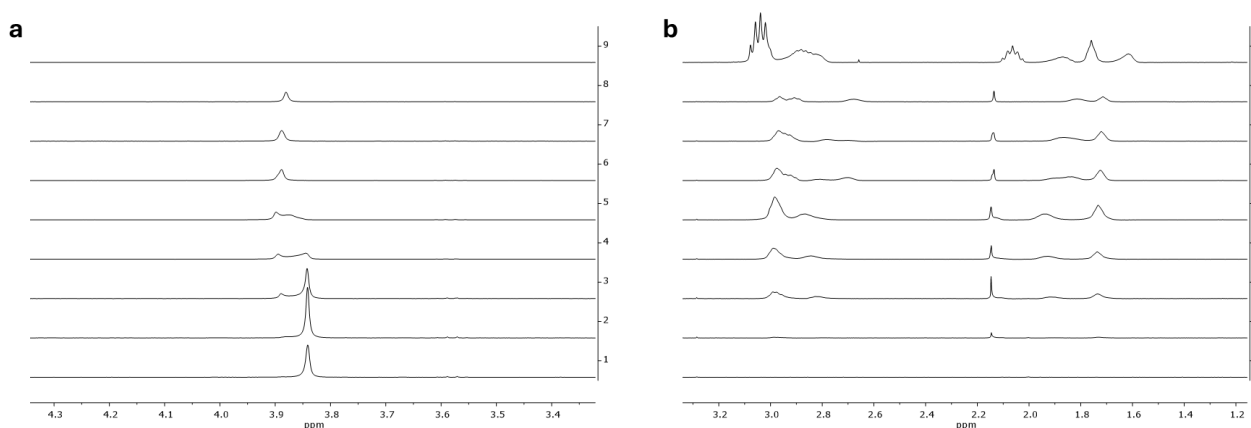


Figure 21. Magnification of a) the host ArCH₂S signal and b) of the methylene region signals of the guest in the titration in Figure 20.

Attempts to identify the stoichiometry of these complexes by using ESI-MS spectrometry have failed either using positive or negative ionization.

2.2.2.2 ¹H NMR titrations with calixarene **2** and polyamines at 50 °C

The presence of two complexes with different exchange rates in the titrations with spermine and spermidine revealed a complicated situation. The same titrations were therefore carried out at 323 K to try to clarify the system. The higher temperatures can indeed increase the exchange rates of the association processes between the free species and the complexes giving simpler spectra and the possibility to use consolidated algorithms to estimate association constants for 1:1 or 1:2 complexes.

Figure 22 shows the titration of **2** with spermidine at 323 K in deuterated phosphate buffer at pD 7.3. Thanks to the higher temperature, the signals of both host and guest are sharper compared to the titration at 298 K, but the peaks split into different signals and become broader during the titration.

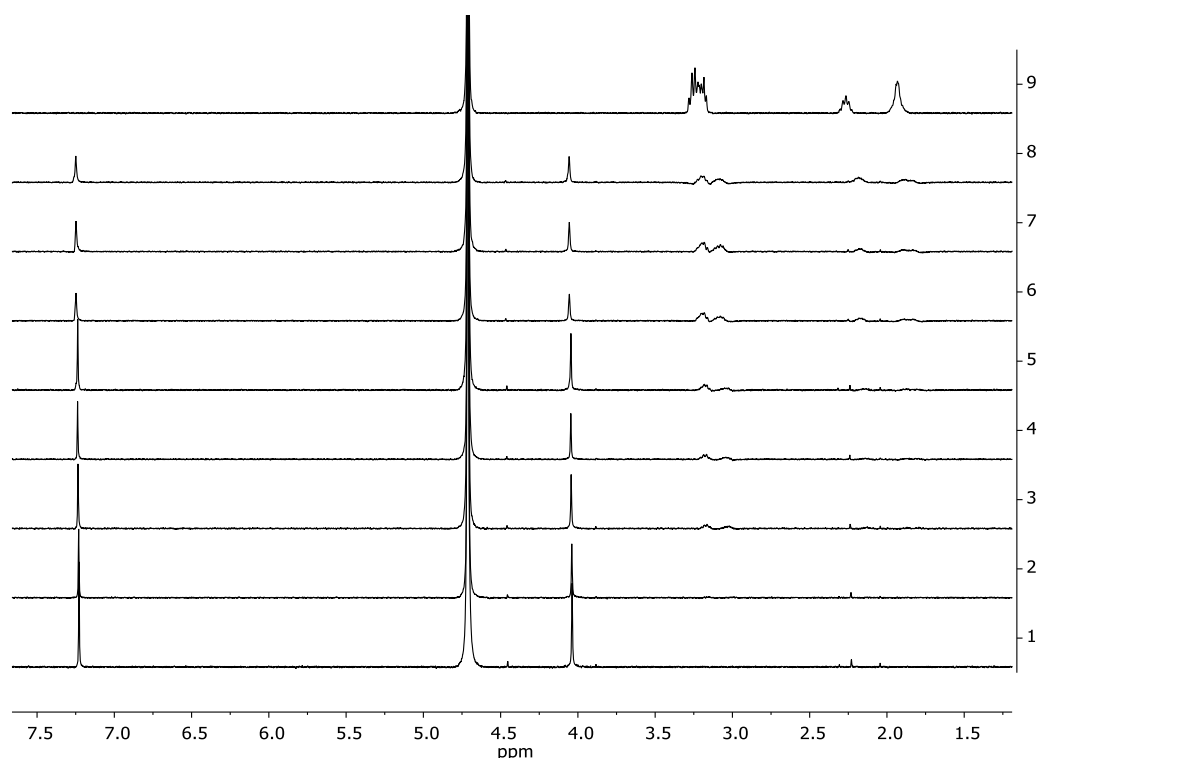


Figure 22. The ^1H NMR spectra (323 K) of the host **2** (0.002 M, track 1) and, from the bottom to the top, an increase of the concentration of the guest (spermidine); track 2) +0.4 eq; 3) +0.8 eq; 4) +1.2 eq; 5) +2 eq; 6) +2.8 eq; 7) +3.6 eq; 8) +4.8 eq. Track 9 corresponds to only to the spermidine spectrum (0.006 M like at the end of the titration, track 8). Spectra were recorded in deuterated phosphate buffer 0.1 M pD 7.3 (400 MHz, 323 K).

Figure 23 shows the comparison between the host methylene bridge ArCH_2Ar signal at 298 K (a) and 323 K (b), where the effect of temperature is more pronounced. Indeed, from track 2 to 5 at 323 K the system appears to be in a fast exchange regime, like in the 298 K titration. The peak at 4.03 ppm moves slightly to lower fields until track 4 (1.2 equivalents of G) and remains in this position also upon addition of other 0.8 equivalents of guest (track 5: total of 2 equivalents of G). With the addition of a further 0.8 equivalents of guest (track 6) the peak reaches 4.06 ppm in one step and starts to broaden until the end of the titration. However, as expected, the shift of the signal at 323 K is less pronounced respect to the titration at 298 K.

The temperature seemed to increase the rate of exchange of the 1:1 complex but it seems that the 1:2 complex exchanges on a scale similar to that of the NMR timescale.

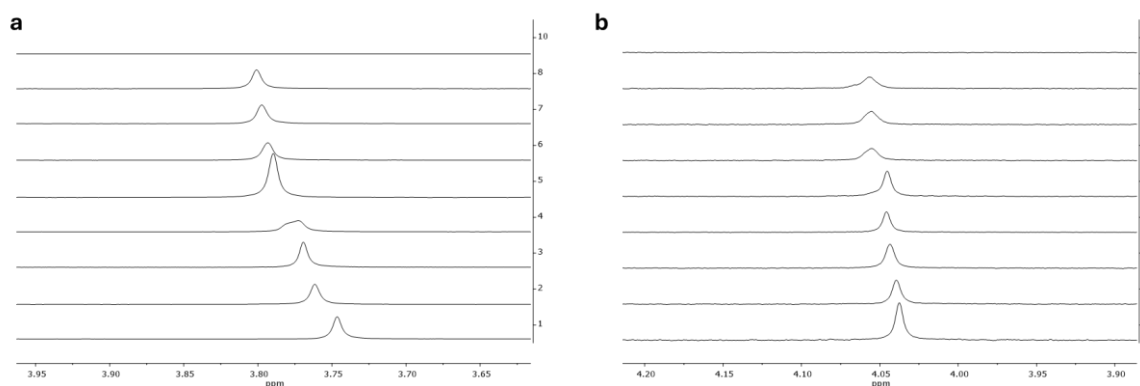


Figure 23. Magnification of a) the host ArCH_2S signal in the titration at 298 K (Figure 17) and b) of the host ArCH_2S signal in the titration at 323 K (Figure 22).

Figure 24 presents the titration of compound **2** with spermine at 323 K in deuterated phosphate buffer at pD 7.3. As in the previous case with spermidine, the elevated temperature makes the signals sharper for both the host and guest compared to the titration at 298 K; however, the peaks become broader as the titration progresses.

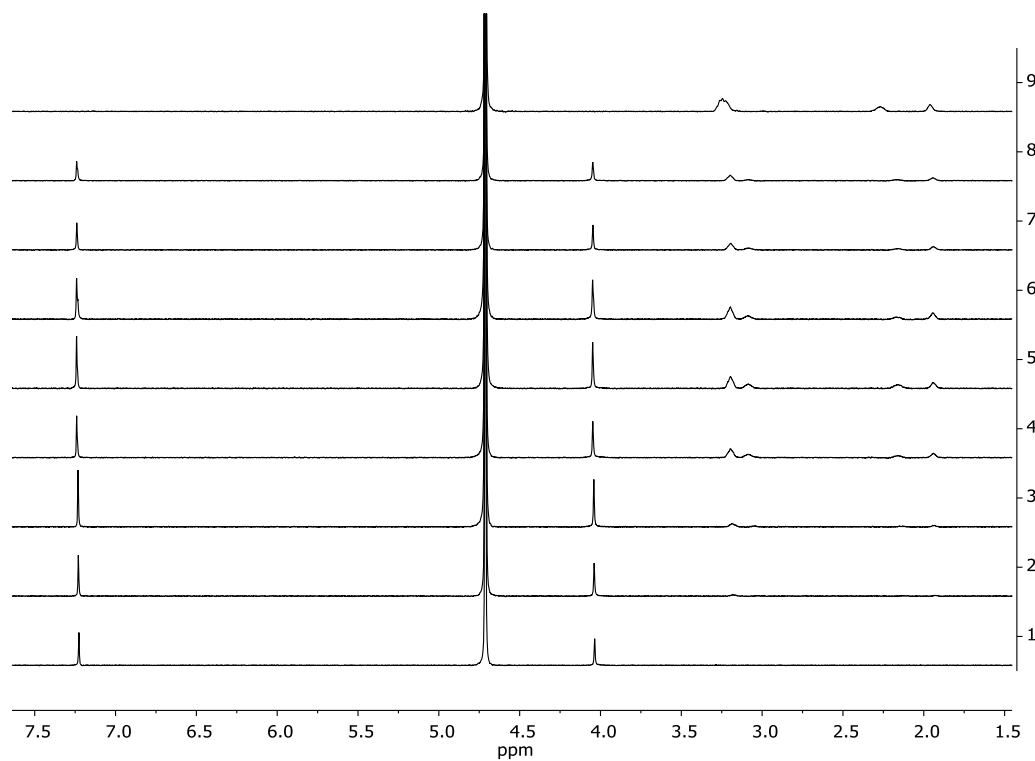


Figure 24. The ^1H NMR spectra (323 K) of the host **2** (0.002 M, track 1) and, from the bottom to the top, an increase of the concentration of the guest (spermine); track 2) +0.4 eq; 3) +0.8 eq; 4) +1.2 eq; 5) +2 eq; 6) +2.8 eq; 7) +3.6 eq; 8) +4.8 eq. Track 9 corresponds to only to the spermine spectrum (0.006 M like at the end of the titration, track 8). Spectra were recorded in deuterated phosphate buffer 0.1 M pD 7.3 (400 MHz, 323 K).

Figure 25 illustrates the comparison of the host methylene bridge signal at 298 K (a) and 323 K (b), where the impact of temperature is more pronounced. At 323 K, the system seems to change to a fast exchange regime between tracks 2 and 3, whereas at room temperature, it remains in a slow exchange regime. The peak at 4.03 ppm gradually shifts to lower fields until it reaches track 4 (+1.2 eq of total Guest) where it is found at 4.05 ppm. For spermine as well, this peak does not move any more with the following additions but broadens significantly.

As in the previous case of spermidine, the effect of temperature seems to be the same: to increase the formation of the initial complex but is not enough to make also the second complexation event fast enough.

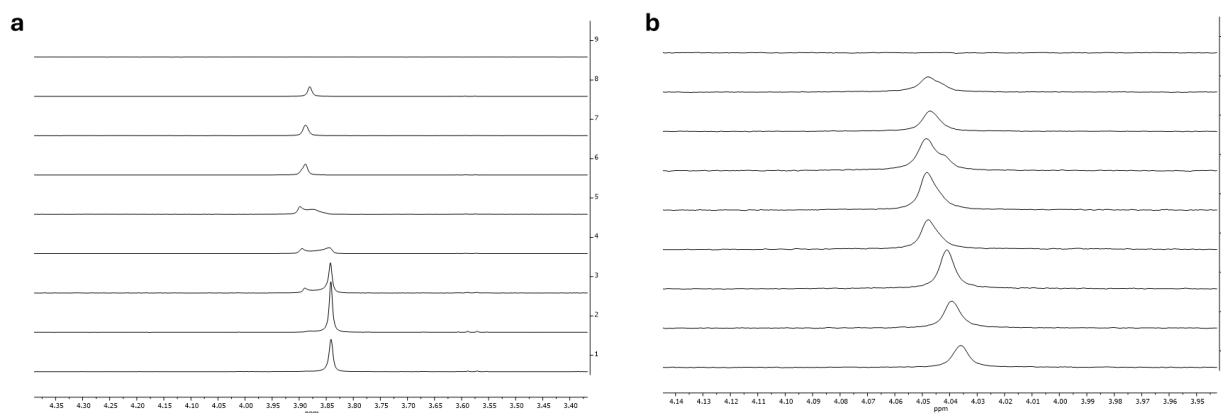


Figure 25. Magnification of a) the host ArCH₂S signal in the titration at 298 K (Figure 20) and b) of the host ArCH₂S signal in the titration at 323 K (Figure 24).

2.2.2.3 ¹H NMR titrations with calixarene **4** and polyamines at 25 °C

Given the affinity of polyamines with phosphate groups (in particular those of nucleic acids^{4,6,22,23}) ¹H NMR titrations were conducted also with calixarene **4**, using the same four guests employed for para-sulfonatomethyl calix[4]arene **2**.

In Figure 26 the titration with **4** as the host and 1,4-diaminobutane as the guest is illustrated.

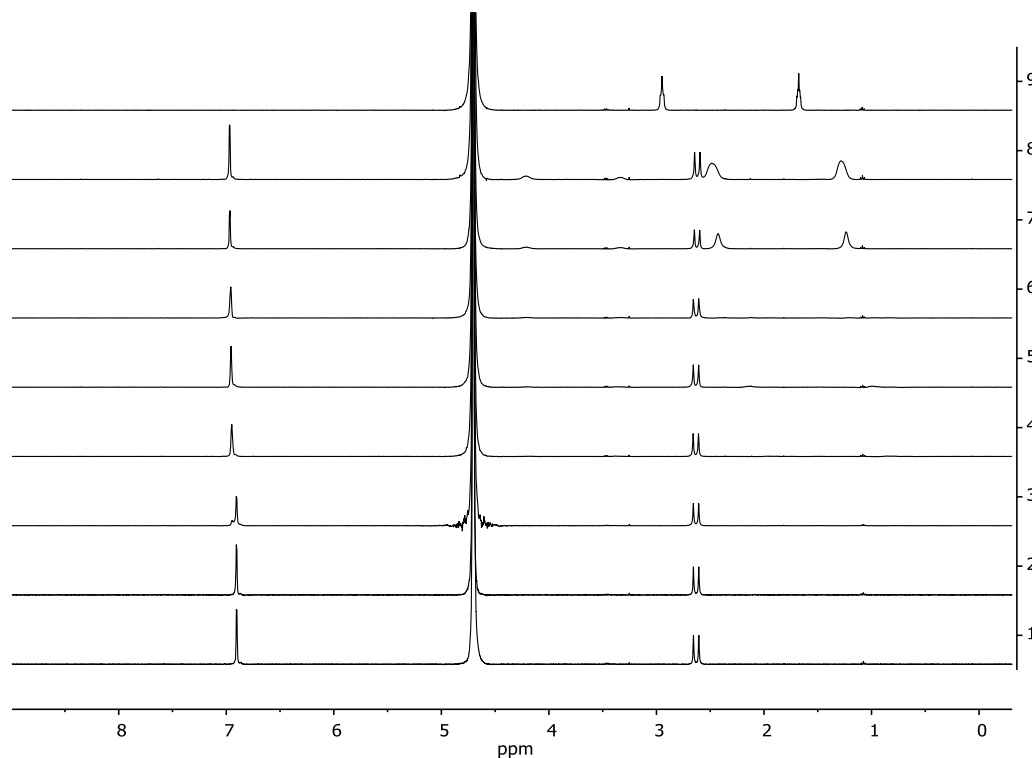


Figure 26. ¹H NMR spectra of the host **2** (0.002 M, track 1) and, from the bottom to the top, an increase of the concentration of the guest (1,4-diamino butane); track 2) +0.2 eq; 3) +0.6 eq; 4) +1.0 eq; 5) +1.5 eq; 6) +2.0 eq; 7) +2.5 eq; 8) +3.0 eq. Track 9 corresponds to only to the 1,4-diamino butane spectrum (0.005 M like at the end of the titration, track 8). Spectra were recorded in deuterated phosphate buffer 0.1 M pD 7.3 (400 MHz, 298 K).

In order to better appreciate the shift of the aromatic signal, magnification is necessary (see Figure 27a). In the initial stages of the titration, up to the addition of 0.6 equivalents of the guest, the system appears to be in a slow exchange regime, as evidenced by the observed decrease in the intensity of the host signal and the appearance of another signal at lower fields. From track 4 (1 equivalent of G), it appears that only one complex is present, exhibiting a broad singlet at 6.95 ppm. The multiplicity of the signal previously attributed to the coupling with P has been lost. The signal thereon slightly shifts to 6.97 ppm (traces 4-7). It is noteworthy that it was still possible to observe a multiplicity for this signal from track 7 (2.5 equivalents of G), and that from that point onwards there is no shift of the signal from 6.97 ppm. This phenomenon is likely attributable to a stiffening of the structural framework, a process that has also been observed in the case of the methylene bridges ArCH_2Ar signals of the calixarene scaffold. These signals are clearly visible in track 7 and 8 (Figure 27b) as two broad signals, as the consequence of their diastereotopicity, at 4.21 ppm (axial protons) and 3.33 ppm (equatorial protons). From the guest point of view, the two signals of the CH_2N protons are rather broad so that they are almost not visible until track 7 (2.5 equivalents of G). Then, in track 8 they undergo a shift to lower fields. Surprisingly, the ArCH_2P signal, visible as a clear doublet due to the coupling with ^{31}P , remains exactly the same in shape during the whole titration, experiencing only a subtle shift from 2.628 to 2.616 ppm.

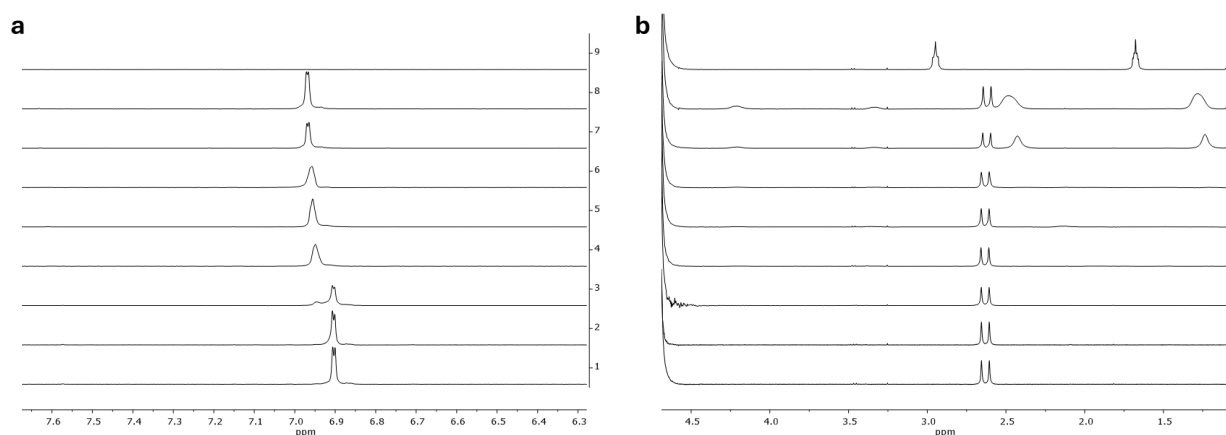


Figure 27. Magnification of a) the host aromatic protons signal and b) one of the guest signals and the ArCH_2P signal in the titration in Figure 26.

The titration of **4** with 1,6-diaminohexane was carried out at 298 K (Figure 28). The system is similar to the previous case, but with a very slight shift of the doublet of the ArCH_2P group and a system in slow exchange regime for the aromatics.

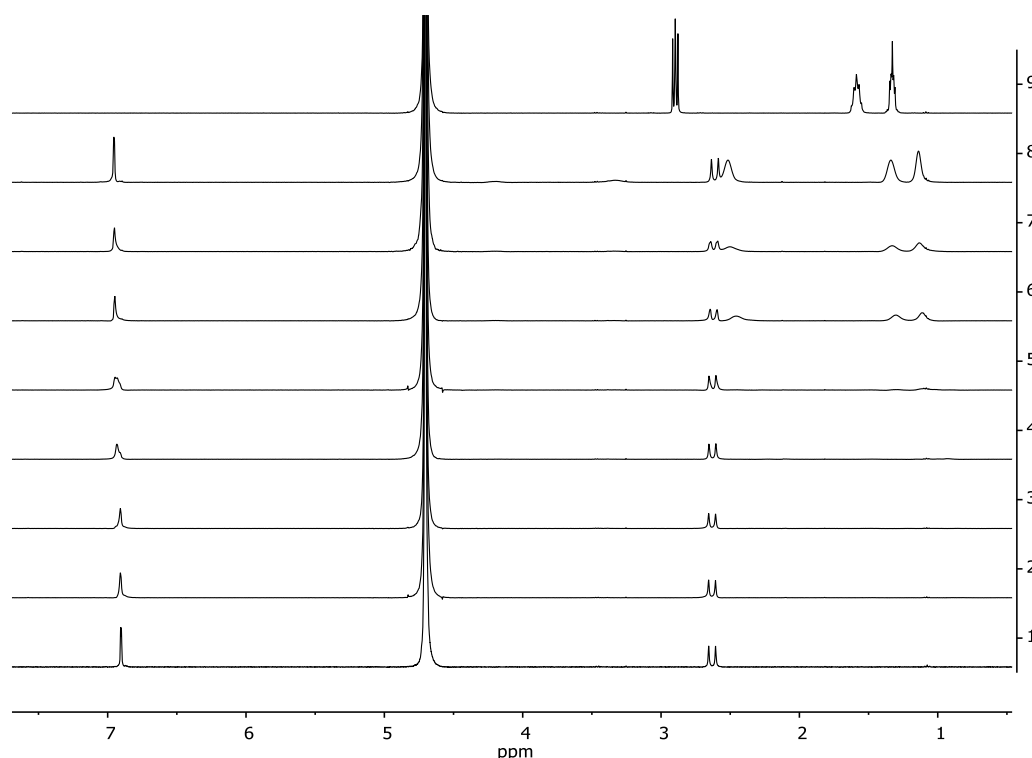


Figure 28. ^1H NMR spectra of the host **4** (0.002 M, track 1) and, from the bottom to the top, an increase of the concentration of the guest (1,6-diamino hexane); track 2) +0.2 eq; 3) +0.6 eq; 4) +1.0 eq; 5) +1.5 eq; 6) +2.0 eq; 7) +2.5 eq; 8) +3.0 eq. Track 9 corresponds to only to the 1,6-diamino hexane spectrum (0.005 M like at the end of the titration, track 8). Spectra were recorded in deuterated phosphate buffer 0.1 M pD 7.3 (400 MHz, 298 K).

As observed in the initial titration of this section, the doublet of the aromatic protons at 6.90 ppm (Figure 29a) loses its multiplicity in track 2 (0.2 equivalents of G) due to the broadening of the signal. From track 3, another peak emerges at 6.93 ppm, while the host signal decreases. Subsequently, the two peaks become broader and merge (track 5, 1.5 eq of G). From track 6 to track 8 (from 2 to 3 equivalents of G), the peak at 6.95 ppm becomes increasingly sharp, ultimately regaining its multiplicity at the end of the titration. As far as the guest signals concern (Figure 29b), they are so broad that at the beginning of the titration (trace 2-5) they are fused with the baseline and are visible only from track 6.

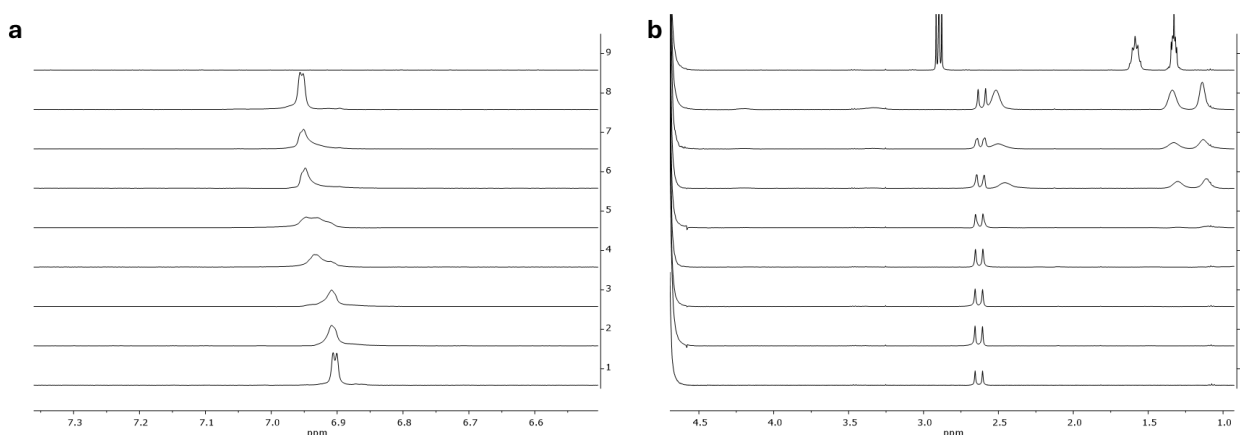


Figure 29. Magnification of a) the host aromatic protons signal and b) one of the guest signals and the ArCH_2P signal in the titration in Figure 28.

Interestingly, all the three sets of signals of the guest are significantly shifted to higher fields even though 2 equivalents of guest are present (trace 6).

The following Figure 30 shows the titration of calixarene **4** with spermidine, a tricationic polyamine. In this case the system is quite different from the previous cases. At 2.10 ppm there is an impurity that remains constant in intensity and at the same chemical shift during the titration, probably an impurity occurred in the buffer preparation.

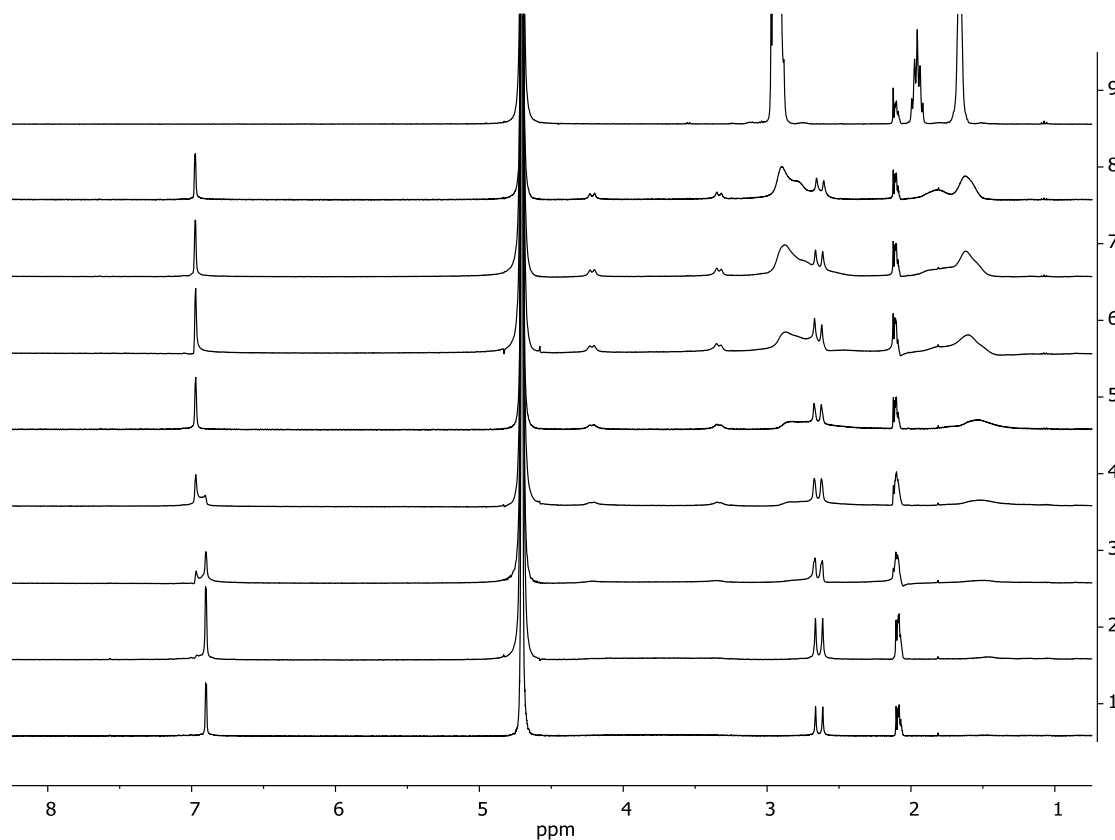


Figure 30. ^1H NMR spectra of the host **4** (0.002 M, track 1) and, from the bottom to the top, an increase of the concentration of the guest (spermidine); track 2) +0.2 eq; 3) +0.6 eq; 4) +1.0 eq; 5) +1.5 eq; 6) +2.0 eq; 7) +2.5 eq; 8) +3.0 eq. Track 9 corresponds to only to the spermidine spectrum (0.005 M like at the end of the titration). Spectra were recorded in deuterated phosphate buffer 0.1 M pD 7.3 (400 MHz, 298 K). The peak at 2.10 ppm remains constant and at the same chemical shift in all the titration, being an impurity in the deuterated phosphate buffer solution.

As illustrated in the magnification of Figure 31a, which depicts the aromatic proton signal, a system exhibiting slow exchange between the free host and the host-guest complex is clearly discernible. Indeed, an additional peak starts to grow in track 3 at 6.97 ppm, which progressively increases during the titration simultaneously to the decrease of the host peak at 6.90 ppm. Furthermore, track 7 illustrates that the peak multiplicity, which was initially absent in this peak, is now restored. Figure 31b also shows the appearance of the peaks of the ArCH_2Ar group as two doublets around 3.32 and 4.20 ppm and of the ArCH_2P doublet that undergoes a weak, but constant shift to higher fields.

The guest signals, shown in Figure 31b, are very broad, slightly high field shifted compared to the free guest (track 9) and clearly visible only from track 5 (1.5 equivalents of G).

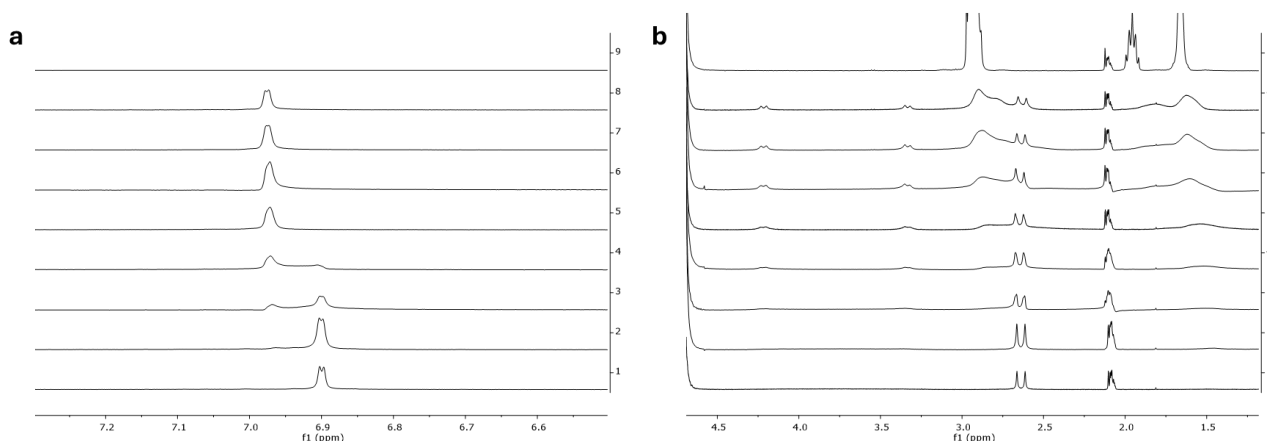


Figure 31. Magnification of a) the host aromatic protons signal and b) one of the guest signals and the ArCH₂P signal in the titration in Figure 30.

Finally, in Figure 32 the titration of **4** with spermine is shown. The titration presents clear analogies with the previous one, with the host aromatic proton signal in slow exchange with the complex one (Figure 33a).

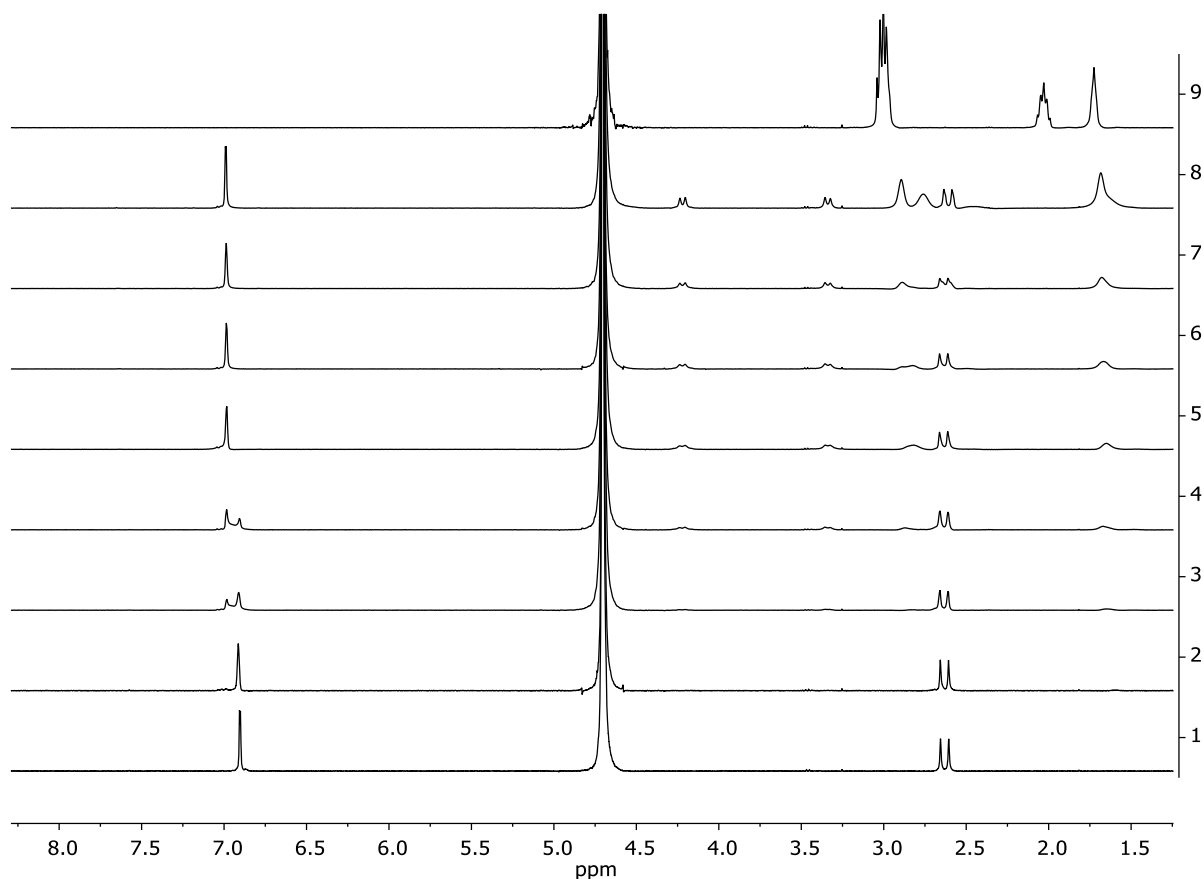


Figure 32. ¹H NMR spectra of the host **4** (0.002 M, track 1) and, from the bottom to the top, an increase of the concentration of the guest (spermine) increases; track 2) +0.2 eq; 3) +0.6 eq; 4) +1.0 eq; 5) +1.5 eq; 6) +2.0 eq; 7) +2.5 eq; 8) +3.0 eq. Track 9 corresponds to only to the spermine spectrum (0.005 M like at the end of the titration). Spectra were recorded in deuterated phosphate buffer 0.1 M pD 7.3 (400 MHz, 298 K).

The evolution of the methylene signals of the host and guest during the titration are illustrated in Figure 33b. The complex formation results in a stiffening of the cone structure, as evidenced by the appearance of the ArCH₂Ar methylene bridge signals of the calixarene scaffold, specifically the doublet at 4.22 and 3.34 ppm in track 8, that in the free ligand are broad and not visible. The ArCH₂P doublet slightly

broadens and shifts to higher fields. The guest signals undergo a slight downfield shift during the titration, and especially the broad signals at 2.8 ppm start to split into two signals from trace 6.

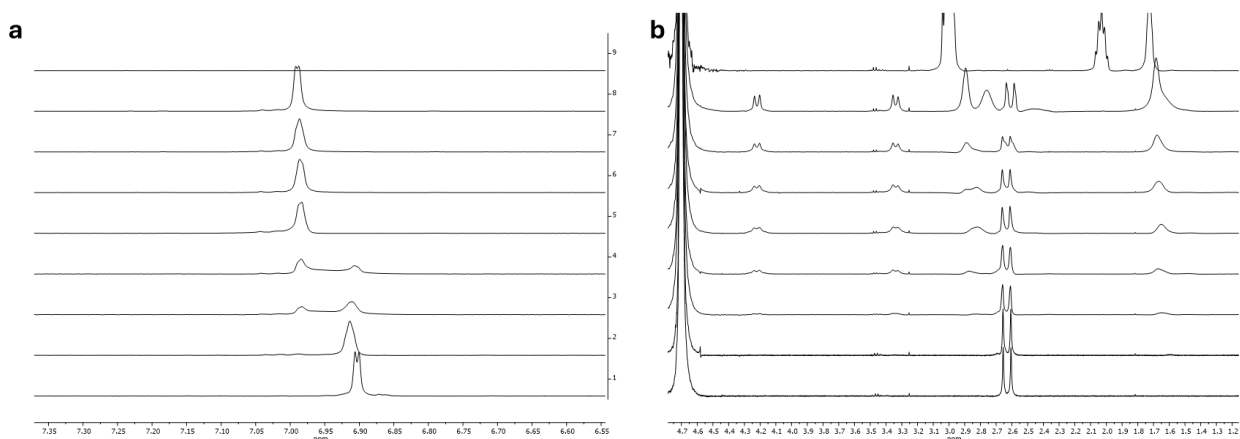


Figure 33. Magnification of a) the host aromatic protons signal and b) one of the guest signals and the ArCH₂P signal in the titration in Figure 32.

2.2.3 Estimation of the association constant for systems in slow exchange

Since the systems are in a slow exchange for ligand **4** and spermidine and spermine, at least at the beginning of the titrations (track 3 and 4) when it is highly reliable that only the free species and the 1:1 complex are present, we tried to roughly evaluate the association constants for these two complexes by using the following formula, also reported in P. Thordarson's review.²⁴

$$K_a = \frac{f_{HG}}{[H]_0(1 - f_{HG}) \left(\frac{[G]_0}{[H]_0} - f_{HG} \right)}$$

With $f_{HG} = \frac{I_{HG}}{I_H + I_{HG}}$ and I_{HG} the integral of the complex signal and I_H the integral of the free host signal.

In Table 2 are reported all the parameters extracted from the NMR spectra and the association constants calculated with the aforementioned formula.

Table 2. Integrals of host (H) and 1:1 complex (HG), initial Host and Guest concentrations and estimation of the association constant K_a (1:1) for the titrations of receptor **4** with spermidine and spermine.

parameters	4 + spermine		4 + spermidine	
	Track 3	Track 4	Track 3	Track 4
I_{HG}	0.23	0.42	0.26	0.73
I_H	0.39	0.15	0.68	0.23
f_{HG}	0.37	0.74	0.28	0.76
H_0 (M)	0.00195	0.00192	0.00191	0.00185
G_0 (M)	0.00117	0.00192	0.00109	0.00189
K_a (M ⁻¹)	1320	5542	681	6568
Average K_a (M ⁻¹)	3400±2100		3600±2900	

The two association constants are certainly affected by a very large error that could be attributed to difficulties associated with the integration process, particularly when the two signals are in close

proximity and their respective tails exhibit partial superposition. Moreover, in traces 3 and 4, especially, peaks are also quite broad.

However, this estimate could suggest that the binding of both spermidine and spermine are of the same order of magnitude and that the presence of the fourth cationic ammonium group in spermine is not affecting too much the binding, probably because, as in Figure 6, only three ammonium groups have space to interact with the four anionic groups of the calixarene.

2.2.4 Photophysical characterization of the complexes

2.2.4.1 Photophysical characterization of the complexes of calixarene **2**

To try to overcome the problems associated with the simultaneous presence of equilibria present in slow exchange and others in fast exchange within the same NMR titration, in collaboration with prof. Montalti and dr. Menichetti from the University of Bologna we undertook the photophysical characterization of the complexes of methyl sulfonate **2** with the different polyamines (1,4-diaminobutane, 1,6-diaminohexane, spermidine, and spermine). The absorption and emission spectra of **2** (Figure 3) show respectively a maximum absorbance peak with a λ_{max} 285 nm and an emission peak with a λ_{max} 460 nm. The absorption and emission spectra in this section were carried out in PBS 0.01 M at pH 7.3, in accordance with the ^1H NMR titrations.

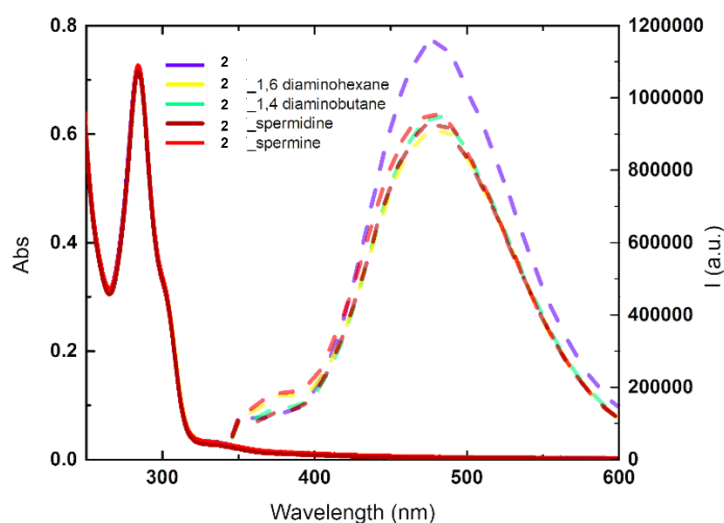


Figure 34. Absorption and emission spectra of **2** (1 mM) alone and in presence of spermine, spermidine, 1,4-diaminobutane and 1,6-diaminohexane (0.1 mM) in PBS 0.01 M pH 7.3.

From the spectra registered by the addition of 10% equivalents of guests to ligand **2**, it can be seen that in presence of all four polyamines studied, the absorption spectrum of calixarene **2** shows no changes, while its emission is quenched. This fluorescence quenching, even if small, can therefore be used to study its interaction of ligand **2** (also called "C" as for Calixarene) with the polyamines (referred to as PA). Figure 35 shows the quenching of **2** with increasing concentrations of 1,6-diaminohexane (a and b) or 1,4-diaminobutane (c and d).

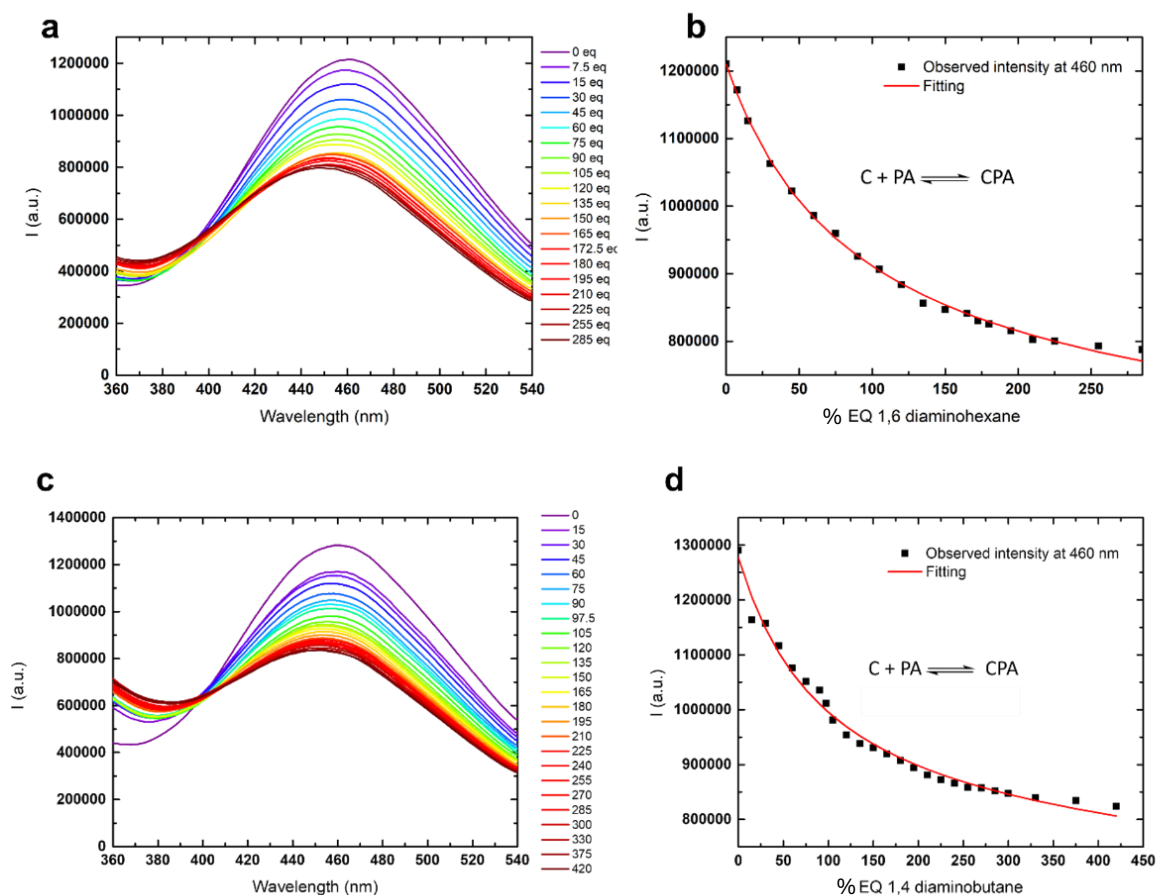


Figure 35. Fluorescence spectra of **2** in the presence of increasing amounts of 1,6-diaminohexane (a) and 1,4-diaminobutane (c) in PBS 0.01 M pH 7.3. Fitting of the emission intensity at 460 nm and the equivalents of 1,6-diaminohexane (b) or 1,4-diaminobutane (d) with the 1:1 binding isotherm.

The presence of these polyamines initially causes a decrease in the emission band, followed by a hypsochromic shift (from 460 nm to 445 nm). This pattern can be properly modeled using a 1:1 interaction, as observed with 1,6-diaminohexane and 1,4-diaminobutane, where only the 1:1 calixarene adduct seems to be formed. However, in the cases of spermidine and spermine, much more consistent decrease in emission peak and the hypsochromic shift are observed, suggesting a more complex scenario. Titration data indicate the presence of a 1:2 adduct, which seems more favored than the 1:1 adduct. This complexity is likely due to the presence of protonated and deprotonated amines, which is influenced by the pH of the system and/or by the higher charge values of these latter guests which might be trications and tetracations, respectively.

The experiments were conducted in a PBS buffer at pH 7.3, where polyamines exhibit varying degrees of protonation based on their pKa values. For 1,4-diaminobutane and 1,6-diaminohexane, nearly all amine moieties are protonated. In contrast, for spermidine and spermine, 8% and 25% of polyamines, respectively, have one deprotonated amine moiety. This suggests the presence of at least two species of polyamines in solution, affecting calixarene complexation. Titrations for spermine and spermidine were fitted by considering the ratio of protonated (PA) to deprotonated polyamines (PAD). The spectral changes observed were attributed to the formation of a 1:1 adduct (CPA) with the fully protonated

polyamine and a 1:2 adduct (CPAPAd) involving calixarene **2**, one protonated polyamine (PA), and one singly deprotonated polyamine (PAd), see Figure 36.

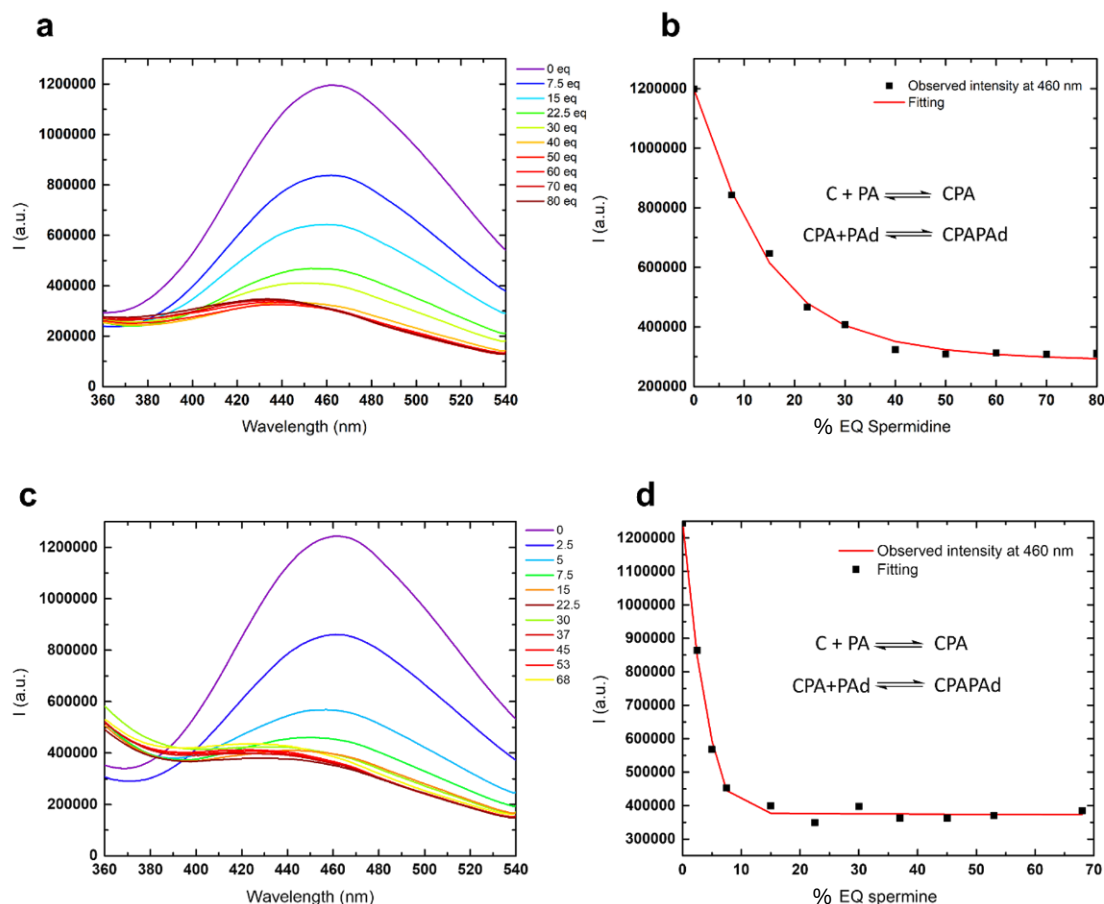


Figure 36. Fluorescence spectra of **2** with spermidine (a) and spermine (b) in PBS 0.01 M pH 7.3. Fitting of the emission intensity at 460 nm and equivalents of spermidine (b) and spermine (d) with the 1:1 and 1:2 model of binding.

Titration curves were then used to calculate the association constants for the 1:1 or 1:2 (when applicable) complexes.

Table 3. Association constants of 1:1 and 1:2 complexes of **2** and polyamines in PBS 0.01 M pH 7.3.

	K_a 1:1 (CPA)	K_a 1:2 (CPAPAd)
2 +1,4-diamino butane	$0.8 \times 10^3 \text{ M}^{-1}$	-
2 +1,6-diamino hexane	$0.9 \times 10^3 \text{ M}^{-1}$	-
2 +spermidine	$3 \times 10^3 \text{ M}^{-1}$	$1 \times 10^5 \text{ M}^{-1}$
2 +spermine	$8 \times 10^3 \text{ M}^{-1}$	$7 \times 10^5 \text{ M}^{-1}$

The results from fitting the titrations of compound **2** with the polyamines (Table 3) indicate that for 1,6-diaminohexane, 1,4-diaminobutane, and spermidine, a 1:1 complex is formed with an association constant of approximately 10^3 M^{-1} . However, the complex formation with spermine is more favoured, as would be expected by the higher charge of this guest, showing an association constant nearly three times higher. While only the 1:1 adduct is observed for 1,6-diaminohexane and 1,4-diaminobutane, the titration curves for spermine and spermidine also indicate the presence of a 1:2 complex, with an

association constant of around 10^5 for spermidine and nearly 10^6 for spermine, exhibiting a relatively strong cooperative effect, since the K_a 1:2 is two order of magnitude higher than the K_a 1:1.

2.2.4.2 Photophysical characterization of the complexes of calixarene **4**

The same photophysical characterization experiments were carried out for calixarene **4**. The absorption maximum for calixarene **4** is at 285 nm (Figure 37a), as for calixarene **2**. The emission spectrum was recorded by excitation of the ligand at this wavelength and show a λ_{max} at 442 nm. The emission spectra (Figure 37b) were recorded adding different solutions of the four polyamines of 10% equivalents (to reach 10^{-3} M of the total polyamine concentration) to a solution of **4** at the concentration 10^{-4} M.

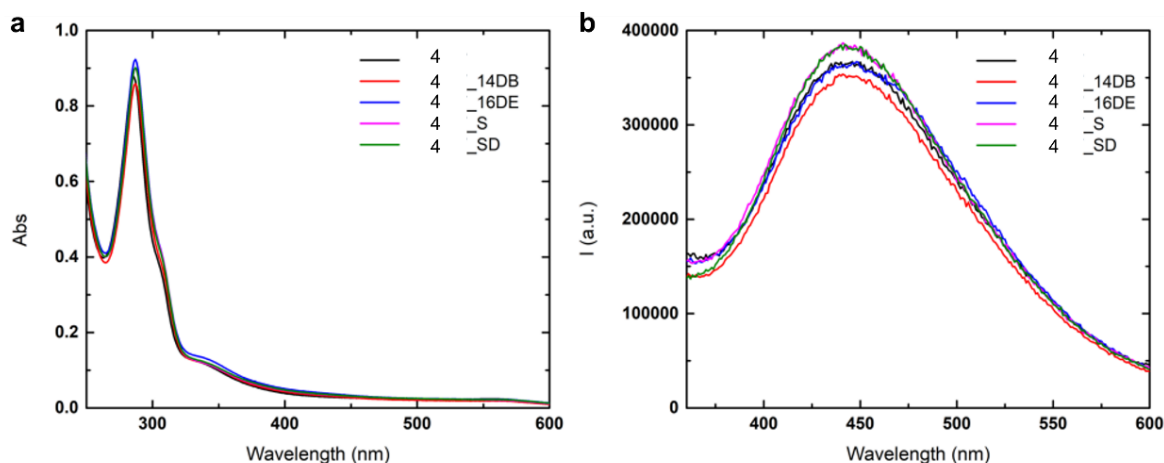


Figure 37. Absorption (a) and emission (b) spectra of receptor **4** alone and in the presence of the four different polyamines (10% of the equivalents) in PBS 0.01 M, pH 7.3. 14DB is 1,4-diaminobutane, 16DE is 1,6-diaminohexane, S is spermine and SD is spermidine.

In this case, it appears that the addition of the polyamine solution does not result in a significant quenching of the emission of **4**, or at least not so evident to allow to carry out titration experiments and K_a determinations with this receptor by fluorescence.

2.3 Conclusions

This chapter presents the investigation of the binding of calixarene **2** and **4** towards 1,4-diaminobutane, 1,6-diaminohexane, spermidine, and spermine. Both ligands interact significantly with these biogenic polyamines. The initial study carried out by ^1H NMR titrations in deuterated phosphate buffer at a pD of 7.3, could reveal the existence of different complex species (1:1 and 1:2) and suggests the existence of electrostatic and/or CH- π interactions to be operative in the complexes.

Calixarene **2** binds to all the four polyamines, yet the evolution of the ^1H NMR spectra during the NMR titrations evidences a very intriguing situation. Indeed, the shift of the host signals in all the titrations indicates the presence of different complexes, possibly 1:1 and 1:2. Furthermore, the titrations with spermidine and spermine presented also a variation in the rate of exchange between the complex and the free species which made the determination and even estimation of K_a impossible at room temperature.

To try to simplify this analysis and bring the complexation event on a fast exchange rate compared to NMR timescale, these titrations were carried out at high (50 °C) temperature. Although at this temperature the first complexation event is sped up and is in a fast exchange regime, the second complexation event remains in slow exchange and therefore the quantification of the K_a was not possible by ^1H NMR spectroscopy.

In collaboration with University of Bologna (Dr. Menichetti and Prof. Montalti) photophysical studies allowed the determination of the association constants of ligand **2** with the different polyamines. A 1:1 complex exclusively forms for the system **2** + 1,4-diaminobutane and **2** + 1,6-diaminohexane having K_a of $0.8 \times 10^3 \text{ M}^{-1}$ and $0.9 \times 10^3 \text{ M}^{-1}$, respectively. The results of the titrations with spermidine and spermine revealed the formation of two complexes with different stoichiometries, namely H:G 1:1 and 1:2. The association constants between the two complexes differ by a factor of two, indicating a relatively strong cooperative effect. The association constants for spermidine and spermine are $3 \times 10^3 \text{ M}^{-1}$ and $8 \times 10^3 \text{ M}^{-1}$ for the complex H:G 1:1 and $1 \times 10^5 \text{ M}^{-1}$ and $7 \times 10^5 \text{ M}^{-1}$ for the complex H:G 1:2, respectively.

Additionally, calixarene **4** also demonstrated to bind with all the four polyamines through ^1H NMR titrations. Like for calixarene **2** the ^1H NMR titrations results were particularly difficult to be interpreted due to a consistent broadening of the signal, particularly in the case of 1,4-diaminobutane and 1,6-diaminohexane. However, the situation with spermine and spermidine is less ambiguous, exhibiting a slow exchange rate on the NMR timescale between the free host and the 1:1 HG complex, at least for the first part of the titration. Binding constants in the range of 10^3 M^{-1} were estimated. Also in these cases, electrostatic interactions and/or CH- π interactions are suggested to be operative in the complexes.

The photophysical studies of the complexes with **4**, indicates that the addition of polyamines to buffered solutions of the ligands does not result in any changes of the emission spectra, and thus, no further experiments could be carried out.

2.4 Experimental part

General information

Commercial reagents and solvents were used as received, without any prior purification or treatment unless otherwise noted. Reactions sensitive to moisture and air were carried out under a nitrogen atmosphere. Dry solvents were prepared following standard protocols and stored with molecular sieves. The progress of synthetic reactions was monitored by thin-layer chromatography (TLC) using 60 F254 silica gel plates for direct-phase and RP-18 F254 silica gel plates for reverse-phase. For detecting reagents and products, TLC plates were sprayed with a 5% ninhydrin solution in ethanol for amines, an FeCl_3 solution in water for phenols, an acidic 2,4-dinitrophenylhydrazine solution in ethanol for aldehydes, and a 0.05% KMnO_4 solution in water for oxidizable compounds. Product purification was achieved using flash chromatography on silica gel 60 (230-400 mesh) under nitrogen pressure, and commercial preparative TLC on 20×20 cm silica gel F254 plates, 0.5 mm thick.

Materials and methods

Absorption spectra were performed using a Perkin Elmer Lambda 650 on **2** solution 1×10^{-4} M in PBS 0.01 M, with the addition of polyamines at 1×10^{-3} M.

Emission spectra were performed using a spectrofluorimeter Horiba Fluoromax-4, on **2** solution 1×10^{-4} M in PBS 0.01 M, with the addition of polyamines at 1×10^{-3} M, exciting at 285 nm.

In emission titrations a compound **2** solution 1×10^{-5} M in PBS 0.05 M (pH 7.3) was used as titrand and polyamines solutions 0.01 M were used as titrants. Emission spectra were performed exciting at 285 nm. The titration data have been elaborated by means of HyperQuad software (HyperSpec).

^1H NMR titrations were conducted on a Bruker AVANCE 400 spectrometer (^1H at 400 MHz, ^{13}C at 100 MHz). A solution of calixarene (**2** or **4**) 0.002 M was prepared in phosphate buffer 0.1 M (pD 7.3) and used as the host. Different aliquots of polyamine solution were added from a mother solution 0.033 M (for host **2**) or 0.05 M (for host **4**).

Product characterization was carried out using ^1H and ^{13}C NMR spectroscopy and mass spectrometry with ESI technique. NMR spectra were recorded on a Bruker AVANCE 400 spectrometer (^1H at 400 MHz, ^{13}C at 100 MHz), with chemical shifts reported in ppm relative to the partially deuterated solvent resonance frequency. Mass spectra were obtained with a Waters single quadrupole SQ detector spectrometer. Melting points were measured using a Gallenkamp apparatus with closed capillaries.

5,11,17,23-tetrakis(bromomethyl)-25,26,27,28-tetrahydroxy calix[4]arene (**1**)

Tetrahydroxy calix[4]arene (1.50 g, 3.23 mmol) and paraformaldehyde (1.95 g, 64.60 mmol) were dissolved in 30 mL of glacial acetic acid in a round bottom flask, giving a pale yellow solution. Hydrobromic acid solution (33% w/w in acetic acid) was then added dropwise (10.00 mL, 129.20 mmol), giving a pale red solution with a suspended solid. The reaction was left stirring at room temperature for 72 hours and monitored via TLC (SiO_2 ; hexane-ethyl acetate 1:1). Once assessed the completion of the reaction the pink precipitate was filtered and washed with H_2O and purified via precipitation with chloroform/petroleum ether. A pale pink solid (2.05 g, 2.58 mmol) was obtained in 80% yield.

^1H NMR (400 MHz, CDCl_3) δ (ppm): 10.11 (s, 4H, OH), 7.12 (s, 8H, ArH), 4.36 (s, 8H, ArCH_2Br), 4.26 (bs, 4H, $\text{ArCH}_{ax}\text{HAr}$), 3.55 (bs, 4H, $\text{ArCHH}_{eq}\text{Ar}$).

The product shows the same spectroscopic data of those reported in literature.²¹

5,11,17,23-tetrakis(sulfonatomethyl)-25,26,27,28- tetrahydroxy calix[4]arene (2)

Compound **1** (0.2 g, 0.25 mmol) was suspended in 1 mL of ethanol in a round bottom flask and a 2 M aqueous solution of sodium sulfite was added (0.14 g, 1.10 mmol): the suspension turns from white to pale blue. The reaction was left stirring at reflux and monitoring via TLC (SiO₂; dichloromethane-methanol 9:1). The solvent was evaporated under reduced pressure and the product was isolated from the inorganic salts, recrystallizing it from methanol/acetone 1:1. It was then dissolved in H₂O and acidified using Amberlit IR-120 column. After recrystallization with a 1:1 EtOH/H₂O mixture a white solid was obtained in a 70% yield (0.14 g, 0.17 mmol).

¹H NMR (400 MHz, D₂O) δ : 7.09 (s, 8H, ArH), 3.87 (bs, 8H, ArCH₂Ar), 3.84 (s, 8H, ArCH₂SO₃Na).

¹³C NMR (101 MHz, D₂O) δ : 148.7 (C_{ipso}), 130.7 (C_{meta}), 128.7 (C_{orto}), 125.2 (C_{para}), 55.9 (ArCH₂SO₃H), 30.3 (ArCH₂Ar).

ESI-MS: 888 m/z [M+H+4Na]⁺, 911 m/z [M+5Na]⁺.

The product shows the same spectroscopic data of those reported in literature.¹⁸

5,11,17,23-tetrakis[(diethoxyphosphonoyl)methyl]-25,26,27,28- tetrahydroxy calix[4]arene (3)

Under nitrogen atmosphere compound **1** (0.20 g, 0.25 mmol) was dissolved in P(EtO)₃ (2.04 mL, 11.30 mmol) and refluxed for 48 hours. The reaction turns from a pale green suspension to colourless solution. The completion of the reaction was assessed via TLC monitoring (SiO₂; ethyl acetate-methanol 9:1), then was P(EtO)₃ distilled under reduced pressure. The product is a yellow oil, due to the presence of residues of P(EtO)₃. The crude product was used as it was for further steps without purification.

The product shows the same spectroscopic data of those reported in literature.²⁰

5,11,17,23-tetrakis[(dihydroxyphosphonoyl)methyl]-25,26,27,28-tetrahydroxy calix[4]arene (4)

Compound **3** (0.4 g) was added to a solution of 37% w/w HCl and stirred at reflux for 24 hours. After completion, as determined by TLC (SiO₂; ethyl acetate-methanol 9:1) and ESI-MS, the solvent was removed under reduced pressure. The black solid obtained was washed with chloroform, methanol and 1 M HCl and then adjusted to basic pH with 1 M NaOH solution. The product was obtained as a purple solid (169 mg, 0.19 mmol) in 77% yield, calculated from the previous step.

¹H NMR (400 MHz, D₂O) δ (ppm): 6.90 (d, J=2.4 Hz, 8H, ArH), 4.27-3.35 (bs, 8H, ArCH₂Ar), 2.63 (d, 8H, J=20.0 Hz, ArCH₂PO(ONa)₂).

ESI-MS: 800 m/z [M+H]⁺, 823 m/z [M+Na]⁺.

The product shows the same spectroscopic data of those reported in literature.²⁰

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Synthesis of zwitterionic calix[4]arenes and PEGylated chains for surface functionalization

3.1 Introduction

In the context of the Bachound project,¹ a recent study carried out by our research group exploits different calix[4]arenes to investigate their interactions with the outer cell wall of Gram-positive bacteria, Gram-negative bacteria, and Mycobacteria.² The calix[4]arenes examined in this work possess different charge states, and thus are zwitterionic, positively, or negatively charged. These compounds were tested on representative bacterial strains of the aforementioned classes using the Saturation Transfer Difference (STD) NMR technique, on live cells, performed by Professor Airoidi and her group at the University of Milano-Bicocca. This powerful technique, originally designed to study ligand binding to biomolecules such as proteins, has been already successfully adapted to investigate interactions with cells, taking advantage of the Magic Angle Spinning (MAS) method that improves resolution and prevents precipitation of the cells during the experiments.³⁻⁵ Among the tested ligands, calix[4]arenes **C1** and **C2**, functionalized with L-proline and L-phenylalanine moieties, respectively, were found to selectively bind to Gram-negative bacteria (*P. putida* and *E. coli*) over Gram-positive bacteria (*S. epidermidis*) and Mycobacteria (*M. smegmatis*).

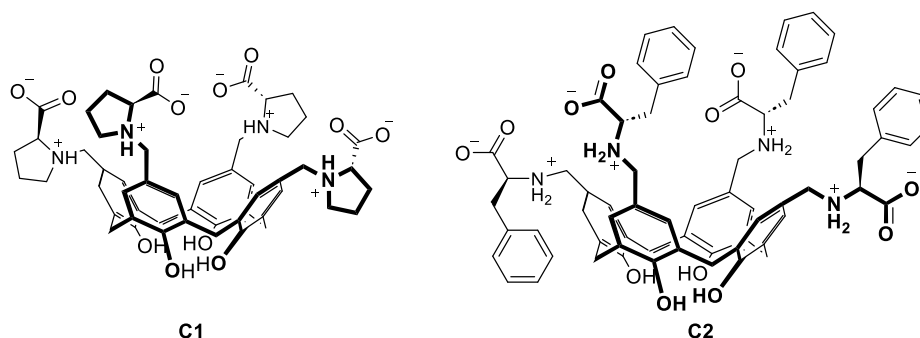
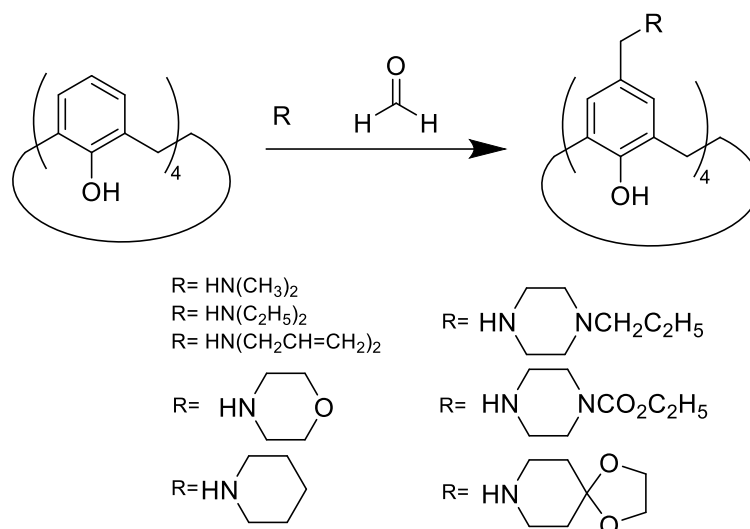


Figure 1. Structure of zwitterionic calix[4]arenes **C1** and **C2**. **C1** is functionalized at the upper rim with L-proline and **C2** with L-phenylalanine.

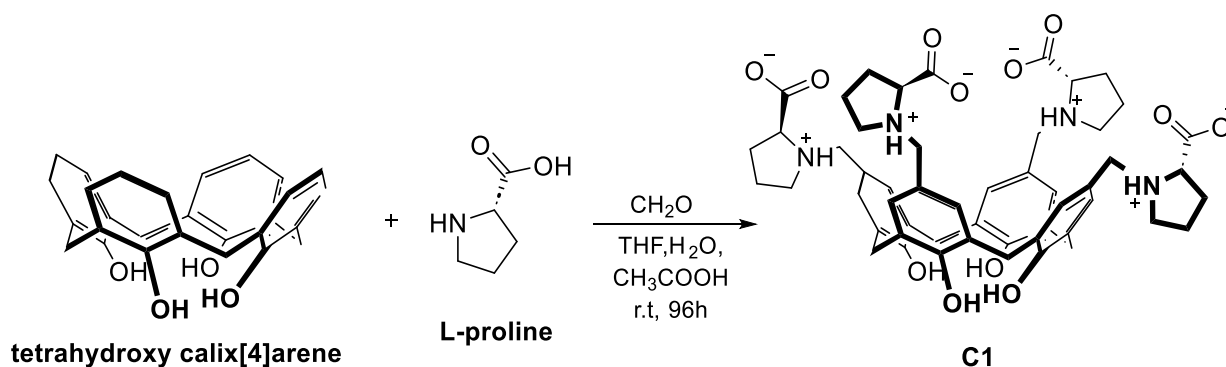
The study further emphasizes the critical role of the zwitterionic motif in mediating the binding. Specifically, the removal of the negative charges, achieved by converting the carboxylate groups into methyl esters, or of the positive charges by preparation of a mimic of **C1** where the proline nitrogen atoms are connected to the calixarene scaffold via amide bonds, result in negligible bacterial binding. Aware of the fact that the outer surface of Gram-negative bacteria cell walls are characterized by a relevant presence of lipopolysaccharide (LPS), and suspecting that this class of macrobiomolecules could be the target of the two calix[4]arenes **C1** and **C2** ligands, STD experiments were carried out using micelles of deuterated sodium dodecylsulfate and the LPS extracted from *E. coli* or *P. putida*. These experiments clearly outlined that calixarene ligands **C1-2** strongly bind LPS, thus suggesting that these lipopolysaccharides are a potential target of the selective recognition event even on the cell walls.⁶

3.1.1 Mannich reaction on calixarenes

One of the most practical way to attach an amine or an amino acid to a calix[4]arene scaffold maintaining the zwitterionic character has been revealed to be the Mannich reaction. Mannich reaction takes place when a CH activated compound (such as a phenol or ketone), a primary or secondary amine, and a non-enolizable aldehyde undergo a reaction. On calixarenes this procedure has been employed mostly with simple amines, through a procedure developed by Gutsche and Nam (Figure 2).⁷

Figure 2. Calix[4]arenes prepared via Mannich reaction by Gutsche and Nam.⁷

Mocerino *et al.*⁸ reported for the first time the possibility to conjugate zwitterionic proline units to the upper rim of tetrahydroxycalix[4]arenes through this rather efficient Mannich reactions. This reaction, however, is taking place only on phenols and not on O-alkylated or O-acylated phenols, limiting the applications to native (OH free) macrocycles. The L-proline-functionalized calix[4]arene is synthesized through a one-step reaction (Figure 3) using formaldehyde and acetic acid, with L-proline at room temperature.

Figure 3. Synthetic strategy for the synthesis of compound **C1**.⁹

Mocerino *et al.* also reported the development of a hydrogel composed of L-proline functionalized calix[4]arene in the presence of specific anions, with its properties varying depending on the cation used. The hydrogel remains stable within a pH range of 0 to 7 but disassembles at pH values above 7. In a separate study by Mocerino's research group, the authors discovered that the L-proline-functionalized calix[4]arene (**C1**) exhibits notable properties as an inhibitor of the assembly of the human papillomavirus 16 L1 pentamer, binding to basic residues on the L1 surface. Comparative binding studies were also conducted using the D-proline-functionalized calix[4]arene enantiomer, which proved to be less effective than the L-enantiomer; this behavior was subsequently confirmed through molecular simulations, which highlighted differences in shape and energy that explained the observed binding disparity.⁹ This discovery paves the way for developing innovative anti-HPV drugs, and the same approach could be adapted to other viruses.

In a recent study conducted by F. Rispoli² *et al.*, the scope of the Mannich reaction on tetrahydroxy calix[4]arenes was expanded to include amino acids beyond proline, such as glycine, L-phenylalanine,

L-leucine, L-sarcosine and L-valine (Figure 4). However, since the net charge of all these derivatives is zero, solubility in water is often an issue and decreases in the series L-Pro >> L-Gly, L-Phe, L-Sar > L-Val, L-Leu indicating that also, but not only, the nature of the amino acid lateral chains is playing a role.

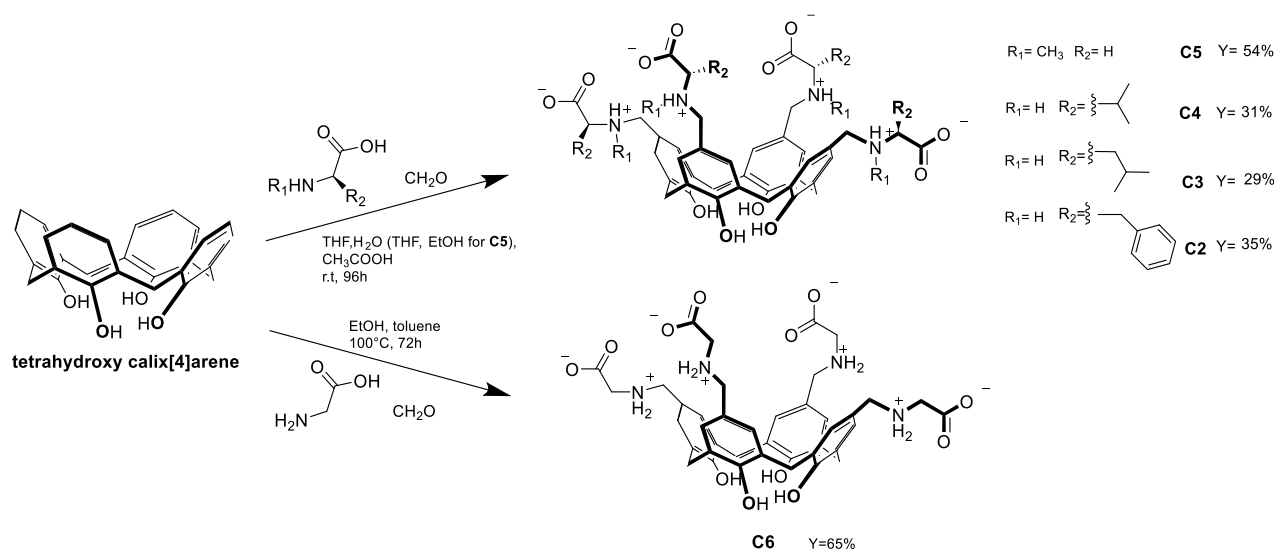


Figure 4. Synthesis for the Mannich reaction on tetrahydroxy calix[4]arene with L-phenylalanine (**C2**), L-leucine (**C3**) and L-valine (**C4**) and sarcosine (**C5**). With a slightly different procedure the glycine derivative **C6** was also synthesized.

Through Mannich reaction it is possible to efficiently functionalize tetrahydroxy calix[4]arenes at the upper rim, but no reaction occurs when the hydroxyl group is alkylated. In the PhD thesis of Goh C. Y.,¹⁰ calix[5]arene and calix[6]arene scaffolds were investigated to achieve exhaustive functionalization with L-proline. The research aimed to determine whether the size of the macrocycle influences the self-assembly properties of these structures, given the differing spatial arrangement of the proline groups. Although the reaction with calix[5]arene produced the desired product, the yield is consistently lower compared to calix[4]arene. In contrast, no product was obtained with calix[6]arene under the same conditions. This is likely due to the poor solubility of the p-H calix[6]arenes and its partially substituted derivatives, which possibly causes the precipitation of reaction intermediates. In the same study, the Mannich reaction was tested on dipropoxy calix[4]arene to selectively functionalize the upper rim of the two aromatic rings containing OH free phenols (Figure 5). However, the reaction was reported to be also unsuccessful, as no product was formed, with the starting material recovered unchanged. This result seems to suggest that effective Mannich reaction at the upper rim requires the presence of the all the four free phenolic OH groups at the lower rim to take place. A possible explanation is that only in the tetrahydroxy calix[4]arenes the first deprotonation is particularly favored ($\text{pK}_{\text{a}1} = 3$) thanks to efficient hydrogen bonding between neighboring phenolic groups and therefore in Mannich reaction conditions the partially deprotonated calix[4]arene is more reactive being present also as phenolate.

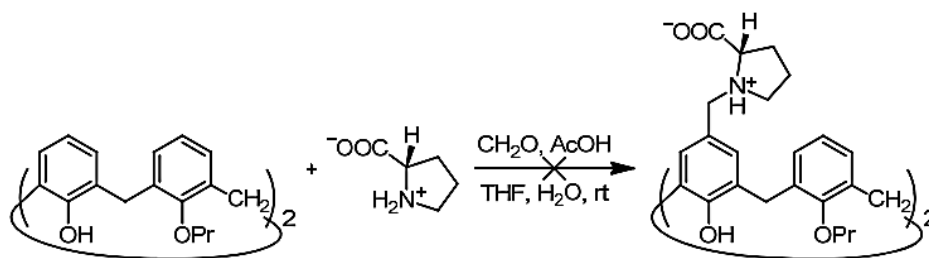


Figure 5. Mannich reaction with 25,27-dipropoxy calix[4]arene, leading to the recovery of the unaltered reagent.¹⁰

Van Loon *et al.*¹¹ have reported a successful Mannich reaction in para to the free phenolic OH groups of 25,27-dimethoxy calix[4]arene with secondary amines such as morpholine or dimethylamine under stringent conditions (refluxing in dioxane for 66 hours). However, Nam and Yoon,¹² using the same procedure, were unable to achieve the aminomethylation of the phenolic nuclei when the lower rim of the calixarene was partially (25,27 positions) acylated or alkylated with benzoyl or allyl groups, respectively. This is consistent with Gutsche's theory,¹³ which proposes that the increased acidity of free phenolic calixarenes under Mannich conditions enhances the reactivity of the para aromatic position.

3.1.2 Aim of this work

The discovery of the class of zwitterionic calix[4]arenes as selective ligands for Gram-negative bacteria discloses the possibility of their applications for the development of both devices able to detect bacteria (see Chapter 4) or of nanoobjects able to selectively deliver drugs (see Chapter 5). In both cases, it would be crucial to introduce at the lower rim of these zwitterionic calixarenes at least one reactive group that would allow the functionalization of surfaces of i) electrodes/QCM for the development of sensors for bacteria (Figure 6a) or ii) of self-assembled nanoobjects able to selectively deliver drugs to bacteria (Figure 6b). Both approaches, with their different impact and perspectives, would help in fighting AMR.

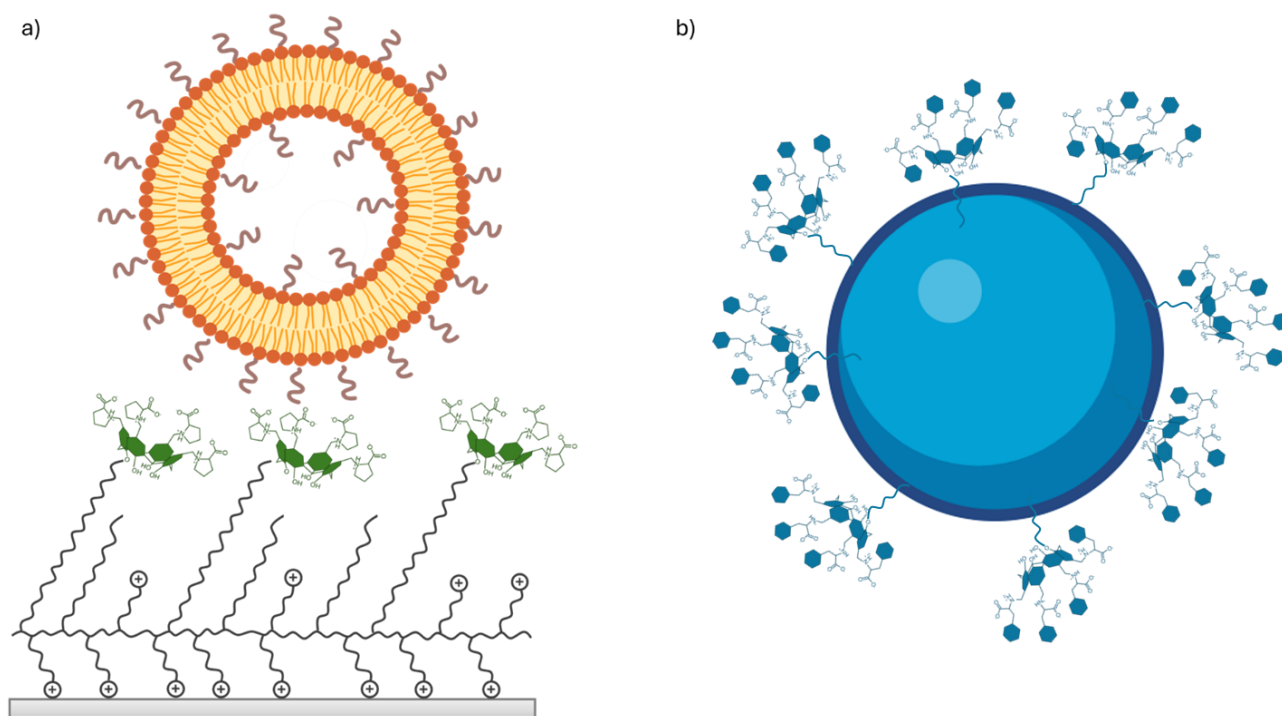


Figure 6. a) QCM-D surface embedded with L-proline calix[4]arene and b) nano-object embedded with L-phenylalanine calix[4]arene.

In this chapter are therefore reported the efforts to synthesize two calix[4]arene functionalized with two amine units at the lower rim, to allow their attachment to surfaces of QCM-D or of nano-objects (Figure 7). These calixarenes are functionalized at the upper rim with four L-prolines (**12**) or four L-phenylalanine moieties (**16**).

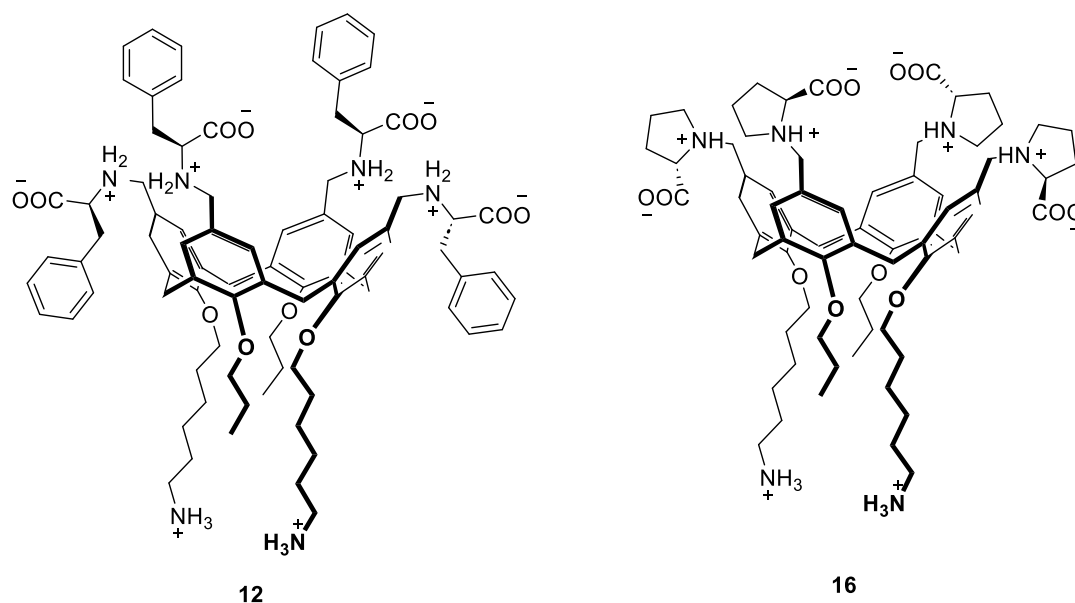


Figure 7. Aminoacid functionalized analogues **12** and **16**, with amine units at the lower rim to allow the functionalization on surfaces.

Since the direct *O*-alkylation or *O*-acylation of phenolic OH groups in the products of Mannich reactions **C1-C6** with a *N*-protected ω -haloalkylamine would have resulted in the functionalization of the amino and/or carboxy group at the upper rim (Figure 8), and being the Mannich reaction on *O*-alkylated calixarenes demonstrated impossible, a study was undertaken to develop a route alternative to the aminomethylation to insert zwitterionic amino acids at the upper rim of *O*-functionalized calixarenes.

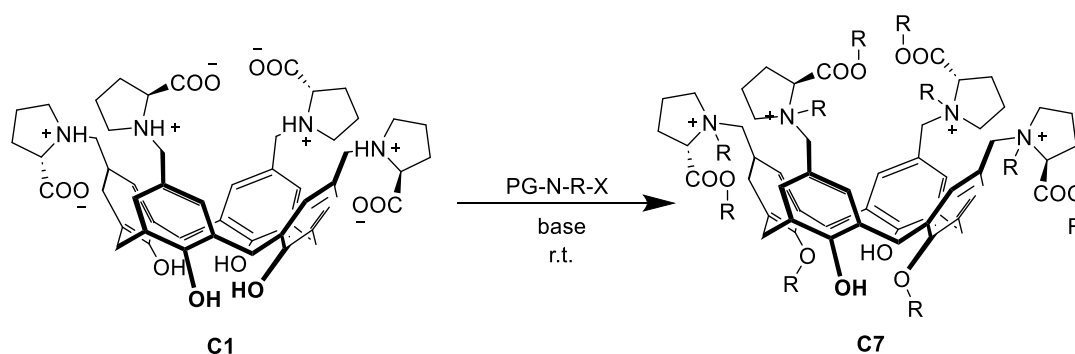


Figure 8. Direct alkylation of **C1** leading to the polyalkylated product **C7**.

With this priority, the tetrapropoxycalix[4]arene was preliminary used as substrate while L-phenylalanine and L-proline were selected as amino acids due to their superior ability to interact with Gram-negative bacteria when linked at the upper rim of **C2** and **C1** respectively (Figure 9).

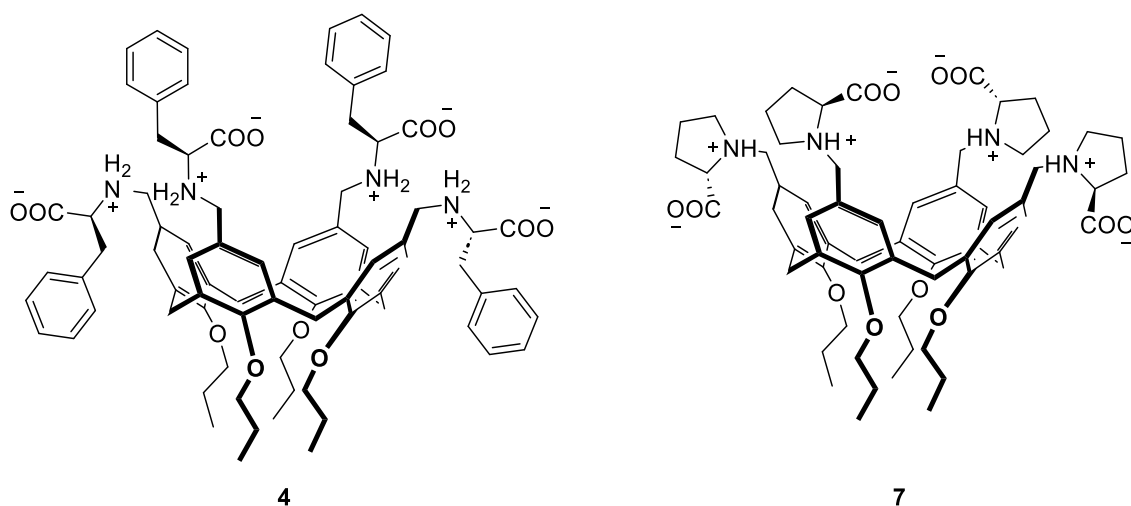


Figure 9. Tetrapropoxy calix[4]arenes functionalized at the upper rim with L-Phe (**4**) and L-Pro (**7**) in their zwitterionic form.

In parallel, in order to prepare the monovalent models of the zwitterionic amino acid-functionalized calixarenes able to be grafted on a polyelectrolyte functionalized surface (see Chapter 4), it was planned to synthesize the L-phenylalanine compound **24** (Figure 10, top) and the L-proline compound **26** (Figure 10, bottom). The terminal part of these models mimics a monovalent model of the calixarenes since they also present the amino acid units connected to a benzene unit via a methylene group. The opposite end of each chain carries an azide group, a reactive unit that enables attachment of these molecules to a surface, for instance, to create a functionalized surface for testing with models of Gram-negative bacterial cell walls. The detailed construction of this system is thoroughly explained in Chapter 4, based on work carried out within Prof. J. Huskens research group at the University of Twente, the Netherlands.

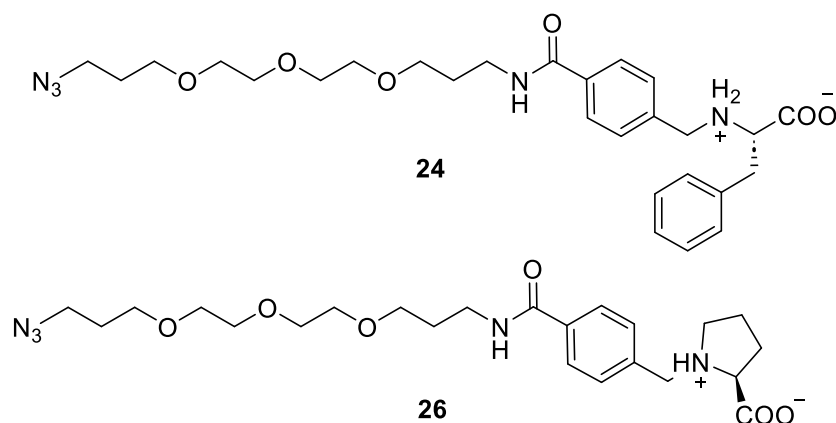


Figure 10. Monovalent models of the calixarene functionalized with L-Phe (**24**) and L-Pro (**26**). These models bear an azide group at one end, to make possible the conjugation with an alkyne on a surface.

3.2 Results and discussion

3.2.1 Synthesis of the tetrapropoxy tetra amino acid functionalized calixarenes **4** and **7**

Since the Mannich reaction with alkylated calixarenes does not yield the desired product, it is necessary to develop alternative synthetic strategies for linking amino acids to the aromatic core of the calixarene via a methylene (CH_2) group, thus preserving the zwitterionic character of the amino acid.

In the case of L-phenylalanine derivative **4** (Figure 11, top), the upper rim of 25,26,27,28-tetrapropoxy calix[4]arene **1** was fully formylated at the upper rim using the Duff reaction, producing 5,11,17,23-tetraformyl-25,26,27,28-tetrapropoxycalix[4]arene **2** in very high yield without any needs for purification. Compound **2** was subsequently subjected to reductive amination with the methyl ester of L-phenylalanine in the presence of sodium cyanoborohydride, triethylamine (TEA), and acetic acid, yielding product **3**. However, the yield was modest (20%) due to extensive purification steps, including flash chromatography and preparative thin-layer chromatography (TLC). The methyl esters of **3** could then be hydrolyzed with lithium hydroxide monohydrate to obtain product **4** as lithium salt.

We also attempted a reductive-amination using the same synthetic strategy as for product **4**, but using L-proline methyl ester; however, this approach did not yield the desired product. Consequently, we adopted the alternative strategy depicted in Figure 11 bottom. The 25,26,27,28-tetrapropoxy calix[4]arene **1** was functionalized at the upper rim with four chloromethyl groups (**5**), enabling a nucleophilic substitution with L-proline methyl ester. After purification by flash chromatography and preparative TLC, compound **6** was obtained in moderate yield (30%). The methyl esters could subsequently be hydrolyzed using lithium hydroxide monohydrate, yielding product **7** as a lithium salt.

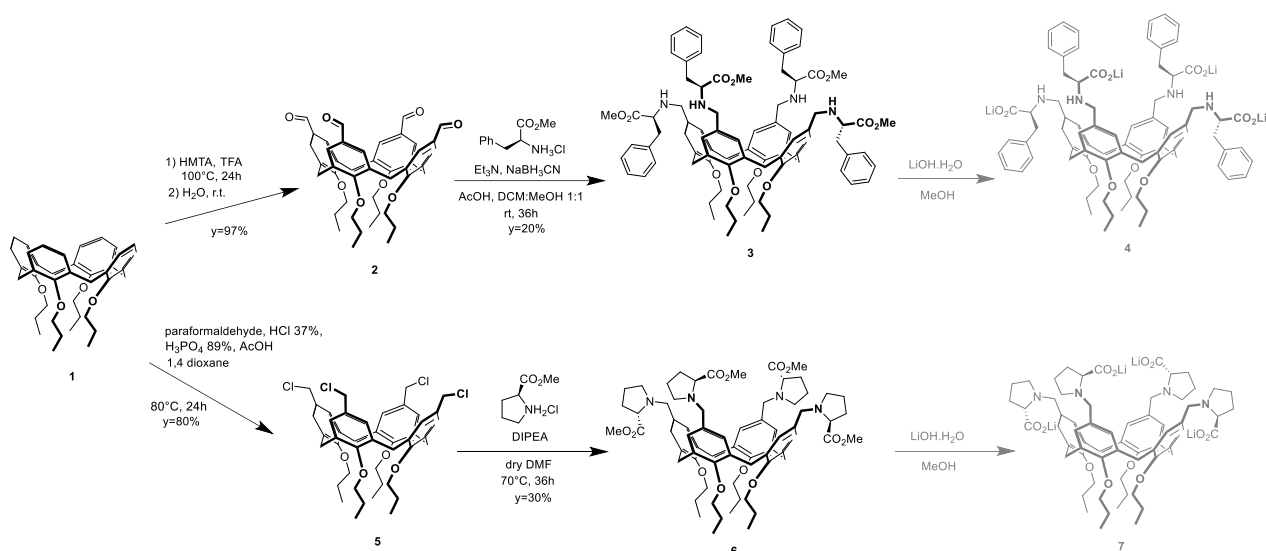


Figure 11. Synthesis scheme to obtain products **4** (top) and **7** (bottom). The hydrolysis of the methyl esters is indicated in grey as it was not yet performed.

In the following figures are reported the ¹H NMR spectra of product **3** (Figure 12) and product **6** (Figure 13).

Product **3** was isolated in pure form through flash chromatography and preparative TLC purification. To confirm its identity, the characteristic signals of phenylalanine were identified in the ¹H NMR spectrum (Figure 12). The aromatic protons appear as a multiplet between 7.30 and 7.14 ppm, while the

methylene protons connecting the amino acid to the calix[4]arene scaffold are diastereotopic, appearing as two doublets at 3.31 and 3.27 ppm. The chiral CH proton is observed as a triplet at 3.47 ppm, while the benzyl CH₂, which is coupled to it and appears as a multiplet, resonates at 2.93 ppm. The multiplet at 6.48-6.42 can be assigned to the calixarene aromatics, and the methylene bridge (ArCH₂Ar) to the two doublets at 4.38 and 3.04 ppm.

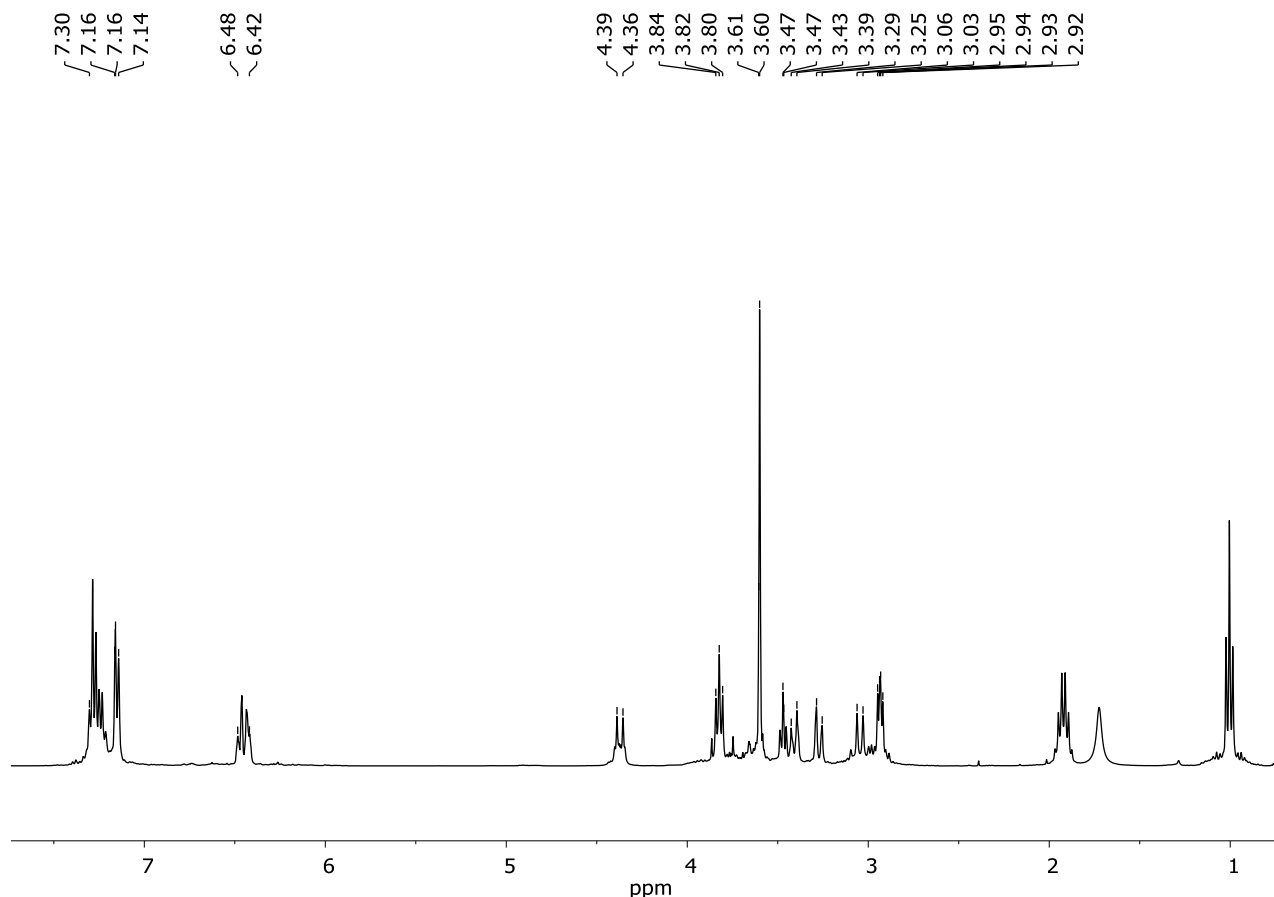
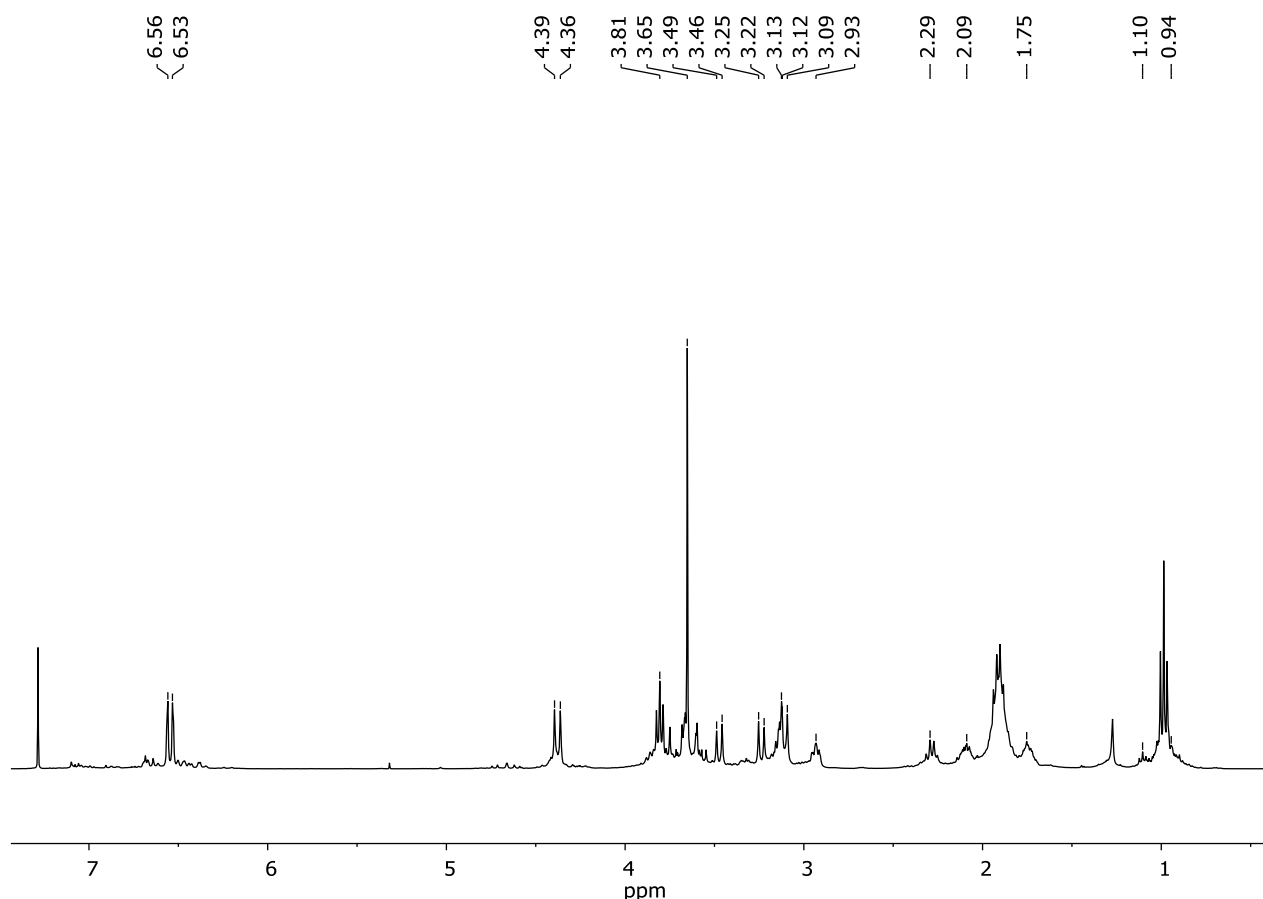


Figure 12. ¹H NMR spectrum of product **3** in CDCl₃ (400 MHz, 298 K).

The ¹H NMR spectrum of product **6** is presented in Figure 13. In this spectrum, the aromatic protons of the calixarene appear as two signals at 6.56 and 6.53 ppm, a result of the chiral center in the amino acid. The methylene protons linking the calixarene scaffold with proline are also diastereotopic, appearing as two doublets at 3.47 and 3.23 ppm. The chiral CH proton is observed at 2.93 ppm. The remaining CH₂ groups in the proline ring are also diastereotopic, appearing as multiplets at 2.29, 2.09, 1.75, 1.10, and 0.94 ppm.

Figure 13. ^1H NMR spectrum of product **6** in CDCl_3 (400 MHz, 298 K).

Upon methyl ester hydrolysis, the two compounds **3** and **6** yield products **4** and **7**, that will be tested using on-cell STD NMR to assess whether the presence of propyl chains at the lower rim and the consequent increased flexibility of the calix[4]arene scaffold influence binding interactions with bacteria.

3.2.2 Synthesis of compounds **12** and **16**

To obtain products **12** and **16**, we employed a strategy similar to that used for products **4** and **7** in modifying the upper rim. The synthesis pathway studied for product **12** is shown in Figure 14. Since the Duff formylation (or chloromethylation) reactions at the upper rim require harsh conditions, a careful selection of a protective group for the amine moiety at the lower rim is crucial. The chosen group must, in fact, withstand conditions (temperatures of 100 °C for 20 hours and a highly acidic pH) required to fully functionalize the upper rim with formyl groups. The same considerations apply to the chloromethylation reaction used to obtain compound **13**.

Stability tests were carried out with fluorenyl-9-methoxycarbonyl (Fmoc), azide and phthalimide, to verify their stability under these conditions: the phthalimide was the only group that remains unaltered. The 25,27-dihydroxy-26,28-dipropoxy calix[4]arene was alkylated at its two hydroxyl groups using *N*-(6-bromohexyl)phthalimide and sodium hydride, yielding compound **8** with a yield of nearly 90%. Subsequently, four formyl groups were introduced at the upper rim of compound **8** via a Duff formylation according to an established procedure, preserving the integrity of the phthalimide group. Compound **10** was synthesized using the same reductive amination protocol applied to compound **3**. By modifying the solvent ratio of dichloromethane to methanol from 1:1 to 2:1 and increasing the equivalents of

NaCNBH_3 , the yield improved to 50%, after purification from reaction intermediates via flash chromatography.

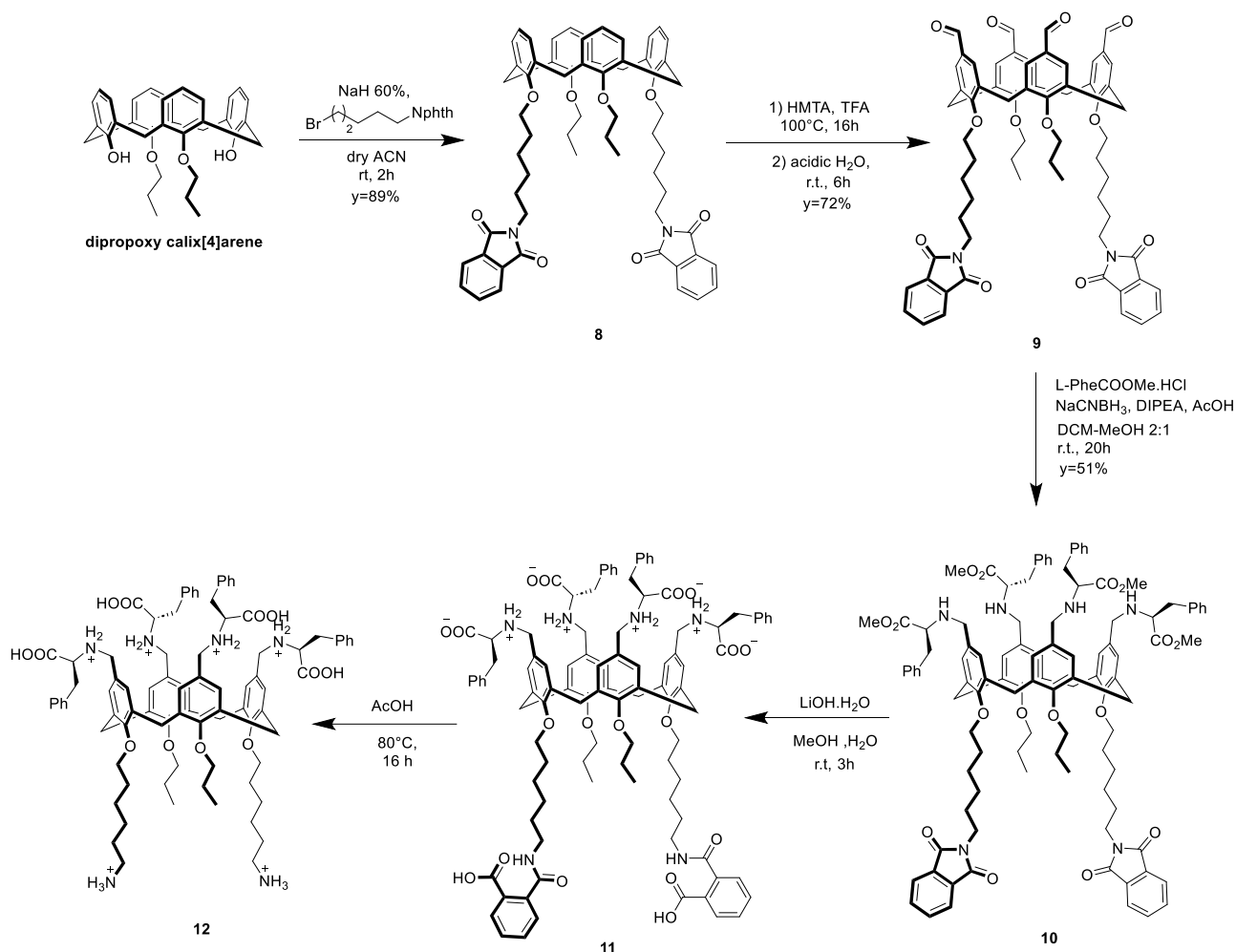


Figure 14. Synthetic scheme for obtaining product **12**.

In Figure 15 the ^1H NMR of compound **10** is shown. The diametrical substitution at the lower rim by the two phthalimides, whose aromatic protons are at 7.83 and 7.72 ppm, influences also the signals of the protons at the upper rim, that split in two signals. The aromatics of the phenylalanine can be found in the multiplet from 7.29 to 7.14 ppm, superimposed to the residual peak of the CDCl_3 at 7.27 ppm, while the calixarene aromatics are the four signals, at 6.48, 6.45, 6.39 and 6.37 ppm, due to the presence of the chiral centers. The two doublets at 4.32 and 3.32 ppm are the signals of the methylene bridge of the calixarene, while the doublets from 3.37 to 3.14 correspond to the signals of the methylene that links the phenylalanine to the calixarene scaffold. In the multiplet from 2.95 to 2.90 resonates the methylene unit of the benzyl groups. The methyl esters give rise to two singlets at 3.60 and 3.58 ppm while the multiplet at 3.44 ppm is the signal of the CH of the chiral carbons.

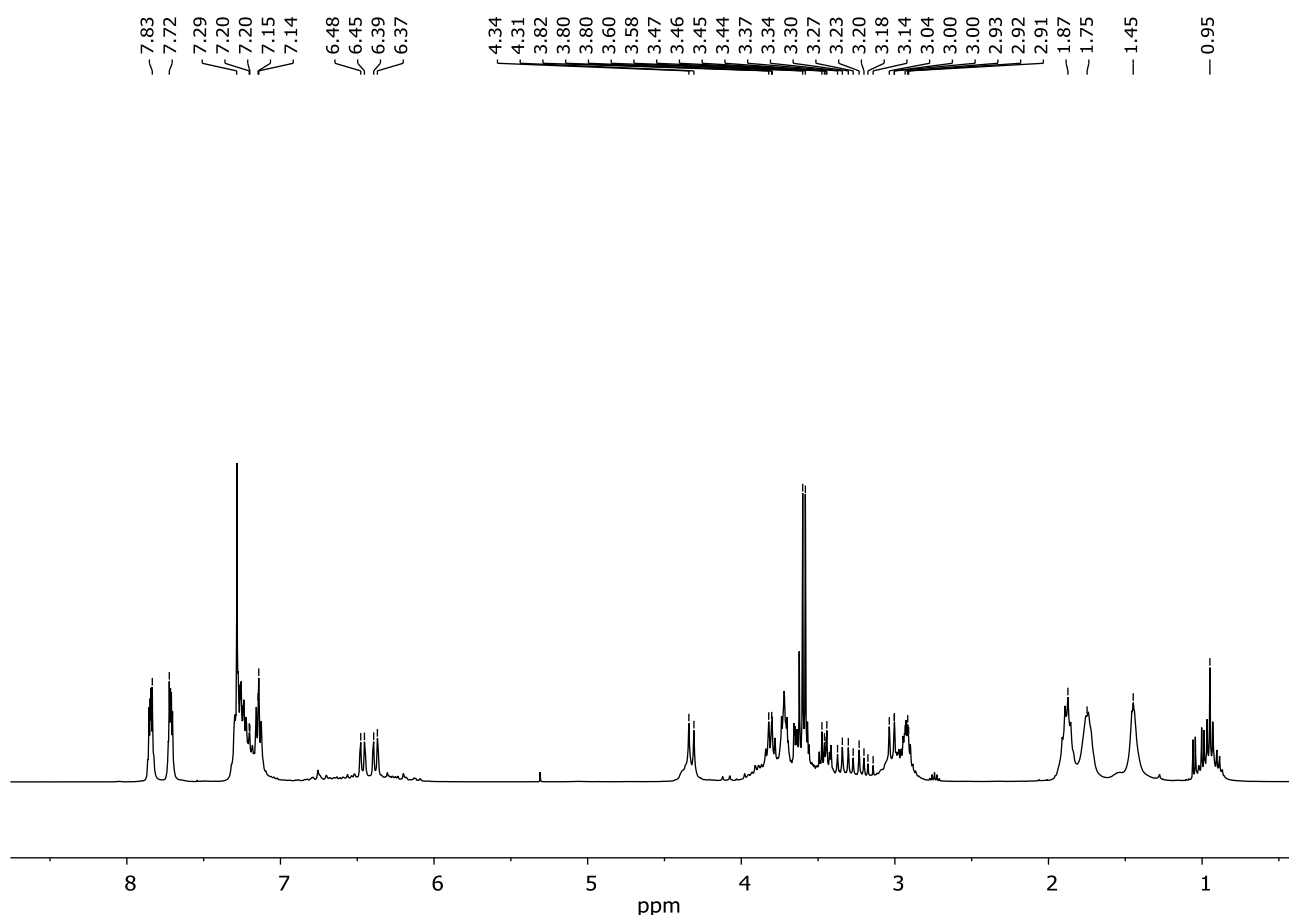


Figure 15. ^1H NMR spectrum of product **10** in CDCl_3 (400 MHz, 298 K). The impurities from 1.04 to 0.90 are the methyl of the DIPEA residues, used in the reaction to deprotonate the L-phenylalanine methyl ester hydrochloride.

Before removing the phthalimide moieties with the classical procedure it was necessary to hydrolyze the methyl esters. The most common procedure for phthalimide removal, in fact, employs hydrate hydrazine in ethanol refluxing, but by reaction with the esters, hydrazine could lead to the formation of hydrazide subproducts.

The methyl esters of compound **10** were then hydrolyzed with 4.4 equivalents lithium hydroxide monohydrate, dissolving the calixarene in methanol and adding a solution of lithium hydroxide in water. The reaction was left stirring at room temperature overnight, and then the solvents were removed under vacuum. The ^1H NMR spectrum of product **11** was recorded (Figure 16). To confirm the positive outcome of the reaction the methyl ester signals are not present around 3.60 ppm. The signals of the phthalimide present as four separated signals, at 7.49, 7.38, 7.30 and 7.23 ppm, indicate that the phthalimide ring was opened (product **11** in the reaction scheme in Figure 14). The partial hydrolysis of the phthalimide ring in basic environment is well-known in literature.¹⁴ Due to the aggregation of the compound in water, the other signals are very broad, losing their multiplicity. Moreover, the product was also poorly soluble in water, or in other solvent mixture tested (methanol, acetonitrile and DMSO). The aromatics of the calixarene in particular are not clearly visible in the region from 6.55 to 6.40 ppm. The ESI-MS analysis has not provided clear data to confirm the identity of the product.

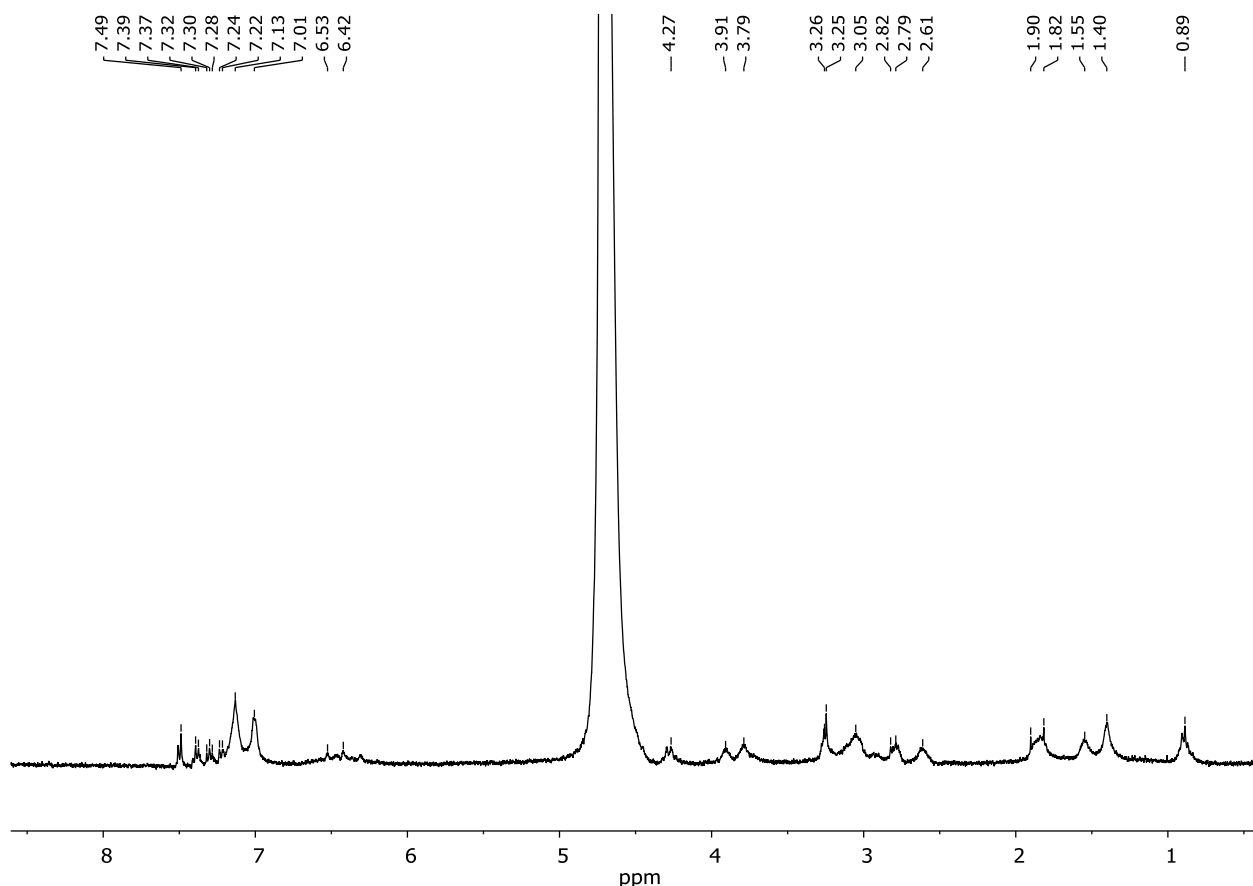
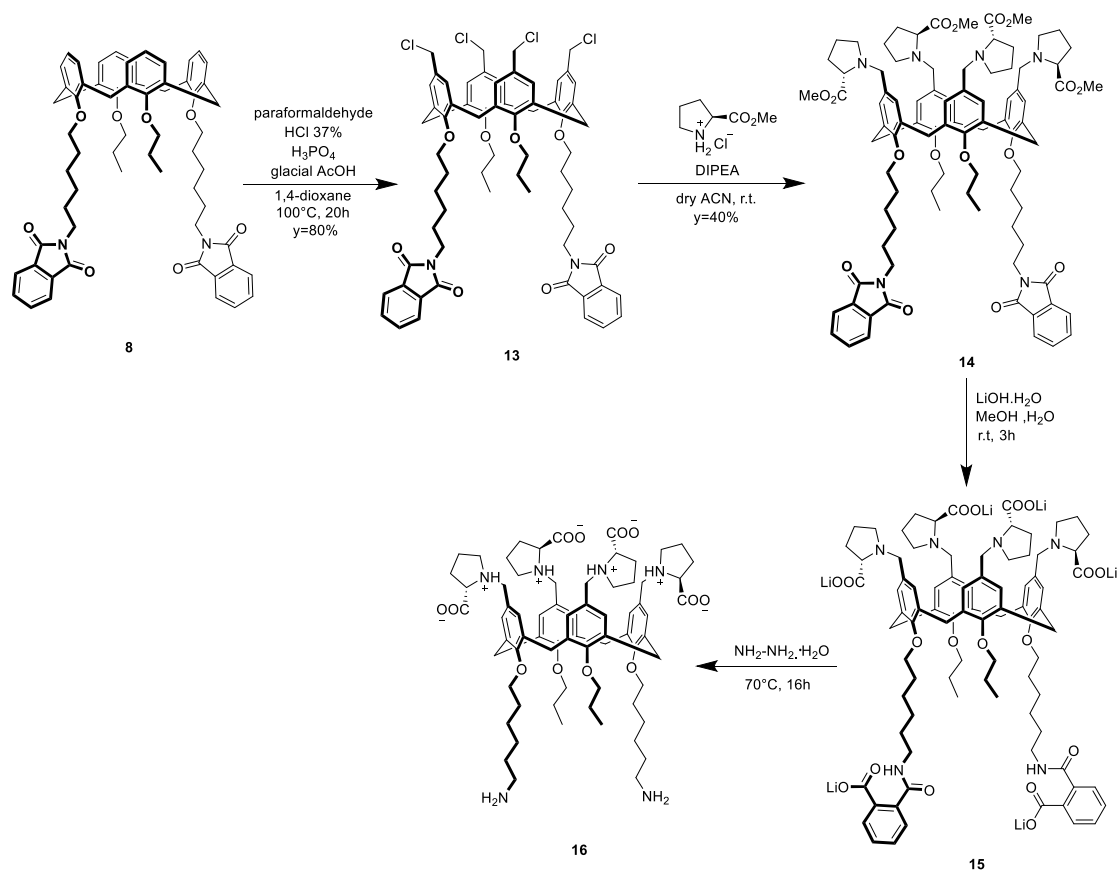


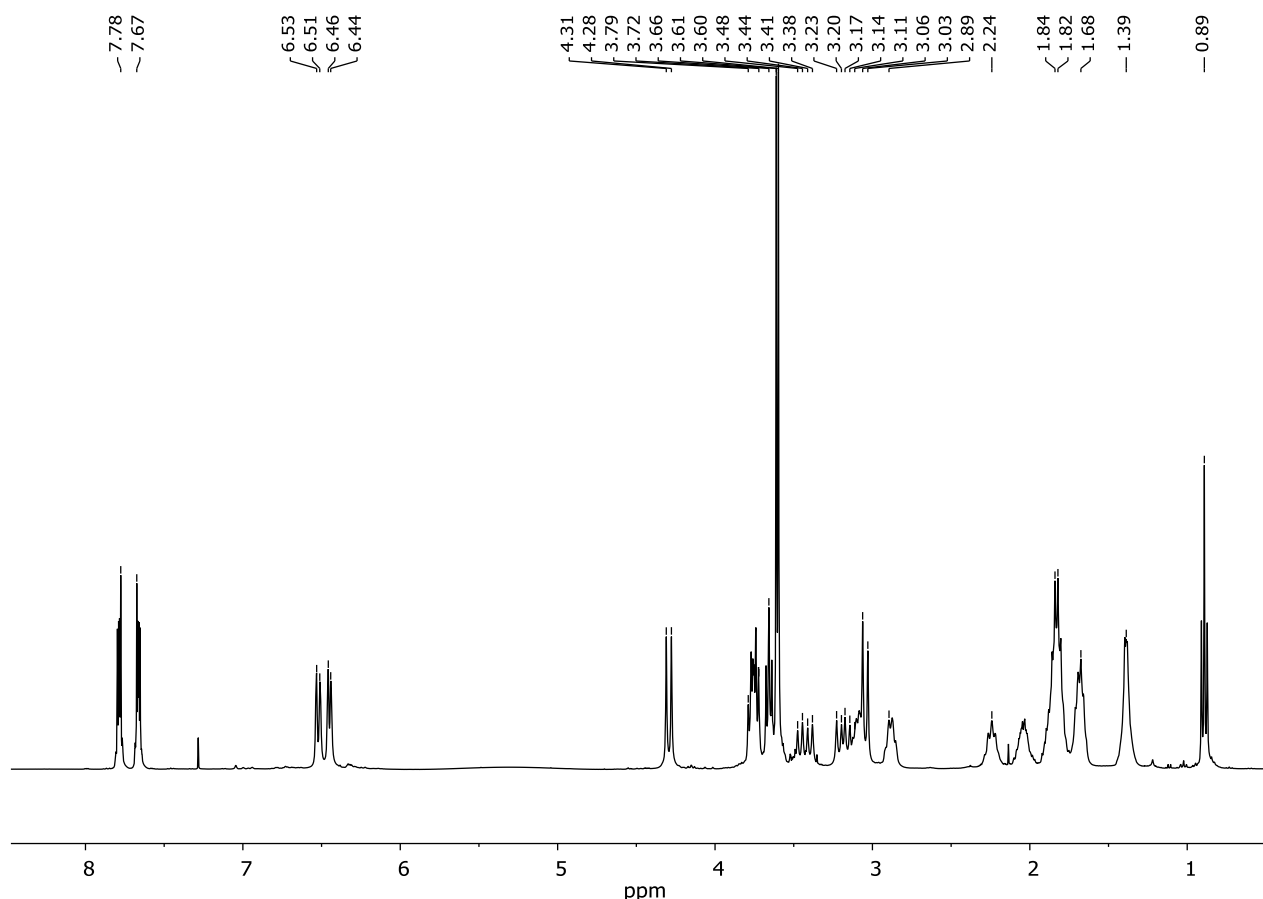
Figure 16. ^1H NMR spectrum of product **11** in D_2O (400 MHz, 298 K).

Following a procedure reported in literature¹⁴ to remove the partially opened phthalimide ring, compound **11** was dissolved in acetic acid and refluxed for almost 20 hours. The spectrum of the crude was analogous to the reagent one. After another night refluxing in acetic acid the obtained sticky solid, that was dissolved in a mixture D_2O - DMSO-d_6 , but from the ^1H NMR spectrum it is possible to see that the partially opened phthalimide ring is still present. Moreover, the mixtures D_2O - DMSO-d_6 and D_2O - CD_3OD were not effective in the disaggregation of the product and in providing a clearer spectrum. Also in this case, ESI-MS analysis did not provide clear data to confirm the identity of the product. Due to time reasons and the small quantity of product obtained it was not possible to try other strategies for phthalimide cleavage.

The synthetic approach used to obtain product **16** is shown in Figure 17. Four chloromethyl groups were introduced at the upper rim of compound **8**, yielding compound **9** with an 80% yield, while preserving the integrity of the phthalimide ring. The nucleophilic substitution with L-proline methyl ester was conducted using the same procedure as applied for the synthesis of product **5**, described in the previous section. Product **14** was obtained with 40% yield, increased with respect to product **7** changing the solvent from dimethyl formamide to acetonitrile.

Figure 17. Synthetic scheme for obtaining product **16**.

The ¹H NMR of product **14** is shown in Figure 18. The two multiplets of the intact phthalimide ring are at 7.78 and 7.67 respectively, while the four signals for the calixarene aromatics are at 6.53, 6.51, 6.46 and 6.44. The two doublets at 4.39 and 3.05 ppm are the signals of the methylene bridge of the calixarene, while the two pair of doublets from 3.48 to 3.14 correspond to the signals of the methylene that links the proline to the calixarene scaffold. The methyl esters are at 3.62 and 3.61 ppm, divided into two singlets for the diametrical functionalization of the lower rim. The presence of signals of the proline ring in the region from 3 to 1.5 ppm allows to confirm the identity of the product.

Figure 18. ^1H NMR spectrum of product **14** in CDCl_3 (400 MHz, 298 K).

The methyl esters of compound **14** were hydrolyzed using lithium hydroxide monohydrate in a methanol-water mixture to obtain product **15**, whose ^1H NMR spectrum is presented in Figure 19. The opening of the phthalimide ring was confirmed also in this case by the signal pattern from 7.55 to 7.29 ppm, resembling that observed in the ^1H NMR spectrum of product **11**. The remaining signals are notably broad, likely due to the aggregation of the product in water, despite its enhanced solubility compared to the phenylalanine derivative **11**.

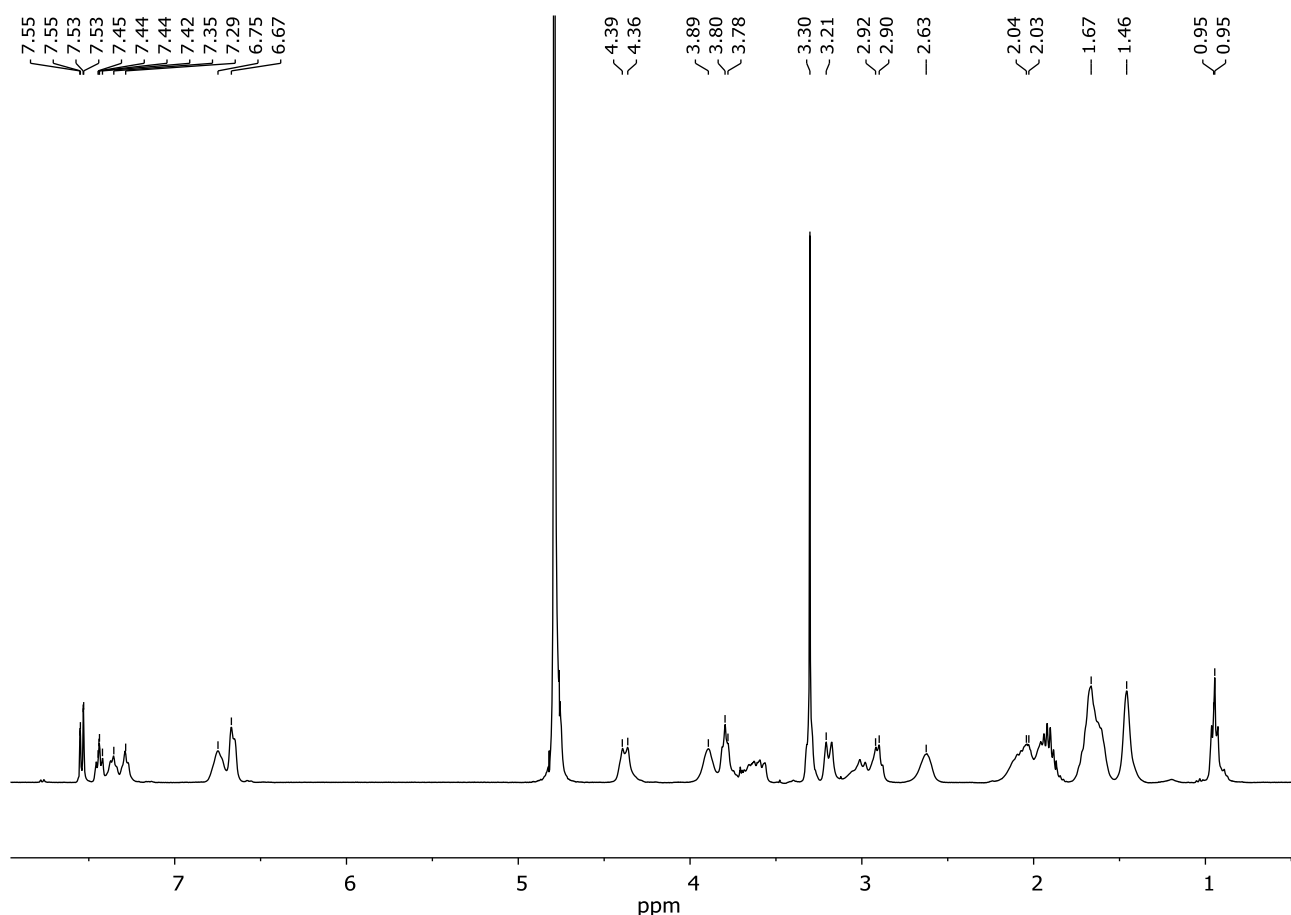


Figure 19. ^1H NMR spectrum of product **15** in D_2O (400 MHz, 298 K). An impurity of methanol is present at 3.30 ppm.

The phthalimide group was removed following a standard procedure using hydrazine in ethanol. After refluxing for 16 hours, the solvent was evaporated under reduced pressure, and the crude product was recrystallized twice in a methanol-diethyl ether mixture. Figure 20 presents the ^1H NMR spectrum of product **16**, recorded in a D_2O - CD_3OD mixture, where the absence of phthalimide proton signals confirms the successful outcome of the reaction. Even in the presence of deuterated methanol and few μL of DCl solution (35% w/w) to enhance the solubility, the signals of product **16** appear broad, likely due to aggregation.

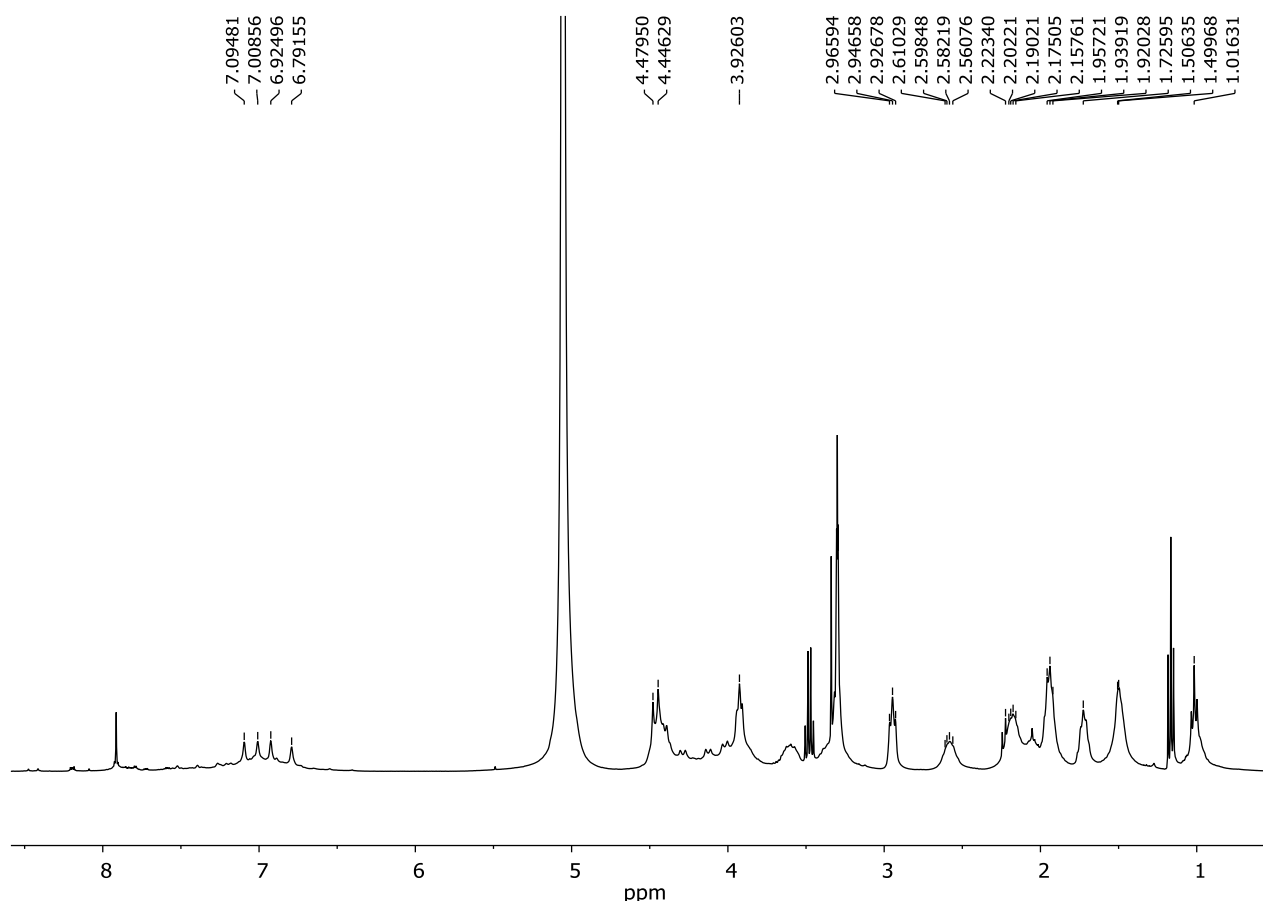
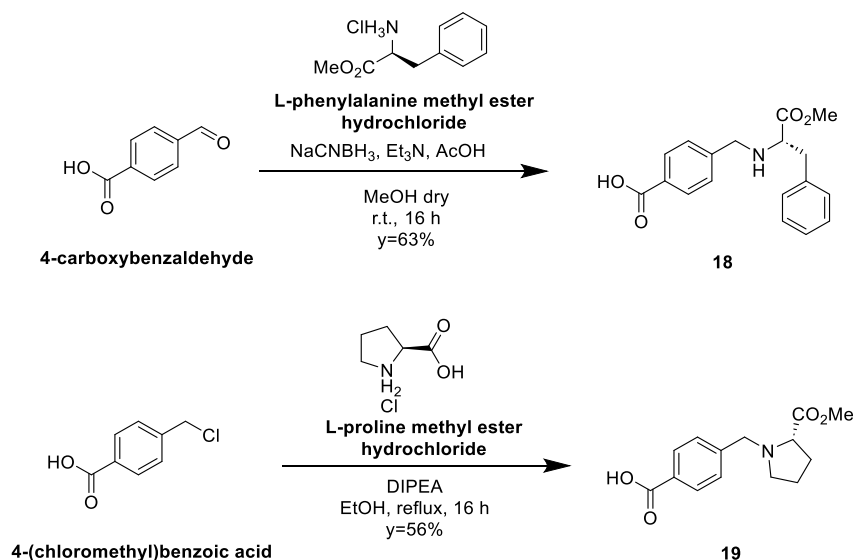


Figure 20. ^1H NMR spectrum of product **16** in $\text{D}_2\text{O}-\text{CD}_3\text{OD}$ (400 MHz, 298 K). Impurities of ethanol are present, with the triplet at 1.16 ppm and the quadruplet at 3.48 ppm, methanol, with the singlet at 3.33 ppm, and DMF, with the singlet at 7.91 ppm.

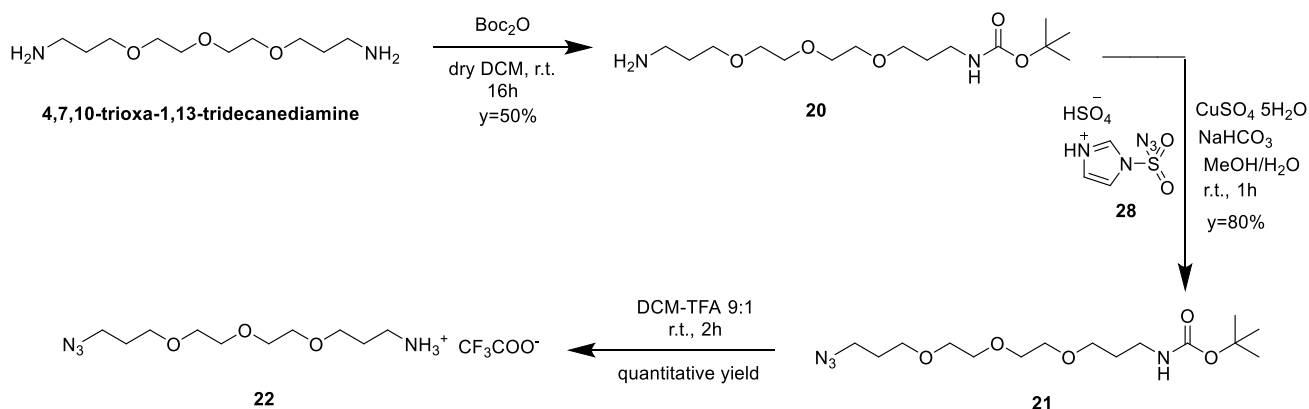
Due to time reasons, it was not possible to substitute the amine with an azide and subsequently attach the calixarene to the polyelectrolyte surface, as originally planned for the functionalization of electrodes in the development of bacterial sensors, but the synthetic strategy developed for compound **16** seems to allow its preparation in larger scale in the future.

3.2.3 Synthesis of the zwitterionic PEGylated monovalent models

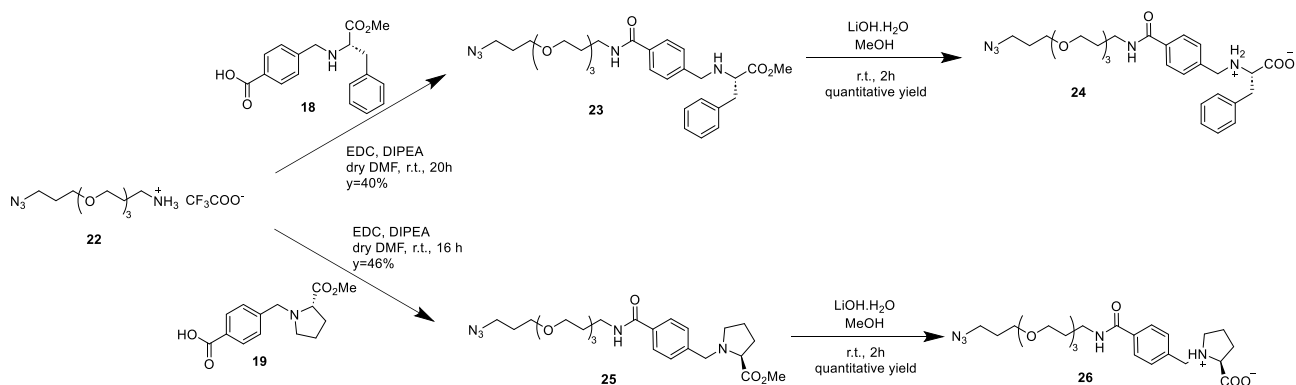
The synthesis of the monovalent model of amino acid-functionalized calix[4]arenes was carried out in two steps. First, the aromatic recognition unit mimicking the calixarene upper rim was functionalized at one end with an amino acid group, as illustrated in Figure 21. L-phenylalanine was conjugated to 4-carboxybenzaldehyde via reductive amination, using the methyl ester form of the amino acid in presence of acetic acid, triethylamine, and sodium cyanoborohydride, yielding product **18** with a 63% yield after flash chromatography (Figure 21, top). The same strategy was exploited for the proline derivative **19**, but unfortunately, as in calixarene case, did not yield the desired product, and the reagent was recovered unchanged. Alternatively, the L-proline derivative **19** was obtained by exploiting the alkylation of the AA nitrogen atom to 4-(chloromethyl)benzoic acid via nucleophilic substitution (Figure 21 bottom), with a 56% yield after purification by flash chromatography.

Figure 21. Synthesis strategies to obtain products **18** and **19**.

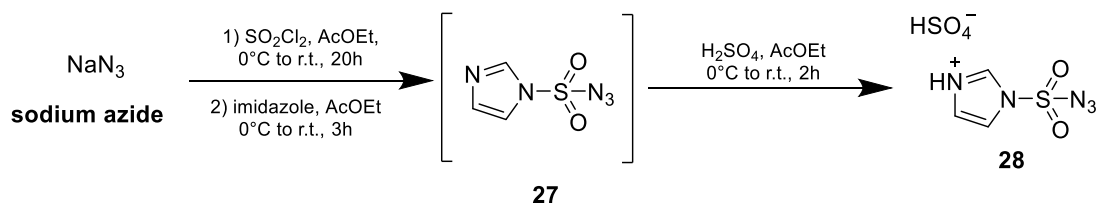
Subsequently, a PEGylated chain bearing an azide group at one end and an amine group at the other (**22** Figure 22), allowing the linkage of the carboxylic acid moieties of compounds **18** and **19** to the PEGylated chain (Figure 22), was synthesized. One of the two amine groups of 4,7,10-trioxa-1,13-tridecanediamine was protected using a Boc group, following a procedure previously reported in the literature.¹⁵ This protection allowed for the conversion of the remaining free primary amine into an azide via a diazotransfer reaction. The diazotransfer reagent **28** was synthesized as described in literature¹⁶ (Figure 13) and stored as a white solid at -18 °C. Product **21** was obtained with a yield of 80% and was used without further purification. The Boc group was subsequently removed to afford product **22**, containing a free amine moiety.

Figure 22. Synthesis scheme to obtain product **22**.

The intermediate **22** was then coupled to compounds **18** and **19** using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide) hydrochloride (EDC) as the coupling agent. The products, **23** and **25**, were obtained with yields of 46% and 40%, respectively, after purification by flash chromatography to remove byproducts derived from the coupling reagent. The methyl esters were then hydrolyzed, and the pure products, **24** and **26**, were isolated following HPLC purification.

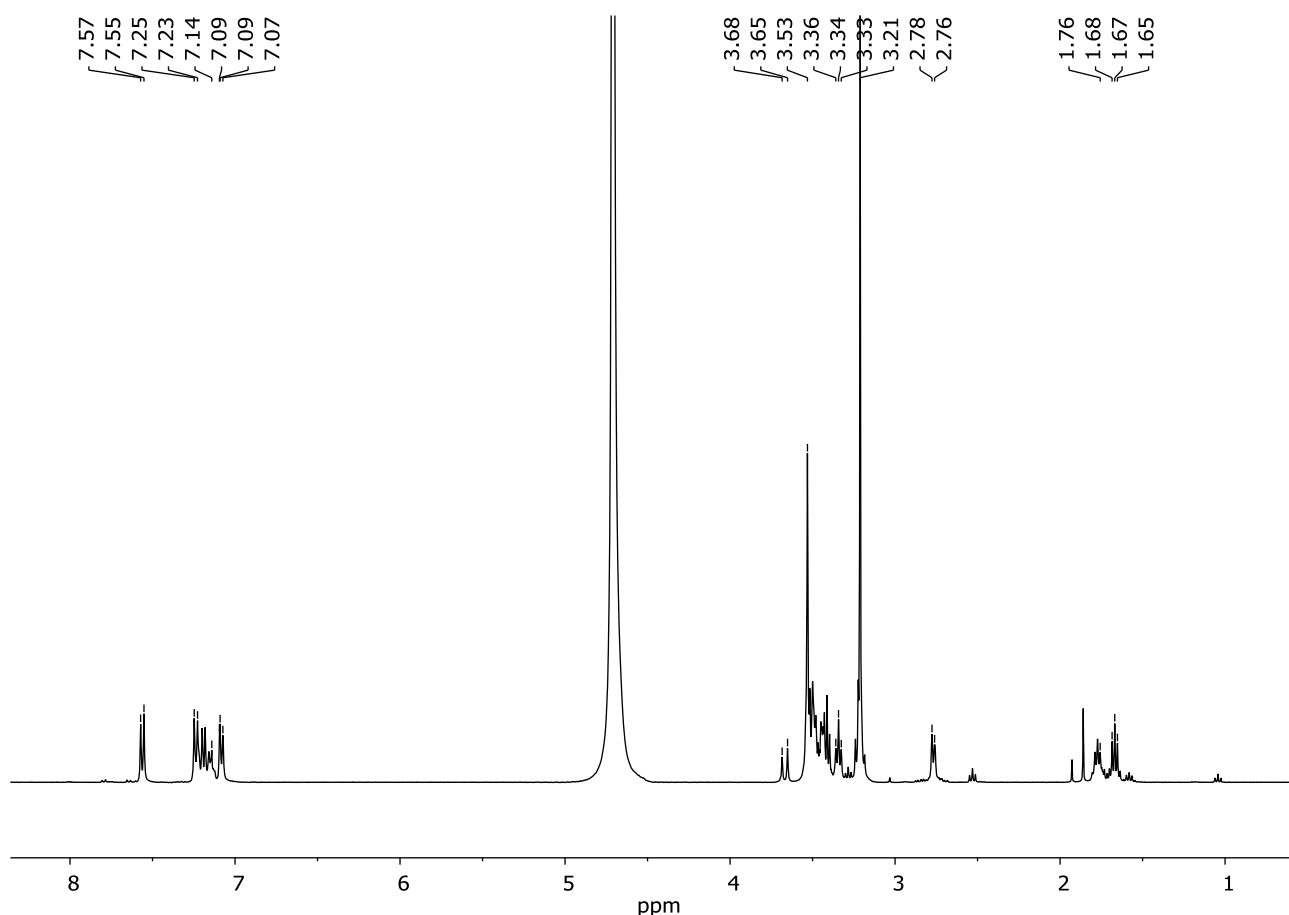
Figure 23. Synthesis scheme to obtain products **24** and **26**.

In Figure 24 the synthesis (CAUTION!) of diazotransfer reagent imidazole-1-sulfonyl azide (**28**) is reported. The procedure needs to be handled carefully because the intermediate **27** is explosive when concentrated. Its hydrogen sulfate salt is reported to be safer to handle and store as solid.

Figure 24. Synthetic scheme to obtain product **28**. The procedure followed was taken from literature.¹⁶

The ^1H NMR spectra of products **24** and **26** are shown in Figure 14 and 15, respectively.

For product **24**, the protons of the aromatic ring of phenylalanine appear as a multiplet between 7.25 and 7.09 ppm. At 3.65 ppm, a doublet corresponding to one diastereotopic proton the CH_2 linking the aromatic ring to the phenylalanine residue can be observed, while a second CH_2 proton is superimposed with the multiplet associated with the PEG chain, spanning from 3.53 to 3.21 ppm. Within this same region, the triplet corresponding to the chiral CH proton of phenylalanine is observed at 3.34 ppm. At 2.77 ppm, one of the protons of the benzyl CH_2 group is detected, coupling with the other proton, which resonates in the PEG multiplet region between 3.28 and 3.18 ppm. Additional signals from the PEG chain appear as a multiplet in the range of 1.76 to 1.65 ppm.

Figure 25. ^1H NMR spectrum of product **24** in $\text{D}_2\text{O}+\text{NaOD}$ (400 MHz, 298 K).

In the NMR spectrum of product **26** (Figure 26) it is possible to identify the aromatic proton at 7.57 and 7.33 ppm which are diastereotopic. At 3.84 ppm, a doublet corresponding to one of the diastereotopic protons of the CH_2 linking the aromatic ring to the proline residue can be observed, while the other is superimposed with the multiplet of the PEG chain, spanning from 3.52 to 3.21 ppm. The triplet corresponding to the chiral CH is observed at 2.85 ppm, while the signals for the diastereotopic CH_2 protons of the proline ring are detected at 2.71, 2.53, 2.18, and 1.97 ppm. Additional signals from the PEG chain appear as a multiplet in the range of 1.76 to 1.65 ppm.

The ESI-TOF analysis revealed peaks corresponding to the compound at m/z 478.29 $[\text{M}+\text{H}]^+$, 484.30 $[\text{M}+\text{Li}]^+$, 500.28 $[\text{M}+\text{Na}]^+$ and 516.24 $[\text{M}+\text{K}]^+$.

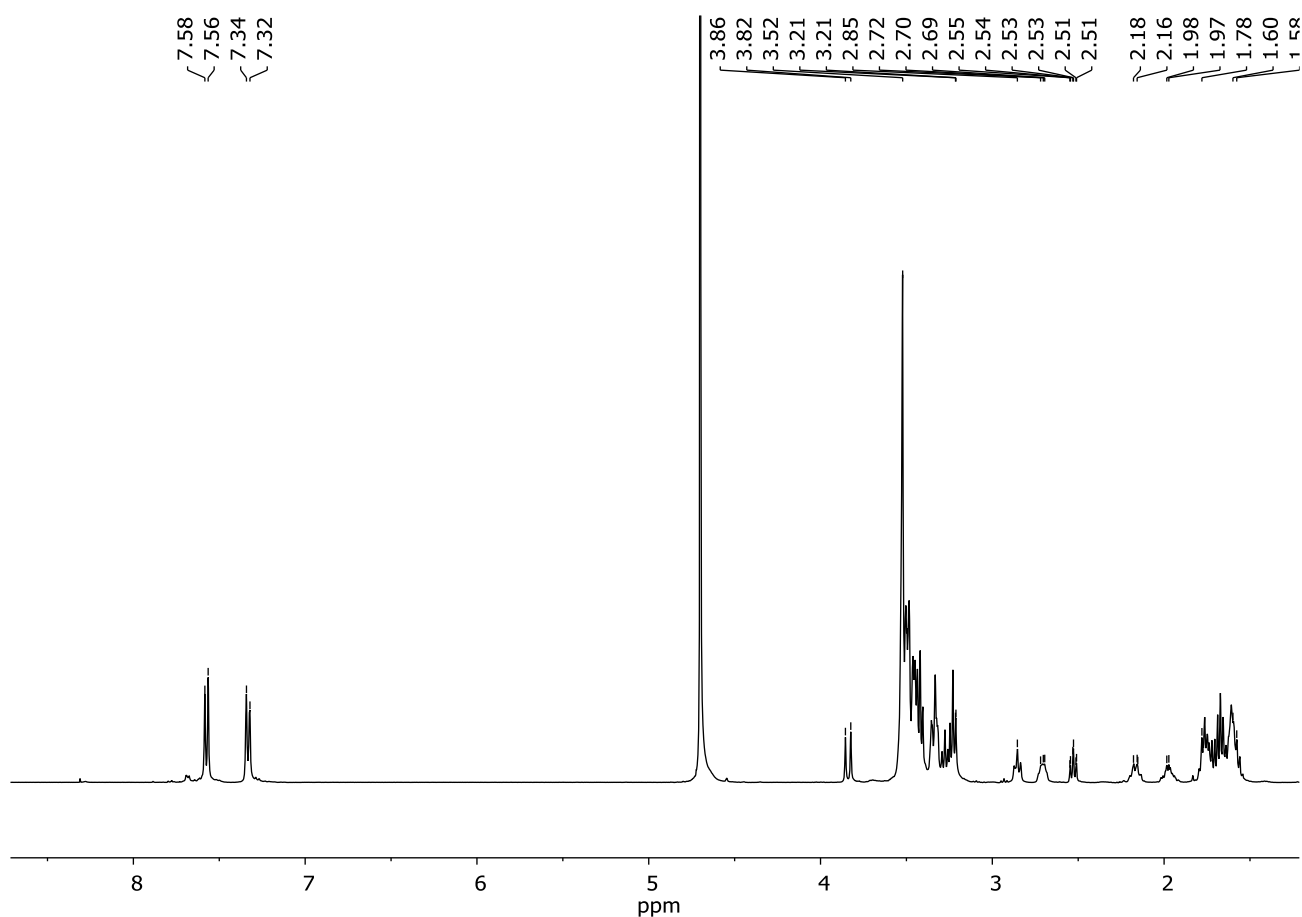


Figure 26. ^1H NMR spectrum of product **26** in $\text{D}_2\text{O}+\text{NaOD}$ (400 MHz, 298 K).

The latter two compounds were designed for attachment to an alkyne moiety through strain-promoted alkyne-azide ligation on a poly-L-lysine-functionalized surface, allowing for testing with a model of a Gram-negative bacterial membrane (see Chapter 4).

3.3 Conclusions

Synthetic approaches for preparing upper-rim analogues of **C1** and **C2** were developed, using a calix[4]arene model with four propyl groups at the lower rim. Two new procedures for the functionalization of the upper rim of tetra alkoxy calix[4]arenes were developed. In the first case employing reductive amination reaction on the tetraformyl precursor for the phenylalanine derivative **3**. The approach was changed to obtain the proline derivative **6** employing tetrachloromethyl derivative as precursor for a nucleophilic substitution. Subsequent hydrolysis of the methyl esters on derivatives **3** and **6** will afford the target compounds **4** and **7**, that could be useful model compounds to test the bacteria recognition properties of surfaces functionalized with calix[4]arenes embedded with such amino acids.

This upper-rim functionalization protocol was also adapted to synthesize the analogues with two amine groups at the lower rim. However, compatibility between reactions at the upper and lower rims needs a refined evaluation of the synthetic strategy. The chosen strategy, using the phthalimide as a masking group for the amine, initially appeared promising, as it allowed for complete functionalization of the upper rim with four amino acid moieties while preserving the phthalimide functionality at the lower rim, yielding intermediates **10** and **14**. However, removing the phthalimide moiety proved more challenging, as the standard removal procedure could not be employed without compromising the zwitterionic amino acid characteristics on the upper rim. Product **16**, containing two amine groups at the lower rim and four proline units at the upper rim, was successfully synthesized, and in future prospective it could be used for the surface functionalization on systems like polyelectrolyte (see Chapter 4) or nano-objects (see Chapter 5) Furthermore, isolating the phenylalanine derivative **12** proved challenging, likely due to its low solubility, which also complicated its characterization. In this case further studies also on the synthetic approach could be useful to be able to use this compound for surface functionalization.

The two oligoethylene chains, each functionalized with a monovalent calixarene model with phenylalanine (compound **24**) or proline (compound **26**) on one end and an azide group on the other, were successfully synthesized. Chapter 4 will present the protocol for their attachment to a functionalized polyelectrolyte surface, as well as the studies on their binding affinity to artificial Gram-negative bacterial membranes.

3.4 Experimental part

3.4.1 General information (see also par. 2.4)

High performance liquid chromatography (HPLC) was performed on Water (2535) set up equipped with analytical and preparative XBridge C₁₈ columns.

Mass spectra were obtained with a Waters single quadrupole SQ detector spectrometer, or a Waters ESI(+)TOF spectrometer (Micromass LCT).

25,26,27,28-tetrapropoxy calix[4]arene (1)

In a two-necked round-bottom flask under a nitrogen atmosphere, tetrahydroxycalix[4]arene (5.00 g, 11.79 mmol) was dissolved in 100 mL of dry dimethylformamide with magnetic stirring. Sodium hydride (60% w/w, 2.70 g, 70.73 mmol) was slowly added, and the reaction mixture was stirred at room temperature for 30 minutes, during which the solution changed from pale yellow to white. Subsequently, *n*-propyl iodide (6.90 mL, 70.73 mmol) was added, and the reaction was allowed to proceed overnight under magnetic stirring. The reaction progress was monitored by TLC (SiO₂, hexane-ethyl acetate 9.8:0.2, revealed with UV lamp and FeCl₃ stain) and quenched by the addition of 1 M HCl (100 mL). The yellow-white precipitate was filtered using a Büchner funnel and recrystallized from DCM/methanol to yield a white solid (5.95 g, 10.04 mmol) with 85% yield.

¹H NMR (400 MHz, CDCl₃) δ : 6.63-6.60 (m, 12H, ArH); 4.47 (d, J = 13.4 Hz, 4H, ArCH_{ax}HAr), 3.87 (t, J = 7.4 Hz, 8H, OCH₂), 3.17 (d, J = 13.4 Hz, 4H, ArCHH_{eq}Ar); 1.95 (sextuplet, 8H, J = 7.2 Hz, OCH₂CH₂CH₃), 1.01 (t, J = 7.4 Hz, 12H, CH₃).

The spectroscopic data found are in agreement with those reported in literature ¹⁷

5,11,17,23-tetraformyl-25,26,27,28-tetrapropoxy calix[4]arene (2)

25,26,27,28-tetrapropoxy calix[4]arene (500 mg, 1.178 mmol), HMTA (6.6 g, 47.120 mmol), and 20 mL of TFA were added in a round bottom flask. The reaction was refluxed overnight under magnetic stirring and monitored by TLC (SiO₂, dichloromethane-methanol 9:1, revealed with UV and 2,4-dinitrophenylhydrazine stain). After allowing the reaction mixture to cool to room temperature 200 mL of water and 100 mL of dichloromethane were added, and the mixture was stirred over 4 hours. The organic phase was washed twice with water and once with a saturated aqueous solution of NaCl, dried over Na₂SO₄, and the solvent was evaporated under reduced pressure. Product **2** was obtained as a yellow solid with 97% yield.

¹H NMR (600 MHz, CDCl₃) δ : 9.57 (s, 4H, CHO), 7.14 (s, 8H, ArH), 4.49 (d, J = 13.8 Hz, 4H, ArCH_{ax}HAr), 3.92 (t, J = 7.5 Hz, 8H, OCH₂), 3.34 (d, J = 13.8 Hz, 4H, ArCHH_{eq}Ar), 1.89 (sextuplet, J = 7.5 Hz, 8H, OCH₂CH₂CH₃), 1.00 (t, J = 7.4 Hz, 12H, CH₃).

The spectroscopic data found are in agreement with those reported in literature ¹⁸

5,11,17,23-(L-phenylalanine methyl ester-*N*-methyl)- 25,26,27,28 tetrapropoxy calix[4]arene (3)

Compound **2** (132.3 mg, 0.188 mmol) was dissolved in 3 mL of dichloromethane, then L-phenylalanine methyl ester hydrochloride (404.8 mg, 1.88 mmol) dissolved in 2 mL of methanol was added. The triethylamine (0.26 mL, 1.88 mmol) was added subsequently, along with sodium cyanoborohydride (82.7 mg, 1.32 mmol). The pH was adjusted to 5 by adding acetic acid dropwise. The reaction was left

stirring at room temperature for 16 hours, monitoring with TLC (SiO₂, hexane-dichloromethane-methanol 3:6:1). Upon completion the solvent was removed under reduced pressure and the reaction was quenched with 3 mL of 1 M HCl solution. The crude was extracted with dichloromethane from the aqueous phase, then the organic phase was washed two times with water and one with brine. The crude was purified via flash chromatography and preparative TLC (SiO₂, hexane-DCM-methanol 3:6:1), and the pure product **3** was obtained as a transparent resin with 20% yield.

¹H NMR (400 MHz, CDCl₃) δ: 7.30-7.14 (m, 20H, ArH Phe), 6.48-6.43 (m, 8H, ArH), 4.38 (d, *J* = 12.8 Hz, 4H, ArCH_{ax}HAr), 3.82 (t, *J* = 8.0 Hz, 8H, OCH₂), 3.60 (s, 12H, COOCH₃), 3.47 (t, *J* = 6.9 Hz, 4H, CH), 3.41 (d, *J* = 12.4 Hz, 4H, ArCHHN), 3.28 (d, *J* = 12.4 Hz, 4H, ArCHHN), 3.05 (d, *J* = 13.1 Hz, 4H, ArCHH_{eq}Ar), 2.95-2.92 (m, 8H, CH₂Ph), 1.97-1.89 (sextuplet, *J* = 7.5 Hz, 8H, OCH₂CH₂), 1.00 (t, *J* = 7.4 Hz, 12H, CH₃).

¹³C NMR (101 MHz, CDCl₃) δ: 175.03 (C=O), 155.67 (C_{ipso}), 137.45 (ArC_q(Phe)), 134.76 (C_{orto}), 132.48 (C_{para}), 129.22 (CH(ArPhe)), 128.40 (CH(ArPhe)), 128.08 (C_{meta}), 127.86 (C_{meta}), 126.63 (CH(ArPhe)), 76.66 (OCH₂), 62.17 (NCH), 51.77 (ArCH₂N), 51.52 (COOCH₃), 39.70 (CH₂Ph), 30.99 (ArCH₂Ar), 23.19 (OCH₂CH₂), 10.34 (CH₃).

5,11,17,23-tetrachloromethyl-25,26,27,28-tetrapropoxy calix[4]arene (**5**)

Compound **1** (261 mg, 0.364 mmol) and paraformaldehyde (219 mg, 7.28 mmol) were suspended in dioxane (15 mL). After the addition of acetic acid (1.32 mL), phosphoric acid (2.50 mL), and HCl (37% w/w, 2.70 mL), the reaction mixture was heated at 80 °C for 20 hours. The reaction progress was monitored via TLC (SiO₂, hexane-ethyl acetate 7:3). Upon completion the reaction was cooled down to room temperature and the mixture was diluted with ice-water and the white precipitate was filtered. Product **5** was obtained with 80% yield after drying under vacuum.

¹H NMR (400 MHz, CDCl₃) δ: 6.67 (s, 8H, ArH), 4.43 (d, *J* = 13.3 Hz, 4H, ArCH_{ax}HAr), 4.32 (s, 8H, CH₂Cl), 3.98 – 3.75 (t, *J* = 7.4 Hz, 8H, OCH₂), 3.16 (d, *J* = 13.3 Hz, 4H, ArCHH_{eq}Ar), 1.93 (q, *J* = 7.5 Hz, 8H, OCH₂CH₂), 1.01 (t, *J* = 7.4 Hz, 12H, CH₃).

The spectroscopic data found are in agreement with those reported in literature.¹⁹

5,11,17,23-tetra(L-proline methyl ester -N-methyl)-25,26,27,28 tetrapropoxy calix[4]arene (**6**)

Compound **5** (117 mg, 0.146 mmol) was dissolved in 2 mL of dry DMF, then L-proline methyl ester hydrochloride (119 mg, 0.730 mmol) and 0.25 mL of DIPEA were added. The solution was stirred for 36 hours at 70 °C and monitored via TLC (SiO₂, hexane-DCM-methanol 4:5:1). The reaction was cooled to room temperature, 3 mL of 1 M HCl were added, and the solution was extracted 2 times with 5 mL of DCM. The organic phase was washed with water and brine, dried over Na₂SO₄ and the solvent was removed under reduced pressure. After flash chromatography product **6** was obtained as a yellow resin with 30% yield.

¹H NMR (400 MHz, CDCl₃) δ: 6.56 (s, 4H, ArH), 6.53 (s, 4H, ArH), 4.38 (d, *J* = 13.0 Hz, 4H, ArCH_{ax}HAr), 3.81 (t, *J* = 7.4 Hz, 8H, OCH₂), 3.65 (s, 12H, COOCH₃), 3.47 (d, *J* = 12.3 Hz, 4H, ArCHHN), 3.24 (d, *J* = 12.3 Hz, 4H, ArCHHN), 3.11 (m, 8H, ArCHH_{eq}Ar, NCH), 2.94 (m, 4H, NCHHCH₂), 2.28 (m, 4H, NCHHCH₂), 2.08 (m, 4H, CHCHH), 1.94-1.88 (m, 16H, CHCHH, NCH₂CHH, OCH₂CH₂), 1.75 (m, 4H, NCH₂CHH), 0.99 (t, *J* = 7.5 Hz, 12H, CH₃).

¹³C NMR (101 MHz, CDCl₃) δ: 174.52 (C=O), 155.81 (C_{ipso}), 134.53 (C_{orto}), 134.46 (C_{orto}), 130.76 (C_{para}), 129.31 (C_{meta}), 76.63 (OCH₂), 65.09 (CH), 58.47 (ArCH₂N), 53.03 (NCH₂), 51.65 (COOCH₃), 30.81 (ArCH₂Ar), 29.37 (CHCH₂), 23.19 (NCH₂CH₂), 22.89 (OCH₂CH₂), 10.35 (CH₃).

25,27-dipropoxy-26,28-bis[(6-phthalimidohexyl)oxy]calix[4]arene (8)

Compound **1** (300 mg, 0.59 mmol) was dissolved in 10 mL of dry DMF under a nitrogen atmosphere. Sodium hydride (60%, 2.36 mmol, 57 mg) was then added, and the mixture was stirred for 10 minutes. Subsequently, 732 mg (2.36 mmol) of *N*-(6-bromohexyl)phthalimide was introduced into the reaction mixture, which was allowed to react at room temperature for 2 hours. The reaction progress was monitored by TLC (SiO₂, hexane - ethyl acetate 8:2, revealed with UV lamp and stain FeCl₃). Upon completion, the reaction was quenched by the addition of 20 mL of a 1 M HCl solution and extracted with dichloromethane. The organic layer was washed successively with brine and water, dried over Na₂SO₄, and the solvent was evaporated under reduced pressure. The crude was then purified using flash chromatography (SiO₂, hexane- ethyl acetate 8:2) to remove the excess of *N*-(6-bromohexyl)phthalimide. A white sticky solid was obtained as product **8** with 89% of yield (506 mg, 0.52 mmol).

ESI-SQ(+): *m/z* calc 967.22, found 989.62 [M+Na]⁺, 1006.60 [M+K]⁺

¹H NMR (400 MHz, CDCl₃) δ: 7.86-7.84 (m, 2H, ArHphth_{orto}), 7.73-7.71 (m, 2H, ArHphth_{meta}), 6.67 (d, *J* = 6.6 Hz, 4H, ArH_{meta}), 6.61 (t, 2H, *J* = 6.6 Hz, ArH_{para}), 6.55 (s, 6H, ArH_{meta}, ArH_{para}), 4.44 (d, *J* = 13.3 Hz, 4H, ArCH_{ax}HAr), 3.90-3.84 (m, 8H, OCH₂), 3.73 (t, *J* = 7.3 Hz, 4H, CH₂Nphth), 3.15 (d, *J* = 13.3 Hz, 4H, ArCHH_{eq}Ar), 1.95-1.87 (m, 8H, OCH₂CH₂), 1.78-1.71 (m, 4H, CH₂CH₂Nphth), 1.52-1.41 (m, 8H, CH₂CH₂CH₂CH₂Nphth), 0.97 (s, 6H, CH₃).

¹³C NMR (100 MHz, CDCl₃) δ: 168.42 (NC=O), 156.72 (C_{ipso}), 156.37 (C_{ipso}), 135.31 (C_{orto}), 134.93 (C_{orto}), 133.86 (CH_{meta} Nphth), 132.19 (C_q Nphth), 128.20 (CH_{meta}), 128.05 (CH_{meta}), 123.17 (ArCH_{orto} phth), 121.92 (ArCH_{para}), 121.86 (ArCH_{para}), 76.67 (OCH₂), 74.81 (OCH₂), 38.02 (CH₂Nphth), 31.02 (ArCH₂Ar), 30.10 (OCH₂CH₂CH₃), 28.78 (CH₂CH₂Nphth), 27.02 (CH₂CH₂CH₂CH₂Nphth), 25.90 (CH₂CH₂, CH₂CH₂CH₂Nphth), 23.29 (CH₂CH₂CH₂Nphth), 10.38 (CH₃).

5,11,17,23-tetraformyl-25,27-dipropoxy-26,28-bis[(6-phthalimidohexyl)oxy]calix[4]arene (9)

Compound **8** (500 mg, 0.52 mmol) and hexamethylene tetramine (1 g, 7.14 mmol) were dissolved in trifluoroacetic acid (15 mL) under magnetic stirring, and the reaction was heated at 100 °C and left for 16 hours. The reaction was monitored via TLC (SiO₂, hexane-ethyl acetate 1:1). Upon completion, after cooling to room temperature 20 mL of 0.1 M HCl and 20 mL of DCM were added to the reaction mixture, and the biphasic mixture was left stirring for 6 hours. The organic phase was removed and washed with water and brine until neutral pH was reached. A yellow sticky resin was obtained as pure product **9** with 72% yield (402 mg, 0.37 mmol).

¹H NMR (400 MHz, CDCl₃) δ: 9.64 (s, 2H, CHO), 9.54 (s, 2H, CHO), 7.85-7.83 (m, 4H, ArHphth), 7.78 – 7.66 (m, 4H, ArHphth), 7.22 (s, 4H, ArH_{meta}), 7.09 (s, 4H, ArH_{meta}), 4.49 (d, *J* = 13.8 Hz, 4H, ArCH_{ax}HAr), 3.95 (m, 8H, OCH₂), 3.71 (t, *J* = 7.2 Hz, 4H, CH₂Nphth), 3.35 (d, *J* = 13.9 Hz, 3H, ArCHH_{eq}Ar), 1.88 (p, *J* = 7.3 Hz, 8H, OCH₂CH₂), 1.73 (p, *J* = 7.3 Hz, 4H, CH₂CH₂Nphth), 1.54 – 1.35 (m, 8H, CH₂CH₂CH₂CH₂Nphth), 0.98 (t, *J* = 7.4 Hz, 6H, CH₃).

¹³C NMR (101 MHz, CDCl₃) δ: 191.36 (CHO), 191.33 (CHO), 168.42 (NHC=O), 162.02 (C_{ipso}), 161.67 (C_{ipso}), 135.71 (C_{orto}), 135.40 (C_{orto}), 133.98 (ArCphth), 132.09 (C_qphth), 131.40 (C_{para}), 131.36 (C_{para}), 130.35 (CH_{meta}), 130.16 (CH_{meta}), 123.19 (ArCphth), 77.22 (OCH₂), 75.41 (OCH₂), 37.83 (CH₂Nphth), 30.94 (ArCH₂Ar), 30.03 (OCH₂CH₂CH₃), 28.64 (CH₂CH₂Nphth), 26.81 (CH₂CH₂CH₂CH₂Nphth), 25.64 (CH₂CH₂CH₂CH₂Nphth), 23.34 (OCH₂CH₂CH₂), 10.26 (CH₃), 10.24 (CH₃).

5,11,17,23-(L-phenylalanine methyl ester-*N*-methyl)-25,27-dipropoxy-26,28-bis[(6-phthalimidohexyl)oxy]calix[4]arene (10)

Compound **9** (200 mg, 0.185 mmol) was dissolved in 3 mL of dichloromethane, then L-phenylalanine methyl ester hydrochloride (800 mg, 3.71 mmol) dissolved in 3 mL of methanol was added. DIPEA (640 μ L) was added subsequently, along with sodium cyanoborohydride (233 mg, 3.71 mmol). The pH was adjusted to 5 by adding acetic acid dropwise. The reaction was left stirring at room temperature for 16 hours, monitoring with TLC (SiO₂, hexane-dichloromethane-methanol 4:5:1). Upon completion the solvent was removed under reduced pressure and the reaction was quenched with 8 mL of 1 M HCl solution. The crude was extracted with dichloromethane from the aqueous phase, then the organic phase was washed two times with water and one with brine. The crude was purified via flash chromatography and preparative TLC (SiO₂, hexane-DCM-methanol 4:5:1), and the pure product **10** was obtained as a transparent resin with 50% yield (160 mg).

¹H NMR (400 MHz, CDCl₃) δ : 7.84 (m, 4H, ArHphth), 7.71 (m, 4H, ArHphth), 7.33–7.07 (m, 20 H, ArHPhe), 6.48 (s, 2H, ArH), 6.45 (s, 2H, ArH), 6.39 (s, 2H, ArH), 6.37 (s, 2H, ArH), 4.32 (d, J = 13.0 Hz, 4H, ArCH_{ax}HAr), 3.81 (m, 8H, OCH₂), 3.72 (m, 4H, CH₂Nphth), 3.67–3.53 (m, 12H, COOCH₃), 3.50–3.40 (m, 6H, NCH, ArCHHN), 3.36 (d, J = 12.6 Hz, 2H, ArCHHN), 3.29 (d, J = 12.6 Hz, 2H, ArCHHN), 3.22 (d, J = 12.6 Hz, 2H, ArCHHN), 3.01, (d, J = 13.0 Hz, 4H, ArCHH_{eq}Ar), 2.93 (m, 8H, CH₂Ph), 1.92–1.80 (m, 8H, OCH₂CH₂), 1.75 (m, 4H, CH₂CH₂Nphth), 1.48–1.40 (m, 8H, CH₂CH₂CH₂CH₂Nphth), 0.95 (t, J = 7.4 Hz, 6H, CH₃).

¹³C NMR (101 MHz, CDCl₃) δ : 175.03 (COOMe), 175.00 (COOMe), 168.44 (CON), 155.75 (C_{ipso}), 155.49 (C_{ipso}), 137.45 (ArC Phe), 134.85 (C_{orto}), 134.78 (C_{orto}), 134.57 (C_{orto}), 134.50 (C_{orto}), 133.86 (ArCphth), 132.50 (C_{para}), 132.47 (C_{para}), 132.17 (ArCphth), 129.22 (ArC Phe), 128.36 (ArC Phe), 128.12 (C_{meta}), 128.02 (C_{meta}), 127.89 (C_{meta}), 127.78 (C_{meta}), 126.63 (ArC Phe), 123.19 (ArCphth), 76.63 (OCH₂), 74.80 (OCH₂), 62.16 (CH), 51.78 (ArCH₂N), 51.74 (ArCH₂N), 51.53 (COOCH₃), 51.51 (COOCH₃), 39.66 (CH₂Ph), 38.00 (CH₂Nphth), 30.98 (ArCH₂Ar), 30.02 (CH₂), 28.78 (CH₂), 27.04 (CH₂), 25.89 (CH₂), 23.20 (CH₂), 22.03, 10.36 (CH₃).

5,11,17,23-(L-phenylalanine-*N*-methyl)-25,27-dipropoxy-26,28-bis[lithium2-((6-hexyl)carbamoyl)benzoate)oxy] calix[4]arene (11)

Compound **10** (90 mg, 0.052 mmol) was dissolved in a 2:1 mixture of methanol-water, then lithium hydroxide monohydrate (22 mg, 0.06 mmol) is added. The solution was left stirring for 4 hours at room temperature. The solvent was removed under vacuum and affording product **11** as a white solid (87 mg).

¹H NMR (400 MHz, D₂O) δ : 7.50 (m, 2H, ArHphth), 7.39 (m, 2H, ArHphth), 7.30 (t, J = 7.4 Hz, 2H, ArHphth), 7.26 (d, J = 7.4 Hz, 2H, ArHphth), 7.18–7.01 (m, 20H, ArH Phe), 6.53–6.42 (m, 8H, ArHcalix), 4.27 (d, 4H, ArCH_{ax}HAr), 3.91–3.79 (m, 8H, ArCH₂N), 3.25 (m, 8H, ArCH₂N), 3.05 (m, 4H, ArCHH_{eq}Ar), 2.82–2.79 (m, 4H), 2.61 (m, 4H, CH₂CH₂N), 1.90–1.82 (m, 8H, OCH₂CH₂), 1.55–1.40 (m, 8H, CH₂CH₂CH₂CH₂N), 0.89 (bs, 6H, CH₃).

5,11,17,23-(L-phenylalanine-*N*-methyl)-25,27-dipropoxy-26,28-bis[lithium 2-(6-aminohexyl)oxy] calix[4]arene (12)

Compound **11** (87 mg) was suspended in 3 mL of glacial acetic acid and left under magnetic stirring at 80 °C for 16 hours. The solvent was then removed under reduced pressure and the crude was recrystallized from methanol-diethyl ether mixture.

The ^1H NMR spectrum has not changed respect to the reagent **11**.

5,11,17,23-tetrachloromethyl-25,27-dipropoxy-26,28-bis[(6-phthalimido)hexyl]oxy]calix[4]arene (13)

Compound **1** (500 mg, 0.517 mmol) and paraformaldehyde (621 mg, 20.7 mmol) were suspended in dioxane (16 mL). After the addition of acetic acid (2.37 mL), phosphoric acid (4.58 mL), and HCl (37% w/w, 5.14 mL), the reaction mixture was heated at 80 °C for 20 hours. The reaction progress was monitored via TLC (SiO_2 , hexane-ethyl acetate 7:3). Upon completion the reaction was cooled down to room temperature and the mixture was diluted with ice-water and extracted with 20 mL of DCM. Product **5** (617 mg) was obtained with 80% yield after drying under vacuum.

^1H NMR (400 MHz, CDCl_3) δ : 7.82 (m, 2H, ArHphth), 7.69 (m, 2H, ArHphth), 6.68 (s, 4H, ArH), 6.59 (s, 4H, ArH), 4.39 (d, $J = 13.3$ Hz, 4H, $\text{ArCH}_{\text{ax}}\text{HAr}$), 4.34 (s, 2H, CH_2Cl), 4.27 (s, 2H, CH_2Cl), 3.85 (m, 8H, OCH_2), 3.71 (t, $J = 7.2$ Hz, 4H, CH_2Nphth), 3.14 (d, $J = 13.4$ Hz, 4H, $\text{ArCHH}_{\text{eq}}\text{Ar}$), 1.88 (m, 8H, $\text{OCH}_2\text{CH}_2\text{CH}_2$), 1.73 (p, $J = 7.1$ Hz, 4H, $\text{OCH}_2\text{CH}_2\text{CH}_3$), 1.52 – 1.37 (m, 8H, $\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{N}$), 0.95 (t, $J = 7.4$ Hz, 6H, CH_3).

5,11,17,23-tetra(L-proline methyl ester -N-methyl) -25,27-dipropoxy-26,28-bis[(6-phthalimido)hexyl]oxy]calix[4]arene (14)

Compound **13** (600 mg, 0.517 mmol) was dissolved in 8 mL of dry acetonitrile, then L-proline methyl ester hydrochloride (1.03 g, 6.20 mmol) and 1.44 mL of DIPEA were added. The solution was stirred for 36 hours at room temperature and monitored via TLC (SiO_2 , DCM-methanol 9:1). The reaction was cooled to room temperature, 10 mL of 1 M HCl were added, and the solution was extracted two times with 10 mL of DCM. The organic phase was washed with water and brine, dried over Na_2SO_4 and the solvent was removed under reduced pressure. After flash chromatography (SiO_2 , DCM-methanol 95:5) product **14** was obtained as a yellow resin with 40% yield (376 mg).

^1H NMR (400 MHz, CDCl_3) δ : 7.80 (m, 2H, ArHphth), 7.67 (m, 2H, ArHphth), 6.54 (s, 2H, ArH), 6.52 (s, 2H, ArH), 6.47 (s, 2H, ArH), 6.45 (s, 2H, ArH), 4.31 (d, $J = 13.1$ Hz, 4H, $\text{ArCH}_{\text{ax}}\text{HAr}$), 3.80 – 3.74 (m, 8H, OCH_2), 3.67 (t, $J = 7.3$ Hz, 4H, CH_2Nphth), 3.62 (s, 6H, OCH_3), 3.61 (s, 6H, OCH_3), 3.47 (d, $J = 12.3$ Hz, 2H, CH_2N), 3.40 (d, $J = 12.3$ Hz, 2H, CH_2N), 3.22 (d, $J = 12.3$ Hz, 2H, CH_2N), 3.16 (d, $J = 12.3$ Hz, 2H, CH_2N), 3.11 (m, 4H, NCH), 3.06 (d, $J = 13.2$ Hz, 4H, $\text{ArCHH}_{\text{eq}}\text{Ar}$), 2.96 – 2.82 (m, 4H, NCH₂), 2.32 – 2.18 (m, 4H, NCH₂), 2.11 – 1.99 (m, 4H, NCH₂CH₂), 1.92 – 1.78 (m, 16H, NCH₂CH₂, OCH_2CH_2), 1.74 – 1.63 (m, 8H, CHCH₂), 1.47 – 1.35 (m, 8H, $\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{N}$), 0.90 (t, $J = 7.5$ Hz, 6H, CH_3).

^{13}C NMR (101 MHz, CDCl_3) δ : 174.47 (COOMe), 174.44 (COOMe), 168.35 (CON), 155.82 (C_{ipso}), 155.59 (C_{ipso}), 134.58 (C_{orto}), 134.51 (C_{orto}), 134.34 (C_{orto}), 134.25 (C_{orto}), 133.82 (ArCphth), 132.14 (ArCphth), 130.75 (C_{para}), 129.33 (C_{meta}), 129.23 (C_{meta}), 129.15 (C_{meta}), 123.11 (ArCphth), 76.57 (OCH_2), 74.72 (OCH_2), 65.02 (CH), 58.39 (ArCH_2N), 52.96 (NCH₂), 51.61 (COOCH₃), 37.95 (CH_2Nphth), 30.80 (ArCH_2Ar), 29.99 (CH_2), 29.32 (CH_2), 28.72 (CH_2), 26.98 (CH_2), 25.86 (CH_2), 22.83 (CH_2), 10.35 (CH_3).

5,11,17,23-tetra(L-proline -N-methyl) -25,27-dipropoxy-26,28-bis[lithium2-((6-hexyl)carbamoyl)benzoate]oxy] calix[4]arene (15)

Compound **14** (130 mg, 0.075 mmol) was dissolved in a 2:1 mixture of methanol-water, then lithium hydroxide monohydrate (31 mg, 0.80 mmol) is added. The solution was left stirring for 4 hours at room temperature. The solvent was removed under vacuum and affording product **15** as a white solid (114 mg).

^1H NMR (400 MHz, D_2O) δ : 7.54 (dd, $J = 7.7, 1.3$ Hz, 2H, ArHphth), 7.47 – 7.40 (m, 2H, ArHphth), 7.39 – 7.33 (m, 2H, ArHphth), 7.31 – 7.25 (m, 2H, ArHphth), 6.81 – 6.70 (m, 4H, ArH), 6.69 – 6.62 (m, 4H, ArH), 4.38 (d, $J = 12.7$ Hz, 4H, $\text{ArCH}_{\text{ax}}\text{HAr}$), 3.89 (bs, 4H, OCH_2), 3.79 (t, $J = 6.8$ Hz, 4H, OCH_2), 3.72 – 3.54 (m, 4H, CH_2Nphth), 3.19 (d, $J = 12.7$ Hz, 4H, $\text{ArCHH}_{\text{eq}}\text{Ar}$), 3.00 (d, $J = 12.6$ Hz, 4H, CH_2N), 2.91 (m, 4H, CH_2N), 2.63 (bs, 4H, CH_2N), 2.20 – 1.81 (m, 16H, NCH_2 , NCH_2CH_2 , OCH_2CH_2), 1.76 – 1.56 (m, 16H, CHCH_2 , NCH_2CHH), 1.50 – 1.42 (m, 8H, $\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{N}$), 0.95 (t, $J = 7.3$ Hz, 6H, CH_3).

5,11,17,23-tetra(L-proline -N-methyl) -25,27-dipropoxy-26,28-bis[6-aminohexyl] oxy] calix[4]arene (16)

Compound **15** (114 mg, 0.085 mmol) was dissolved into 10 mL of ethanol, then hydrate hydrazine was added (0.160 mmol, 80 μL). The solution was refluxed for 4 hours. Upon completion the reaction was cooled to room temperature and the solvent was removed under reduced pressure. The crude was recrystallized from a 1:1 mixture of methanol-diethyl ether. Product **16** was obtained with 80% yield.

^1H NMR (400 MHz, Methanol- d_4) δ : 7.09 – 6.79 (m, 8H, ArH), 4.46 (d, $J = 13.3$ Hz, 4H, $\text{ArCH}_{\text{ax}}\text{HAr}$), 4.42 – 4.35 (m, 2H, CH_2N), 4.29 (d, $J = 12.9$ Hz, 2H, CH_2N), 4.13 (d, $J = 12.2$ Hz, 2H, CH_2N), 4.02 (d, $J = 12.4$ Hz, 2H, CH_2N), 3.93 (m, 6H, CH_2N , OCH_2), 3.72 – 3.52 (m, 4H, $\text{ArCHH}_{\text{eq}}\text{Ar}$), 3.37 – 3.26 (m, 8H,), 2.95 (m, 4H, CH_2NH_2), 2.58 (bs, 4H, NH_2), 2.27 – 2.00 (m, 8H,), 2.00 – 1.86 (m, 8H, NCH_2 , NCH_2CH_2), 1.78 – 1.67 (m, 4H, $\text{OCH}_2\text{CH}_2\text{CH}_3$), 1.57 – 1.40 (m, 8H, NH_2), 1.02 (t, $J = 7.5$ Hz, 6H, CH_3).

(S)-4-(((1-methoxy-1-oxo-3-phenylpropan-2-yl)amino)methyl)benzoic acid (18)

4-formylbenzoic acid (200 mg, 1.33 mmol) was dissolved in 5 mL of methanol, then L-phenylalanine methyl ester hydrochloride (578 mg, 2.66 mmol) was added. The DIPEA (0.464 mL, 2.66 mmol) was added subsequently, along with sodium cyanoborohydride (335 mg, 5.33 mmol). The pH was adjusted to 5 by adding acetic acid dropwise. The reaction was left stirring at room temperature for 16 hours, monitoring with TLC (SiO_2 , dichloromethane-methanol 9:1). Upon completion the solvent was removed under reduced pressure and the reaction was quenched with 3 mL of 1 M HCl solution. The crude was extracted with dichloromethane from the aqueous phase, then the organic phase was washed two times with water and one with brine. The crude was purified via flash chromatography and preparative TLC (SiO_2 , dichloromethane-methanol 9:1), and the pure product **18** (322 mg) was obtained as a transparent resin with 77% yield.

^1H NMR (400 MHz, CDCl_3) δ : 8.04 (d, $J = 8.1$ Hz, 2H, ArH), 7.88 (bs, 1H, NH), 7.37 – 7.20 (m, 5H, ArH(Phe)), 7.19 (d, $J = 8.1$ Hz, 2H, ArH), 3.95 (d, $J = 13.9$ Hz, 1H, ArCHHN), 3.82 (d, $J = 13.9$ Hz, 1H, ArCHHN), 3.76 (s, 3H, CH_3), 3.67 – 3.59 (m, 2H, CH, CHCHHAr), 3.06 (d, $J = 6.8$ Hz, 1H, CHCHHAr).

(S)-4-((2-(methoxycarbonyl)pyrrolidin-1-yl)methyl)benzoic acid (19)

4-(chloromethyl)benzoic acid (500 mg, 2.93 mmol) was dissolved in 15 mL of ethanol. L-proline methyl ester hydrochloride (1.94 g, 11.7 mmol) and triethylamine (1.19 g, 11.7 mmol) were added. The reaction was refluxed for 20 hours and monitored via TLC (SiO_2 , DCM-methanol 9:1). Upon completion of the solvent was removed under reduced pressure, the crude was dissolved in DCM and washed with 1M HCl, water and brine. The crude was then purified via flash chromatography (SiO_2 , DCM-methanol 9:1). Product **19** (with 20% of ethyl ester analogue) was obtained as a brown oil (309 mg) with 56% yield.

^1H NMR (400 MHz, CDCl_3) methyl ester δ : 8.03 (d, $J = 8.1$ Hz, 2H, ArH), 7.73 (bs, 1H, NH), 7.43 (d, $J = 8.1$ Hz, 2H, ArH), 4.00 (d, $J = 13.0$ Hz, 1H, ArCHHN), 3.71 (d, $J = 13.1$ Hz, 1H, ArCHHN), 3.65 (s, 3H, CH_3),

3.38 – 3.30 (m, 1H, CH), 3.13 – 3.05 (m, 1H, CH₂NCHH), 2.53 – 2.44 (m, 1H, CH₂NCHH), 2.22 – 2.11 (m, 1H, CHCHH), 2.04 – 1.87 (m, 2H, NCH₂CHH, CHCHH), 1.85 – 1.76 (m, 1H, NCH₂CHH).

¹³C NMR (101 MHz, CDCl₃) methyl ester δ: 174.03 (COOCH₃), 170.69 (COOH), 143.22 (ArC), 130.09 (ArC), 129.35 (ArC), 129.25 (ArC), 64.95 (CH), 58.00 (ArCH₂N), 53.10 (CH₂NCH₂), 51.90 (CH₃), 29.15 (CHCH₂), 22.80 (NCH₂CH₂).

Tert-butyl N-(3-{2-[2-(3-aminopropoxy)ethoxy]ethoxy}propyl)carbamate (20)

The reagent 4,7,10-trioxa-1,13-tridecanediamine (20 mL, 91.2 mmol) was dissolved in 100 mL of dry dichloromethane and cooled down to 0 °C into an ice bath. The di-*tert*-butyl decarbonate (2 g, 91.6 mmol) was dissolved in 50 mL of dichloromethane and was added to the solution at 0 °C dropwise, within 3 hours. After that the solution was left reacting for 16 hours, monitored by TLC (SiO₂, dichloromethane-methanol 8:2, revealed with ninhydrine). After that time 150 mL of water were added and the organic solution was washed six times. The solvent of the organic phase was removed under reduced pressure, obtaining the product as a transparent oil (3.1 g, 9.3 mmol).

¹H NMR (400 MHz, CDCl₃) δ: 5.12 (bs, 1H, NHBoc), 3.67-3.54 (m, 12H, (PEG)CH₂), 3.25 (m, 2H, CH₂NHBoc), 2.88 (m, 2H, CH₂NH₂), 1.79-1.76 (m, 4H, NH₂CH₂CH₂, BocNHCH₂CH₂), 1.45 (s, 9H, CH₃).

The spectroscopic data found are in agreement with those reported in literature.¹⁵

Tert-butyl (3-(2-(2-(3-azidopropoxy)ethoxy)ethoxy)propyl)carbamate (21)

Compound **20** (1g, 3.12 mmol) was dissolved in 28 mL of methanol, after which CuSO₄·5H₂O (15 mg, 0.06 mmol) and sodium bicarbonate, dissolved in 14 mL of water, were sequentially added. Compound **28** (1.014 g, 3.74 mmol) was then introduced in portions. Upon the addition of compound **28**, the initially light blue solution darkened. The reaction was monitored via TLC (SiO₂, dichloromethane-methanol 9:1, revealed with UV lamp and ninhydrin). The solution was left stirring for 5 hours. The reaction mixture was stirred for 5 hours. After completion, the solvent was removed under reduced pressure, and the crude product was taken up in 20 mL of ethyl acetate. The organic phase was washed twice with a 1 M KHSO₄ solution and twice with water. The organic layer was dried over Na₂SO₄, and the solvent was evaporated under reduced pressure. The product was obtained as a clear oil (1.148 g, 3.14 mmol) with a quantitative yield.

¹H NMR (400 MHz, CDCl₃) δ: 4.99 (bs, 1H, NHBoc), 3.68-3.55 (m, 12H, (PEG)CH₂), 3.42 (t, *J* = 6.1 Hz, 2H, CH₂N₃), 3.26-3.24 (m, 2H, CH₂NHBoc), 1.91-1.85 (m, 2H, CH₂CH₂NHBoc), 1.80- 1.77 (m, 2H, CH₂CH₂N₃), 1.46 (s, 9H, CH₃).

The spectroscopic data found are in agreement with those reported in literature.¹⁵

3-(2-(2-(3-azidopropoxy)ethoxy)ethoxy)propan-1-amine (22)

Compound **11** (1.148 g, 3.14 mmol) was dissolved in 9 mL of dichloromethane and 1 mL of trifluoroacetic acid (TFA) was added. The resulting mixture was stirred at room temperature for four hours, monitoring via TLC (SiO₂, dichloromethane-methanol 9:1, revealed with ninhydrin). The solvent and TFA were removed under reduced pressure to afford product **12** as trifluoroacetic salt of in quantitative yield.

¹H NMR (400 MHz, CDCl₃) δ: 8.13 (bs, 3H, NH₃⁺), 3.82-3.78 (m, 2H, OCH₂CH₂CH₂NH₃⁺), 3.70-3.59 (m, 10H, (PEG)CH₂), 3.40 (t, *J* = 6.1 Hz, 2H, CH₂N₃), 3.32-3.25 (sextuplet, 2H, *J* = 5.3 Hz, CH₂NH₃⁺), 1.99 (p, *J* = 5.3 Hz, 2H, CH₂CH₂NH₃⁺), 1.86 (p, *J* = 6.1 Hz, 2H, CH₂CH₂N₃).

The spectroscopic data found are in agreement with those reported in literature.¹⁵

Methyl (4-((3-(2-(2-(3-azidopropoxy)ethoxy)ethoxy)propyl)carbamoyl)benzyl)-L-phenylalaninate (23)

Compound **18** (200 mg, 0.638 mmol) was dissolved in 5 mL of dry dimethyl formamide, followed by the addition of EDC (135 mg, 0.702 mmol). The mixture was stirred for 5 minutes. Subsequently, compound **22** (460 mg, 1.28 mmol) and 0.4 mL of DIPEA were added. The reaction was stirred at room temperature for 16 hours, with progress monitored via TLC (SiO₂, dichloromethane-methanol 9:1, revealed with UV lamp and ninhydrin). Upon completion, the reaction was quenched with 10 mL of water, and the product was extracted with dichloromethane. The organic phase was washed twice with water and brine, dried over Na₂SO₄, and the solvent was removed under reduced pressure. The crude product was purified by flash chromatography (SiO₂, dichloromethane/methanol 9:1), yielding a brown oil (**23**, 140 mg, 0.26 mmol) with a 40% yield.

¹H NMR (400 MHz, CDCl₃) δ: 7.72 (d, *J* = 8.3, 2H, ArH), 7.41 – 7.05 (m, 7H, ArH), 3.91 (d, *J* = 13.0 Hz, 1H, ArCHHN), 3.76 (d, *J* = 13.0 Hz, 1H, ArCHHN), 3.71 – 3.45 (m, 19H, CH, CHCHHAr, CH₃, CH₂NHCO, (OEG)CH₂), 3.40 – 3.31 (m, 2H, CH₂N₃), 3.06 (m, 2H, CHHAr), 1.97 – 1.75 (m, 6H, CH₂CH₂N₃, CH₂CH₂NH).

¹³C NMR (101 MHz, CDCl₃) δ: 173.81 (COOMe), 166.96 (CONH), 143.72 (ArC), 136.60 (ArC), 136.45 (ArC), 133.90 (ArC), 129.25 (ArC), 128.51 (ArC), 127.17 (ArC), 126.94 (ArC), 70.66 ((OEG)CH₂), 70.46 ((OEG)CH₂), 70.30 ((OEG)CH₂), 70.24 ((OEG)CH₂), 67.85 (CH₂O), 61.73 (CH), 51.93 (COOCH₃), 51.26 (ArCH₂N), 48.39 (CH₂N₃), 38.89 (CH₂Ar), 37.02 (CH₂NH), 29.04 (CH₂CH₂NH), 28.83 (CH₂CH₂N₃).

(S)-2-((4-((3-(2-(2-(3-azidopropoxy)ethoxy)ethoxy)propyl)carbamoyl)benzyl)ammonio)-3-phenylpropanoate (24)

Compound **23** (100 mg) was dissolved in a 2:1 mixture of methanol-water, then lithium hydroxide monohydrate (31 mg, 0.080 mmol) is added. The solution was left stirring for 4 hours at room temperature. The solvent was removed under vacuum and affording product **24** as a white solid (98 mg).

¹H NMR (400 MHz, D₂O) δ: 7.56 (d, *J* = 8.2 Hz, 2H, ArH), 7.27 – 7.11 (m, 6H, ArH), 7.08 (d, *J* = 6.9 Hz, 2H, ArH), 3.67 (d, *J* = 13.2 Hz, 1H, ArCHHN), 3.58 – 3.38 (m, 15H, CH₂NHCO, ArCHHN, (OEG)CH₂), 3.34 (t, *J* = 6.5 Hz, 2H, CH₂N₃), 3.21 – 3.17 (m, 1H, CH), 2.77 (m, 2H, CH₂Ar), 1.79 – 1.71 (m, 2H, CH₂CH₂NH), 1.71 – 1.61 (m, 2H, CH₂CH₂N₃).

¹³C NMR (101 MHz, D₂O) δ: 170.45 (COOH), 168.33 (CONH), 142.86 (ArC), 137.92 (ArC), 132.53 (ArC), 129.35 (ArC), 128.72 (ArC), 127.14 (ArC), 126.55 (ArC), 69.53 ((OEG)CH₂), 69.34 ((OEG)CH₂), 69.21 ((OEG)CH₂), 68.75 ((OEG)CH₂), 67.79 (CH₂O), 64.36 (CH), 50.61 (ArCH₂N), 48.77 (CH₂N₃), 38.97 (CH₂Ar), 37.24 (CH₂NHCO), 28.18 (CH₂CH₂NH), 27.89 (CH₂CH₂N₃).

Methyl (4-((3-(2-(2-(3-azidopropoxy)ethoxy)ethoxy)propyl)carbamoyl)benzyl)-L-prolinate (25)

Compound **19** (309 mg, 1.17 mmol) was dissolved in 5 mL of dry dimethyl formamide, followed by the addition of EDC (247 mg, 1.29 mmol). The mixture was stirred for 5 minutes. Subsequently, compound **23** (791 mg, 2.28 mmol) and 0.4 mL of DIPEA were added. The reaction was stirred at room temperature for 16 hours, with progress monitored via TLC (SiO₂, dichloromethane-methanol 9:1, revealed with UV lamp and ninhydrin). Upon completion, the reaction was quenched with 10 mL of water, and the product was extracted with dichloromethane. The organic phase was washed twice with water and brine, dried over Na₂SO₄, and the solvent was removed under reduced pressure. The crude was purified by flash

chromatography (SiO₂, dichloromethane/methanol 9:1), yielding a brown oil (**25**, 250 mg, 0.51 mmol) with a 46% yield.

ESI-TOF: calc. 477.2 found: *m/z* 478.29 [M+H]⁺, 484.30 [M+Li]⁺, 500.28 [M+Na]⁺, 516.24 [M+K]⁺.

¹H NMR (400 MHz, CDCl₃) (methyl ester) δ : 7.73 (d, *J* = 8.0 Hz, 2H, ArH), 7.37 (d, *J* = 8.1 Hz, 2H, ArH), 7.10 (bs, 1H, NH), 3.95 (d, *J* = 13.1, 1H, ArCHHN), 3.69 – 3.41 (m, 18H, CH₂NH, ArCHHN, CH₃, (OEG)CH₂), 3.41 – 3.28 (m, 2H, CH₂N₃), 3.30 – 3.17 (m, 1H, CH), 3.03 – 2.91 (m, 1H, NCHH), 2.44 – 2.28 (m, 1H, NCHH), 2.20 – 2.04 (m, 1H, CHCHH), 1.96 – 1.73 (m, 5H, CHCHH, CH₂CH₂N₃, CH₂CH₂NH).

¹³C NMR (101 MHz, CDCl₃) δ : 174.42 (COOMe), 167.09 (CONH), 142.09 (ArC), 133.56 (ArC), 129.04 (ArC), 126.91 (ArC), 70.61 ((OEG)CH₂), 70.56 ((OEG)CH₂), 70.46 ((OEG)CH₂), 70.44 ((OEG)CH₂), 70.43 ((OEG)CH₂), 67.82 ((OEG)CH₂), 65.33 (CH), 58.24 (ArCH₂N), 53.15 (NCH₂), 51.72 (CH₃), 48.38 (CH₂N₃), 38.81 (CH₂NH), 29.28 (CHCH₂), 29.03 (CH₂CH₂N₃), 28.84 (CH₂CH₂NH), 23.01 (NCH₂CH₂), 22.98 (CH₂CH₂CH₂).

(2S)-1-(4-((3-(2-(2-(3-azidopropoxy)ethoxy)ethoxy)propyl)carbamoyl)benzyl)pyrrolidin-1-ium-2-carboxylate (26**)**

Compound **25** (100 mg) was dissolved in a 2:1 mixture of methanol-water, then lithium hydroxide monohydrate (31 mg, 0.080 mmol) is added. The solution was left stirring for 4 hours at room temperature. The solvent was removed under vacuum and affording product **26** as a white solid (98 mg).

¹H NMR (400 MHz, D₂O) δ : 7.57 (d, *J* = 7.4 Hz, 2H, ArH), 7.33 (d, *J* = 7.0 Hz, 2H, ArH), 3.84 (d, *J* = 12.5 Hz, 1H, ArCHHN), 3.59 – 3.37 (m, 12H, (OEG)CH₂), 3.37 – 3.30 (m, 3H, ArCHHN, OCH₂CH₂CH₂NH), 3.30 – 3.18 (m, 2H, CH₂N₃), 2.85 (t, *J* = 6.9 Hz, 1H, CH), 2.75 – 2.68 (m, 1H, NCHHCH₂), 2.59 – 2.49 (m, 2H, CH₂NH), 2.25 – 2.10 (m, 1H, NCHHCH₂), 2.05 – 1.90 (m, 1H, CHCHH), 1.82 – 1.52 (m, 5H, CHCHH, NCH₂CH₂, CH₂CH₂CH₂).

¹³C NMR (101 MHz, D₂O) δ : 181.45 (COOH), 170.44 (CONH), 141.72 (ArC), 132.53 (ArC), 130.11 (ArC), 126.85 (ArC), 69.54 ((OEG)CH₂), 69.49 ((OEG)CH₂), 69.34 ((OEG)CH₂), 69.26 ((OEG)CH₂), 69.22 ((OEG)CH₂), 68.69 (CH), 68.22 ((OEG)CH₂), 57.39 (ArCH₂N), 52.04 (NCH₂CH₂), 47.98 (CH₂N₃), 37.16 (CH₂NH), 31.37 (CH₂CH₂N₃), 28.86 (CHCH₂), 23.21 (NCH₂CH₂), 21.56 (CH₂CH₂CH₂).

Imidazole-1-sulfonyl azide hydrogensulfate (28**)**

Sodium azide (4 g, 61.5 mmol) was placed in 70 mL of dry ethyl acetate under nitrogen atmosphere. The resulting suspension was cooled to 0 °C. Under a nitrogen atmosphere, sulfonyl chloride (4.93 mL, 61.5 mmol) was added dropwise over 15 minutes with constant stirring. The mixture was then allowed to gradually warm to room temperature and stirred for 20 hours. The suspension was then cooled back to 0 °C, and under nitrogen atmosphere, imidazole (8.37 g, 123.1 mmol) was added continuously over 5 minutes. The resulting thick suspension was stirred for 5 hours at 0 °C and subsequently basified by adding 150 mL of saturated aqueous sodium bicarbonate (NaHCO₃). The organic phase was separated, washed with 150 mL of water, and dried over Na₂SO₄. The solution was filtered, cooled to 0 °C, and maintained under a nitrogen atmosphere. Concentrated sulfuric acid (3.29 mL, 61.5 mmol) was added dropwise over 5 minutes. The mixture gradually warmed up to room temperature with vigorous stirring. After 30 minutes, a white precipitate formed. The product was isolated by filtration, washed with cold ethyl acetate, yielding the hydrogensulfate salt with 80% yield (13 g, 50 mmol).

The spectroscopic data found are in agreement with those reported in literature.¹⁶

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Study of the Interactions Between Multivalent Zwitterionic Surfaces and Gram-Negative Bacterial Membrane Models

*This work has been carried out in the research group of Prof. Jurriaan Huskens at University of Twente
(Enschede, The Netherlands)

4.1 Introduction

This chapter reports the work performed, in a six-month period in Prof. J. Huskens laboratories to investigate the interaction between multivalent zwitterionic systems and models of the bacteria cell wall. Among the techniques that could be used for the study of these interactions, quartz crystal microbalance (QCM) can be a useful tool for its ability to quantify low concentrations of analytes and its ability to perform real-time monitoring (see paragraph 1.9 in Chapter 1).¹

4.1.1 Aim of this work

In the introduction of Chapter 3, paragraph 3.1, it was shown that zwitterionic amino acid functionalized calix[4]arenes selectively interact with the cell wall of Gram-negative bacteria, being able to distinguish these bacteria among the other families of Gram-positive and Mycobacteria. The proline and phenylalanine derivatives (**1** and **2**, respectively, Figure 1) demonstrated to interact with Gram-negative bacteria thanks to the use of on-cell saturation transfer difference nuclear magnetic resonance (STD-NMR) techniques. This technique also allowed to identify the upper rim of the calixarene, and especially the zwitterionic amino acid residues, as epitopes of binding, i.e. the region of the ligand most involved in the binding with the biomolecules present on bacteria cell wall. It was also proved, again by using STD-NMR studies, that calixarenes **1** and **2** can recognize micelles of lipopolysaccharides (LPSs), macrobiomolecules that are highly abundant on the outer membrane of only Gram-negative bacteria. This also suggests that LPS is a possible target element of this selective recognition event.

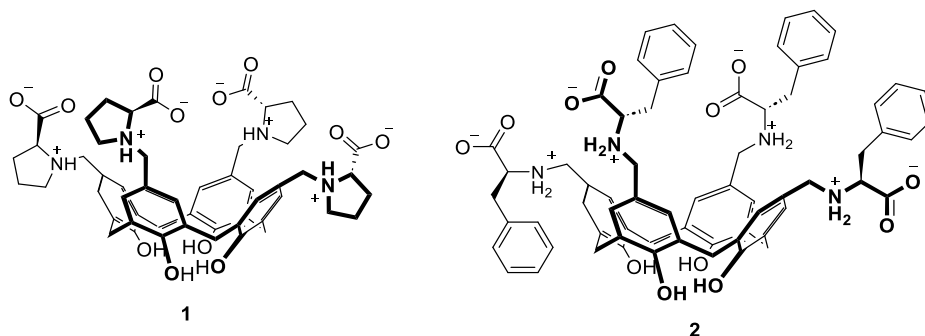


Figure 1. Structure of zwitterionic calix[4]arenes **1** and **2**. Ligand **1** is functionalized at the upper rim with L-proline and **2** with L-phenylalanine.

The aim of the work reported in this chapter is to try to set up conditions on QCM systems to allow the study of the interaction of multivalent calixarenes carrying zwitterionic amino acids with surfaces containing LPS molecules, possibly mimicking the recognition events which take place with ligands **1** and **2** onto Gram-negative bacteria.

We used two different approaches:

- Initially, we mimicked the outer bacterial cell membrane by assembling onto the surface of a QCM a supported lipid bilayer comprising *E. Coli* lipopolysaccharide, and flowed solutions of calixarenes **1** and **2** (Figure 2). The supported lipid bilayer is fabricated on a QCM chip surface, by assembling a principal lipid component (POPC), depicted in red, and the lipopolysaccharide, shown in yellow, with the majority of the saccharide units exposed outside of the bilayer.

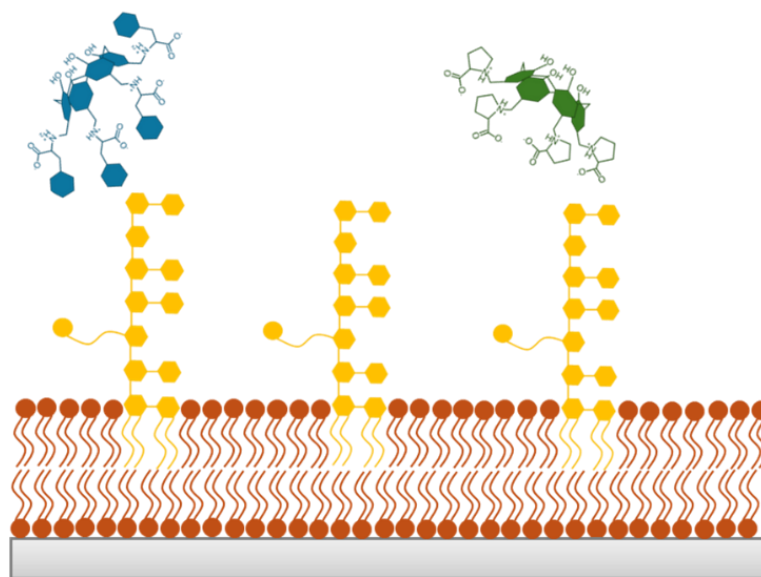


Figure 2. Schematic representation of the supported lipid bilayer incorporating LPS, and its interaction with calixarene **1** (in green) and **2** (in blue). The QCM chip surface (SiO_2) is represented in grey.

- In an alternative approach, we planned to assemble multivalent zwitterionic amino acidic assemblies (or the multivalent zwitterionic calixarenes **1** and **2**) on the QCM surface, and flowed a dispersion of vesicles functionalized with LPS, which mimic the membranes of Gram-negative bacteria. The study aimed to investigate both calixarenes (Figure 3a) and their monovalent mimics (Figure 3b). In each case, the zwitterionic component (the calixarene or the monovalent mimic) is covalently bound to a polycationic polyelectrolyte, poly-L-lysine (PLL). The zwitterionic component is functionalized with an azide group, enabling its attachment to the PLL, which has been suitably modified with clickable alkyne units.

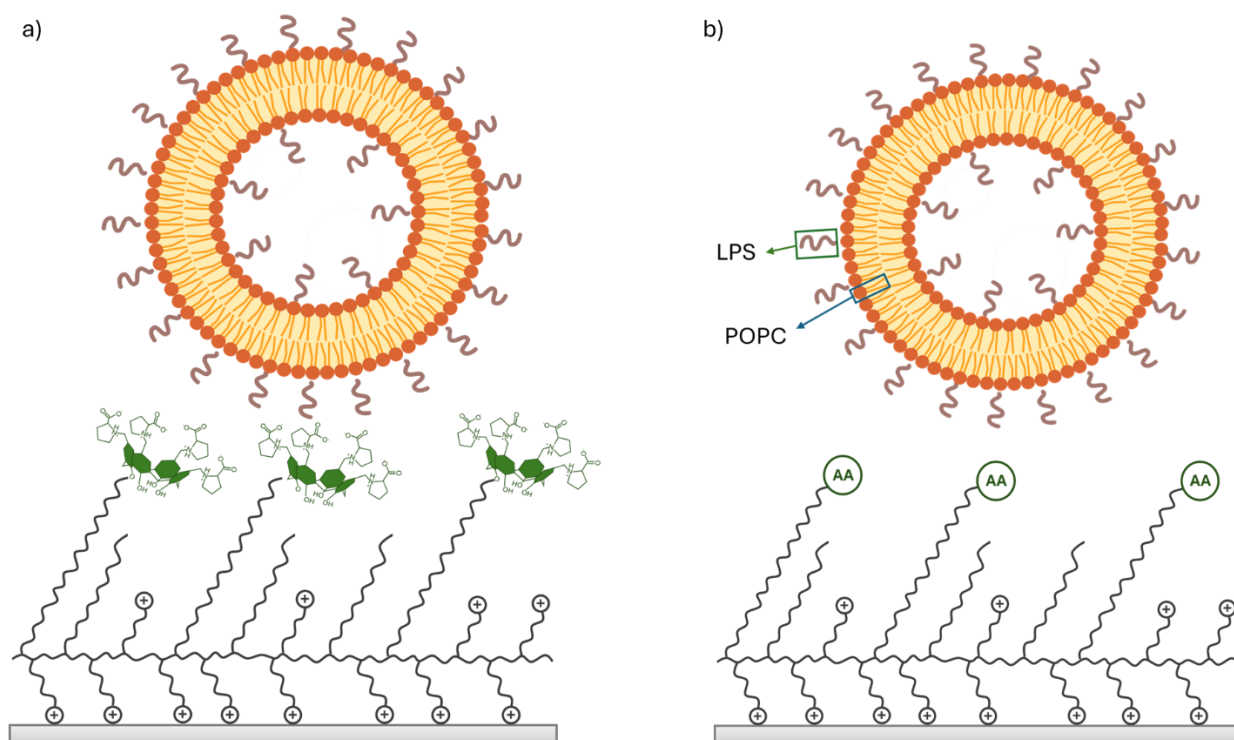


Figure 3. Multivalent zwitterionic amino acid (AA) surfaces on QCM interacting with vesicles functionalized with LPS, mimicking the membranes of Gram-negative bacteria. (a) Derivatives of calixarenes **1** and **2** are bound to a poly-L-lysine-coated surface. (b) In contrast, monovalent mimics of calixarenes **1** and **2** are bound to the surface.

Due to time constraints and problems in the synthesis (see paragraph 3.2.2, Chapter 3) of the complex calixarene scaffold functionalized with amino acids at the upper rim and an azide group at the lower rim, we were only able to study the two monovalent compounds **3** and **4**. To mimic as much as possible the multivalent disposition of amino acids presents on the calixarene backbone, we conjugated the PLL with two PEG chains bearing a benzyl unit (representing the calixarene aromatic unit) bound to a proline or a phenylalanine moiety (respectively **3** and **4**, presented in Figure 4).

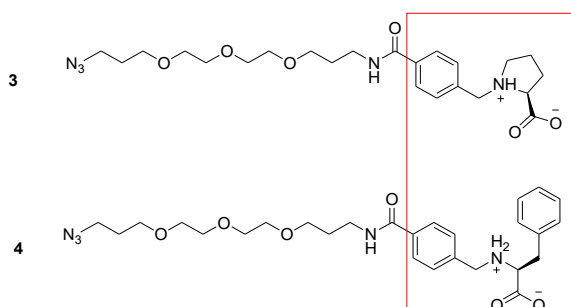


Figure 4. Zwitterionic ligands **3** and **4**, bearing an azide for conjugation to the PLL at one end. The region of these ligands highlighted in the red rectangle mimics the epitope of binding of the zwitterionic calixarenes.

After the deposition of the cationic polyelectrolyte (poly-L-Lys, PLL) functionalized with the amino acid units **3** and **4** on a QCM-D chip surface, interaction tests were conducted with vesicles that mimic the outer membrane of Gram-negative bacteria by exposing LPSs. By fabricating vesicles without LPS and with different LPS concentrations, it would be also possible to verify whether LPS is a real binding site and how the multivalent interaction is influenced by the LPS density on the vesicles.

4.1.2 Supported lipid bilayer as mimic of the bacterial membrane

The aim of our project was to adopt an alternative methodology to that used in biosensors that exploit antibodies and classic sandwich-type assays to sense bacteria. In the first approach of this study, we planned to mimic the outer bacterial cell membrane by assembling Supported Lipid Bilayers (SLBs) comprising LPSs onto the surface of a QCM (Figure 4). The cell membrane plays a pivotal role in targeting bacteria, serving as the site of interaction for bacterial detection. Indeed, the cell membrane is fundamental for both structure and functions of living organisms. In particular, the outer membrane serves as a physio-chemical barrier, maintaining a separation between the cell and its external environment while providing protein systems able to regulate communication and transport processes of molecules essential for the cell life.² The arrangement of these components creates a robust permeable barrier that controls the transport of different types of molecules (see the introduction in Chapter 1).

Due to the complexity of natural cell membranes, including those of microorganisms and bacteria, simplified artificial model systems are often used to study the properties of membrane constituents and the interactions of microorganism with external systems.² Techniques like surface plasmon resonance spectroscopy, waveguide spectroscopy, and quartz crystal microbalance with dissipation monitoring are commonly employed to explore the interactions of such simplified artificial model systems under controlled conditions.

Supported lipid bilayer (SLB) systems, have been often used as simplified models of the cell membranes being formed by a planar bilayer of lipids on a solid substrate. The conventional approach³ to form SLBs entails the rupture and fusion of lipid vesicles. This involves exposing a suitable surface to a solution of lipid vesicles, which then undergo a rupture and a fusion process, ultimately leading to the formation of a planar supported lipid bilayer. Figure 5 illustrates the schematic representation of this process, with the three steps of the SLB formation.

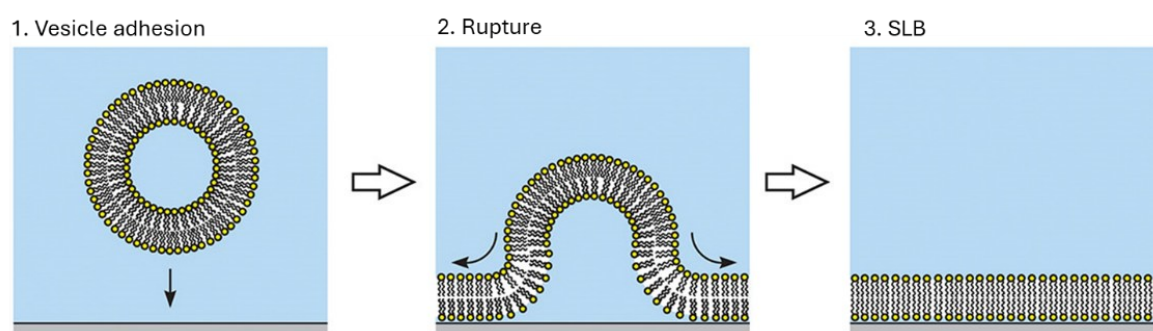


Figure 5. Scheme of the vesicle adhesion process, the vesicle rupture and the formation of the SLB. Figure adopted from reference 4.

The solid surface is typically composed of titanium oxide or silicon oxide, given that liposomes are known to undergo spontaneous decomposition on these surfaces, under physiological conditions. The addition of ions such as Ca^{2+} can be useful to promote the SLB formation, in particular with titanium oxide surfaces, due to their ability to act as a bridge between the negative charges of the head of the lipids (typically carboxylate or phosphate groups) or the negatively charged surface.⁵

Figure 6 depicts the typical QCM-D graph observed during the fabrication of an SLB. The vesicles utilized in this example are composed of pure 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPC), a lipid that is frequently employed to mimic the cell membrane.²

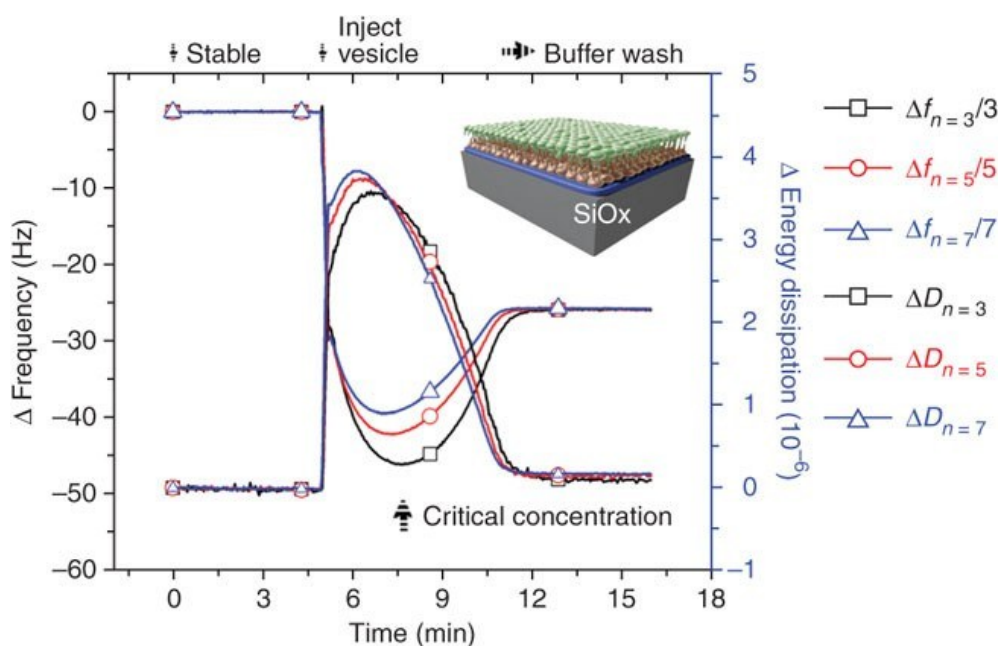


Figure 6. Example of QCM-D graph of the SLB fabrication with a solution 0.1 mg/mL vesicles of pure POPC, obtained with a Tris buffer (pH 7.0). In the graph only the third, fifth and seventh harmonics in frequency and dissipation are reported. The frequency harmonics start at the top of the graph at time 0 and move downward, eventually stabilizing around -25 Hz. In contrast, the dissipation harmonics start at the bottom of the graph at time 0 and move upward, eventually stabilizing between 0 and 0.5×10^{-6} . Figure adopted from reference 6.

In the graph of Figure 6, all the steps explained earlier are present, each one characterized by a different trend of the curves. The adsorption of intact vesicles leads to the formation of a viscoelastic film, as evidenced by a significant increase in dissipation in the central region of the graph and by the presence of not overlapping overtones in the same region. This is due to the flexible structure of the vesicles and the solvent interactions both within and between them. The observed negative shift in frequency reflects the added mass, which lowers the crystal resonance frequency. Initially, vesicle rupture produces bilayer patches with hydrophobic edges, which promotes further vesicle rupture until the lipid bilayer is fully formed, minimizing edge exposure. Once the bilayer is established, dissipation returns close to zero and the overtones overlap again, indicating the bilayer rigidity and the minimal presence of bound water molecules. The frequency shift decreases due to the loss of solvent previously trapped inside the vesicles.⁶

To more accurately mimic the outer membrane of Gram-negative bacteria, it is essential to incorporate a specific percentage of lipopolysaccharide (LPS) in our artificial vesicles. This is because the outer membrane of Gram-negative bacteria is composed approximately of 20–25% phospholipids, 20–30% lipopolysaccharides (LPS), and 45–50% proteins.⁷ The structure of *Escherichia Coli* LPS is showed in Figure 7. It consists of the Lipid A, the inner core and the O-antigen regions. The inner core of LPS consists of KDO, heptose, hexuronic acid, or phosphate, while the outer core is made of branched oligosaccharides of neutral sugars along with the repeating oligosaccharide units of the O-specific chain. Based on the structure of their LPS, bacteria are classified as "deep-rough" if they possess only lipid A and the inner core, as "rough" if they have an outer core as well, and as "smooth" if their membrane includes O-antigen repeats.

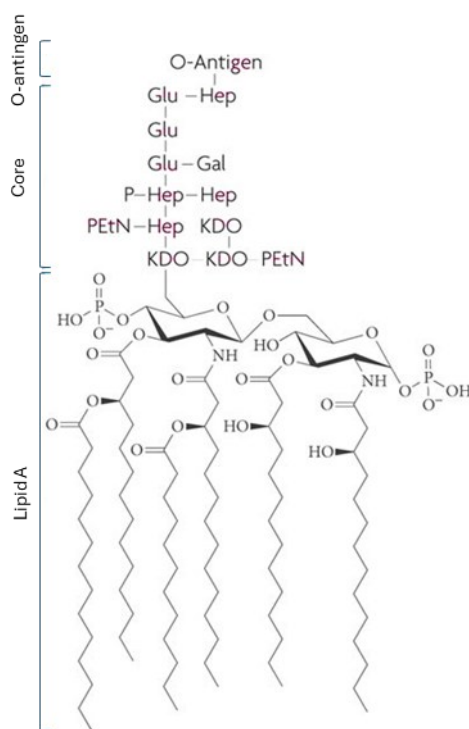


Figure 7. Structure of *E. Coli* lipopolysaccharide.⁷

S. Kaufmann *et al.*⁸ presented a work in which they prepared supported lipid bilayers made with POPC and LPS from *Escherichia Coli* or *Salmonella enterica sv typhimurium* to study the saccharide-lectine proteins interaction and the structure of LPS in the membrane. We took inspiration from this work for the first part of this chapter and prepared SLB with *E. coli* LPS, which should mimic the bacterial membrane.

4.1.3 Functionalization of poly-L-lysine and its adsorption on SiO₂ surfaces

In the second approach of our study (see Figure 3 in paragraph 4.1.1) we planned to graft the calixarenes **1** and **2** or amino acid units **3** and **4** to a surface on QCM-D chips and utilize LPS-containing vesicles as a mimic of the Gram-negative bacteria cell-wall. The objective is to examine the adhesion of the vesicles on the surface. Due to the long synthetic procedures needed to functionalize calixarenes **1** and **2** with the azide groups needed for the grafting onto the QCM chip, in this chapter are reported only the studies which exploit the zwitterionic ligands **3** and **4**.

A typical substrate suitable for being attached to a silicon oxide chip and capable of being easily modified is the poly-L-lysine (PLL), a polyelectrolyte positively charged in physiological conditions, that can strongly adsorb to different surfaces thanks to the multivalent interactions of the positively charged ammonium cations ($pK_a \sim 10$) of the lysine lateral chains and the negatively charged surface.⁹ The adsorption behavior of polyelectrolytes on metal oxide surfaces has been well-documented,^{10,11} showing that polycations, especially, create stable adsorbed layers on negatively charged oxides such as silicon dioxide and titanium dioxide. In addition, PLL is a biocompatible material, making it a suitable candidate for surface modification and biosensing applications. Functionalization of poly-L-lysine with polyethylene glycol (PEG) chains has been shown to increase selectivity and sensitivity in biosensing applications while reducing non-specific protein interactions.¹² PLL grafting can be readily accomplished by a nucleophilic attack between its primary amino group and an amine-reactive

functional group, such as N-hydroxy succinimide (NHS) esters. This reaction (Figure 8) is facilitated at a mildly basic pH, forming an amide bond and releasing the NHS group.¹³

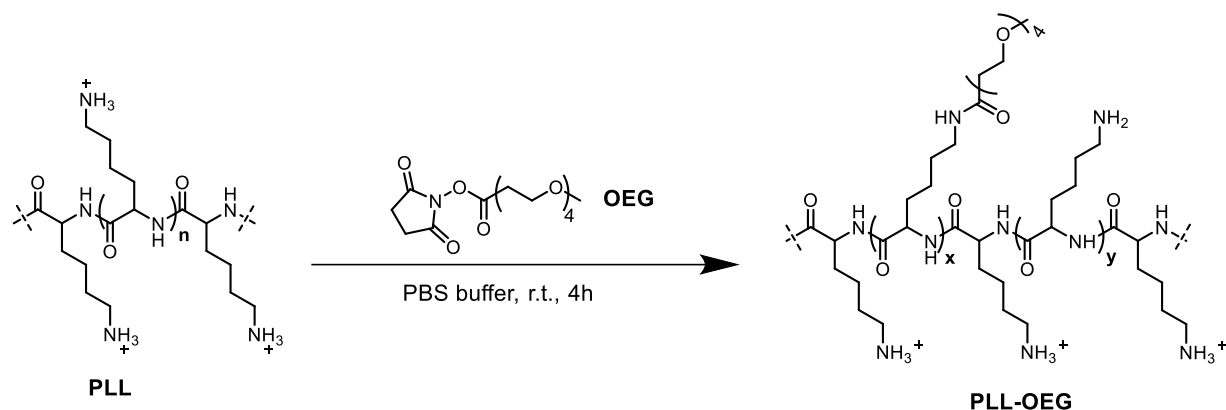


Figure 8. PLL functionalization scheme with OEG-NHS ester.

The degree of functionalization of PLL with oligo(ethylene glycol) (OEG) or other substituents can be controlled by adjusting the molar ratios of the components during the conjugation reaction and can be estimated using ^1H NMR spectroscopy.¹³ By knowing the degree of functionalization of PLL (measured in solution), direct control over the receptor density on the surface during PLL adsorption can be achieved. It is also important to maintain the degree of functionalization of the PLL below 30-40% to ensure good surface adhesion while allowing for sufficient OEG to prevent non-specific interactions.⁹

Various receptors/recognition elements have been linked to PLL, including biotin (for the binding with streptavidin),¹⁴ RGD peptide (to facilitate cell adhesion),¹⁵ and PNA (for the binding of natural nucleic acids) providing control over surface density.⁹

Among all the possible strategies of biorthogonal reaction which could be exploited to the functionalization of the PLL layer, a pivotal role is occupied by “click reactions”. The field of click chemistry encompasses a group of reactions that are characterized by their efficiency, modularity, and stereospecificity. These reactions, being orthogonal to those present on biomolecules (biorthogonality), often result in high yields with minimal and safe byproducts and, even more importantly, do not alter the structure of the biomolecules which are conjugated.¹⁶ A significant advantage of these reactions is that they can be carried out under mild conditions, such as room temperature and physiological conditions, and have demonstrated to be efficient in the conjugation of biomolecules, including enzymes, antibodies, and nucleic acids, to surfaces. These latter molecules can therefore be supported onto the surface of different devices, subsequently becoming available for further interactions for biosensing applications.

We planned to use the Huisgen 1,3-dipolar cycloaddition of azides and alkynes. Azides are particularly useful due to their stability against hydrolysis. The reaction typically requires a copper catalyst to occur at room temperature (CuAAC). Since the cytotoxicity of copper presents a challenge in biological systems, copper-free alternatives have been developed. The key to eliminating the need for a catalyst lies in the use of ring strained alkynes, such as cyclooctyne.¹⁶ Cyclooctyne exhibits reactivity in strain-promoted azide-alkyne cycloaddition (SPAAC, Figure 9) due to the notable deformation of the alkyne angle from 180° to 163° . In fact, the ground state is destabilized in comparison to the transition state, resulting in a considerable acceleration of the reaction rate.¹⁷

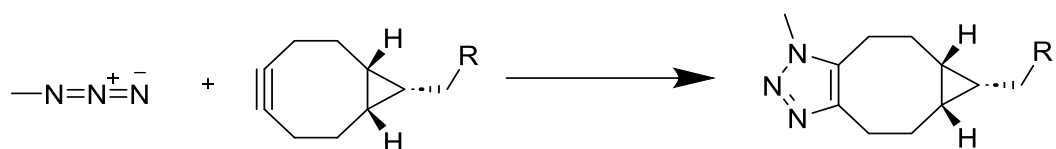


Figure 9. Scheme of strain-promoted azide-alkyne 1,3-dipolar cycloaddition (SPAAC) between an azide and BCN.

Bicyclo[6.1.0]non-4-yne (BCN) is a promising substrate for SPAAC reactions since it offers a reaction rate constant of $0.7 \text{ M}^{-1}\text{s}^{-1}$ with aliphatic azides and can reach up to $6 \text{ M}^{-1}\text{s}^{-1}$ with aromatic azides.¹⁸ BCN is particularly appealing due to its reduced hydrophobicity while maintaining a high reactivity.¹⁹

4.2 Results and discussion

4.2.1 LPS-functionalized SLBs on QCM chips and their interactions with calixarenes **1** or **2** in solution

4.2.1.1 Lipopolysaccharide-containing vesicles

To test the affinity of a multivalent object exposing zwitterionic amino acid residues from its structure (multivalent calixarenes **1** and **2**) and to add other clues to our hypothesis that Gram-negative bacteria are recognized through the binding of the lipopolysaccharide (LPS) moieties, we first prepared a suitable artificial membrane that mimics the Gram-negative bacteria outer cell wall. We chose to use 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPC), which has been frequently employed in the literature^{2,8,20} to mimic the phospholipids of the bacterial outer membrane, and the *Escherichia coli* Ra mutant lipopolysaccharide (Figure 10).

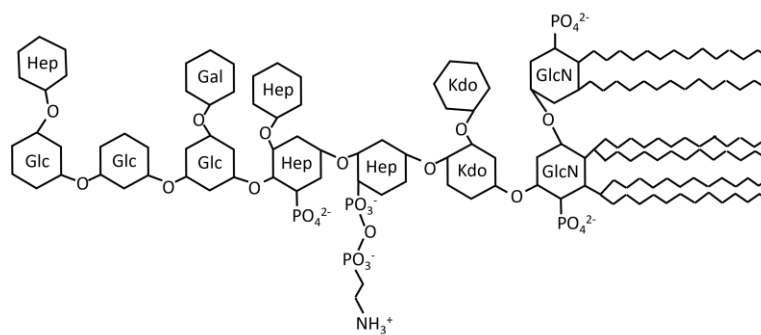


Figure 10. Lipopolysaccharide (rough strains), from *Escherichia coli* EH100 (Ra mutant).

Supported lipid bilayers (SLBs), which are extended membranes assembled on solid surfaces, offer a versatile model system for studying membranes. Furthermore, research has demonstrated that POPC (Figure 11) is capable of facilitating the fusion of liposomes, leading to a fast and reproducible formation of SLBs on silica surfaces.²¹

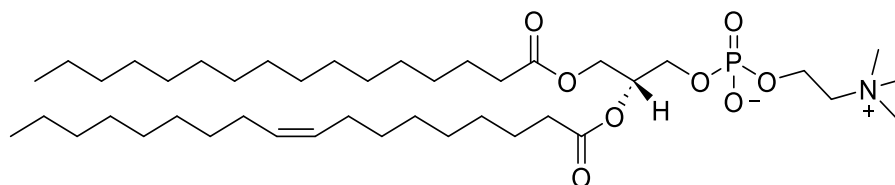


Figure 11. Structure of 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPC).

In accordance with the protocols defined in the literature,⁸ vesicles of pure 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPC) and those containing varying percentages of LPS were prepared. The required amount of POPC dissolved in chloroform was added to a vial and a gentle nitrogen flux was used to evaporate the solvent, forming a lipidic film on the vial walls. The appropriate amount of LPS was then added with MQ water and the solution stirred, resulting in a solution of vesicles at a concentration of 1 mg/mL in MQ. The obtained vesicles were then extruded with a 100 nm polycarbonate membrane and characterized with dynamic light scattering (DLS).

The DLS analysis of the vesicle size was repeated for all the types of vesicles, with comparable outcomes. For the POPC and 10wt% of LPS reported in Figure 12 the average diameter was 113.5 ± 1.1 nm and the polydispersity index 0.048 ± 0.022 .

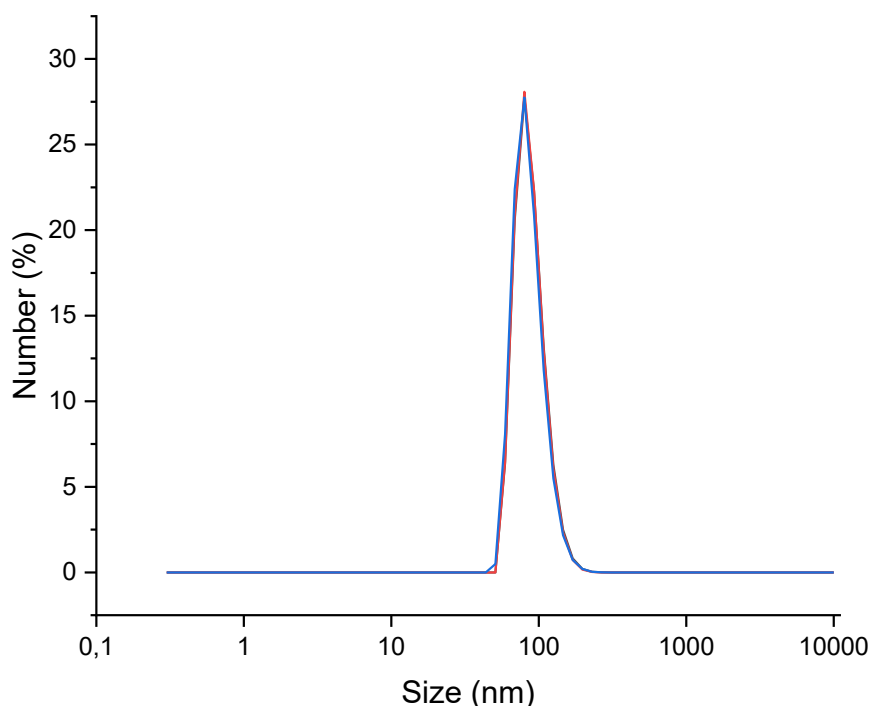


Figure 12. DLS analysis of POPC+10wt% of LPS vesicles.

4.2.1.2 Supported lipid bilayer formation and experiments with calixarenes **1** and **2**

The vesicle solution was diluted in Tris buffer solution (TBS, pH 7.3) and flowed over the quartz crystal microbalance (QCM-D) chip to facilitate the formation of the supported lipid bilayer (SLB). The grey area in Figure 13 indicates the point at which the vesicle solution was flowed in. Initially, there is a steep decrease in frequency (in blue) due to the absorption of the vesicles on the surface. Concurrently, the dissipation (in red) increases because vesicles are a non-rigid material. Subsequently, the frequency begins to increase due to the rupture of the vesicles, and consequently, the dissipation decreases. Then, dissipation and frequency reach a stable equilibrium, indicating the formation of a supported lipid bilayer, a highly rigid material as evidenced by a close-to-zero dissipation signal.

Once the SLB was formed, different concentrations of solution of the calixarenes were added, to test if an interaction occurred. In Figure 13 the QCM-D graph with the formation of the SLB from vesicles with POPC+2wt% of LPS is presented. In the first green section a solution of **1** 1 $\mu\text{g/mL}$ (0.0011 mM), the second one 10 $\mu\text{g/mL}$ (0.011 mM) and the third one 100 $\mu\text{g/mL}$ (0.11 mM).

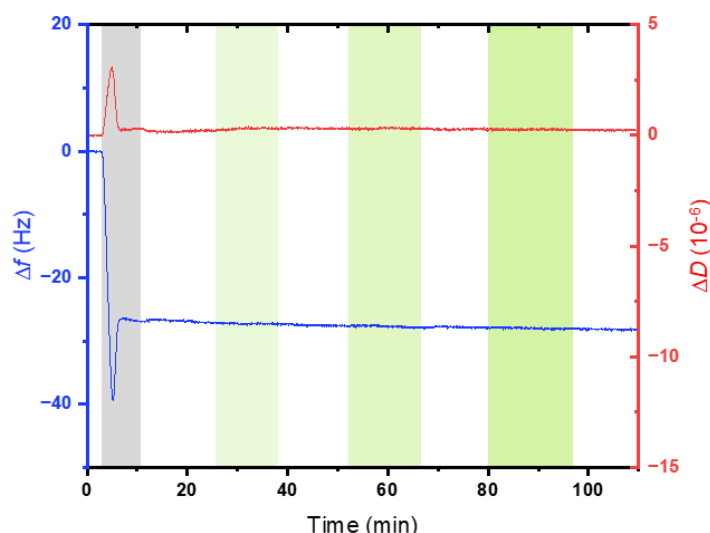


Figure 13. QCM-D graph of the experiment with POPC+2wt% of LPS vesicles (formation of the SLB in grey) and the addition of solutions of **1** in TBS (pH 7.3) in green at different concentrations: 1 $\mu\text{g/mL}$ (0.0011 mM), 10 $\mu\text{g/mL}$ (0.011 mM) and 100 $\mu\text{g/mL}$ (0.11 mM).

No changes in frequency or dissipation could be registered in this case, when calixarene **1** flowed over a POPC+2% LPS bilayer. The situation is similar with the different percentages of LPS-containing bilayers, and also with the pure POPC bilayer. There is no evidence of binding of **1** with any of the bilayers prepared, as there is no apparent change in frequency and dissipation when the solution of **1** is flowed across the SLBs. The QCM graphs obtained fabricating SLB from vesicles of pure POPC, POPC+5wt% of LPS and POPC+10wt% of LPS and then flowing solutions of calixarene **1** can be found in the Appendix of this chapter.

The same experiment was run with calixarene **2**, as shown in Figure 14a. The vesicle solution was flowed, and the SLB was formed in TBS. Due to the poor solubility of **2** in TBS, it was necessary to add 10% DMSO to the buffer solution. Thus, after the formation of the SLB and the proper rinse with TBS, a solution of TBS with the addition of 10% DMSO was flowed, resulting in a decrease in frequency and an increase in dissipation. After the equilibration of the system, the solution of calixarene **2** (1 $\mu\text{g/mL}$) was flowed, resulting in a discernible decrease in frequency accompanied by an increase in dissipation, similar to that of TBS+DMSO. The rinsing with the buffer containing 10% DMSO resulted in a rapid return to the initial frequency value prior to the addition of the further injection of calixarene. The same process was observed with the other two solutions (10 $\mu\text{g/mL}$ and 100 $\mu\text{g/mL}$), with no significant difference in the frequency reached after the addition of the calixarene despite its higher concentrations. The observed alterations in frequency and dissipation can be attributed to a buffer effect, whereby the QCM-D is sufficiently sensitive to detect the varying quantities of DMSO present in the calixarene **2** solution and the buffer used for rinsing. This hypothesis is corroborated by the observation that all other graphs exhibit a similar pattern, also the one with SLB made using vesicles of pure POPC. The experiment conducted with SLBs of pure POPC (Figure 14b) demonstrates a distinctive outcome, as evidenced by the increase in frequency with the third solution and the subsequent return to the initial value following rinsing. It is likely that the quantity of DMSO was less than that of the buffer. The remaining QCM graphs obtained fabricating SLB from vesicles of POPC+5wt% of LPS and POPC+10wt% of LPS and then fluxing solutions of calixarene **2** can be found in the Appendix of this chapter.

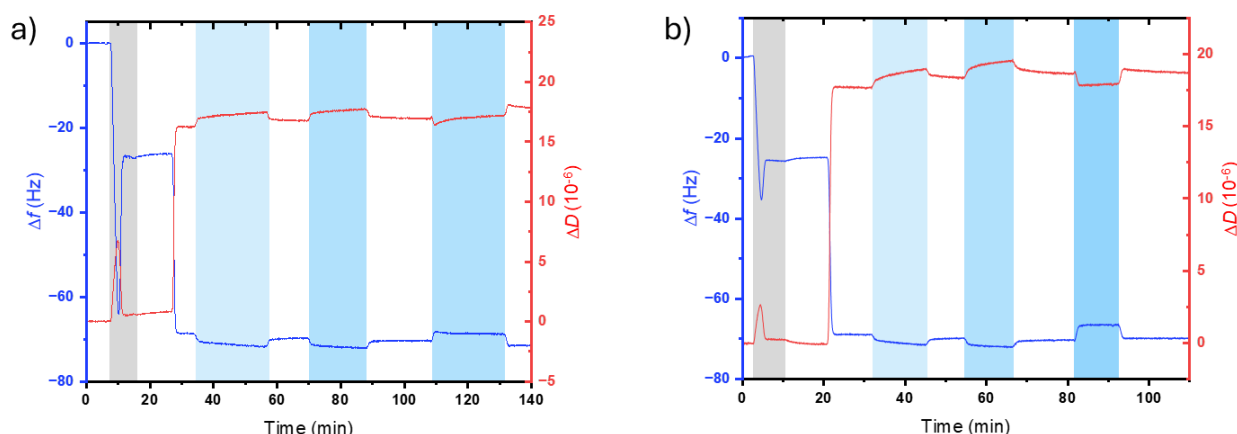


Figure 14. QCM-D graph of the experiment with a) POPC+2wt% of LPS vesicles and b) pure POPC vesicles (formation of the SLB in grey). The addition of solutions of **2** in TBS (pH 7.3) +10% of DMSO in light blue at different concentrations: 1 $\mu\text{g/mL}$ (0.0011 mM), 10 $\mu\text{g/mL}$ (0.011 mM) and 100 $\mu\text{g/mL}$ (0.11 mM).

In conclusion, the interaction of calixarene **1** or **2** with any of the bilayers prepared could not be shown. It is proposed that the amounts of calixarene ligands, which have modest molecular mass adsorbing are too small to influence the frequency of resonance of the crystal and therefore QCM is not sufficiently sensitive to detect the interaction. A too low affinity between LPS and the calixarenes is the most likely reason for this result. Possibly, higher concentrations of calixarenes could have shown some frequency/dissipation variations, but the poor solubility of ligands **1** and **2** impair further studies. Alternatively, the kinetics of this interaction may be so slow as to render it undetectable by the QCM.

4.2.2 Poly-L-lysine surface functionalized with zwitterionic amino acids for LPS detection by QCM-D monitoring

4.2.2.1 Clickable zwitterionic PEG chains

The alternative approach we aimed to establish focused on investigating the interaction between a multivalent surface of zwitterionic amino acids and a model of the Gram-negative bacterial cell wall. This approach entailed immobilizing the recognition moieties, zwitterionic calixarenes or zwitterionic amino acids, on the surface of a QCM chip and then fluxing vesicles of POPC adorned with given percentages of LPS. Ultimately, this strategy could facilitate the application of surface chemistry in detecting Gram-negative bacteria through appropriate sensing techniques.

Given the complexity of the synthesis of analogues of calixarenes **1** and **2** and bearing a clickable group at the lower rim, and the necessity to evaluate optimal conditions for their preparation (see Chapter 3, paragraph 3.2.2), we opted to start this study with the incorporation in the SLB of a polyether chain incorporating only a zwitterionic amino acid moiety. This approach would possibly allow us to assess the role of a multivalent zwitterionic amino acidic surface of SLBs in the recognition of LPS-functionalized liposomes and in the future, when clickable calixarenes will be obtained, to shed light on the possible role of the calixarene macrocycle, e.g. in preorganization of the zwitterionic motif on the calixarene template. For now, the PLL chemistry outlined below allows to control the amino acid density presented at the surface. The structure of the amino acid functionalized polyether chains **3** and **4** were presented in the introduction of this chapter, in paragraph 4.1.1. The benzyl units present between the polyether chains and the amino acid moieties, were inserted to replicate, as much as possible, the monomeric units of calixarene ligands **1** and **2**.

The detailed synthetic procedures for the synthesis of **3** and **4** are reported in Chapter 3 (paragraph 3.2.3).

4.2.2.2 Synthesis of modified poly-L-lysine containing units **3** or **4**

The poly-L-lysine was modified in accordance with the protocols described in the literature.⁹ The PLL-HBr salt was dissolved in PBS at pH 7.4 and added to a vial. The proper quantity (in relation to the lysine monomer) of the methyl-OEG₄-N-hydroxy succinimide (NHS) ester (24 equivalents) and the *endo*-BCN-OEG₄-N-hydroxy succinimide (NHS) ester (6 equivalents) linker (both dissolved in DMSO) were added simultaneously to the solution, which was then left to stir overnight at room temperature. The equivalents were calculated based on the number of L-lysine monomers in the sample. The reaction scheme is presented in Figure 15. The percentage of functionalization of the amino groups of PLL in product **5** was determined by NMR to be: 24% with methyl-OEG₄ and 4% with *endo*-BCN-OEG₄.

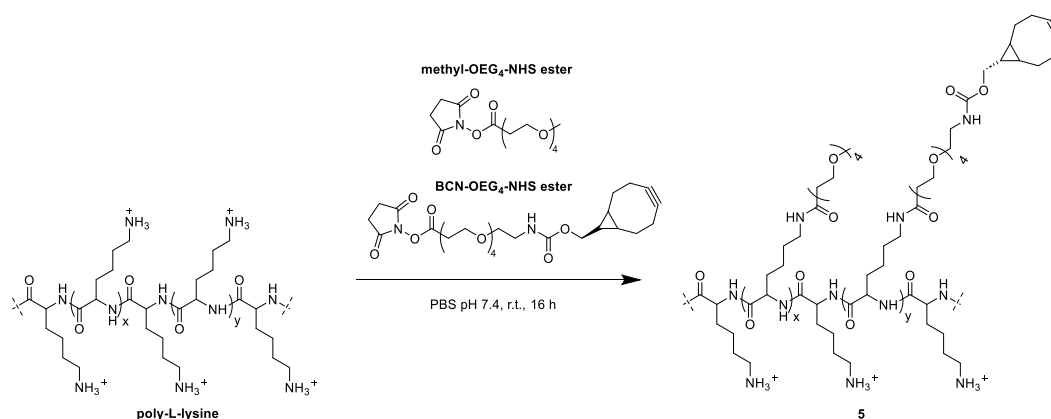


Figure 15. Synthesis of the PLL-OEG-BCN **5**. PLL reacts with appropriate proportions of BCN-OEG₄-NHS and methyl-OEG₄-NHS ester to reach the final modified PLL with the target levels of functionalization.

From the ¹H NMR spectrum of product **5**, in Figure 16, the degree of functionalization can be derived by normalizing the integral of the CH of the lysine backbone (broad signal at 4.17 ppm, labelled as **a**) and integrating the signal of the functionalized (**b**) and unfunctionalized (**c**) lysine ε CH₂ group in the lateral chain. The cyclopropane signal of BCN was also integrated and is labelled as **d**. The equation used for the quantification of OEG and OEG-BCN is shown later in this text and was taken from the paper of J. Movilli *et al.*⁹

$$BCN\% = \left(\frac{d}{b + c} \right) * 100$$

$$OEG\% = \left(\frac{b}{b + c} \right) * 100 - BCN\%$$

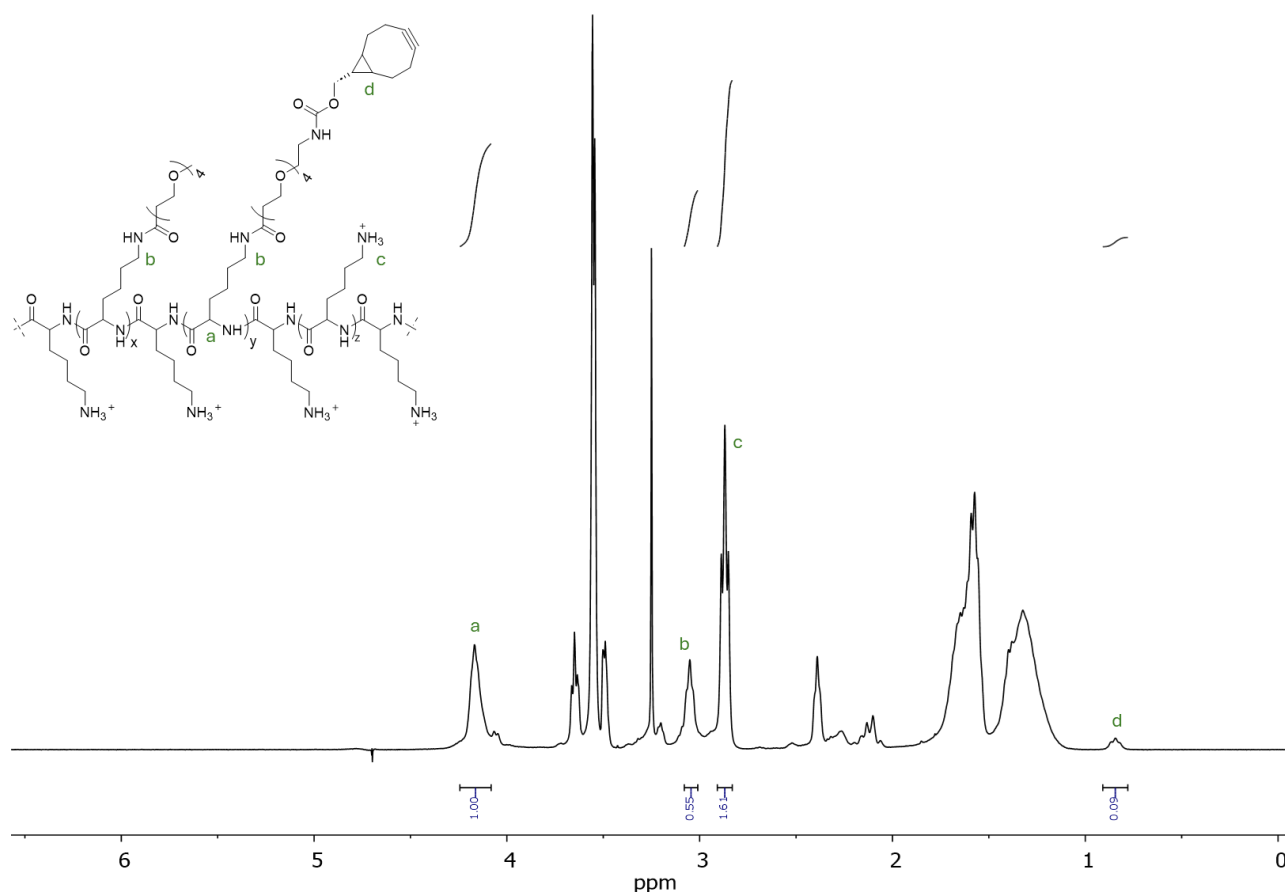


Figure 16. ^1H NMR of PLL-OEG-BCN **5**, recorded in D_2O at 400 MHz (298 K). Water residue signal was removed with water suppression. The signals needed for the quantification are reported in the spectrum.

From the equation shown above the PLL-OEG-BCN turned out to be functionalized for a 24% with OEG and 4% with OEG-BCN. The total degree of functionalization is below 30% which is good to ensure strong adhesion to an appropriately activated surface.

To create a poly-L-lysine layer devoid of a clickable moiety as a control, the same experiment was conducted without the BCN group (Figure 17), to obtain PLL-OEG **6**.

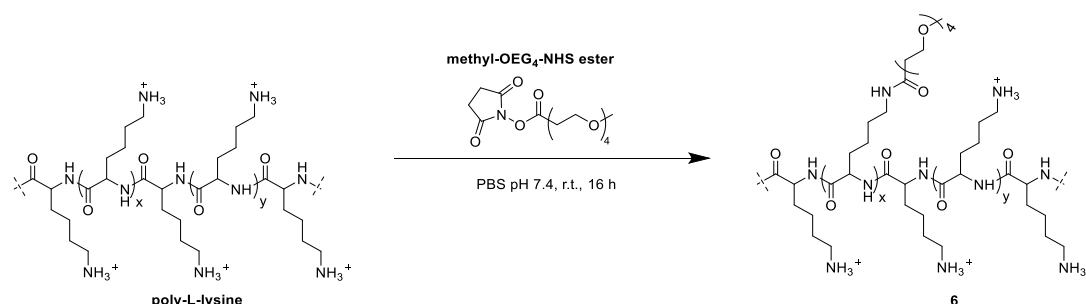


Figure 17. Synthesis of the PLL-OEG **6**.

The quantification of the PLL modified with a single group is considerably more straightforward, as it requires a comparison of the integrals of the modified (**b**) and unmodified (**c**) $\epsilon\text{-CH}_2$ groups of Lys in PLL. The ^1H NMR spectrum is reported in Figure 18. The total of modifications with OEG₄ groups amounts to 28%.

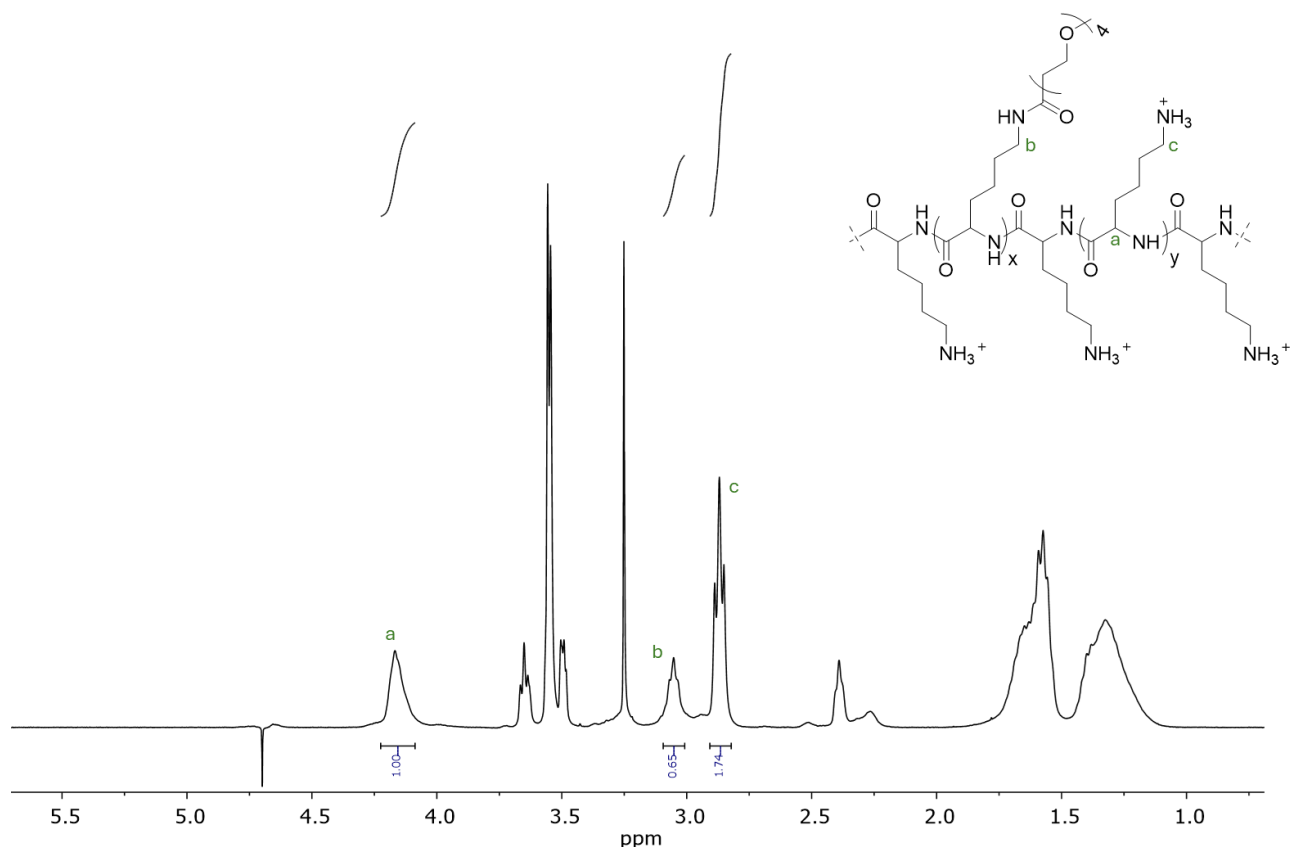


Figure 18. ^1H NMR of PLL-OEG **6**, recorded in D_2O at 400 MHz (298 K). Water residue signal was removed with water suppression. The signals needed for the quantification are reported in the spectrum.

Product **5** reacted with the zwitterionic PEG chains **3** and **4** bearing an azide unit at one end and L-Pro or L-Phe at the other end to yield compound **7** and **8**, respectively (Figure 19). The azide group reacts with the BCN group of **5** by a strain-promoted azide-alkyne cycloaddition (SPAAC). To exclude the presence of non-specific interactions between the LPS-functionalized vesicles and the QCM surfaces, PLL functionalized with only PEG chains, PLL-OEG **6**, and lacking the amino acids units were also synthesized.

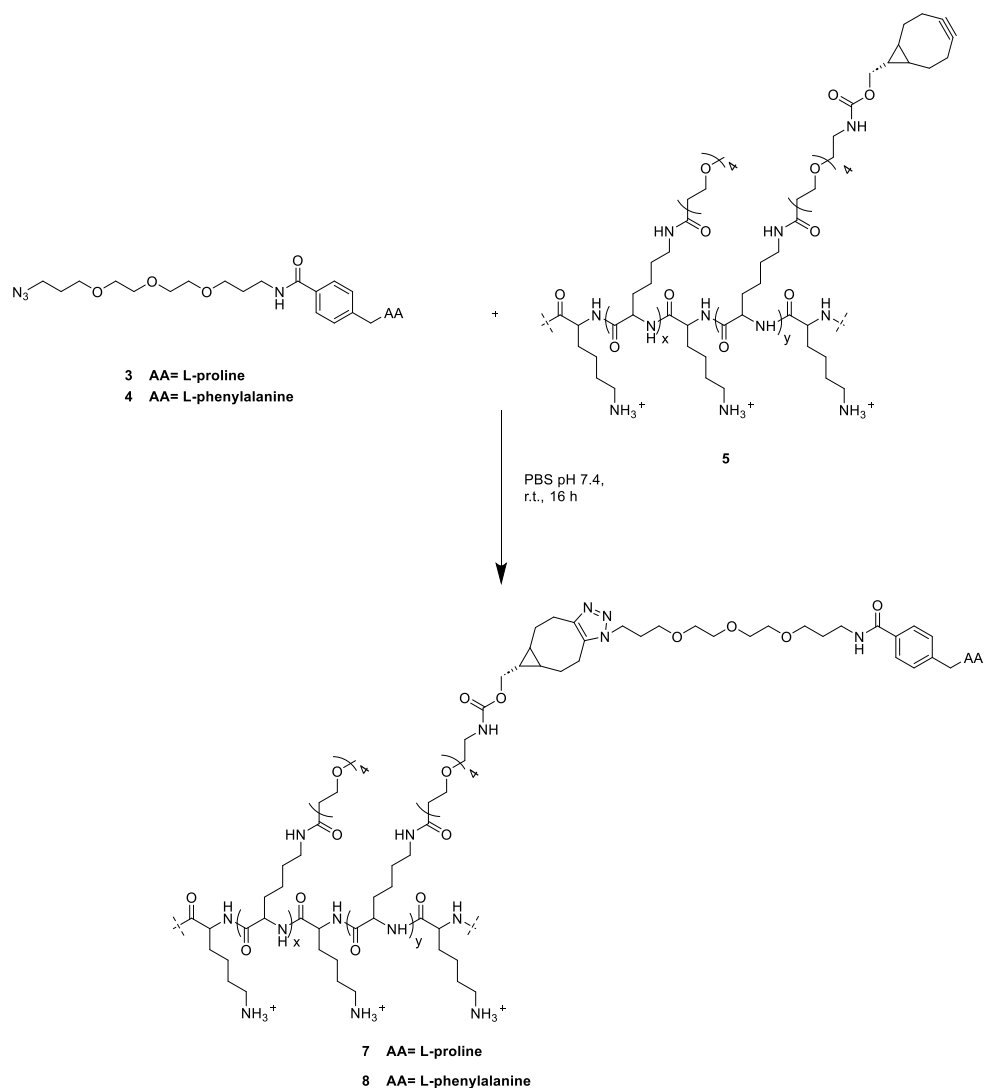


Figure 19. Reaction scheme for the BCN-azide cycloaddition between the two PEG chains **3** and **4** and the modified PLL **5**.

The yield of the reaction is calculated by comparing the integral of the CH of the lysine with the integral of the aromatic protons b of compounds **7** and **8**, since these signals are in a region free of polymer backbone signals (Figure 20 and 21). Given that the degree of conjugation of compounds **3** and **4** to PLL **5** was 4%, it can be concluded that all the BCN groups underwent conjugation with the azide. Therefore, the yield can be considered quantitative.

Chapter 4: Study of the Interactions Between Multivalent Zwitterionic Surfaces and Gram-Negative Bacterial Membrane Models

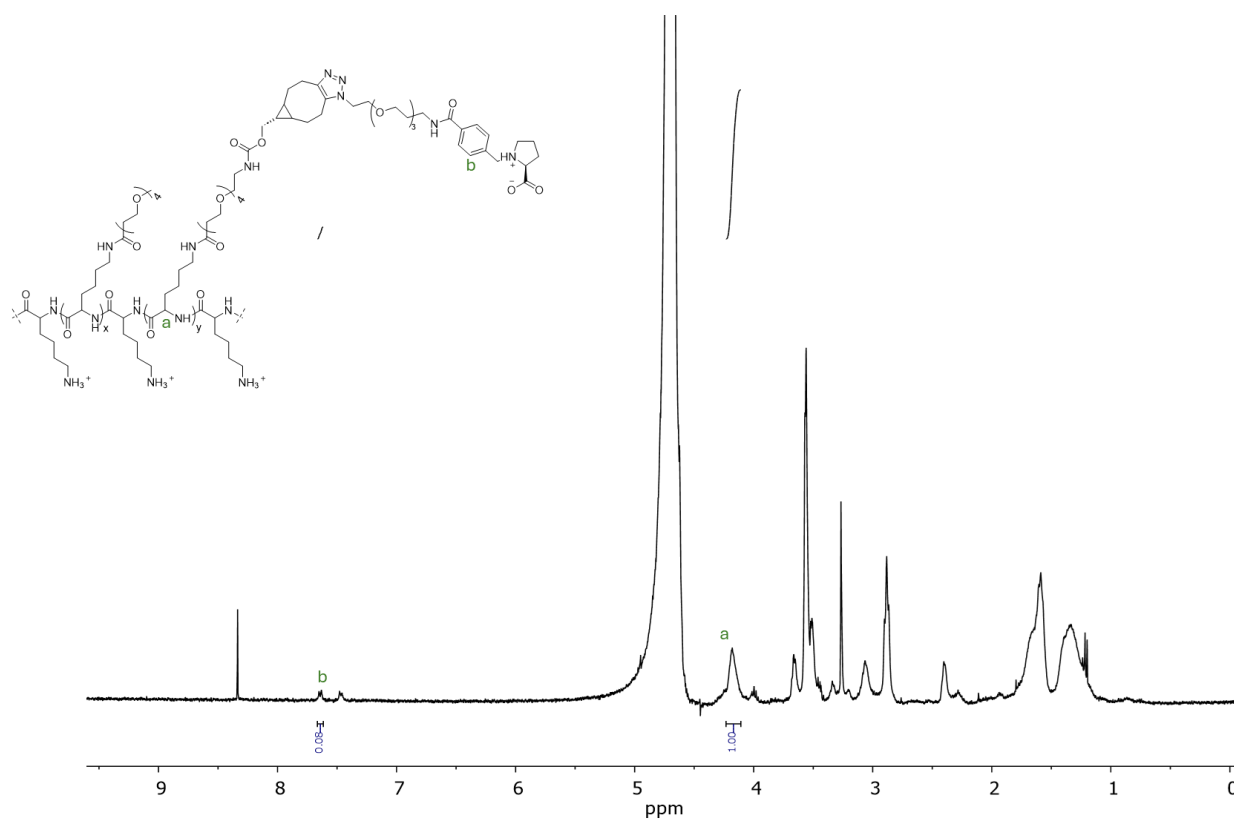


Figure 20. ^1H NMR of product **7**, recorded in D_2O at 400 MHz (298 K). The integrals present were used for the determination of the yield of the SPAAC reaction.

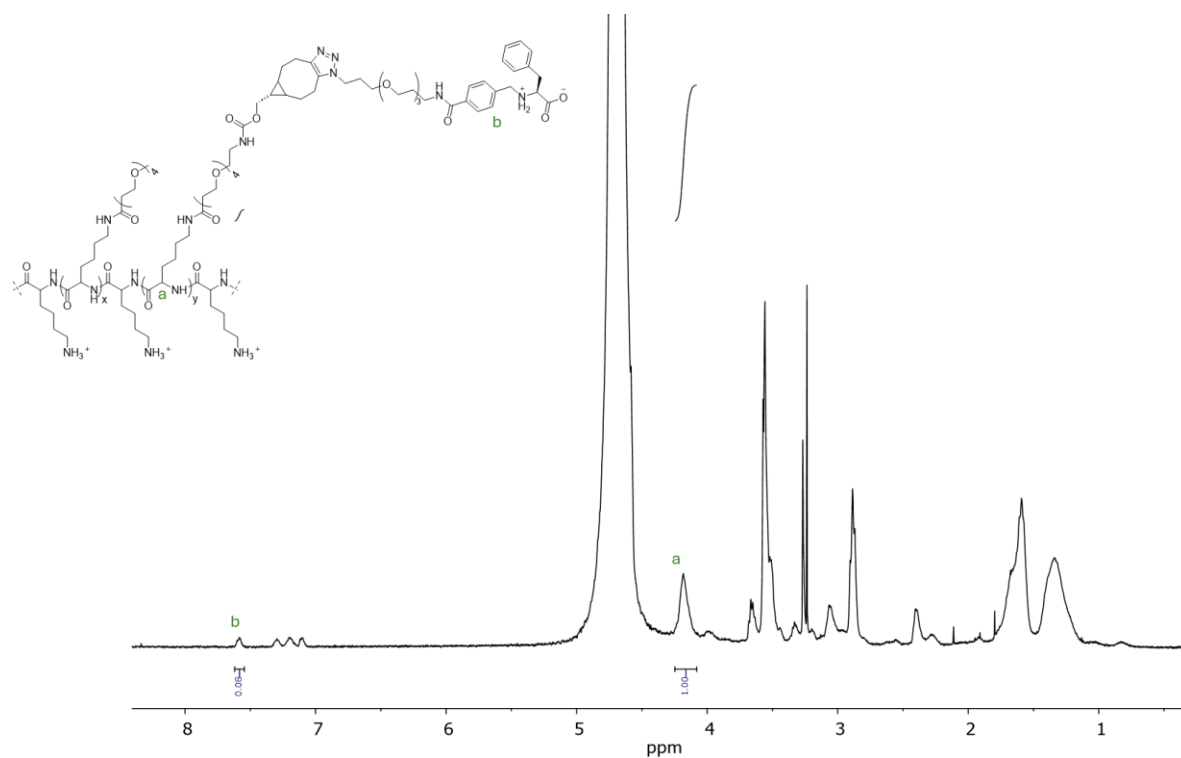


Figure 21. ^1H NMR of product **8**, recorded in D_2O at 400 MHz (298 K). The integrals present were used for the determination of the yield of the SPAAC reaction.

4.2.2.3 QCM-D experiments with AA modified poly-L-lysines surfaces and LPS functionalized vesicles

The partial functionalization of PLL with amino acids, described in the previous section, enables the exploitation of the remaining positively charged ammonium cations of polymers **6-8** for the adhesion to QCM-D chips, properly activated with UV-ozone to expose silanol groups. The ammonium groups in PLL facilitated the electrostatic interaction between the negative charges of the activated SiO₂.

The modified PLL (Figure 22) with 28% of OEG (**6**) and with 24% of OEG and 4% of zwitterionic PEG chain (**7** and **8**) were dissolved at a concentration of 0.1 mg/mL in phosphate-buffered saline (PBS) at a pH of 7.4, and the solution was flowed onto the QCM-D chip. Subsequently, a solution of vesicles flowed onto the PLL layer. The vesicles were identical to those described in section 2.1 and were constructed to mimic the outer membrane of Gram-negative bacteria. The vesicles were prepared in a batch, comprising pure POPC, POPC with 2wt% LPS, POPC with 5wt% LPS, and POPC with 10wt% LPS.

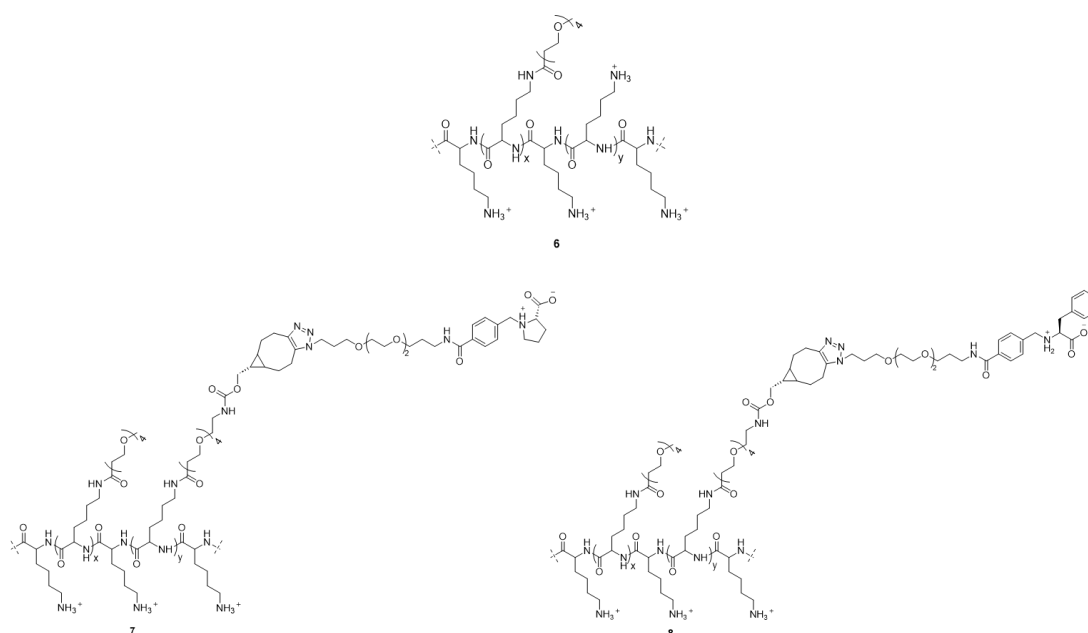


Figure 22. Modified PLL with only OEG (**6**) and with the two zwitterionic chains (**7** and **8**). Product **7** is modified with a L-proline and **8** is modified with a L-phenylalanine.

In the QCM-D graph in Figure 23a is illustrated the deposition of PLL-OEG **6** (section in aquamarine), on which a solution of pure POPC vesicles was flowed (section in peach). The same experiment (Figure 23b) was conducted with the modified PLL **7**, embedded with the PEG chain and a proline.

As illustrated in the graph (Figure 22a), there is a discernible decrease in frequency following the flowing of the vesicles (peach zone). However, after 15 min, the frequency stabilizes at -10.5 Hz and exhibits a downward drift solely due to the buffer rinsing. Additionally, the dissipation exhibited a change of +0.63 μ Hz (to 0.84 μ Hz), which corroborates the attachment of the vesicle to the surface. Nevertheless, it appears that the vesicles remain intact, as evidenced by the sustained dissipation at 0.84 μ Hz and the absence of a return to the initial value.

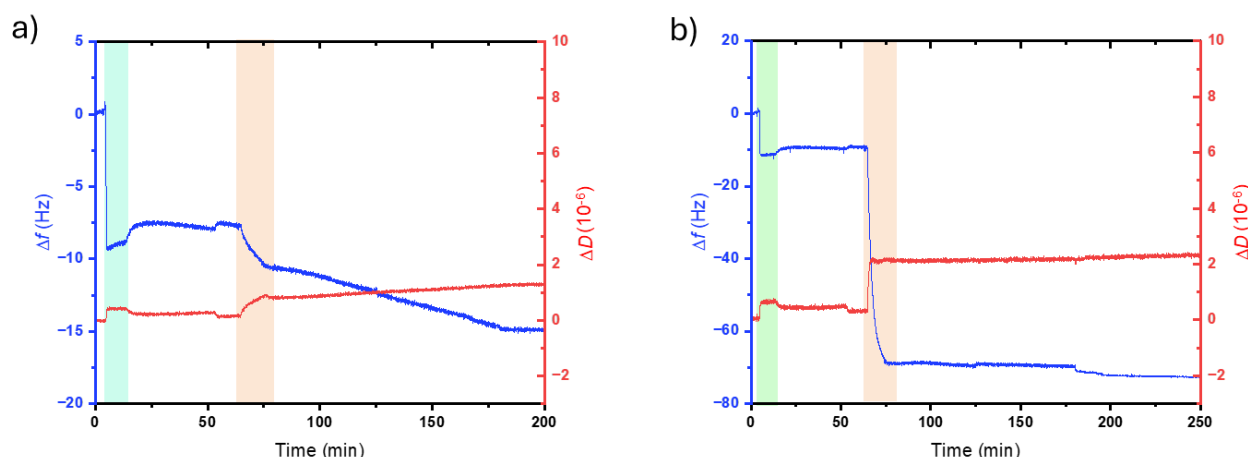


Figure 23. QCM-D graphs illustrating the interactions between PLL-based coatings and pure POPC vesicles: (a) Interaction between PLL-OEG **6** and pure POPC vesicles (PLL deposition highlighted in aquamarine and vesicle deposition in peach). (b) Interaction between modified PLL **7** and pure POPC vesicles (PLL deposition highlighted in green and vesicle deposition in peach).

The graph (Figure 23b) depicts a noticeable decrease in frequency, with a reduction from -8.85 to -68.35 Hz subsequent to the introduction of pure POPC vesicles to the PLL layer. Despite prolonged rinsing with buffer for over three hours, the vesicles remain adhered to the layer. The attachment of vesicles to the surface is evident from the observed increase in dissipation. The dissipation remains stable due to the absence of rupture/detachment of vesicles.

The flux of a 0.1 mg/mL solution of vesicles made with POPC+10wt% of LPS results in a reversed behavior for the modified PLL **6** and **7**. Figure 26 illustrates the impact of introducing POPC vesicles with a 10 wt% of LPS to a PLL-OEG layer **6** (24a) and functionalized PLL layer **7** (24b).

A comparison of this graph with the one in Figure 23a (PLL OEG **6** and pure POPC vesicles) reveals a decrease in frequency, from -10.45 Hz to -67.49 Hz, which can be attributed to the flowing of the vesicle solution. With regards to the interaction with pure POPC vesicles, it appears that the interaction is more pronounced with PLL OEG **6** than with PLL layer **7**. This interaction is likely due to the presence of a slightly higher number of free ammonium cations on the PLL OEG **6** layer, which can electrostatically interact with the negatively charged LPS. Consequently, dissipation increases significantly with the addition of the vesicles, due to their adhesion to the PLL layer. In the case of PLL layer **7**, the frequency also decreases significantly because of the adhesion of the vesicles, however, the decrease is not as pronounced as in the case of PLL-OEG **6**. In fact, the frequency has decreased from -11.70 to -17.70 Hz. The dissipation increases to 1.30 μ Hz and remains stable, indicating that the vesicles remain intact.

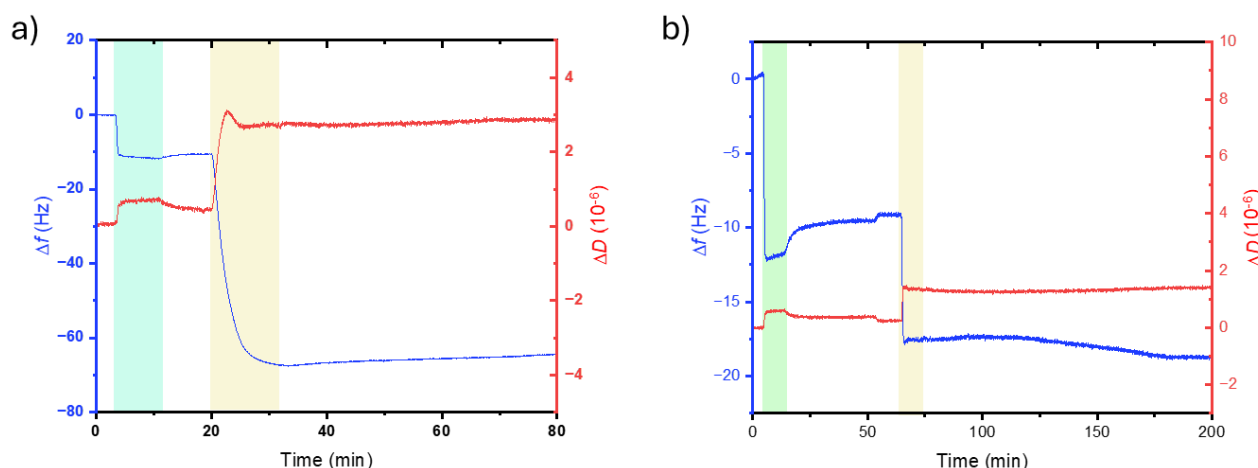


Figure 24. QCM-D graphs illustrating the interactions between PLL-based coatings and POPC+10 wt% LPS vesicles: (a) Interaction between PLL-OEG **6** and POPC+10 wt% LPS vesicles (PLL deposition highlighted in aquamarine and vesicle deposition in yellow). (b) Interaction between modified PLL **7** and POPC+10 wt% LPS vesicles (PLL deposition highlighted in green and vesicle deposition in yellow).

In light of the aforementioned considerations, it can be guessed that the interaction is predominantly governed by the electrostatic interactions between the positively charged PLL layers and the negatively charged vesicles. It is noteworthy that both PLL layers exhibit positive net charges, given that only 28% of the amines are functionalized with neutral OEG or zwitterionic amino acids. The ammonium cations present in solution allow the PLL to tightly attach to the silicon oxide chip, but part of these $\varepsilon\text{-NH}_3^+$ groups is exposed to the solution and prone to interact with the analyte that flowed over it. The PLL-OEG **6** exhibits a robust interaction with POPC+LPS vesicles, which are negatively charged due to the presence of LPS, characterized by the presence of several phosphate groups. Conversely, the modified PLL with the zwitterionic chain **7** demonstrates a pronounced interaction with the pure POPC vesicles, which are also zwitterionic.

4.2.2.4 QCM-D experiments with POPC+POPG (10%) vesicles

To corroborate our hypothesis, additional experiments were run, modifying the LPS percentage within the vesicles or creating mixed vesicles with a percentage of a negatively charged lipid, 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (POPG, Figure 25).

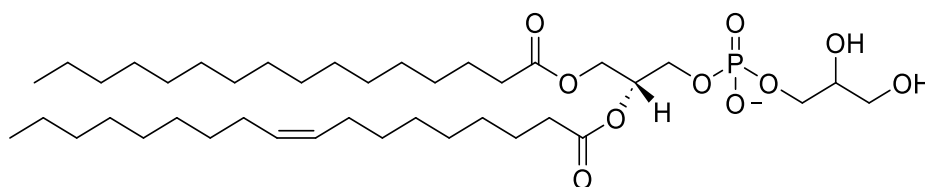


Figure 25. Structure of POPG, 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1'-rac-glycerol).

Vesicles with a POPG concentration of 10 wt% were prepared using the same methodology as previously described. The solutions of the lipids in chloroform were combined in the appropriate ratio within a vial and dried with a nitrogen flux, resulting in the formation of a film on the vial walls. The buffer was then added to the vial and mixed, after which the vesicle solution was extruded through a polycarbonate membrane to obtain a monodisperse solution of vesicles.

In this instance, the deposition of the PLL layer was carried out on the chip without the QCM-D monitoring apparatus because of the use of a well-established protocol. A solution of 1 mg/mL PLL-

OEG **6** in PBS was deposited onto a UV-ozone activated QCM-D chip. Given the rapid reaction rate, the solution was left to sit in a humidity chamber for 15 minutes, after which it was rinsed with PBS and Milli-Q. Once the solution had been adequately dried, it was placed in the QCM-D, and the vesicles were flowed onto the surface. Figure 26 depicts the QCM-D graph, which illustrates the introduction of POPC+POPG 10wt% vesicles on (a) PLL-OEG **6**, (b) modified PLL **7** and (c) modified PLL **8**.

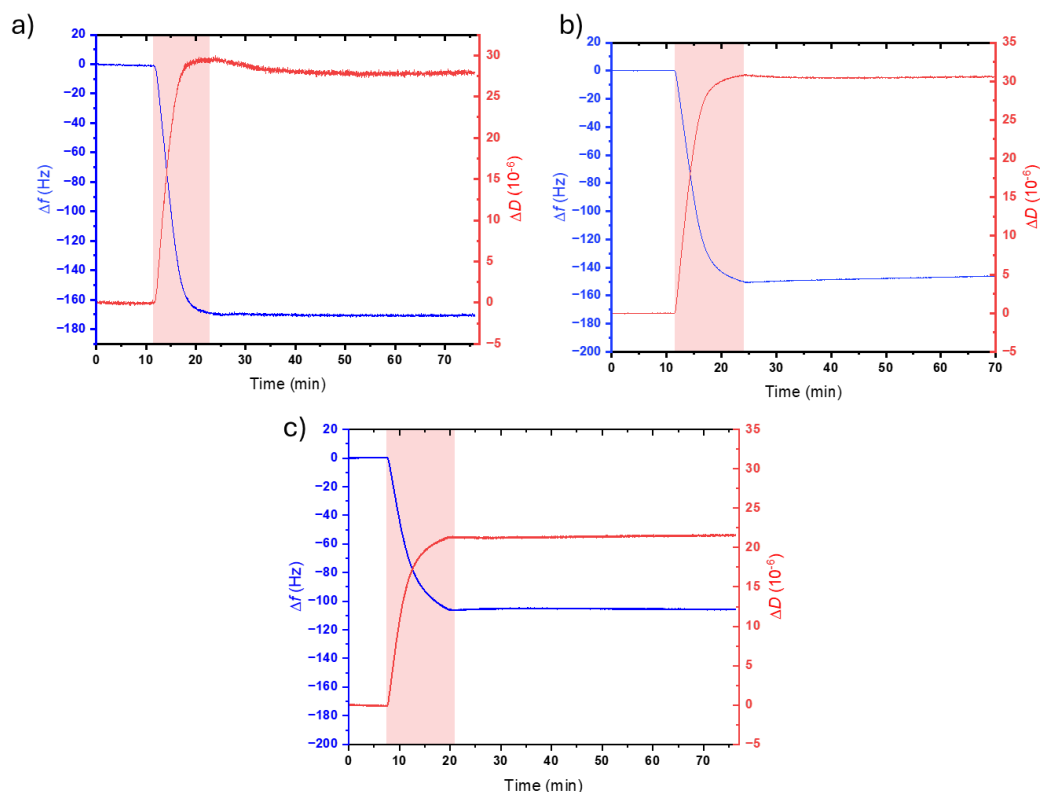


Figure 26. QCM-D graphs showing the interactions between modified PLL variants and POPC+POPG 10 wt% vesicles: (a) Interaction between PLL-OEG **6** and POPC+POPG 10 wt% vesicles (b) Interaction between modified PLL **7** and POPC+POPG 10 wt% vesicles. (c) Interaction between modified PLL **8** and POPC+POPG 10 wt% vesicles. The vesicles introduction is highlighted in light red.

As illustrated in the preceding graphs, it appears that the negative charges of the vesicles exert a significant influence on the interaction with the positively charged PLL, functioning as the primary interaction that occurs between the PLL and the vesicles. To address this challenge, we determined that it was essential to initially engage the primary ammonium ions in a stable interaction to prevent their involvement non-specific interactions with vesicles.

4.2.2.5 QCM-D experiments using polyacrylic acid to impair non-specific interactions

To address the issue of non-specific interactions between positively charged PLL layer and negatively charged PLS, we selected polyacrylic acid (PAA) as its sodium salt, basing our choice on the protocols employed by Prof. Huskens's research group. PAA (Figure 27) is a commonly utilized compound for this purpose, as it can effectively prevent undesired interactions between PLL and the analyte. Its relatively simple structure and small dimensions allow it to counterbalance the ammonium cation charges of PLL without hindering the exposure of other groups present in the layer.

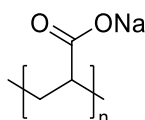


Figure 27. Structure of polyacrylic acid sodium salt (PAA).

Figure 28a depicts the graph of the QCM-D experiment with PLL-OEG **6**, PAA and pure POPC vesicles. Following the deposition of PLL on the chip, as previously described, a solution of PAA in PBS (highlighted in grey) was introduced until a stable frequency was achieved. Subsequently, the chip was rinsed with PBS until the frequency exhibited stability, after which a solution of pure POPC vesicles was flowed (highlighted in peach). The outcome is noteworthy. When the contribution of ammonium cations is impaired by rinsing PAA, it appears that the PLL-OEG exhibits no affinity for pure POPC vesicles, as evidenced by the absence of a change in frequency during their fluxing.

In light of the encouraging outcome, a similar experiment was conducted with amino acid-modified PLL **7** and **8**, respectively shown in Figure 28b and 28c, employing the same methodology.

In the case of proline derivative **7**, it appears that an interaction is taking place; however, the reduction in frequency is gradual, and it requires approximately an hour to reach saturation. Nevertheless, the rinsing with the buffer did not result in the detachment of the vesicles, as the baseline remained stable. A similar pattern is evident in the case of the phenylalanine derivative **8**, with a gradual decline in frequency. However, in this instance, the application of the buffer solution resulted in a gradual increase in frequency, which appeared to stabilize after 40 min, probably due to a partial detachment of the vesicles from the surface, and therefore a weaker binding.

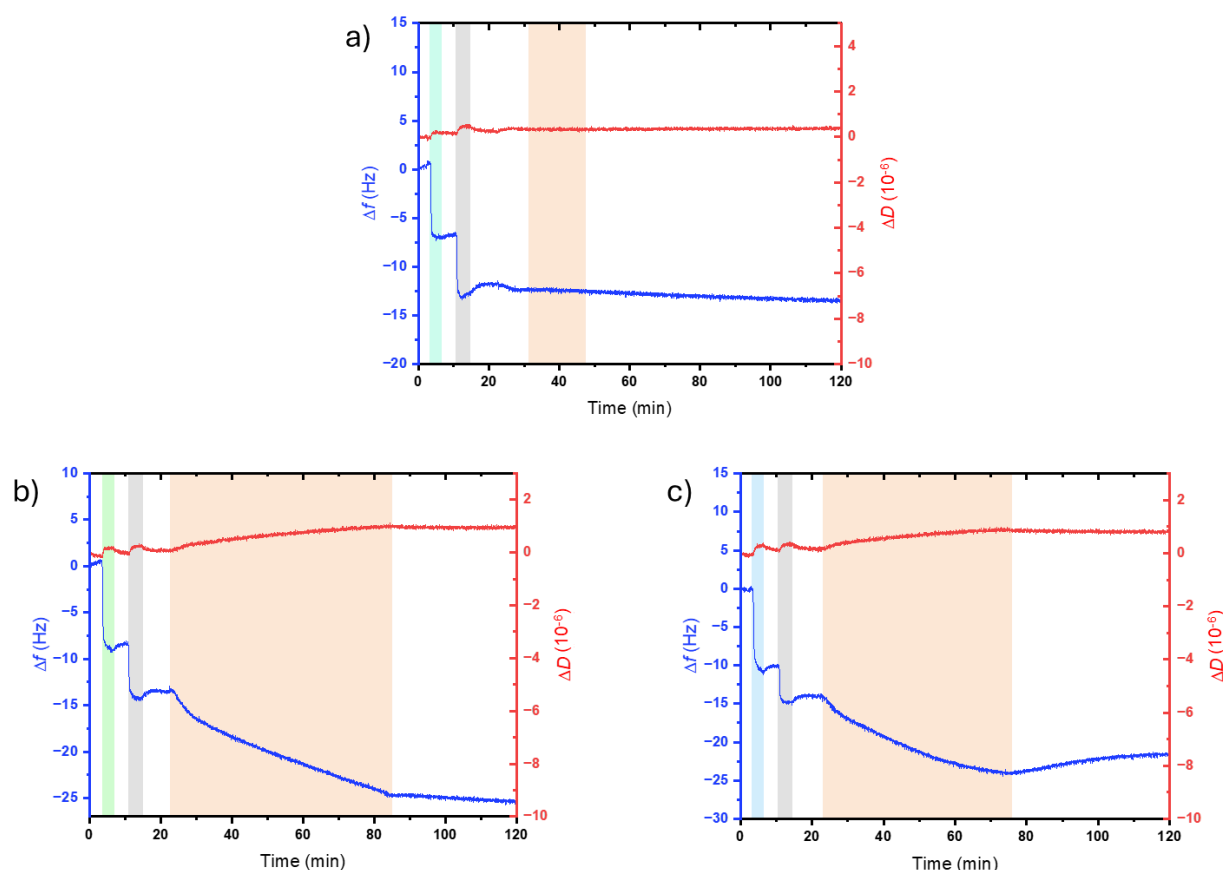


Figure 28. QCM-D graphs showing the interactions between modified PLL coatings and pure POPC vesicles: (a) Interaction between PLL-OEG 6 and pure POPC vesicles, with PLL deposition highlighted in aquamarine. (b) Interaction between L-proline-modified PLL 7 and pure POPC vesicles, with PLL deposition highlighted in green. (c) Interaction between L-phenylalanine-modified PLL 8 and pure POPC vesicles, with PLL deposition highlighted in blue. The PAA addition is highlighted in grey and vesicle deposition in peach.

The same methodology was then employed for the three PLLs with vesicles of POPC+10wt% LPS, with all graphs shown in Figure 29. It seems that in absence of the contribution of the ammonium cations the interaction between PLL-OEG 6 and POPC+10wt% LPS vesicles does not take place, since no variation of the frequency occurs upon the flowing of the vesicles on the surface (Figure 29a, yellow section).

Figure 29b illustrates the interaction between POPC+10wt% LPS vesicles and L-proline-modified PLL 7. The decline in frequency is not particularly pronounced, as the contribution of the ammonium cation has been removed. The frequency signal stabilizes after approximately one hour of fluxing vesicles. A further examination of the same experiment with L-phenylalanine-modified PLL 8, as illustrated in Figure 29c, reveals a more intriguing outcome. The frequency decline is notably more pronounced in comparison to the pure POPC vesicles, and also occurs more rapidly, reaching saturation within a half-hour. The interaction appears to be more pronounced with regard to L-proline 7, with a greater number of vesicles adhering to the PLL layer.

Following the rinse with the buffer, the frequency remains stable due to the absence of vesicle detachment within the analysis time frame. Furthermore, as in the other experiments, it is noteworthy that the dissipation increases when the vesicles are flowing and does not decrease over time, indicating that the vesicles remain intact.

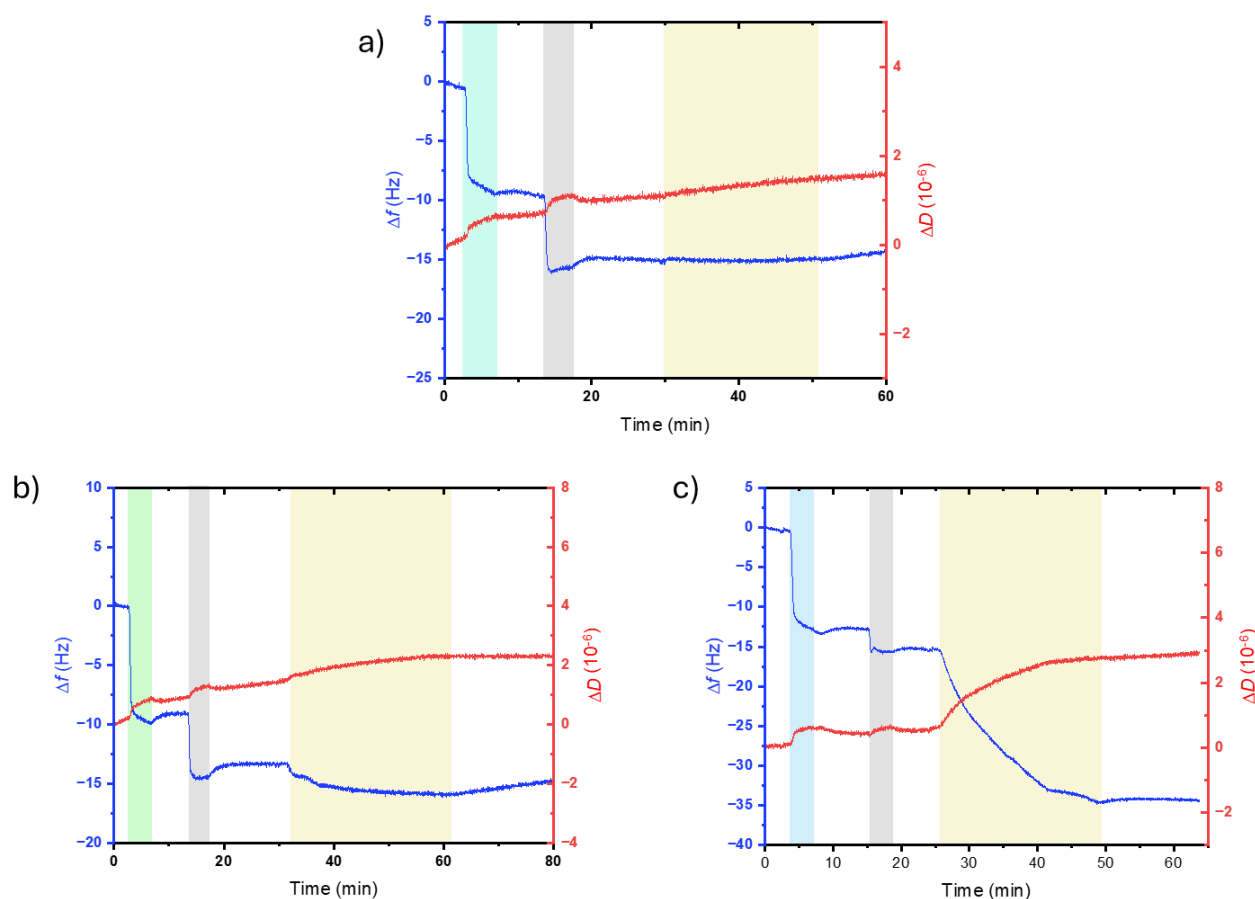


Figure 29. QCM-D graphs showing the interactions between various PLL modifications and POPC+10 wt% LPS vesicles: (a) Interaction between PLL-OEG **6** and POPC+10 wt% LPS vesicles, with PLL deposition highlighted in aquamarine. (b) Interaction between L-proline-modified PLL **7** and POPC+10 wt% LPS vesicles, with PLL deposition highlighted in green. (c) Interaction between L-phenylalanine-modified PLL **8** and POPC+10 wt% LPS vesicles, with PLL deposition highlighted in blue. The PAA addition is highlighted in grey, and vesicle deposition in yellow.

Further experiments were conducted with 5wt% of LPS vesicles, the results of which are presented in Figure 30a for the L-proline derivative **7** and Figure 30b for the L-phenylalanine derivative **8**.

In this case, the interactions of both modified PLL derivatives, **7** and **8**, with 5 wt% LPS vesicles are comparable, exhibiting a frequency shift of -14 Hz for derivative 7 and -16 Hz for derivative 8. Saturation occurs within approximately forty minutes for both derivatives and rinsing with buffer results in a slight frequency increase (+3–4 Hz), indicating that the binding affinity is relatively strong.

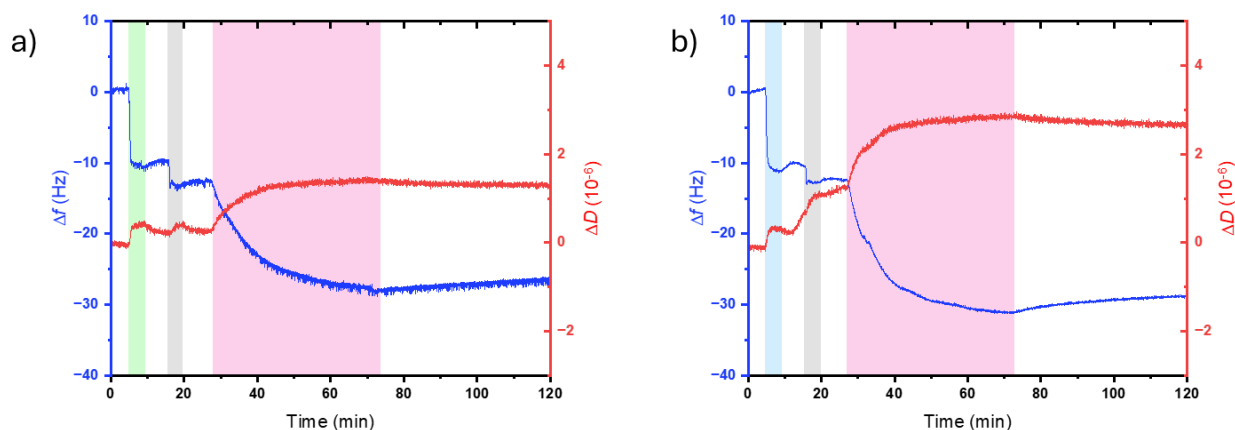


Figure 30. QCM-D graphs depicting the interactions between modified PLL coatings and POPC+5 wt% LPS vesicles: (a) Interaction between L-proline-modified PLL **7** and POPC+5 wt% LPS vesicles, with PLL deposition highlighted in green, PAA addition in grey. (b) Interaction between L-phenylalanine-modified PLL **8** and POPC+5 wt% LPS vesicles, with PLL deposition highlighted in blue, PAA addition in grey. Vesicle deposition is highlighted in pink.

The graph reported in Figure 31 is an overview of the frequency data reported in this section, as a function of the percentage of LPS present in the vesicles, for the three PLL modified surfaces. The black line refers to the PLL-OEG **6**, while the green to proline modified PLL **7** and the blue to phenylalanine modified PLL **8**.

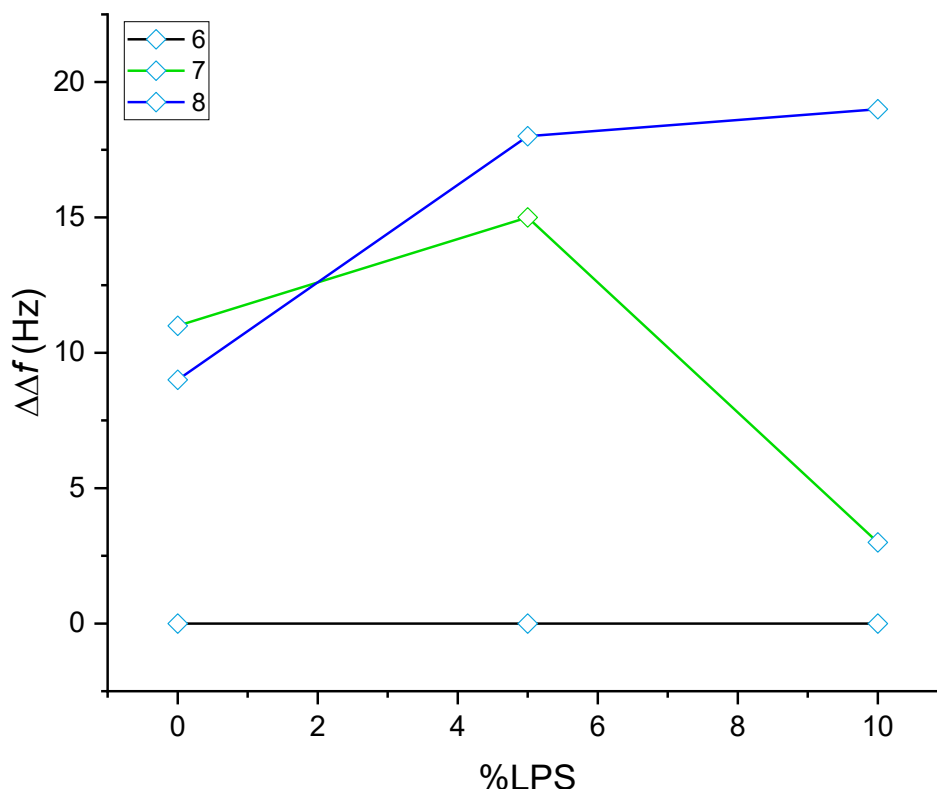


Figure 31. Graph illustrating the trend in frequency difference ($\Delta\Delta f$) recorded after the vesicle insertion (respect to the value after the PAA rinsing) in the previously presented QCM-D graphs.

Upon examination of the graph, several key observations can be made to summarize the findings, always taking into consideration that these measurements were performed only once and should be repeated. First, PLL-OEG **6** exhibits no significant affinity for any vesicles, showing no discrimination even in the presence of LPS. In contrast, proline-modified PLL **7** and phenylalanine-modified PLL **8**

display similar interactions with pure POPC vesicles, each showing a $\Delta\Delta f$ of approximately 10 Hz. This trend is consistent, with only minor differences of a few Hz, when 5 wt% LPS is introduced into the POPC vesicles. However, when the LPS concentration is increased to 10 wt%, the interaction profiles diverge considerably: while phenylalanine-modified PLL **8** maintains an affinity comparable to that observed with 5 wt% LPS vesicles, proline-modified PLL **7** shows a reduced affinity, reflected by a $\Delta\Delta f$ of only 3 Hz.

Further data collection is required to confirm reproducibility, particularly for this system, as viscoelastic films are highly sensitive to external conditions. Variations in hydration, sample preparation, and even minor sensor surface defects can greatly impact the measurements.

4.3 Conclusions

This chapter presents two distinct methodologies for achieving a singular objective.

The fabrication of supported lipid bilayers that could mimic the bacterial cell wall has been successfully completed, and their interaction with zwitterionic calixarenes **1** and **2** has been evaluated. The results show no evidence of interactions between the calixarenes and the LPS-functionalized monolayer: this is likely due to the slow kinetic of the interaction, which was not captured by the quartz crystal microbalance with dissipation monitoring, or to the low molecular weight of the calixarene ligands that makes the QCM-D detection not sensitive enough.

In a complementary approach, two modified poly-L-lysines were synthesized: PLL-OEG **6**, with 28% OEG modification, and PLL-OEG-BCN **5**, with 24% OEG and 4% BCN modification. To PLL **5**, zwitterionic chains **3** and **4** were reacted via a strain promoted azide-alkyne cycloaddition, resulting in the functionalized PLL **7** and **8**, which comprise proline and phenylalanine, respectively, in their zwitterionic form. PLL **6**, **7**, and **8** were tested with vesicles of pure POPC and POPC containing 5 wt% or 10 wt% of *E. coli* LPS. The observed interaction was found to be primarily due to non-specific electrostatic interactions between the negatively charged LPS and zwitterionic POPC, as well as the positively charged residues of the polyelectrolyte PLL.

When the Lysine ammonium residues present on the QCM chip were capped using polyacrylic acid, the non-specific interactions could be minimized allowing to collect stable and reproducible results with QCM-D. When the amino acid functionalized PLL **7** and **8** are present on the QCM chip, the microbalance could sense, to different extents, the presence of vesicles functionalized with variable % of LPS. Indeed, the two PLL **7** and **8** exhibit disparate behaviors in the presence of LPS-containing vesicles. The phenylalanine derivative **8** appears to exhibit a stronger interaction with POPC vesicles as the percentage of LPS present is increased. In contrast, the proline derivative **7** displays a greater propensity to interact with vesicles containing 5% LPS.

Although further investigations are needed to reproduce data and elucidate the different behavior observed between the two PLL derivatives, the present work allows to set-up conditions on QCM-D to study the interactions of a multivalent amino acid functionalized PLL surface with vesicles adorned with different % of LPS.

4.4 Experimental part

4.4.1 Materials

Buffers

Water utilized in the preparation of buffers, suspensions, and for substrate cleaning was sourced from a Merck Millipore Milli-Q Advantage A10 (resistivity: 18.2 M Ω · cm at 25 °C) purification system.

Tris-buffered saline (TBS) was prepared by dissolving 10 mM Tris(hydroxymethyl)aminomethane (Sigma, Switzerland) and 150 mM sodium chloride in water. The pH was adjusted to 7.3 by adding 2 M hydrochloric acid. The buffers were filtered through 0.22 μ m filters (Sartorius, France) and stored at 4 °C until use.

PBS (0.01 M sodium dihydrogen phosphate and 0.15 M sodium chloride, pH 7.4) was prepared dissolving commercial PBS tablets in ultrapure water, and then filtered.

Lipids

1-palmitoyl-2-oleoyl-glycero-3-phosphocholine and 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) were either purchased from Avanti Polar Lipids or Sigma and stored in chloroform at -30 °C. The Lipopolysaccharide Ra mutant (rough strain) from *Escherichia Coli* was obtained from Sigma Aldrich.

Poly-L-lysine

Poly(L-lysine) hydrobromide (MW = 15-30 kDa by viscosity), was purchased from Sigma-Aldrich. Methyl-OEG₄-N-hydroxy succinimide (NHS) ester and *endo*-BCN-OEG₄-N-hydroxy succinimide (NHS) ester were purchased from Thermo-Fischer Scientific. Methyl-OEG₄-N-hydroxy succinimide (NHS) ester was stored at -30 °C in 10 mg/mL PBS solution, while *endo*-BCN-OEG₄-N-hydroxy succinimide (NHS) ester was stored at -30 °C in 10 mg/mL DMSO.

Quartz Crystal Microbalance with Dissipation Monitoring (QCM-D)

Silica-coated (50 nm, QSX303) sensors, sourced from LOT-Quantum, were cleaned by rinsing with water and 2% sodium dodecyl sulfate solution (SDS; Sigma). After drying with a nitrogen stream, the sensors were activated using UV-ozone system for 1 hour.

QCM-D measurements were conducted on a Q-Sense E4 4-channel quartz crystal microbalance equipped with a peristaltic pump (Biolin Scientific) at a flow rate of 50 μ L/min. The 3rd, 5th, 7th, 9th, 11th, and 13th overtones were recorded simultaneously, with the 5th overtone used for all comparisons in this chapter.

3.1.1 Vesicles preparation

The lipids were stored at -30 °C in 10 mg/mL solutions in chloroform. The Lipopolysaccharide Ra mutant (rough strain) was stored at 4 °C in Milli-Q solution 5 mg/mL. The desired quantity of lipid was added to a clean vial and the solution dried under vacuum for 2 hours. The dried lipid was resuspended in the appropriate buffer solution, in a concentration of 1 mg/mL, after the addition of LPS. Unilamellar vesicles were produced by extruding the solution 15 times through a polycarbonate membrane (100 nm

pore size). The extruded vesicles were checked by dynamic light scattering (DLS, Malvern Zetasizer), stored at 4 °C and used within a week.

4.4.2 Supported lipid bilayer deposition on QCM-D

The solution of extruded vesicles at a concentration of 1 mg/mL in buffer or milli-Q was diluted to reach a concentration of 0.1 mg/mL in the desired buffer. Following a two-hour cleaning and activation process with UV-ozone, the QCM-D chips were placed in the QCM device, and the buffer was flowed. Subsequently, the signal was allowed to stabilize for an additional period until the baseline was observed to be flat. Subsequently, the vesicle solution was flowed until the SLB was formed, after which it was rinsed with the buffer until a stable baseline was reached.

This protocol was followed for all the SLB prepared in this chapter.

4.4.3 Poly-L-lysine modification **5** and **6**

1 mL of PLL.HBr (molecular weight of 15-30 kDa, Sigma Aldrich, 10 mg/mL in PBS) was dissolved in 1 mL of filtered PBS (pH 7.4). 24 equivalents (respect to the lysine monomer) of methyl- OEG₄-NHS ester (ThermoFischer Scientific, dissolved in DMSO) were added to obtain PLL-OEG **6**. To obtain PLL-OEG-BCN **5**, methyl- OEG₄-NHS ester (ThermoFischer Scientific, dissolved in DMSO) and 6 equivalents *endo*-BCN-OEG₄-NHS ester (BroadPharm, dissolved in DMSO) were mixed in a clean and dry vial, and then 1 mL of PLL.HBr dissolved in 1 mL of filtered PBS (pH 7.4) was added. The vial was left under vigorous stirring overnight (16 hours) at the shaker. After the reaction time, the solution was removed and placed into a dialysis membrane (SpectraPor, 6-8 kDa MWCO, diameter 6.4 mm) and left in a beaker containing 1 L of 70% PBS. Subsequently, the solution was replaced after a minimum of six hours, with the concentration reduced to 50% PBS, and 30% PBS, followed by two exchanges with milli-Q water. The solution in the membrane was then removed and freeze-dried to obtain the product as a white solid. The product was dissolved in D₂O and the ¹H NMR spectrum was recorded. The deuterium oxide was removed with freeze-drying and the product was stored at -30 °C in PBS solution 1 mg/mL until use.

Product **5** ¹H NMR (400 MHz, D₂O) δ (ppm): 0.84 (CH of cyclopropane of BCN group), 1.26–1.55 (CH₂ from cyclooctyne ring and lysine γ -CH₂), 1.63–1.83 (lysine β,δ CH₂), 2.50 (ethylene glycol CH₂ from both OEG and BCN coupled, -CH₂-C(=O)-NH), 3.00 (free lysine, H₂N-CH₂), 3.16 (ethylene glycol CH₂ of coupled lysine from both OEG and BCN, C(=O)-NH-CH₂-), 3.25 (OEG methoxy, -O-CH₃), 3.65 (oligo ethylene glycol from both OEG, CH₂-O-), 4.29 (lysine backbone, NH-CH-C(=O)-).

Product **6** ¹H NMR (400 MHz, D₂O) δ (ppm): 1.26–1.55 (lysine γ -CH₂), 1.63–1.83 (lysine β,δ CH₂), 2.50 (ethylene glycol CH₂ OEG, -CH₂-C(=O)-NH), 2.99 (free lysine, H₂N-CH₂), 3.16 (ethylene glycol CH₂ of coupled lysine from OEG, C(=O)-NH-CH₂-), 3.25 (OEG methoxy, -O-CH₃), 3.65 (oligo ethylene glycol from OEG, CH₂-O-), 4.29 (lysine backbone, NH-CH-C(=O)-).

4.4.4 Poly-L-lysine modification **7** and **8**

To a solution of **5** (1 mL, 5 mM in PBS pH 7.4) a solution of zwitterionic chain (**3** or **4**) in PBS was added, considering 10 equivalents of zwitterionic chain for every BCN-OEG unit (4%). The vial was left under vigorous stirring overnight (16 hours) at the shaker. After the reaction time, the solution was removed and placed into a dialysis membrane (SpectraPor, 6-8 kDa MWCO, diameter 6.4 mm) and left in a beaker containing 1 L of 70% PBS. Subsequently, the solution was replaced after a minimum of six hours, with the concentration reduced to 50% PBS, and 30% PBS, followed by two exchanges with milli-Q water. The solution in the membrane was then removed and freeze-dried to obtain the product as a white solid.

The product was dissolved in D₂O and the ¹H NMR spectrum was recorded. The deuterium oxide was removed with freeze-drying and the product was stored at -30 °C in PBS solution 1 mg/mL until use.

Product **7** ¹H NMR (400 MHz, D₂O) δ (ppm): 0.84 (CH of cyclopropane of BCN group), 1.26–1.55 (CH₂ from cyclooctyne ring, CH₂ of proline ring, lysine γ-CH₂), 1.63–1.83 (CH₂ of proline ring, lysine β,δ CH₂), 2.50 (ethylene glycol CH₂ from OEG and cyclooctene coupled, -CH₂-C(=O)-NH), 3.00 (free lysine, H₂N-CH₂), 3.16 (ethylene glycol CH₂ of coupled lysine from both OEG and cyclooctene, C(=O)-NH-CH₂-), 3.20 (Ar-CH₂-N proline), 3.25 (OEG methoxy, -O-CH₃), 3.35 (), 3.65 (oligo ethylene glycol from OEG, CH₂-O-), 4.29 (lysine backbone, NH-CH-C(=O)-).

Product **8** ¹H NMR (400 MHz, D₂O) δ (ppm): 0.84 (CH of cyclopropane of BCN group), 1.26–1.55 (CH₂ from cyclooctyne ring and lysine γ-CH₂), 1.63–1.83 (lysine β,δ CH₂), 2.50 (ethylene glycol CH₂ from both OEG and BCN coupled, -CH₂-C(=O)-NH), 3.00 (free lysine, H₂N-CH₂), 3.16 (ethylene glycol CH₂ of coupled lysine from both OEG and BCN, C(=O)-NH-CH₂-), 3.25 (OEG methoxy, -O-CH₃), 3.65 (oligo ethylene glycol from OEG, CH₂-O-), 4.29 (lysine backbone, NH-CH-C(=O)-).

4.5 Appendix

QCM-D graph with SLB and different percentages of LPS

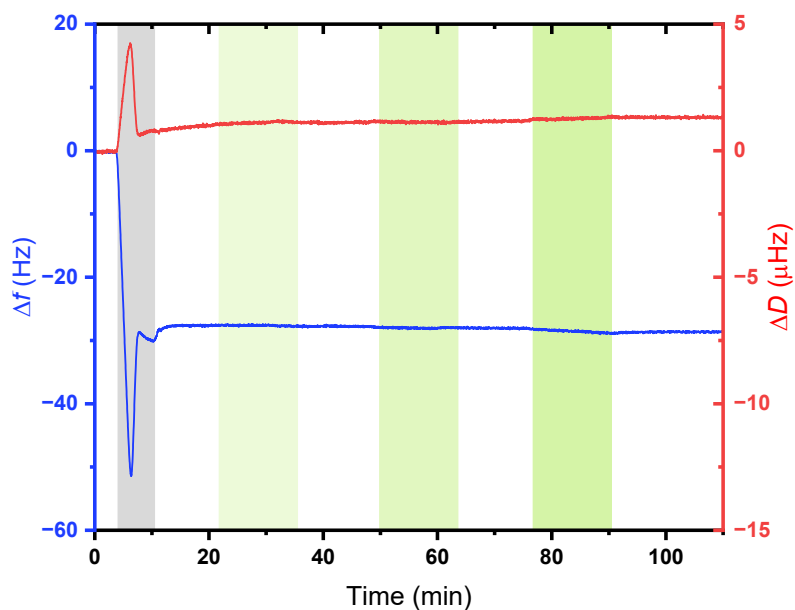


Figure 32. QCM-D graph of the experiment with POPC vesicles (formation of the SLB in grey) and the addition of solutions of **1** in TBS (pH 7.3) in green at different concentrations: 1 $\mu\text{g/mL}$ (0.0011 mM), 10 $\mu\text{g/mL}$ (0.011 mM) and 100 $\mu\text{g/mL}$ (0.11 mM).

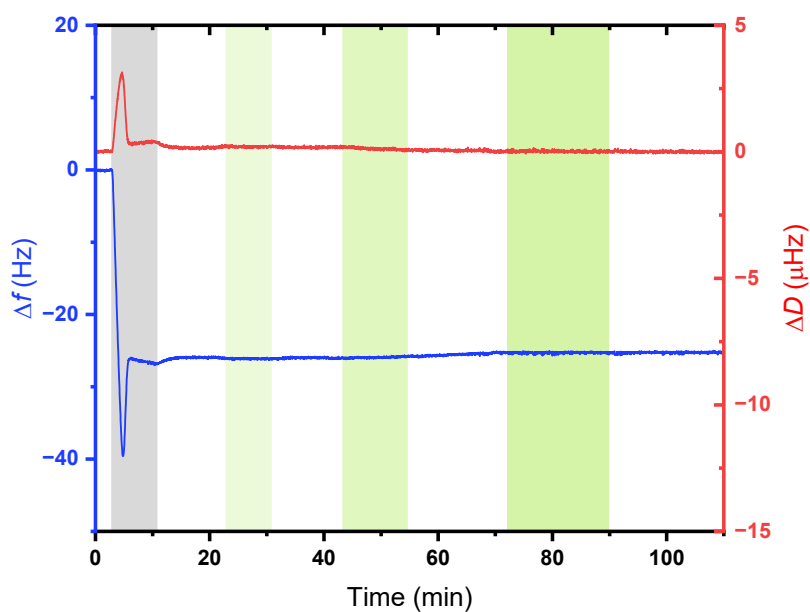


Figure 33. QCM-D graph of the experiment with POPC+5wt% of LPS vesicles (formation of the SLB in grey) and the addition of solutions of **1** in TBS (pH 7.3) in green at different concentrations: 1 $\mu\text{g/mL}$ (0.0011 mM), 10 $\mu\text{g/mL}$ (0.011 mM) and 100 $\mu\text{g/mL}$ (0.11 mM).

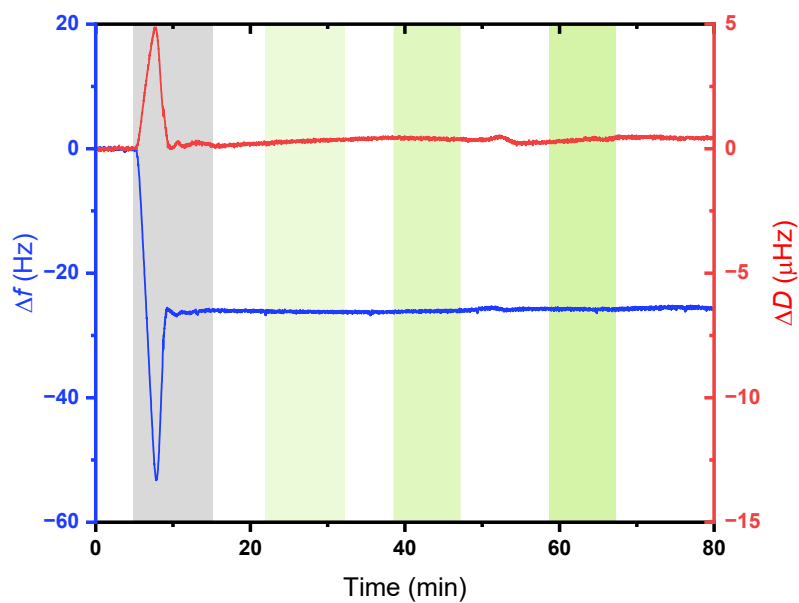


Figure 34. QCM-D graph of the experiment with POPC+10wt% of LPS vesicles (formation of the SLB in grey) and the addition of solutions of **1** in TBS (pH 7.3) in green at different concentrations: 1 $\mu\text{g/mL}$ (0.0011 mM), 10 $\mu\text{g/mL}$ (0.011 mM) and 100 $\mu\text{g/mL}$ (0.11 mM).

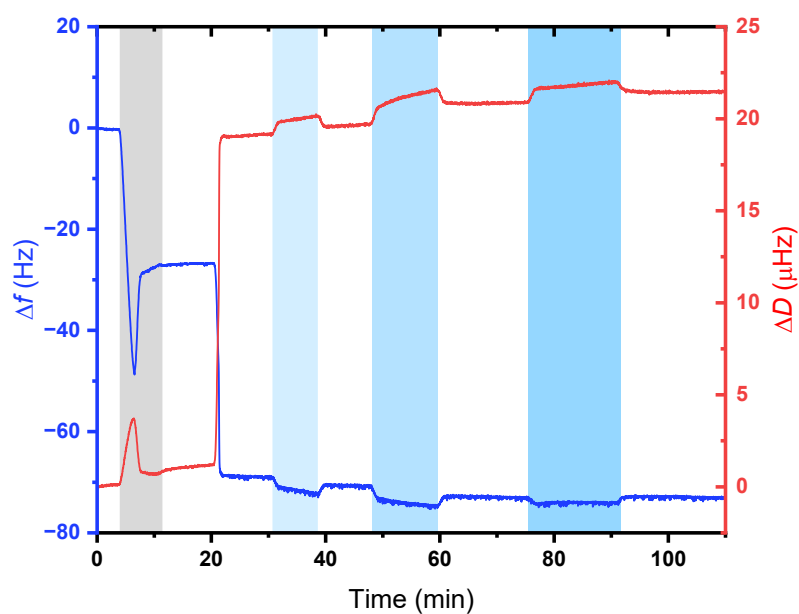


Figure 35. QCM-D graph of the experiment with POPC+10wt% of LPS vesicles (formation of the SLB in grey) and the addition of solutions of **2** in TBS (pH 7.3) +10% of DMSO in light blue at different concentrations: 1 $\mu\text{g/mL}$ (0.0011 mM), 10 $\mu\text{g/mL}$ (0.011 mM) and 100 $\mu\text{g/mL}$ (0.11 mM).

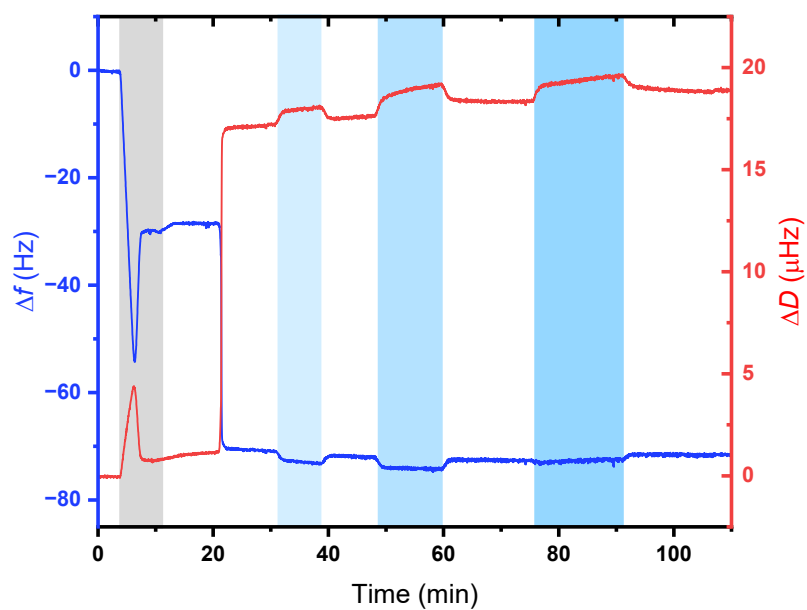


Figure 36. QCM-D graph of the experiment with POPC+5wt% of LPS vesicles (formation of the SLB in grey) and the addition of solutions of **2** in TBS (pH 7.3) +10% of DMSO in light blue at different concentrations: 1 μ g/mL (0.0011 mM), 10 μ g/mL (0.011 mM) and 100 μ g/mL (0.11 mM).

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RAFT Polymerization of NIPAA Using Calixarenes as Chain Transfer Agents

*This work was conducted in collaboration with Prof. Pierangelo Gobbo's group at the University of Trieste.

5.1 Introduction

The national research project SAMBA,¹ within which this chapter is contextualized, aims to fight bacterial infection by developing multivalent objects at the mesoscopic scale (ranging from 20 nm to 100 μm) embedded within multifunctional systems able to both selectively detect bacteria through fluorescent probes and treat the infection by releasing antibiotics close to the microorganisms.

Conventional molecular drug-delivery systems, such as small molecules, dendrimers, macrocycles, and polymers, exhibit several limitations, including low valency, limited loading capacity due to the small number of molecules they can carry, and low sensitivity. To address these challenges, the project proposes the use of nanomaterials structured as microcapsules with mesoscale dimensions. At the core of the SAMBA project lies a self-assembly technique known as RAFT-PISA (Reversible Addition-Fragmentation Chain Transfer coupled with the Polymerization-Induced Self-Assembly).² This technique will be employed to synthesize a small library of nano-objects (NOs) that share the same chemical functionalities but exhibit diverse morphologies (Figure 1).

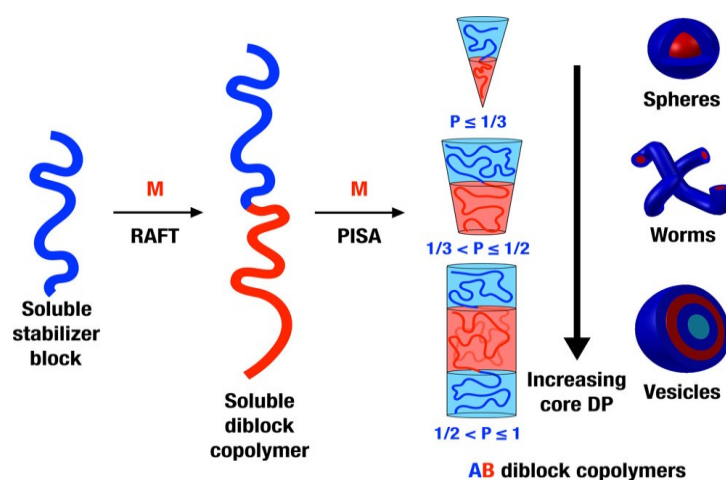


Figure 1. Schematic representation of the RAFT-PISA process to build nano-objects of different shapes. In blue the water-soluble block, in red the hydrophobic block. Depending on the degree of polymerization it is possible to obtain AB diblock copolymers with different Packing parameters (P) and therefore able to self-assemble in nanoobject of different morphologies. Figure adopted from reference 2.

The bacteria targeting properties will be granted by multivalent ligands covalently bound to the surfaces of the nano-objects. In previous studies carried out in our research group, calixarenes capable of interacting with D-Ala-D-Ala and displaying antimicrobial activity against Gram-positive bacteria comparable to that of vancomycin were synthesized.³ In addition, as already mentioned in the introduction at paragraph 3.1 of Chapter 3, calixarenes functionalized with zwitterionic amino acids, such as L-phenylalanine, L-proline and glycine at their upper rim, selectively recognize Gram-negative bacteria, over Gram-positive bacteria or mycobacteria. The RAFT-PISA technique should be utilized to synthesize materials with varying morphologies and sizes and bearing calixarene ligands at the top of the hydrophilic block polymer. These materials will subsequently be organized into protocell structures through the Pickering emulsion technique,⁴ achieving dimensions ranging from 1 to 100 μm .

Three distinct strategies might be envisaged for the functionalization of the NOs with monovalent or multivalent ligands will be explored:

- a pre-polymerization approach, in which the ligand is covalently attached to the RAFT agent, followed by the application of the RAFT-PISA technique to produce the NOs;
- a post-polymerization approach, where the ligand is covalently grafted to pre-formed NOs through click reactions such as azide-alkyne cycloaddition, amide bond formation, or thiol-ene addition;
- a non-covalent approach, in which an amphiphilic ligand interacts non-covalently with the NOs, leveraging hydrophobic interactions between the lipophilic segment of the ligand and the lipophilic core of the NOs.

5.1.1 Aim of this work

In this thesis, it was explored the possibility of using the pre-polymerization approach to manufacture nanoobjects functionalized with calixarene macrocycles. The project is certainly challenging since there are very limited examples in the literature of RAFT polymerization starting from calixarenes functionalized with RAFT agents. Four distinct calix[4]arenes (compounds **4**, **9** and **12**, Figure 2), having on the lower rim one, two or four alkyl chains of different length and ending with the RAFT agents will be synthesized.

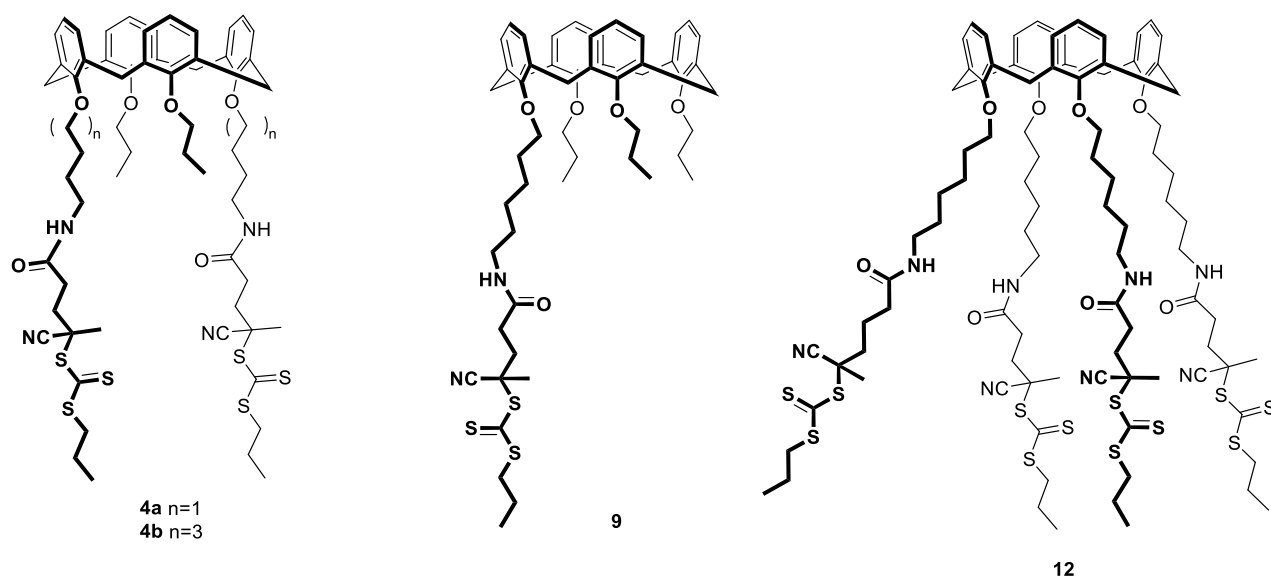


Figure 2. Calixarene-based chain transfer agent (CTA) synthesized in this chapter.

The aim of this preliminary work is to study the polymerization reaction having calixarenes as chain transfer agent (CTA), with particular attention in identifying the optimal reaction conditions and investigating the properties of the resulting polymers. It was decided to begin with polymers derived from N-isopropyl acrylamide (NIPAA), a highly versatile monomer whose polymers usually exhibit solubility in both organic and aqueous solvents.

5.1.2 RAFT polymerization

RAFT polymerization is a form of reversible deactivation radical polymerization (RDRP) that, under precise control of reagents and reaction conditions, can display several features commonly associated with living polymerization. A key characteristic of living polymerization is the absence of termination and chain transfer reactions, which allows the polymerization process to continue when additional monomer is introduced. This process is usually characterized by low molar mass dispersity, high end-

group retention, the ability for continuous chain growth, and the production of complex polymer architectures. In many applications, the uniform chemical composition, low molar mass dispersity, and high fidelity of end groups achieved through RAFT polymerization are essential for ensuring consistent, reproducible, and reliable performance.⁵

The monomers used in RAFT polymerization can be categorized into two groups based on their reactivity in the polymerization process. LAMs, or "less activated monomers," refer to monomers where the olefinic double bond is adjacent to a heteroatom, such as oxygen, nitrogen, halogen, or sulfur. In contrast, MAMs, or "more activated monomers," include molecules where the double bond is conjugated with aromatic groups, carbonyls, or nitriles.⁵ By selecting the appropriate RAFT agent for the specific monomers to be employed, RAFT polymerization proves to be a versatile technique applicable to all the above-mentioned class of monomers.⁶

The process of reversible deactivation in RAFT polymerization is illustrated in Figure 3. The process is initiated by the addition of a radical initiator, which allows the formation of the active polymer, a propagating radical species. The function of the RAFT agent is to capture this radical, generating an intermediate where the radical is relatively stable, with the active polymer covalently bound to the RAFT agent. Through a kinetic constant k_β , the radical is transferred to another propagating radical, resulting in the formation of a dormant polymer, a species in which the polymer remains attached to thioester group of the RAFT agent. The chain transfer steps that establish the main equilibria are termed degenerate because they simply involve the exchange of functionality between propagating radicals and dormant polymer chains. The primary distinction between the species on either side of the equilibrium (the two polymer chains P_n and P_m) lies in their degrees of polymerization (n and m). Given that in a RAFT polymerization process the rate of propagation (k_p) is expected to be lower than the rate of the main equilibria (k_β), the two degrees of polymerization should be comparable.

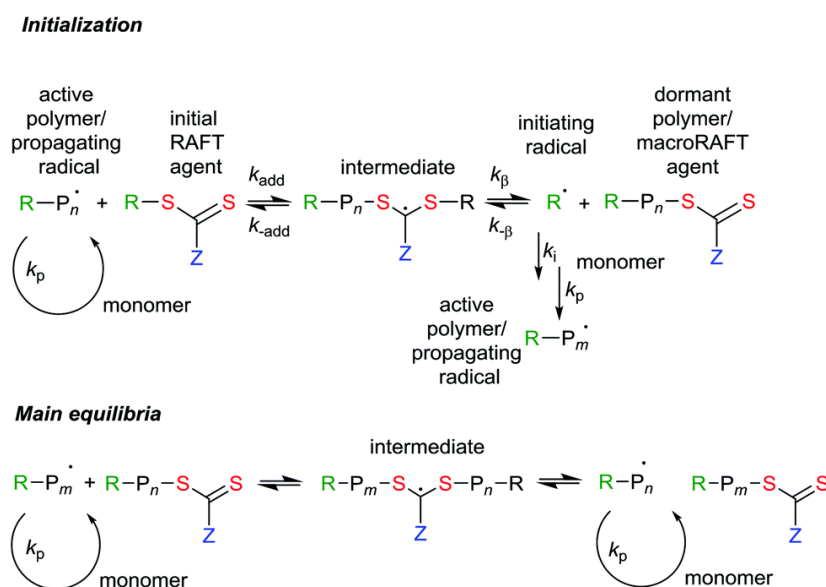


Figure 3. Mechanism of the RAFT polymerization; the formation of the propagating radical requires a radical initiator; this first step is not shown in the image. Figure adopted by reference 5.

In theory, any propagating radical, originating from the monomer, initiator, or growing polymer chain, can be involved in a termination reaction with another radical species. However, by carefully adjusting the reaction conditions, the likelihood of such termination events can be significantly reduced. Since

the polymer grows between the R group and the thioester of the RAFT agent, it is known that each polymer chain contains a RAFT agent group at its end (Figure 4).

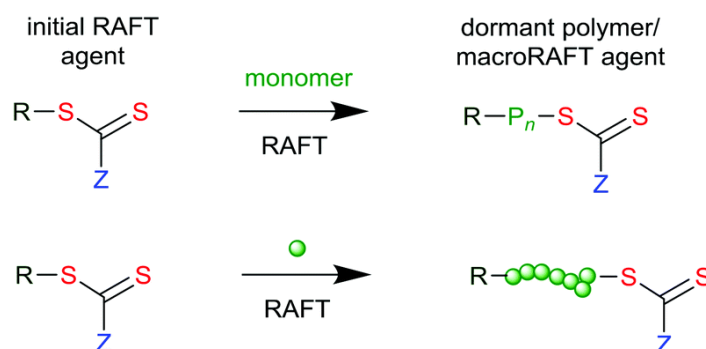


Figure 4. Scheme of the RAFT polymerization. Figure adopted by reference 5.

This polymer behaves as a macro-RAFT agent, allowing for the formation of a diblock copolymer by copolymerizing an additional monomer using the macro-RAFT agent. This characteristic is particularly valuable for a range of applications, especially in the RAFT-PISA process.

5.1.3 RAFT-PISA

Polymerization-induced self-assembly (PISA) is a technique employed to produce block copolymer nano-objects. This process involves the addition of a comonomer to a homopolymer through RAFT polymerization, performed in an appropriate solvent. The resulting diblock copolymer must become insoluble upon synthesis, thereby promoting in-situ self-assembly to form nano-objects of various shapes and dimensions (Figure 5).² This process can also be extended to more than two blocks copolymer.

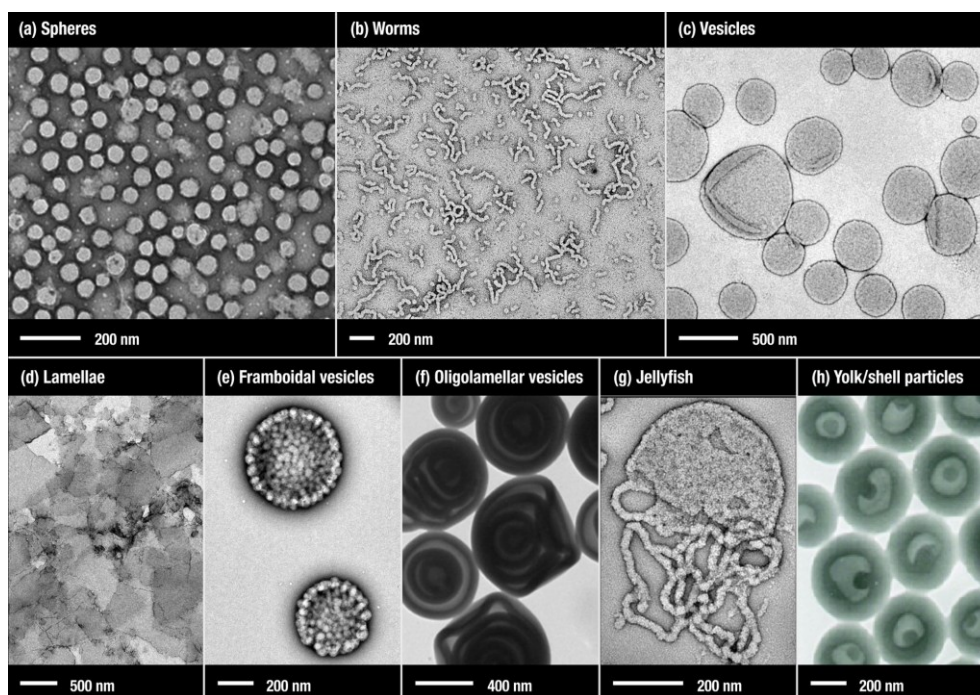


Figure 5. Transmission electron microscopy images of example of morphologies, synthesized by RAFT-PISA. Figure adopted from reference 2.

The final morphology of the nano-object is governed by the relative volume fractions of the copolymer blocks, which are described by the packing parameter P .⁷ The most common morphologies are vesicles,

spheres and worms, but also framboidal vesicles, lamellae, yolk/shell particles and space concentric vesicles.

A significant advantage of PISA achieved through RAFT polymerization lies in the broad range of functional monomers and solvents that can be employed. Noteworthy among the solvents that can be utilized are water,^{8,9} alcohols (Figure 6), and other polar solvents,^{10–12} as well as n-alkanes, mineral oils, other apolar solvents,^{13–15} and even ionic liquids.¹⁶ This broad solvent compatibility enhances the applicability of PISA across a wide range of chemical environments.

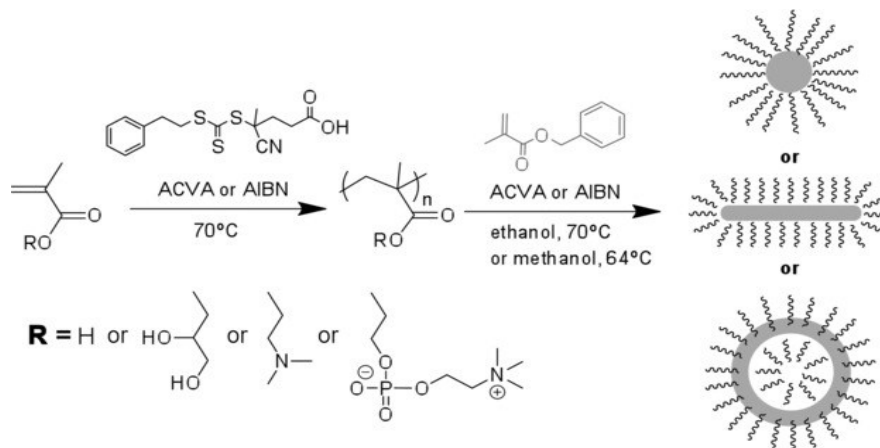


Figure 6. Example of a RAFT-PISA process in alcohol solvent, to get morphologies like spheres, worms or vesicles. Figure adopted from reference 10.

5.1.4 Macrocycle-based chain transfer agents

As previously stated, there are only a limited number of examples in literature where a RAFT agent has been integrated into a macrocycle to allow RAFT polymerization.

With a primary focus on the synthesis of star-shaped polymers,¹⁷ Nitta *et al.*¹⁸ employed a dimeric cavitand capsule, formed by the self-assembly of two cavitands obtained by the coordination of four silver cations to the bipyridyl arms of the macrocycle. Chain transfer agents were anchored to the lower rim of the cavitand, enabling the RAFT polymerization process to produce the star polymer, with styrene serving as the monomer. The formation of the capsule in the presence of silver tetrafluoroborate was evaluated before and after polymerization via NMR spectroscopy. In the latter case, the NMR spectra confirmed the successful formation of an eight-armed supramolecular star polymer (Figure 7).

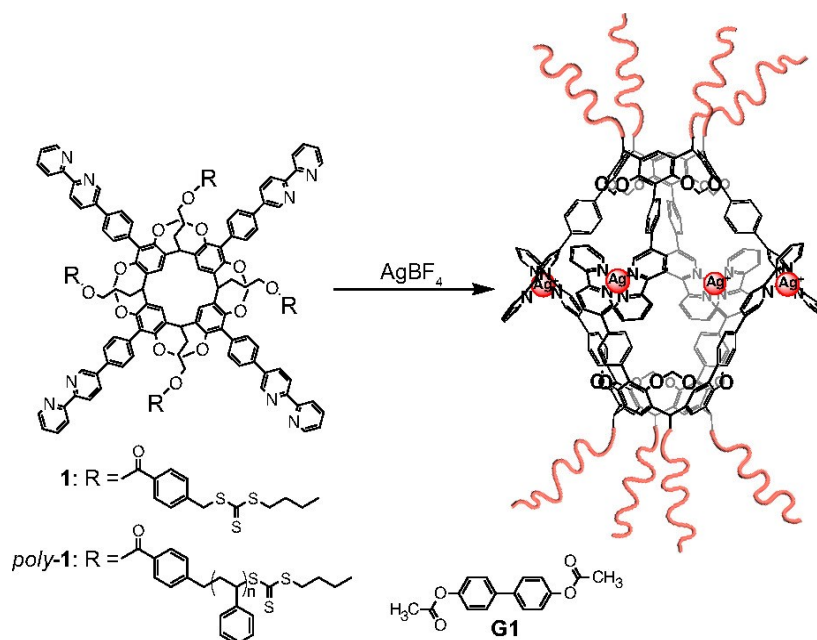


Figure 7. Formation of the capsule by complexation of Ag^+ with the cavitands **1** and the cavitands with polymers attached at the lower rim poly-**1**. Figure adopted from reference 18.

Other commonly used supramolecular precursors for the introduction of chain transfer agents are pillarenes. For instance, a pillar[6]arene derivative (**P8**, Figure 8), mono-functionalized with an oligoethylene glycol bearing a RAFT transfer agent at its end was reacted with N-isopropyl acrylamide, resulting in the formation of pillar[6]arene-modified poly(N-isopropyl acrylamide) (PNIPAA) (**H12**, Figure 8).¹⁹

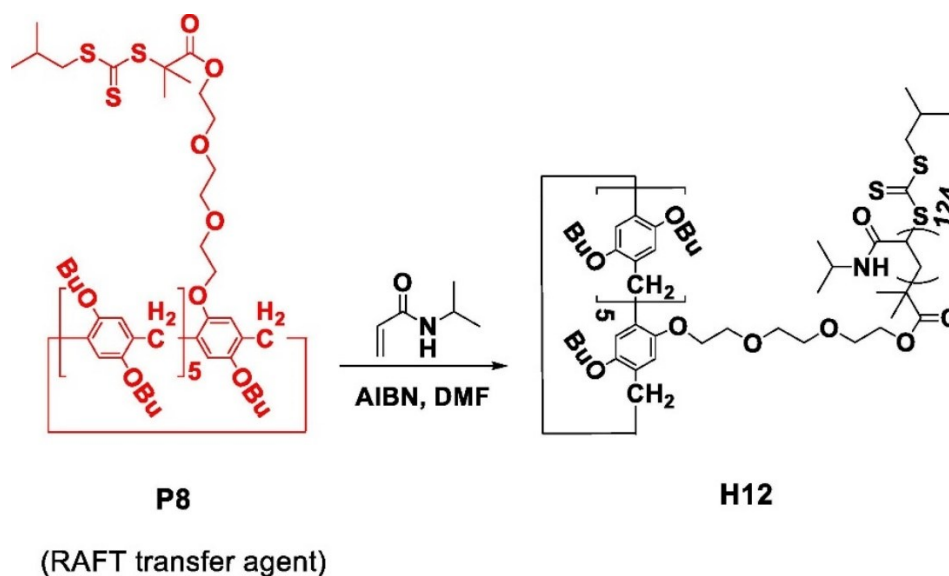


Figure 8. Example of a conjugation between a pillar[6]arene and a RAFT agent (**P8**), and subsequent polymerization, with NIPAA as monomer, to obtain polymer **H12**. Figure adopted by reference 20.

Another study carried out by Laggoune *et al.*²¹ exploits the use of pillarene, also bearing a single arms containing the trithiocarbonate-type RAFT agent, **MePilla-CTA**, for the polymerization of dimethylacrylamide (DMAC). This allows for the formation of the methylated pillar[5]arene-functionalized polydimethylacrylamide (PDMAC) (**MePilla-PDMAC**) reported in Figure 9. A key attribute of the PDMAC chain is its ability to enhance the solubility of the methylated pillar[5]arene scaffold in

both aqueous and organic solvents, a useful feature exploited in the study for the complexation of electron deficient guest molecules.

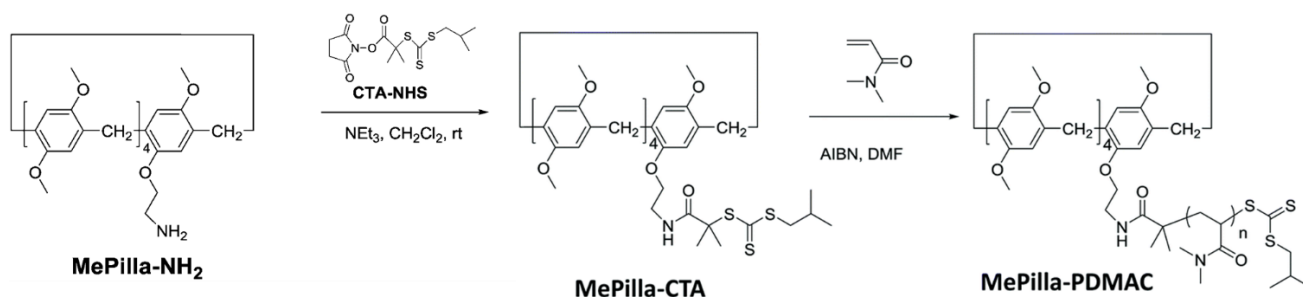


Figure 9. Example of a conjugation between a pillar[5]arene and a RAFT agent (**MePilla-CTA**), and subsequent polymerization, with dimethylacrylamide (DMAC) as monomer, to obtain polymer **MePilla-PDMAC**. Figure adopted from reference 21.

5.1.5 Thermoresponsive NIPAA polymers

Poly(N-isopropylacrylamide) (PNIPAA) is a widely recognized thermo-sensitive polymer that experiences a reversible shift from a coil to a globule conformation in water at approximately 32 °C, which represents its lower critical solution temperature (LCST).²² When the temperature exceeds the LCST, PNIPAA displays hydrophobic characteristics and forms colloidal structures as a result of the breaking of intermolecular hydrogen bonds with water, a process that leads to the aggregation of PNIPAA chains. In contrast, below the LCST, PNIPAA becomes soluble in water, adopting a random coil structure, where the hydrogen bonds between water and PNIPAA are restored.²³

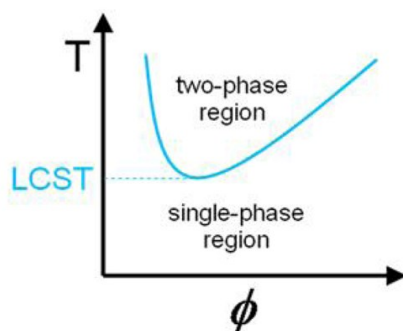


Figure 10. Temperature versus polymer volume fraction, ϕ . Schematic diagram illustrating the phase behavior of a polymer solution with lower critical solution temperature (LCST) characteristics.

This interesting feature is well studied in literature, and its applications can be found in drug delivery, gene delivery and tissue engineering.²⁴

5.2 Results and discussion

Although the final goal of the project is to polymerize different types of monomers at the lower rim of calix[4]arenes functionalized with zwitterionic amino acids at the upper rim, in this thesis work the problem is simplified and only calix[4]arenes having no substituents at the upper rim will be used. The RAFT agent will be conjugated at the lower rim of calix[4]arenes and polymerization reaction with NIPAA will be explored in order to optimize the experimental protocols. Two different lengths of the linkers between the phenolic oxygen atom and the RAFT agent will be used. Moreover, calixarenes bearing one or two RAFT agents will be polymerized to understand what the influence of the number of chain-transfer agents on the polymer structure is.

5.2.1 Synthesis of CTA calixarenes

The synthesis of target products **4a** and **4b**, illustrated in Figure 11, was not previously documented in the literature. Consequently, we elected to adhere to established protocols within our research group for the initial two steps. Following the selective alkylation of two phenolic units in 1,3 distal positions, the exhaustive alkylation of compound **1** was performed using N-(4-bromobutyl)phthalimide as the alkylating agent, resulting in the formation of product **2a**. Similarly, phthalimide N-(6-bromohexyl)phthalimide was used to obtain product **2b**. These reactions were conducted at room temperature for a period of two hours, to prevent the formation of byproducts resulting from the opening of the phthalimide ring, probably by hydrolysis. The phthalimide was then cleaved by refluxing the products **2a,b** with hydrated hydrazine in ethanol for four hours according to the protocol described in the literature,²⁵ giving **3a** and **3b** in yields of around 60%. The products were reacted with 4-cyano-4-(propylsulfanyltiocarbonyl)sulfanylpentanoic acid mercaptothiazoline amide (**activated RAFT agent**) in dry dichloromethane and in presence of diisopropylethylamine (DIPEA) to give the final calix-RAFT conjugates **4a** and **4b** after purification by flash chromatography.

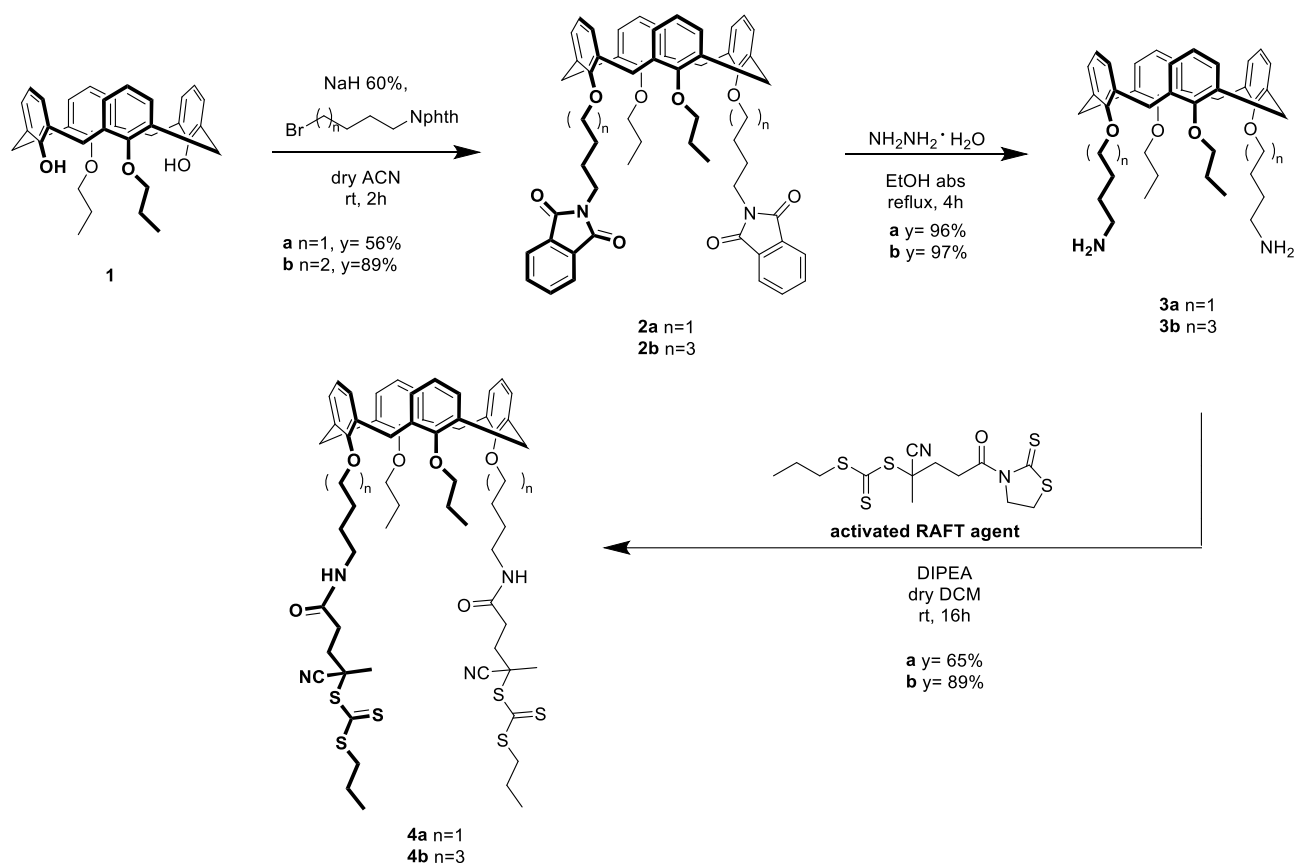
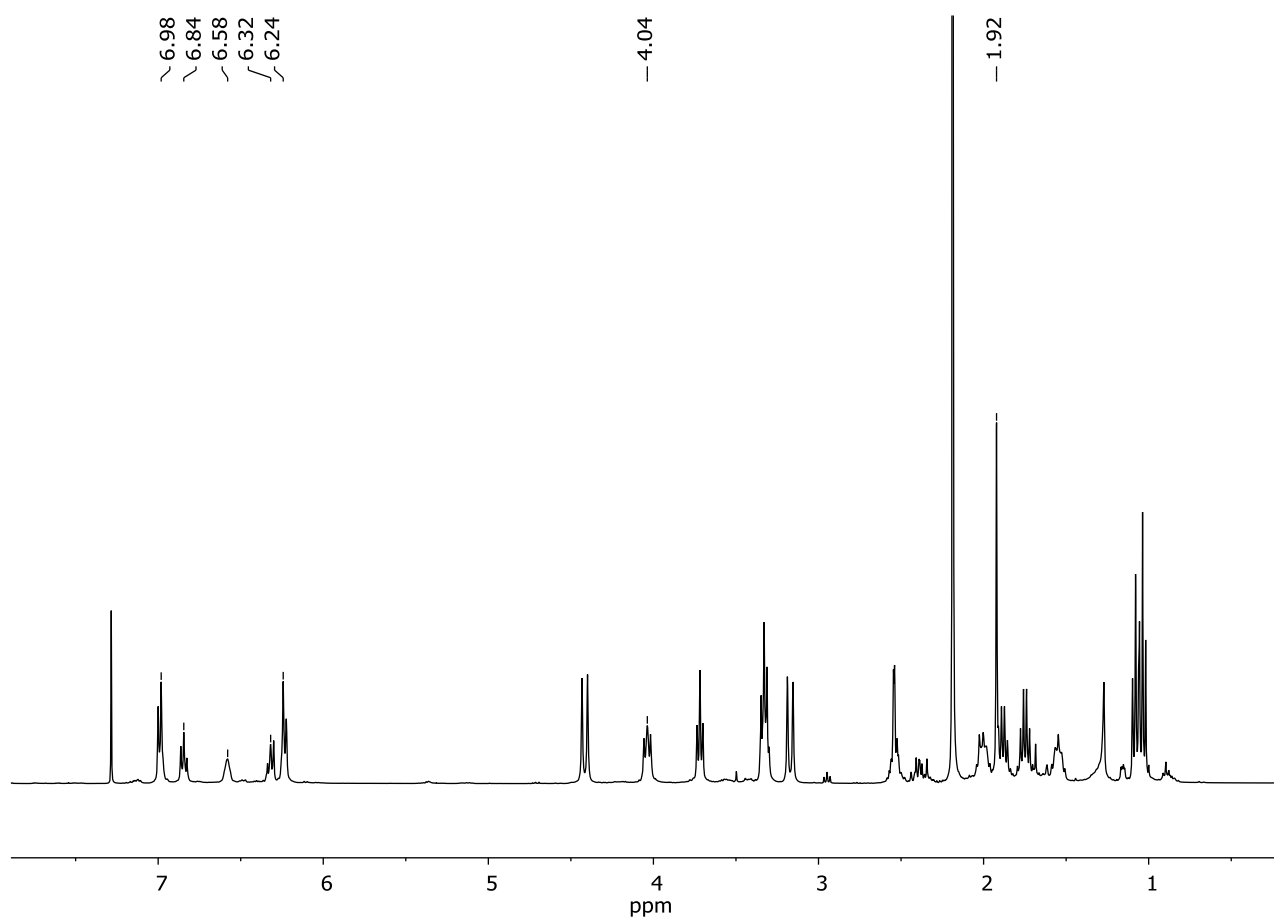
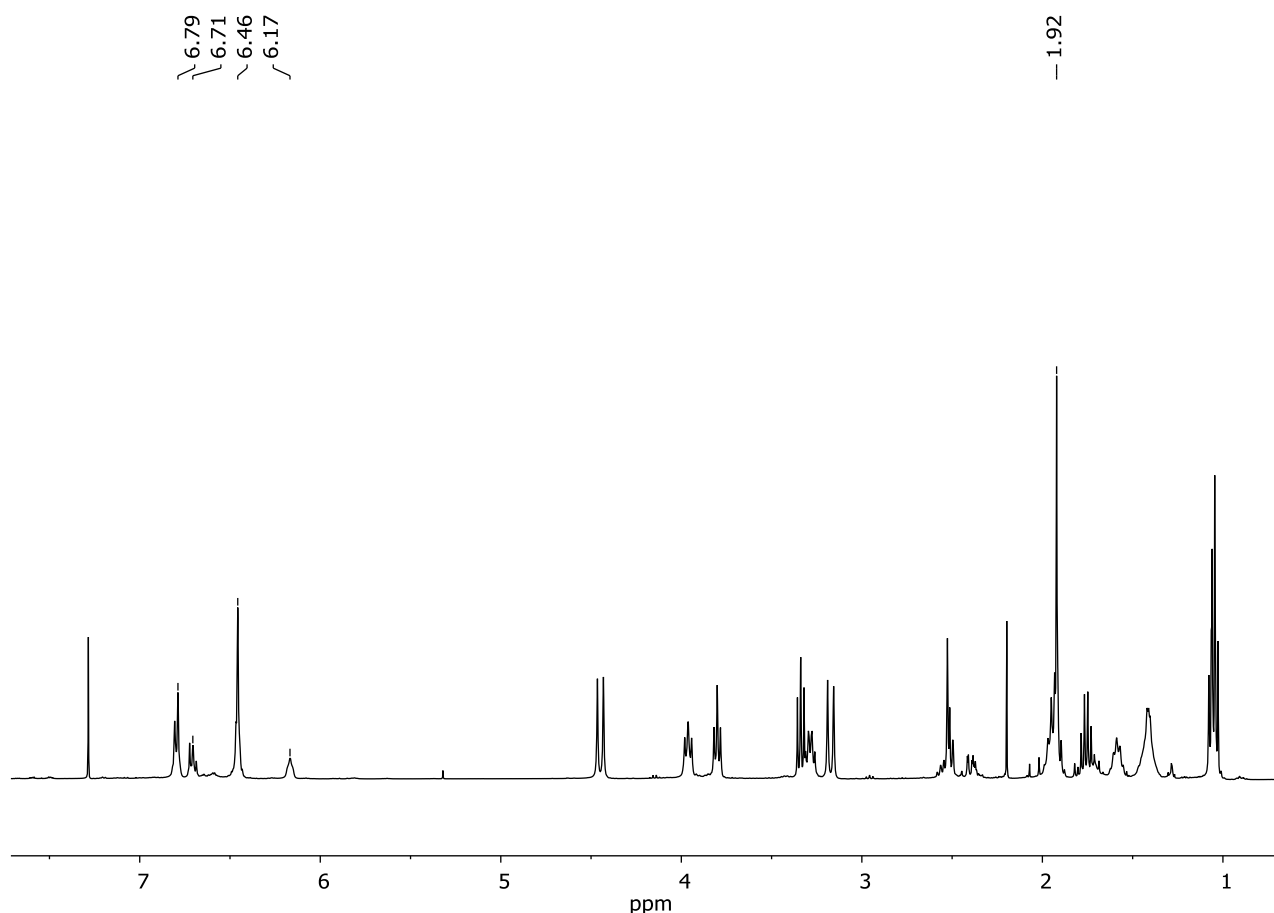
Figure 11. Scheme of synthesis for compounds **4a** and **4b**.

Figure 12 shows the ^1H NMR spectrum of compound **4a**. Diagnostic signals confirming the identity of the product are the broad singlet of the amide proton (6.58 ppm) and the appearance of the RAFT-agent signals, all present in the ppm range from 3.5 to 1. In particular, the methyl proton in α respect to the cyanide group of the RAFT agent can be identified as the singlet at 1.92 ppm.

Figure 12. ^1H NMR (CDCl_3 , 400 MHz) of compound **4a**.

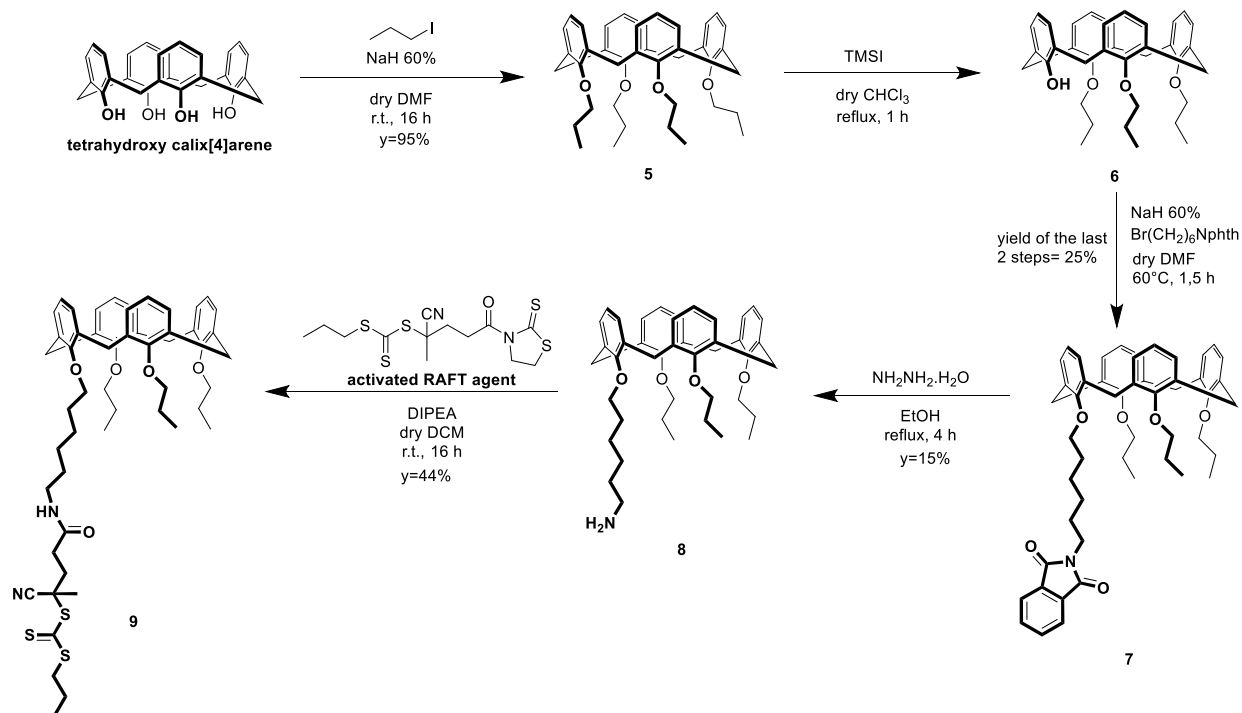
The ^1H NMR of product **4b** (Figure 13) exhibits a high degree of similarity to that of **4a**. However, a discernible difference is observed in the pattern of aromatic signals. In the first case, compound **4a**, the two pairs of aromatic rings, which are differently functionalized at the lower rim, present a distinctive signal pattern comprising two pairs of triplet and doublet signals, respectively at 6.98 and 6.84 ppm, and 6.32 and 6.24 ppm. Compound **4b** instead presents a triplet and doublet at 6.79 and 6.71 ppm, while the other six aromatic protons are nearly isochronous and give rise to a singlet at 6.46 ppm.

In this case, the amide signal, observed at 6.17 ppm and the signal of the methyl in α to the cyanide of the RAFT agent at 1.92 ppm serve to corroborate the favorable outcome of the coupling reaction.

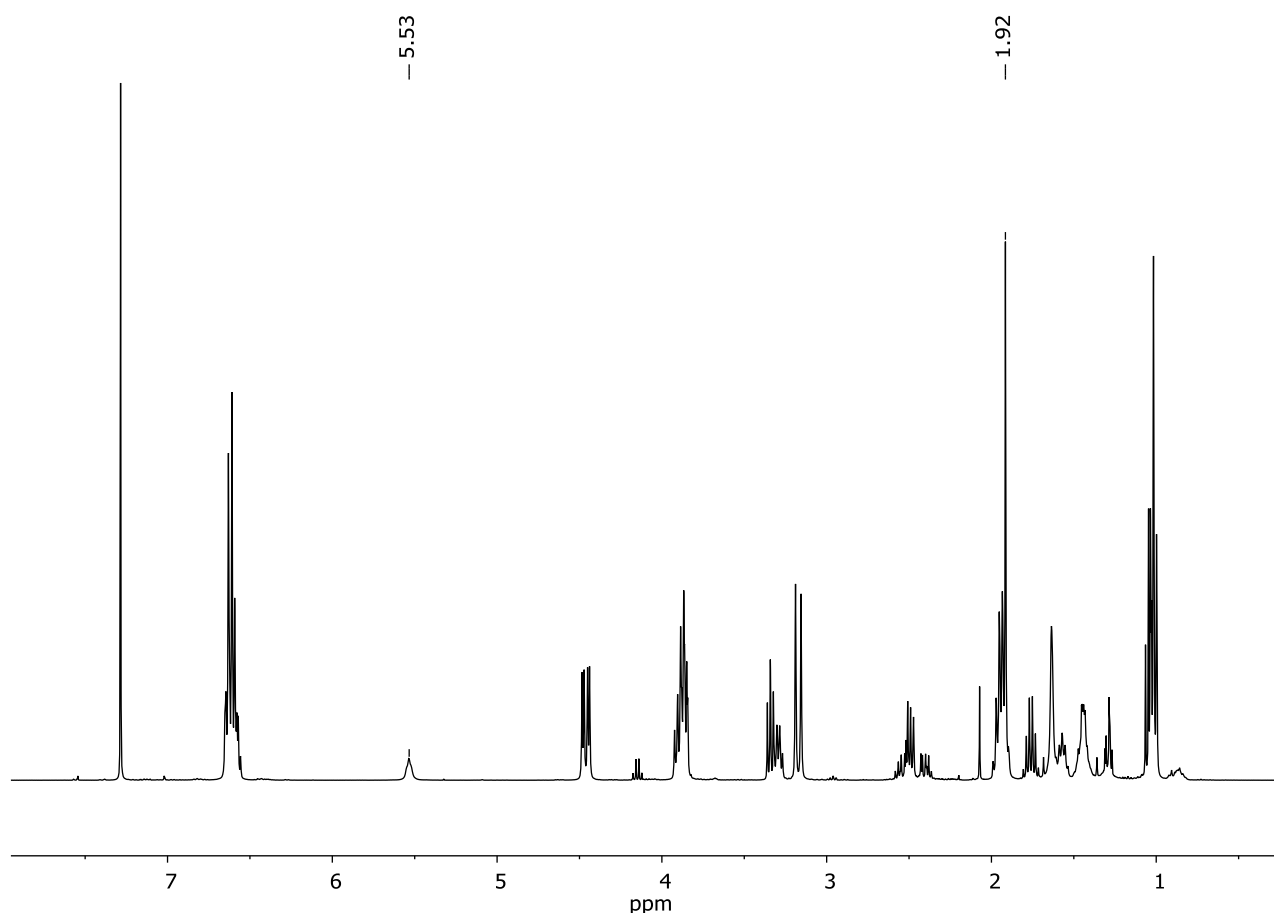
Figure 13. ^1H NMR (CDCl_3 , 400 MHz) of compound **4b**.

In the case of the asymmetric mono-functionalized calixarene **9**, the synthetic pathway (Figure 14) requires two additional steps in order to reach the phthalimide derivative **7**. The tetrahydroxy calix[4]arene was subjected to exhaustive alkylation with four *n*-propyl groups, after which one of the alkyl groups was cleaved with trimethylsilyl iodide. This resulted in the trialkylated product **6**, which was obtained in mixture with the reagent **5**. Since it was impossible to separate the tripropylated (**6**) from the tetrapropylated (**5**) calix[4]arene with either recrystallization or flash chromatography, this crude was used for the following reaction and a successful separation was then achieved after the alkylation of the free hydroxyl group of **6** with *N*-(6-bromohexyl)phthalimide. This indirect, longer strategy was preferred over the standard procedure²⁶ of direct trialkylation of the tetrahydroxy calix[4]arene with BaO_2 and $\text{Ba}(\text{OH})_2$ since the latter one, in our hands, brought to the formation of only small quantities of tri-functionalized products in comparison to the tetra-alkylated ones.

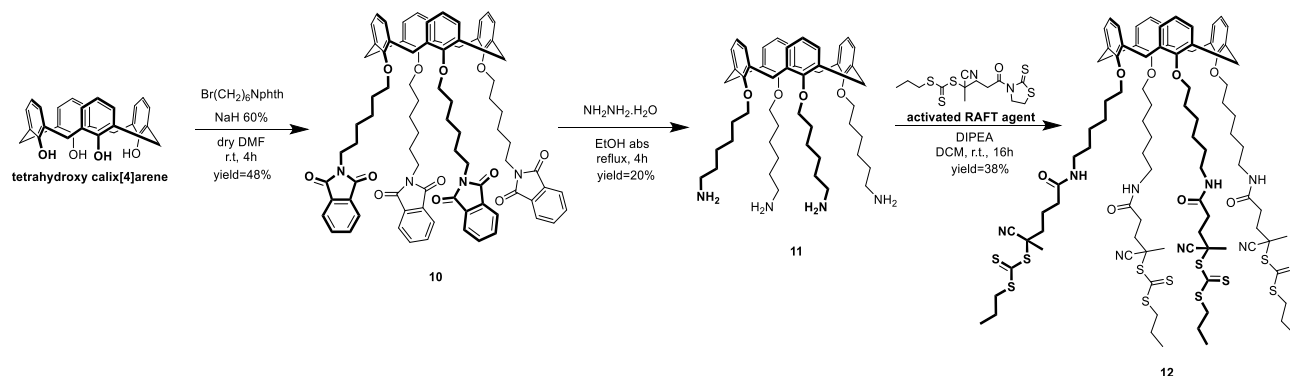
The phthalimide group of compound **7** was cleaved using hydrazine hydrate, yielding product **8**, though with a modest isolated yield. The coupling reaction to obtain product **9** was then performed with the **activated RAFT agent** in the presence of DIPEA, and after purification through flash chromatography, product **9** was obtained in a 44% yield.

Figure 14. Scheme for the synthesis of compound **9**.

In the ^1H NMR spectrum of product **9**, shown in Figure 15, the amide signal is observed at 5.53 ppm, confirming the successful outcome of the reaction. Additionally, the proton signals corresponding to the RAFT agent, including the methyl group at 1.92 ppm, could be also identified.

Figure 15. ^1H NMR (CDCl_3 , 400 MHz) of compound **9**.

The tetra functionalized calix[4]arene **12** was synthesized as shown in Figure 16. The tetrahydroxy calix[4]arene was exhaustively alkylated using N-(6-bromohexyl)phthalimide and sodium hydride. The reaction was stirred at room temperature for four hours and subsequently quenched, despite incomplete conversion of the reagent. This was necessary due to the formation of a byproduct resulting from phthalimide ring opening. Once purified from the crude, the phthalimide groups of compound **10** were then cleaved using hydrazine hydrate to afford product **11**. The modest yield of the latter step is probably due to problems in the isolation of the tetramine **11**, likely due to its partial solubility in water during the work-up. The coupling reaction was subsequently carried out with the **activated RAFT agent** in the presence of DIPEA, and following purification by flash chromatography, product **12** was obtained with a 38% yield.

Figure 16. Scheme for the synthesis of compound **12**.

In the ^1H NMR spectrum of product **12**, shown in Figure 17, the amide signal is observed at 6.86 ppm, confirming the successful outcome of the reaction. Additionally, the proton signals corresponding to the RAFT agent, including the methyl group at 1.92 ppm, are also identified.

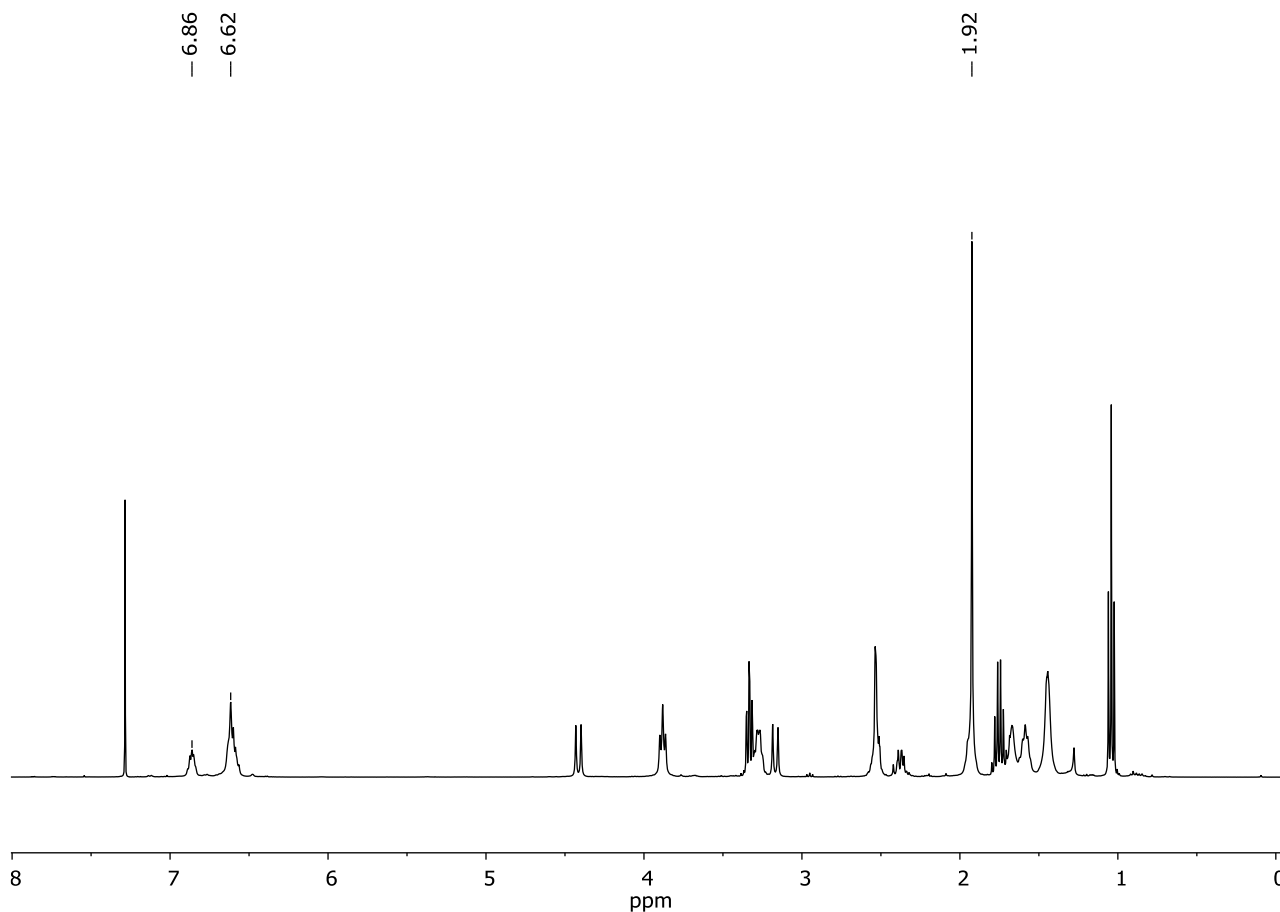


Figure 17. ^1H NMR (CDCl_3 , 400 MHz) of compound **12**.

5.2.2 Polymers synthesis

After the RAFT agent was successfully conjugated to the calixarene scaffolds, the polymerization reaction was carried out using N-isopropylacrylamide (NIPAA) as the primary monomer. The optimized molar ratios between the RAFT agent (in this case, the functionalized calixarene), the specific monomer and radical initiator had been previously established by Prof. Pierangelo Gobbo's group and were therefore adopted without further modification. The concentration of the monomer in acetonitrile is 2 M, and the equivalents of radical initiator and RAFT agent are calculated from the monomer quantity. The millimoles of radical initiator are 0.18% of the monomer, while the RAFT agent is 5 times the initiator, therefore 0.9% of the monomer. The effective quantity of chain transfer agent, the properly conjugated calixarene in this case, should take into account that for each calixarene more than one RAFT agent moiety is present for products **4a**, **4b**, **9** and **12**.

The first three polymers discussed in this section (**13-15**) were synthesized in Pierangelo Gobbo's laboratories at the University of Trieste, where I spent three weeks gaining practical experience in managing RAFT polymerization.

The initial trial aimed to obtain a double-branched polymer, designated as polymer **13**, with a theoretical molecular weight of 22,000 Da (denoted as $M_n(\text{theor})$). This target molecular weight, $M_n(\text{theor})$, represents the ideal value, achievable by carefully balancing the quantities of monomer and chain

transfer agent (CTA) in the reaction. The polymerization was carried out in acetonitrile for an unusually long reaction time of 24 hours, an arbitrary time chosen due to the unknown kinetics of the reaction on such macrocycles. Azobisisobutyronitrile (AIBN) was used as the radical initiator, with the temperature maintained at 65 °C, the temperature at which AIBN undergoes homolytic decomposition. The monomer, the chain transfer agent (CTA), in this case compound **4b**, the radical initiator and the internal standard DMF were dissolved in acetonitrile in a Schlenk tube. The gas dissolved in the solution was removed via five freeze-pump-thaw cycles, then filled with argon and closed. The Schlenk tube was soaked into an oil bath at 65 °C, to activate the radical initiator and start the polymerization.

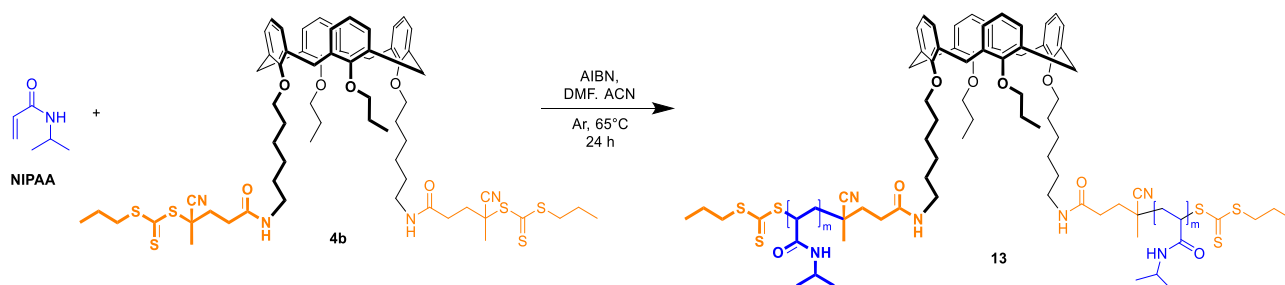


Figure 18. Reaction scheme to obtain polymer **13**.

Before initiating the polymerization reaction, a 40 μL aliquot of the reaction mixture was collected as the t_0 sample to enable calculation of monomer conversion and residual monomer content using NMR analysis.

Upon completion of the reaction, the mixture was rapidly frozen in liquid nitrogen to stop the polymerization, and the Schlenk tube cap was removed to allow air to displace the internal argon atmosphere, and the oxygen present to effectively quench the radicals responsible for chain growth. After returning the mixture to room temperature, a second 40 μL aliquot (the t_f sample) was collected from the crude for comparison with the t_0 sample. Both aliquots were diluted with deuterated chloroform, and their ^1H NMR spectra were recorded, shown in Figure 19.

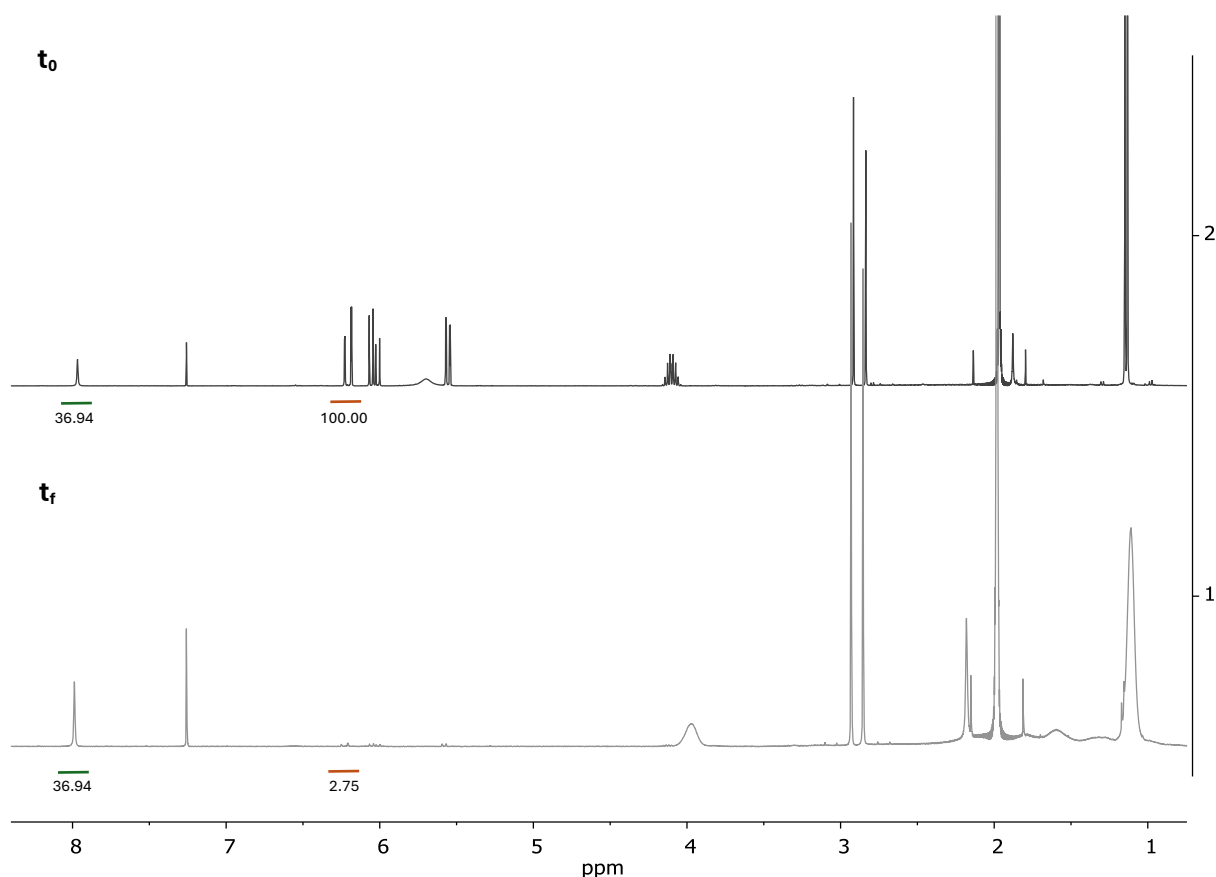


Figure 19. ^1H NMR (CDCl_3 , 400 MHz) spectra of the t_0 (track 2) and t_f (track 1) for the polymerization reaction in Figure 18. The residual monomer content indicates a conversion degree of 97%.

The monomer conversion degree was estimated to be 97%, which is to be considered too high for RAFT polymerization, where the optimal conversion is typically around 80%. Beyond this point, the likelihood of radical recombination between growing chains (star-to-star and branch-to-branch) increases significantly, resulting in a loss of polymer monodispersity and of the trithiocarbonyl groups from the polymers, event that prevents the possibility to add further polymers blocks. The degree of conversion was quantified by employing DMF as an internal standard. This was achieved by comparing the singlet of the formamide HCO proton at 8.01 ppm with the signal of the monomer vinyl proton at 6.25 ppm at the initial (t_0) and final (t_f) time. In the t_0 spectrum, the integral of the selected monomer proton (highlighted in red) was standardized to 100.00, after which the DMF proton (highlighted in green) was integrated. Given that DMF was added as a stable internal standard, its quantity remained constant in both aliquots, t_0 and t_f . Consequently, the same DMF signal was integrated in the t_f spectrum and set to the same reference value as in t_0 , enabling a direct and accurate quantification of the residual monomer signal based on the integration of its respective proton. The conversion degree is calculated by the following equation:

$$\text{conversion degree} = \left(100 - I_{\text{mon}}(t_f)\right) * 100$$

Where $I_{\text{mon}}(t_f)$ is the intensity of the integral (calibrated in comparison with the spectrum at t_0 as previously described) of the residual monomer signal in the t_f ^1H NMR spectrum.

Using the conversion degree, it is possible to give an estimation of the average molecular weight of the polymer, here denoted as $M_n(\text{conv})$, because it is calculated from the monomer conversion. Knowing the initial number of moles of monomer (n_{NIPAA}), it is possible to calculate how many monomers on

average are attached, and knowing their molecular weight, to estimate the average M_n of the polymer. The effective moles (n_e) of NIPAA that have polymerized are calculated according to the following equation:

$$n_{e\text{ NIPAA}} = n_{\text{NIPAA}} * \text{conv degree}$$

The number of monomers units (n_{monNIPAA}) in every polymer chain can be calculated considering that in RAFT polymerization the RAFT agent is the end group of every chain:

$$n_{\text{monNIPAA}} = n_{e\text{ NIPAA}} / n_{e\text{ calixarene}}$$

where $n_{e\text{RAFTagent}}$ is the number of moles of calixarene-based RAFT reagent (independently from the number of CTA per calixarene).

The average molecular weight of the polymer is therefore:

$$M_n(\text{conv}) = n_{\text{monNIPAA}} * PM_{\text{NIPAA}} + (1) * PM_{\text{calixarene}}$$

This is the general equation to be used when RAFT agent bears a single CTA. However, in the case of core with multiple (m) CTA agent moieties (branches) the last equation becomes:

$$M_n(\text{conv}) = m * n_{\text{monNIPAA}} * PM_{\text{NIPAA}} + (1) * PM_{\text{RAFT}}$$

Considering that in the case of polymer **13**, for every chain transfer agent (the calixarene) two chains of polymer are generated, with the equations shown above, the $M_n(\text{conv})$ of the polymer obtained is ca. 24,000 Da, that corresponds to approximately 196 monomers per calixarene unit.

The polymer was precipitated as a yellow solid and purified from unreacted monomer and solvent by adding the reaction mixture to a 1:1 mixture of hexane and diethyl ether. After centrifugation, the supernatant was removed, and the pale-yellow resin was dissolved in a minimal amount of dichloromethane, followed by a second precipitation in the hexane-diethyl ether mixture. The polymer was then dried under vacuum and its ^1H NMR spectrum was recorded (Figure 20).

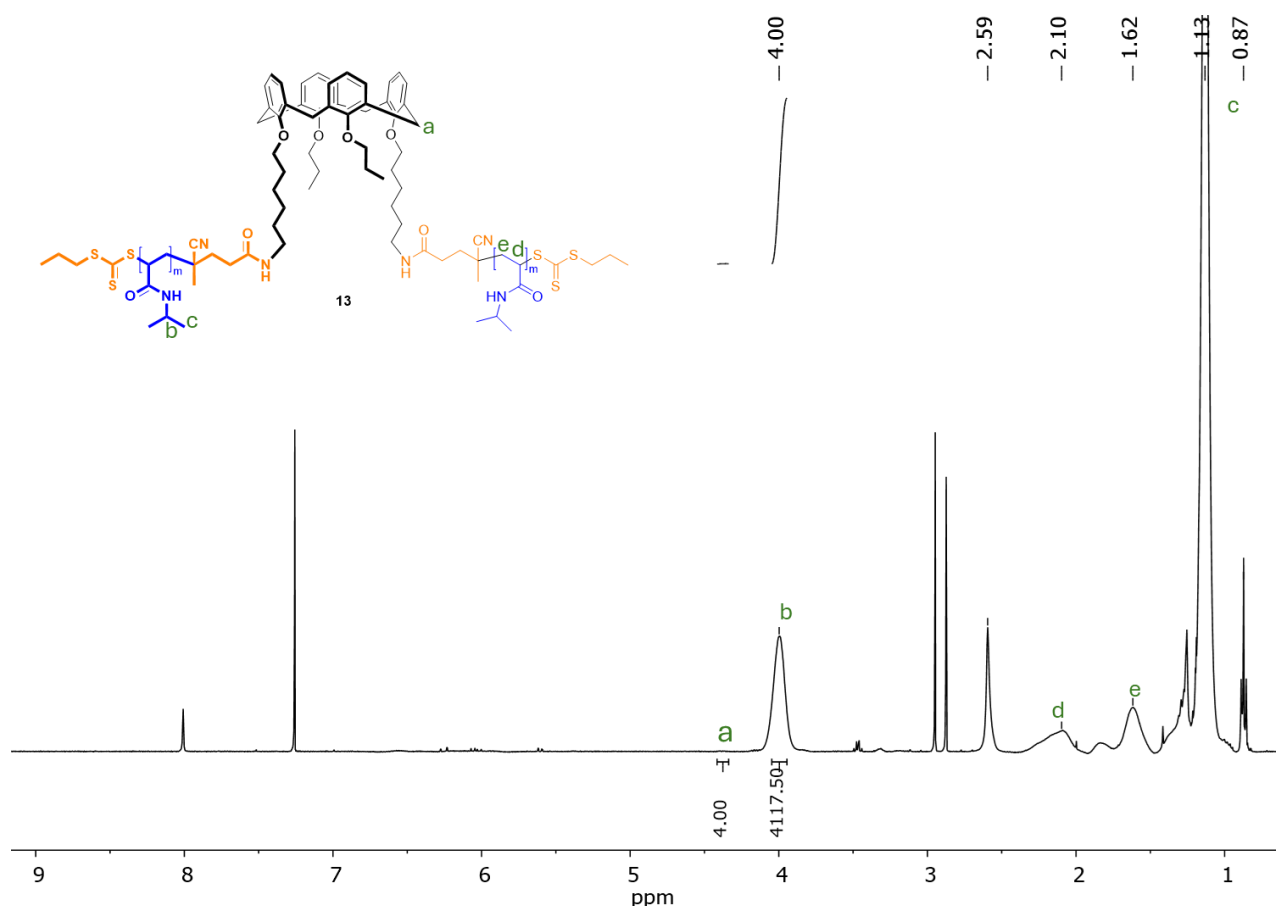


Figure 20. ^1H NMR (CDCl_3 , 400 MHz) spectrum of polymer **13** after precipitation.

In the ^1H NMR spectrum of the polymer, the protons of the methylene bridge (ArCH_2Ar) in the calixarene can be identified, albeit with some difficulty because of the low signal intensity, with the axial protons resonating at 4.38 ppm and the equatorial protons at 3.17 ppm. The presence of calixarene in the spectrum supports its inclusion in the polymer and that the macrocycle does not interfere with the RAFT polymerization process. Moreover, the polymerization reaction time using the NIPAA and this difunctionalized calixarene-based chain transfer agent (CTA) seems not to be significantly decreased compared to the polymerization time observed with the conventional monofunctional RAFT agents. The ^1H NMR spectrum of the polymer provides an estimation of the number-average molecular weight, here denoted as $M_n(\text{NMR})$ because it is calculated from the integral in the spectrum. This is achieved by determining n , the number of repeating units (monomers) in the polymer chain from the integrals of the NMR spectra and multiplying this value by the molecular weight of the monomer,²⁷ according to the equation:

$$M_n = n * (\text{NIPAA molar mass})$$

The value of n is obtained from the integral of the signal at 4.00 ppm, corresponding to the CH group of the isopropyl moiety in the NIPAA units (Figure 20), setting the signal of the axial protons of the methylene bridge of the calixarene as 4 protons (at 4.38 ppm), considering that the calixarene is included in the polymer. The integral calculated in this case is much higher than what would be expected from the conversion, reaching a value over 400,000 Da. This is likely attributable to the recombination of radicals generated during the reaction, as the conversion went over the optimal threshold, thereby increasing the likelihood of recombination and termination processes.

From the data obtained until now it is not possible to assess whether the two RAFT agents attached to the calixarene give rise to the growth of two identically long chains. Steric hindrance problems arise if a chain is growing much faster than the other, may impair or completely inhibit the elongation of the second polymer chains. It is, however, reasonable to think that, if this takes place, it happens at the beginning of the chain elongation since as far as the degree of polymerization increases the chains are probably going to diverge and to grow far apart each from the other and independently. Since each of the axial and equatorial $\text{ArCH}_{\text{ax}}\text{H}_{\text{eq}}\text{Ar}$ signals appear as single doublets, it does not seem that polymerization is blocked on one arm, at least not close to the calixarene lower rim.

In this latter case, where polymerization has proceeded to nearly completion, leaving only a minimal amount of residual monomer, no other signals of the hexyl spacer, of the CH_2CH_2 groups of the RAFT agent or of the calixarene scaffold, apart from the methylene bridge, are observable also because superimposed by the huge signals of the polymer protons.

Given the high average M_n of the polymer, we decided not to conduct further studies on this specific sample. A more comprehensive analysis, including the calculation of the weight-average molecular weight (M_w) based on gel permeation chromatography (GPC) data, is currently underway in University of Trieste, by Prof. Gobbo's group.

A kinetic study of this reaction (Figure 21) was performed by taking four 0.6 mL aliquots from a polymerization mixture containing all the necessary reagents, after taking a t_0 sample to calculate the conversion. Each of the four samples was transferred into a separate Schlenk tube. Each Schlenk tube underwent freeze-pump-thaw cycles obtaining air substitution with Argon. Then all the Schlenk tubes were simultaneously placed in an oil bath at 65 °C to initiate the polymerization. This procedure is extremely sensitive, as even a small amount of oxygen inside the Schlenk tube can impair the reaction by quenching the radical chain, given the very little amount of radical initiator present. Therefore, every step, from the freeze-pump-thaw cycles to the filling of the Schlenk tube with argon, must be executed with great care. The simultaneous handling of four Schlenk tubes adds further complexity to the procedure. Each polymerization reaction was allowed to proceed for a fixed time interval (1, 3, 5, and 7 hours, respectively). After the designated time had elapsed, the corresponding Schlenk tube was removed from the oil bath, and the reaction was stopped by freezing the solution with liquid nitrogen. Opening the cap of the Schlenk and letting air enter allow the reaction to be quenched, and the final time point (t_f) was registered.

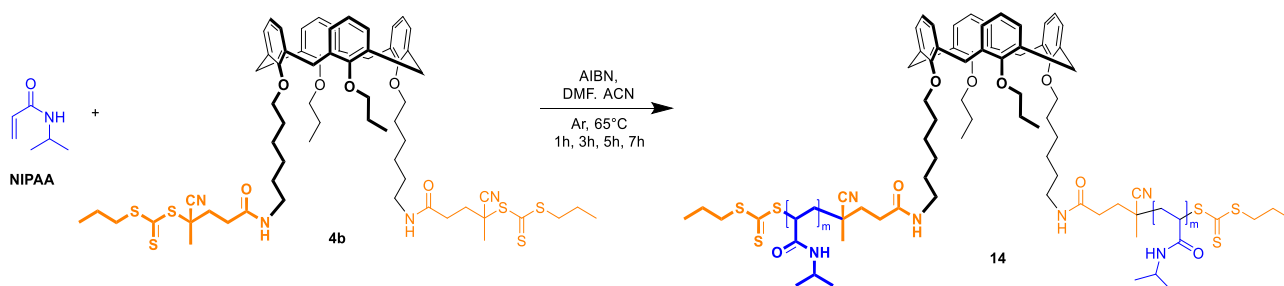


Figure 21. The kinetic study conducted to obtain polymer **14**. The polymerization was carried out in four separated Schlenk tubes using the same original mixture of reactants, solvent and initiator. Each tube was quenched separately after 1 hour, 3 hours, 5 hours and 7 hours.

Likely due to the presence of air in the first two Schlenk tubes, the reactions quenched after 1 and 3 hours showed no evidence of polymer formation, as the ^1H NMR spectra were identical to those of the initial time point (t_0). In the following Figure 22 are shown the t_0 (track 3), the t_{f3} (track 2, collected after 5 hours) and t_{f4} (track 1, collected after 7 hours).

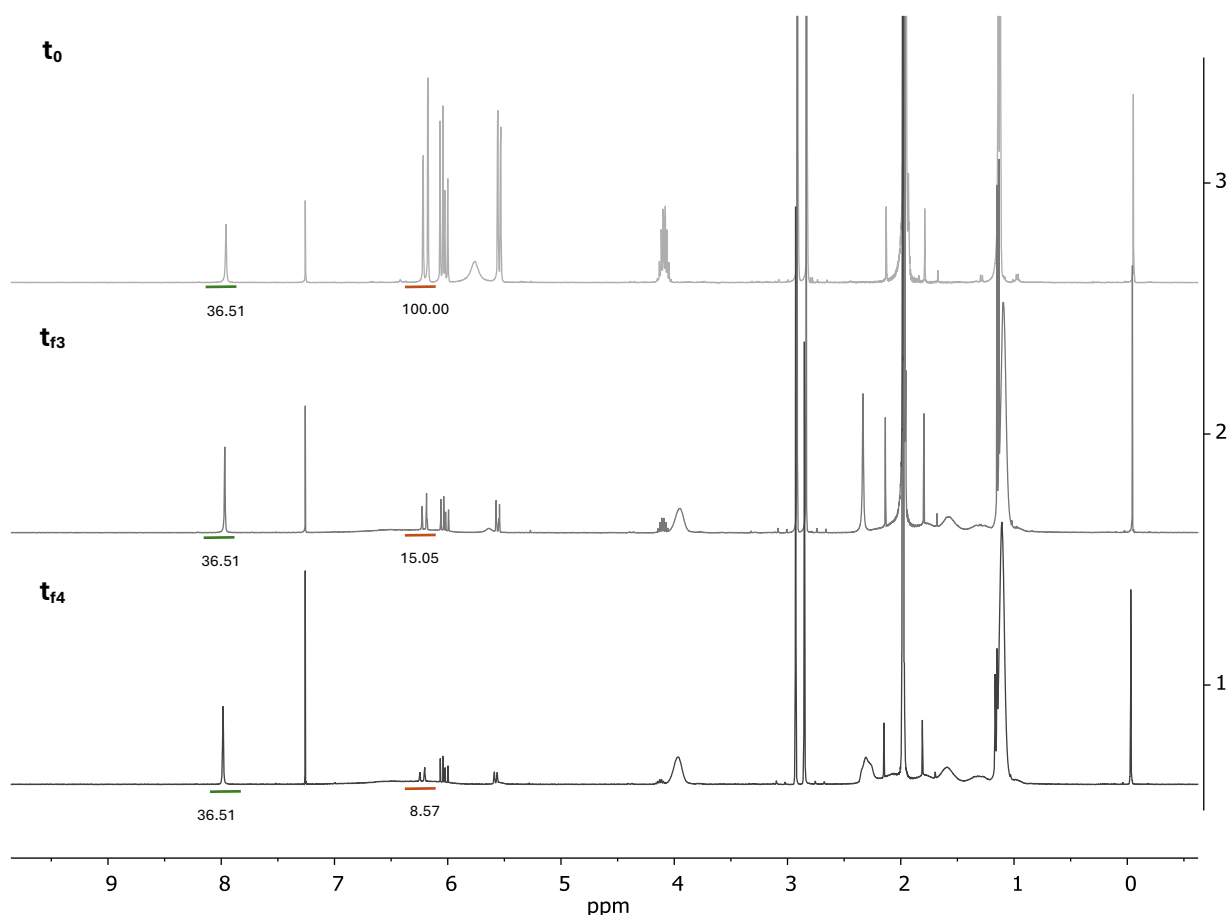


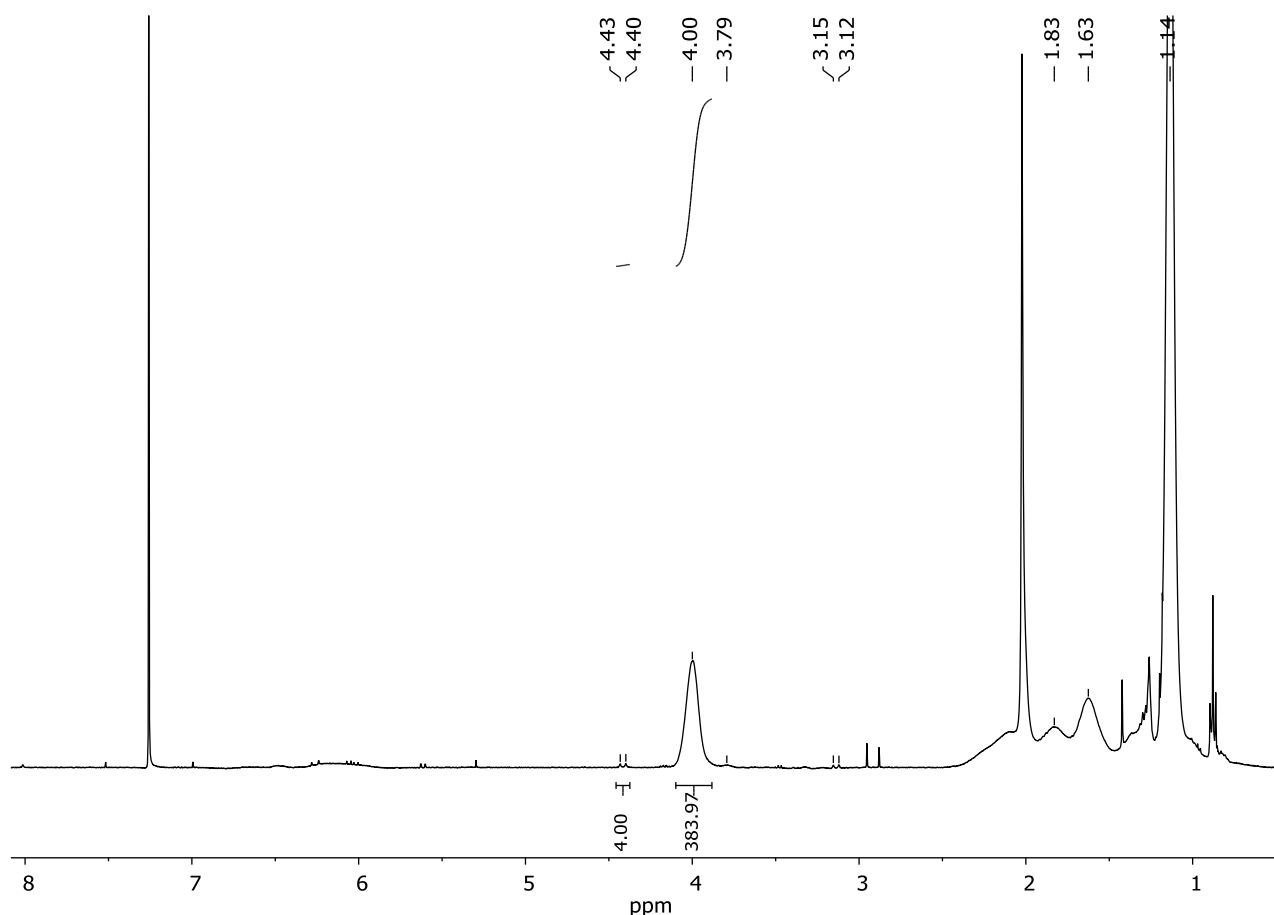
Figure 22. ^1H NMR (CDCl_3 , 400 MHz) spectra of the t_0 (track 3; 0 hours), t_{f3} (track 2; 5 hours) and t_{f4} (track 1; 7 hours) for the polymerization reaction in Figure 21. After 5 hours the monomer conversion was 84%, while after 7 hours the conversion was 91%.

The conversion after 5 hours was 84%, close to the optimal value for RAFT polymerization (80%). From the residual monomer quantity it was possible to estimate the $M_n(\text{conv})$ of polymer **14**, with the equation

$$M_n(\text{conv}) = 2 * n^{\circ} \text{mon}_{\text{NIPAA}} * PM_{\text{NIPAM}} + (1) * PM_{\text{RAFT}}$$

obtaining a $M_n(\text{conv})$ of 22,000 Da, with 186 monomers per calixarene.

The obtained polymer **14** from the reaction quenched after 5 hours was purified as described before, obtaining a yellow powder. Its ^1H NMR spectrum is shown in Figure 23. From the spectrum, it is possible to estimate a molecular weight $M_n(\text{NMR})$ of 43,000 Da (roughly 340 repeating units in the polymer and an average of 170 for each branch), considering that two polymer chains are attached to the two RAFT agent at the calixarene lower rim. Although this value deviates from the target theoretical molecular weight (11,000 Da per RAFT agent unit, that is an average of 22,000 Da per calixarene) programmed for this synthesis on the basis of the ratio between calixarene and monomer with a yield of 80%, this discrepancy is acceptable, as the error in the integral calculation might be relatively high since the integral of the ArCH_2Ar signals in the calixarene is considerably smaller than that of the polymer.

Figure 23. ^1H NMR (CDCl_3 , 400 MHz) spectrum of polymer **14** after precipitation.

In this polymer ^1H NMR spectrum, the doublets corresponding to both the axial (4.42 ppm) and equatorial (3.13 ppm) protons of the calixarene methylene bridge are more distinct compared to the previous case. This enhanced resolution allows for the calculation of the coupling constant, J , which is found to be 13.3 Hz, consistent with that observed in product **4b**. The remaining signals of the calixarene scaffold are not visible, except for a broad signal at 3.79 ppm, likely attributable by the chemical shift to the methylene (OCH_2) group of the propyl chain of the calixarene. The presence of polymer signals in the aliphatic region masks the less intense signals of the calixarene alkyl chains. Moreover, the aromatics are not visible due to the correction of the baseline applied on the spectrum in a region where the broad and intense peak of the NH of the polymer is also present.

The following polymerization reaction was carried out using the calixarene **4a** as CTA, having a shorter linker than compound **4b** (butyl chains instead of hexyl chains). The reaction was conducted under the same conditions, but the reaction time was limited to 5 hours in order to achieve an optimal monomer conversion of 80%, while all the other parameters remained unchanged. The reaction scheme for obtaining product **15** is shown in Figure 24.

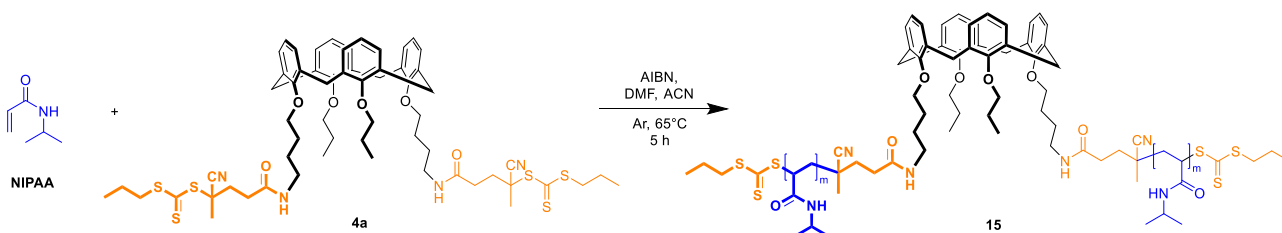
Figure 24. Reaction scheme to obtain polymer **15**, with a reaction time of 5 hours.

Figure 25 displays the ^1H NMR spectra for the reaction at t_0 (track 2) and t_f (track 1), recorded before the initiation of the reaction (t_0) and after quenching the radicals (t_f). In this case, the estimated monomer conversion was 86%, again closer to the optimal conversion value for RAFT polymerization. Therefore, under these conditions, the optimal polymerization time is approximately 4.5 to 5 hours. Interestingly, the similar kinetics and conversion degree starting from **4a** and **4b** seems to indicate that the different linker length between the calixarene and the RAFT agent does not affect the polymerization reaction.

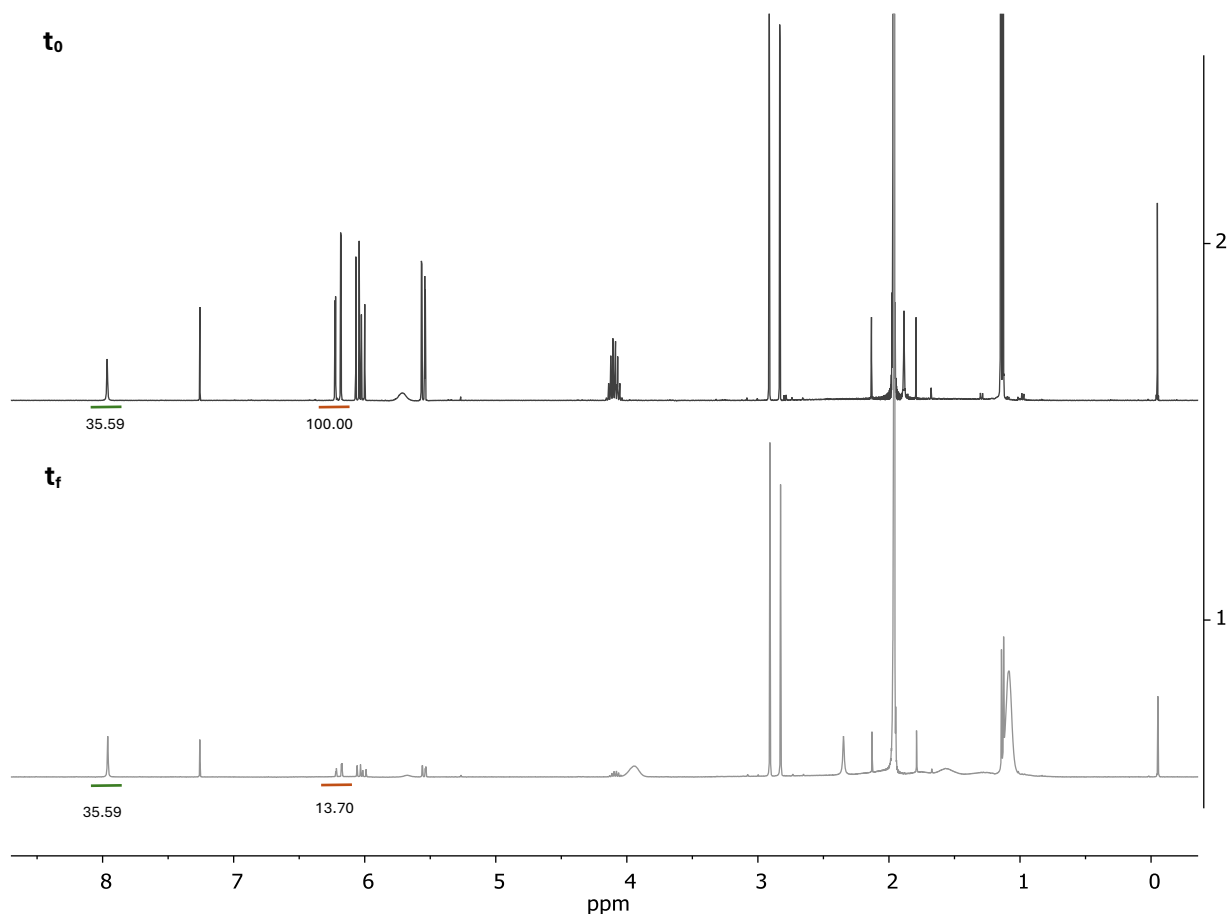


Figure 25. ^1H NMR (CDCl_3 , 400 MHz) spectra of the t_0 (track 2) and t_f (track 1) for the polymerization reaction in Figure 24. The residual monomer content indicates a conversion degree of 86%.

The $M_n(\text{conv})$ calculated by the residual monomer quantity is around 23,000 Da, with around 191 monomers per calixarene.

The reaction mixture was treated in the same way as described before, obtaining the polymer **15** as a yellow solid. The ^1H NMR spectrum was then recorded (Figure 26).

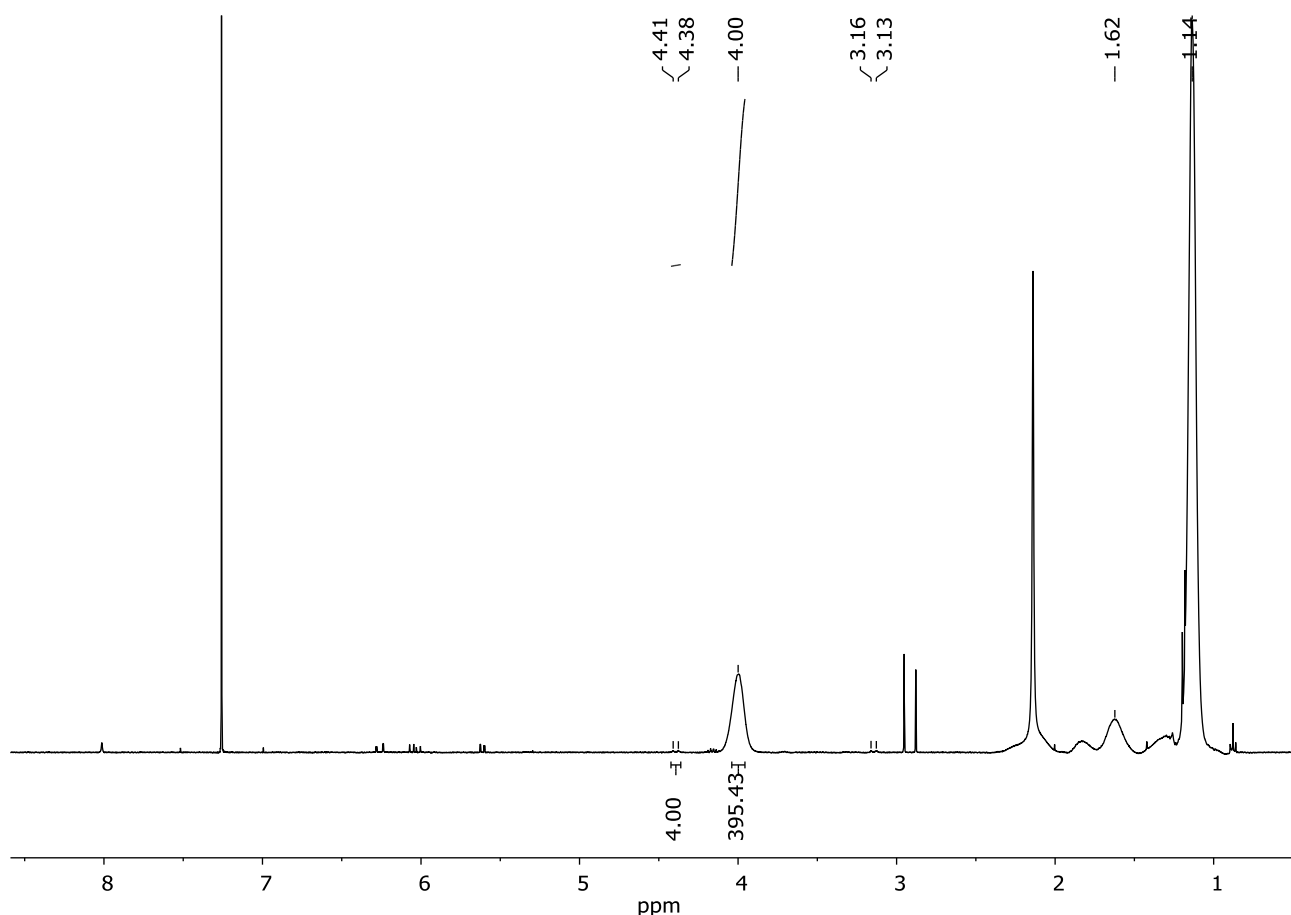


Figure 26. ^1H NMR (CDCl_3 , 400 MHz) spectrum of polymer **15** after precipitation.

In this case, the methylene bridge signals of the calixarene are more clearly visible in the spectrum, and they were used to estimate the M_n molecular weight of the obtained polymer **15**. For polymer **15**, the average molecular weight estimated by NMR, $M_n(\text{NMR})$ appears to be approximately 45,000 Da (roughly 400 repeating units in the polymer and an average of 200 for each branch). This value is higher than anticipated; however, it should be noted that this is merely a rough estimate, with only the order of magnitude being relevant for our purposes. For a more detailed analysis gel permeation chromatography (GPC) data are needed.

The subsequent two polymers were synthesized in our research group's laboratories at the University of Parma. The procedure followed was identical to that previously described, except for a change in the radical initiator. Specifically, 4,4'-azobis(4-cyanovaleric acid) (ACVA) was used, which undergoes thermally activated homolytic decomposition at 70 °C, as opposed to 65 °C for AIBN. This substitution was made due to the unavailability of AIBN in our laboratory, and because ACVA possesses comparable characteristics.

The polymerization reaction was conducted under the same conditions with calixarene **4b**, at the activation temperature of ACVA (70 °C), for a duration of 5 hours (Figure 27).

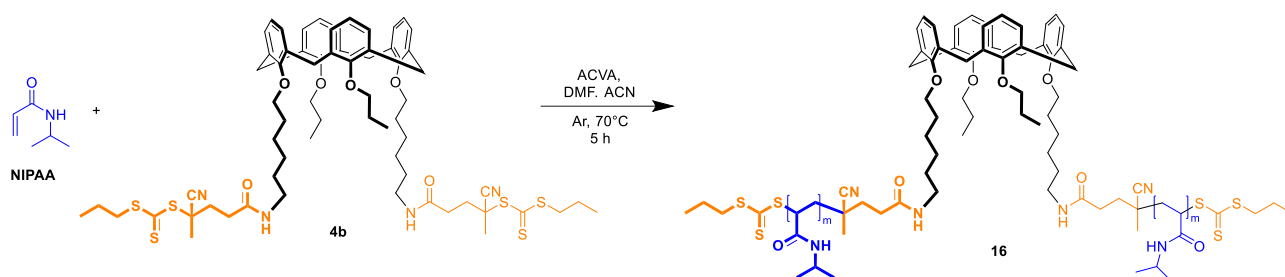
Figure 27. Reaction scheme to obtain polymer **16**, with a reaction time of 5 hours.

Figure 28 displays the ^1H NMR spectra for the reaction at t_0 (track 2) and t_f (track 1), recorded before the initiation of the reaction (t_0) and after quenching the radicals (t_f). In this case, the estimated monomer conversion was 88%, probably due to the higher temperature used.

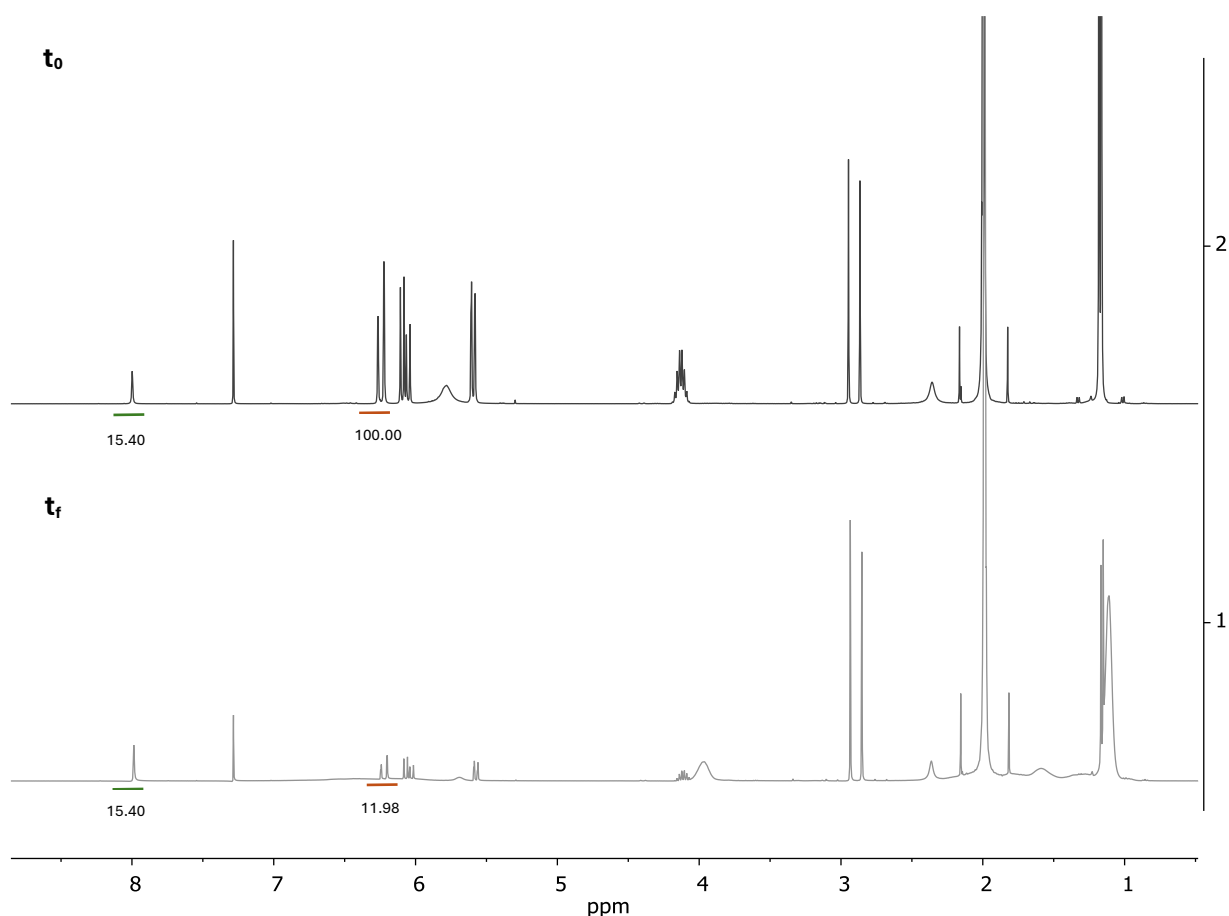


Figure 28. ^1H NMR (CDCl_3 , 400 MHz) spectra of the t_0 (track 2) and t_f (track 1) for the polymerization reaction in Figure 27. The residual monomer content indicates a conversion degree of 88%.

The $M_n(\text{conv})$ calculated by the residual monomer quantity is around 21,000 Da, with around 176 monomers per calixarene.

The obtained product **16** was purified from the excess of monomer and ^1H NMR recorded (Figure 29). The axial protons of the methylene bridge of the calixarene are visible as a doublet at 4.43 ppm, and the equatorial at 3.16 ppm. From the integral it seems that the polymer has a M_n of 51,000 Da (considering the overall two polymer chains attached to the calixarene), higher by a factor of two respect to the predicted one. This value means roughly 450 repeating units in the polymer and an average of 225 for each branch.

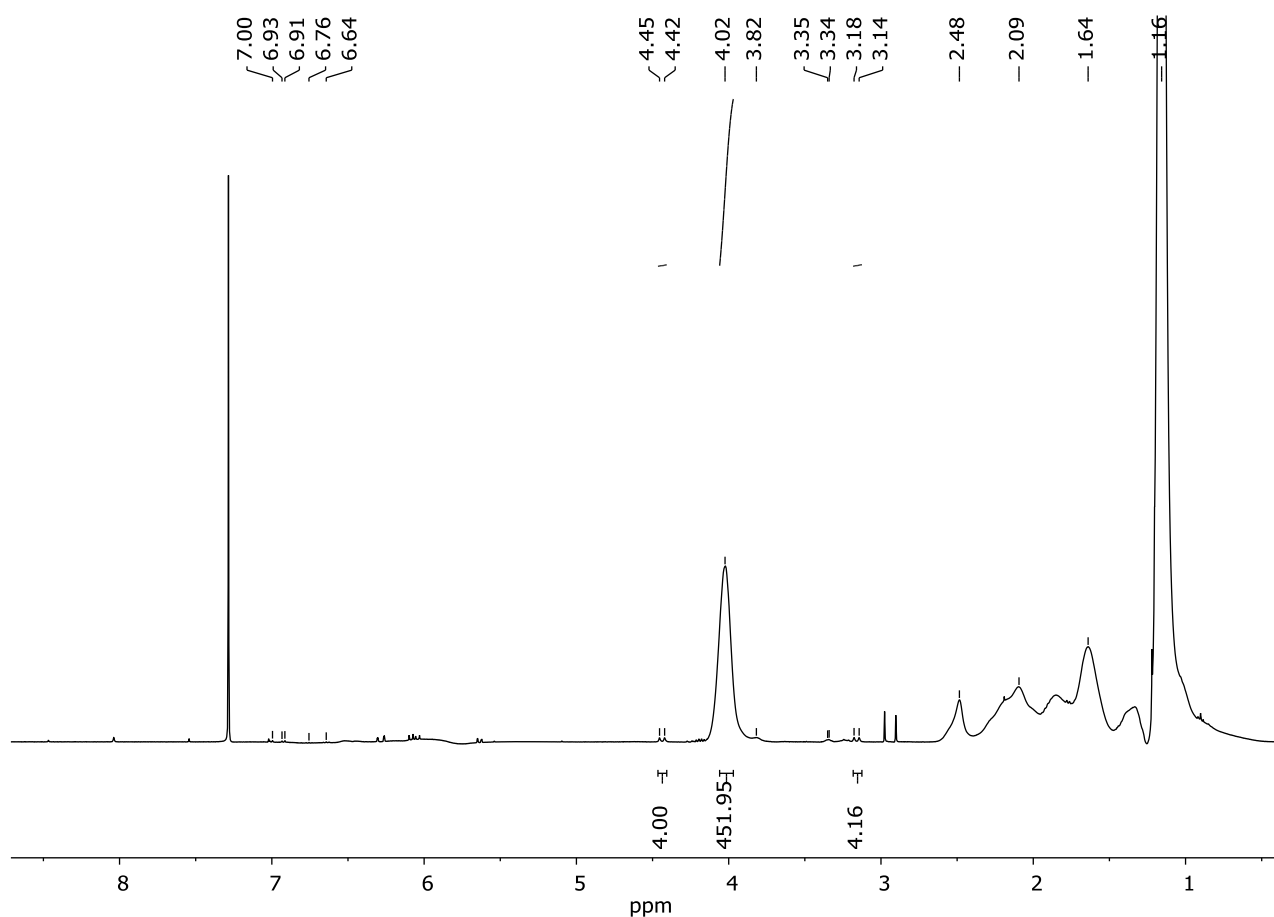


Figure 29. ^1H NMR (CDCl_3 , 400 MHz) spectrum of polymer **16** after precipitation.

In this case, additional signals corresponding to the calixarene scaffold can be identified, beyond those of the methylene bridge, including signals from the aromatic protons (Figure 30). The two doublets of the ArH_{meta} appear at 7.00 and 6.92 ppm, while the triplets are observed at 6.76 and 6.64 ppm, respectively. Moreover, the OCH_2 signal of the calixarene propyl chains and the SCH_2 of the RAFT agent can be identified, two broad signals at 3.82 and 3.34 ppm respectively.

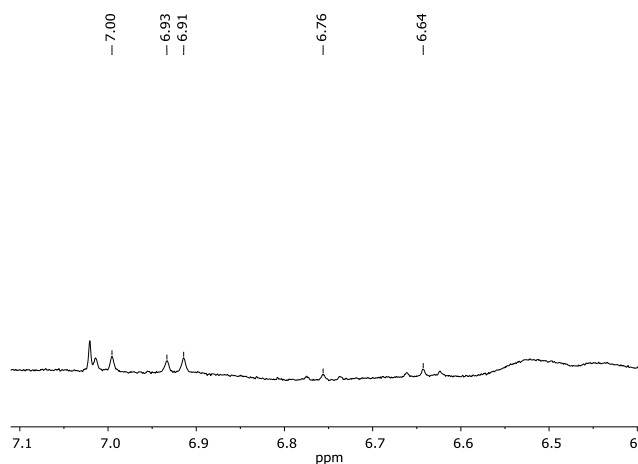


Figure 30. Magnification of the aromatic region of the ^1H NMR spectrum of polymer **16**.

We also attempted to synthesize a polymer with a lower molecular weight (6,000 Da) compared to the previous ones, by adjusting the quantity of monomer utilized. The experimental conditions remained consistent with those used in the prior synthesis, employing ACVA as the radical initiator and

maintaining the temperature at 70 °C. The polymerization process (Figure 31) was conducted for 4.5 hours.

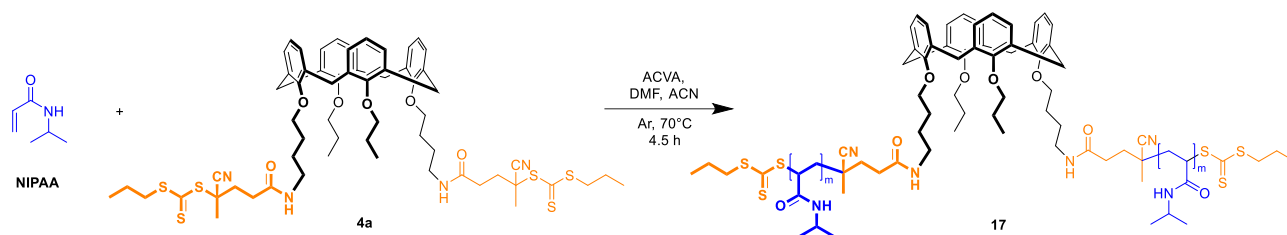


Figure 31. Reaction scheme to obtain polymer **17**, with a reaction time of 4.5 hours.

Figure 32 displays the ^1H NMR spectra for the reaction at t_0 (track 2) and t_f (track 1), recorded before the initiation of the reaction (t_0) and after quenching the radicals (t_f). Also in this case, the estimated monomer conversion was 88%.

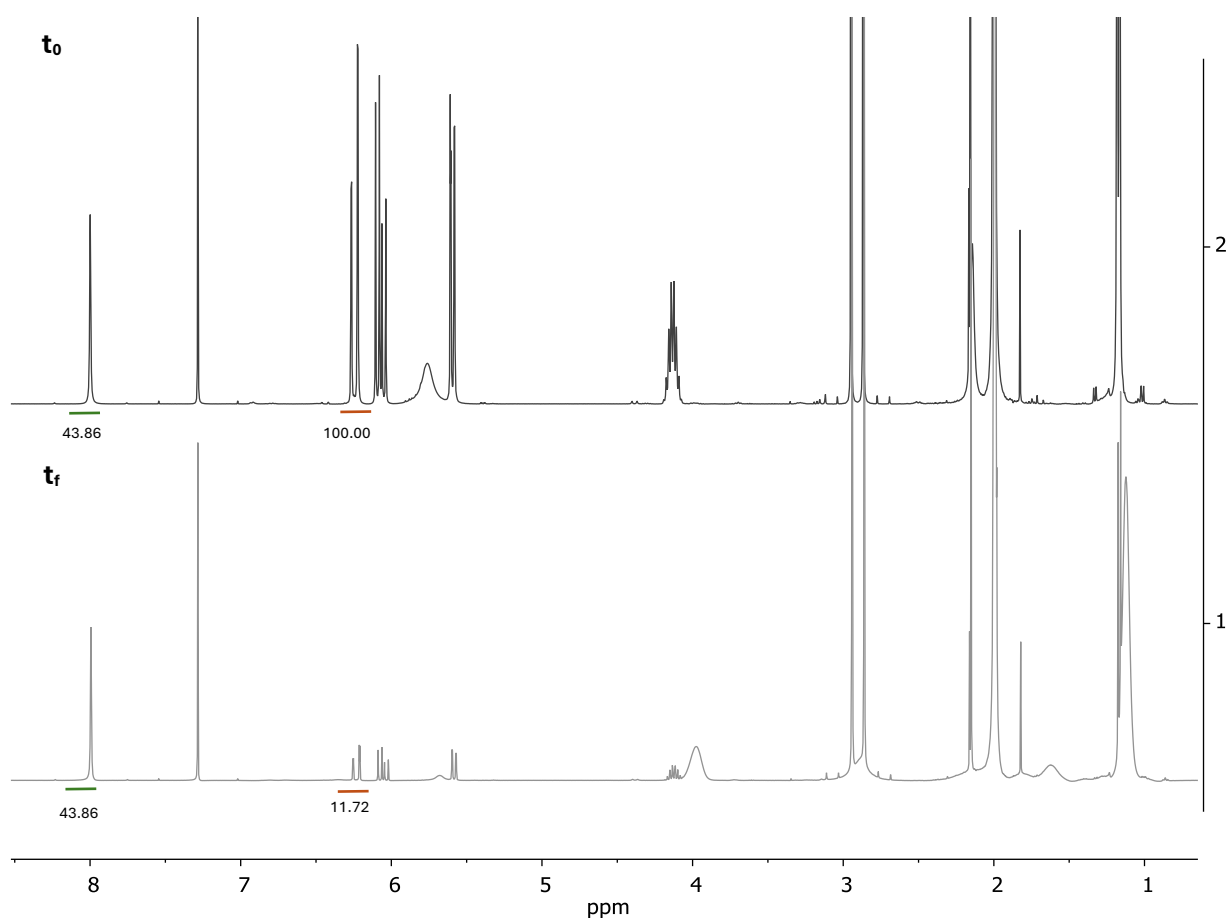


Figure 32. ^1H NMR (CDCl_3 , 400 MHz) spectra of the t_0 (track 2) and t_f (track 1) for the polymerization reaction in Figure 31. The residual monomer content indicates a conversion degree of 88%.

The M_n calculated by the residual monomer quantity is around 16,000 Da, that means around 133 monomers per calixarene.

The obtained product **17** was purified to remove the excess monomer, and its ^1H NMR spectrum was recorded (Figure 33). The methylene bridge of the calixarene, axial protons at 4.42 and the equatorials at 3.16 ppm, were used to normalize the integral, as in previous cases. The other two broad signals are identified belonging to the calixarene scaffold, OCH_2 at 3.73 ppm and SCH_2 at 3.34 ppm. The polymer signal CH of the isopropyl group is used for the estimation of the M_n of the polymer. From the integral it

appears that the polymer has a M_n of 33,000 Da (roughly 290 monomers per calixarene unit, that means 145 per branch), considerably higher than the predicted one.

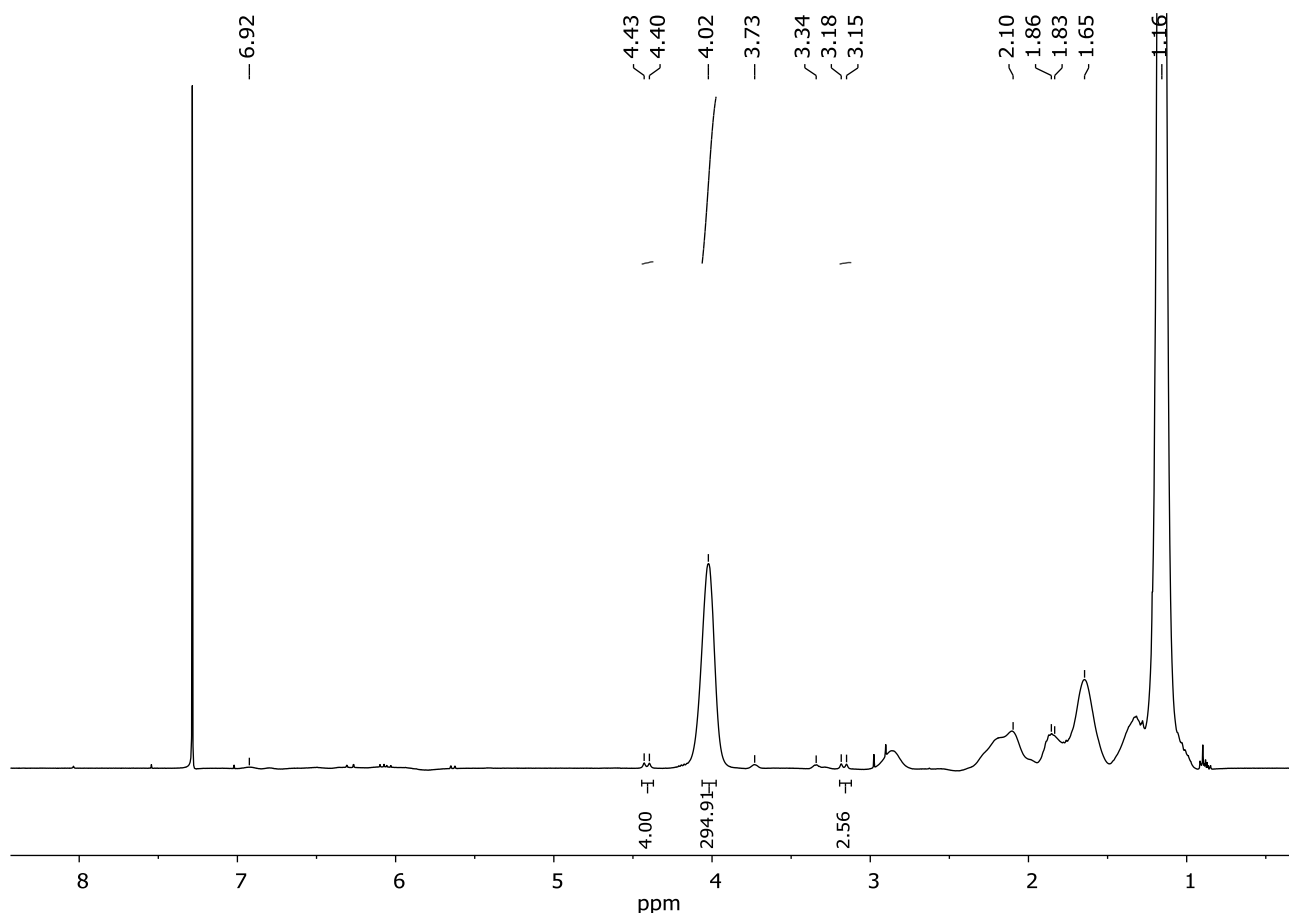


Figure 33. ^1H NMR (CDCl_3 , 400 MHz) spectrum of polymer **17** after precipitation.

In this spectrum the aromatics, present as two broad signals, are confused in the baseline from the spectrum with the baseline correction in the area from 7 to 5.5 ppm.

5.2.3 Thermoresponsive properties of p-NIPAA

As reported in paragraph 5.1.5, it is well known that polymers based on N-isopropylacrylamide (NIPAA) have characteristics of thermoresponsivity. The polymers **13-17** also show thermoresponsivity properties, despite having a calixarene backbone in the polymeric structures. Below 32 °C, these polymers are well-dissolved in water due to the formation of an extensive network of hydrogen bonds with surrounding water molecules. When the solution is heated to 32 °C, reaching the lower critical solution temperature (LCST), these hydrogen bonds break, releasing water molecules and allowing the polymer to form globular particles, thereby inducing phase separation (Figure 34). It is worth noting that, despite the presence of the calixarene backbone, the NIPAA polymers grown on calixarenes maintain the same thermoresponsive properties.

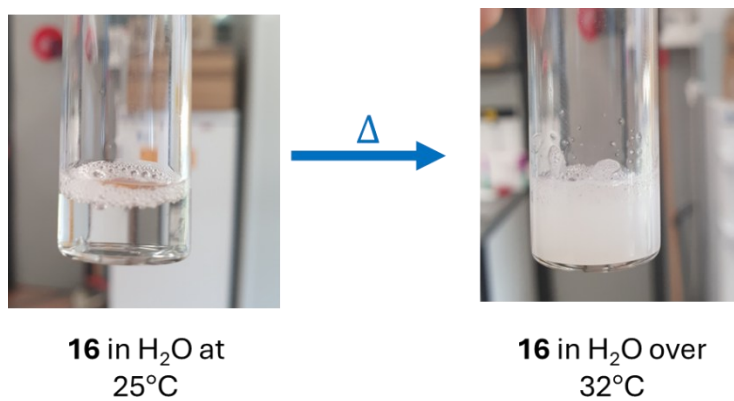


Figure 34. Example of thermoresponsivity of the pNIPAA 16, whose synthesis was discussed in this chapter.

5.3 Conclusions

This work reports, for the first time, the synthesis of four calix[4]arenes functionalized with one, two or four RAFT agent units at the lower rim and the first examples of RAFT polymerization on a calix[4]arene having two chain-transfer agents per macrocyclic structure and using NIPAA as monomer.

It was possible to set reaction conditions in terms of equivalents of initiator, monomer (NIPAA), temperature and reaction times to reach the optimal 80% conversion of monomers into polymers.

The polymers were isolated, purified from the unreacted monomers and characterized by using ^1H NMR, that also allowed to estimate an average molecular weight (M_n) of the polymers. When conversion reaches values above 85-90% conversion, the average molecular weights (M_n) of the polymers calculated by NMR remarkably exceeded the average molecular weights estimated by the unreacted monomer, which clearly indicates that chain recombination has taken place.

Remarkably, NMR data also showed that the calixarenes have been retained into the polymeric structures and that apparently the polymer chains are growing homogeneously on the two branches. No indications of the inhibition of the growth of one chain in the presence of the other due to steric hindrance could be collected, but this point should be investigated more in detail in the future.

The average molecular weights M_n estimated by ^1H NMR are in all cases higher than expected and also than the values calculated from the residual monomer quantity. A more comprehensive analysis, including the calculation of the weight-average molecular weight (M_w) based on gel permeation chromatography (GPC) data is needed to clarify the NMR data, and it is currently performed by Prof. Gobbo's group in University of Trieste.

5.4 Experimental part

25,27-dihydroxy-26,28-dipropoxycalix[4]arene (1)

Tetrahydroxy calix[4]arene (2 g, 4.71 mmol) was suspended in 20 mL of acetonitrile, followed by the addition of 2.6 g (18.8 mmol) of K_2CO_3 . After 10 minutes, 1.84 mL (18.8 mmol) of 1-iodopropane was introduced to the mixture, and the reaction was stirred at room temperature for 16 hours. The progress of the reaction was monitored via TLC (SiO_2 , hexane-ethyl acetate 7:3, revelation with UV lamp). Upon completion of the reaction, 20 mL of a 1 M aqueous HCl solution was added to quench the reaction, allowing the precipitation of a white solid. Product **1** was purified through filtration and recrystallization using a 1:1 mixture of dichloromethane and methanol, yielding a white crystalline solid with an 84% yield (0.39 mmol, 2 g).

^1H NMR (400 MHz, $CDCl_3$) δ : 8.33 (s, 2H, OH), 7.08 (d, J = 7.5 Hz, 4H, ArH_{meta}), 6.95 (d, J = 7.5 Hz, 4H, ArH_{meta}), 6.77 (t, J = 7.5 Hz, 2H, ArH_{para}), 6.66 (t, J = 7.5 Hz, 2H, ArH_{para}), 4.34 (d, J = 13 Hz, 4H, $ArCH_{ax}HAr$), 4.00 (t, J = 6.3 Hz, 4H, OCH_2), 3.41 (d, J = 13 Hz, 4H, $ArCHH_{eq}Ar$), 2.14-2.05 (m, 4H, OCH_2CH_2), 1.34 (t, J = 7.4 Hz, 6H, CH_3).

The spectroscopic data found are in agreement with those reported in literature.²⁸

25,27-dipropoxy-26,28-bis[(4-phthalimidobutyl)oxy]calix[4]arene (2a)

Compound **1** (300 mg, 0.59 mmol) was dissolved in 10 mL of dry DMF under a nitrogen atmosphere. Sodium hydride (60%, 2.36 mmol, 57 mg) was then added, and the mixture was stirred for 10 minutes. Subsequently, 665 mg (2.36 mmol) of *N*-(4-bromobutyl)phthalimide was introduced into the reaction mixture, which was allowed to react at room temperature for 2 hours. The reaction progress was monitored by TLC (SiO_2 , hexane-ethyl acetate 8:2, revealed with UV lamp and stain $FeCl_3$). Upon completion, the reaction was quenched by the addition of 20 mL of a 1 M HCl solution and extracted with dichloromethane. The organic layer was washed successively with brine and water, dried over Na_2SO_4 , and the solvent was evaporated under reduced pressure. The crude was then purified using flash chromatography (SiO_2 , hexane-ethyl acetate 8:2) to remove the excess of *N*-(4-bromobutyl)phthalimide. A white sticky solid was obtained as product **2a** with a 60% yield (322 mg, 0.35 mmol).

ESI-SQ(+): m/z calc 911.11, found 933.54 $[M+Na]^+$, 949.86 $[M+K]^+$

^1H NMR (400 MHz, $CDCl_3$) δ : 7.83 (m, 2H, $ArH_{phth_{orto}}$), 7.72 (m, 2H, $ArH_{phth_{meta}}$), 6.71 (d, J = 6.6 Hz, 4H, ArH_{meta}), 6.64 (t, 2H, ArH_{para}), 6.53 (s, 6H, ArH_{meta} , ArH_{para}), 4.44 (d, J = 13.3 Hz, 4H, $ArCH_{ax}HAr$), 3.98-3.85 (m, 8H, OCH_2), 3.80 (t, J = 7.3 Hz, 4H, CH_2N_{phth}), 3.17 (d, J = 13.3 Hz, 4H, $ArCHH_{eq}Ar$), 2.03-1.82 (m, 12H, $OCH_2CH_2CH_3$, $OCH_2CH_2CH_2CH_2N_{phth}$), 0.96 (t, J = 7.4 Hz 6H, CH_3).

^{13}C NMR (100 MHz, $CDCl_3$) δ : 168.30 (NC=O), 156.79 (C_{ipso}), 156.06 (C_{ipso}), 135.40 (C_{orto}), 134.75 (C_{orto}), 133.85 ($ArCH_{meta}$ phth), 132.22 (ArC phth), 128.31 ($ArCH_{meta}$), 128.06 ($ArCH_{meta}$), 123.18 ($ArCH_{orto}$ phth), 122.12 ($ArCH_{para}$), 121.90 ($ArCH_{para}$), 76.70 (OCH_2), 74.26 (OCH_2), 37.96 (CH_2N_{phth}), 31.03 ($ArCH_2Ar$), 27.65 ($OCH_2CH_2CH_3$), 25.48 ($OCH_2CH_2CH_2CH_2N_{phth}$), 23.31 ($OCH_2CH_2CH_2CH_2N_{phth}$), 10.29 (CH_3).

25,27-dipropoxy-26,28-bis[(6-phthalimidoethyl)oxy]calix[4]arene (2b)

Compound **1** (300 mg, 0.59 mmol) was dissolved in 10 mL of dry DMF under a nitrogen atmosphere. Sodium hydride (60%, 2.36 mmol, 57 mg) was then added, and the mixture was stirred for 10 minutes. Subsequently, 732 mg (2.36 mmol) of *N*-(6-bromoethyl)phthalimide was introduced into the reaction

mixture, which was allowed to react at room temperature for 2 hours. The reaction progress was monitored by TLC (SiO₂, hexane- ethyl acetate 8:2, revealed with UV lamp and stain FeCl₃). Upon completion, the reaction was quenched by the addition of 20 mL of a 1 M HCl solution and extracted with dichloromethane. The organic layer was washed successively with brine and water, dried over Na₂SO₄, and the solvent was evaporated under reduced pressure. The crude was then purified using flash chromatography (SiO₂, hexane- ethyl acetate 8:2) to remove the excess of *N*-(6-bromohexyl)phthalimide. A white sticky solid was obtained as product **2b** with 89% of yield (506 mg, 0.52 mmol).

ESI-SQ(+): m/z calc 967.22, found 989.62 [M+Na]⁺, 1006.60 [M+K]⁺

¹H NMR (400 MHz, CDCl₃) δ: 7.86-7.84 (m, 2H, ArHphth_{orto}), 7.73-7.71(m, 2H, ArHphth_{meta}), 6.67 (d, *J* = 6.6 Hz, 4H, ArH_{meta}), 6.61 (t, 2H, *J* = 6.6 Hz, ArH_{para}), 6.55 (s, 6H, ArH_{meta}, ArH_{para}), 4.44 (d, *J* = 13.3 Hz, 4H, ArCH_{ax}HAr), 3.90-3.84 (m, 8H, OCH₂), 3.73 (t, *J* = 7.3 Hz, 4H, CH₂Nphth), 3.15 (d, *J* = 13.3 Hz, 4H, ArCHH_{eq}Ar), 1.95-1.87 (m, 8H, OCH₂CH₂), 1.78-1.71 (m, 4H, CH₂CH₂Nphth), 1.52-1.41 (m, 8H, CH₂CH₂CH₂CH₂Nphth), 0.97 (t, *J* = 7.4 Hz 6H, CH₃).

¹³C NMR (100 MHz, CDCl₃) δ: 168.42 (NC=O), 156.72 (C_{ipso}), 156.37 (C_{ipso}), 135.31 (C_{orto}), 134.93 (C_{orto}), 133.86 (CH_{meta} Nphth), 132.19(C_q Nphth), 128.20 (CH_{meta}), 128.05 (CH_{meta}), 123.17 (ArCH_{orto} phth), 121.92 (ArCH_{para}), 121.86 (ArCH_{para}), 76.67 (OCH₂), 74.81 (OCH₂), 38.02 (CH₂Nphth), 31.02 (ArCH₂Ar), 30.10 (OCH₂CH₂CH₃), 28.78 (CH₂CH₂Nphth), 27.02 (CH₂CH₂CH₂CH₂Nphth), 25.90 (CH₂CH₂CH₂CH₂Nphth), 23.29 (CH₂CH₂CH₂Nphth), 10.38 (CH₃).

25,27-dipropoxy-26,28-bis[(4-aminobutyl)oxy]calix[4]arene (3a)

Compound **2a** (202 mg, 0.22 mmol) was suspended in 8 mL of ethanol, then 0.28 mL of hydrazine hydrate (285 mg, 8.88 mmol) were added. The reaction mixture was refluxed for 3 hours and was monitored with TLC (SiO₂, hexane-ethyl acetate-methanol 3:6:1, revelation with UV lamp and stain ninhydrin) until a white suspended solid was observed. The suspension was concentrated under reduced pressure, then water was added, dissolving the white solid. The aqueous phase was extracted with dichloromethane two times, then the organic phases were dried over Na₂SO₄ and the solvent was evaporated under reduced pressure, obtaining a white solid. Product **3a** (139 mg, 0.21 mmol) was obtained with 96% yield.

ESI-SQ(+): m/z calc 650.90, found 652.0 [M+H]⁺

¹H NMR (400 MHz, CDCl₃) δ: 6.79 – 6.46 (m, 12H, ArH), 4.48 (d, *J* = 13.3 Hz, 4H, ArCH_{ax}HAr), 3.98 (t, *J* = 7.6 Hz, 4H, OCH₂), 3.86 (t, *J* = 7.4 Hz, 4H, OCH₂), 3.20 (d, *J* = 13.4 Hz, 4H, ArCHH_{eq}Ar), 2.81 (t, *J* = 7.2 Hz, 4H, CH₂NH₂), 2.04 – 1.90 (m, 8H, OCH₂CH₂), 1.68 – 1.50 (m, 8H, NH₂, CH₂CH₂NH₂), 1.06 (t, *J* = 7.4 Hz, 6H, CH₃).

¹³C NMR (101 MHz, CDCl₃) δ: 156.61 (C_{ipso}), 156.34 (C_{ipso}), 135.38 (C_{orto}), 134.80 (C_{orto}), 128.30 (C_{meta}), 128.07 (C_{orto}), 122.03 (C_{para}), 122.01 (C_{para}), 76.73 (OCH₂), 74.70 (OCH₂), 42.31 (CH₂NH₂), 31.04 (ArCH₂Ar), 30.39 (CH₂CH₂NH₂), 27.57 (CH₂CH₂CH₂NH₂), 23.40 (OCH₂CH₂), 10.55 (CH₃).

25,27-dipropoxy-26,28-bis[(6-aminohexyl)oxy]calix[4]arene (3b)

Compound **2b** (270 mg, 0.28 mmol) was suspended in 12 mL of ethanol, then 0.26 mL of hydrazine hydrate (268 mg, 8.37 mmol) were added. The reaction mixture was refluxed for 3 hours and was monitored with TLC (SiO₂, hexane-ethyl acetate-methanol 3:6:1) until a white suspended solid was observed. The suspension was concentrated under reduced pressure, then water was added, dissolving

the white solid. The aqueous phase was extracted with dichloromethane two times, then the organic phases were dried over Na_2SO_4 and the solvent was evaporated under reduced pressure, obtaining a white solid. Product **3b** (192 mg, 0.27 mmol) was obtained in 97% yield.

ESI-SQ(+): m/z calc 707.01, found 707.61 $[\text{M}+\text{H}]^+$, 729.97 $[\text{M}+\text{Na}]^+$

^1H NMR (400 MHz, CDCl_3) δ : 6.67- 6.58 (m, 12H, ArH), 4.50 (d, J = 13.3 Hz, 4H, $\text{ArCH}_{ax}\text{HAr}$), 3.96-3.88 (m, 8H, OCH_2), 3.20 (d, J = 13.3 Hz, 4H, $\text{ArCHH}_{eq}\text{Ar}$), 2.74 (t, J = 6.9 Hz, 4H, CH_2NH_2), 1.98 - 1.87 (m, 8H, OCH_2CH_2), 1.75 - 1.61 (bs, 4H, NH_2), 1.55 - 1.43 (m, 12H, $\text{OCH}_2\text{CH}_2\text{CH}_2\text{CH}_2$, $\text{CH}_2\text{CH}_2\text{CH}_2\text{NH}_2$, $\text{CH}_2\text{CH}_2\text{NH}_2$), 1.01 (t, J = 7.4 Hz, 6H, CH_3).

^{13}C NMR (101 MHz, CDCl_3) δ : 156.65 (C_{ipso}), 156.47 (C_{ipso}), 135.23 (C_{orto}), 135.03 (C_{orto}), 128.17 (CH_{meta}), 128.09 (CH_{meta}), 121.93 (CH_{para}), 77.23 (OCH_2), 74.95 (OCH_2), 42.15 (CH_2NH_2), 33.74 ($\text{CH}_2\text{CH}_2\text{NH}_2$), 31.01 (ArCH_2Ar), 30.24 (OCH_2CH_2), 27.09 ($\text{CH}_2\text{CH}_2\text{CH}_2\text{NH}_2$), 26.16 ($\text{OCH}_2\text{CH}_2\text{CH}_2\text{CH}_2$), 23.30 (OCH_2CH_2), 10.44 (CH_3).

25,27-dipropoxy-26,28-bis[(4(4-cyano-4-(((dodecylthio)carbonothioyl)thio)pentanoic) amidobutyl)-oxy]calix[4]arene (4a)

Compound **3a** (33.3 mg, 0.051 mmol) was dissolved in 2 mL dichloromethane, then 4-cyano-4-(propylsulfanyltiocarbonyl)sulfanylpentanoic acid mercaptothiazoline amide (RAFT agent) (35.5 mg, 0.093 mg) and DIPEA (8 μL , 0.046 mmol) were added. The reaction was stirred for 16 hours at room temperature and was monitored via TLC (SiO_2 , dichloromethane-methanol 95:5, UV lamp). Upon completion the crude was washed with a solution of saturated Na_2CO_3 , the solvent of the organic phase was evaporated under reduced pressure. The product **4a** was obtained as a yellow resin and purified with flash chromatography (SiO_2 , dichloromethane-methanol 95:5). It was obtained with 65% yield (39 mg).

ESI-SQ(+): m/z calc (1169.70), found 1170.91 $[\text{M}+\text{H}]^+$, 1192.52 $[\text{M}+\text{Na}]^+$

^1H NMR (400 MHz, CDCl_3) δ : 6.99 (d, J = 7.3 Hz, 4H, ArH_{meta}), 6.84 (t, J = 7.3 Hz, 2H, ArH_{para}), 6.58 (bs, 2H, NHC=O), 6.32 (t, J = 7.3 Hz, 2H, ArH_{para}), 6.23 (d, J = 7.3 Hz, 4H, ArH_{meta}), 4.42 (d, J = 13.3 Hz, 4H, $\text{ArCH}_{ax}\text{HAr}$), 4.04 (t, J = 8.0 Hz, 4H, $\text{OCH}_2(\text{butyl})$), 3.72 (t, J = 6.8 Hz, 4H, $\text{OCH}_2(\text{propyl})$), 3.33 (m, 8H, CH_2NHCO , SCH_2), 3.17 (d, J = 13.3 Hz, 4H, $\text{ArCHH}_{eq}\text{Ar}$), 2.57-2.51 (m, 6H, COCH_2CHHC), 2.43-2.34 (m, 2H, COCH_2CHHC), 2.03-1.95 (m, 4H, OCH_2CH_2), 1.92 (m, 10H, $\text{OCH}_2\text{CH}_2\text{CH}_3$, CCH_3), 1.75 (sextuplet, J = 7.3 Hz, 4H, SCH_2CH_2), 1.58-1.50 (m, 4H, $\text{CH}_2\text{CH}_2\text{NH}$), 1.10-1.02 (m, 12H, CH_3).

^{13}C NMR (100 MHz, CDCl_3) δ : 217.36 (C=S), 170.5 (C=ONH), 157.5 (C_{ipso}), 155.4 (C_{ipso}), 136.4 (C_{orto}), 133.6 (C_{orto}), 128.8 (C_{meta}), 127.6 (C_{meta}), 122.1 (C_{para}), 122.1 (C_{para}), 119.27 (CN), 76.9 ($\text{OCH}_2(\text{propyl})$), 74.5 ($\text{OCH}_2(\text{butyl})$), 46.9 (C_q), 40.1 (CH_2NHCO), 38.8 (SCH_2), 34.7 ($\text{COCH}_2\text{CH}_2\text{C}$), 31.8 ($\text{COCH}_2\text{CH}_2\text{C}$), 31.00 (ArCH_2Ar), 27.7 (OCH_2CH_2), 26.2 ($\text{CH}_2\text{CH}_2\text{NH}$), 25.0 (CCH_3), 23.5 ($\text{OCH}_2\text{CH}_2\text{CH}_3$), 21.3 (SCH_2CH_2), 13.5 (CH_3), 10.9 (CH_3).

25,27-dipropoxy-26,28-bis[(6(4-cyano-4-(((dodecylthio)carbonothioyl)thio)pentanoic) amidoheptyl)-oxy]calix[4]arene (4b)

Compound **3b** (30 mg, 0.042 mmol) was dissolved in 2 mL dichloromethane, then 4-cyano-4-(propylsulfanyltiocarbonyl)sulfanylpentanoic acid mercaptothiazoline amide (RAFT agent) (35.5 mg, 0.093 mg) and DIPEA (7 μL , 0.042 mmol) were added. The reaction was stirred for 16 hours at room temperature and was monitored via TLC (SiO_2 , dichloromethane-methanol 95:5, UV lamp). Upon completion the crude was washed with a solution of saturated Na_2CO_3 , the solvent of the organic phase

was evaporated under reduced pressure. The product **4b** was obtained as a yellow resin and purified with flash chromatography (SiO₂, dichloromethane-methanol 95:5). It was obtained with 89% yield (46 mg).

ESI-SQ(+): *m/z* calc 1225.81, found 1226.9 [M+H]⁺, 1249.4 [M+Na]⁺

¹H NMR (400 MHz, CDCl₃) δ: 6.80 (d, *J* = 7.3 Hz, 4H, ArH_{meta}), 6.71 (t, *J* = 7.3 Hz, 2H, ArH_{para}), 6.46 (s, 6H, ArH_{meta}+ ArH_{para}), 6.17 (s, 2H, C=ONH), 4.45 (d, *J* = 13.3 Hz, 4H, ArCH_{ax}HAr), 3.95 (t, *J* = 7.6 Hz, 4H, OCH₂(propyl)), 3.80 (t, *J* = 7.2 Hz, 4H, OCH₂), 3.34 (t, *J* = 7.2 Hz, 4H, SCH₂), 3.28 (q, *J* = 7.5 Hz, 4H, CH₂NHCO), 3.17 (d, *J* = 13.4 Hz, 4H, ArCH_{eq}Ar), 2.56–2.37 (m, 8H, C=OCH₂CH₂C), 1.93 (m, 15H, OCH₂CH₂, CCH₃), 1.75 (sextuplet, *J* = 7.6 Hz, 4H, SCH₂CH₂), 1.62–1.55 (m, 4H, CH₂CH₂NH), 1.46–1.36 (m, 8H, CH₂CH₂CH₂CH₂NHCO), 1.08–1.03 (m, 12H, CH₂CH₃).

¹³C NMR (101 MHz, CDCl₃) δ: 217.34 (C=S), 170.4 (C=ONH), 157.0 (C_{ipso}), 156.0 (C_{ipso}), 135.8 (C_{orto}), 134.4 (C_{orto}), 128.4 (C_{meta}), 127.9 (C_{meta}), 121.9 (C_{para}), 121.8 (C_{para}), 119.3 (CN), 76.9 (OCH₂), 74.8 (OCH₂), 46.8 (CCN), 39.9 (CH₂NHCO), 38.9 (SCH₂), 34.7 (C=OCH₂CH₂C), 31.9 (C=OCH₂CH₂C), 31.01 (ArCH₂Ar), 29.97 (CH₂CH₂NHCO), 27.26 (CH₂CH₂CH₂NHCO), 25.98 (CH₂CH₂CH₂CH₂NHCO), 25.05 (CCH₃), 23.43 (CH₂CH₂CH₂CH₂NHCO), 21.3 (OCH₂CH₂), 13.51 (CH₃), 10.76 (CH₃).

25,26,27,28-tetrapropoxy calix[4]arene (5)

In a two-neck flask under a nitrogen atmosphere, tetrahydroxycalix[4]arene (5.00 g, 11.79 mmol) was dissolved in 100 mL of dry dimethylformamide. NaH 60% w/w (2.70 g, 70.73 mmol) was added, and the mixture was left stirring at room temperature for 15 minutes. 1-iodopropane (6.90 mL, 70.73 mmol) was added and the reaction was left stirring for 16 h. The reaction progress was monitored by TLC (eluent: Hexane-Ethyl Acetate 9.8:0.2; detection: UV lamp and FeCl₃ stain). Upon completion the reaction was quenched with 1 M HCl (100 mL). The yellow-white precipitate is filtered using a Büchner funnel and recrystallized with DCM/methanol, yielding a white solid (5.95 g, 10.04 mmol) with 85% yield.

¹H NMR (400 MHz, CDCl₃) δ: 6.63–6.60 (m, 12H, ArH), 4.47 (d, *J* = 13.4 Hz, 4H, ArCH_{ax}HAr), 3.87 (t, *J* = 7.4 Hz, 8H, OCH₂), 3.17 (d, *J* = 13.4 Hz, 4H, ArCH_{eq}HAr), 1.95 (m, 8H, *J* = 7.2 Hz, CH₂CH₃), 1.01 (t, *J* = 7.4 Hz, 12H, CH₃).

The spectroscopic data found are in agreement with those reported in literature.²⁹

25,26,27-tripropoxy calix[4]arene (6)

Compound 5 (300 mg, 0.506 mmol) was dissolved in 20 mL of chloroform in three necks round bottom flask under nitrogen atmosphere. The iodotrimethylsilane was dissolved in 20 mL of chloroform and added dropwise to the calixarene solution, then was refluxed for 90 minutes. The reaction was monitored via TLC (SiO₂, hexane-ethyl acetate 9:1, UV) and it was quenched when the dipropoxy calix[4]arene started to form. 20 mL of 1M HCl solution were added, the organic phase was then washed with a solution of Na₂S₂O₃ 0.5 M. The crude mixture contained both the starting reagent and tripropoxy calix[4]arene **6**. Attempts at purification via flash chromatography were unsuccessful; therefore, the subsequent step was carried out using the crude mixture.

25,26,27-tripropoxy-28-mono[(6-phthalimido)hexyl]oxy calix[4]arene (7)

The crude **6** (177 mg) was dissolved in 10 mL of dry DMF under a nitrogen atmosphere and NaH 60% w/w (14 mg, 0.273 mmol). After 10 minutes of stirring the N-(6-bromohexyl)phthalimide (113 mg, 0.364 mmol) was added. The reaction was monitored via TLC (SiO₂, hexane-ethyl acetate 9:1, UV), and

quenched with 5 mL of 1 M HCl and the organic phase washed with water and brine. The crude was purified via flash chromatography (SiO₂, hexane-ethyl acetate 9:1). The product was obtained as a sticky resin (100 mg), with a yield of the two steps overall of 25%.

¹H NMR (400 MHz, CDCl₃) δ: 7.86 (m, 2H, , ArH_{phth}), 7.73 (m, 2H, , ArH_{phth}), 6.72 – 6.44 (m, 12H, ArH), 4.45 (m, 4H, ArCH_{ax}HAr), 3.86 (t, *J* = 7.5 Hz, 2H, OCH₂(CH₂)₅Nphth), 3.70 (t, *J* = 7.2 Hz, 4H, OCH₂), 3.41 (t, *J* = 6.8 Hz, 2H, CH₂Nphth), 3.15 (d, *J* = 13.4, 4H, ArCHH_{eq}Ar), 1.95-1.83 (m, 2H, OCH₂CH₂CH₂), 1.77-1.66 (m, 2H, CH₂CH₂Nphth), 1.60-1.33 (m, 4H, OCH₂CH₂CH₂CH₂), 0.92 (t, *J* = 7.4 Hz, 9H, CH₃).

¹³C NMR (101 MHz, CDCl₃) δ: 170.4 (C=ONH), 157.0 (C_{ipso}), 156.0 (C_{ipso}), 135.8 (C_{orto}), 134.4 (C_{orto}), 128.4 (C_{meta}), 127.9 (C_{meta}), 121.9 (C_{para}), 119.3 (CN), 76.9 (OCH₂(propyl)), 74.8 (OCH₂ (hexyl)), 46.8 (CCN), 39.9 (CH₂NHCO), 38.9 (SCH₂), 34.7 (C=OCH₂CH₂C), 31.9 (C=OCH₂CH₂C), 31.01 (ArCH₂Ar), 29.97 (CH₂CH₂NHCO), 27.26 (CH₂CH₂CH₂NHCO), 25.98 (CH₂CH₂CH₂CH₂NHCO), 25.05 (CCH₃), 23.43 (CH₂CH₂CH₂CH₂NHCO), 21.3 (OCH₂CH₂), 13.51 (CH₃), 10.76 (CH₃).

25,26,27-tripropoxy-28-mono[(6-aminohexyl)oxy]calix[4]arene (8)

Compound **7** (100 mg, 0.13 mmol) was suspended in 12 mL of ethanol, then 0.26 mL of hydrate hydrazine (41 mg, 1.28 mmol) were added. The reaction mixture was refluxed for 3 hours and was monitored with TLC (SiO₂, hexane-ethyl acetate-methanol 3:6:1) until a white suspended solid was observed. The suspension was concentrated under reduced pressure, then water was added, dissolving the white solid. The aqueous phase was extracted with dichloromethane two times, then the organic phases were dried over Na₂SO₄ and the solvent was evaporated under reduced pressure, obtaining a white solid with 15% yield.

¹H NMR (400 MHz, CDCl₃) δ: 6.61 (m, 12H, ArH), 4.45 (m, 4H, ArCH_{ax}HAr), 3.93 – 3.73 (m, 8H, OCH₂), 3.17 (d, *J* = 13.5 Hz, 4H, ArCHH_{eq}Ar), 2.96 (t, *J* = 7.8 Hz, 2H, CH₂NH₂), 1.93 (m, 8H, OCH₂CH₂), 1.75 (t, *J* = 7.5 Hz, 2H, NH₂), 1.56 (m, 2H, OCH₂CH₂CH₂), 1.30 (m, 4H, CH₂CH₂NH₂, OCH₂CH₂CH₂CH₂), 1.01 (m, 9H, CH₃).

¹³C NMR (101 MHz, CDCl₃) δ: 156.60 (C_{ipso}), 156.46 (C_{ipso}), 156.52 (C_{ipso}), 135.21 (C_{orto}), 135.11 (C_{orto}), 135.08 (C_{orto}), 135.07 (C_{orto}), 135.02 (C_{orto}), 128.17 (CH_{meta}), 128.12 (CH_{meta}), 122.00 (CH_{para}), 121.92 (CH_{para}), 77.23 (OCH₂), 74.85 (OCH₂), 74.64 (OCH₂), 40.30 (CH₂NH₂), 31.00 (ArCH₂Ar), 30.40 (ArCH₂Ar), 30.13 (ArCH₂Ar), 28.72 (OCH₂CH₂), 27.02 (OCH₂CH₂CH₂), 26.76 (OCH₂CH₂CH₂CH₂), 25.95 (CH₂CH₂NH₂), 25.66, 23.30 (OCH₂CH₂), 23.28 (OCH₂CH₂), 10.46 (CH₃).

25,26,27-tripropoxy-28-mono[(6(4-cyano-4-(((dodecylthio)carbonothioyl)thio)pentanoic) amido hexyl)oxy]calix[4]arene (9)

Compound **8** (30 mg, 0.042 mmol) was dissolved in 2 mL dichloromethane, then 4-cyano-4-(propylsulfanythiocarbonyl)sulfanylpentanoic acid mercaptothiazoline amide (RAFT agent) (17 mg, 0.043 mg) and DIPEA (7 μL, 0.042 mmol) were added. The reaction was stirred for 16 hours at room temperature and was monitored via TLC (SiO₂, dichloromethane-methanol 95:5, UV lamp). Upon completion the crude was washed with a solution of saturated Na₂CO₃, the solvent of the organic phase was evaporated under reduced pressure. Product **9** was obtained as a yellow resin and purified with flash chromatography (SiO₂, dichloromethane-methanol 98:2). It was obtained with 44% yield (17 mg).

¹H NMR (400 MHz, CDCl₃) δ: 6.73 – 6.46 (m, 12H, ArH), 5.54 (bs, 1H, NH), 4.46 (dd, *J* = 13.3, 4H, ArCH_{ax}HAr), 3.96 – 3.70 (m, 8H, OCH₂), 3.44 – 3.23 (m, 4H, CH₂NHCO, SCH₂), 3.17 (d, *J* = 13.4 Hz, 4H, ArCHH_{eq}Ar), 2.56 – 2.47 (m, 3H, COCH₂CHH), 2.43 – 2.38 (m, 1H, COCH₂CHH), 1.99 – 1.86 (m, 10H,

OCH₂CH₂, CCH₃), 1.76 (q, *J* = 7.3 Hz, 2H, SCH₂CH₂), 1.59 – 1.52 (m, 2H, OCH₂CH₂CH₂), 1.49 – 1.40 (m, 4H, CH₂CH₂NH), 1.32 – 1.21 (m, 2H, CH₂CH₂CH₂NH), 1.20 – 0.86 (m, 12H, CH₃).

¹³C NMR (101 MHz, CDCl₃) δ: 217.38 (C=S), 170.43 (C=ONH), 157.00 (C_{ipso}), 156.07 (C_{ipso}), 136.81 (C_{orto}), 135.75 (C_{orto}), 134.45 (C_{orto}), 133.41 (C_{orto}), 128.89 (C_{meta}), 128.43 (C_{meta}), 127.89 (C_{meta}), 127.41 (C_{meta}), 122.01 (C_{para}), 121.94 (C_{para}), 119.29 (CN), 77.31 (OCH₂), 76.74 (OCH₂), 74.85 (OCH₂), 46.84 (CCN), 39.85 (CH₂NHCO), 38.85 (SCH₂), 34.67 (C=OCH₂CH₂C), 31.84 (C=OCH₂CH₂C), 31.20 (ArCH₂Ar), 31.01 (ArCH₂Ar), 29.96 (CH₂CH₂NHCO), 27.27 (CH₂CH₂CH₂NHCO), 25.99 (CH₂CH₂CH₂CH₂NHCO), 25.03 (CCH₃), 23.44 (CH₂CH₂CH₂CH₂CH₂NHCO), 21.33 (OCH₂CH₂), 13.51 (CH₃), 10.95 (CH₃).

25,26,27,28-[(6-phthalimidohexyl)oxy]calix[4]arene (10)

In a two-necked flask under a nitrogen atmosphere previously dried, NaH 60% (136 mg, 5.654 mmol) and 25,26,27,28-tetrahydroxy calix[4]arene (300 mg, 0.707 mmol) were added, dissolved in 10 mL of dry DMF. After 15 minutes of stirring, N-(6-bromohexyl)phthalimide (997 mg, 3.534 mmol) was introduced. The solution transitioned from clear to yellow. It was stirred at room temperature for four hours. The reaction progress was monitored by TLC (SiO₂, hexane-ethyl acetate 6:4, revealed with UV lamp and stain FeCl₃). Upon completion the reaction was quenched with 1M HCl and extracted twice with dichloromethane. The organic phase was washed four times with a saturated aqueous NaCl solution, dried over Na₂SO₄, and the solvent was evaporated under reduced pressure. The crude product was purified by flash column chromatography (SiO₂, hexane-ethyl acetate 7:3), and obtained as a sticky white solid with 48% yield (454 mg, 0.34 mmol).

¹H NMR (400 MHz, CDCl₃) δ: 7.80-7.78 (m, 4H, ArHphth_{orto}), 7.67-7.65 (m, 4H, ArHphth_{meta}), 6.59-6.53 (m, 12H, ArH), 4.41 (d, *J* = 13.3 Hz, 4H, ArCH_{ax}HAr), 3.88 (t, *J* = 6.8 Hz, 8H, OCH₂), 3.68 (t, *J* = 7.0 Hz, 8H, CH₂Nphth), 3.13 (d, *J* = 13.3 Hz, 4H, ArCHH_{eq}Ar), 1.94-1.86 (m, 8H, OCH₂CH₂), 1.75-1.68 (m, 4H, CH₂CH₂Nphth), 1.47-1.38 (m, 16H, CH₂CH₂CH₂CH₂Nphth).

¹³C NMR (100 MHz, CDCl₃) δ: 168.27 (NC=O), 156.50 (C_{ipso}), 135.07 (C_{orto}), 133.75 (ArCH_{meta} phth), 132.19 (ArC phth), 128.12 (ArCH_{meta}), 123.08 (ArCH_{orto} phth), 121.90 (ArCH_{para}), 74.82 (OCH₂), 37.97 (CH₂Nphth), 31.08 (ArCH₂Ar), 30.07 (OCH₂CH₂), 28.78 (CH₂CH₂Nphth), 27.01 (OCH₂CH₂CH₂), 25.83 (CH₂CH₂CH₂Nphth).

25,26,27,28-[(6-aminohexyl)oxy]calix[4]arene (11)

Compound **10** (270 mg, 0.28 mmol) was suspended in 12 mL of ethanol, then 0.26 mL of hydrazine hydrate (268 mg, 8.37 mmol) were added. The reaction mixture was refluxed for 4 hours and was monitored with TLC (SiO₂, dichloromethane-methanol 9:1) until a white suspended solid was observed. The suspension was concentrated under reduced pressure, then water was added, dissolving the white solid. The aqueous phase was extracted with dichloromethane two times, then the organic phases were dried over Na₂SO₄ anhydrous, and the solvent was evaporated under reduced pressure, obtaining a white solid. Product **11** (192 mg, 0.27 mmol) was obtained with 20% yield.

ESI-SQ: *m/z* calc.821.20, found 822.5 [M+H]⁺

¹H NMR (400 MHz, CDCl₃) δ: 6.70 – 6.49 (m, 12H, ArH), 4.44 (d, *J* = 13.3 Hz, 4H, ArCH_{ax}HAr), 3.90 (t, *J* = 7.5 Hz, 8H, OCH₂), 3.17 (d, *J* = 13.4 Hz, 4H, ArCHH_{eq}Ar), 2.72 (t, *J* = 6.8 Hz, 8H, CH₂NH₂), 1.93 (m, 8H, OCH₂CH₂), 1.64 – 1.37 (m, 24H, OCH₂CH₂CH₂CH₂CH₂NH₂).

25,26,27,28- tetra[(6(4-cyano-4-(((dodecylthio)carbonothioyl)thio)pentanoic) amido hexyl -)oxy] calix[4]arene (12)

Compound **11** (30 mg, 0.042 mmol) was dissolved in 2 mL dichloromethane, then 4-cyano-4-(propylsulfanyltiocarbonyl)sulfanylpentanoic acid mercaptothiazoline amide (RAFT agent) (35.5 mg, 0.093 mg) and DIPEA (7 μ L, 0.042 mmol) were added. The reaction was stirred for 16 hours at room temperature and was monitored via TLC (SiO_2 , dichloromethane-methanol 95:5, UV lamp). Upon completion the crude was washed with a solution of saturated Na_2CO_3 , the solvent of the organic phase was evaporated under reduced pressure. Product **12** was obtained as a yellow resin and purified with flash chromatography (SiO_2 , dichloromethane-methanol 95:5). It was obtained with 36% yield.

^1H NMR (400 MHz, CDCl_3) δ : 7.02 (bs, 4H, NH), 6.61 (m, 12H, ArH), 4.41 (d, $J = 13.3$ Hz, 4H, $\text{ArCH}_{ax}\text{HAr}$), 3.88 (t, $J = 7.3$ Hz, 8H, OCH_2), 3.32 (m, 16H, CH_2NHCO , SCH_2), 3.17 (d, $J = 13.4$ Hz, 4H, $\text{ArCHH}_{eq}\text{Ar}$), 2.47 – 2.20 (m, 16H, $\text{COCH}_2\text{CH}_2\text{C}$), 1.93 (s, 12H, CCH_3), 1.75 (q, $J = 7.3$ Hz, 8H, SCH_2CH_2), 1.61 (bs, 8H, OCH_2CH_2), 1.45 (bs, 16H, $\text{CH}_2\text{CH}_2\text{NH}$, $\text{OCH}_2\text{CH}_2\text{CH}_2$), 1.29 (s, 8H, $\text{CH}_2\text{CH}_2\text{CH}_2\text{NH}$), 1.04 (t, $J = 7.3$ Hz, 12H, CH_3).

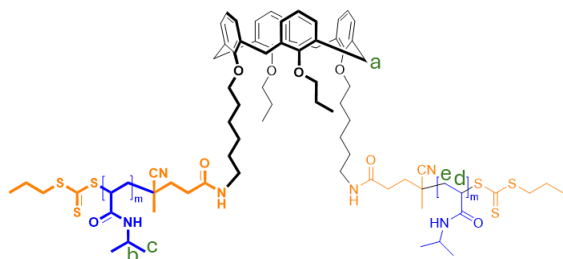
^{13}C NMR (101 MHz, CDCl_3) δ : 217.41 (C=S), 171.0 (C=ONH), 156.42 (C_{ipso}), 135.05 (C_{orto}), 128.19 (C_{meta}), 122.07 (C_{para}), 119.30 (CN), 74.94 (OCH_2), 46.84 (CCN), 40.06 (CH_2NHCO), 38.87 (SCH_2), 34.74 (C= $\text{OCH}_2\text{CH}_2\text{C}$), 31.94 (C= $\text{OCH}_2\text{CH}_2\text{C}$), 30.96 (ArCH_2Ar), 29.95 ($\text{CH}_2\text{CH}_2\text{NHCO}$), 27.52 ($\text{CH}_2\text{CH}_2\text{CH}_2\text{NHCO}$), 26.30 ($\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{NHCO}$), 25.01 (CCH_3), 22.71 ($\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{NHCO}$), 21.33 (OCH_2CH_2), 13.52 (CH_3).

General procedure for the synthesis of polymers 13-17

The monomer (NIPAA) and the radical initiator AIBN were recrystallized by hexane and methanol, respectively. ACVA was used without the need of recrystallization. The NIPAA, RAFT-Agent and AIBN were dissolved in acetonitrile and DMF were added. The reaction mixture was homogenized. The first aliquot 40 μ L of reaction mixture was taken for NMR as t_0 . Five cycles of freeze-pump-thaw (pump per 10 min), then the reaction was put under argon. The reaction was left under stir at 65 $^\circ\text{C}$ (when AIBN was used) and at 70 $^\circ\text{C}$ (when ACVA was used). Once the determined time was passed, the reaction was quenched with N_2 liquid and an aliquot of 40 μ L for NMR of t_f was taken. The product was precipitated pouring the reaction mixture in a solution 1:1 hexane-diethyl ether.

Synthesis of polymer 13

The NIPAA (412 mg, 3.65 mmol), AIBN (1.075 mg, 0.0065 mmol), **4b** (22.36 mg, 0.014 mmol) were dissolved in 2.367 mL of acetonitrile, then 150 μ L of DMF were added. The reaction was quenched after 24 hours. Polymer **13** was obtained as a pale yellow solid.



^1H NMR (400 MHz, CDCl_3) δ : 8.01 (HCO DMF), 4.00 (H_b), 2.95 (CH_3 DMF), 2.87 (CH_3 DMF), 2.10 (H_e), 1.62 (H_d), 1.13 (H_c), 0.87 (CH_3 hexane).

Synthesis of polymer 14

The NIPAA (448 mg, 3.06 mmol), AIBN (1.18 mg, 0.0072 mmol), **4b** (22.065 mg, 0.013 mmol) were dissolved in 2.370 mL of acetonitrile, then 150 μ L of DMF were added. The solution was equally divided into four Schlenk tubes, on which the same procedure was performed. The same procedure was followed for every Schlenk tube. The reactions were quenched after 1, 3, 5, 7 hours, respectively. The polymer **14** (with a reaction time of 5 hours) was obtained as a pale yellow solid.

^1H NMR (400 MHz, CDCl_3) δ : 4.42 (d, $J=13.3$ Hz, $\text{ArCH}_{\text{ax}}\text{HAr}$), 4.00 (H_b), 3.79 (bs, OCH_2), 3.13 (d, $J=13.3$ Hz, $\text{ArCHH}_{\text{eq}}\text{Ar}$), 2.95 (CH_3 DMF), 2.88 (CH_3 DMF), 1.83 (H_e), 1.63 (H_d), 1.14 (H_c), 0.88 (CH_3 hexane).

Synthesis of polymer 15

The NIPAA (453 mg, 4.01 mmol), AIBN (1.18 mg, 0.0072 mmol), **4a** (21.05 mg, 0.012 mmol) were dissolved in 2 mL of acetonitrile, then 150 μ L of DMF were added. The reaction was quenched after 5 hours. Polymer **15** was obtained as a pale yellow solid.

^1H NMR (400 MHz, cdcl_3) δ : 4.40 (d, $J=13.3$ Hz, $\text{ArCH}_{\text{ax}}\text{HAr}$), 4.00 (H_b), 3.14 (d, $J=13.3$ Hz, $\text{ArCHH}_{\text{eq}}\text{Ar}$), 2.95 (CH_3 DMF), 2.88 (CH_3 DMF), 1.83 (H_e), 1.62 (H_d), 1.14 (H_c).

Synthesis of polymer 16

The NIPAA (1028 mg, 9.09 mmol), ACVA (5.096 mg, 0.0182 mmol), **4b** (27.85 mg, 0.0227 mmol) were dissolved in 5.38 mL of acetonitrile, then 150 μ L of DMF were added. The reaction was quenched after 5 hours. Polymer **16** was obtained as a pale yellow solid.

^1H NMR (400 MHz, CDCl_3) δ : 7.00 (d, $J=7.7$ Hz, ArH_{meta}), 6.92 (d, $J=7.7$ Hz, ArH_{meta}), 6.76 (t, $J=7.7$ Hz, ArH_{para}), 6.64 (t, $J=7.7$ Hz, ArH_{para}), 4.43 (d, $J=13.3$ Hz, $\text{ArCH}_{\text{ax}}\text{HAr}$), 4.02 (H_b), 3.82 (bs, OCH_2), 3.34 (bs, SCH_2), 3.16 (d, $J=13.3$ Hz, $\text{ArCHH}_{\text{eq}}\text{Ar}$), 2.09 (H_e), 1.64 (H_d), 1.16 (H_c).

Synthesis of polymer 17

The NIPAA (147 mg, 1.30 mmol), ACVA (0.655 mg, 0.0023 mmol), **4a** (11.42 mg, 0.0056 mmol) were dissolved in 1.19 mL of acetonitrile, then 150 μ L of DMF were added. The reaction was quenched after 4.5 hours. Polymer **16** was obtained as a pale yellow solid.

^1H NMR (400 MHz, CDCl_3) δ : 6.92 (bs, ArH), 4.41 (d, $J=13.3$ Hz, $\text{ArCH}_{\text{ax}}\text{HAr}$), 4.02 (H_b), 3.73 (bs, OCH_2), 3.34 (bs, SCH_2), 3.17 (d, $J=13.3$ Hz, $\text{ArCHH}_{\text{eq}}\text{Ar}$), 2.10 (H_e), 1.65 (H_d), 1.16 (H_c).

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