SYNTHESIS OF MODIFIED POLYDIACETYLENE (PDA) POLYMERS FOR THE DETECTION OF INFECTIVE SPECIES

By

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Abstract

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Polydiacetylene (PDAs) polymers are receiving more attention as biosensors due to their ability to change absorption and fluorescence properties upon sensing various chemical and physical changes in the environment. In chapter 1, the introduction to PDA polymers have been described along with their optical properties. Diacetylene (DA) monomers can be synthesized to tune photochemical and sensing properties of PDAs. DAs self-polymerize upon UV-irradiation and show fluorescence upon stimulation. They have been reported as biological and chemical sensors, capable of visible color changes (e.g. blue to red) upon certain environmental factors such as temperature, pH changes, and upon binding with biological species or other analytes. Chapter 2 discussed two important previously published studies of PDA material as biosensors. it is reviewed that PDA modified with different receptors and substrates were able to detect target biological species. In Chapter 3 we have described our interest and approach for PDA polymers synthesis using 2 different antibiotics and a sialic acid. In chapter 4 we described the synthesis of new derivatives of 10,12-pentacosadiyonic acid (PCDA) that are coupled to different biological inhibitors such as vancomycin, sialic acid, and

polymixin B. Following literature, in chapter 5 we observed the PDA binds to specific metals as well from their color change property. So far we have successfully coupled vancomycin with PCDA and are in a process of investigating its function as a detector and inhibitor for gram positive bacteria *Staphylococcus aureus (S. aureus)*. Additionally, various diamine linkers will be investigated to optimize PDA-inhibitor linkage and function. The completed PDAs are processed into liposomes, which bind to specific classes of bacteria or viruses, potentially inhibit their growth, and give an identifying fluorescence signal to indicate the presence of infection.

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LIST OF ABBREVIATIONS

ACN	Acetonitrile
BOC	tert-butyloxycarbonyl protecting group
C18	Carbon 18
^{o}C	Degree Celsius
DCM	Dicholormethane
DMF	Dimethylformamide
EDA	Ethylene diamine
EDC. HCl	*N*-(3-Dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride
FT-IR	Fourier Transform Infrared Spectroscopy
g	Gram (s)
Hz	Hertz (cycles per sound)
HOBT xH_2O	1-Hydroxybenzotriazole hydrate
J	Coupling constant (NMR)
LB	Langmuir-Blodgett
LPS	Lipopolyscchride
LC-MS	Liquid chromatography-Mass spectrometry
MeOH	Methanol
mmol	millimole
mol	Mole(s)
MIC	Minimum inhibitory concentration
NHS	*N*-Hydroxysuccinimide
NMR	Nuclear magnetic resonance
PDA	Polydiacetylene
PCDA	10, 12-Pentacosadiyonic acid
PCDA-EDA	Pentacosadiyonic acid-Ethylenediamine

PCDA-Van	Pentacosadiyonic acid-Vancomycin
PMB	Polymyxin B
PyBOP	Benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate
RP	Reverse phase
rt	Room temperature
SA	Sialic acid
SAM	Self-assembled monolayers
Temp	Temperature
TFA	Trifluoro acetic acid
TLC	Thin layer chromatography
UV	Ultra violet

CHAPTER 1

Introduction

1.1 Introduction to Polydiacetylene (PDA)

Polydiacetylene (PDA) materials are conjugated polymers due to the presence of alternate double and triple bonds. These polymers have great optical properties, which are used for sensing different materials including biological species [1]. PDA upon interacting with environmental factors such as: temperature, pressure, organic solvents, mechanical stress, or different biological species can change their blue color to red which additionally brings the fluorescence in the polymer as well [2].

In 1969, Wegner synthesized first PDA material [3]. Different methods can be used to prepare PDA polymers such as: ultra-violet (UV) light irradiation, free radical methods, and plasma treatment. The polymerization takes place in PDA material as the monomers self-assemble at 80 ^{o}C or as they are irradiated with UV light of 254 nm wavelength at room temperature; both cause 1,4-addition between monomers. This addition gives ene-yne alternate bonds as the backbone for PDA polymers, which because of conjugation are usually blue colored material. Absorption spectra for PDA reveals a maximum wavelength close to λ_{max} = 640 nm [4].

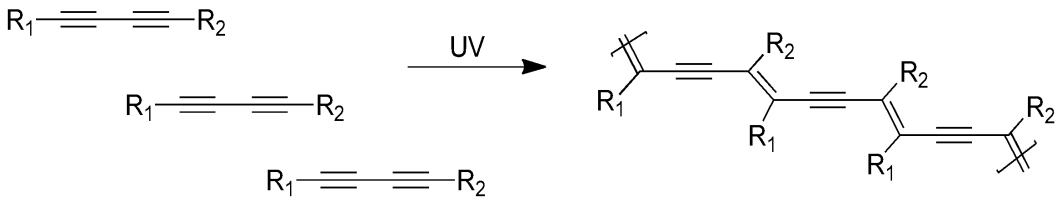
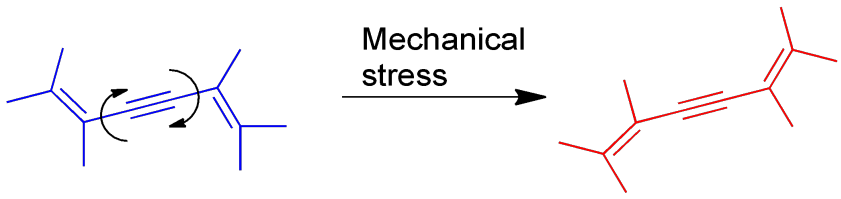


Figure 1.1: Photopolymerization of self assembled diacetylenes monomers upon UV irradiation

The formed PDA polymers are non-fluorescent but upon environmental influence such as heat or binding to biological species (bacteria, viruses) or biomolecule, the PDA blue color changes to red color which brings fluorescence in them. Blue PDA polymers are in planar structural conformation but under such environmental stimuli the conformation changes to non-planar arrangement which causes color change in them figure 1.2. [4].



(Non-fluorescent) (Fluorescent)

Figure 1.2: Backbone rotation in PDA results in color change of blue phase to red phase

1.2 PDA Liposomes:

Synthesis of PDA polymers can be accomplished in many interesting structures which includes Langmuir-Blodgett (LB) films, self-assembled monolayers (SAM), and liposomes.

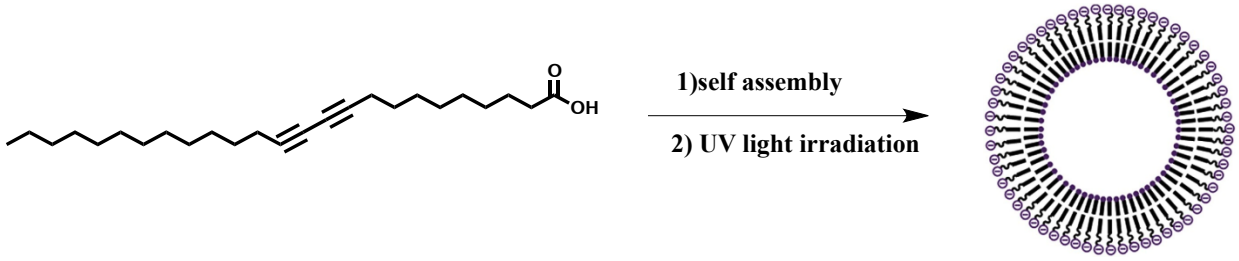
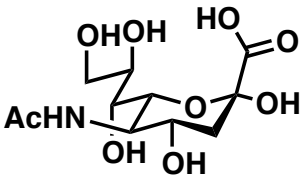


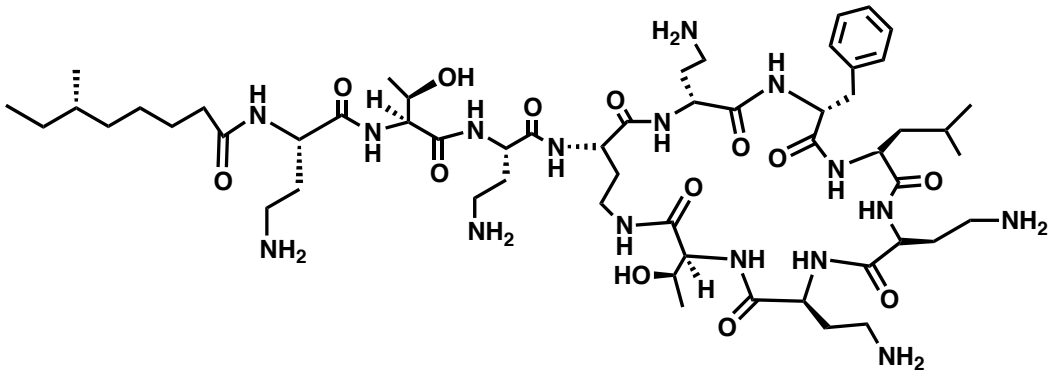
Figure 1.2.1: Liposomes formation from 10,12-Pentacosadiyonic acid[5]

1.3 PDA as biological sensors

Due to the property of PDA polymer to go under color transition from blue to red upon certain exposure to environmental stimuli and giving rise to fluorescent material, PDA polymers are used as biological sensors [6] and may provide relatively sensitive detection. As biological sensors PDA can be used for the detection of different species such as: bacteria (gram negative and positive), viruses, enzymes, different peptides, and DNA's [7]. PDA can be coupled to different antibiotics for example polymyxin B and vancomycin for the detection of different bacterial infectious species. Similarly they can be coupled to different small molecules such as sialic acid to detect influenza A virus [6].



(a)



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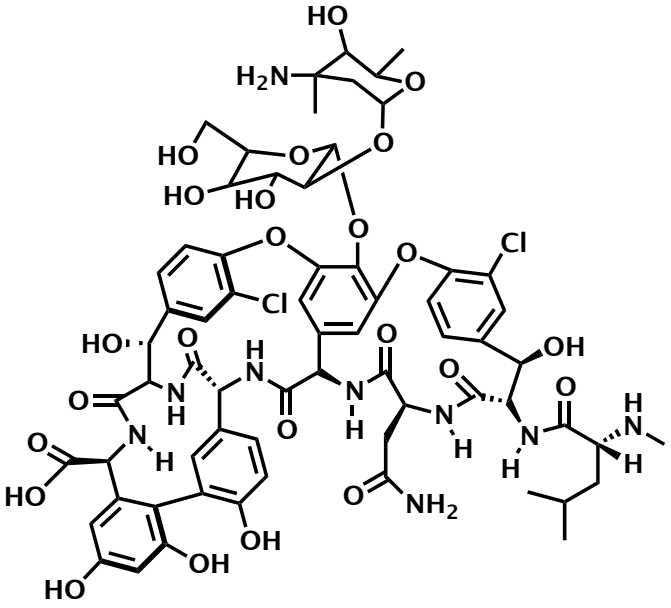


(c)

Figure 1.3.1: Structures of (a) Sialic acid (b) Polymyxin B (c) Vancomycin

In this project, our aim was to synthesize liposomes of modified PDA materials bearing detection systems for infective agents and evaluate them against different selected bacteria (*S. aureous*) and viruses.

Specifically, the research project was focused on the following aims:

I. Synthesis of new 10,12-pentacosadiyonic acid (PCDA) derivatives with diamine linkers.
II. Coupling of biological inhibitors capable of detecting the surface of infective species, such as vancomycin, sialic acid, and polymyxin B with PCDA derivatives.
III. The investigation of diamine linker length and composition for the optimization of PDA-inhibitor's linkage and function.

CHAPTER 2

2.1 Previous Studies on the synthesis and function of PDA sensors

Charych *et al.* were the first to report PDA liposomes as biological sensors. In their study they modified PDA head groups with sialic acid which is host molecule of influenza A virus. They used varying concentrations of sialic acid modified PCDA (1-10%) with original reactant PCDA. The polymers were synthesized on a glass film and were blue in color when incubated with virus the instant color change was observed. Using colorimetric response (CR) equation they found 47% color change in 8 minutes and 87% color change in 24 min. So their study concluded with detection of target infective species [6].

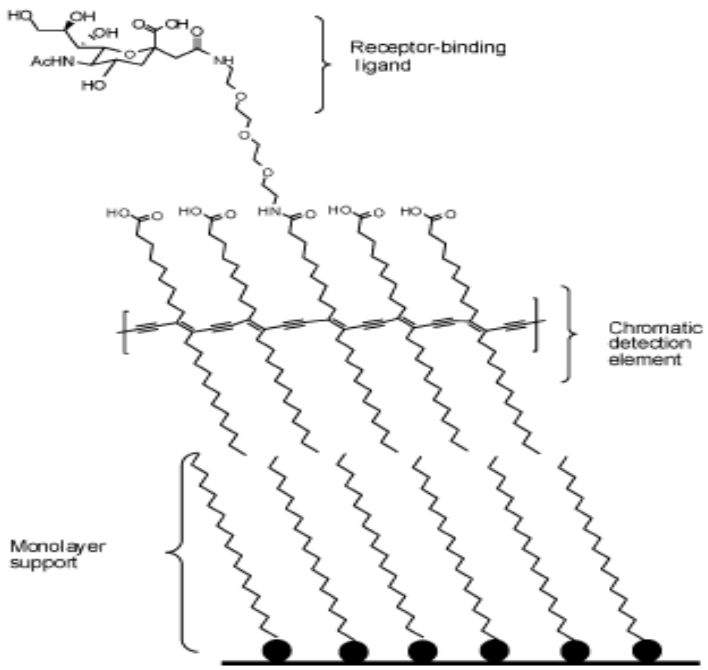


Figure 2.1.1: Polymer film on glass slide of PCDA coupled sialic acid receptor of influenza A virus[6]

After that, a lot of work was done on PDA as biosensors. In 2016, Lee *at al.* published a paper in which they reported imidazolium and imidazole modified PCDA polymers. The synthesized PDA modified complex were tested against different bacterial strains *Staphylococcus aureus* and *Eschericha coli*. In their studies the PDA polymers changed their blue color to red upon interacting with bacterial species. Also the group found that these sensors were able to kill bacterial growth as well [12].

In 2019 J. Yun *et al.* published paper in which they used PDA modified liposomes for detection of glutathione S-transferase (GST) recombinant protein using

colorimetric method instead of instrumental techniques. They modified PDA terminal head (COOH) with glutathione (GSH) which is a substrate for GST and synthesized blue liposomes after exposure to UV light. These liposomes successfully detected the GST protein which was obvious from color change of these liposomes from blue to purple. This happens as the GST substrate attached to the PDA molecules interacts with the binding site on the protein via secondary forces (hydrogen bonding) and causes color change of the solution [13].

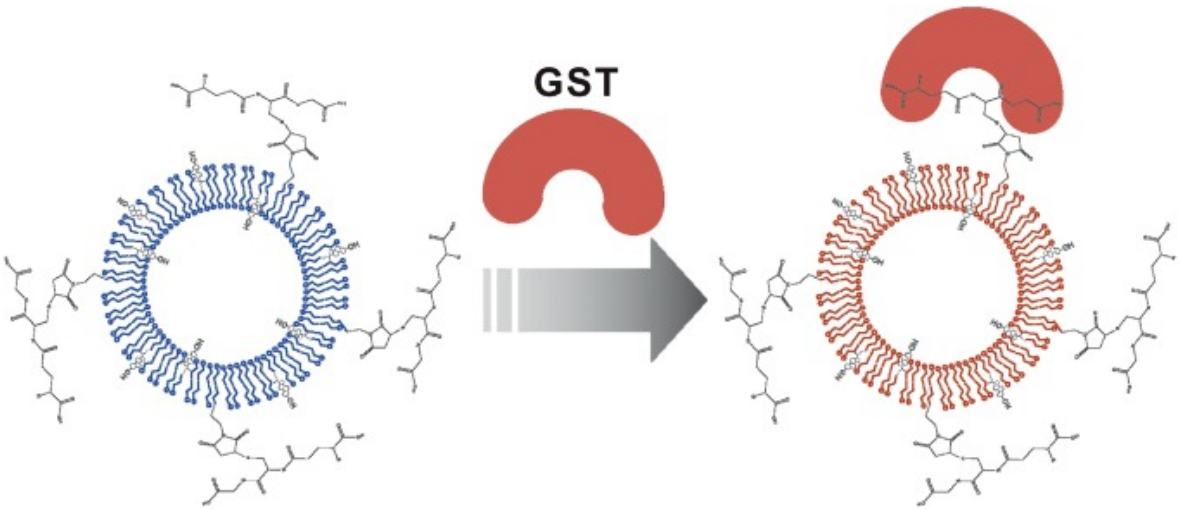


Figure 2.1.2: Schematic diagram for detection of GST protein with modified PDA liposomes[13]

The synthesized liposomes detected the GST-hGH protein within 15 min with PDA liposomes and color transition was observed. Use of PDA as protein detection is useful as it can be used as an alternate to different biological techniques of protein detection such as SDS-PAGE gel electrophoresis and others. PDA based sensors are more robust [13].

CHAPTER 3

Research Interest and Background

3.1 Introduction to our synthetic strategy

10,12-Pentacosatiyonic acid (PCDA) was our main building block in this project. Polymerization of PCDA molecules using UV irradiation method is relatively straight and well described in the literature, requiring no additional catalyst or initiator, also the polymer's synthesis ends without any byproducts [4].

We planned to synthesize different PCDA derivatives with diamine linkers and then attach vancomycin, polymyxin B and sialic acid to these derivatives. Finally, mixing different ratios of non-binding PCDA monomers and PCDA-antibiotic monomers for the formation of liposomes of varying consistencies and subsequently investigating their biological properties. According to previous studies [1][4][6] the liposomes from these monomers should give blue color and when binds to biological species (bacteria or virus) it should change to a red fluorescent polymer or a more reversible purple/fluorescent phase. Ultimately, these functional liposomes are intended to be incorporated as component of sol-gels as embedded in woven materials, such as wound dressings. Potentially, such smart wound dressing would alert medical professionals to infection at the site of healing.

3.2 Antibiotics and small molecule sialic acid

3.2.1 Vancomycin

Vancomycin was discovered in 1956 and is representative of a major class of natural and synthetic compounds called glycopeptides. It is considered as a last resort drug and is only prescribed in the clinic when other treatments have failed. *In vitro* studies have shown the minimum inhibitory concentration (MIC) for vancomycin is 0.25-10.0 μg mL^{-1} against *S. aureus* [8]. Vancomycin is proposed to bind to the *D*-Ala-*D*-Ala terminus sequence on the growing bacterial cell wall via a series of hydrogen bonding events, these interactions cause bacterial death [9]. Interestingly, a single amino acid mutation, from *D*-ala to *D*-serine or *D*-lactic acid can result in a potency drop of up to 1000 times for vancomycin, showing the importance of an individual hydrogen bonding event and the corresponding conformational arrangement.

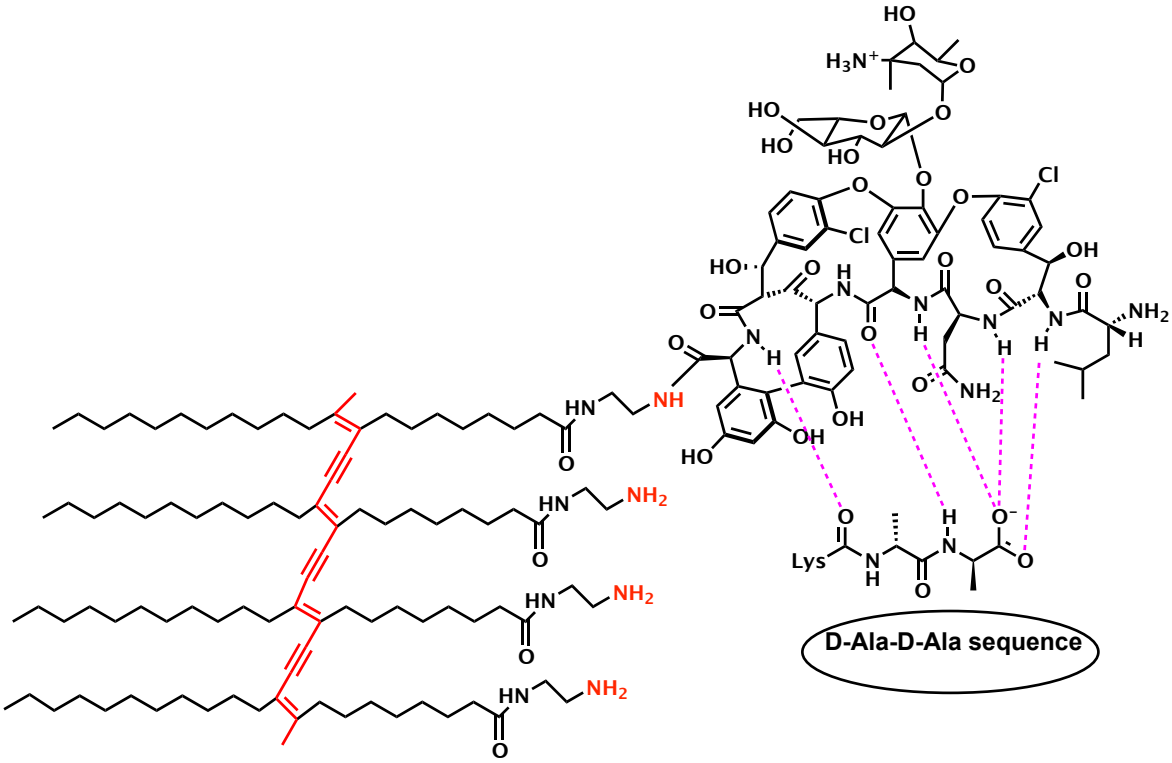


Figure 3.2.1: Hypothesized model of PCDA-Vancomycin (PCDA-Van) PCDA-diamine (PCDA-EDA) linker with 1:3 ratio respectively. Polymers binding to bacterial cell wall (Lys-D-Ala-D-Ala) sequence by making non-covalent bond with it and inhibiting bacterial growth

3.2.2 Polymyxin B and Sialic acid

Polymyxin B (PMB) is another antibiotic containing a cyclic peptide in its structure, due to the presence of charged lysine residue at biological pH, it is often referred to as a cationic antibiotic. Structurally, PMB is a decapeptide possessing heptapeptide in a cyclic loop, a side chain consisting of a tripeptide, and a long fatty acid chain within its structure (Fig. 4b). PMB inhibits the Gram-negative bacterial infection by killing it. PMB has strong affinity to lipopolysacchride

(LPS), which is the outer monolayer in bacteria and this membrane is important for the bacteria and its ability to resist toxic materials [10]. PMB's mode of action is proposed to begin by its binding to LPS of bacterial outer wall and then lysis of its membrane, which ultimately causes bacterial death [11].

Another molecule in our study is sialic acid (SA) which is a small carbohydrate molecule. In influenza A viral infection the glycoprotein hemagglutinin (HA) binds to SA on membrane surface via glycosidic linkage causing infection in the host cell [6].

In summary, our study focuses on the detection of bacteria (gram positive and negative) and influenza A virus. These infective species have critical challenges to human health. The molecules will be synthesized and tested for their inhibition and detection of such infective growth or life, as well as their ability to provide specific and selective colorimetric and/or fluorometric response.

CHAPTER 4

Experimental Data

4.1 General Experimental Data

All the compounds were purchased from sigma aldrich. Anhydrous solvent purchased from the millimore through VWR. All the chemicals were used as obtained from different vendors. The reactions were run under nitrogen gas unless otherwise reported. Bacteria used in this study were *S. aureus* (25904) ordered from ATCC. They were incubated in MHB media (Sigma). 500 MHz ^{1}H and 125 MHz ^{13}C NMR was used for different experiments and they were performed on JEOL ECX 500 instrument. Chemical shifts were recorded in reference to residual solvent peaks ($CDCl_3$ = 7.26 ppm, D_2O = 4.79 and CD_3OD = 3.31). For NMR representation of data these abbreviation were used for description of each peak: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, quin = quintet, bs = broad singlet. For reaction monitoring, TLC was performed on EMD Merck F254 with thickness 254 μm. For flash chromatography P60 silica gel (mesh 230-400), basic activated alumina (58 angstrom pore size), and reverse phase (C18) silica gel (particle size 100 angstrom). For mass spectrometry, the samples were run in Shimadzu center for Advance Analytical Chemistry LCMS IT-TOF Mass Spectrometer. For IR, Bruker Alpha instrument IR was used to get absorption peaks of different functional groups.

4.2.1 Synthesis of tert-Butyl (5-aminopentyl)carbamate

In 0.94 mL (8 mmol) cadaverine $CHCl_3$ (3 mL) was added and kept at 0 ^oC. The solution of di-*tert*-butyl dicarbonate 0.37 mL (1.6 mmol) in 2 mL $CHCl_3$ was added drop wise over a period of 40 min. After complete addition the solution was kept stirred at room temperature for 24 h. The precipitates were filtered off and organic filtrate was washed twice with water and brine. The organic layer was dried over $MgSO_4$ and filtered [14]. The product was dense oily liquid and yield was 86%. ^1H NMR (500 MHz, $CDCl_3$) δ 4.78 (br, 1H), 3.01 (d, J = 6.1 Hz, 2H), 2.59 (t, J = 6.85 Hz, 2H), 1.45 – 1.21 (m, 15H).

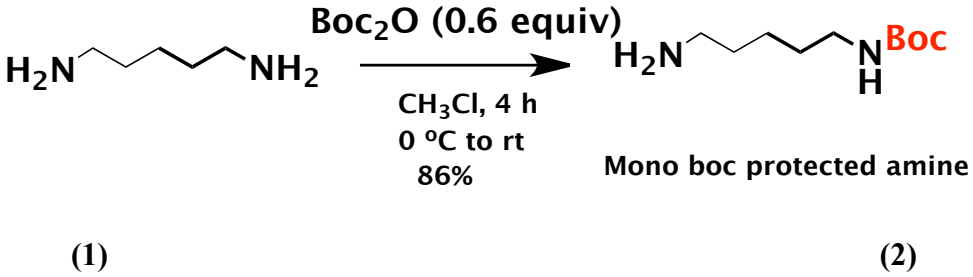


Figure 4.1.1: Synthesis of mono-boc protected amine

4.2.2 Sytnhesis of PCDA amide from tert-Butyl (5-aminopentyle) carbamate and 10,12-Pentacosadiyonic acid (PCDA)

The solution of 153 mg Boc-protected amine (1) in 4 mL anhydrous Dichloromethane was prepared and kept for stirring at 0^{o}C. In separate flask PCDA 225 mg (0.6 mmol), PyBOP 390 mg (0.75 mmol), and 3 mL triethyl amine were added. The mixture was slowly added to the above boc-amine and stirred for 30 min at 0 ^{o}C and then brought to room temperature and stirred for 24 h under nitrogen. The solvent was evaporated and product was extracted with EtOAc and washed thoroughly with water and then with brine. Organic layer was dried over $MgSO_4$ and was filtered off [14]. The product was purified using basic activated alumina (1% MeOH/DCM followed by 10% MeOH/DCM). The product obtained yield was 45%. ^{1}H NMR (500 MHz, CD_3OD) δ 3.49 (t, J = 6.8 Hz, 2H), 3.40 (t, J = 6.9 Hz, 2H), 2.32 (t, J = 7.6 Hz, 2H), 2.24 (t, J = 6.9 Hz, 4H), 1.97 (dd, J = 13.5, 6.7 Hz, 2H), 1.93 – 1.84 (m, 2H), 1.80 – 1.72 (m, 2H), 1.67 – 1.58 (m, 4H), 1.56 – 1.46 (m, 6H), 1.33 – 1.27 (m, 36H); ^{13}C NMR (125 MHz, CD_3OD) δ 174.6, 172.9, 76.4, 76.4, 65.1, 46.7, 45.5, 34.1, 31.7, 31.4, 29.4, 29.3, 29.3, 29.1, 29.0, 29.0, 28.8, 28.7, 28.5, 28.4, 28.2, 28.1, 25.6, 24.7, 24.0, 22.4, 19.5, 18.3, 13.1, 13.1.

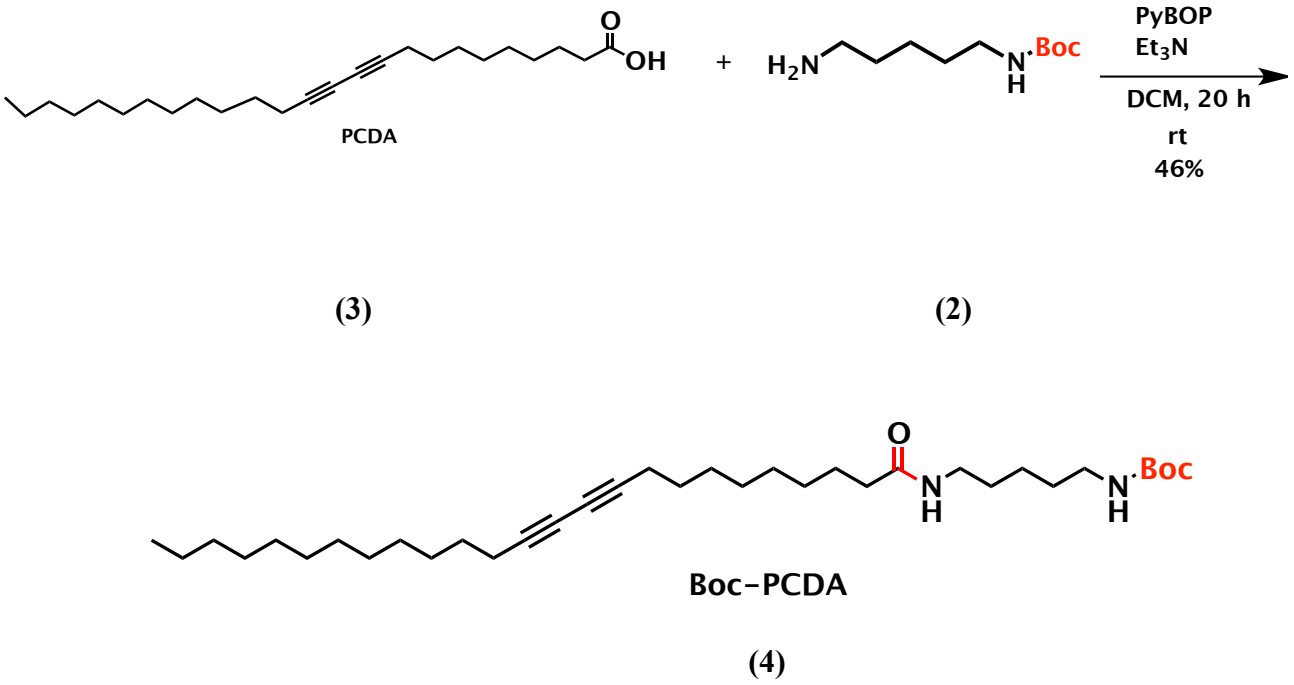


Figure 4.2.2: Synthesis of Amide bond from PCDA and free amine

4.2.3 Synthesis of N-(5-aminopentyl)-10,12-pentacosadiynamide

In a stirred solution of above (2) 83.6 mg (0.16 mmol) in 5 mL of dichloromethane the 10 equivalent of trifluoroacetic acid was added dropwise at 0 ^{o}C. Reaction was basified with sodium bicarbonate stirred for 4 h and the solvent was evaporated and the precipitates were dissolved in dichloromethane and were washed twice with water and brine. Organic layer was further dried over $MgSO_4$ and the solvent was evaporated [15]. The product was purified using column chromatography with basic activated alumina as stationary phase (1% MeOH/DCM followed by 10% MeOH/DCM). The product was bluish solid and obtained in 80% (95 mg) yield.

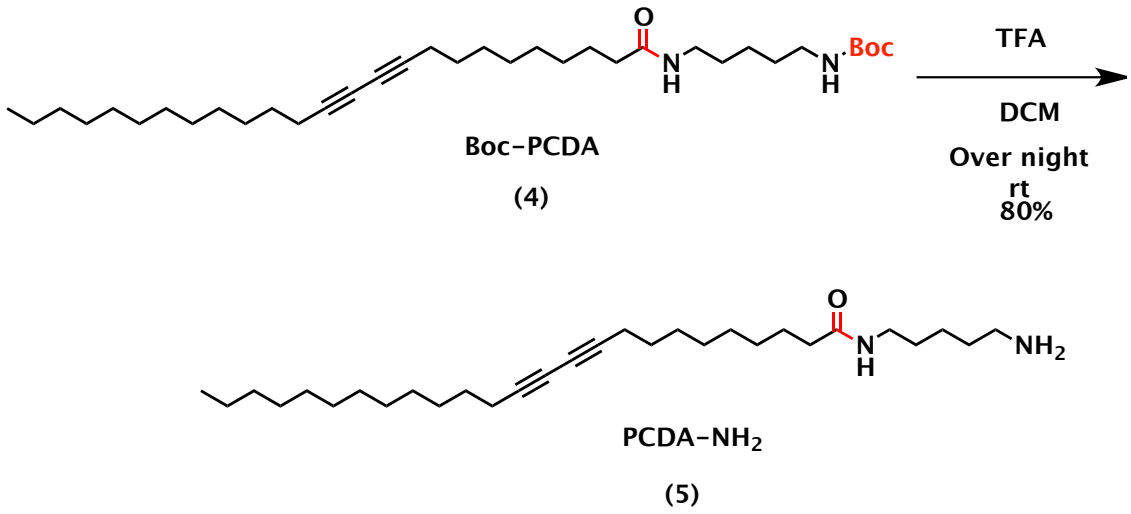


Figure 4.2.3: De-protection of Boc group from Boc-PCDA

4.3.1 Synthesis of 10,12-Polydiacetylene N-hydroxysuccinimide (NHS-PCDA) ester (6):

376 mg (1 mmol) of PCDA was dissolved in 5 ml of dichloromethane. In the stirred solution of PCDA, 129 mg (1.12 mmol) N-hydroxysuccinimide (NHS), and 224 mg (1.17 mmol) 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) were added. The solution was stirred under nitrogen gas for 4 hours at room temperature. The solvent was evaporated under vacuo and the product was extracted using 13 ml ethyl acetate and it was washed with water four times. Remaining water drops were removed from organic layer using magnesium sulfate [16]. The product was white powder and obtained with 86% yield. **^{1}H NMR** (500 MHz, $CDCl_3$) δ 2.82 (s, 4H), 2.58 (t, J = 7.4 Hz, 2H), 2.22 (t, J = 6.8 Hz, 4H), 1.82 – 1.65 (m, 2H), 1.59 – 1.45 (m, 4H), 1.40 – 1.14 (m, 25H), 0.86 (t, J = 6.8 Hz, 3H); **^{13}C NMR** (125 MHz, $CDCl_3$) δ 169.3, 168.7, 77.7, 77.5, 65.4, 65.3, 32.0, 31.0, 29.7, 29.7, 29.7, 29.5, 29.4, 29.2, 28.9, 28.9, 28.8, 28.8, 28.4, 28.37, 25.70, 24.64, 22.79, 19.30, 19.2, 19.2, 14.2.

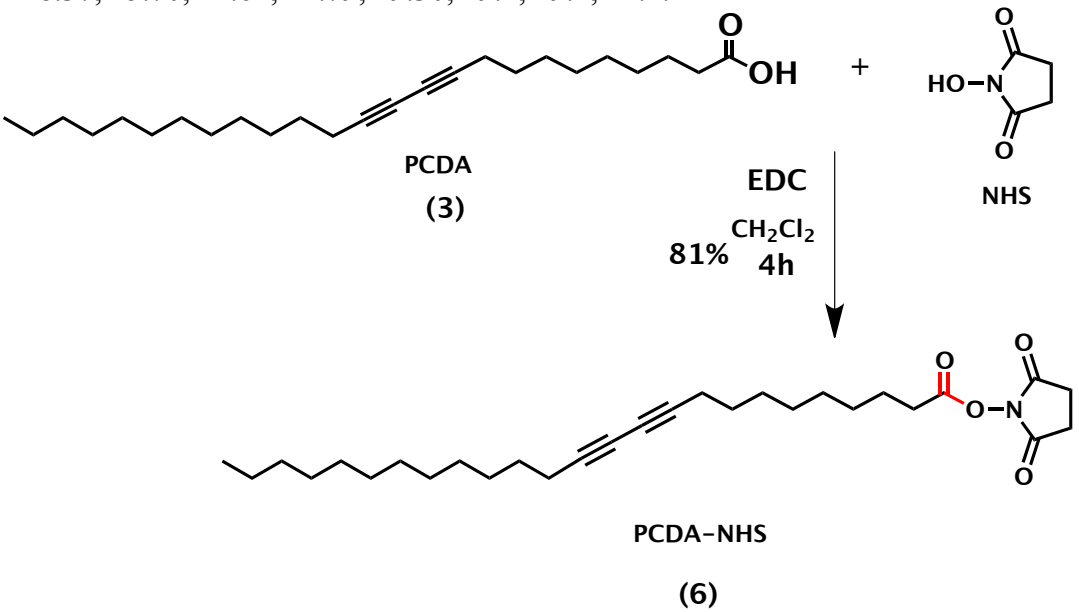


Figure 4.3.1: Syntheis of PCDA-NHS ester

4.3.2 Synthesis of N-(2-aminoethyl)-10,12-pentacosadiynamide from NHS-PCDA and ethylene diamine (NH2-PCDA) (8)

1 ml ethylene diamine was dissolve in 2 ml anhydrous dichloromethane and was stirred at 0 ^{o}C. 150 mg (0.31 mmol) NHS-PCDA was dissolved in 5 ml anhydrous dichloromethane and 3 ml of triethylamine was added in it. This solution was added dropwise over a period of 10 minutes in cold ethylene diamine solution. After complete addition the solution was brought to room temperature and was stirred for 8 h. Solvent was evaporated under vacuum and precipitates were dissolved in 20 mL dichloromethane and washed with water and brine three times each. The organic layer was dried over anhydrous $MgSO_4$ [16]. The solution was concentrated and was purified using column chromatography with basic activated alumina as stationary phase (1% MeOH/DCM followed by 10% MeOH/DCM). The product was white precipitates. IR (neat cm-1) 3291, 2917, 2847, 1638. ^{1}**H NMR** (500 MHz, CDCl3) δ 3.30 (dd, J = 11.7, 5.8 Hz, 2H), 2.83 (t, J = 5.9 Hz, 2H), 2.31 – 2.18 (m, 4H), 2.19 (dd, J = 18.2, 10.7 Hz, 2H), 1.62 (m, 2H), 1.56 – 1.45 (m, 12H), 1.41 – 1.18 (m, 28H), 0.88 (t, J = 6.9 Hz, 3H). ^{13}**C NMR** (126 MHz, CDCl3) δ 174.3, 77.7, 77.5, 65.4, 65.3, 40.7, 36.7, 32.0, 29.7, 29.7, 29.7, 29.6, 29.5, 29.4, 29.3, 29.2, 29.1, 29.1, 29.0, 28.9, 28.9, 28.5, 28.4, 25.7, 22.8, 19.3, 14.2.

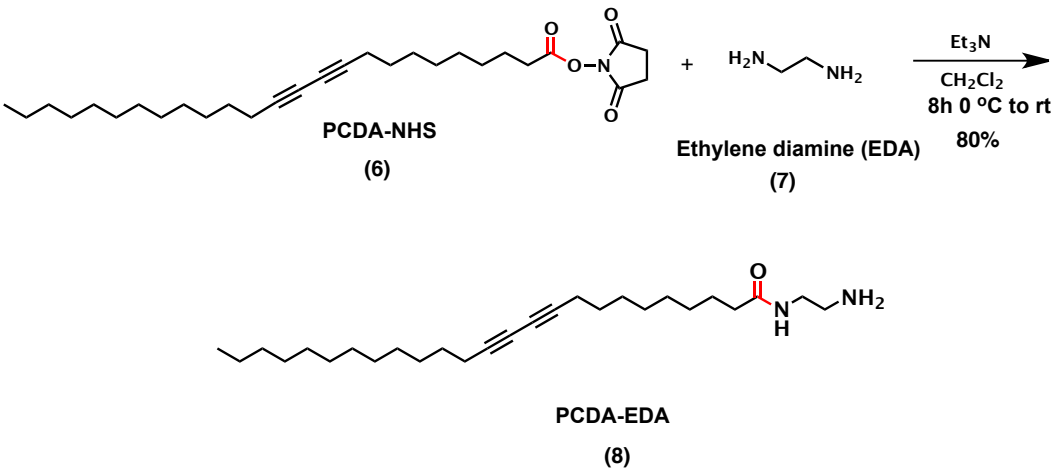


Figure 4.3.2: Synthesis of PCDA-EDA from PCDA-NHS ester and ethylene diamine

4.3.3 Synthesis of vancomycin monomer from N-(2-aminoethyl)-10,12-pentacosadiyonamide and vancomycin (10)

Vancomycin.HCl 235 mg (0.158 mmol), EDC 31 mg (0.2 mmol) and 1-hydroxy-7-azabenzotriazole 27 mg (0.2 mmol) all dissolved in dimethylformamide (DMF) and were stirred for 10 minutes. In this stirred solution, NH_2-PCDA 80 mg (0.19 mmol) in 2 mL DMF was added. *N*-methyl morpholine was added to pH 8 and the resulting solution was stirred overnight at room temperature [17]. The reaction was monitored with reverse phase C18 end capped silica gel glass thin layer chromatography (60% ACN/H_2O, 0.1 % formic acid). The solvent was evaporated in vacuo. The product was purified with reverse phase C18 flash chromatography (20% ACN/H_2O, 0.1% formic acid) followed by (50% ACN/H_2O, 0.1% formic acid). The obtained product was yellowish solid with 45% yield. ESI-MS *m*/*z*: calcd for $C_{94}H_{124}Cl_2N_{10}O_{24}$ $[M-H]^-$ 1846.8, found 1846.8.

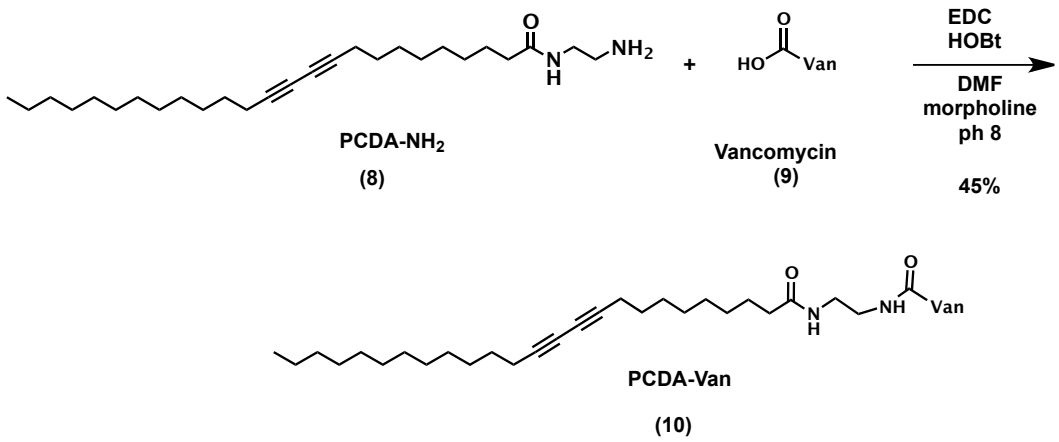


Figure 4.3.3: Synthesis of PCDA-Vancomycin monomer from PCDA-NH_2 and vancomycin ester and ethylene diamine [16]

4.3.4 Preparation of Liposomes

Liposomes were prepared with different ratios/percentages of monomer 5 and 6. For the preparation of 20% liposomes of PCDA-Van **(6)** with PCDA-NH_2 (PCDA-EDA) **(5),** in a round bottom flask the PCDA-NH_2 (4.0 mg, 10 μmol) and

PCDA-Van (3.69 mg) were dissolved in (1 mL) dichloromethane. The solvent was evaporated in vacuo, the precipitates were mixed with ultrapure water (10 mL) to make lipid concentration 1 mM. The suspension was probe sonicated (digital Pro ultra sonicator) for an hour at 80 ^{o}C. The hot cloudy suspension was immediately filtered through 0.8 μm cellulose filter paper to get rid of undispersed lipids aggregates. The solution was stored below 4 ^{o}C for 12 h. After that photopolymerization was done using hand held 254 nm Ultra violet (UV) lamp (1mW cm^{-2}) at room temperature for 10 minutes [16].Using dialysis tubing the dialysis was performed [Mw cut off: 10,000] against deionized water The blue liposomes were stored below 4 ^{o}C and were stable enough to work for 2 weeks.

4.3.5 Metal detection by PCDA/PCDA-EDA (1:1) of 1 mM liposomes

PCDA/PCDA-EDA (1:1) Liposomes were prepared of 1 mM concentration in HEPES buffer having pH 7.4. Then, 1 mM, 5 mM and 10 mM concentrations of metal salts; barium perchlorate, copper sulfate, and zinc chloride were prepared in HEPES buffer. These metal solutions were titrated with liposomes and allowed to equilibrate for 30 min. In 1 mM solution of all three salts no color change was observed. Whereas, 5 mM and 10 mM concentrations of barium and zinc solutions gave purple color change. No color change was observed for any concentration of copper salt.

4.3.6 Bacterial sensing test

Gram negative bacteria *S. aureus* was inoculated into MHB media under constant shaking at 100 rpm at 37 ^{o}C for 8 h to mid-exponential growth phase. The bacterial suspension was centrifuged at 8000 x g for 5 min and the bacterial

pellets were re-suspended in distilled water. The procedure was repeated three times to completely remove all the media, and the final bacteria suspended was adjusted to 10^{10} CFU/mL. Liposomes at varying concentrations (10 mM, 5 mM and 2.5 mM) with 20% PCDA-Vancomycin monomer with PCDA-EDA monomer and 40% PCDA-Vancomycin monomer with PCDA-EDA. The 50 mL liposomes liposome solution and 50 mL bacterial suspension was mixed in 96-well microtiter plate, each concentration have 3 replicates. The phenomenon was observed after overnight incubation under constant shaking at 100 rpm at room temperature.

CHAPTER 5

5.1 Results and Discussion

5.1.1 Scheme 1

The Main monomer is N-(2-aminoethyl)-10,12-pentacosadiynamide (PCDA-NH_2). In our studies initially we followed scheme 1 to synthesize monomer PCDA-NH_2. Following this scheme we used cadaverine (1,5-diaminopentane) as our linker and protected its one amine with Boc group using di-*tert*-butyl dicarbonate in chloroform. The product was oily dense liquid. Mono-boc amine of cadaverine was achieved with 86% yield. Proton NMR was taken for structural analysis. In the next step, we treated the free amine with 10,12-Pentacosadiyonic acid (PCDA) using coupling reagent pyBOP (benzotiazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate) in the presence of base triethyl amine. The reaction was monitored by thin layer chromatography (TLC), an additional spot was observed. The product was separated using silica gel column chromatography and was characterized by proton NMR. The product yield was only 40%. After that we tried to deprotect Boc group using trifluoro acetic acid (TFA) 5 equivalent 8 hours stirring at 40 ^{o}C. Upon evaporating the solvent and characterizing with NMR and infrared (FT-IR) spectroscopy we found the product was decomposed. Probably because PCDA compounds are temperature sensitive. On TLC we see 4 spots and none of them was corresponding to any reactant. On IR spectra there was no peak for free amine around 3200 cm^{-1}. So the deprotection was done again with 10 equivalent TFA at room temperature overnight stirring. NMR and IR spectra of the product was taken. On IR spectra the 1^{o} amine peak around 3291 cm^{-1} was observed

confirming the presence of free amine. PCDA-NH_2 was achieved in high yield 85%. But because the previous reaction gave yield only 40% so we switched to scheme 2.

5.1.2 Scheme 2

Using scheme 2 first carboxyl group of PCDA was activated by converting it to ester. For this purpose PCDA was treated with N-hydroxysuccinimide (NHS) in the presence of coupling reagent 1-Ethyle-3-(3-dimehtylaminopropyl) carbodiimide hydrochloride (EDC) the reaction was stirred for 8 hours and the product PCDA-NHS ester was obtained with 85% yield. The reaction was clean as the by-product was only urea which was removed from reaction mixture during work up with water. Once the PCDA head group was activated in the form of PCDA-NHS ester it was treated with ethylene diamine (EDA) linker with triethyl amine as base the reaction was stirred overnight at room temp. The reaction was monitored by TLC. One extra spot was observed on the TLC which was very small spot. Probably the by product was the one where both amines of EDA was coupled with PCDA. Initially the product was tried to separate using silica gel column chromatography but each time the product got stuck with the column after getting rid of impurities. Three solvent system was also tried (10% MeOH/DCM with 20% triethlamine) but it did not make any difference. Also we tried to flush 40% triethylamine in hexane through the silica gel and then flushing 5% MeOH in DCM but even it did not work. Finally the stationary phase was replaced with basic activated alumina and the product was separated with 10% MeOH/DCM solvent system. The product was obtained with 80% yield. The NMR and FTIR spectra was obtained. FTIR spectra we saw amine peak around 3200 cm^{-1}.

In the next step the antibiotic vancomycin was reacted with the free amine of PCDA-EDA monomer. Amine group was coupled with the COOH group of

vancomycin. For this purpose the vancomycin was stirred in dimethylformamide (DMF) with coupling reagent EDC and HOBT (Hydroxybenzotriazole) and base n-methyl morpholine to make pH close to 8. The reaction was monitored with normal phase (NP) and reverse phase (RP) C18 TLC. NP TLC confirmed the absence of reactant PCDA-EDA and on RP an extra product spot was observed. The RP TLC was ran three times in the solvent system (60% ACN: 40% H_2O, 0.1 % formic acid) to make product RF value to 0.3. Increasing water percentage from 40% the solvent system does not move on RP TLC because of non-polar C18 stationary phase, and by decreasing it the product spot did not move on the TLC. After reaction completion the DMF was evaporated on rotary evaporator by making its azeotropic mixture with toluene at 38 ^{o}C. The crude gummy mixture was obtained and the product was separated using RP C18 column chromatography. The product was yellowish solid and obtained with 45% yield. The structure was analyze using by comparing the NMR of vancomycin reactant and product and by mass spectrometry (MS) results.

5.2.1 Liposmes preparation

Liposomes were synthesized with different percentages of PCDA-Vancomycin monomer with PCDA-EDA monomer, such as 20% PCDA-Van/PCDA-EDA and 40% PCDA-Van / PCDA-EDA. Liposomes synthesis was done by self-assembly of monomers using sonication at 80 ^{o}C for 40 min. Once the sonication was done milky suspension was observed. Heating the solution causes the self-assembly of monomers and also helps in polymer synthesis. After that the hot solution was filtered off quickly using 8 μm pore size cellulose filter paper. The purpose of this filtration was to get rid of unreacted monomers. After that the solution is kept

below 4 ^{o}C for at least 12 h. Then the UV light of wavelength 254 nm was irradiated using hand held UV-lamp, which gave blue color in 30 sec [16].

5.2.2 PCDA-EDA liposomes color change upon heating

Liposomes of PCDA original reactant and ethylene diamine (EDA) linker modified PCDA-NH_2 with 1:1 having 1 mM concentration were synthesized. The blue color was observed. When they are subjected to heat above 60 ^{o}C, their blue color started changing to red color as soon as temperature reaches close to 60 ^{o}C and it gains fluorescence. Liposomes were heated for 10 min at 100 ^{o}C precipitation of liposomes was observed [18].

Figure 5.2.2: Schematic representation of PCDA-EDA (PCDA-NH_2) and PCDA (1:1) liposomes synthesis and change of color upon heating

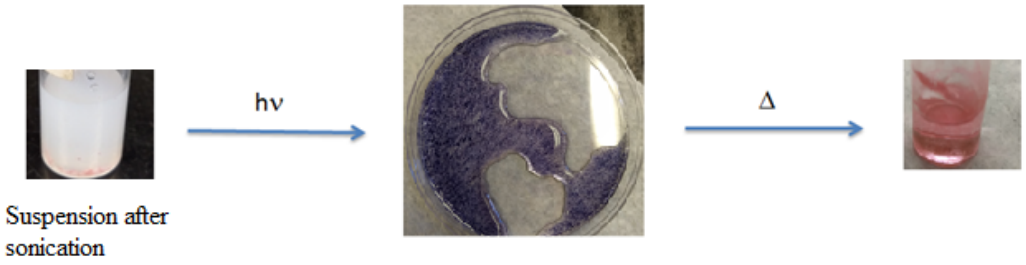


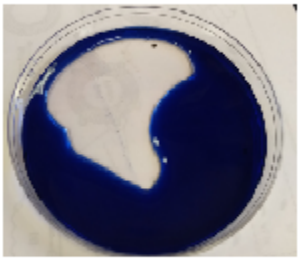
Figure 5.2.2: Cloudy suspension was observed upon sonication at 80 ^{o}C and UV irradiation gave blue color which changed to red color when heated to 100 ^{o}C

Temp(^{o}C)	30	40	50	60	70	80	90	100
Color	Blue	No change	No change	Color change light purple	Red slightly	Red	Red	Red ppt

Table 5.2.1: Temperature profile for color change of blue liposomes to red color

Changing the carboxyl (COOH) head group of PCDA to some other groups gives more intense or bright blue color liposomes [18]. So when the 1mM liposomes of PCDA-EDA were it was observed that the PCDA-EDA liposomes were more intense in color as compared to the previously synthesized 1:1 liposomes. Similarly when these liposomes were subjected to heat more intense red color was observed [18].

(A)



At room temperature
PCDA-EDA liposomes

(B)

After heating
PCDA-EDA liposomes

Figure 5.2.3: (A) PCDA-EDA (PCDA-NH_2) 1 mM blue liposomes (B) change to red color upon heating to 80 ^oC

5.3 PCDA-EDA as metal sensor

In many different studies [20][21] researcher have reported different PCDA liposomes as metal detector. These PCDA molecules upon binding with different metals change their blue color to purple or red depending on concentration [21]. Considering the same concept we thought to try to see what type of metals our linker modified PCDA can detect. Following the reported procedure in which they reported PCDA-EDA with original reactant PCDA having 1:1 ratios and tested copper (Cu), zinc (Zn) and barium (Ba) [22]. Different concentration of these metal salts (barium perchlorate, zinc chloride and copper sulfate) were prepared (1 mM, 5 mM and 10 mM) for each salt in HEPES buffer. It was observed from the resluts that Ba and Zn lower concentrations 1 mM gave no change in color but for 5 mM light purple color was observed and for 10 mM intense purple color was observed. But for copper there was no change in color for any concentration in our range. The results confirm that these liposomes are selective for some metals.

Concentration (mM)	Ba^{+2}	Zn^{+2}	Cu^{+2}
1	No change	No change	No change
5	Light purple	Light purple	No change
10	Conc. purple	Conc. purple	No change

Table 5.2.2: Metal detection using PCDA-EDA and PCDA (1:1) blue liposomes with different concentration of $Ba(ClO_4)_2$, $ZnCl_2$, and $CuSO_4$. Control was 1 mM (1:1) ratio of PCDA-EDA with PCDA blue liposomes

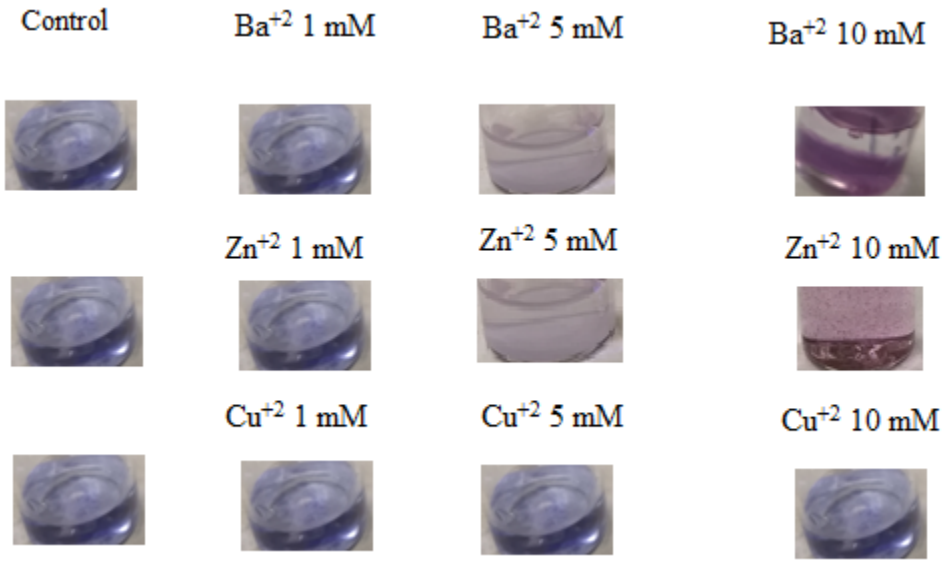


Figure 5.3.1: Ba^{+2} and Zn^{+2} ions were detected with liposomes as indicated by change in color. But for Cu^{+2} ions no change in color was observed

5.4 Anti-bacteria assay

For bacterial studies we collaborated with Dr. Dong's lab, they assisted us in detecting bacterial specie (*S. aureus*) with vancomycin liposomes. Following the previously described methods [23][24] we tested 5 mM and 10 mM concentrations of 20%, and 40% PCDA-Van with PCDA-EDA liposome with gram positive bacteria (*S. aureus*). PCDA-EDA (without antibiotic) liposomes were set as control. Also, PCDA-Van of each percentage were set as control. For 5 mM both 20% and 40% PCDA-Van / PCDA-EDA we did not see change in color after addition of bacterial species. Rather results from 5 mM concentration of liposomes shows that modified PCDA-EDA control liposomes are sensitive even at room temperature that they change their blue color to pinkish. 20% PCDA-Vancomycin liposomes did not change their color with or without bacteria.

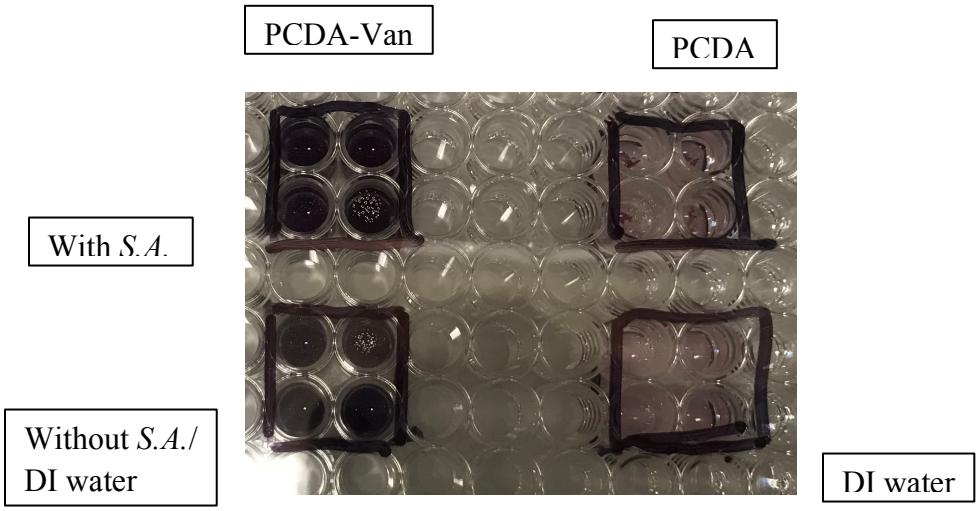


Figure 5.4.1: 5 mM 20% PCDA-Van and 80% PCDA-EDA with and without bacteria. Control was 5 mM PCDA-EDA liposomes

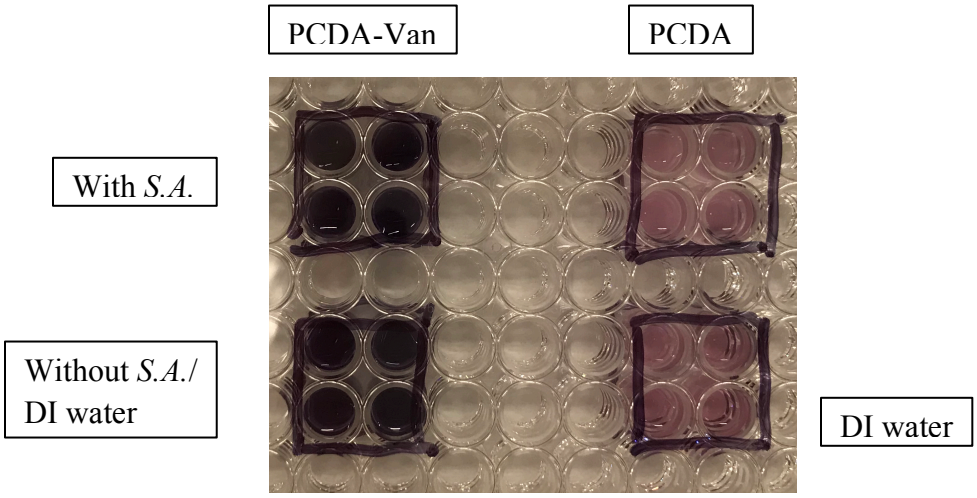


Figure 5.4.2: 10 mM 20% PCDA-Van and 80% PCDA-EDA with and without bacteria. Control was 10 mM PCDA-EDA liposomes

After that 40% PCDA-Van with 60% PCDA-EDA with 10 mM concentration were synthesized and again bacterial studies was done with *S. Aureus*. But the results from these new high concentration PCDA-Van and PCDA-EDA liposomes were not different than the previously tested 20% PCDA-Van liposomes.

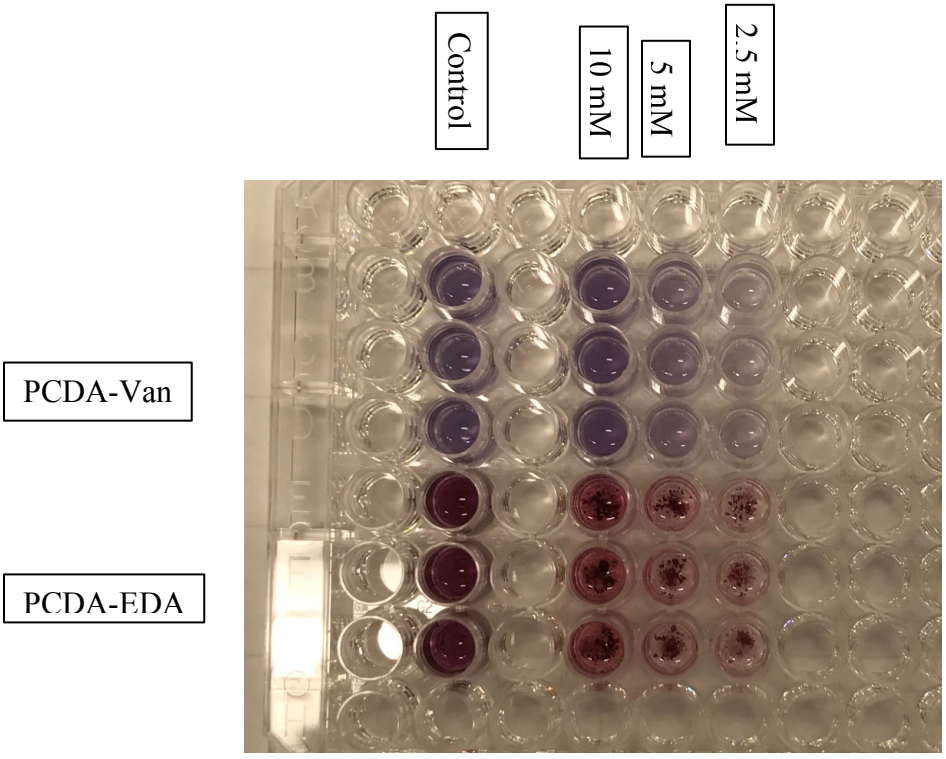


Figure 5.4.3: 10 mM, 5 mM and 2.5 mM 40% PCDA-Van and 60% PCDA-EDA with and without bacteria. Control 10 mM PCDA-EDA liposomes

CHAPTER 6

Future Work

We have synthesized one PCDA monomer with antibiotic vancomycin successfully and now we are in a process to find exact condition suitable for its working biosensor and inhibitor for gram positive bacteria. Along with this we are also planning on coupling of our other antibiotic Polymyxin B (PMB) with PCDA which is very effective in inhibiting gram negative bacteria. The approach we are planning with PMB is to protect most basic amine groups using Boc [25][26] and then coupling of free amine with COOH group of PCDA followed by deprotection of Boc groups using TFA.

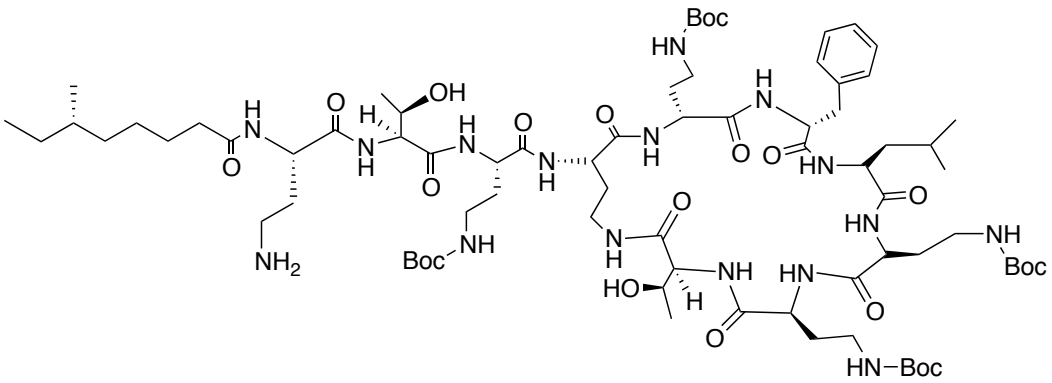


Figure 6.1: Protection of four amines of 2, 4-Diaminobutaric acid (Dab) with Boc protected groups of polymyxin B

We also have a plan to work with sialic acid molecule which is very effective in influenza A virus detection. Our approach would be to couple PCDA-EDA amine group with COOH group of sialic acid (fig. 4a) using coupling reagent such as EDC [27]. Once we synthesize PMB and sialic acid bound PCDA monomers we would work on finding the exact concentrations and conditions for their use as biosensors and inhibition of gram negative bacteria and for influenza A virus. After successful results we would do their UV-visible and fluorescence spectroscopy as well.

CHAPTER 7

Conclusion

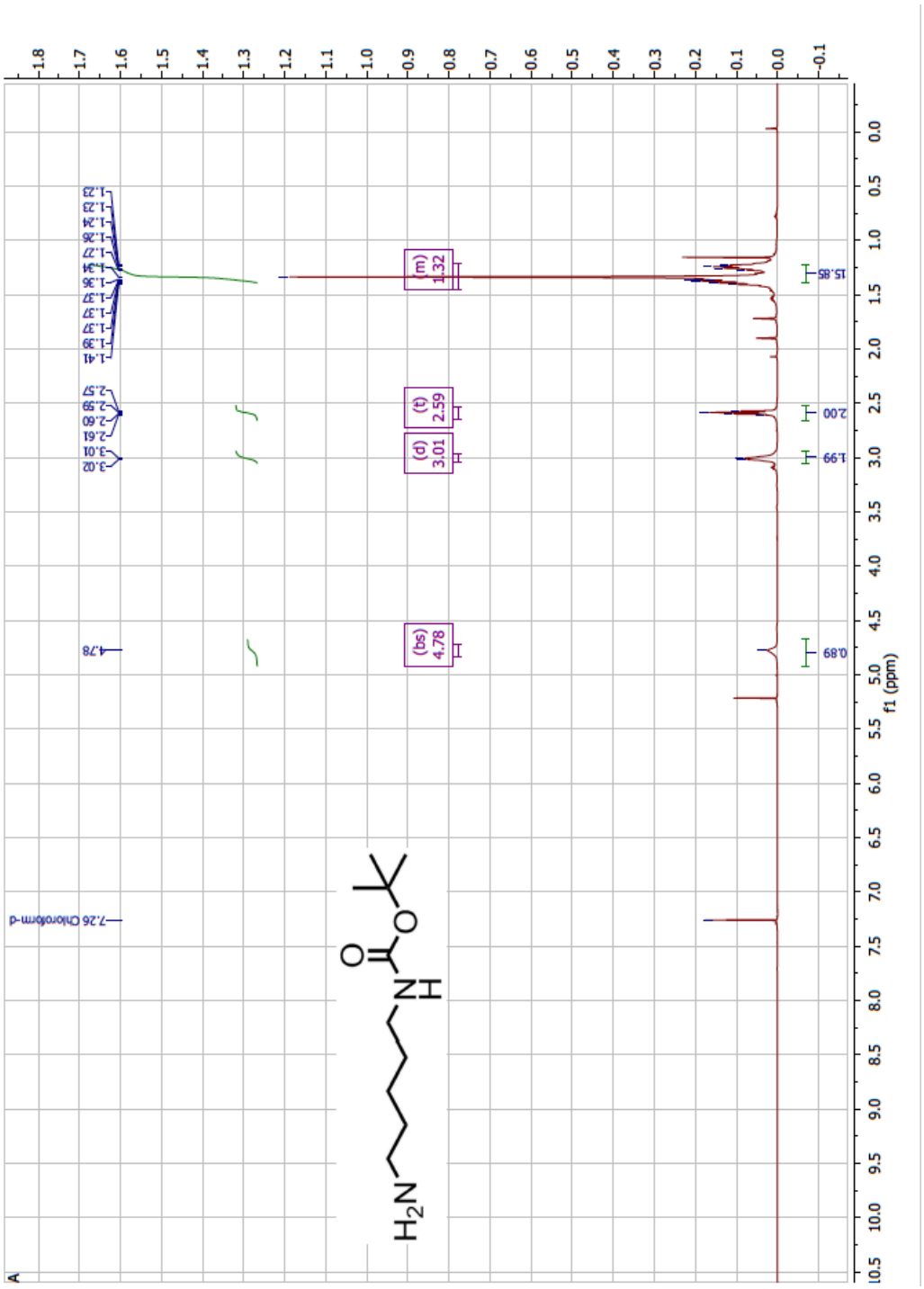
PDA polymers are great way for sensing infective specie such as bacteria and viruses. In our studies so far we have modified one PCDA monomer with linker ethylene diamine (PCDA-NH_2) using two different scheme. We started working with scheme 1 with cadaverine protected its one amine group using Boc protection group and treated the other free amine with PCDA carboxyl group using pyBOP coupling reagent to form peptide bond. The yield from this reaction was 40%. Then we tried to de-protect the Boc group at 40 ^{o}C with 4 equivalent but it did not work mainly because we used temperature and PCDA are sensitive to temperature. After that Boc-deprotection was successfully done by avoiding temperature and increasing the equivalence of TFA. But as the yield for peptide bond formation reaction was lower so we shifted to scheme 2. In this scheme we first activated the PCDA carboxyl group by converting it to NHS ester (PCDA-NHS) and then using 10 equivalent of linker ethylene diamine the peptide bond was formed with one amine of linker. The yield was 80%, the other 20% probably was where both amine of linker are coupled to PCDA. The product (PCDA-EDA) was successfully separated using basic activated alumina as stationary phase. In next step the PCDA-EDA was treated with COOH group of vancomycin. The product was obtained with 40% yield. We characterize PCDA-Vancomycin compound with Mass spectrometry (LC-MS). In last step of synthesis with one antibiotic we synthesize liposomes of 20% and 40% PCDA-Van with PCDA-EDA and gave few trials for their anti-bacterial studies with *S. aureus.* gram

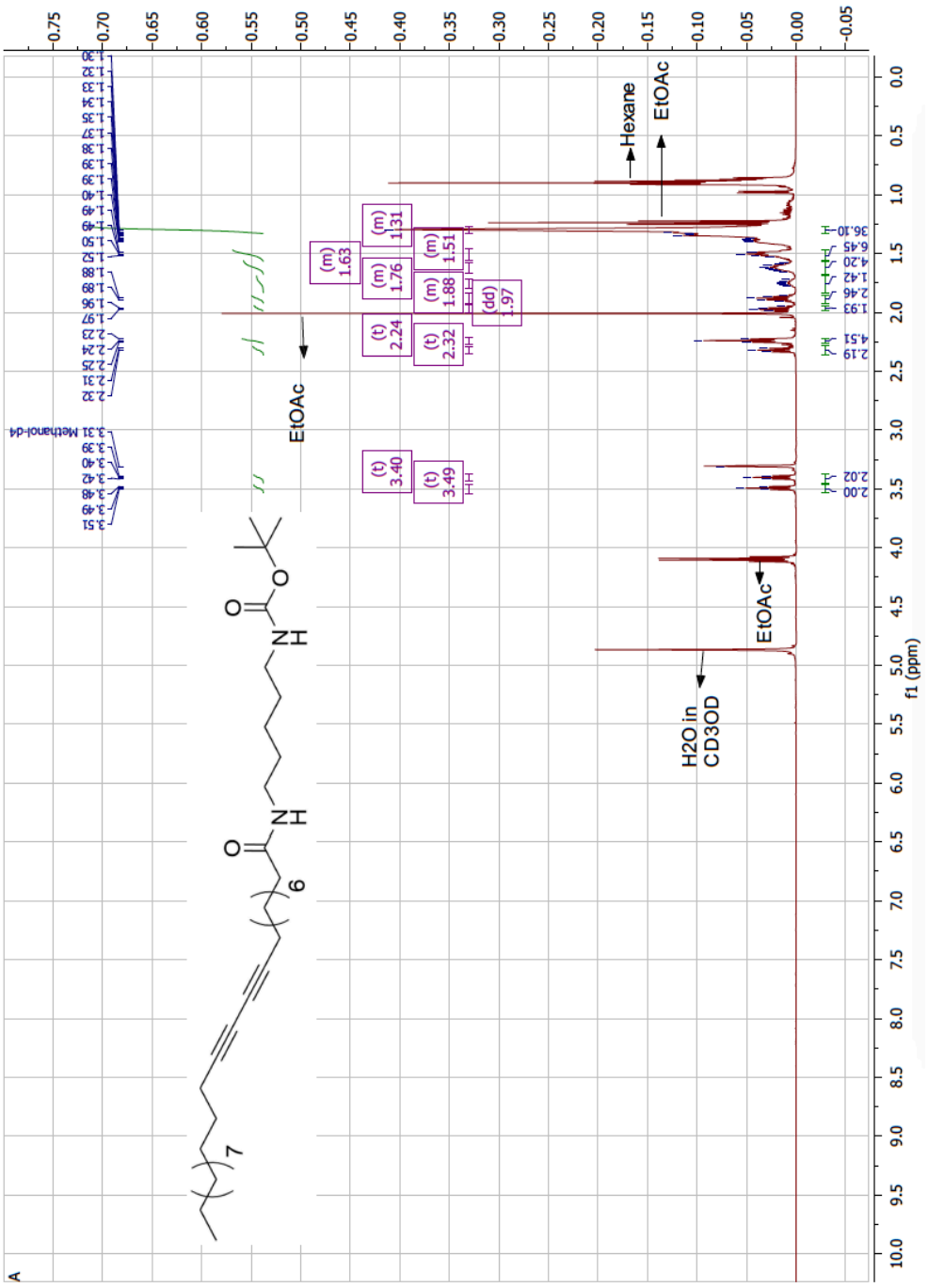
positive bacteria. Still we did not see any positive results but we are in a process to maintain the exact conditions like concentrations and some other factors to make our PCDA-Van liposomes to change their blue color to purple or red once they bind to bacteria.

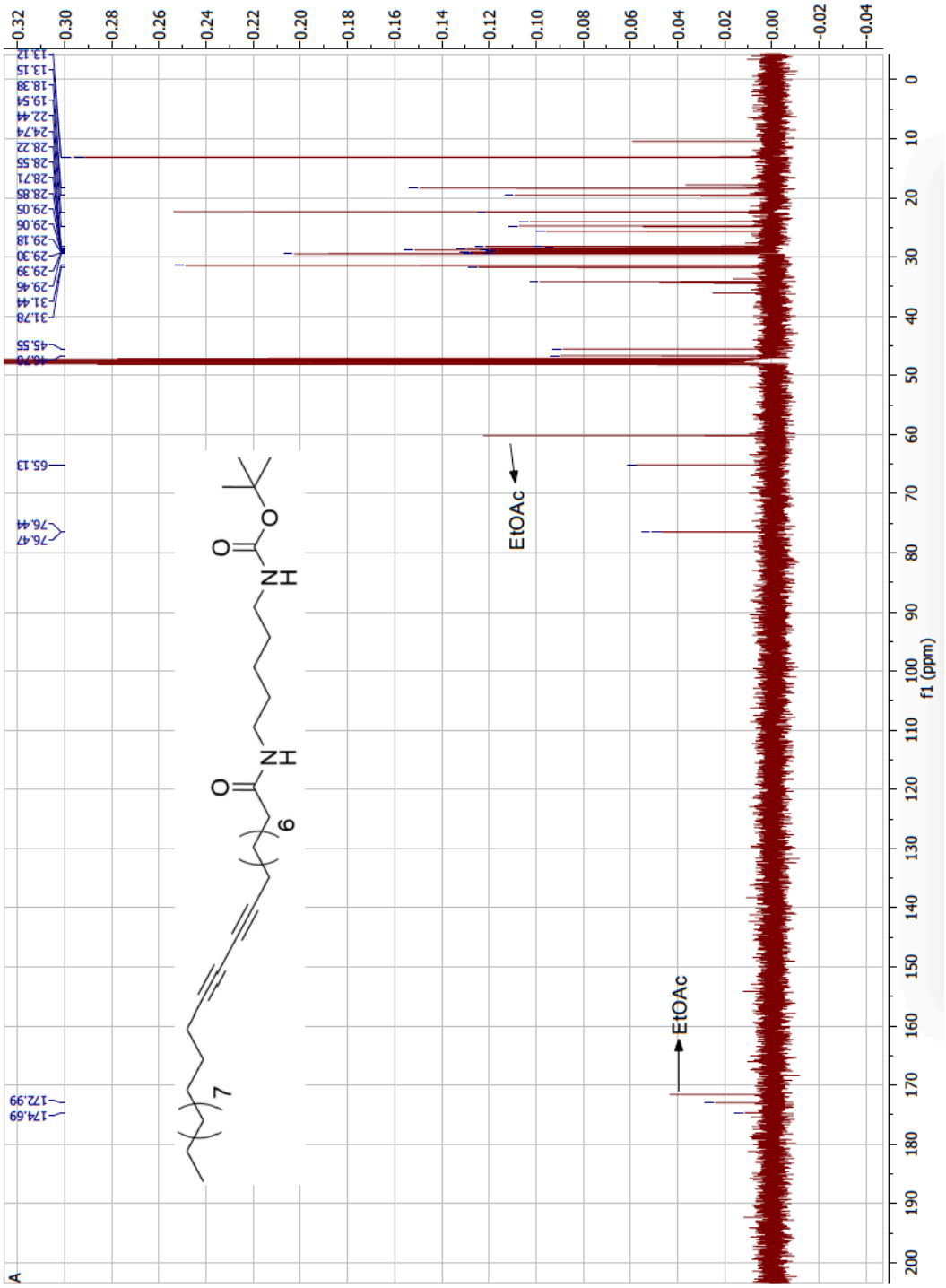
To see our synthesized PCDA modified monomers are showing same reported properties [17][22] such as change in color to purple or red upon heating and binding to different metals. We synthesized liposomes of original reactant PCDA and linker modified PCDA-EDA with 1:1 ratio having concentration of 1 mM. We saw blue color liposomes changed to red when heating to 80 ^{o}C for 10 min. Also we saw change in color from blue to purple upon binding of these liposomes with Zn^{+2} and Ba^{+2} metals as reported in the literature.

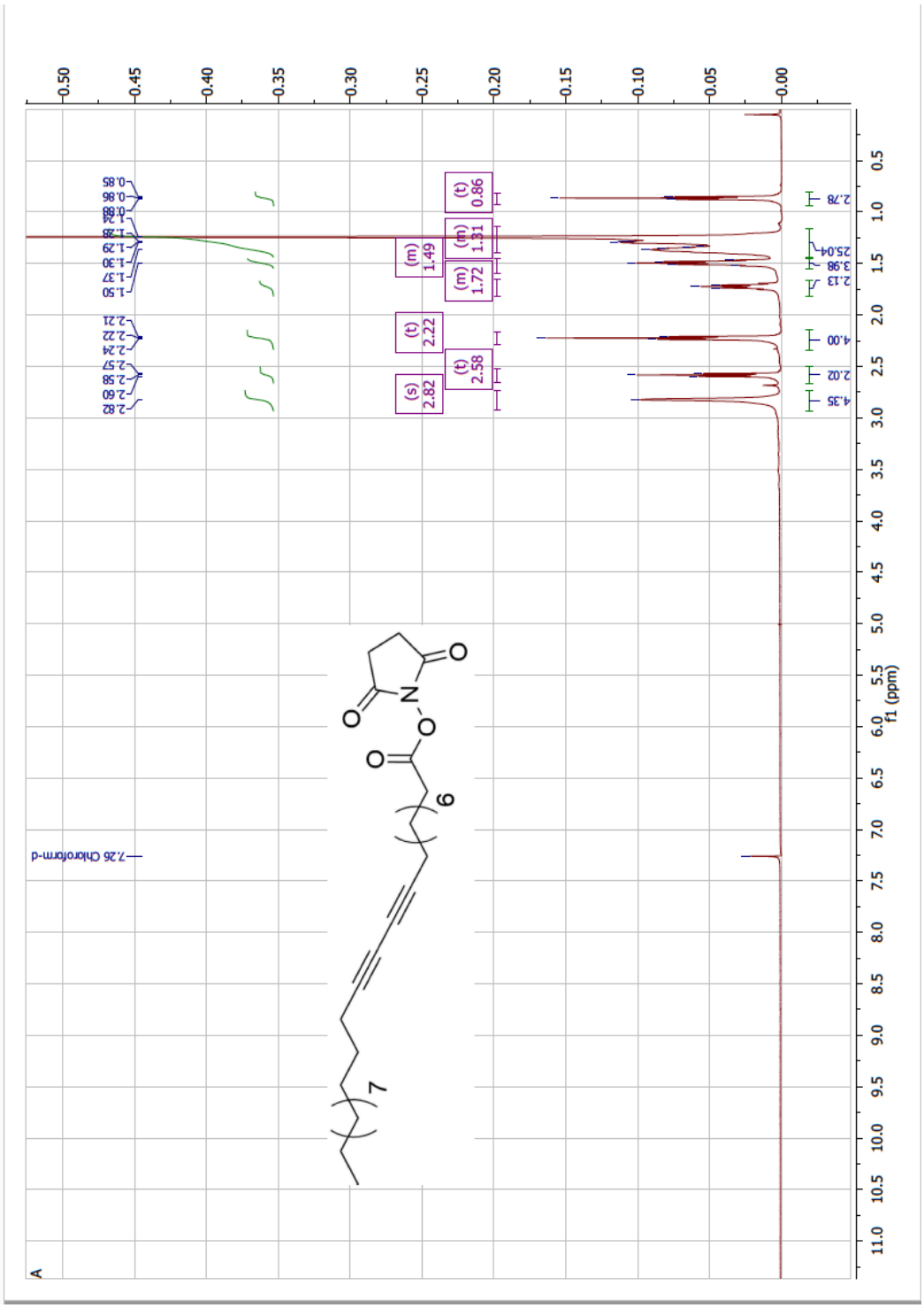
Appendix A

NMR and IR spectra


A
7.26 Chloroform-d
4.78
(bs) 4.78
(d) 3.01
(t) 2.59
(m) 1.32
0.89
1.99
2.00
15.85
f1 (ppm)


Hexane
EtOAc
EtOAc
EtOAc
H2O in CD3OD
Methanol-d4
f1 (ppm)


EtOAc
EtOAc
f1 (ppm)


A
f1 (ppm)
7.26 Chloroform-d
(t) 0.86
(m) 1.31
(m) 1.72
(m) 1.49
(t) 2.22
(t) 2.58
(s) 2.82
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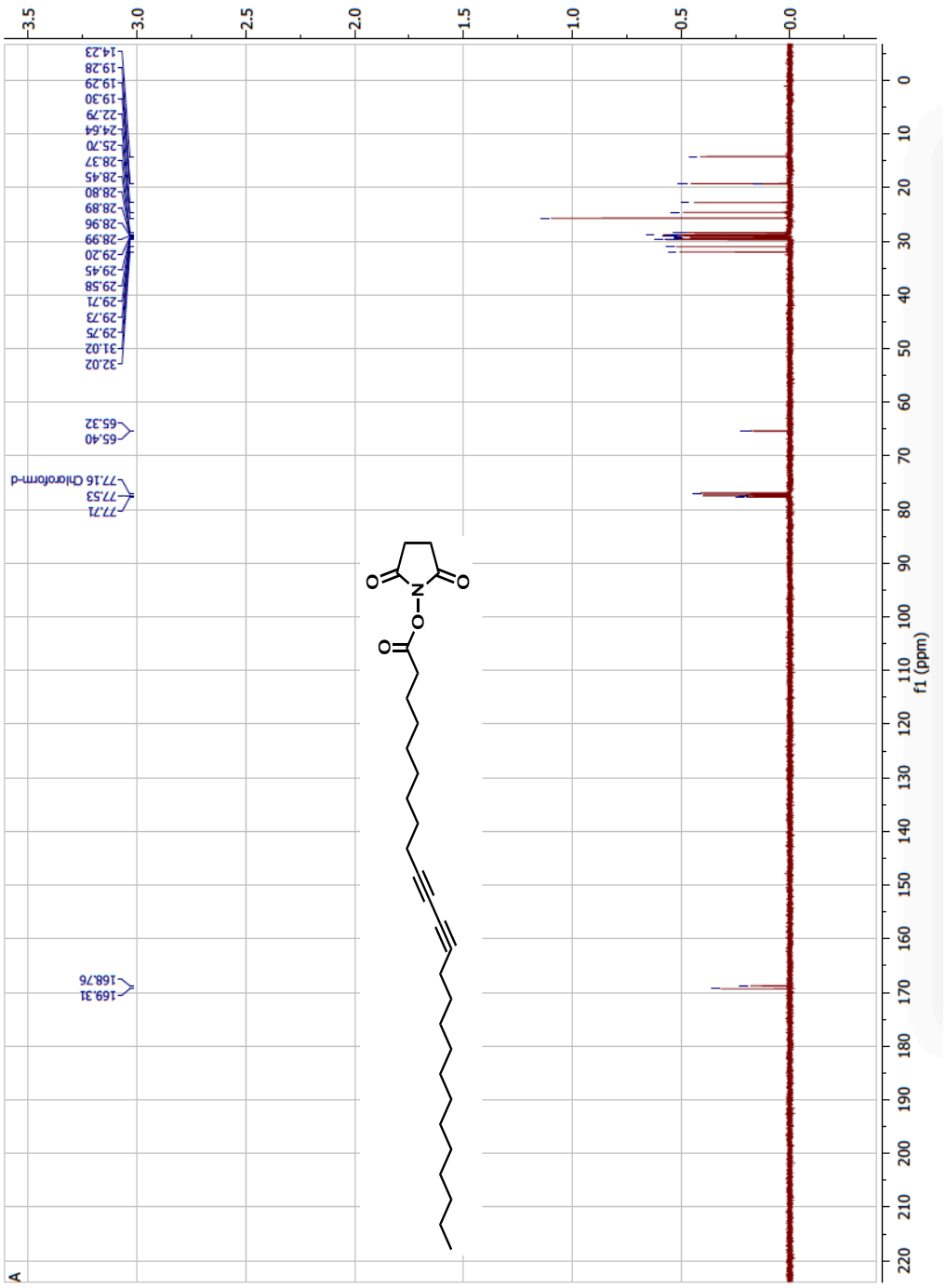
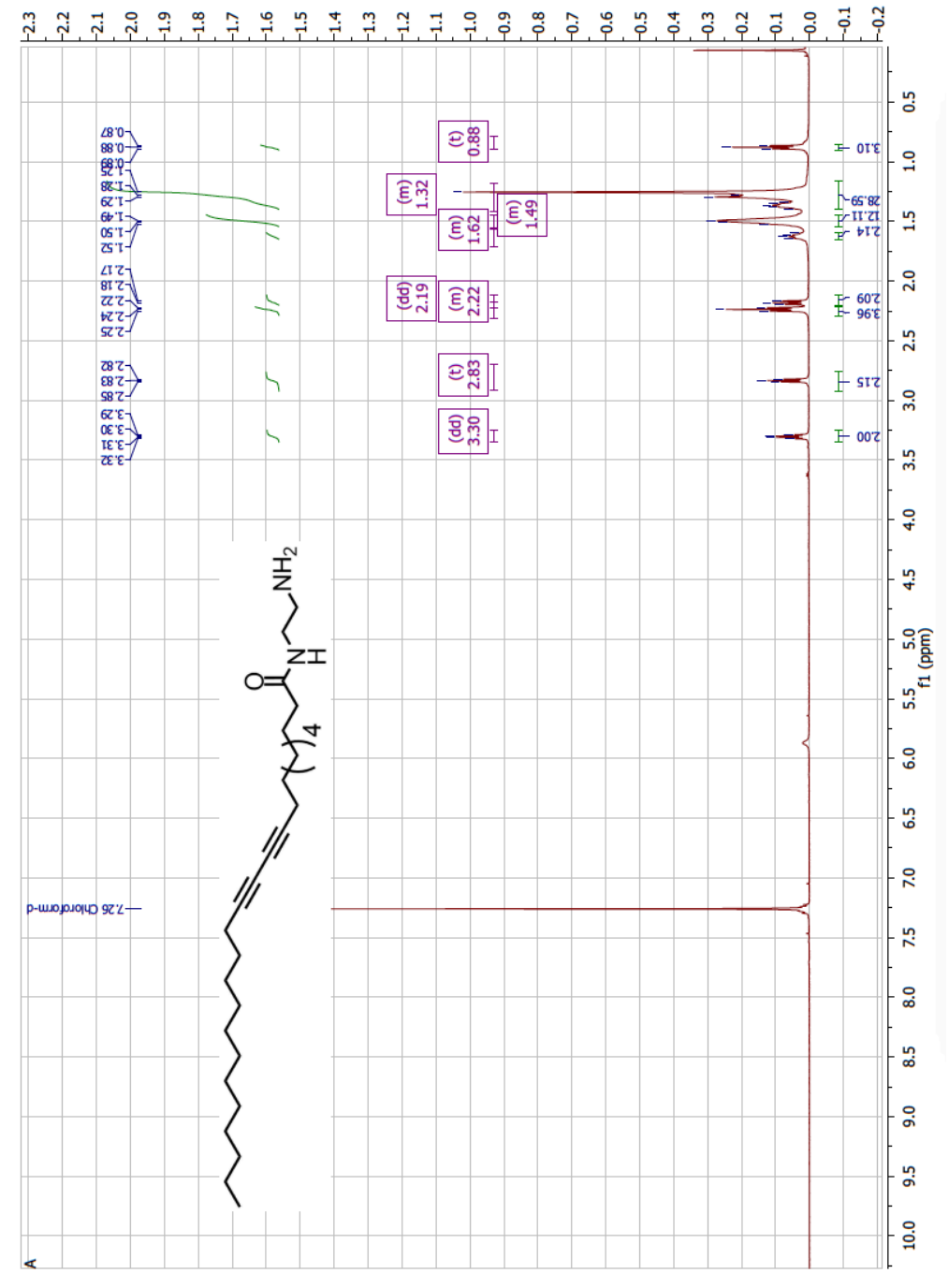
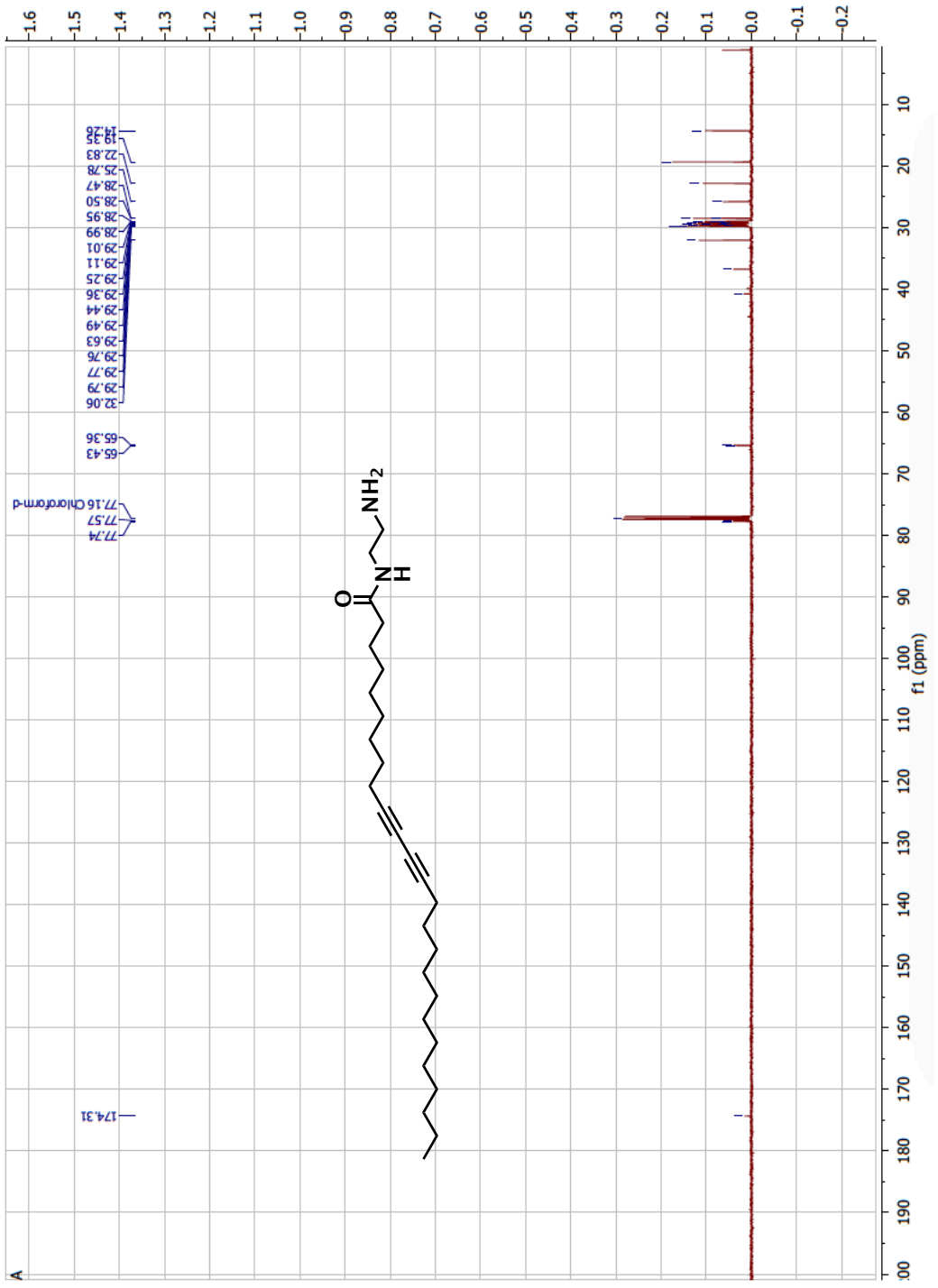
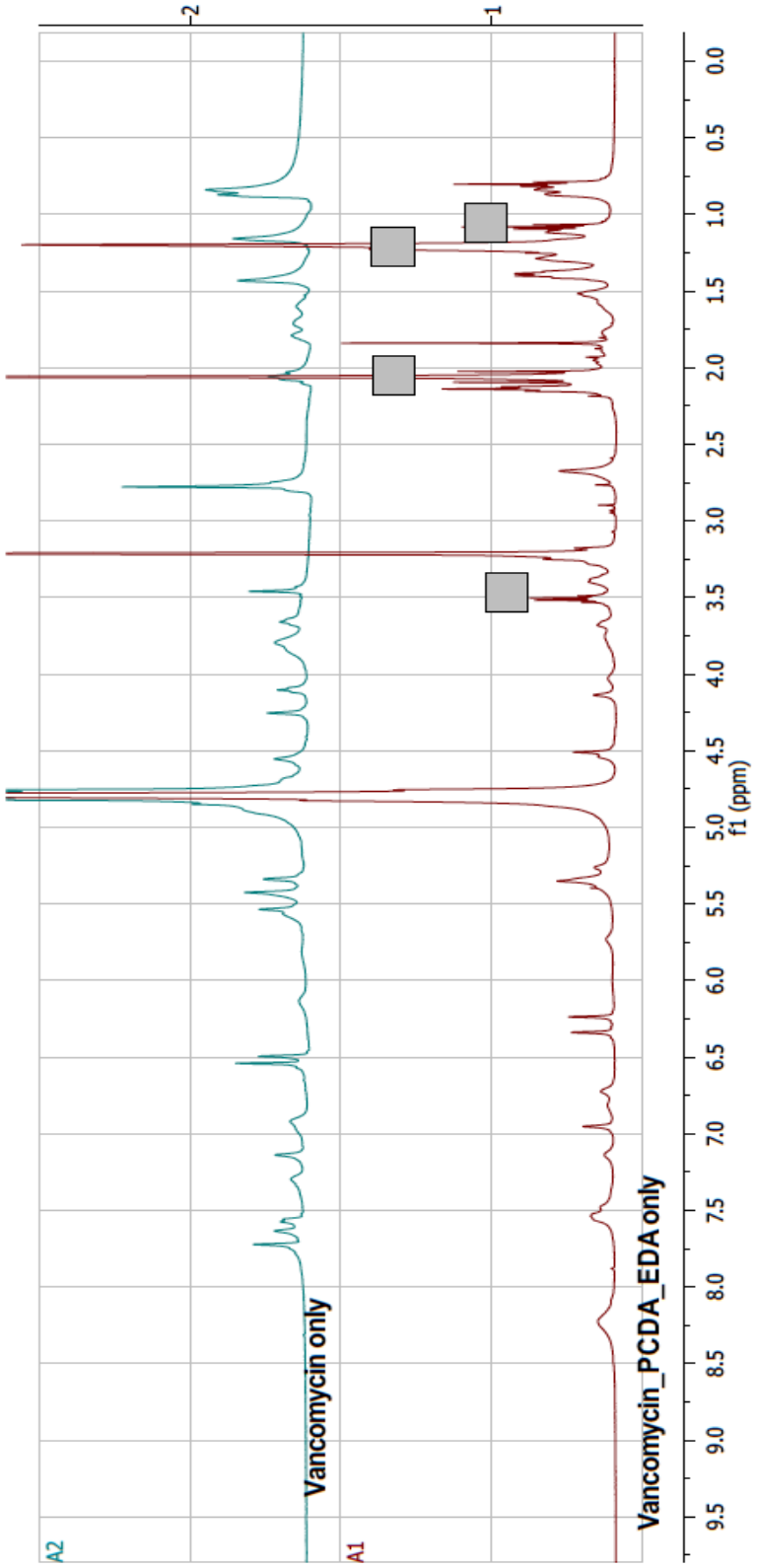
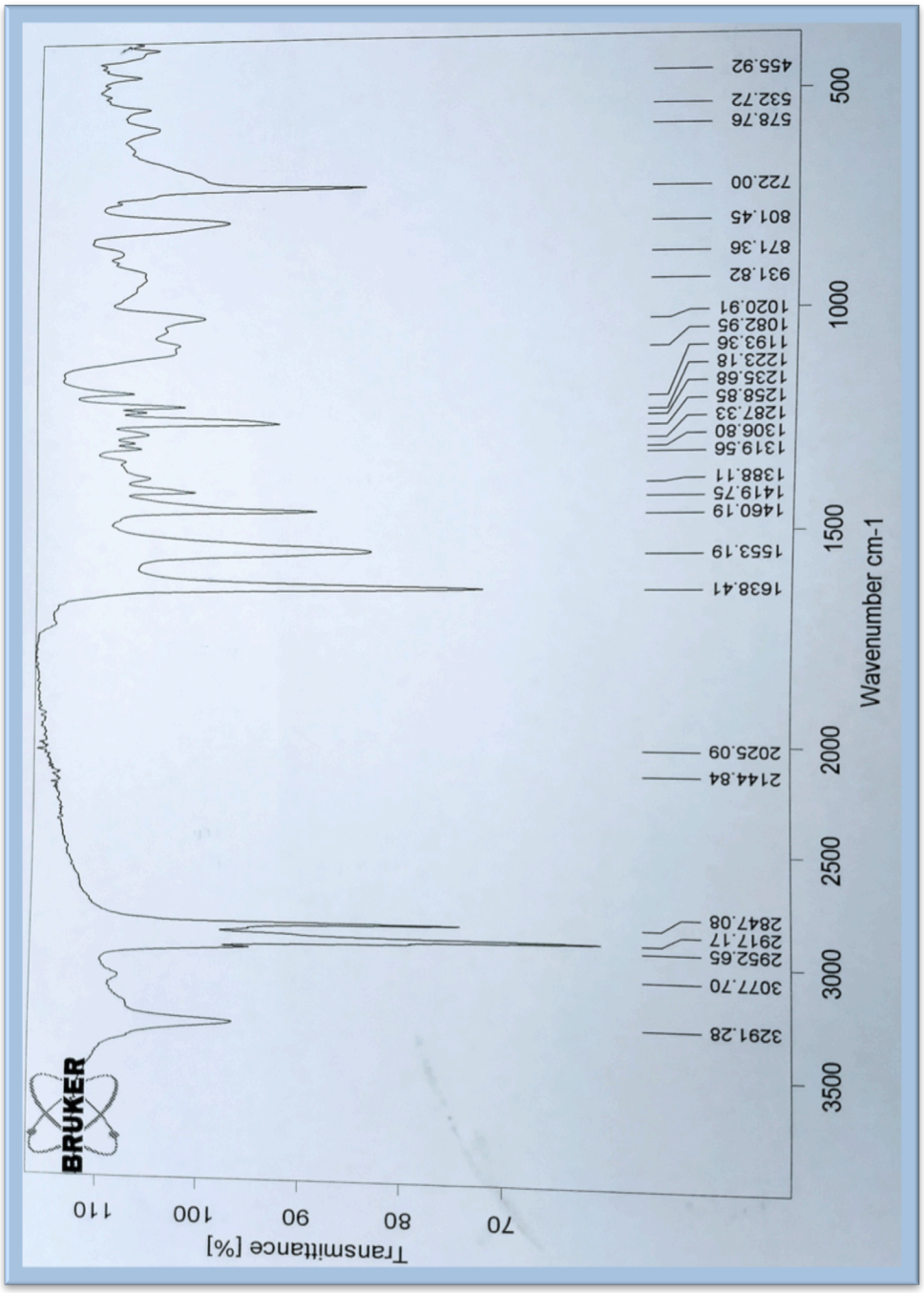
4.35
O
N
O
O
O
7
6


14.23
19.28
19.29
19.30
22.79
24.64
25.70
28.37
28.45
28.80
28.89
28.96
28.99
29.20
29.45
29.58
29.71
29.73
29.75
31.02
32.02
65.32
65.40
77.16 Chloroform-d
77.53
77.71
168.76
169.31
f1 (ppm)


7.26 Chloroform-d
NH2
N
H
O
4
(t) 0.88
(m) 1.32
(m) 1.62
(m) 1.49
(dd) 2.19
(m) 2.22
(t) 2.83
(dd) 3.30
3.10
28.59
12.11
2.14
3.96
2.09
2.15
2.00
f1 (ppm)


A
f1 (ppm)
NH2
O
N
H
Chloroform-d


2
1
A2
A1
Vancomycin only
Vancomycin_PCDA_EDA only
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
f1 (ppm)


BRUKER
Transmittance [%]
110
100
90
80
70
Wavenumber cm-1
3500
3000
2500
2000
1500
1000
500
3291.28
3077.70
2952.65
2917.17
2847.08
2144.84
2025.09
1638.41
1553.19
1460.19
1419.75
1388.11
1319.56
1306.80
1287.33
1258.85
1235.68
1223.18
1193.36
1082.95
1020.91
931.82
871.36
801.45
722.00
578.76
532.72
455.92

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