Synthesis of tetrasulfonated calix[4]arenes designed for carbon-nanotube grafting and acetylcholine sensing

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Abstract

A tetrasulfonated calix[4]arene with a methylene-bridge substituent was prepared from a 2-(ω-haloalkyl)-substituted tetrahydroxy-*p*-tert-butylcalix[4]arene (**1**). Calixarene **1** was converted by ipso-sulfonation to tetra(*p*-sulfonato)calix[4]arene (**2**) by treatment with concentrated sulfuric acid at room temperature for approximately 5 h. Compound **2** was converted to its sodium salt form (**3**) by addition of aqueous NaOH. Conversion of the ω-halide to an ω-azide (**4**) was achieved by treatment of **3** with 2 equiv NaN_3 in H_2O for 48 h at 100 °C. These conversions afforded pure product in ca. 46% and 90% yield, respectfully, as judged by ^{1}H NMR spectroscopy. Aqueous association constants of **3** and **4** with acetylcholine chloride were studied via NMR-titration and isothermal calorimetry (ITC) experiments. Efforts to covalently attach compound **4** to multiwall carbon nanotubes (MWCNT) by thermal decomposition of the azide and reaction between an intermediate nitrene and MWCNT, and the subsequent characterization of these products will be described.

NOTE: *numbering notation of calixarenes defined alternatively in this abstract relative to rest of paper.*

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General Introduction

The development of molecular sensing devices is essential for monitoring one's health, detecting toxins, and sensing the presence of disease-causing agents. The accuracy, precision, and economic suitability of such sensors can potentially limit their application and use in the world, and therefore, there is a need for enhancing modern sensors and developing new, more effective ones. One specific molecule that requires a more economically-friendly sensing device is acetylcholine (ACh), a neurotransmitter.[1] This project seeks to create a novel sensing device for ACh through the incorporation of a commonly used ACh sensor-molecule, *p*-sulfonatocalix[4]arene, into a carbon-nanotube (CNT) device. Prior to the discussion of this target device and the goals for this project (Section 2), previous work in relevant fields – calixarenes (1.1), ACh & its sensing (1.2), and CNT devices (1.3) – must first be summarized.

1.1 Calixarenes

Though they can easily be introduced within the parameters of ACh sensing, calixarenes are deserving of a broader discussion due to their wide applications beyond ACh, which in turn heightens the potential implications of this study. These extended implications are discussed within Section 2.

Calix[*n*]arene is a macrocycle or cyclic oligomer composed of *n* number of phenol derivatives linked via methylene substituents (or "bridges") at the ortho position. This paper focuses on derivatives of *p-tert*-butylcalix[4]arene (**1**), which is easily synthesized at large scale via the treatment of *p-tert*-butylphenol and formaldehyde with base.[2] Since the discovery of this high yielding procedure by Gutsche *et al.*, numerous derivatives have been synthesized.[3] Each targets complex formation with various analytes through

the modification of calixarene's three distinct regions: upper rim, lower rim, and methylene bridge (Figure 1).[3] Examples include chiral calixarenes serving as enantioselective receptors for asymmetric catalysis[4], amphiphilic cation calixarenes servings as counter-ion activators for DNA[5], and fluorophore-linked calixarenes used to sense toxic metals[6]. Another important modification of calixarene is its immobilization onto insoluble or chromatography-applied materials for the separation of target molecules.[7]

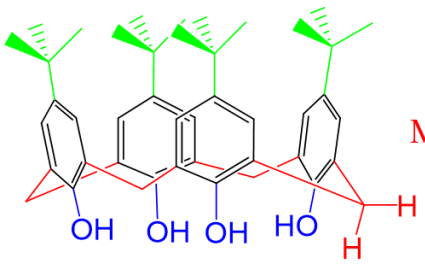

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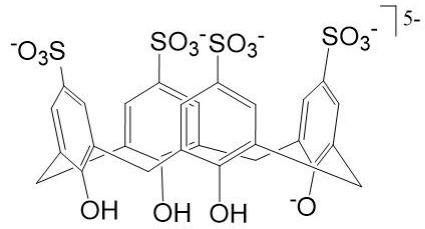
Figure 1. *p*-tert-butylcalix[4]arene and the three major regions of functionalization

One widely studied derivative of **1** possesses a substituted upper-rim with sulfonate groups. This *p*-sulfonatocalix[4]arene (**2**) has been shown to sense a variety of molecules:



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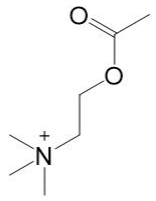
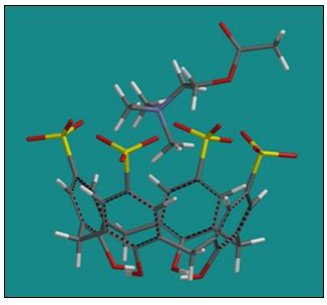


ACh



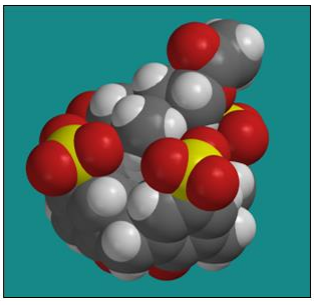


Figure 2. *p*-sulfonatocalix[4]arene (top left), acetylcholine (top right) drawn under neutral conditions (pH = 7). Complex of calixarene and ACh is shown below – depictions only differ in 3D molecular model used.

amino acids[8,9], quaternary ammonium cations[10], phosphonium cations[11], alcohols[12], and metal ions[13] (not an exhaustive list). Calixarene **2** is in fact the major "host" molecule used in sensing ACh.[1,10,14-18] It exhibits a strong binding affinity toward ACh in water due to combined effects of electrostatic forces from formal charges as well as hydrophobic effects (Figure 2). Thus, the bucket-like geometry of calixarene establishes an ideal binding pocket that is thermodynamically stable for ACh.

1.2 Acetylcholine

Sensing ACh in solution carries significance due to its role as a neurotransmitter, serving as regulator for the electrical signal transduction along cells' axons in the central nervous system.[14] ACh concentrations are closely controlled by the body and imbalances are related to multiple disorders: high concentrations lead to overstimulation of the neurons and muscle spasms;[14] or the blocking of ACh receptors leads to inactivity and accumulation of ACh.[19] The latter is known as *Myasthenia gravis* (MG) (Greek for 'serious muscle weakness'), which affects 20 in 100,000 people worldwide. In fact, this is considered as a huge underestimate of the true number of persons affected because of the poor diagnosis methods.[19] Altogether, it is useful to test for ACh concentration, but current methods are economically inadequate.[1,14]

As introduced in Section 1.1, calixarene **2** is frequently used to sense ACh. The most commonly used technique utilizes a fluorescent dye switch; here, ACh must displace a competitive fluorescent dye that is bound by calixarene **2**. Because the dye's fluorescence is quenched when bound by **2**, its displacement can be measured quantitatively by a fluorescence spectrometer. This experimental setup can detect ACh at a limit of 10^{-6} M.[17]

For reference, ACh is present at 10^{-9} M for normal levels in synaptic clefts, while at 10^{-3} M when accumulated due to MG.[10,17] Therefore, sensing at an ideal minimum of 10^{-5} M is sufficient for medical purposes (this minimum allows at least a 100-fold difference in detected ACh levels of 'normal' versus 'accumulated', which is sufficient for preliminary diagnosis testing and still allows for blood dilution). Fluorescence-switch meets this minimum, but it is still not applicable in a medical setting. The technique requires an expensive fluorescence spectrometer unavailable outside laboratory settings; in addition, detecting fluorescence in blood samples compromises sensing limits unless samples are diluted greatly.[20] Thus, a new ACh sensor is needed.

1.3 Carbon-nanotube Devices

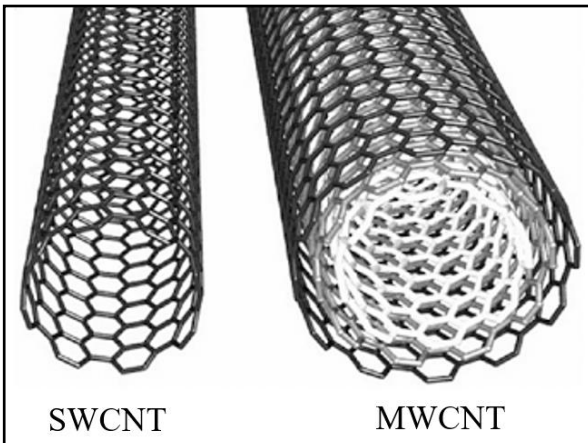


Figure 3. Visualization of single-walled (left) and multi-walled (right) carbon nanotubes. Image adapted from Kreupl *et al*.[21]

Carbon nanotubes (CNTs) have been an increasing interest in scientific research due to their unique electrical and mechanical properties.[21] Often described as a tube of graphene rolled up, CNTs come in multiple forms; there are single-walled CNTs (SWCNTs) and the more economically suitable multi-walled CNTs (MWCNTs) (Figure 3). The tunability of CNT characteristics through modification permits numerous desirable characteristics, such as altering electrical sensitivity, flexibility in design, and most importantly their chemical sensing abilities.[21] The ability of CNTs to serve as a low-cost chemiresisters drives the interest for further research in their potential applications.

Devices that employ CNTs are taking advantage of the electrical resistivity of the nanotubes, and that the resistivity changes in a reproducible way when the environment of the CNT is changed.[22-27] More specifically, the CNTs can be modified to sense specific analytes through the covalent attachment (or "grafting") of common sensor molecules. If a molecule (the “host”) with specific affinity for another molecule (the “guest”) is attached to a CNT, the resistivity of the CNT will change depending on whether a guest is bound up to the host molecule on the CNT. This variable resistivity to presence of guest molecule is the basis for sensing analyte with a CNT-device.

Many labs have fashioned a CNT-device using this principle of variable resistance[22-25]; however, the N-methylammonium sensor from Dionisio *et. al.* 2012 will be specifically discussed for illustrative purposes due to its similarity to the project described below.[25] There are multiple strategies of grafting molecules onto CNTs, and one common method is through the thermal decomposition of an azide into a nitrene and the subsequent reaction of the nitrene with the carbon-carbon double bonds on the CNT

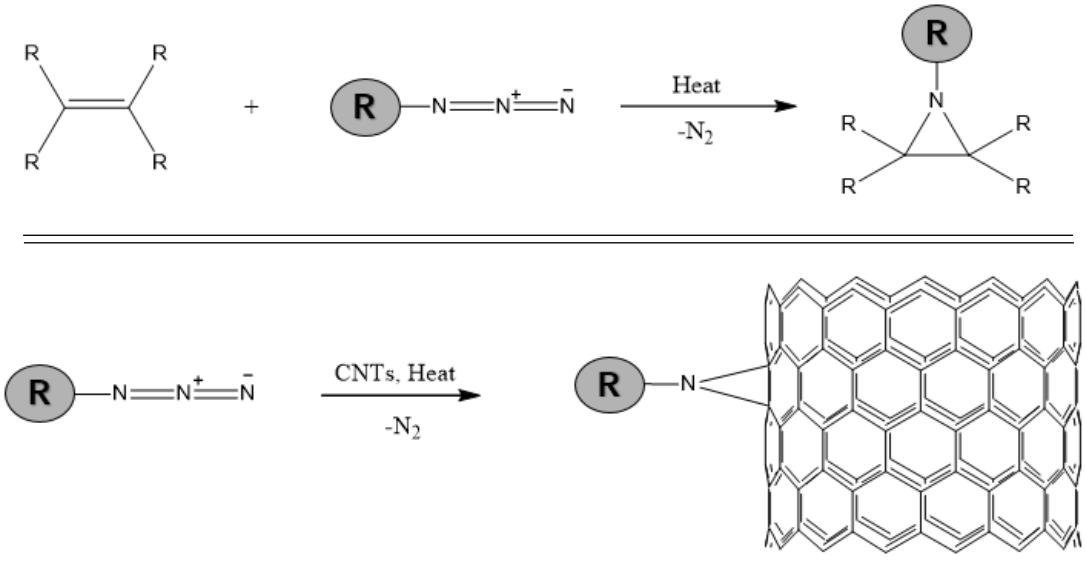


Figure 4. Thermal decomposition of an organic azide and the subsequent reaction with c-c double bond. General scheme (top) and when grafting to CNTs (bottom)

surface (Figure 4).[26-27] The resulting grafted-CNTs are directly incorporated into the sensing device. On a gold-coated slide with a 1 mm metal-free strip in the center, the grafted-CNT material completes the conducting circuit and serves as the variable resistor (Figure 5). In this device, solvent flow passes over the CNT-network, and analyte injections are made at set time intervals– the exposure of this analyte affects the resistance of the grafted-CNTs, which can be quantitatively recorded (Figure 5). In the case of N-methylammonium, the CNT-device was capable of sensing at 10^{-5} M, where $K_a = 10^5$ M^{-1} for the complexation in CNT-free system.[25] Analogous devices and measurements were made for sensing propranolol hydrochloride[23] and ethylene gas[24].

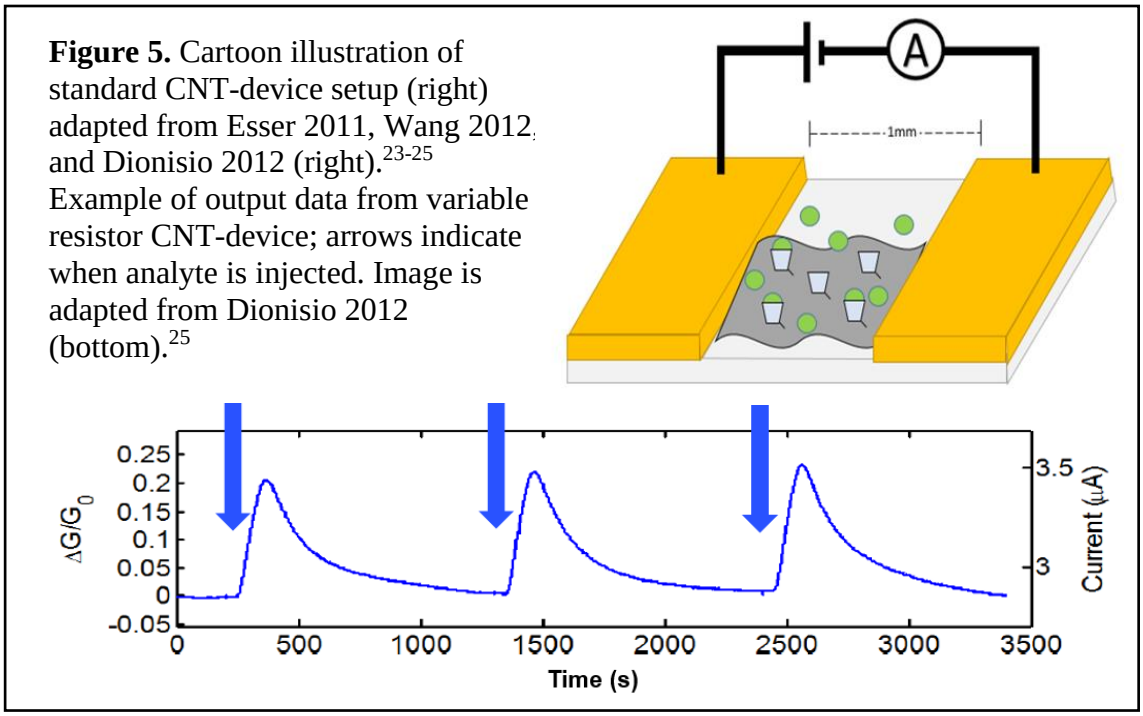


Figure 5. Cartoon illustration of standard CNT-device setup (right) adapted from Esser 2011, Wang 2012, and Dionisio 2012 (right).[23-25] Example of output data from variable resistor CNT-device; arrows indicate when analyte is injected. Image is adapted from Dionisio 2012 (bottom).[25]

Project Overview

This paper herein describes the motivation and progress towards a novel ACh-sensing device through the synthesis of a modified sulfonated calixarene. Such a project

brings together past works of the Fantini lab (calixarene modification), ACh-sensing with sulfonated calixarene, and modified CNT devices. A combination of aforementioned works yields this project's synthetic aim: the grafting of a sulfonated calixarene onto CNTs (Figure 6).

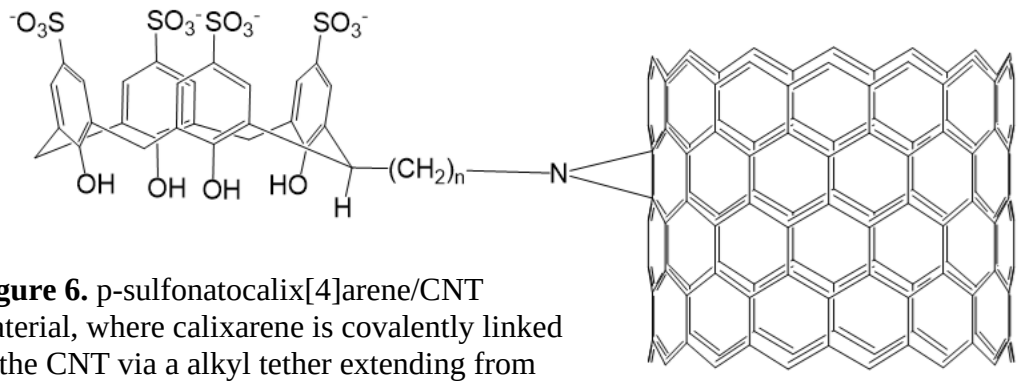


Figure 6. p-sulfonatocalix[4]arene/CNT material, where calixarene is covalently linked to the CNT via a alkyl tether extending from the methylene bridge

The grafting of calixarene onto CNTs will utilize an identical strategy as used by Dionisio and others – that is through the thermal decomposition of an azide tether (Figure 4).[25] While the clear majority of tethered calixarenes are modified at the lower rim, the Fantini group instead specializes in the modification of the methylene bridge.[7] Modification at the methylene bridge avoids surrendering a hydroxy lower-rim position, which plays a vital role in maintaining the cone formation of the calixarene (see Appendix A: Calix[4]arene Conformational Isomerism).[28] Thus, the target *p*-sulfonatocalix[4]arene of this project (Figure 7) possesses an alkyl azido tether attached at the methylene-bridge position.

The synthetic route for **7** begins with tetramethoxy-*p*-tert-butylcalix[4]arene and involves four modification steps: alkylation, demethylation, sulfonation, and azidination (Figure 7). Alkylation and demethylation procedures for **4** and **5** (Steps 1-2) have been

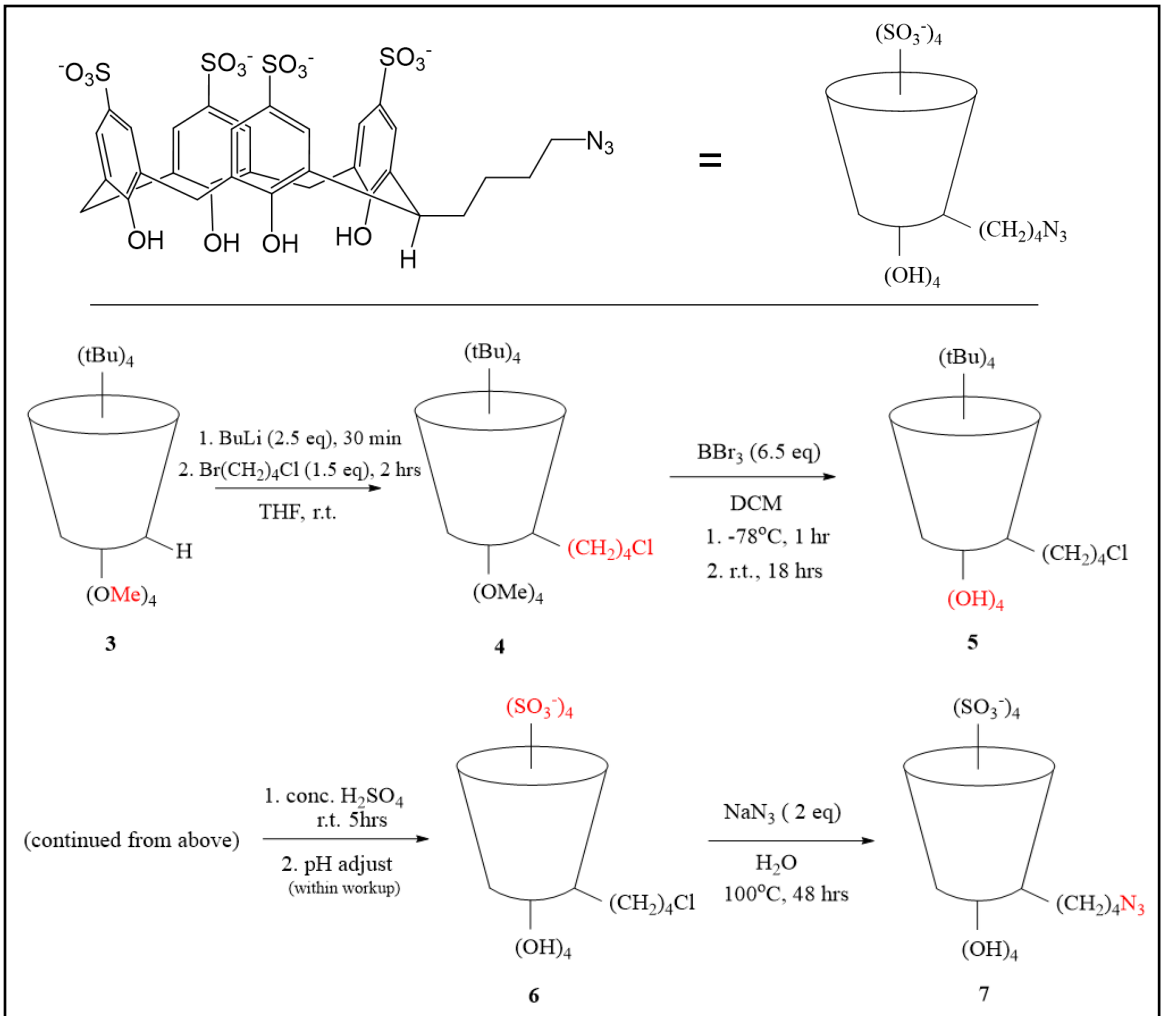


Figure 7. Target calixarene to be used for CNT-grafting and it corresponding short-hand representation (top). Synthetic scheme used to synthesize target calixarene (**7**). All sulfonated calixarenes exist as a pentasodium sodium salt (counter-cations not shown) – calixarenes are drawn as tetra-anions for simplicity.

previously published by Fantini *et al.* 2011, and the below procedures (3.2 and 3.3) use these reaction conditions with alternative workup/recrystallization methods.[28-29] The sulfonation and azidination for **6** and **7** (Steps 3-4) represent original procedures, which utilize reaction conditions similar to other calixarene sulfonations[8-13] and water-soluble azidinations[30] respectfully.

To confirm the resulting calixarene is still capable of acting as an ACh-sensor molecule, the binding affinity of **7** with ACh must be comparable to that of **2**. The

molecule can only be incorporated into a CNT-device in such a case, and this project applies NMR-titration and ITC techniques to investigate this aspect.

It then follows to graft **7** onto CNTs via azide decomposition described above. Whereas some devices involve grafting of calixarene onto SWCNTs, this project focuses on MWCNTs in its current state, largely due to MWCNTs being an inexpensive alternative for preliminary experiments – comparison to a SWCNT-material will be explored in a forthcoming project.[24-25] Common characterization techniques for confirming successful grafting include TEM imaging and even suspension of CNTs in solvent.[23-25,27] In the case of solvent-suspension, sonicating modified-CNTs in solvent (which the grafted material is soluble in) results in extended suspension of modified-CNTs relative to pristine-CNTs; modified-CNTs will remain suspended for weeks while pristine-CNTs settle within a few hours.[27] Indirect confirmation of attachment through interaction with analyte can also be used.[23-24] Solvent suspension and indirect characterization techniques will be described in this paper.

The resulting modified-CNT material can then be incorporated into a variable resistor device (Figure 5) for the sensing of ACh. Though the current project does not investigate device assembly, discussion of the potential device is warranted as it encompasses the justification and future for this project.

As mentioned before, the best current ACh-sensor involves a fluorescent-dye switch, which can detect ACh at a limit of 10^{-6} M.[19] Comparatively, the CNT-device from Dionisio *et al.* 2011 senses N-methylammonium at 10^{-5} M, where $K_a = 10^5\ M^{-1}$ for the analyte/macrocycle complexation in a CNT-free system; based on these data and the similar binding constant for ACh & **2** ($K_a = 10^5\ M^{-1}$), it can be hypothesized that this

project's target device would detect ACh at 10^{-5} M concentrations.[10,25] Note that this is factor of 10 worse than the fluorescent-dye method. However, the CNT-device has the added advantages of using solid state material and testing not requiring expensive equipment: the solid state is desirable for easier handling, and CNT-material can easily be recovered and washed; further, a variable-resistor device simply requires an ampere meter, which is small inexpensive device relative to a fluorescence spectrometer. Therefore, the targeted CNT-device can serve beyond demonstration of a novel ACh-sensing device.

The calixarene-CNT material can be further applied with other analytes as well. As discussed in Section 1.1, calixarene **2** can bind to amino acids, quaternary ammonium cations, phosphonium cations, alcohols, and metal ions. While this project strictly investigates ACh, the CNT-device can easily be tested with these other analytes, each with a range of applications.[8-12] Furthermore, the immobilization of calixarene onto CNTs establishes a novel solid-state material that can be used analogously to other calixarene-immobilized devices for separation and purification techniques.[7] For example, the target CNT-material of this study can be used to remove toxic metals from a solution or used in pesticide detoxicification.[12,32] Altogether, this project investigates the synthesis of a novel calixarene-CNT material that contends a large promise for ACh-sensing and other applications.

Methods and Materials

3.1. Materials

All reagents and compounds were attained from Sigma-Aldrich apart from concentrated sulfuric acid and buffer used in ITC experiments, which were purchased from Fischer Scientific. A 400 MHz JEOL spectrometer was used for all NMR experiments; Eppendorf 5804 R for centrifugations; Thermo scientific Nicolet iS5 with diamond iD5 ATR for IR spectroscopy. Calixarenes **1** and **3** is synthesized by methods previously reported.[2,3]

3.2. Synthesis of 2-(4-chlorobutyl)tetramethoxy-*p*-tert-butylcalix[4]arene (4)

Calixarene **3** (8 mmol, 5.640 g) was added to a flame-dried Schleck vessel and filled with N_2. The vessel was kept under inert gas for the remainder of reaction. The calixarene was fully dissolved in anhydrous THF (100 mL), which was added by cannulation, yielding a clear, yellow-tinted solution. n-Butyllithium (1.5 M in hexanes) was added via syringe dropwise until the solution remained red in color for more than 5 seconds. n-Butyllithium (1.5 M in hexanes, 12.48 mL, 20 mmol) was then added in equivalent amount giving a blood-red solution. The solution was left to stir under N_2 at room temperature for 30 minutes. The following alkylation was done in situ: 1-bromo,4-chlorobutane (1.38 mL, 12 mmol) was added by syringe, and the solution was left to stir for an additional 2 hours – the solution gradually changed from the initial blood-red to a yellow/orange solution during this time. The excess reagent was then quenched by adding aqueous sodium bicarbonate (saturated sol., 3 mL). THF was removed via rotary evaporation. The resulting yellow gel was dissolved in dichloromethane (DCM) (75 mL) and separatory-washed with sat. sodium bicarbonate (3x), deionized water (1x), and brine

(1x) (100 mL's each, 15 mL DCM backwash for each aq. wash). The final organic solution was dried with $MgSO_4$ and then solvent was evaporated down to 10 mL. Solution was added slowly into stirring ethanol (95%, 175 mL) and then brought to a boil, which was sustained until the solution reached 160 mL. Solution was slowly cooled and crystals began forming after 15 minutes at -2 °C – it was kept at this temperature for a total of 24 hours. Product was filtered by vacuum yielding white crystals. 2nd and 3rd crops attained by further evaporation of ethanol solution also yielded white crystals. Product was dried in vacuo at 80 °C for 18 hrs (5.914 g, 7.433 mmol, 92.9% yield). ^{1}H NMR ($CDCl_3$/CD_3CN/NaI) δ (ppm) 7.20 (br, 8H, aryl), 4.59 (t, J = 11.6 Hz, 1H, Ar_2CHR) ,4.20 (d, J = 8.7 Hz, 3H, Ar_2CH_2 ax.), 4.055 (s, 6H, ArOMe), 4.042 (s, 6H, ArOMe), 3.54 (t, J = 9.3 Hz, 2H, 4-alkyl), 3.43 (d, J = 8.8 Hz, 3H, Ar_2CH_2 eq.), 2.11 (q, J = 7.9 Hz, 2H, 1-alkyl), 1.819 (m, J = 9.9 Hz, 2H, 3-alkyl), 1.44 (m, J = 11.0 Hz, 2H, 2-alkyl), 1.15 (s, 36H, ^{t}Bu)

3.3. Synthesis of 2-(4-chlorobutyl)tetrahydroxy-*p*-tert-butylcalix[4]arene (5)

Calixarene **4** (4.829 g, 6.07 mmol) was added to an oven-dried Schleck vessel and dissolved in DCM (75 mL). Vessel was filled with N_2 and kept under inert gas for the remainder of the reaction. Vessel was placed in dry-ice/acetone bath and 15 minutes were allowed for temperature equilibration. Concentrated BBr_3 (3.69 mL, 39.5 mmol) was added to the solution via syringe, changing the solution from colorless to brown. Solution was stirred for 1 hour, and it was then removed from the bath+N_2 and left to stir at room temperature with a drying tube for 18 hours. The reaction was quenched by adding 20 mL sat. sodium bicarbonate solution. The mixture was transferred for separatory washing, where additional washes included sat. sodium bicarbonate (3x), deionized water (1x), and

brine (1x) (100 mL's each, 15 mL DCM backwash for each aq. wash). Final organic solution was dried with $MgSO_4$ and then rotary evaporated down to 10 mL. Solution was transferred into stirring methanol (90 mL), causing the product to precipitate. White product was vacuum filtered off and dried in vacuo at 80 °C for 18 hrs (3.530 g, 4.77 mmol, 78.6% yield). A sample of the precipitated product (300 mg) was further recrystallized in ethanol (95%, 30 mL), but showed no discernable increase in purity relative to the precipitated product as determined by NMR. ^{1}H NMR ($CDCl_3$) δ (ppm) 10.270 (s, 4H, ArOH), 7.08 (d, J = 1.7 Hz, 2H, aryl), 7.06 (d, J = 1.4 Hz, 2H, aryl), 7.04 (d, J = 1.7 Hz, 2H, aryl), 6.98 (d, J = 1.4 Hz, 2H, aryl), 4.48 (t, J = 11.0 Hz, 1H, Ar_2CHR), 4.24 (dd, J = 5.9 Hz, 3H, Ar_2CH_2 ax.), 3.50 (br, 5H, 4-alkyl and Ar_2CH_2 eq.), 2.20 (q, J = 5.4 Hz, 2H, 1-alkyl), 1.84 (m, J = 10.2 Hz, 2H, 3-alkyl), 1.46 (m, J = 10.4 Hz, 2H, 2-alkyl), 1.203 (s, 18H, ^{t}Bu), 1.188 (s, 18H, ^{t}Bu).

3.4. Synthesis of 5 Na^+ ·[2-(4-chlorobutyl)tetrahydroxy-*p*-sulfonatocalix[4]arene]^5 (6)

Concentrated H_2SO_4 (40 mL) was added to a round bottom flask containing calixarene **5** (1.975 g, 2.67 mmol). The solution was vigorously stirred for 5 hours, open to atmosphere and at room temperature, after which a sample from the solution fully dissolved in water indicating completeness. During the 5 hours, the calixarene gradually dissolved in the sulfuric acid, and the mixture transitioned from colorless to red to dark orange over time. The solution was slowly added into deionized water (40 mL) in a chilled water/ice bath – the calixarene remained fully dissolved at first but began to precipitate when approximately half of the H_2SO_4/calixarene mixture was added. The precipitate was filtered off by vacuo yielding a pink solid that slowly turned purple when exposed to air (>3 g recovered – wet). Solid was then dissolved in deionized water (20

mL) giving a purple solution. Aqueous NaOH (10 M, 12 mL) was slowly added to the calixarene solution while monitoring the pH. At pH = 5.5, a more diluted NaOH solution (2 M) was used until the solution reached a pH = 7.0. Between pH = 6.5 and 7.0, the solution's color drastically shifted from a dark purple to a light yellow. The resulting aqueous solution was left in large beaker to evaporate off water (~30 hours). Methanol (100 mL) was added to the remaining yellow gel & white powder combination and brought to a boil (white solid remained insoluble), cooled slowly, and stored at -2 °C for 24 hours. The white solid was filtered from solution by vacuum filtration and dried in vacuo at 80 °C for 24 hours. NMR of the solid (100 mg in 1 mL D_2O) indicated only noise-level amounts of calixarene present, and thus the solid was considered Na_2SO_4 waste. The remaining methanol/calixarene solution was rotary-evaporated completely. The remaining dark yellow gel was dissolved in 10 mL deionized water and slowly added into stirring ethanol (100%, 70 mL) causing a fine, white precipitate to form. After storing the solution at -2 °C for 24 hours, the suspended precipitate was centrifuged off (5k rpm, 4 °C, 30 min). The supernatant was decanted off, and the pellet was resuspended in acetone (40 mL), followed by a second centrifugation at identical settings. The resulting white pellet was dried for 12 hours in vacuo without heat, during which the solid attained a slight brown tint, making it off-white in color. The pellet was grinded with a mortar & pestle and dried for an additional 96 hours in vacuo without heat, yielding our final product (1.185 g, 1.19 mmol, 44.6% yield). ^{1}H NMR (D_2O) δ (ppm) 7.42 (br, 4H, aryl), 7.39 (dd, J = 1.7 Hz, 2H, aryl), 7.33 (dd, J = 1.7 Hz, 2H, aryl), 4.83 (t, J = 10.4 Hz, 1H, Ar_2CHR), 4.17 (br, 3H, Ar_2CH_2 ax.), 3.47 (br, 5H, 4-alkyl and Ar_2CH_2

eq.), 2.07 (q, J = 5.4 Hz, 2H, 1-alkyl), 1.72 (m, J = 5.1 Hz, 2H, 3-alkyl), 1.32 (m, J = 10.1 Hz, 2H, 2-alkyl).

3.5. Synthesis of 5 Na^+ · [2-(4-azidobutyl)tetrahydroxy-*p*-sulfonatocalix[4]arene]$^{5-}$ (7)

Calixarene **6** (500 mg, 0.503 mmol) was dissolved in deionized water (20 mL), and the solution's pH was tested to confirm neutral conditions safe for inorganic azide. NaN_3 (65.7 mg, 1.01 mmol) was then dissolved in solution. The solution was refluxed (open) for 48 hours in an oil bath. Once cooled to room temperature, a sample (0.5 mL) was taken as an IR "pre-dialysis" reference. The remainder was moved into a prepped dialysis tubing (BioTech CE Tubing; MW 100-500 D) where a series of 5 washes in deionized water (500 mL baths, t = 1, 1.5, 2, 5, 12 hours sequentially) were done. The calixarene solution increased by roughly 200% volume during the final two washes. Another sample (1 mL) was taken for IR comparison to the 'pre-dialysis' sample – the dried samples indicated successful removal of the inorganic azide. The remaining solution was left in large beaker to evaporate off all water (~48 hours). The resulting off-white/yellow solid was grinded with mortar & pestle and dried in vacuo at 80 °C for 8 hours without further purification (429 mg, 0.450 mmol, 89.8% yield). ^{1}H NMR (D_2O) δ (ppm) ^{1}H NMR (D_2O) δ (ppm) 7.54 (br, 8H, aryl), 4.54 (t, J = 10.7 Hz, 1H, Ar_2CHR), 4.17 (d, J = 10.2 Hz, 3H, Ar_2CH_2 ax.), 3.67 (d, J = 9.6 Hz, 3H, Ar_2CH_2 eq.), 3.53 (t, J = 9.3 Hz, 2H, 4-alkyl), 2.20 (q, J = 5.0 Hz, 2H, 1-alkyl), 1.79 (m, J = 5.1 Hz, 2H, 3-alkyl), 1.35 (m, J = 10.4 Hz, 2H, 2-alkyl).

3.6. Synthesis of 5 Na^+ · [tetrahydroxy-*p*-sulfonatocalix[4]arene]$^{5-}$ (2)

Calixarene **1** (2.596 g, 4 mmol) was added to concentrated H_2SO_4 (40 mL) and heated to 80 °C for 3 hours with vigorous stirring. Following, the brown/black solution

was cooled to room temperature and then transferred into chilled deionized water (100 mL) in an water/ice bath. The now brown solution did not produce a precipitate. Solid NaCl (20 g) was added slowly until the solution became cloudy with a white precipitate. The precipitate was separated via vacuum filtration and washed with ethanol (95%, 3 mL) yielding a pink solid. The solid was left to airdry for 48 hours, during which the solid turned brown. The solid was dissolved in 20 mL and put through ion exchange resin: Amberlite IR 120 Na^+-form (10 g) was charged with brine (50 mL) followed by deionized water (50 mL), and then calixarene solution was put through resin. This was repeated 5x, during which the pH transitioned from 2 to 7. During the final two 'exchanges', the calixarene solution's color shifted from dark purple to light yellow. The remaining solution was left in large beaker to evaporate off all water (~24 hours). The resulting yellow solid was recrystallized in methanol (30 mL) that was dried in vacuo at 80 °C for 8 hours to yield a final white product (1.598 g, 1.85 mmol, 46%). Effective mol. wt. = 863.1 g/mol. ^{1}H NMR (D_2O) δ (ppm) 7.44 (s, 8H, aryl), 3.17 (s, 4H, ArOH).

3.7. Grafting of 5 Na^+ · [2-(4-azidobutyl)tetrahydroxy-*p*-sulfonatocalix[4]arene]$^{5-}$ (7) onto MWCNTs

Calixarene **7** (30 mg, 0.031 mmol) and MWCNTs (30 mg) were added to deuterated DMSO (5.00 mL). Solution was sonicated for 2 hours, where CNTs were settled to start and then fully suspended/homogenous at finish. A sample (0.5 mL) was taken to serve as t = 0 for later analysis. Solution was heated to 180 °C (oil bath, condenser, drying tube) for 96 hours. Samples (0.5 mL each) were taken at t = 24, 48, 96 hours during this period. All four samples where centrifuged (12k rpm, 15 min) to remove CNTs, and remaining supernatants were analyzed by ^{1}H NMR. Spectra showed disappearance of calixarene in

samples. Supernatants displayed gradient color change over time, where t=0 was colorless solution and t=96 h was dark purple. Remaining solution reaction was centrifuged (12k rpm, 15 min) and supernatant decanted off. The remaining CNT-pellet was resuspended in DMSO (3 mL) and centrifuged again ((12k rpm, 20 min), followed by decanting of the solvent; this was repeated 3x. One last wash of the CNTs was done with deionized water (3 mL). The final washed CNTs were dried in vacuo at 80 °C for 24 hours (33 mg). Characterization will be described.

3.8. Isothermal Calorimetry

Phosphate buffer (pH = 7.2) purchased was reported as containing the following: KH_2PO_4 (1.544 mM), $Na_2HPO_4 \cdot 7H_2O$ (2.709 mM), NaCl (155.17 mM). The experiments were carried out by starting with a calixarene/buffer solution (between 0.403 and 0.811 mM) and then titrating in aliquots of acetylcholine chloride/buffer solution (7.43 mM) in select amounts.

3.9. NMR Titrations

D_2O buffer was prepared containing $Na_2HPO_4 \cdot 7H_2O$ (10 mM) and $NaOAc \cdot 3H_2O$ (4 mM) and pH adjusted using DCl/D_2O solution to pH = 7.0. This buffer solution was used to prepare a ‘guest solution’ containing prior compounds and added acetylcholine chloride (2.5 mM). This guest solution was in turn used to make a ‘host solution’ containing prior compounds and added sulfonated calixarene (11 mM). The experiments were carried out by starting with guest solution and then titrating in aliquots of host solution in select amounts. Optimized fits were made using 1:1 stoichiometric assumptions and supramolecular.org. Reported data (molar ratios) are based on NMR

calculations against the sodium acetate internal standard, not measurements during solution preparation.

Results and Discussion

4.1. Synthesis of Target Calixarene

Of the four-step synthesis, the first two steps had previously been published by the Fantini group.[28-29] The first (alkylation) involves the deprotonation of a methylene hydrogen followed by *in situ* alkylation. Because tetramethoxy calixarene **3** is used rather than **1** with hydroxy lower-rim groups, the protons at the methylene bridge position become the most acidic hydrogens; interestingly, only 1 out of 8 methylene hydrogens is deprotonated even with equivalent excess of butyllithium base added – as found previously. This paper, however, reports an improved workup procedure relative to Fantini *et al.* 2011. The previous procedure uses a methylene chloride and methanol mixture for recrystallization at 78.8% overall reaction yield, whereas it has since been found that a methylene chloride and ethanol (95%) mixture gives 92.9% overall yield.[28] Furthermore, recrystallization often failed in methanol solution due to rapid fallout of the product, causing the material to gel; no such difficulties have been encountered with ethanol-based recrystallization. This is a surprising find due to *p*-tert-butlycalix[4]arene's extremely hydrophobic nature and sensitivity to small amounts of water in solution, but the results are nevertheless useful. The following demethylation reaction had identical findings for an ethanol-based recrystallization procedure, and yields were improved from 50.4% to 78.6%.[29]

The following sulfonation of **5** encompassed the most difficult and lowest yielding step of the synthetic route. Because of this, it was preferred to change the tether from chloro- to azido- first and have the sulfonation last in the synthetic route; however, it was found that the azide would not survive the sulfonation condition. Thus, sulfonation had to be carried out third. All literature reports the sulfonation of **1** into **2** at 80 °C for 3 hours, including the adapted procedure reported in this paper (Section 3.6). When ran at room temperature, no detectable reaction progress was made within 8 hours. Conversely, the sulfonation of **5** into **6** required no applied heat and went to completion after 5 hours at room temperature. The solubility of **5** as caused by its methylene-bridge substituent is believed to have a major role in this outcome; **5** slowly dissolves in H_2SO_4 whereas **1** does not. Additionally, the increased solubility of **6** required an alternative, more extensive recovery procedure (i.e. whereas **2** can be simply filtered off at reaction completion, **6** was fully dissolved in H_2SO_4). Despite its breadth, a recovery procedure for the **5** into **6** sulfonation was found with comparable yield to the **1** into **2** sulfonation (44.6% and 46.0% yields, respectfully). A recrystallization procedure to replace the precipitation in ethanol has yet to be found. ^{1}H NMR confirmed the successful synthesis of **6**, where the calixarene exists entirely in the cone formation.

Finally, the conversion of **6** into **7** has also been successfully shown. Though Fantini *et al*. have previously reported azidination procedures for *p*-tert-butylcalix[4]arene, an alternative procedure had to be found due to **6** being insoluble in most organic solvents. Instead, reaction conditions were adapted from azidination of water-soluble compounds.[30] The reaction was first monitored in D_2O, where aliquots were taken over time for characterization by ^1H NMR (Figure 8). The conversion of the chloro- to an

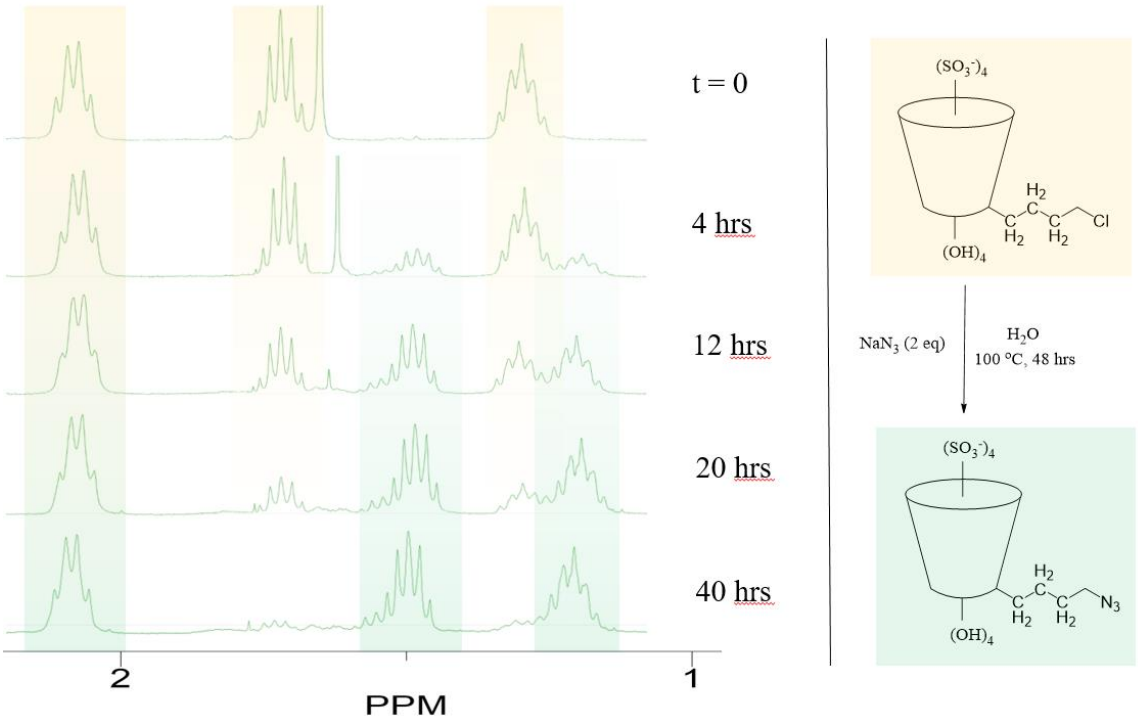


Figure 8. ^1H NMR spectra indicating the conversion of **6** into **7** over time. The peaks shown correspond to the three pairs of protons on the alkyl-tether of the methylene bridge. The three pairs visible are explicitly written out in the shorthand structures (right). The singlet at ~1.6 ppm corresponds to trace ethanol solvent, which evaporates off during the reaction.

azido-tether was clearly seen as the reaction progressed. At completion, the excess sodium azide could not be removed by separatory washing due to **7**'s water-solubility, so dialysis washing was used. Comparison of pre- and post-dialysis samples by IR-spectroscopy further confirmed the synthesis of the azido calixarene as well as the

successful removal of excess inorganic azide (Figure 9). The 90% yield reports the product in crude form, and a recrystallization procedure has yet to be established.

4.2. Acetylcholine Binding

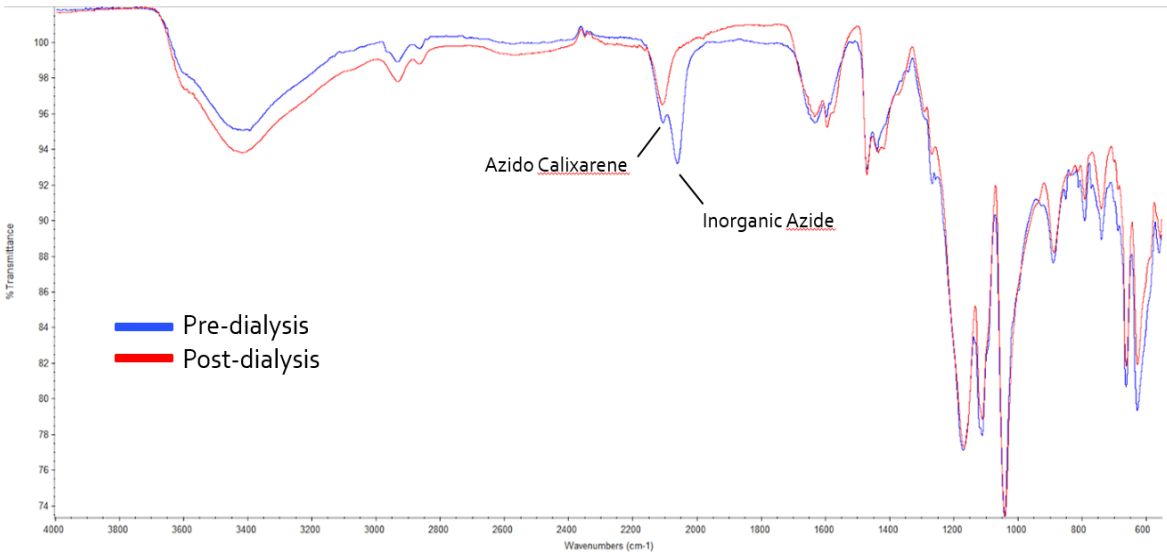


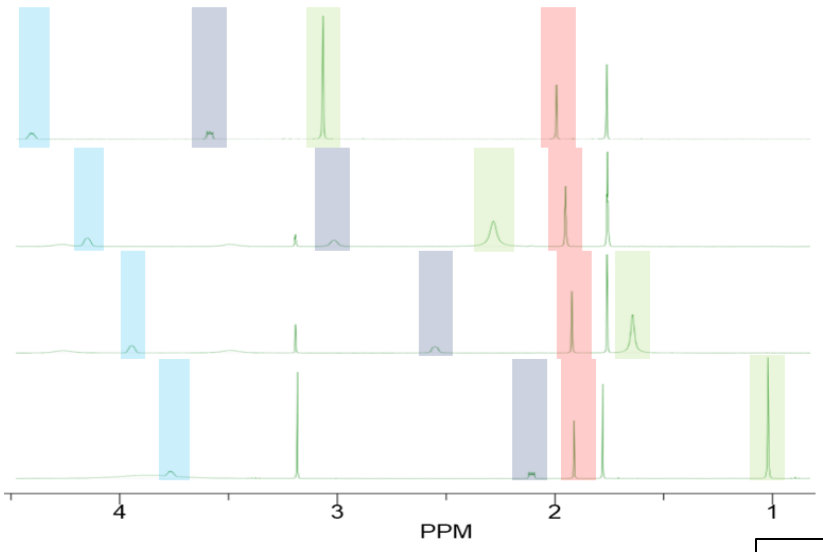
Figure 9. Characterization of **7** by IR-spectroscopy.

The association constants (K_a) of **6** and **7** with ACh have been investigated, using **2** as a control/reference. Both NMR titrations and ITC experiments were carried out under neutral pH conditions.

NMR titrations entail having a set solution of ACh and slowly adding aliquots of a calixarene solution – note that the experimental setup described in Section 3.9 keeps the concentration of ACh constant throughout the experiment, while slowly increasing the concentration of calixarene present. When encapsulated (or 'bound') within calixarene's cavity (Figure 2), ACh's protons exist in a distinct environment relative to its unbound state. More specifically, the aromatic ring of calixarenes induce an upfield shifts in ACh protons; the further in the cavity the proton, the larger the shift. The ratio of calixarene

(host) to ACh (guest) ([H]/[G]) increases, the molefraction of ACh bound (χ) increases as well and a chemical shift in NMR (Δδ) is observed. Only one peak represents both the bound and unbound state for a given proton due to the rapid association and dissociation of the complex – thus an 'average' chemical shift is observed. These phenomena are depicted in Figure 10.

Though NMR titrations have been done for **2**, **6**, and **7**, the experiments and corresponding data must all be treated as preliminary. Titration curves for both



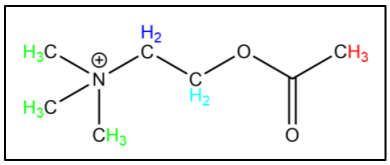


Figure 10. ^{1}H NMR spectra of NMR-titration experiment with **2**. ACh proton peaks are highlighted and correspond to respective colors as labeled in the ACh structure shown.

experimental curves (**6**,**7**) and the control (**2**) match predicted trends, as discussed in the previous paragraph (results for **2** are shown in Figure 11 as example. All curves can be found in Appendix B: Figure B.6). However, when Δδ curves are fitted, optimized fits still possess large percentage errors and non-random residual plots. This is due to the unoptimized distribution of [H]/[G] ratios used.[32] This can easily be seen by looking at χ-distribution and seeing most data (more than 5 out of 10 data points in all cases) are

recorded at $\chi_{HG} > 0.90$. Adjustments were made with each following NMR titration, and percent errors in K_a's were decreased from 397.1% to 13.5% in the first experiment to the most recent, respectfully (Table B.1). These preliminary data do propose the expected $K_a = 10^5\ M^{-1}$ to ACh with minimal to no loss in affinity of **7** relative to **2**; again, these data are still not conclusive due to the large percent errors, and further optimization is required.

ITC on the other hand involves a highly sensitive, thermally-conducting probe used to detect heat changes. In the experiment, the probe maintains a constant temperature and

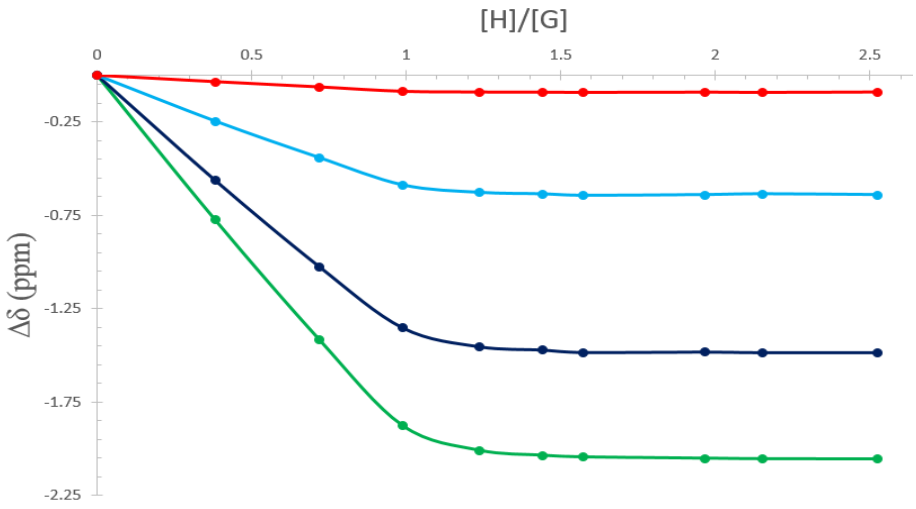
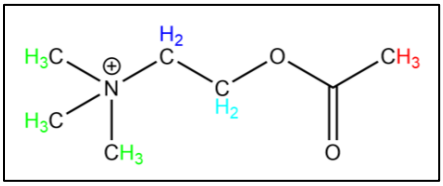


Figure 11. Results of NMR-titration of **2** into ACh (2.5 mM), where each is H and G, respectfully. Each curve corresponds to one ACh proton peak and the chemical shifts (Δδ) it went through throughout the titration. Curves correspond to respective colors as labeled in the ACh structure shown.



records the input of power required to do so. As guest is titrated into the host solution, heat is either released or absorbed causing the power input to adjust accordingly. (Note: this is the reverse of NMR titration described above, where host is titrated into guest. The distinction between host and guest is often arbitrary, and thermodynamic values are

unchanged when the labels are reversed.[32]) Integration of power-input responses, followed by a fitting of the corresponding curve, produces values for the association constant (K_a) stoichiometry (n), and enthalpy of complexation (ΔH). The remaining thermodynamic values of Gibbs energy (ΔG) and entropy (ΔS) can then be calculated through $\Delta G = -RT \ln(K_a) = \Delta H - T\Delta S$ (where R = gas constant, T = absolute temperature). Results for **2** are shown in Figure 12 as example, and all curves can be found in Appendix B: Figures B.7.

The data for all ITC experiments are summarized in Table 1 with a comparative literature reference that also ran ITC on **2**.[18] The first major aspect that must be addressed

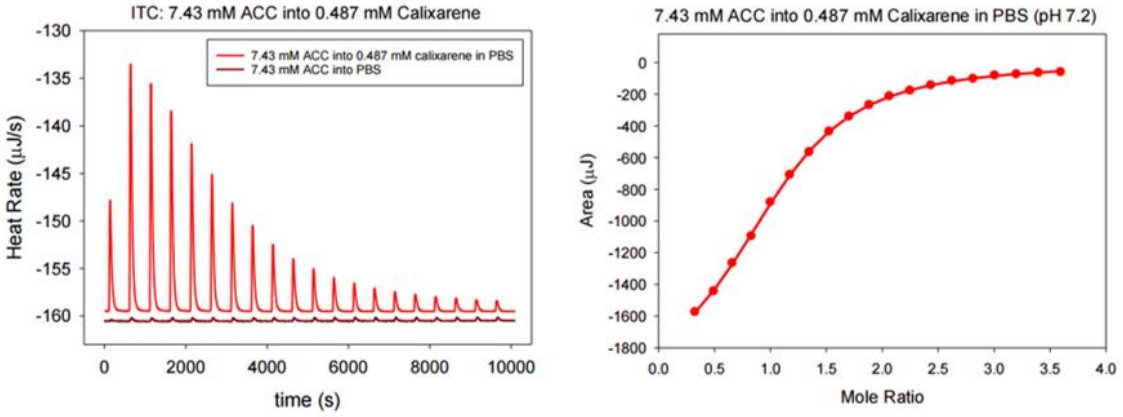


Figure 12. Results of ITC-titration of ACh into **2**.

is the binding constant, which is an order of magnitude lower than the $K_a = 10^5\ M^{-1}$ referenced throughout this paper. Literature suggests that this loss in binding affinity is by virtue of the large salt concentration present in solution – likely through the competitive binding of the high concentration of cations (Na^+ and K^+) that causes the stabilization of unbound host/guest ions and hinder complexation.[13,17] For reference, ITC buffer contained 155 mM NaCl relative to the 300-800 µM calixarene and 7.43 mM ACh solutions used. In total, cations 'stabilizing' calixarene were $[Na^+] \approx 160$ mM and $[K^+] \approx 2$

mM, where their association constants to calixarene are K_a = 85 and 115, respectfully.[13] Thus, the excess of inorganic salt in solution is believed to electrostatically stabilize unbound calixarene and ACh and thus hinders their complexation by an order of magnitude. Further experiments will need to be done to confirm this hypothesis with certainty.

Beyond the reduced K_a values, ITC experiments produced very promising data. The small amount of error permits analysis of these data with confidence. Further, the experiments ran with **6** at different concentrations are in agreement (as to the overlap of their 95% confidence intervals). The negative entropic and enthalpic values match expectations of an association of two molecules ($\Delta S<0$) into a more thermodynamically stable complex ($\Delta H<0$). Comparatively, **6** and **7** binding appears to occur at slightly reduced but still competitive levels relative to **2** (av. 3.60% difference in ΔG).

Table 1. Results of ITC experiments. 95%-confidence intervals for association constants and enthalpy of associations are shown

Calixarene	log(K_a)	ΔH (kJ/mol)	-TΔS (kJ/mol)	ΔG (kJ/mol)
2 (lit. reported[18])	4.14	-30.12	6.49	-23.63
2	4.007 ± 0.013	-27.96 ± 0.26	5.082	-22.87
6 (403 μM)	3.889 ± 0.029	-28.73 ± 1.15	6.538	-22.20
6 (803 μM)	3.860 ± 0.017	-27.59 ± 0.38	5.557	-22.03
7	3.839 ± 0.019	-26.74 ± 0.34	4.827	-21.91

4.3. MWCNT Grafting

As previously discussed, major techniques used to characterize the grafting of compounds onto CNTs are TEM, solvent suspension, and indirect confirmation (see Project Overview). Only solvent suspension and indirect characterization techniques have been investigated thus far.

Due to the water-solubility of **7**, deionized water was selected as the solvent for testing solvent suspension. After sonication for 2 hours, both pristine and **7**-grafted MWCNTs were fully suspended in the aqueous solvent. Pristine MWCNTs settled entirely within 7 hours, while grafted-MWCNTs remained entirely suspended for over 1 month. This is in agreement with published findings that found extended suspension indicated successful grafting of material.[27]

Indirect characterization was also attempted via ITC with **7**-grafted MWCNTs serving as the "host" compound. While the concentration of CNTs suspended in buffer was known, the experimental data was treated with [Host] as an unknown variable, which was later calculated by forcing the stoichiometric ratio of complexation to be 1:1. This allows for an indirect measure of the amount of **7** grafted per mass MWCNTs. This approach assumes there is no loss in **7** binding affinity once grafted. With a grafted-CNT concentration of 2.67 mg/mL, optimized data found [Host] = 0.105 mM = 99.2 μg/mL. Thus, data suggests the grafted-MWCNTs are 3.72% calixarene by mass. However, this calculation does not confirm attachment for two reasons: (1) there is no negative control such as pristine MWCNTs or even benzenesulfonic acid grafted MWCNTs for comparison; (2) the titration curve for ACh into grafted-CNTs does not clearly indicate a complexation pattern (Figure B.8). The power-input's response for this titration are extremely close to the noise-level measurements. Only the first 3-4 peaks clearly show a temperature response, which again cannot be confidently attributed to calixarene/ACh complexation without a negative control. Thus, the data reported for this indirect characterization is inconclusive.

Conclusions

It has been reported that the synthesis of **7** has been achieved via a 4-step synthetic route. Through the improved yields of previously reported procedures and optimization of those newly reported, the overall scheme gives **7** in 29.3% net yield.

Investigation of ACh binding by **7** has also been investigated. NMR titrations provide only preliminary data, but development of these titration procedures is trending toward low-error, precise data. ITC experiments indicate association constants of ~10^4 M^{-1} for sulfonated calixarenes **2** and **7**, where the decrease in K_a is attributed to excessive inorganic salt present in the buffer solution. Nevertheless, **7** shows minimal loss in affinity toward ACh, relative to **2**, as indicated by a 4% difference in ΔG of association.

Efforts to graft **7** onto the surface of MWCNTs is ongoing. The strongest evidence of attachment pertains to the extended suspension of treated CNTs in water, relative to pristine CNTs. Strategies to indirectly confirm attachment are inconclusive due to the lack of a negative control.

Altogether, this paper reports significant advancements toward a calixarene-based material designed for an ACh-sensing CNT-device.

References

1. Pradhan, Tuhin; Jung, H.S.; Jang, J.H.; Kim, T.W.; Kang, C.; Kim, J.S. Chemical sensing of neurotransmitters. *Chem. Soc. Rev*., **2014**, 43, 4684-4713
2. Gutsche, David C.; Iqbal, Muzaffer; Stewart, Donald. Calixarenes. 18. Synthesis Procedures for *p*-tert-Butylcalix[4]arene. *J. Org. Chem*. **1986**, 51, 742-745
3. Gutsche, David C. *Calixarenes: An Introduction (2)*. **2008**, 116-146
4. Li, Shao-Yong; Xu, Yao-Wei; Liu, Jun-Min; Su, Cheng-Yong. Inherently Chiral Calixarenes: Synthesis, Optical Resolution, Chiral Recognition and Asymmetric Catalysis. *Int. J. Mol. Sci*. **2011**, 12, 429-455
5. Takeuchi, Toshihide; Bagnacani, Valentina; Sansone, Francesco; Matile, Stefan. Amphiphilic Counterion Activators for DNA: Stimuli-Responsive Cation Transporters and Biosensors in Bulkand Lipid Bilayer Membranes. *ChemBioChem* **2009**, 10, 2793-2799
6. Leray, Isabell; Valeur, Bernard. Calixarene-Based Fluorescent Molecular Sensors for Toxic Metals. *Eur. J. Inorg. Chem*. **2009**, 3525-3535
7. Gezici, Orhan; Bayrakci, Gezici. Calixarene-engineered surfaces and separation science. *J. Incl. Phenom. Macrocycl. Chem*. **2015**, 83, 1-18
8. Atwood, Jerry L.; Ness, Timothy; Nichols, Peter J.; Raston, Colin L.. Confinement of Amino Acids in Tetra-p-Sulfonated Calix[4]arene Bilayers. *Crystal Growth & Design* **2002**, 2, 171-176.
9. Buschmann, HJ.; Mutihac, L.; Schollmeyer, E. Complexation of Some Amino Acids and Peptides by p-Sulfonatocalix[4]arene and Hexasodium *p*-Sulfonatocalix[6]arene in Aqueous Solution. *J. Incl. Phenom.* **2003**, 46, 133-137
10. Lehn, Jean-Marie; Meric, Robert; Vigneron, Jean-Pierre; Cesario, Michele; Guilhem, Jean; Pascard, Claudine. Binding of acetylcholine and other quaternary ammonium cations by sulfonated calixarenes. Crystal structure of a [choline-tetrasulfonated calix[4]arene] complex. *Supramol. Chem.* **1995**, 5, 97-103
11. Abraham, W. Inclusion of Organic Cations by Calix[n]arenes. *J. Incl. Phenom. Macrocycl. Chem*. **2002**, 43, 159-174
12. Perret, Florent; Morel, Jean-Pierre, Morel-Desrosiers, Nicole. Thermodynamics of the Complexation of the p-Sulfonatocalix[4]arene with Simple Model Guests in Water: a Microcalorimetric Study. *Supramol. Chem*. **2003**, 3, 199-206
13. Bakirci, Huseyin; Koner, Apurba L.; Nau, Werner M.. Binding of inorganic cations by p-sulfonatocalix[4]arene monitored through competitive fluorophore displacement in aqueous solution. *Chem. Commun.* **2005**, 5411-5413

14. Korbakov, Nina. *Optical and electrical sensing of acetylcholine.* **2007**.

15. Korbakov, Nina; Timmerman; Lidich; Urbach; Sa'ar; Yitzchaik. Acetylcholine Detection at Micromolar Concentrations with the Use of an Artificial Receptor-Based Fluorescence Switch. *Langmuir*, **2008**, 24, 2580-2587

16. Ghale, Garima; Nau, Werner M.. Dynamically Analyte-Responsive Macrocyclic Host−Fluorophore Systems. *Acc. Chem. Res.* **2014**, 47, 2150−2159

17. Guo, Dong-Sheng; Uzunova, V.D.; Su, X.; Liu, Y.; Nau, W.M. Operational calixarene-based fluorescent sensing systems for choline and acetylcholine and their application to enzymatic reactions. *Chem. Sci.*, **2011**, 2, 1722-1734

18. Wang, Li-Hua; Guo, Dong-Sheng; Chen, Yong; Liu, Yu. Thermodynamics of interactions between organic ammonium ions and sulfonatocalixarenes. *Thermochimica Acta* **2006**, 443, 132-135

19. "Myastjenia gravis Foundation of America." myasthenia.org, National Health Council. **2010**. Web

20. Lowndes, Shannely. *Blood interference in fluorescence spectrum – Experiment, analysis and comparison with intraoperative measurements on brain tumor.* **2010**

21. Schnorr, J.M.; Swager, T.M.. Emerging Applications of Carbon Nanotubes. *Chem. Mater.* **2011**, 23, 646–657

22. Wang, Fei; Swager, Timothy M.. Diverse Chemiresistors Based upon Covalently Modified Multiwalled Carbon Nanotubes. *J. Am. Chem. Soc*. **2011**, 133, 11181–11193.

23. Kun, Zhao; Y. Shuai; T. Dongmei; Z. Yuyang. Electrochemical behavior of propanolol hydrochloride in nuetral solution on calixarene/multi-walled carbon nanotubes modified glassy carbon electrode. *J. Electroanalyt Chem* **2013**, 709, 99–105

24. Esser, Birgit; Schnorr, Jan M.; Swager, Timothy M.. Selective Detection of Ethylene Gas Using Carbon Nanotube-based Devices: Utility in Determination of Fruit Ripeness. *Angew. Chem. Int. Ed*. **2012**, 51, 5752–5756

25. Dionisio, Marco, Jan M. Schnorr, Vladimir K. Michaelis, Robert G. Griffin, Timothy M. Swager, and Enrico Dalcanale. Cavitand-Functionalized SWCNTs for N-Methylammonium Detection. *Am. Chem. Soc.* **2012**, 134, 6540−6543

26. Qin, S.; Qin, D.; Ford, W. T.; Resasco, D. E.; Herrera, J. E., Functionalization of single-walled carbon nanotubes with polystyrene via grafting to and grafting from methods. *Macromolecules* **2004**, 37, 752-757

27. Kong, Na; M. Shimpi; O. Ramström; M. Yan. Carbohydrate conjugation through microwave-assisted functionalization of single-walled carbon nanotubes using perfluorophenyl azides. *Carbohydr Res.* **2014**, 405, 33-38

28. Fantini, Jordan L.; Hardman, M.J.; Thomas, A.M.; Carroll, L.T.; Williams, L.C.; Parkin, S. Synthesis and 'click' cycloaddition reactions of tetramethoxy- and tetrapropoxy-2-(ω-azidoalkyl)calix[4]arenes. *Tetrahedron* **2011**, 67, 7027-7034

29. Fantini, Jordan L.; Hertel, M.P.; Behrle, A.C.; Williams, S.A.; Schmidt, J.A.R. Synthesis of amine, halide, and pyridinium terminated 2-alkyl-p-tert-butylcalix[4]arenes. *Tetrahedron* **2009**, 65, 8657-8667

30. Tanaka, Tomonari; Nagai, Hikaru; Noguchi, Masto. One-step conversion of unprotected sugars to β-glycosyl azides using 2-chloroimidazolinium salt in aqueous solution. *Chem Commun.* **2009**, 3378-3379

31. Guo, Dong-Sheng; Liu, Yu. Supramolecular Chemistry of *p*-Sulfonatocalix[n]arenes and Its Biological Applications. *Acc. Chem. Res.* **2014**, 47, 1925-1934

32. Thordarson, Pall. Determining association constants from titration experiments in supramolecular chemistry. *Chem. Soc. Rev*. **2010**

Appendix A: Calix[4]arene Conformational Isomerism

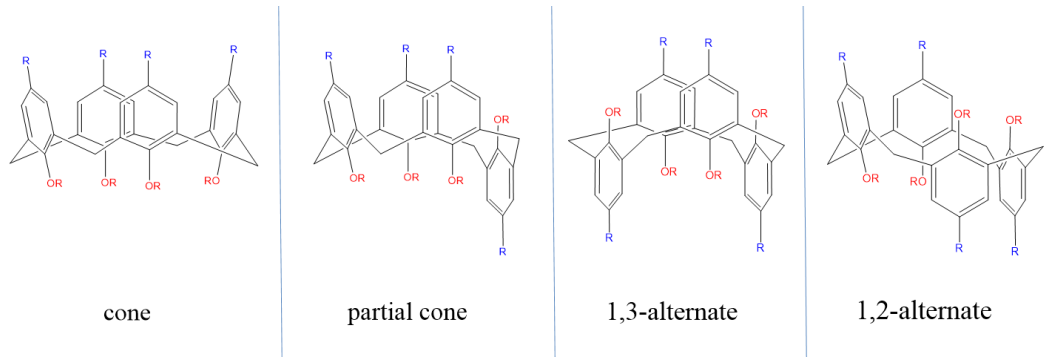


Figure A.1. The four possible conformational isomers for calix[4]arene

Calix[4]arene possesses a methylene bridge where aromatic groups can rotate freely about the Ar-C-Ar linkage. Because of this, calix[4]arene can exist as any of four conformational isomers (Figure A.1). There are many exceptions, however. Large substituents on the upper and/or lower rim can prevent the 'flipping' of a calixarene's aromatic, thus locking the calixarene in given state. With smaller substituents, the calixarene can be free to rotate about its conformational isomers, making NMR spectra very difficult to analyze (Figure A.2). However, introduction of a guest salt (such as NaI) can interact with the smaller substituents, causing the calix[4]ene to lock calixarene in the cone formation (Figure A.3). Fortunately for this study, tetrahydroxy-*p*-tert-butylcalix[4]arene and tetrahydroxy-*p*-sulfonatocalix[4]arene both lock in cone formation due to the hydrogenbonding of the hydroxy lower-rims with one another.

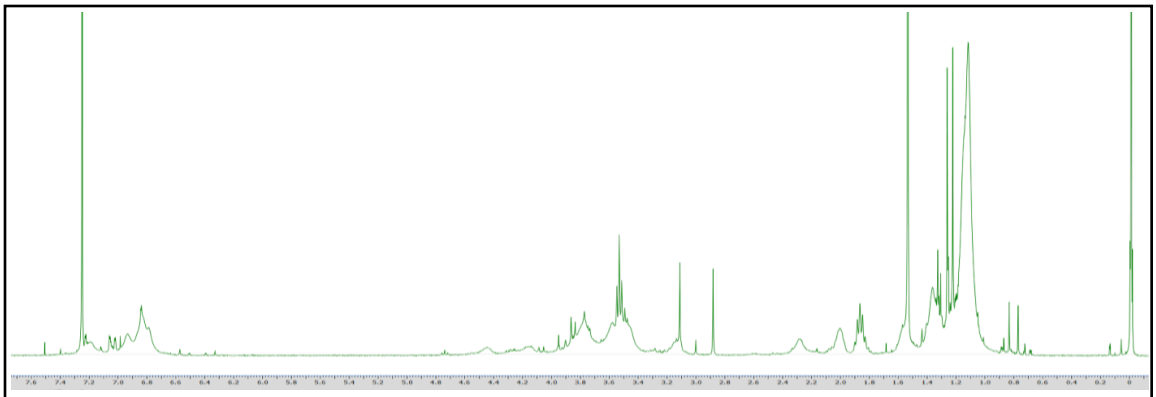


Figure A.2. ^{1}H NMR of 2-(4-chlorobutyl)tetramethoxy-*p*-tert-butylcalix[4]arene in $CDCl_3$

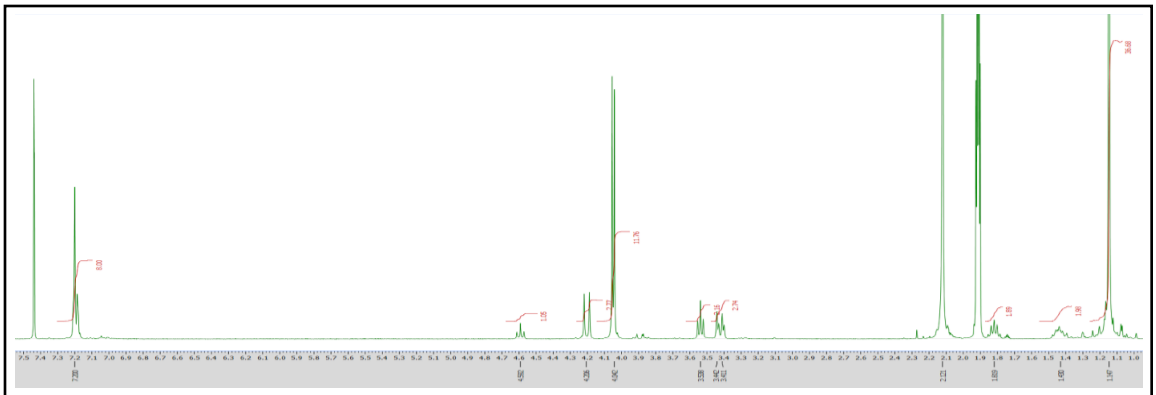


Figure A.3. ^{1}H NMR of 2-(4-chlorobutyl)tetramethoxy-*p*-tert-butylcalix[4]arene in $CDCl_3$:CD_3CN:NaI

Appendix B: Additional Spectra and Titration Curves

^{1}H NMR Spectra:

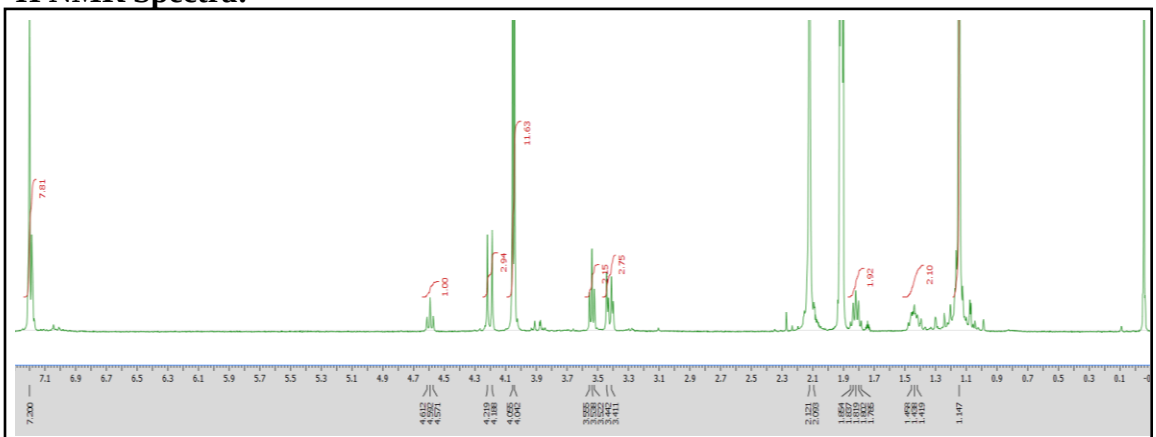


Figure B.1. ^{1}H NMR of 2-(4-chlorobutyl)tetramethoxy-*p*-tert-butylcalix[4]arene in $CDCl_3$:CD_3CN:NaI

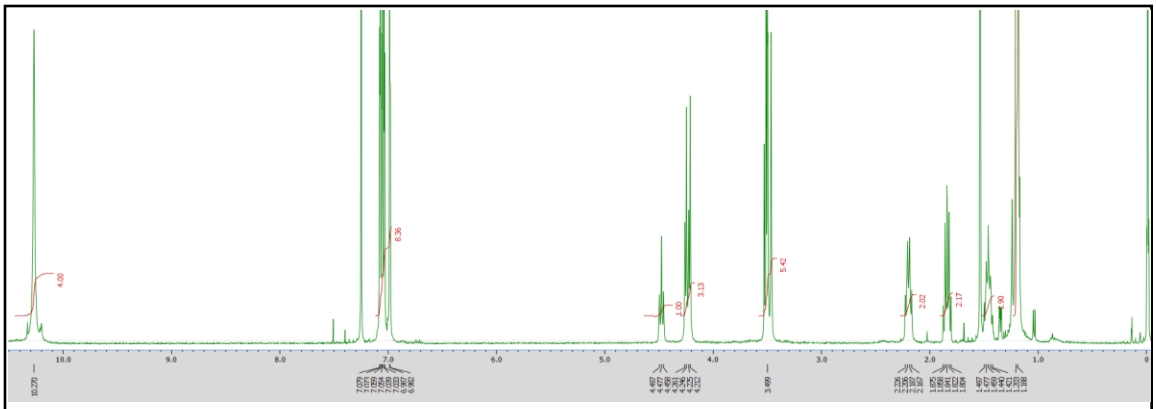


Figure B.2. ^{1}H NMR of 2-(4-chlorobutyl)tetrahydroxy-*p*-tert-butylcalix[4]arene in $CDCl_3$

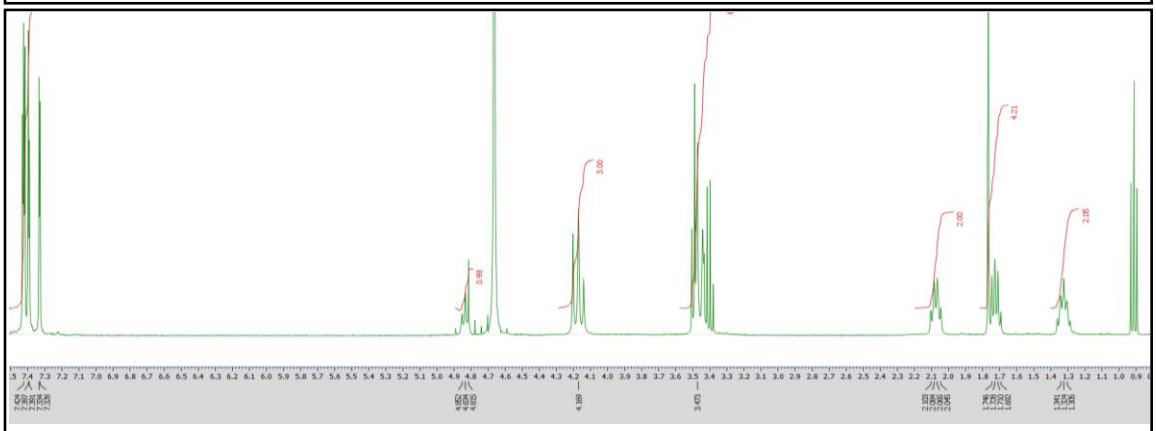


Figure B.3. ^{1}H NMR of 2-(4-chlorobutyl)tetrahydroxy-*p*-sulfonato[4]arene in D_2O

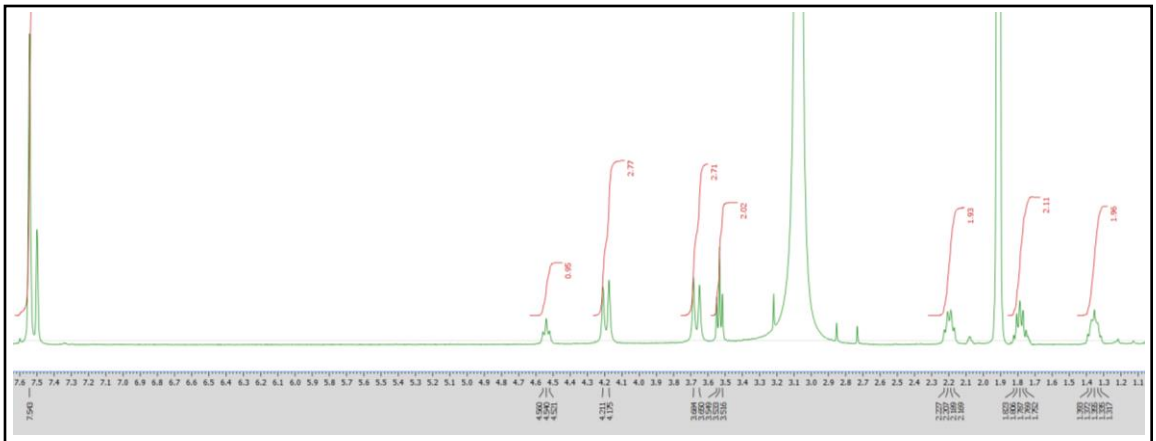


Figure B.4. ^{1}H NMR of 2-(4-azidobutyl)tetrahydroxy-*p*-sulfonato[4]arene in D_2O

Figure B.5. ^{1}H NMR of tetrahydroxy-*p*-sulfonato[4]arene in D_2O

NMR Titration Data:

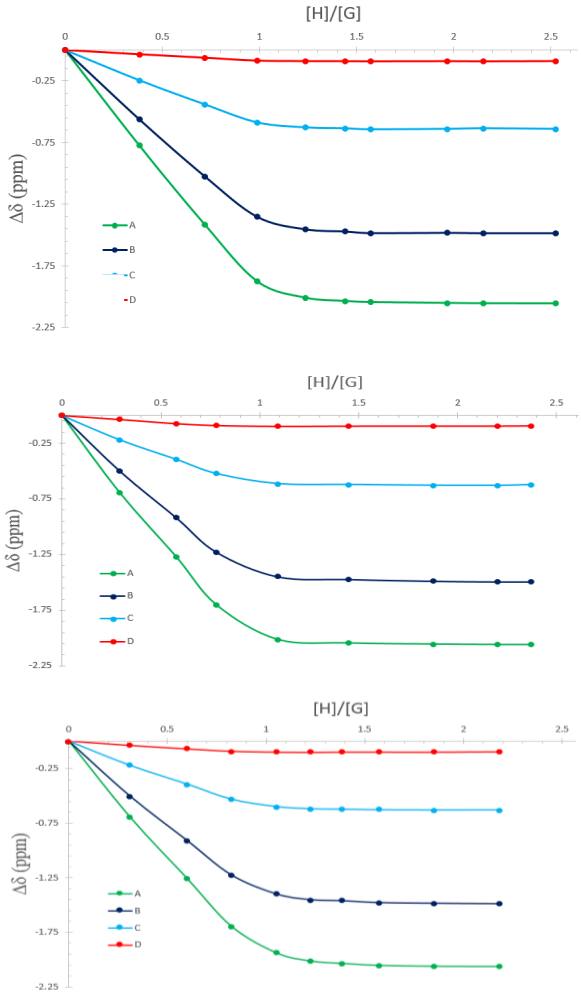


Figure B.6. Results of NMR-titration of **2** (top), **6** (middle), and **7** (bottom) into ACh (2.5 mM), where each is H and G, respectfully. Each curve corresponds to one ACh proton peak and the chemical shifts (Δδ) it went through throughout the titration. Curves correspond to respective colors as labeled in the ACh structure shown.

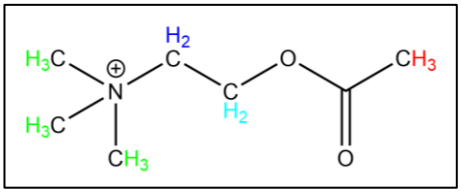


Table B.1. Resulting data from the fitting of NMR titration

Tether	K (M^{-1})	e_K (%)	ΔG (kJ/mol)	ΔG (kcal/mol)
Chloro	4.06E+05	397.1	32.0	7.65
Azide	8.24E+04	51.9	28.0	6.70
Control	8.36E+04	13.5	28.1	6.71

**assumed 298 K*

ITC Curves:

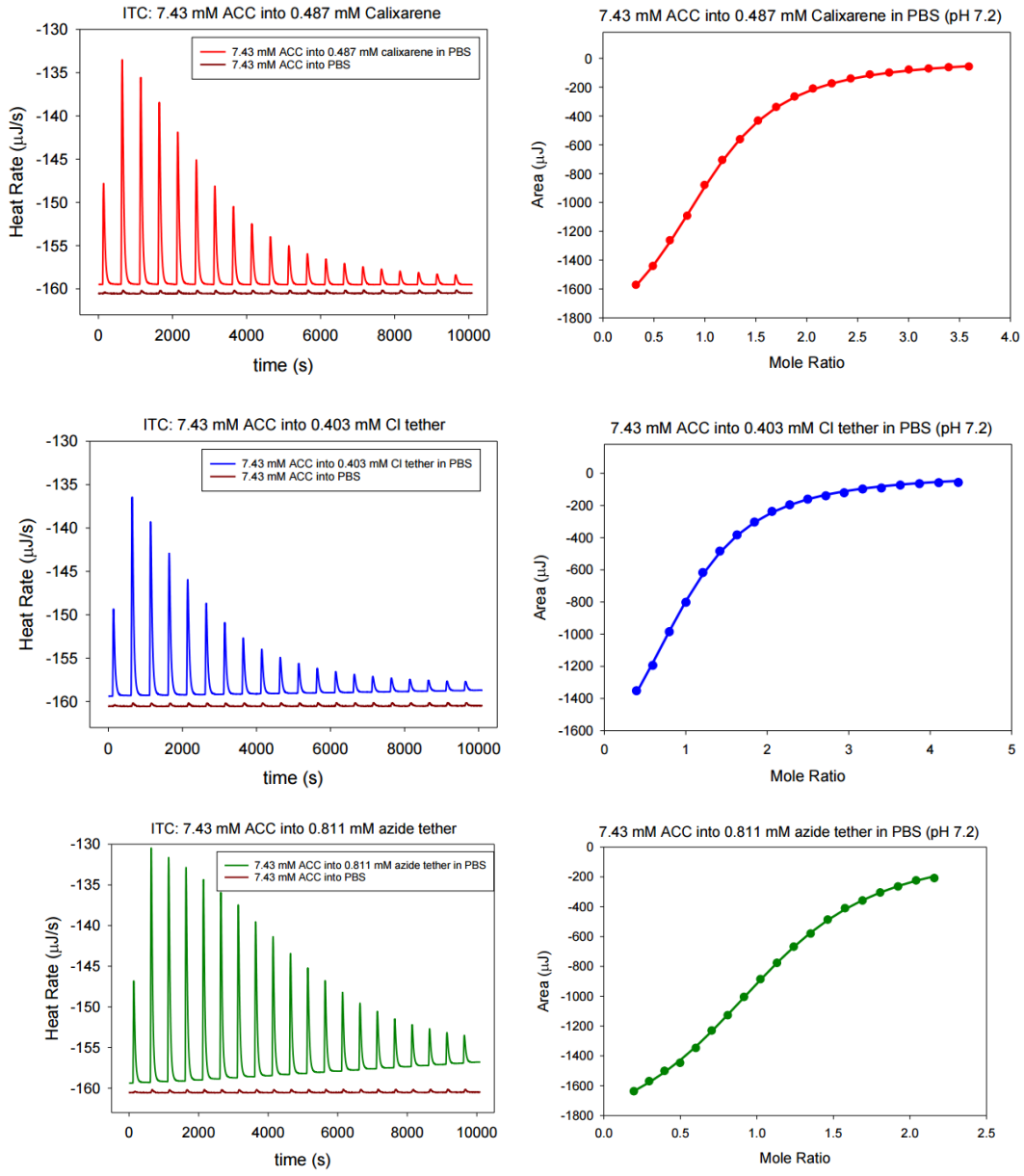


Figure B.7. Results of ITC experiments of **2** (top), **6** (middle), and **7** (bottom) with ACh

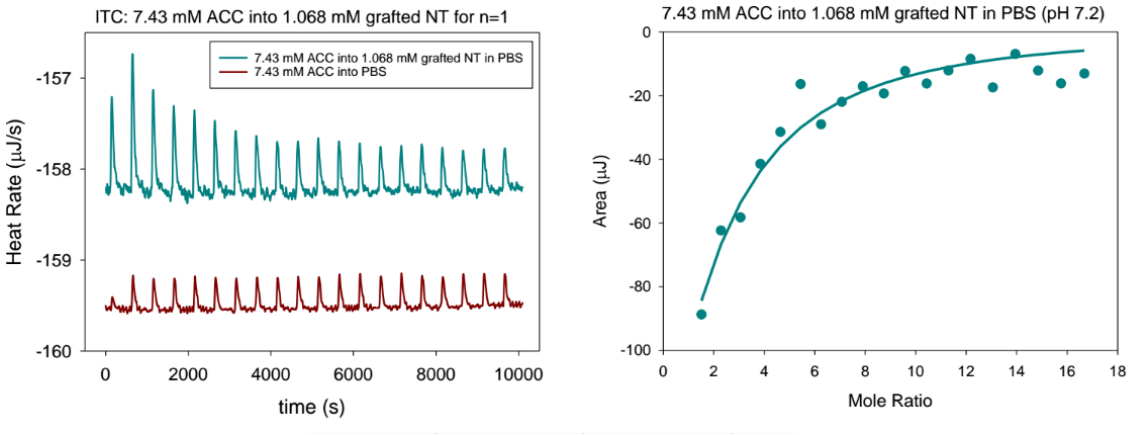


Figure B.8. Results of ITC experiment of **7**-grafted MWCNTs with ACh