Polycaprolactone based clickable nano-assemblies for antimicrobial activity

Submission of MS Thesis Report for partial fulfilment of
BS-MS dual degree program



By

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CERTIFICATE

"This is to certify that this dissertation entitled ***Polycaprolactone based clickable nano-assemblies for antimicrobial activity*** towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents work carried out by Keerthana M at IISER Pune under the supervision of Prof. M. Jayakannan, Professor, Department of chemistry during the academic year 2021-2022."

Date: 09-04-2022
Place: Pune

Signature of student

Date: 09-04-2022
Place: Pune

Signature of supervisor

DECLARATION

"I hereby declare that the matter embodied in the report entitled ***Polycaprolactone based clickable nano-assemblies for antimicrobial activity*** are the results of the work carried out by me at the Department of Chemistry, IISER Pune, under the supervision of Prof. M. Jayakannan and the same has not been submitted elsewhere for any other degree."

Date: 09-04-2022
Place: Pune



Signature of student

Date: 09-04-2022
Place: Pune



Signature of supervisor

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Abstract

Microbial infection is an all-time threat to global public health which includes the ever-increasing infections caused by bacteria, parasites, viruses and fungi. The scientific community have been developing various strategies and techniques to tackle microbial infections and help us build a healthy community. The microbes developing drug resistance have always been a problem for us, there comes the importance of cationic polymeric compounds as promising candidates for anti-microbial agents with a decreased potential for resistance development. Along with its intrinsic antimicrobial action through membrane permeabilization, polymeric matrix is also responsible for encapsulating antibiotics for dual action.

This MS Project is directed towards the study of polycaprolactone based Biodegradable Cationic block copolymers. We aim to develop a PEGylated cationic polycaprolactone polymer for enhanced solubility, decreased immunogenicity and boost the pharmacokinetic behaviour of the cationic nano-assembly. Along with this we are also trying to bring the concept of 'click chemistry' for the synthesis of these cationic polymers. PEG-5K monomethyl ether and tri-ethylene glycol monomethyl ether were used as the initiators for successful ring opening polymerization of an alkyne substituted caprolactone monomer which was further clicked with a halogen bearing small molecule and then quaternized with triethyl amine to form PEG-containing cationic polymers. PEG was utilized to improve the selectivity of the cationic polymers towards bacterial over mammalian cells. The quaternized polymers were checked for antibacterial activity and we got 80% killing in 50 microgram/mL concentration of the block copolymer whereas homo polymer showed no killing. To make the direct visualization of this antimicrobial activity possible, we also developed an azide functionalized fluorophore conjugate which can be attached to the polymeric backbone through click reaction.

1.Introduction

1.1 Antimicrobial Polymers:

Antimicrobial polymers are the synthetic mimics of antimicrobial peptides which are responsible for providing immune response towards invasion of pathogenic organisms [1-3]. They are important classes of macromolecular nano-assemblies which exhibits bacterial cell membrane disruption. These cationic polymers were predominantly built on appropriate hydrophobic and hydrophilic balance which is responsible for its mechanism of action [4,5]. The cationic units are responsible for initial adhesion to negatively charged bacterial membrane while hydrophobic part inserts into the lipid bilayer membrane. The interaction of hydrophobic polymeric chain to hydrophobic counterparts of the membrane causes lesions on the surface of membrane thus contributing to membrane breaching. The basic principle behind the antimicrobial activity of AMPs is the cellular composition and surface charge of bacterial membranes. The surface of eukaryotic cell is composed of zwitterionic phosphatidyl choline due to which antimicrobial polymers have no effect on mammalian cells [6]. At the same time, the bacterial cell membranes have a net negative charge on their surface because of the presence of teichoic acid and lipopolysaccharides [7]. As a result, AMPs can attach on bacterial cell membrane and can cause surface disruption through electrostatic and hydrophobic interactions (figure 1.1). These are the 'preliminary mechanism' responsible for the antimicrobial activity of AMPs [8].

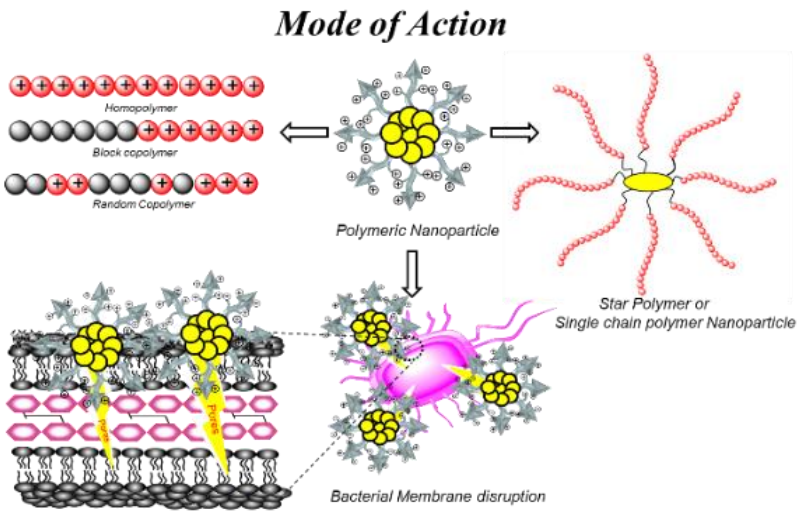


Figure 1.1 Mode of action of cationic polymers

Based on the mechanisms of action of the AMPs, they are classified into two

groups. That is “non-membrane acting and membrane acting peptides” [8]. The various mechanisms of action of AMPs are

a) *The barrel-stave mechanism:* “In this mechanism the AMPs are inserted into the biological membrane, the hydrophobic substance will flip inward and it will create pores producing trans-membrane helical bundles” [9].
b) *Carpet-like mechanism:* In this, the destruction of the cell membranes assembly by the AMPs are through their collaborative activity. The AMPs will reach near the bacterial membrane’s phospholipids-rich areas located on the lipid bilayer and get self-associated onto them. As soon as they reach a specific threshold of concentration, they get permeated into the membranes. [10]
c) *Toroidal mechanism:* This is the mechanism of construction of “toroidal pore” in the bilayer membrane. These pores are oriented vertically onto the membranes. In other words, “the AMPs cumulate and impel the lipid monolayers for continuous bending through the pore so that both lipid head groups as well as inserted peptides line the water core” [11].

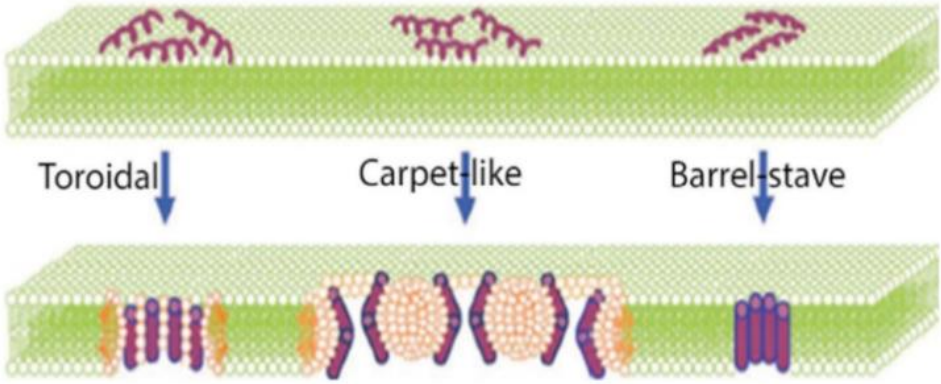


Figure 1.2 Mechanism of action [27]

These mechanisms of AMPs activity are illustrated in the figure 1.2. LL-37, magainins, defensin and melittin are the examples for AMPs inducing “trans-membrane pores” on the membrane of target cells. AMPs like mersacidin, pleurocidin, HNP-1, dermaseptin, indolicidin and pyrrhocidin are examples for AMPs that suppress the important cellular activities by translocation across the biological membranes resulting in bacterial cells death. There are also some AMPs like melittin, papiliocin, lactoferrin and

histatin which act by the reactive oxygen species (ROS) formation. In the case of AMP mimicking polymers, let it be a homo polymer, block co-polymer, random co-polymer or a non-linear polymeric structure like a single chain star polymer, self-assembled to form a polymeric nano particle, these polymers can mimic the AMPs due to their structure and charge distribution and these nano particles gets attached to the outer leaflet of bacterial cell membranes through electrostatic interactions. Then they can induce the membrane perforations through any of the various mechanisms explained above and it results in the thinning of the outer leaflet. “The surface area of the outer leaflet expands relative to the inner leaflet resulting in strain within the bilayer. Transient pore formations happen and collapses the cell membranes to fragments causing physical disruption of target cell membrane”.

1.2 Ring opening polymerization (ROP):

ROP being a living polymerization technique, overcomes the limitations of other polymerization methods and brings a good control over molecular weights and dispersity properties, it can give rise to narrow molecular weight distribution of polymers. Since it has a good control over molecular weights it can be used to design block copolymers using various monomers. Various bio compactible polymers including poly caprolactones, poly lactides, and polyglycolides were synthesized using ROP[12-14] . Depending on the catalysts and initiators used, there are four different mechanisms for ROP.

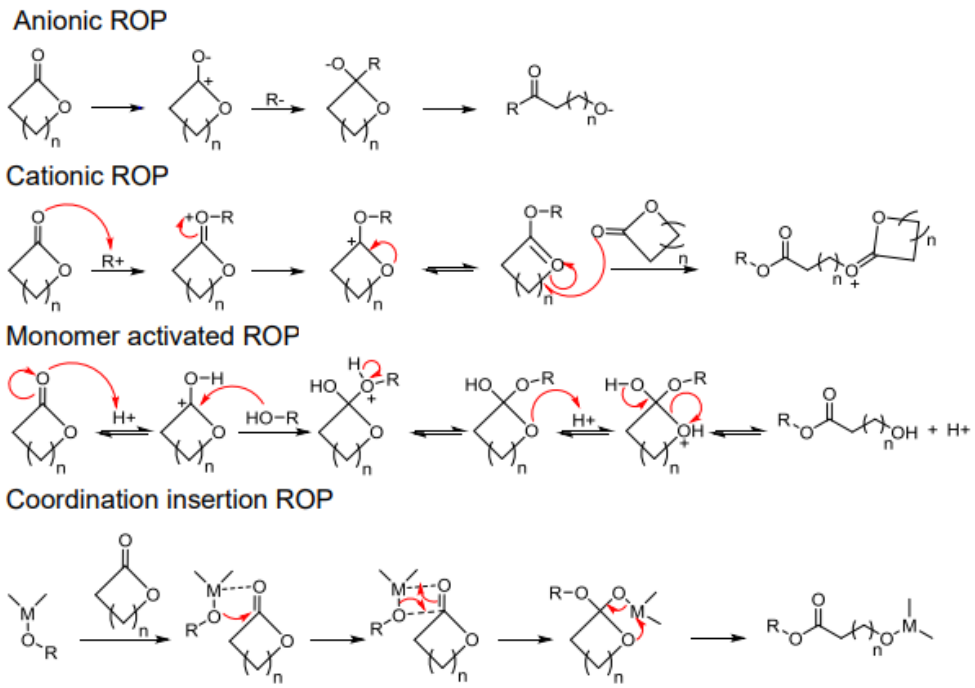


Figure 1.3 Different mechanisms of ROP[26]

They are cationic,anionic, monomer activated and coordination insertion mechanisms. Among these we are using metal catalyzed ROP which follows the coordination insertion mechanisms.The different mechanisms of ROP are illustrated in figure 1.3[15].

1.3 Block copolymers:

These polymers consist of blocks of more than one monomer units. Different block copolymer architechtures are known which includes linear, centipede, star, brush etc. Various controlled polymerization techniques as well as post polymerizatiomodifications made it possible to make these many types of block copolymers. Figure 1.4 illustrates various types of block copolymers like (a) AB block, (b) ABA type block, (c) BAB type, (d) ABABAB… type, (e) palm tree, and (f) star block copolymer. In this project we are trying to bring the concept of block copolymer in order to increase the hydrophilicity of the polymer and there by increasing solubility of the polymer in hydrophilic environments or cellular environments. For that we are using PEG-5k as an initiator to synthesize amphiphilic block copolymer which is then modified to obtain cationic block copolymer which is antibacterial in nature.

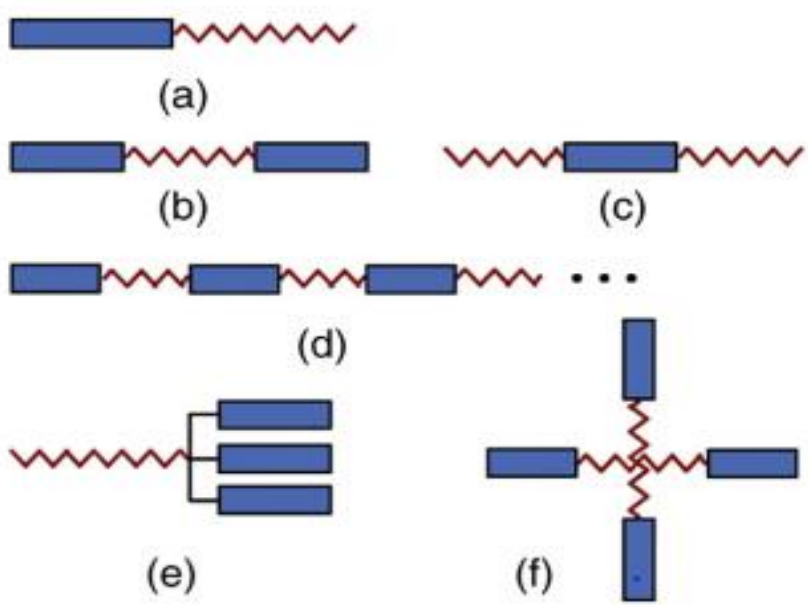


Figure 1.4 Various Block-copolymer architectures [14]

1.4 Polycaprolactones and Substituted polycaprolactones:

Polycaprolactones (PCL) are biodegradable polyester polymers composed of hexanoate repeating units. Other than biodegradability the important properties of PCL includes its mechanical properties like young's modulus, crystallinity and decomposition temperature. It is a semi crystalline solid which can reach a degree of crystallinity of around 69% and it has a very high decomposition temperature of around 350°C. Even though it has all these benefits, the hydrophobicity and the crystallinity of PCL are limitations for it to be used in many biomedical applications. In order to grow from these limitations, we started synthesizing substituted polycaprolactones in which the properties of the polymers were remodified by the presence of particular substitution groups. By this we were able to bring desired properties in these polymers and still maintain the biodegradability nature of it. The structures of few of the substituted caprolactone monomers are shown in figure 1.5. It facilitates the possibility of conjugating other molecules like drugs, fluorophores and targeting ligands to the polymer system which paves way to a wide range of applications for these polymers.

From our group, a carboxyl functionalized caprolactone monomer was developed which was used for pH responsive delivery of anti-cancer drugs [16-22]. It was also used as a cargo to co-deliver cisplatin and doxorubicin through combination therapy [23-24]. Another caprolactone monomer substituted by a chlorine group was developed which was then quaternized to obtain positively charged polymer which is antimicrobial in nature. The following are some of the examples for substituted caprolactone monomers.

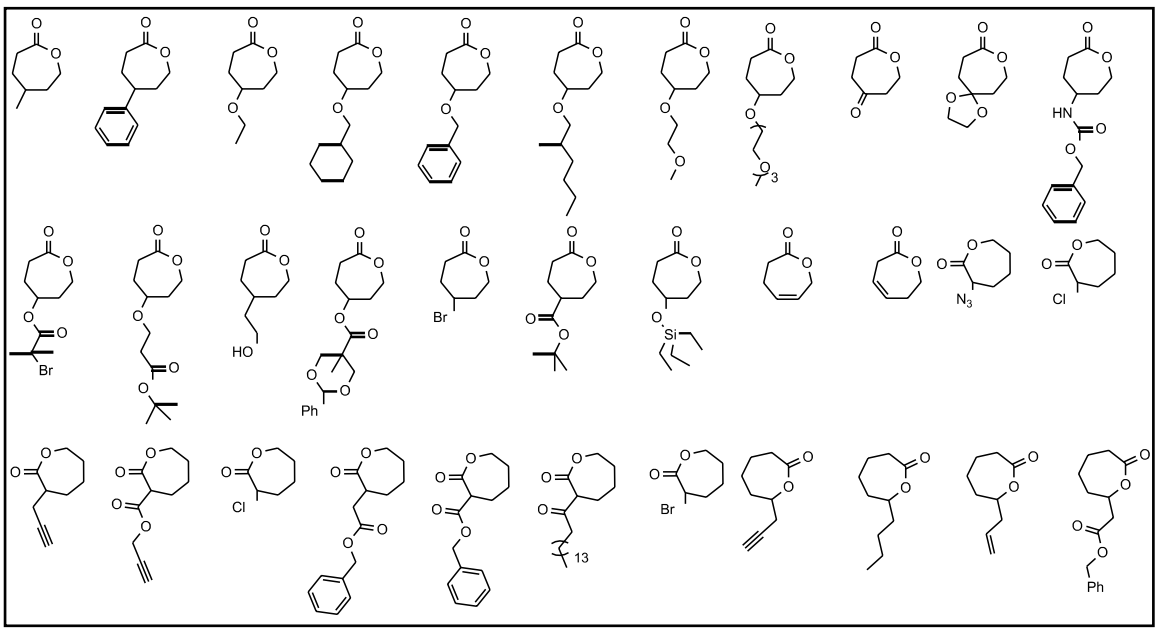
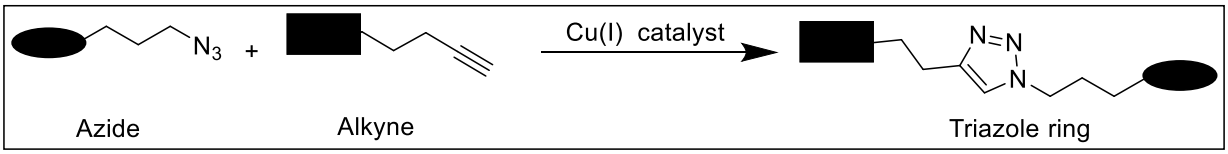


Figure 1.5 Various types of substituted caprolactone monomers[26]

1.5 Click chemistry

The 1,3-dipolar cycloaddition of an azide and alkyne resulting in the formation of a 1,2,3-triazole ring is called a click reaction. Due to the simplicity in work up, purification steps of the reaction as well as because of the rapid product formation and green chemistry, it have a wide range of applications.



Scheme 1.1 Click chemistry

The active species catalysing this reaction is Cu(I) and it can either be added directly to the reaction mixture or generated in situ. We are using sodium ascorbate as a reducing agent to generate Cu(I) in situ from $CuSO_4.5H_2O$ which can then accelerate the reaction.

1.6 Aim of the project:

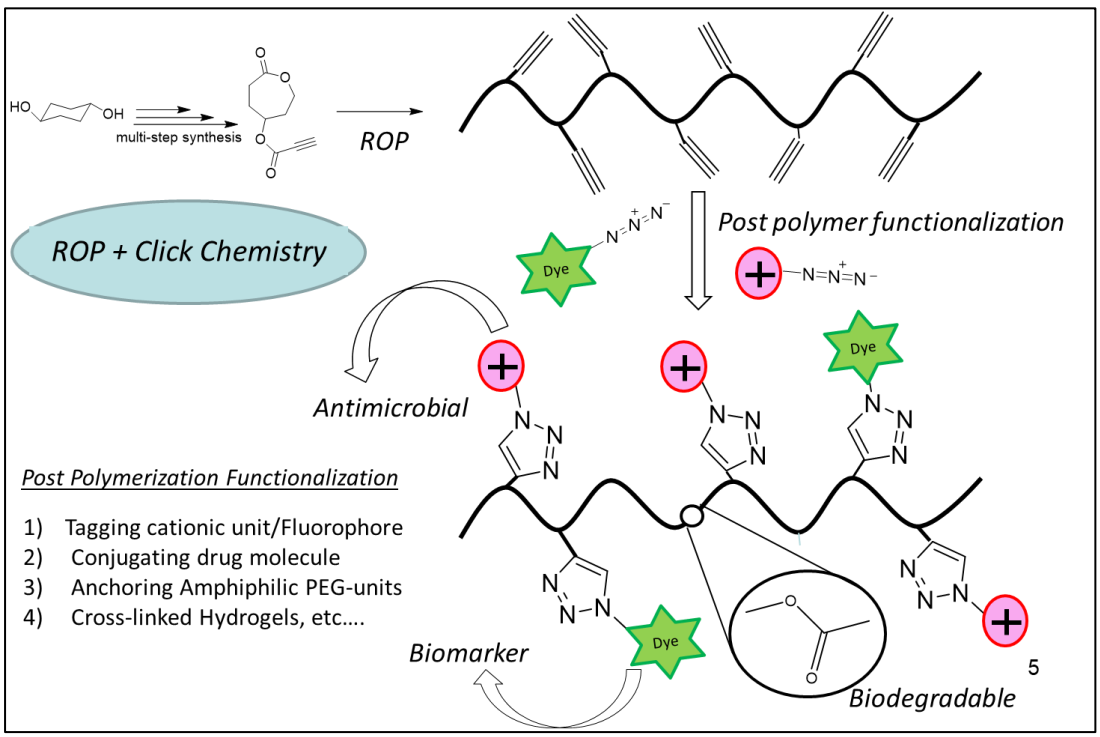


Figure 1.6 Schematic represenatation of aim of the project

This thesis aims at the designing and synthesis of polycaprolactone based biodegradable cationic clickable polymers with antimicrobial activity. For this a new class alkyne substituted caprolactone monomer was synthesized which was stable up to 130 ˚C. This monomer was ring open polymerised to develop alkyne group containing

caprolactone polymers which were then clicked with small clickable linkers. This clicked polymers would further be quaternized with triethyl amine to develop quaternary ammonium containing cationic polymers. These cationic polymers can be checked for their antimicrobial activity. Fluorophore tagging is also possible which would lead to development of fluorophore conjugated polymers which can be used directly for visualizing the real-time attack of polymer on the surface of bacteria.

2. Experimental methods

2.1 Materials:

1,4-Cyclohexanediol, propiolic acid, pyridinium chlorochromate (PCC), molecular sieves (4Å), per benzoic acid (m-CPBA), tin(II)2-ethyl hexanoate ($Sn(oct)_2$), Tri-ethylene glycol mono-methyl ether(TEG), Polyethylene glycol mono-methyl ether(PEG5000), Ethylenediaminetetraacetic acid(EDTA), 1,4-dibromo butane, fluorescein, were Sigma Aldrich products. Common salts like Na_2SO_4, $NaHCO_3$, $Na_2S_2O_3.5H_2O$, HCl, H_2SO_4, Silica gel, Sodium ascorbate, Copper sulphate pentahydrate ($CuSO_4.5H_2O$), sodium azide, Triethylamine, and potassium carbonate were purchased from AVRA and SD Fine Chemicals. Toluene, diethyl ether, Dimethyl sulphoxide (DMSO), Dimethyl Formamide (DMF), petroleum ether, Tetrahydrofuran (THF), Dichloromethane (DCM), Trichloromethane (Chloroform) were used.

***Methods**:*

^{1}H and ^{13}C NMR samples were recorded using 400 and 100 MHz respectively in BRUKER and JEOL spectrophotometer in $CDCl_3$ or DMSO-D_6 solvent. Gel permeation chromatography (GPC) was carried out using triple detectors at 25ºC. $CHCl_3$ was used for sample preparation. Thermal Stability and thermal analysis were carried out by using Perkin Elmer STA 6000 Stimulus Thermal Analyzer and TA Q-20 Differential Scanning Calorimeter respectively with the heating and cooling rate of 10 °C/min with continuous purging of nitrogen from 30°C to 800°C. Nano ZS-90 DLS instrument was used for size determination using 633 nm red laser. PerkinElmer multimode plate reader EnSight™ instrument was used to measure the absorbance of bacterial cells incubated with cationic polymers.

2.2 Synthesis:

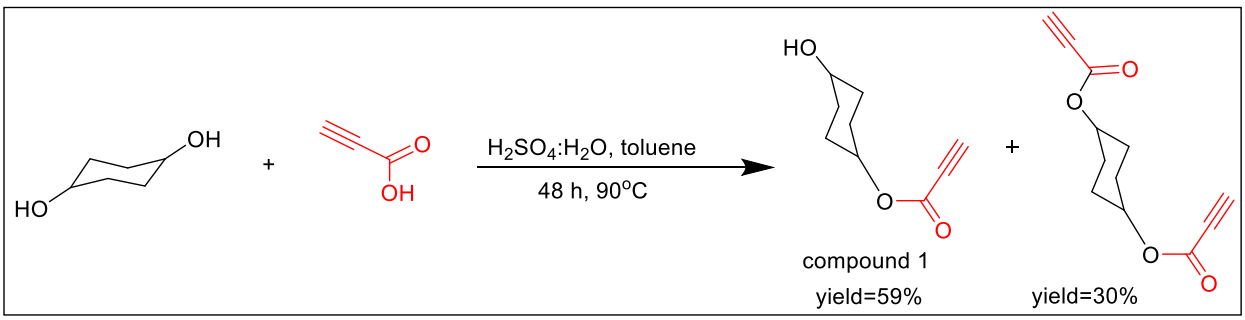
2.2.1 Multistep synthesis of alkyne substituted caprolactone monomer

a) 4-hydroxycyclohexyl propiolate (compound 1):

To a 250 ml round bottom flask equipped with a magnetic bead, cyclohexane diol (4.0g, 34 mmol) was taken and toluene (100 ml) was added to the same. No solubility was observed. A solution of 72 µL H_2SO_4 in 3mL of water was added to the reaction mixture and stirred at room temperature. The diol is soluble in water and so the turbidity in toluene disappears as we add water to the reaction mixture. We can see diol containing microdroplets of water in toluene. Propiolic acid (2.412g, 34 mmol) was dissolved in

toluene (30 ml) and was added dropwise over an hour to the reaction mixture under ice-cold conditions using a dropping funnel. Once the addition was completed, the reaction was set up for reflux at 90°C for 48 hours. After completion of the reaction, toluene was completely evaporated on a rota evaporator and 100 ml water was added to it. Workup was done using ethyl acetate (3×150 ml), the organic layer was collected and combined organic layer washed with 100 ml water, passed over sodium sulphate and rota evaporated to collect the brown coloured crude product. Column chromatography was done using pet ether + ethyl acetate as eluent. The by-product (off white solid powder) was eluted at 10% ethyl acetate in pet ether (v/v) and the product (light yellowish brown viscous liquid) was eluted at 30% ethyl acetate in pet ether(v/v) Yield: 3.4 g (59%).

^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 4.87-4.96 (m, 1 H), 3.76 (m, 1H), 2.86 (s, 1 H), 1.45-2.02 (m, 8 H).^{13}C NMR (100 MHz, $CDCl_3$) δ ppm: 152.3, 74.5, 72.7, 68.7, 67.6, 31.9, 30.4, 28.2, 27.1. FT-IR (cm^{-1}): 3278, 2946, 2867, 2115, 1700, 1448, 1373, 1232, 1062, 1033, 1015, 963, 947, 910, 895, 756, 685. HRMS (ESI+): m/z [M + H+] for $C_9H_{12}O_3$: 168.4912(Expected Mass: 168.0786).

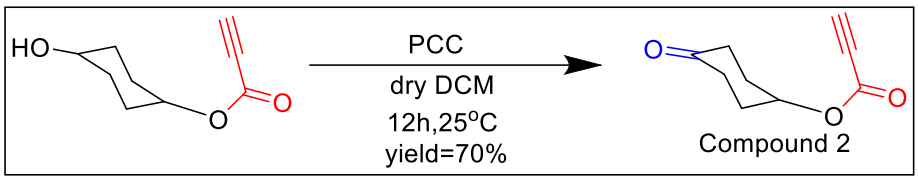


Scheme 2.1 Synthesis of compound 1

b) Synthesis of 4-oxocyclohexyl propiolate (compound 2):

Compound 1 (2.85g, 17 mmol) was weighed and taken in a 250 ml round bottom flask equipped with a magnetic bead. To this 100 ml, dry DCM was added along with freshly dried molecular sieves. PCC (7.3g, 34 mmol) was added to this reaction mixture and stirred at room temperature for 6 hours under nitrogen atmosphere. After the reaction was complete, the reaction mixture was filtered by Buchner filtration to collect the dark brown coloured filtrate which was passed over sodium sulphate and evaporated to get the crude product. Product (off white solid) was eluted at 20% ethyl acetate in pet ether (v/v) system. Yield: 1.923g (70%).

^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 5.28 (m, 1 H), 2.93 (s, 1 H), 2.59 (m, 2 H), 2.40 (m, 2H), 2.15 (m, 4H).^{13}C NMR (100 MHz, $CDCl_3$) δ ppm: 209.2, 152.1, 75.2, 74.7,71.0, 37.1, 30.2. FT-IR (cm^{-1}): 3222, 2958, 2919, 2107, 1702, 1452,1419, 1373, 1330, 1246, 1176, 1116, 1070, 1007, 955, 899, 853, 758, 722. HRMS (ESI+): m/z [M + H+] for $C_9H_{10}O_3$: 165.9912(Expected Mass-166.0630).

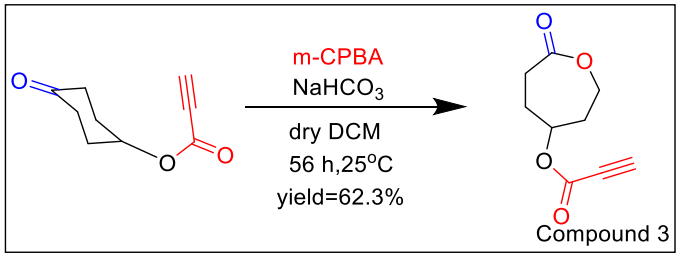


Scheme 2.2 Synthesis of compound 2

c) Synthesis of 7-oxooxepan-4-yl propiolate (compound 3):

Compound 2 (1.9 g, 11.4 mmol) was weighed and taken in a 250 ml round bottom flask equipped with a magnetic bead and dissolved in 60 mL of dry DCM. $NaHCO_3$(2.88 g, 34.3 mmol) and m-CPBA (5.9 g, 34.3 mmol) were added into the reaction mixture under nitrogen atmosphere and stirred at 25 °C for 48 h. Once the reaction was completed the reaction was neutralised with $NaHCO_3$ and $Na_2S_2O_3$ solution. Workup was done using ethyl acetate (3×150 ml. Column chromatography was done using pet ether + ethyl acetate as eluent. white solid product was extracted at 30% ethyl acetate in pet ether solution (v/v). Yield: 1.3 g (63%).

^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 5.24 (m, 1 H), 4.45 (m, 1 H), 4.17(m, 1H), 2.95 (m, 1H), 2.95 (s, 1H), 2.56 (m, 1H), 2.04-2.14 (m, 4H).^{13}C NMR (100 MHz, $CDCl_3$) δ ppm: 174.7, 151.6, 75.6, 74.4, 72.3,63.2, 33.8, 28.3, and 27.3. FT-IR (cm^{-1}): 3774, 3660, 3252, 2958, 2114, 1703, 1477, 1440, 1395, 1365, 1341, 1324, 1291, 1220, 1189, 1149, 1058, 1023, 1007, 996, 968, 934, 880, 755, 703, 602, 570, 535, 516. HRMS (ESI+): m/z [M + H+] for $C_9H_{10}O_4$: 183.0694(Expected Mass- 182.0579).



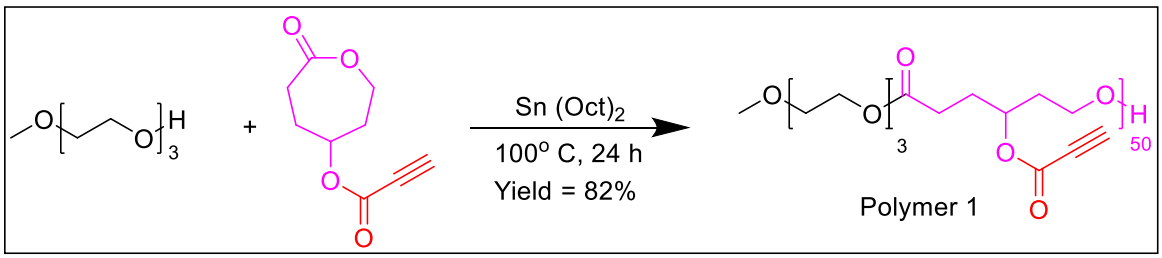
Scheme 2.3 Synthesis of compound 3

2.2.2 Synthesis of alkyne substituted polycaprolactone through ROP process:

a) Synthesis of Tri ethylene glycol monomethyl ether (TEG) initiated homopolymer (polymer 1):

The typical ring-opening polymerization through melt route was followed to make the polymer of compound 3 using Tri ethylene glycol monomethyl ether (TEG) as the initiator. In a flame-dried Schlenk tube equipped with a star-shaped magnetic bead, tri ethylene glycol monomethyl ether (TEG, 9 mg, 0.06 mmol as initiator) was weighed and. Sn(Oct)2 (11 mg, 0.03 mmol, as catalyst) was added along with compound 3 as monomer (500g, 3 mmol). The Schlenk tube was sealed with a glass cap and vacuum were applied for 40 minutes (till the pressure reaches in the order of 10^{-2} millibar), the tube was vacuum sealed properly and immersed in a pre-heated oil bath at 100°C, the reaction was continued for 24 h with constant stirring. After 24 hours polymer solution was dispersed in 400µL of HPLC grade THF and precipitated in 50 mL of 0.5:2.5, diethyl ether in hexane solution, kept in the refrigerator for 8 hours and then decanted the supernatant to collect the polymer. Any solvent remaining was removed on rota evaporator and high vacuum to obtain a brown viscous liquid product. Yield: 416mg (82%).

^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 4.62 (m, 1 H), 4.35 (m, 2 H), 3.66-3.89(m,12 H), 3.37(s, 3 H), 2.92 (s, 1H), 2.54 (m, 2H), 2.39 (m, 1H), 2.04 (m, 2H), 1.90 (m, 1H), ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm: 176.6, 152.4, 75.6, 75.3, 74.4, 62.5, 34.5, 32.7, 30.1 – 29.9, 29.9 – 28.5, 28.1. FT-IR (cm^{-1}): 3251, 2958, 2116, 1768, 1708, 1461, 1421, 1354, 1222, 1180, 1033, 969, 903, 755, 699. GPC molecular weights: M_n = 1200g/mol, M_w = 2100 g/mol, and M_w/M_n = 1.63.

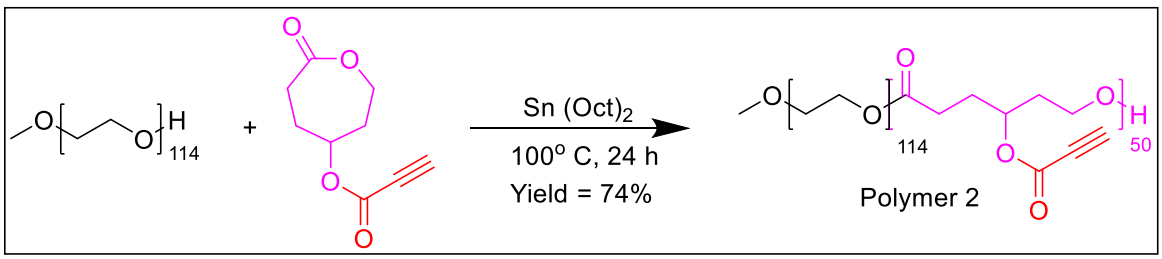


Scheme 2.4 Homo polymer (polymer 1) synthesis

b) Synthesis of PEG_{5K} initiated block-copolymer (polymer 2):

The typical ring-opening polymerization through melt route using over-head stirrer set up was followed to synthesize the polymer of compound 3 using PEG-5K as initiator. Over-head stirrer set up was used due to the viscous nature of PEG-5k. It ensures proper mechanical stirring throughout the reaction period. Polyethylene glycol monomethyl ether (PEG-5K, 384.1 mg, 0.077 mmol as initiator) was weighed and taken. Sn(Oct)2 (15.56 mg, 0.0384 mmol, as catalyst) was added and also compound 3 (700 mg, 3.84 mmol) was weighed and added to the same. Vacuum was applied for 40 minutes (till the pressure reaches in the order of 10^{-2} millibar), the tube was vacuum sealed properly, immersed in a pre-heated oil bath at 100°C and stirred. Reaction continued for 24 h. After 24 hours polymer solution was dispersed in 400µL of HPLC grade THF and precipitated in 80 mL of 0.5:2.5, diethyl ether in hexane solution, kept in the refrigerator for 8 hours and then decanted the supernatant to collect the polymer. Any solvent remaining was removed on rota evaporator and high vacuum to obtain a dark olive-green amorphous polymer. Yield: 800mg (74%).

^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 4.63 (m, 1 H), 4.36 (m, 2 H), 3.64 (s, 455-700 H), 3.37(s, 3 H), 2.92 (s, 1 H), 2.57 (m, 2 H), 2.40 (m, 1 H), 2.05 (m, 2 H), 1.94 (m, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm: 176.6, 152.5, 77.3, 77.2, 75.3, 74.4, 70.6, 62.5, 34.5, 28.7, and 28.1. FT-IR (cm^{-1}): 3517, 3216, 2912, 2873, 2113, 1771, 1713, 1460, 1353, 1232, 1180, 1150, 1099, 968, 914, 853, 805, 757, 704, 656. GPC molecular weights: M_n = 8100g/mol, M_w = 11100 g/mol, and M_w/M_n = 1.37.



Scheme 2.5 Synthesis of polymer 2

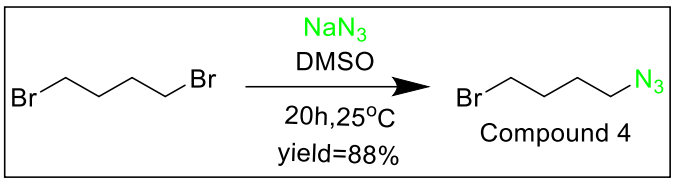
2.2.3 Synthesizing clicking units

a) Synthesis of 1-azido-4-bromobutane (Compound 4):

NaN_3 (1.2 g, 18.526 mmol) was weighed in a 100 ml round bottom flask. To this, 50 ml of DMSO was added and was sonicated to completely dissolve it. 1,4-

dibromobutane (4 g, 18.526 mmol) solution in 10 ml DMSO was added to it and stirred vigorously at 25°C for 20 hours. At the end of the reaction, 100 ml of H_2O was added to quench the reaction, allowed to cool down and extracted 3 times using 60 ml of diethyl ether (3*60), the combined organic layer was washed once with 50 ml of water (1*50), the organic layer was collected, passed over sodium sulphate and evaporated to obtain a light yellow oily liquid product. Yield: 2.91 g (88%).

^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 3.44 (dt, 2 H), 3.34 (dt, 2 H), 2.04 (p, 1H), 1.94 (p, 1 H), 1.76 (p, 1 H), 1.69 (p, 1 H). ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm: 51.0, 50.6, 32.9, 32.6, 31.0, 29.8, 27.5, 26.2. FT-IR (cm^{-1}): 2942, 2871, 2090, 1447, 1350, 1258, 893, 747, 645. HRMS (ESI+): m/z [M + H+] for $C_4H_8BrN_3$: 178.1233 (Expected Mass- 176.9902).

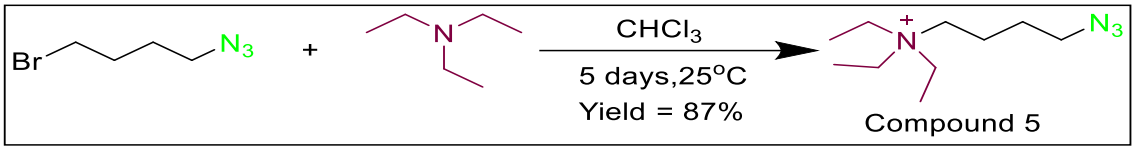


Scheme 2.6 Synthesis of compound 4

b) Synthesis of 4-azido-N, N, N-triethylbutan-1-aminium (Compound 5):

Triethylamine (956 mg, 9.45 mmol) was weighed in a 25 ml round bottom flask and 1ml HPLC grade $CHCl_3$ was added to it and stirred at 25 °C. Compound 4 (2g, 11.24 mmol) was dissolved in 2 ml of HPLC grade $CHCl_3$ and added to the reaction mixture, The reaction was stirred at 25°C for 5 days. As the reaction proceeds, the product starts getting precipitated. At the end of the reaction, chloroform was evaporated completely and washed several times using diethyl ether to remove unreacted reagents and to obtain a white solid hygroscopic product. Yield: 1.954 g (87%).

^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 3.47 (m, 10 H), 2.05 (p, 1 H), 1.93(p, 1H), 1.85 (p, 1 H), 1.74 (p, 1 H), 1.39 (m, 9 H). ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm: 57.1, 53.7,50.6, 25.8, 20.6, 19.6, 8.2. FT-IR (cm^{-1}): 3838, 3768, 3414, 2981, 2953, 2095, 1625, 1482, 1458, 1395, 1257, 1185, 1159, 1108, 1075, 1005, 939, 794, 747, 648. HRMS (ESI+): m/z [M + H+] for $C_{10}H_{23}N_4^+$: 199.1925 (Expected Mass-199.1917).

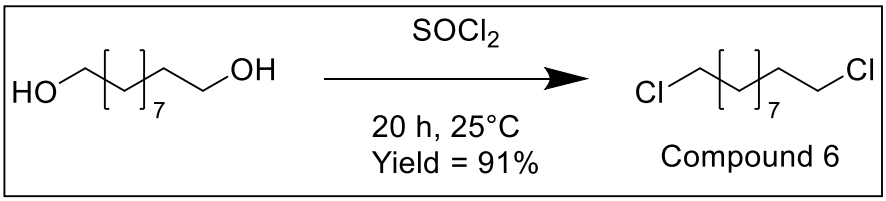


Scheme 2.7 Synthesis of compound 5

c) Synthesis of 1,10-dichlorodecane (Compound 6):

In a melt tube equipped with a magnetic bead, decane diol (6 g, 34.43 mmol) was weighed and taken. This was immersed in a pre-heated oil bath at 100°C and waited for the diol to completely melt. After the diol completely melts, thionyl chloride (12.3 g, 103.3 mmol) was added to the reaction mixture dropwise and was stirred at 100°C for 20 hours under nitrogen atmosphere. At the end of the reaction work up was done using ethyl acetate and H_2O, the combined organic layer was washed several times with water to completely remove thionyl chloride, organic layer passed over anhy. sodium sulphate and evaporated on rota to obtain brown liquid product. Yield = 6.6 g (91%).

^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 3.53 (t, 4H), 1.77 (p, 4H), 1.42 (p, 4H), 1.30 (s, 8H). FT-IR (cm−1): 3333, 2923, 2854, 1736, 1460, 1376, 1284, 1188, 1054, 970, 821, 723, 652.

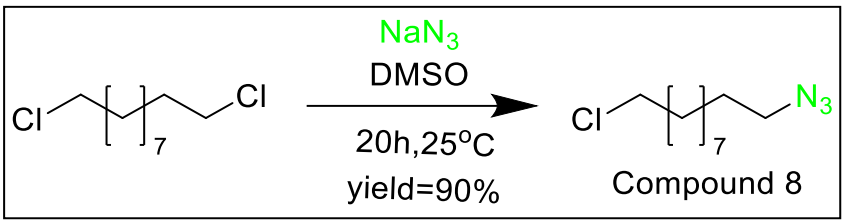


Scheme 2.8 Synthesis of compound 6

d) Synthesis of 1-azido-10-chlorodecane (Compound 7):

NaN_3 (923mg, 14.21 mmol) was weighed in a 100 ml RB and 30 ml of DMSO was added to it, sonicated to completely dissolve it. 1,10-dibromodecane (3 g, 14.21 mmol) dissolved in 5 ml DMSO was added to the same and stirred vigorously at 25°C for 20 hours. At the end of the reaction, 100 ml of H_2O was added to quench the reaction, allowed to cool down and extracted 3 times using 60 ml of diethyl ether (3*60), the combined organic layer was washed once with 50 ml of water (1*50), the organic layer was collected, passed over sodium sulphate and evaporated to obtain a light brown oily liquid product. Yield: 2.8 g (90%).

^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 3.52 (s, 2H), 3.25 (s, 2H), 1.76 (s, 2H), 1.59 (s, 2H), 1.42-1.29 (m, 12 H).^{13}C NMR (100 MHz, $CDCl_3$) δ ppm: 63.1, 62.4, 51.5, 45.2, 32.8, 29.5, 29.2, 28.9, 26.8, 25.8. FT-IR (cm^{-1}): 3333, 2926, 2855, 2091, 1736, 1459, 1349, 1257, 1051, 895, 723, 650.



Scheme 2.9 Synthesis of compound 7

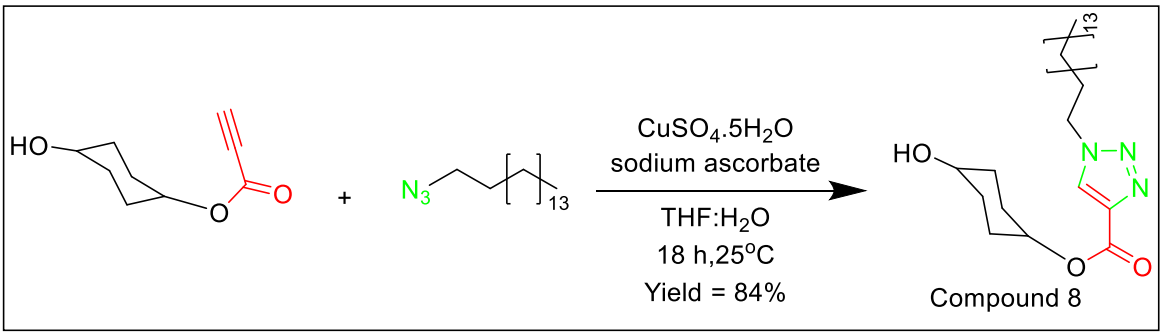
2.2.4 Optimising click chemistry:

2.2.4.1 Click chemistry in small molecules – conjugating hexadecane azide and 4-hydroxycyclohexyl propiolate (compound 8):

Compound 1 (100 mg, 0.5945 mmol) was weighed and taken in a melt tube equipped with a star magnetic bead. 1 ml of HPLC grade THF was added and sonicated to solubilize it completely. Hexadecane diol (191 mg, 0.7134 mmol) was weighed and taken in a pear-shaped round bottom flask, solubilized in 1 ml of HPLC grade THF, added to the reaction mixture and stirred at room temperature. In the meantime, copper sulphate pentahydrate (75 mg, 0.3 mmol) solution and sodium ascorbate (176.6 mg, 0.9 mmol) solution in 750 microlitres of H_2O each were freshly prepared and added to the reaction mixture one after the other. The reaction was continued for 48 hours. After the completion of the reaction, THF was evaporated completely and then work up was done using DCM and water. A pinch of EDTA was added to chelate with copper which facilitates the complete removal of copper from the organic layer. The organic layer was collected and passed over Na_2SO_4 and evaporated on rota evaporator to obtain the crude product. From TLC and ^{1}H NMR of crude product presence of unreacted azide was found. Column chromatography was done using methanol and DCM as eluent to remove unreacted azide and the product was eluted at 3% methanol in DCM system(v/v). Yield: 218 mg (84%).

^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 8.04 (s, 1 H), 5.04-5.16 (m, 1 H), 4.40 (t, 2 H), 3.76–3.85 (m, 1 H), 2.14 (m, 1 H), 2.04 (m, 2 H), 1.93 (m, 2 H), 1.78 (m, 4 H), 1.47 (m, 2 H),

1.25 (m, 25 H), 0.87 (t, 3 H). ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm:160.4, 140.5, 127.3, 72.9, 71.2, 69.1, 67.6, 50.8, 32.0, 30.3, 29.8, 29.6, 29.4, 29.1, 14.2. FT-IR (cm^{-1}): 3405, 3119, 2954, 2916, 2849, 1723, 1528, 1467, 1366, 1208, 1118, 1070, 1049, 968, 937, 884, 782, 720. HRMS (ESI+): m/z [M + H+] for $C_{25}H_{45}N_3O_3$: 436.3544 (Expected Mass-435.3461).



Scheme 2.10 Synthesis of compound 8

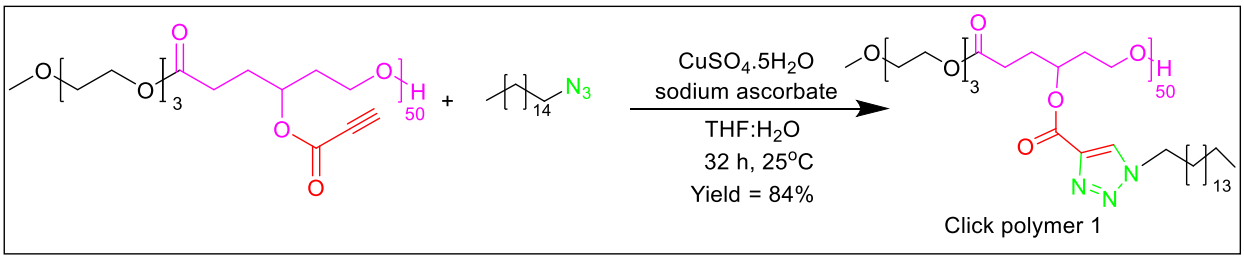
2.2.4.2 Applying click chemistry in polymers

a) Conjugating hexadecane azide to polymer 1 (Click polymer 1):

Polymer 1 (100 mg, 0.549 mmol) was weighed and taken in a melt tube equipped with a star magnetic bead. 1.5 ml of HPLC grade THF was added and sonicated to solubilize it completely. Hexadecane azide (176.24 mg, 0.659 mmol) was weighed and taken in a pear-shaped round bottom flask, solubilized in 1.5 ml of HPLC grade THF, added to the reaction mixture and stirred at room temperature. In the meantime, copper sulphate pentahydrate (68.5 mg, 0.274 mmol) solution and sodium ascorbate (162.84 mg, 0.822 mmol) solution in 750 microlitres of H_2O each were freshly prepared and added to the reaction mixture one after the other. The reaction was stirred at room temperature for 48 hours. After the completion of the reaction, THF was evaporated completely and then worked up was done using DCM and water. A pinch of EDTA was added to chelate with copper which facilitates the complete removal of copper from the organic layer. The aqueous layer was extracted three times with DCM and the combined organic layer was washed once with H_2O. The organic layer was collected passed over Na_2SO_4 and evaporated on rota evaporator to obtain the crude product which was then dissolved in 400 microlitres of HPLC grade THF and precipitated with stirring at room temperature in 30 ml of HPLC grade hexane. Stirred for 10 minutes, then packed properly and kept undisturbed for overnight. The next day product was filtered out using Buchner filter paper and washed with hexane to remove any free azide present. The product obtained (off

white solid) was air-dried, transferred to a 10 ml RB and dried on rota at 60°C for 30 minutes and submitted for NMR. Yield: 208 mg (84%).

^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 8.09 (s, 1 H), 4.62–4.72 (m, 1 H), 4.26,4.51 (m, 2H), 4.40 (t, 2 H), 3.75–3.91 (m, 12 H), 3.36 (s, 3 H), 2.56 (m, 2 H), 2.44 (m, 1 H), 2.17 (m, 2 H), 1.92 (m, 3 H), 1.24 (m, 26 H), 0.87 (t, 3 H). ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm: 176.9, 160.8, 127.6, 77.2, 61.6, 50.9, 34.8, 32.1, 30.3, 30.1 – 29.5, 29.1, 28.8, 28.2, 26.5, 22.8, 14.2. FT-IR (cm^{-1}): 3116, 2955, 2917, 2848, 1773, 1721, 1528, 1464, 1365, 1218, 1186, 1123, 1050, 967, 910, 779, 722, 654. GPC molecular weights: M_n = 2900 g/mol, M_w = 3900 g/mol, and M_w/M_n = 1.3.



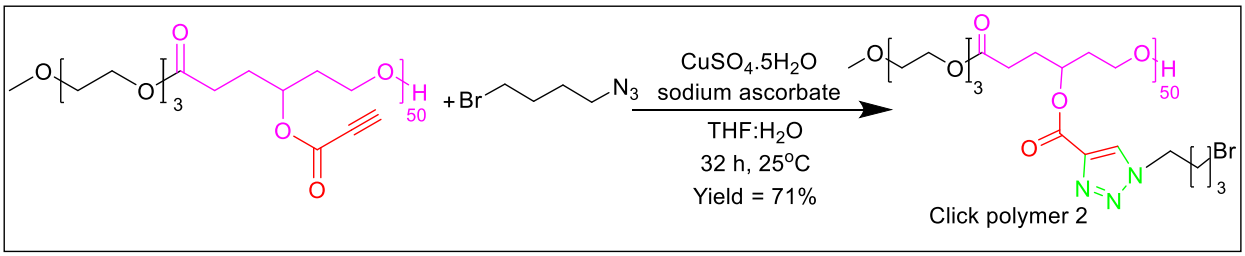
Scheme 2.11 Synthesis of click polymer 1

b) Conjugating 1-azido-4-bromobutane (Compound 4) to polymer 1 (click polymer 2):

Polymer 1 (100 mg, 0.549 mmol) was weighed and taken in a melt tube equipped with a star magnetic bead. 1.5 ml of HPLC grade THF was added and sonicated to solubilize it completely. 1-azido-4-bromobutane (Compound 4) (117.24 mg, 0.659 mmol) was weighed and taken in a pear-shaped round bottom flask, solubilized in 1.5 ml of HPLC grade THF, added to the reaction mixture and stirred at room temperature. In the meantime, copper sulphate pentahydrate (68.5 mg, 0.274 mmol) solution and sodium ascorbate (163mg, 0.824 mmol) solution in 750 microlitres of H_2O each were freshly prepared and added to the reaction mixture one after the other. The reaction was stirred at room temperature for 48 hours. After the completion of the reaction, THF was evaporated completely and then work up was done using DCM and water. A pinch of EDTA was added to chelate with copper which facilitates the complete removal of copper from the organic layer. The aqueous layer was extracted three times with DCM and the combined organic layer was washed once with H_2O. The organic layer was collected passed over Na_2SO_4 and evaporated on rota evaporator to obtain the crude product which was then dissolved in 400 microlitres of HPLC grade $CHCl_3$ and precipitated with stirring

at 0°C in 40 ml of HPLC grade diethyl ether in hexane solution (17:93), Stirred for 10 minutes, then packed properly and kept undisturbed in the refrigerator for 8 hours. The next day supernatant was decanted out to collect the product (light brown sticky solid), dried on rota evaporator at 60°C for 30 minutes and submitted for NMR. Yield: 140 mg (71%).

^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 8.13 (s, 1 H), 4.72 (p, 1 H), 4.53 (m, 2H), 4.47 (t, 2 H), 3.74,3.43,3.36(m, 5H), 3.64 (m, 12 H), 2.57 (m, 2 H), 2.43 (m, 1 H), 2.16 (m, 2 H), 1.85-2.05 (m, 5H). ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm:176.9, 160.6, 127.8, 77.7, 61.6, 50.7, 50.3, 49.9, 49.8, 34.8, 32.3, 29.2, 28.8, 27.6, 27.0, and 0.1. FT-IR (cm^{-1}): 3514, 3135, 2956, 2099, 1769, 1726, 1540, 1459 ,1357, 1182, 1118, 1043, 966, 908, 776, 698, 652.



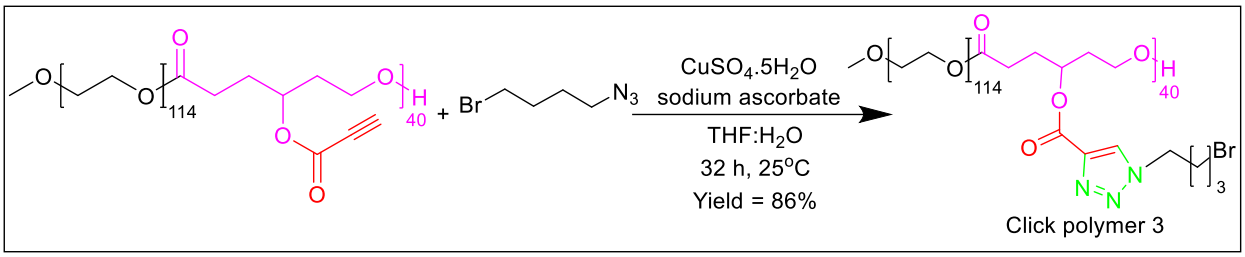
Scheme 2.12 Synthesis of click polymer 2

c) Conjugating 1-azido-4-bromobutane (Compound 4) to polymer 2 (click polymer 3):

Polymer 2 (100 mg, 0.353 mmol, active mass =64.28 mg) was weighed and taken in a melt tube equipped with a star magnetic bead. 1.5 ml of HPLC grade THF was added and sonicated and heated in the water bath at 60°C to solubilize it completely. 1-azido-4-bromobutane (Compound 4) (94.21 mg, 0.529 mmol) was weighed and taken in a pear-shaped round bottom flask, solubilized in 1.5 ml of HPLC grade THF, added to the reaction mixture and stirred at room temperature. In the meantime, copper sulphate pentahydrate (132.2 mg, 0.529 mmol) solution and sodium ascorbate (140mg, 0.706 mmol) solution in 750 microlitres of H_2O each were freshly prepared and added to the reaction mixture one after the other. The reaction was stirred at room temperature for 48 hours. After the completion of the reaction, THF was evaporated completely and then work up was done using DCM and water. A pinch of EDTA was added to chelate with copper which facilitates the complete removal of copper from the organic layer. The aqueous layer was extracted three times with DCM and the combined organic layer was washed once with H_2O. The organic layer was collected passed over Na_2SO_4 and evaporated on rota evaporator to obtain the crude product which was then dissolved in

400 microlitres of HPLC grade THF and precipitated with stirring at 0°C in 40 ml of HPLC grade diethyl ether in hexane solution (17:93), Stirred for 10 minutes, then packed properly and kept undisturbed in the refrigerator for 8 hours. The next day supernatant was decanted out to collect the product (light olive-green amorphous polymer), dried on rota evaporator at 60°C for 30 minutes and submitted for NMR. Yield: 140 mg (86%).

^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 8.13 (s, 1 H), 4.72 (p, 1 H), 4.53 (m, 2H), 4.47 (t, 2 H), 3.74,3.43,3.38(m, 5H), 3.64 (m, PEG protons), 2.57 (m, 2 H), 2.43 (m, 1 H), 2.16 (m, 2 H), 1.85-2.05 (m, 5H). FT-IR (cm^{-1}): 3490, 3131, 2869, 2098, 1770, 1726, 1539, 1458, 1353, 1179, 1097, 1041, 959, 910, 853, 807, 777, 652. GPC molecular weights: M_n = 4900 g/mol, M_w = 6900 g/mol, and M_w/M_n =1.4.



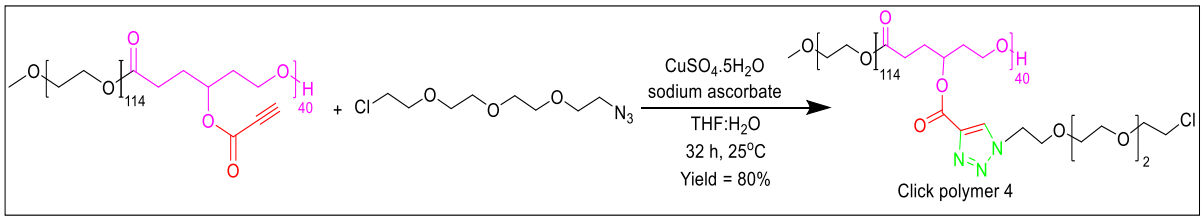
Scheme 2.13 Synthesis of click polymer 3

d) Conjugating 1-azido-2-(2-(2-(2-chloroethoxy)ethoxy)ethoxy)ethane to polymer 2 (click polymer 4):

Polymer 2 (100 mg, 0.353 mmol, active mass =64.28 mg) was weighed and taken in a melt tube equipped with a star magnetic bead. 1.5 ml of HPLC grade THF was added and sonicated and heated in the water bath at 60°C to solubilize it completely. 1-azido-2-(2-(2-(2-chloroethoxy)ethoxy)ethoxy)ethane(100.4 mg, 0.424 mmol) was weighed and taken in a pear-shaped round bottom flask, solubilized in 1.5 ml of HPLC grade THF, added to the reaction mixture and stirred at room temperature. In the meantime, copper sulphate pentahydrate (132.2 mg, 0.529 mmol) solution and sodium ascorbate (140mg, 0.706 mmol) solution in 750 microlitres of H_2O each were freshly prepared and added to the reaction mixture one after the other. The reaction was stirred at room temperature for 48 hours. After the completion of the reaction, THF was evaporated completely and then work up was done using DCM and water. A pinch of EDTA was added to chelate with copper which facilitates the complete removal of copper from the organic layer. The aqueous layer was extracted three times with DCM and the combined organic layer was washed once with H_2O.The organic layer was collected passed over Na_2SO_4 and

evaporated on rota evaporator to obtain the crude product which was then dissolved in 400 microlitres of HPLC grade THF and precipitated with stirring at 0°C in 40 ml of HPLC grade diethyl ether in hexane solution (17:93), Stirred for 10 minutes, then packed properly and kept undisturbed in the refrigerator for 8 hours. The next day supernatant was decanted out to collect the product, dried on rota evaporator at 60°C for 30 minutes and submitted for NMR. Yield: 80%

^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 8.37 (s, 1 H), 4.71 (p, 1 H), 4.61 (m, 2H), 4.49 (t, 2 H), 3.90,3.76,3.38(m, 5H), 3.64 (m, PEG and TEG protons), 2.57 (m, 2 H), 2.43 (m, 1 H), 2.17 (m, 2 H), 1.94 (m, 5H). FT-IR (cm^{-1}): 3496, 3132, 2916, 2870, 2104, 1771, 1726, 1545, 1459, 1353, 1174, 1100 ,1041, 962, 911, 848, 776, 731, 654.



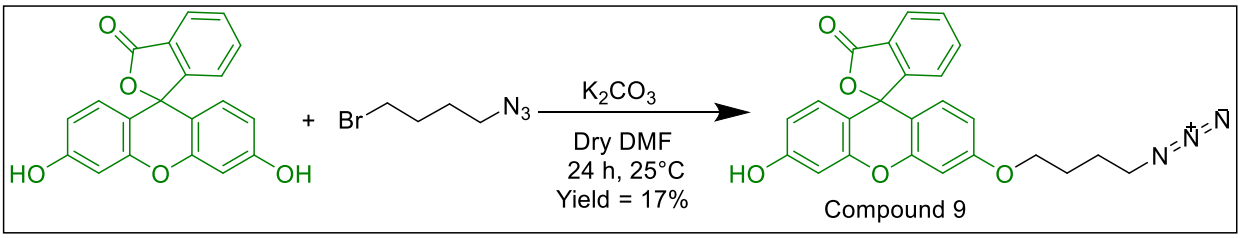
Scheme 2.14 Synthesis of click polymer 4

2.2.5 Fluorescein azide synthesis:

Conjugating 1-azido-4-bromobutane (Compound 4) to fluorescein (compound 9):

In to a two necked round bottom flask equipped with a magnetic bead, fluorescein (2 g, 6.02 mmol) was weighed and added. Completely dried K_2CO_3 (8.3 g, 60.18 mmol) was also weighed and added to the reaction mixture. 20 ml of dry DMF was added using needle and syringe set up and the reaction mixture was stirred at 25°C for 45 minutes under nitrogen atmosphere. After 45 minutes, compound 4 (1.1 g, 6.02 mmol) was weighed and dissolved in 10 ml dry DMF, added dropwise to the reaction mixture at ice cold condition. The reaction was continued to stir for 48 hours at 25°C. After the completion of the reaction DMF was completely removed using vacuum distillation and then an acid work up was done using 100 ml of 0.1 N HCL, ethyl acetate and water. Aqueous fraction was extracted thrice using 100 ml of ethyl acetate (3*100) and the combined organic layer was washed once with water, passed over anhydrous sodium sulphate and dried on rota evaporator to obtain the crude product. Pure product was separated and collected using column chromatography in ethyl acetate/ pet ether system (v/v). Product (orange sticky solid) was eluted out in 23% ethyl acetate in pet ether (v/v). Yield = 528 mg (17 %).

^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 8.01 (d, 1H), 7.67 (m, 2H), 7.15 (d, 1H), 6.60-6.73(m, 6 H), 4.02 (t, 2H), 3.50 (s, 1H), 3.37(t, 3H), 1.81-1.87(m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm: 162.9, 160.6, 152.6, 135.1, 129.7, 129.2, 125.1, 124.1, 112.5, 111.9, 111.3, 103.1, 101.4, 67.6, 51.2, 36.7, 31.7, 29.8, 26.4, 25.8. FT-IR (cm^{-1}): 3327, 2924, 2096, 1729, 1609, 1503, 1461, 1429, 1342 ,1283, 1248, 1178, 1109, 1007, 847, 792, 759, 733, 692 ,628. HRMS (ESI+): m/z [M + H+] for $C_{25}H_{45}N_3O_3$: 430.1404 (Expected Mass-429.1325).

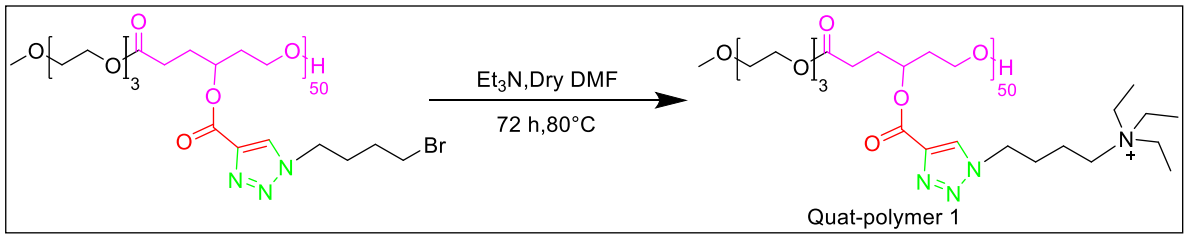


Scheme 2.15 Synthesis of fluorophore conjugate

2.2.6 Quaternisation of clicked polymers:

a) Quaternization of click polymer 2 (quat-polymer 1):

In to a melt tube equipped with a magnetic bead, polymer 4(50 mg, 0.1388 mmol) dissolved in 0.5 ml of dry DMF was added, dry conditions were maintained and stirred with nitrogen purging at room temperature for 10 minutes. After 10 minutes triethyl amine (0.193 ml, 1.388 mmol, dry) was added to the reaction mixture and immersed the melt tube in an oil bath maintained at 80°C. The reaction mixture was continued stirring for 72 hours under dry conditions. Polymer was precipitated in diethyl ether in hexane (20:80) system and kept undisturbed in the refrigerator for 8 hours, decanted to remove the supernatant and dried on rota evaporator at 60°C for an hour and submitted for NMR.
^{1}H NMR (400 MHz, $CDCl_3$): 8.68 (s, 1H), 8.16 (s, 1H), 4.72 (m, 1H), 4.50 (m, 4H), 3.39-3.83(m, 17H), 3.12 (q, 6H), 2.55 (m, 2H), 2.42 (m, 1H), 2.16 (m, 2H), 1.99 (m, 5H), 1.43 (t, 9H). FT-IR (cm^{-1}): 3439, 3134, 2956, 2099, 1767, 1726, 1541, 1459, 1390, 1358, 1181, 1116, 1042, 966, 909, 802, 777, 730, 699, 651.

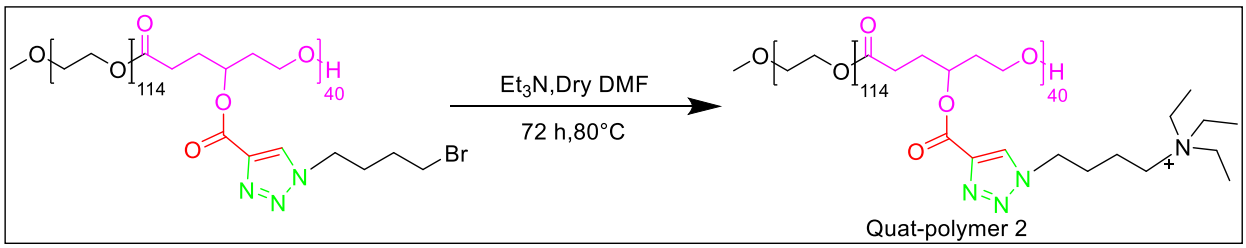


Scheme 2.16 Quaternization of click polymer 2

b) Quaternization of click polymer 3 (quat-polymer 2):

In to a melt tube equipped with a magnetic bead, polymer 4(76 mg, 0.1616 mmol) dissolved in 1 ml of dry DMF was added, dry conditions were maintained and stirred with nitrogen purging at room temperature for 10 minutes. After 10 minutes triethyl amine (0.167 ml, 1.198 mmol, dry) was added to the reaction mixture and immersed the melt tube in an oil bath maintained at 80˚C. The reaction mixture was continued stirring for 72 hours under dry conditions. Precipitation was done in diethyl ether in hexane (20:80) system and kept undisturbed in the refrigerator for 8 hours, decanted to remove the supernatant and dried on rota evaporator at 60°C for an hour and submitted for NMR.

^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 8.58 (s, 1H), 8.12 (s, 1H), 4.72 (m, 1H), 4.52 (m, 4H), 3.38-3.83(m, 5H), 3.14 (q, 6H), 2.56 (m, 2H), 2.41 (m, 1H), 2.16 (m, 2H), 1.99 (m, 5H), 1.41 (t, 9H). FT-IR (cm^{-1}): 3441, 3128, 2917, 2871, 2098, 1770, 1728 ,1667, 1542, 1459, 1355, 1181, 1101, 1044, 960, 851, 778, 732, 654.



Scheme 2.17 Quaternization of click polymer 3

2.2.7 Determination of size and surface charge of self-assemblized polymers:

2.2.7.1 Self-assembly: In order to self-assemblies the quaternized polymers, a homogeneous solution was prepared in DMSO. For that, 5 mg of polymer (homo and block) were dissolved in 0.5 mL of DMSO and stirred for one hour. Then, 4.5 mL water was added to the solution in dropwise manner and resulting solution was left to stir at room temperature for an hour. Then, they were transferred to 3.5kD dialysis bags and dialysed against water for 24 h.

2.2.7.2 *Size and surface charge:* 100 µL of 1mg/mL polymeric solution in water was diluted in 900 µL of water and hydrodynamic size and zeta potential was determined. It is an important parameter for characterisation of polymeric nano-assemblies.

2.2.8 Inhibition of bacteria (MIC)

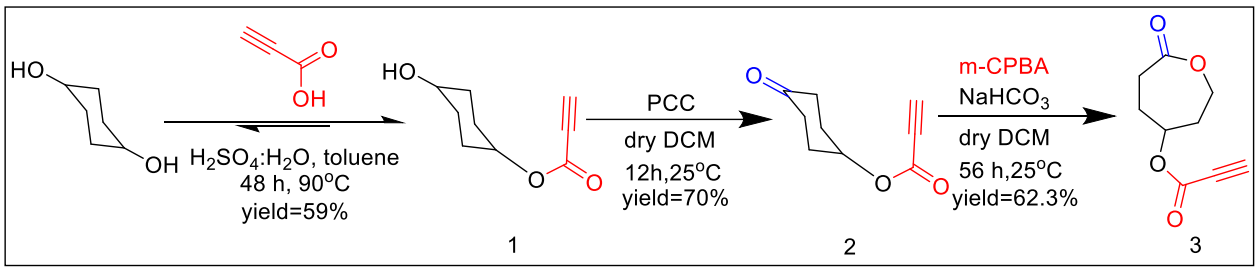
To check the antimicrobial action of the polymers, MIC was determined using colony suspension method as mentioned in our previous report [25] with the help of Ruma (senior member of our group). The optical density was determined using PerkinElmer EnSight™ multimode plate reader. Temperature was maintained at 37 °C and excitation wavelength was 600 nm. 15 flashes were given for one measurement with orbital shaking. Number of measurements were 216 with each measurement starting after 5 minutes.

3. Results and Discussions

3.1 Polymer synthesis and characterization

3.1.1 Monomer Synthesis:

A new alkyne substituted caprolactone monomer (compound 3) was synthesized from cyclohexane diol and propiolic acid in 3 steps as shown in scheme 3.1. Synthesis of compound 1 was interfacial reaction as the diol was soluble in water and the propiolic acid was soluble in toluene. Coupling reaction occurs at the interface of water and toluene (two immiscible liquids). The reaction yields two products, the mono and di substituted molecules, and the desired product is separated through column chromatography. The monosubstituted product was then oxidized using pyridinium chlorochromate (PCC) in dry DCM to obtain compound 2 which was then oxidised to alkyne-substituted ε-caprolactone monomer (compound 3) using m-CPBA. Column chromatography technique was used for purification of the products in each step. Characterisation was done using ^{1}H NMR, ^{13}C NMR, HRMS and FTIR.



Scheme 3.1

Synthetic scheme for Monomer

Figure 3.1 shows the ^{1}H-NMR spectra of the intermediate 1, 2 and monomer (3). The formation of compound 1 was confirmed by the downfield shifting of proton ‘a’ and the appearance of new peak at 2.86 ppm (proton-c). Also, by comparing the ratio of peaks corresponding to proton ‘c’ and proton ‘a’ we can confirm the product formation. Formation of compound 2 was confirmed by the vanishing of the proton peak at 3.76 ppm corresponding to proton ‘c’. The appearance of 2 new peaks (4.46 ppm, 4.18 ppm) corresponding to the down fielded e$^{'}$ and e$^{''}$ protons (each peak integrating to one, diastereotopic splitting) confirms the formation of compound 3. The alkyne substituted caprolactone monomer was a white solid compound which was then polymerized using various initiators to obtain alkyne substituted homo and block-copolymers of caprolactone.

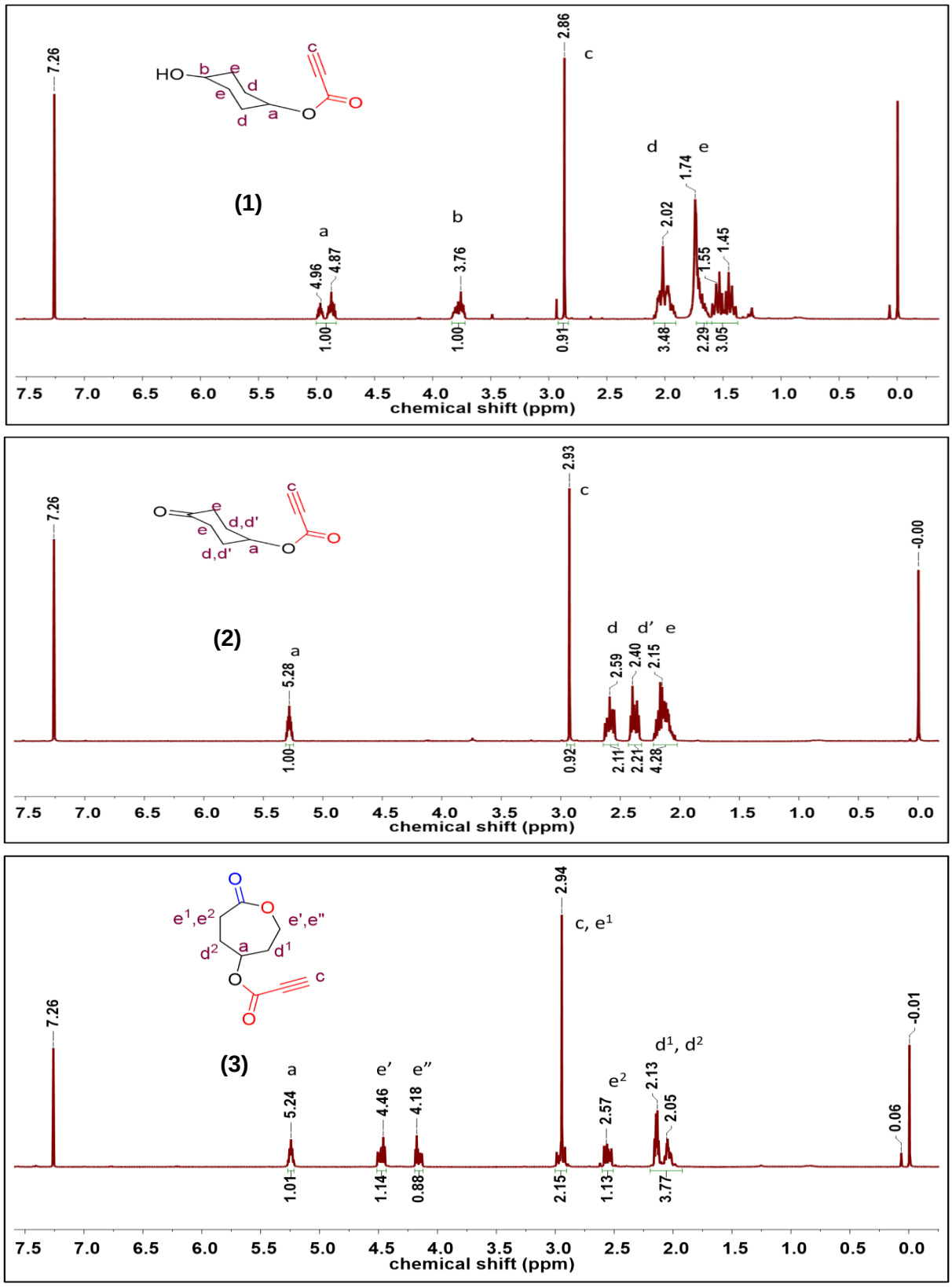


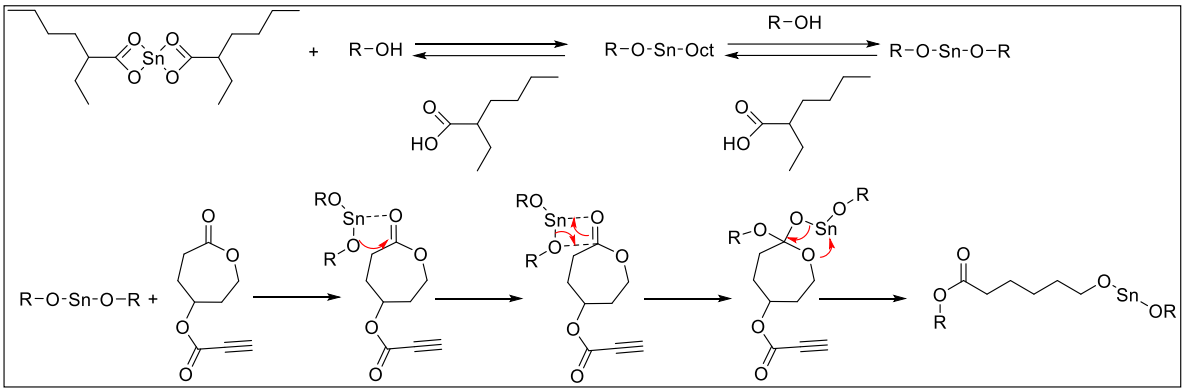
Figure 3.1. ^{1}H NMR compound 1,2 and 3

3.1.2 Homo Polymer synthesis and characterisation:

The alkyne substituted caprolactone monomer (3) underwent Ring opening polymerisation with the initiator Tri ethylene glycol mono methyl ether (TEG) following the melt route (solvent free polymerization) at 100°C for 24 hours (scheme 3.2). Temperature for the polymerization was optimised based on the degradation temperature of monomer which was found from thermo-galvanometric analysis (TGA). The catalyst used was Tin(II) 2-ethylhexanoate. The reaction goes through a metal mediated coordination insertion mechanism. ROP being a controlled polymerization technique, we were able to control the degree of polymerization by varying the monomer to initiator ratio.

$$X_n = P \times M/I$$

So, assuming the P (extend of reaction) to be 100% we can vary the monomer to initiator ratio (M = 1 equivalents, I =1/ 50 equivalents) such that we can get a desired degree of polymerization (X_n = 50). Since one catalyst molecule could activate 2 initiators, only half the quantity of initiator taken was used (1/100 equivalents).



Scheme 3.2: ROP mechanism of Sn(Oct)$_2$ catalyst

^{1}H NMR spectra of the polymer in $CDCl_3$ is shown in the figure 3.2. The peak appearing at 3.37 ppm which corresponds to the 3 protons in -O-CH3 group of initiator group was used as reference to interpret the integrals in NMR. By comparing the ratio of this end group to any other peak corresponding to a particular species of proton in the polymer we can find the number averaged molecular weight (M_n) or number averaged degree of polymerization (DP_n). Let's consider the peak at 4.62 ppm, it corresponds to the proton 'a' (single proton), We can see that giving an integration value of 3 to the peak 'f' results in an integration of around 50 for the peak 'a', that means our number averaged degree of polymerization is 50. We can confirm this by comparing the ratios of other peaks as well. Vanishing of the monomer peak

at 5.24 ppm in the polymer ^{1}H NMR spectra confirms the polymerization. Other peaks corresponding to monomers were unchanged suggesting that other functional groups were stable under melt polymerization. The other initiator peaks of TEG in polymer 1 could be found between 3.38-3.89 ppm which integrates to give 12 protons. We can also see the diastereotopic splitting of 2 protons (up field) in the polymer NMR spectra. This corresponds to the d_2 and d_2' protons of the monomer back bone.

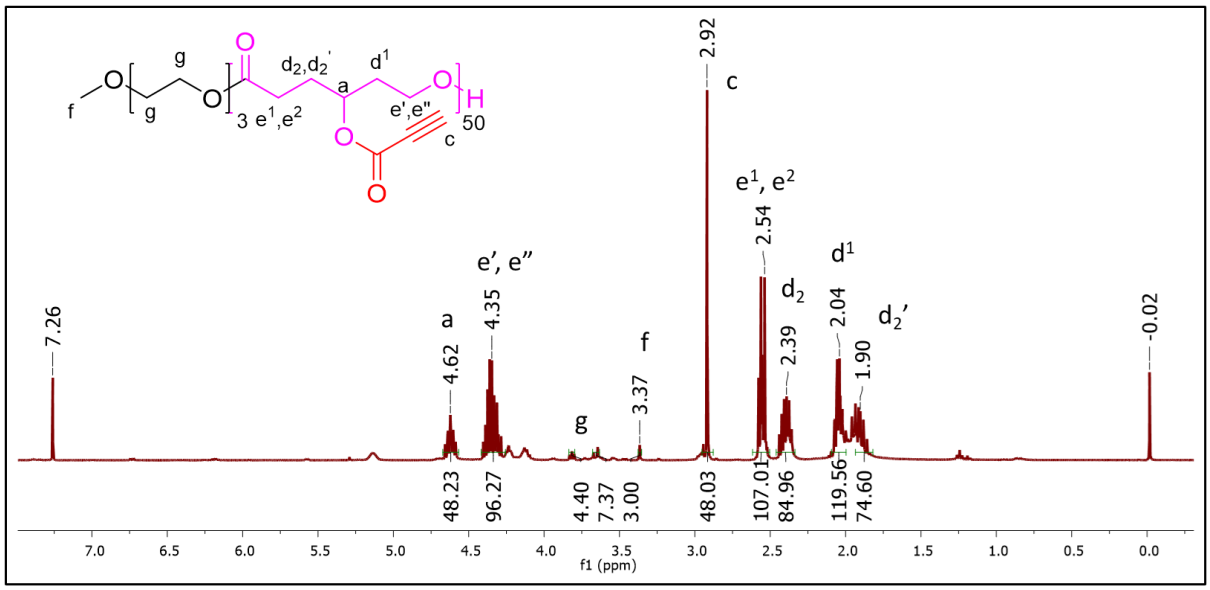


Figure 3.2. ^{1}H NMR of polymer 1

3.1.3 Block co-polymer synthesis and characterisation:

The synthesis of block copolymer was done using the overhead stirrer set up through melt route. Mechanical stirring was required due to the viscous nature of PEG $_{5K}$. For that we had to use the overhead stirrer set up. PEG $_{5k}$ was used as the initiator to ring open the alkyne substituted monomer and the reaction scheme follows the same as in the case of homo polymer (scheme 3.2). Stirring was maintained at 130 rotations per minute. Temperature was maintained at 100°C and reaction was continued for 24 hours. As similar to how we found the degree of polymerization in homo polymer, here also we used the methyl end group protons (3 H) at 3.37 ppm as reference and compared its ratio to the polymer peak at 4.65 ppm which corresponds to 'a' proton, it gave an integration of 43 showing the formation of 40 units of polymer. Ratio of integral values of peaks corresponding to e' and e'' protons to the integral value of proton 'a' was 2, which again confirmed the DP value to be 40. This shows that number of repeating units were 40. Similarly, all other peaks were assigned and ratio of integrals was compared to that of peak

corresponding to proton ‘a’ to confirm the formation of 40-unit block co-polymer.

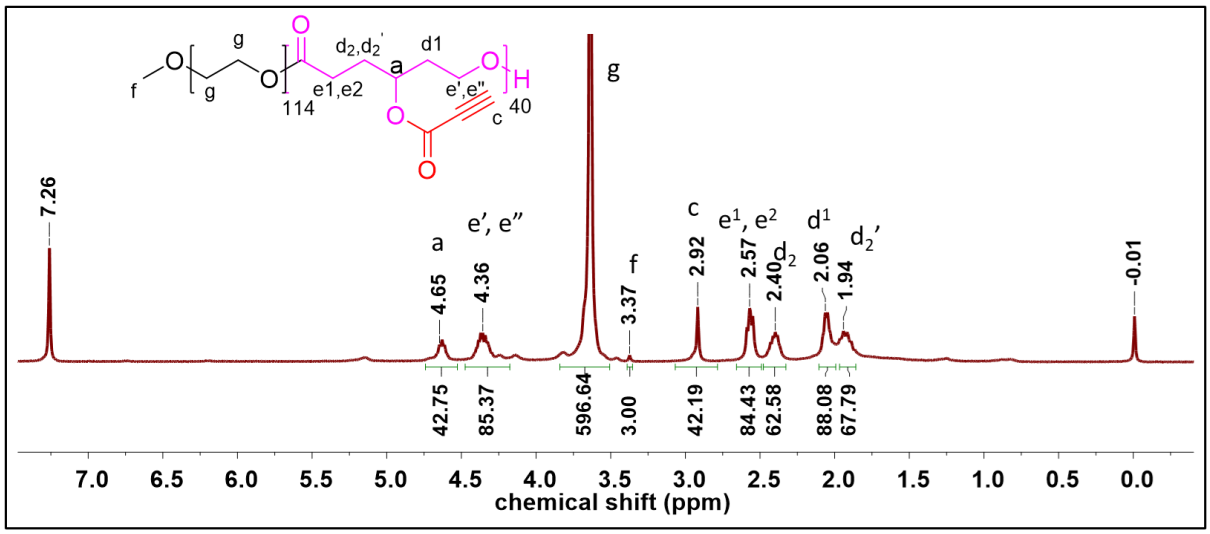


Figure 3.3. ^{1}H NMR of polymer 2

3.1.4 Molecular weight determination of polymers using gel permeation chromatography (GPC):

Gel permeation chromatography, a type of size exclusion chromatography is a powerful characterization technique which provides molecular weight of the polymers along with their polydispersity index as shown in table 3.1. Chloroform was used as the solvent. In gel permeation chromatography the polymer chains depending on their molecular weights moves through the column and the higher molecular weight polymers elute faster as they do not pass through the polystyrene beads resulting in a lower retention time compared to the lower molecular weight polymers.

SI NO	Polymer	NMR			GPC		
		DP_n		M_n	M_n	M_w	PDI
		Feed	Incorporated	(g/mol)	(g/mol)	(g/mol)	
1	Homo polymer	50	50	9000	1200	2100	1.63
2	Block polymer	50	40	12200	8100	11100	1.37

Table 3.1

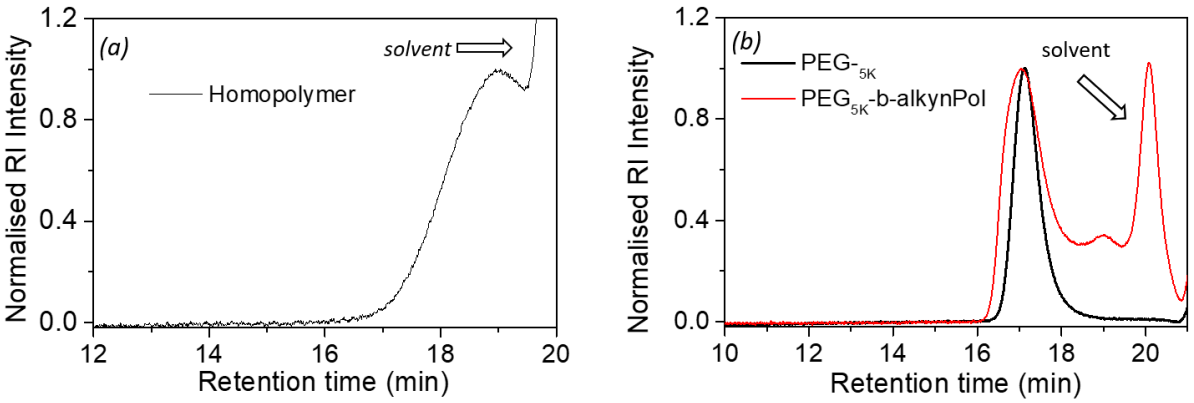


Figure 3.4 GPC chromatograms of (a) homo polymer and (b) PEG-5k Macroinitiator and PEG5k-block copolymer

The block co-polymer has a retention volume of 18.967 whereas the homo polymer retention time is 17.05. The theoretical molecular weights of the polymers were calculated from ^{1}H NMR as 9000 and 12200 g/mol. Comparing the molecular weights obtained from GPC to the calculated molecular weights of polymers, we could see that the M_n and M_w determined through GPC were highly under estimated. This is because the standardization was done using polystyrene, which is structurally very much different from alkyne substituted poly caprolactone polymers. GPC gives a relative molecular weight of polymers, in order to find the absolute molecular weight of polymers we need to follow the triple detector absolute molecular weight determination method using RALS, LALS and RI signals of the polymers.

3.1.5 Determination of absolute molecular weights of polymers using triple detector:

From GPC using polystyrene standards we could see that the molecular weight of the polymers was highly under estimated. In order to find the absolute molecular weight of the polymer triple detector molecular weight determination method was utilized. Triple detection is a technique in which three different detectors are used simultaneously to analyse a sample in a single sample run. The combined results will give more accurate value for the molecular weight of polymers. The three detectors used in this experiment were the RALS (right angle light scattering), LALS (low-angle light scattering and RI (refractive index). A RALS signal provide information about the intensity of light scattered at 90^{o} to the incident beam. The molecular weight is then calculated directly from the intensity measured and the sample concentration. The RALS detector offers the best sensitivity for small

molecules. LALS detector measures the intensity of light scattered at an angle that is as close to 0°, the intensity will be very close to the intensity at zero degree and so it gives an advantage of calculated molecular weight with a good match with actual molecular weight of the polymer. These detector offers best sensitivity for larger molecules. The RI detector which is sensitive to the changes in concentration of sample measures the change in refractive index as the sample flows through the column. The first step of triple detection method was the determination of dn/dc value of polyethylene glycol in chloroform.

Determination of dn/dc of PEG-5k:

For this the RALS, LALS and RI signals of three different concentrations of PEG-5k (2,4 and 8 mg/ml) were recorded. PEG-99k was used as the standard and the dn/dc value of poly ethylene glycol in chloroform was determined to be *0.047889*.

Determination of absolute molecular weight of block copolymer:

The dn/dc values of PEG-5k and polycaprolactone in chloroform was utilized for the absolute molecular weight determination of the block copolymer. The following method was used.

*dn/dc of block copolymer = (weight fraction of PEG-5k * dn/dc of PEG-5k) + (weight fraction of PCL * dn/dc of PCL)*

*dn/dc of block copolymer = (0.357*0.047889) + (0.642 * 0.1250) = 0.09735*

This dn/dc value was used in the calibration plot to obtain the absolute molecular weight of block copolymer as 12700(Mn), 19000 (Mw) and polydispersity index as 1.5. The molecular weight obtained were similar to Mn obtained from NMR.

3.1.6 Thermal stability of monomer and polymers:

Thermal stability of the monomer and polymers were analysed using Thermogravimetric analysis (TGA). Temperature was increased at a rate of 10°C per minute from 30 °C to 600 °C under nitrogen atmosphere.

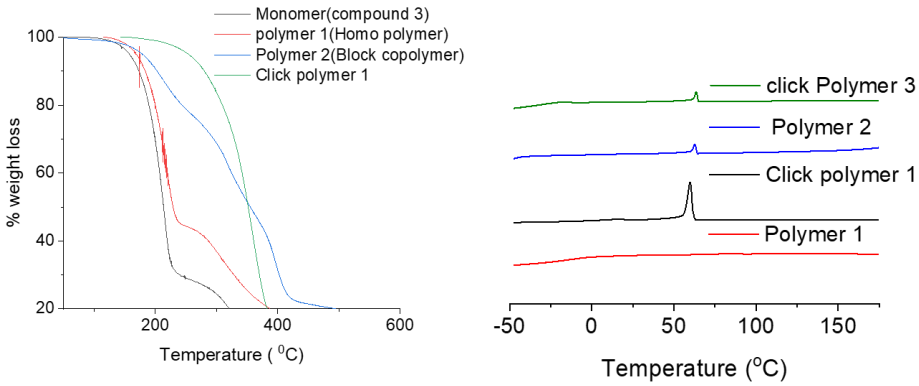
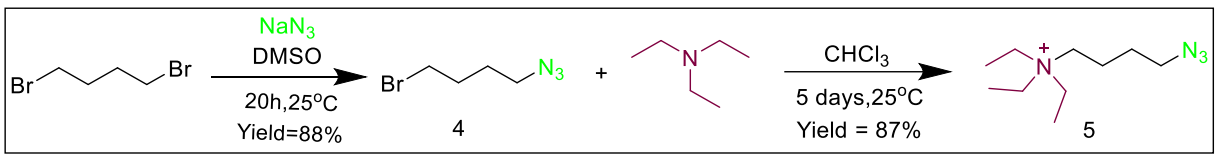


Figure 3.5 a) TGA plots of monomer and polymers b) DSC plots of polymers

The monomer was stable up to 130 °C, it started degrading beyond this temperature. So, the ring opening polymerization of the monomer was carried out at 100 °C. From TGA analysis of polymers it was found that the homo (polymer 1), block (polymer 2) and the clicked homopolymer (click polymer 1) are stable up to temperature of 140,145 and 190°C respectively (as shown in figure 3.5 a). This result shows that the thermal stability of polymer is increasing by the clicking of aliphatic carbon chains to the polymer side chains. Thermal properties of polymers were also studied using DSC. DSC thermograms were recorded with nitrogen purging and heating/cooling rates of 10°C/min. thermograms corresponds to heating cycle is shown in Figure 3.5 b. DSC plots revealed that these polymers were amorphous in nature and the glass transition temperatures for the homopolymer are in the range of -3.33°C (figure 3.5 b). And for block copolymer it was in the range of 25°C.

3.2 Synthesis and characterisation of cationic units:

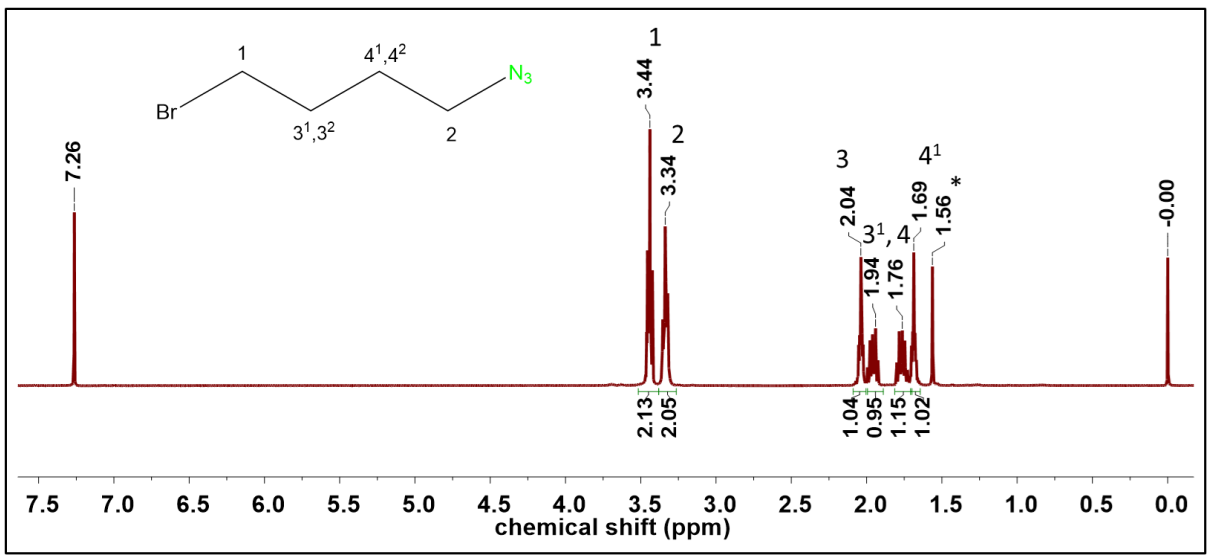
Triethyl amine based small molecule cationic unit was synthesized following the scheme 3.3 in two steps.



Scheme 3.3: Synthesis of cationic clickable conjugate 1

The formation and purity of the products were confirmed through ^{1}H NMR spectroscopy and HRMS. The conversion from dibromide to mono azide disturbed the symmetry of the molecule and the proton 2 got slightly up fielded to 3.34 ppm from 3.44 ppm in the reagent stage. Also, the asymmetry caused in a splitting pattern

in the protons 3 and 4 as well. In the second step compound 4 was quaternized using triethyl amine in chloroform as solvent. The reaction was carried out at room temperature and was continued for 5 days to obtain the product with 88% yield. The quaternized product gets precipitated as the reaction proceeds and it was separated and purified by first evaporating the chloroform and then washing it with diethyl ether several times to remove the unreacted reagents. The product formation was confirmed through ^{1}H NMR and HRMS. After quaternization the protons 1 and 2 gets shifted up field and appeared along with the proton 5 of the triethyl amine group. The peak at 1.39 which corresponds to the 9 protons of the triethyl amine group was used as the reference for the ^{1}H NMR analysis. This white solid product formed was highly hygroscopic in nature and so was stored carefully in air dry conditions. The ^{1}H NMR spectra of compounds 4 and 5 are provided in figure 3.6 below.



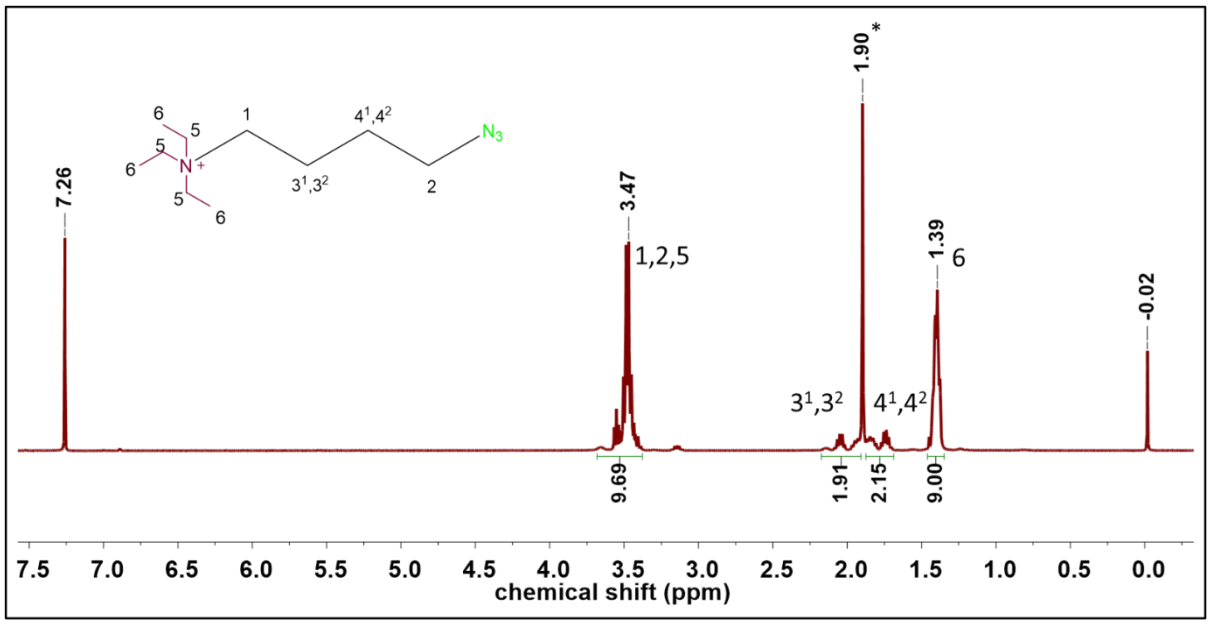
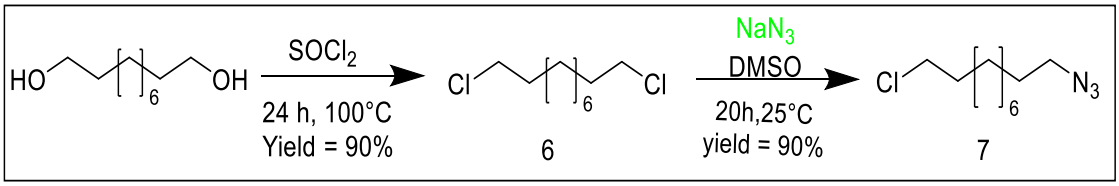


Figure 3.6. [1]H NMR of compounds 4 and 5

Similarly, a chlorine based 10-carbon aliphatic chain mono azide was also synthesized following the scheme 3.4. The product formation and purity were confirmed through [1]H NMR spectra and HRMS at each step of the reaction.



Scheme 3.4: Synthesis of clickable conjugate 2

In the first step decane diol was converted to dichloride by using thionyl chloride ($SOCl_2$) and the reaction was carried out at 100°C for 20 hours. [1]H NMR was analysed and we could see that the protons a, b, and c got down filed shifted by the introduction of electronegative chlorine groups. In the second step the dichloride was converted to mono azide by using sodium azide in DMSO. Reaction was carried out at room temperature and in order to avoid or minimise di substitution the reaction was carried out under dilute conditions. The product formation was confirmed by the up field shifting of proton a" and b" by the introduction of azide group. The [1]H NMR spectra of compounds 6 and 7 are provided in figure 3.7.

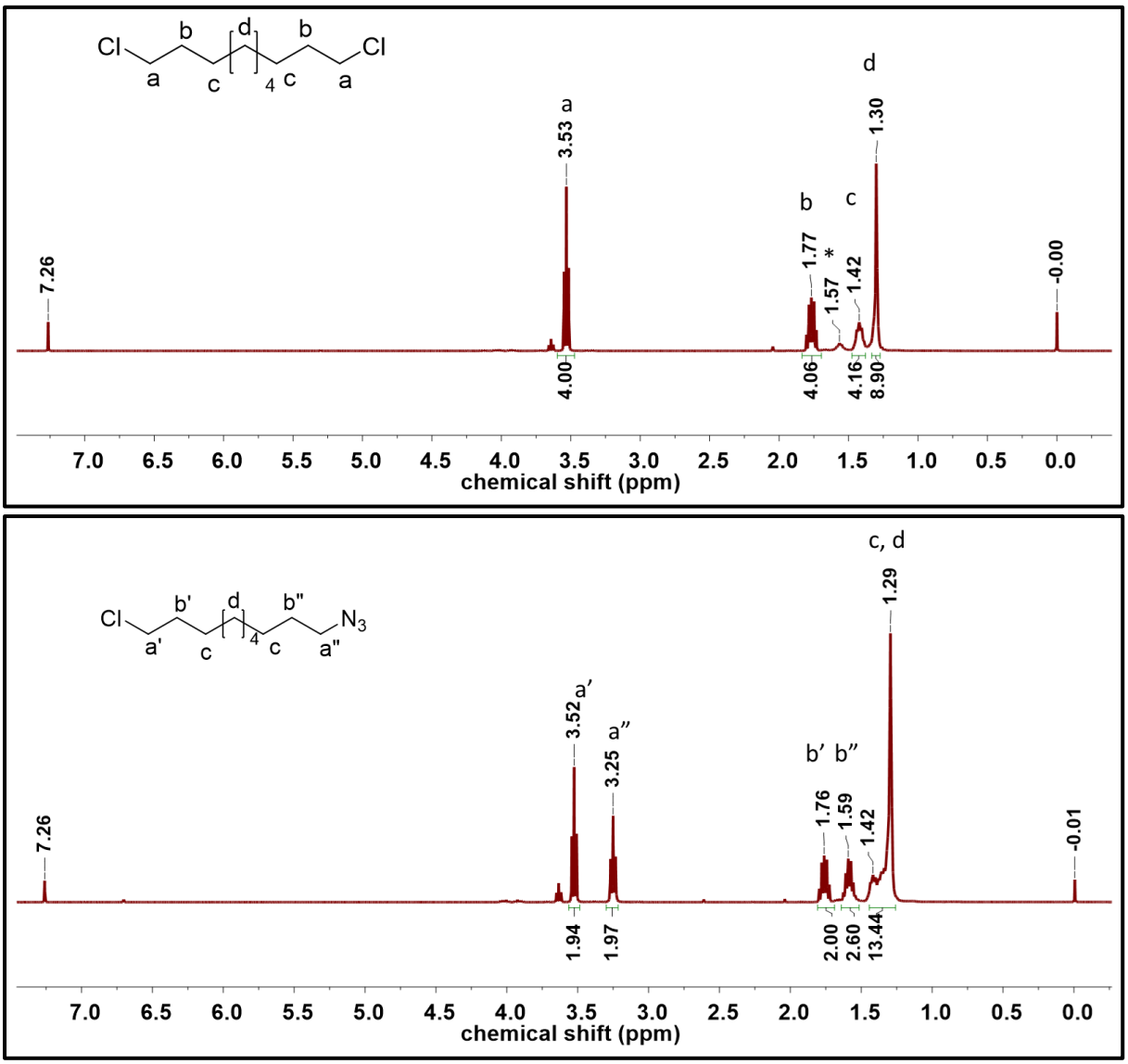


Figure 3.7. [1]H NMR of compounds 6 and 7

3.3 Introduction of click chemistry in polymers:

3.3.1 Optimisation of click chemistry in small molecules:

In order to optimise the synthetic procedure for clicking the azide unit to the polymer, as a first step we optimised the conditions for click reaction in a small molecule, i.e; in the compound 1. Compound 4 was conjugated to compound 1 via click reaction. The solvent system used was THF and water in 2:1 ratio combination and other reagents used were $CuSO_4.5H_2O$ as the source of catalyst and sodium ascorbate as reducing agent. The reaction was carried out at room temperature for 2 days. The addition of sodium ascorbate indicated the formation of reactive catalytic species Cu(I) by giving a colour change from blue of copper sulphate to yellow and then to green. At the end of the reaction THF was eveporated and product was

collected by work up using DCM. A pinch of EDTA was used during work up to remove the copper from organic layer completely by chelation with EDTA.

The completion of reaction was confirmed through ^{1}H NMR spectroscopy aswell as FTIR. The down field shifting of the triazole proton to 8.04 ppm as well as the

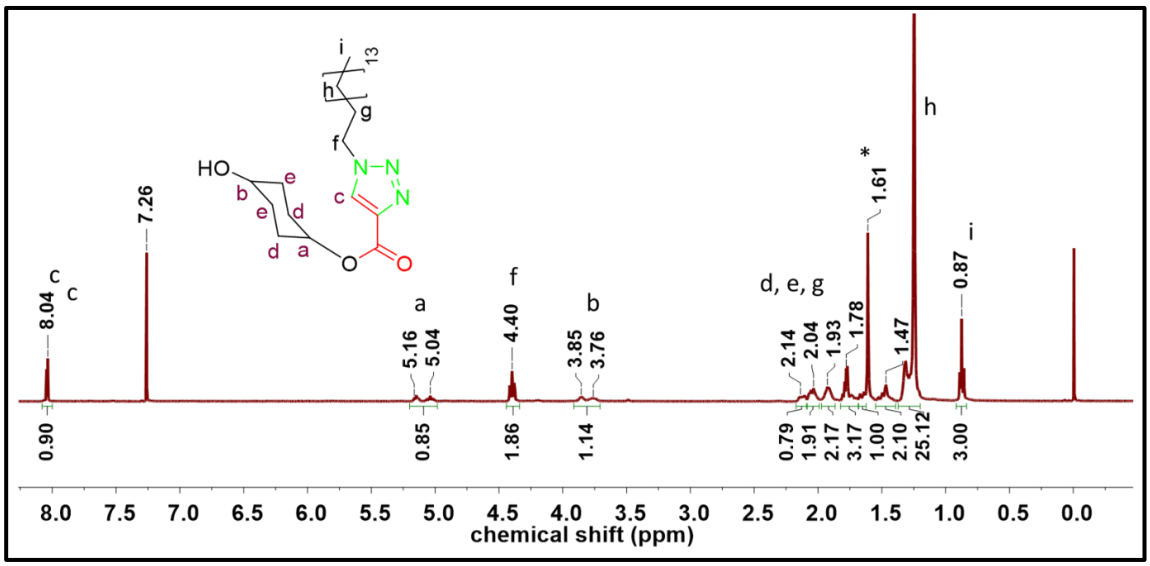


Figure 3.8. ^{1}H NMR of compounds 8

disappearance of alkyne proton at 2.86 ppm confirmed that the reaction has gone 100%. The ratio of protons a, b, c, i were also compared to confirm the product formation and purity(figure 3.8). Also FTIR was analyzed for the confirmation of the reaction. The disappearance of the azide stretching frequency and C≡C stretching frequency around 2100 cm^{-1} and the alkyne C-H stretching frequency around 3200 cm^{-1} region confirmed the reaction(figure 3.9).

3.3.2 Optimisation of click chemistry in polymers:

Once the click reaction scheme was optimised in small molecule, we tried replicating the same in the case of polymers. We started with conjugating hexadecane azide to polymer 1 and the reaction went successfully. Same conditions as in the case of small molecule were maintained. Reaction was carried out in THF and water in 2:1 ratio and reaction was continued for 2 days.Polymers were precipitated in diethyl ether and hexane after work up was done. Precipitation increases the purity of the polymer. An off white solid product was obtained which was characterized using ^{1}H NMR spectra and FTIR.

Once click chemistry was optimised in polymer our plan was to click the small molecule cationic unit we synthesized, i.e; the compound 5 to the polymeric chains,

but it did not work as shown in figure 3.9. Various conditions were also tried towards solving the problem but the influence of quaternary unit on click reaction could not be substantiated. Further analysis is needed to be put in this aspect to understand the poblem and solving the same.

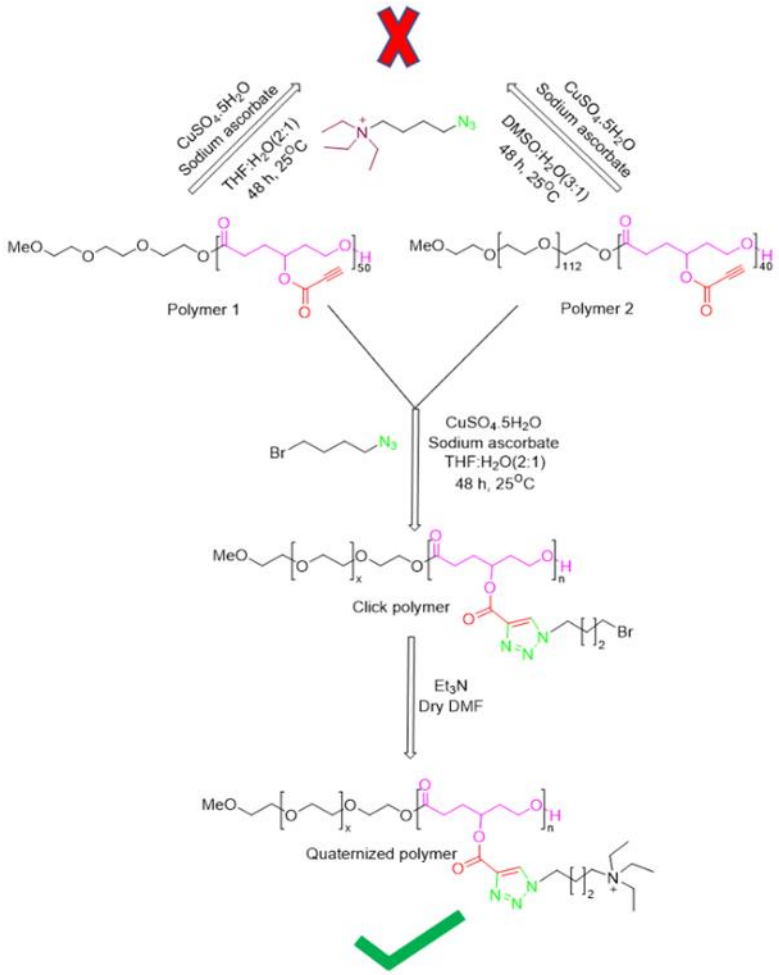
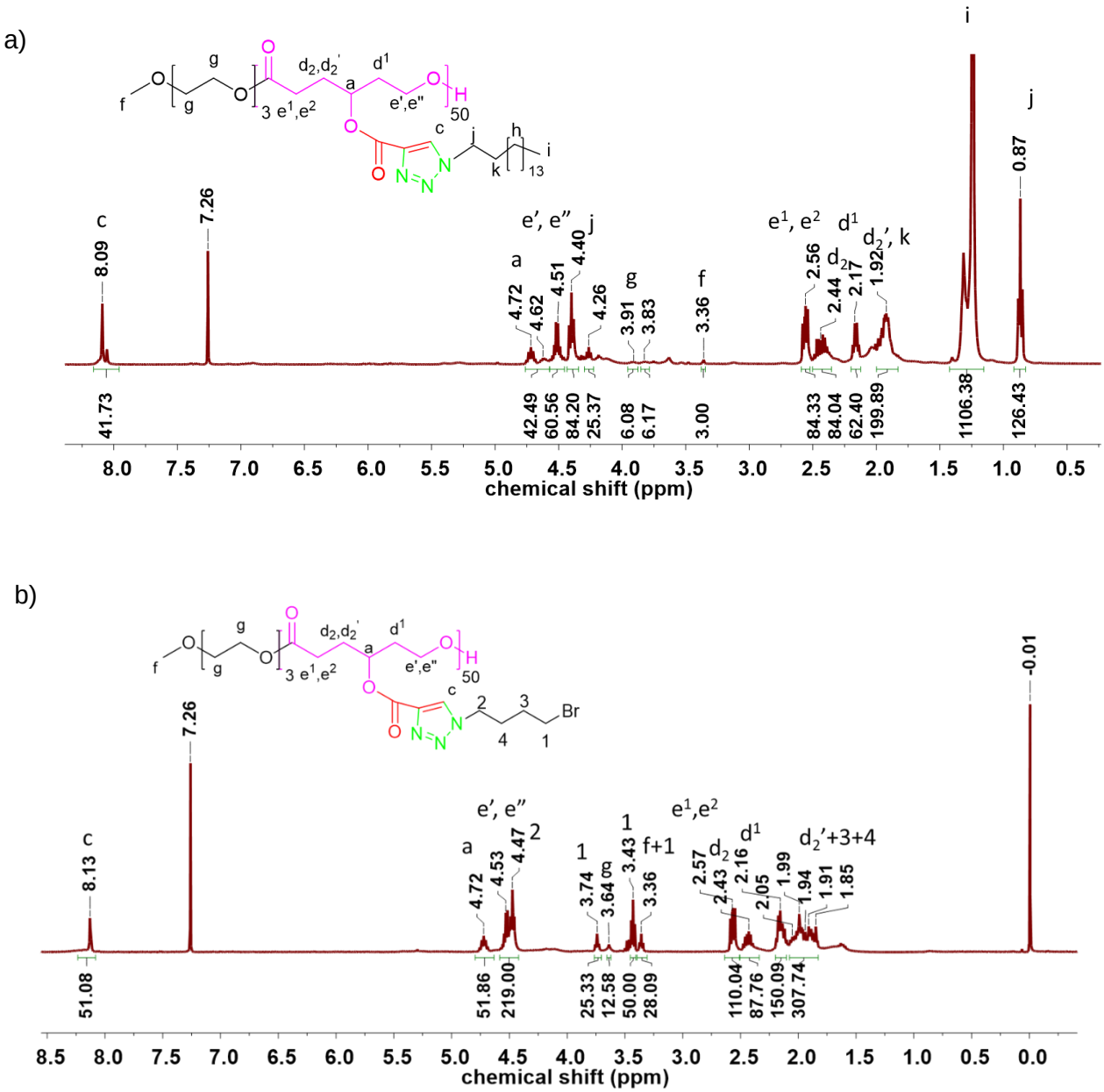


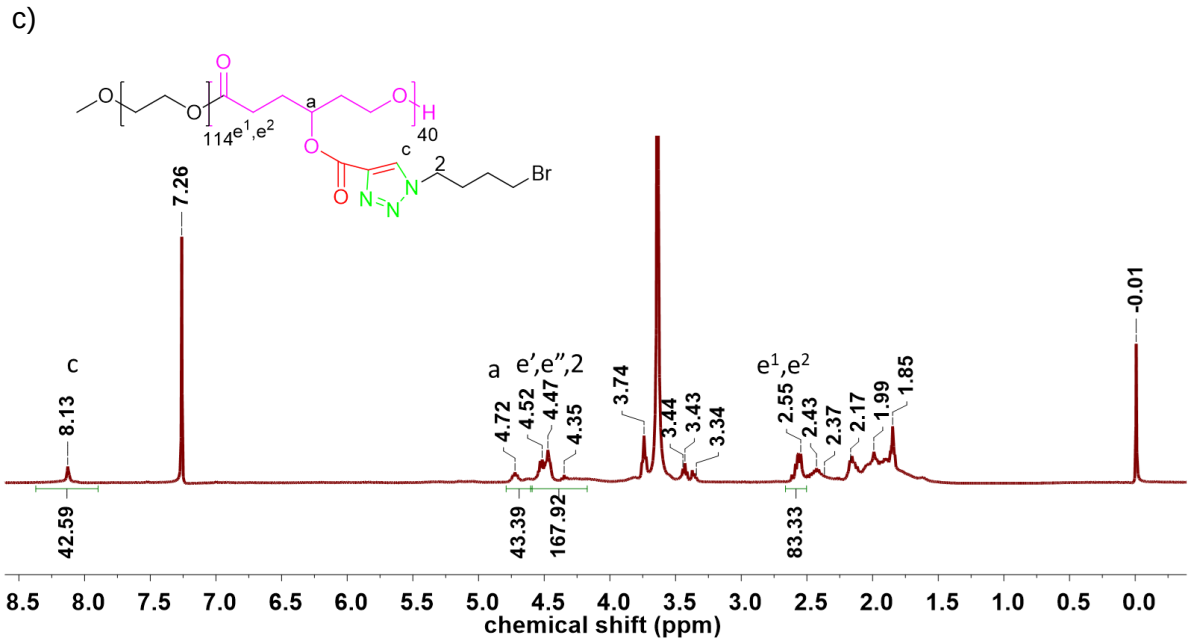
Figure 3.9. Schematic representation of clickable units to polymers

So we decided to take forward the project through another route, which was to first click the azide functionalized neutral molecules to the polymer and then quaternize them. Following the same click chemistry conditions optimised before, the compound 4 was conjugated to both homo (polymer 1) and block copolymers (polymer 2). Also we clicked a tetra ethylene glycol mono azide with chloride group on the other end to the block copolymer.

In all these cases product formation was confirmed through the disappearance of alkyne proton peak at 2.92 ppm and the appearance of more down

shielded triazole ring proton in the ^{1}H NMR spectra (Figure 3.10). The reaction was also confirmed through FTIR in which the alkyne C-H and C≡C stretching frequencies as well as the azide stretching frequencies where monitored. With the completion of the reaction the azide and C≡C stretching frequencies around 2100 cm^{-1} and the alkyne C-H stretching frequency around 3200 cm^{-1} disappears. Also the appearance of an alkene proton stretching frequency in the region 3100 cm^{-1} corresponding to the triazole ring C-H can be monitored (figure 3.11). This confirms that click chemistry worked nicely.





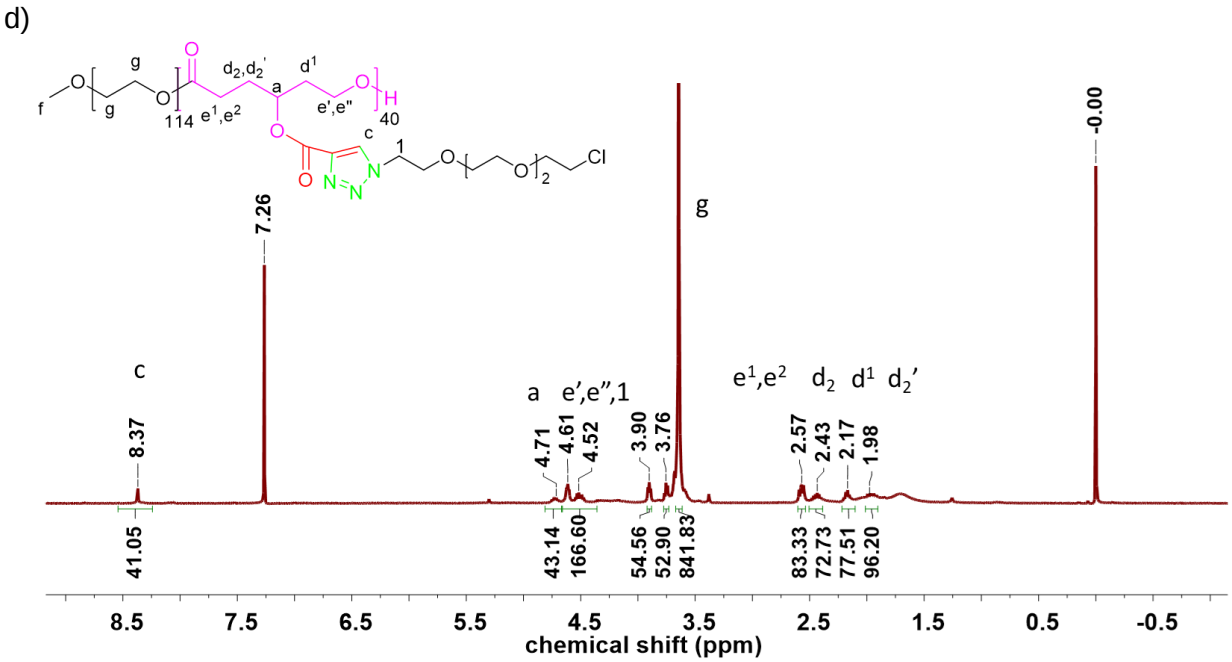


Figure 3.10. ^{1}H NMR of click polymer 1 to 4

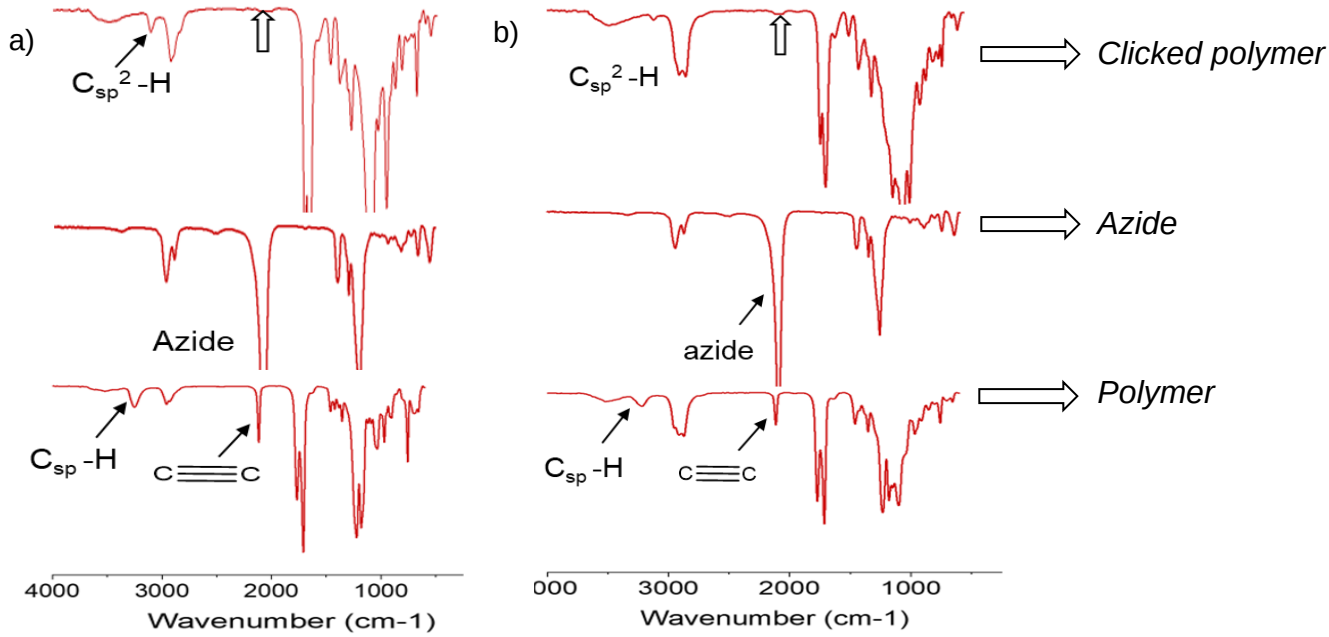
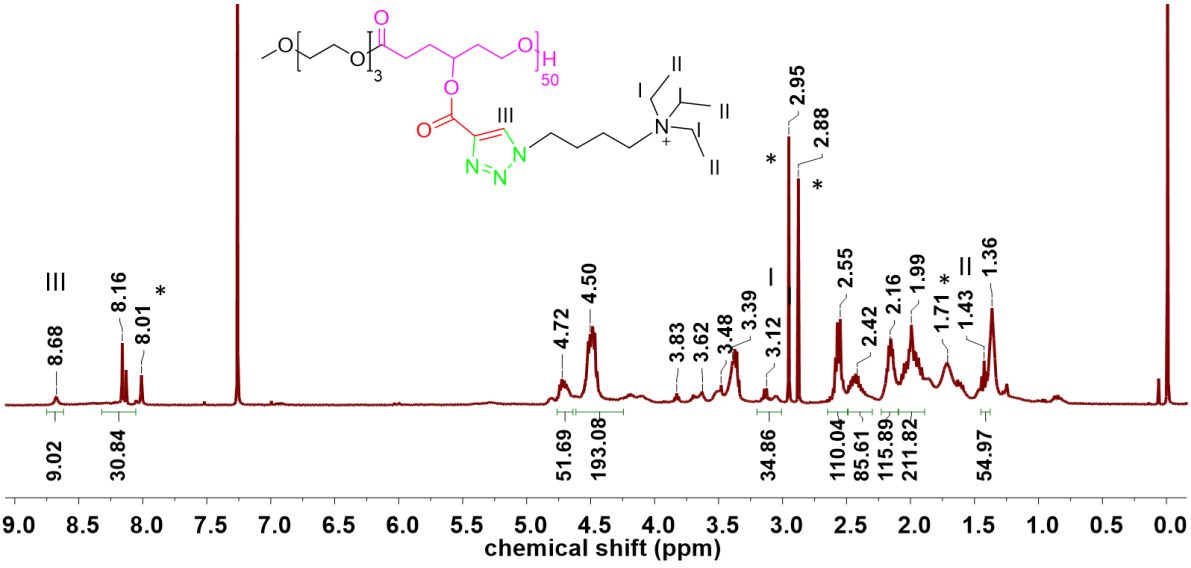


Figure 3.11. FTIR -comparison before and after click a) click in Homo polymer (click polymer 2) b) click in Block copolymer (click polymer 3)

3.4 Quaternization of polymers:

The polymers, Both homo and block after clicking the halogen bearing small molecule was then quaternized using triethyl amine in DMF at 80°C for 72 hours. The quaternized units were confirmed by the appearance of a quartet at 3.12 ppm (I) and a tripplet at 1.43 ppm (II). By comparing the ratio of these peaks to that of the polymers it was found that 11- 15% quaternization has happened. In the case of both homo and block copolymers, 5-9 units were quaternized. The ^{1}H NMR spectra of quaternization in homo and block copolymers are given in figure 3.11.



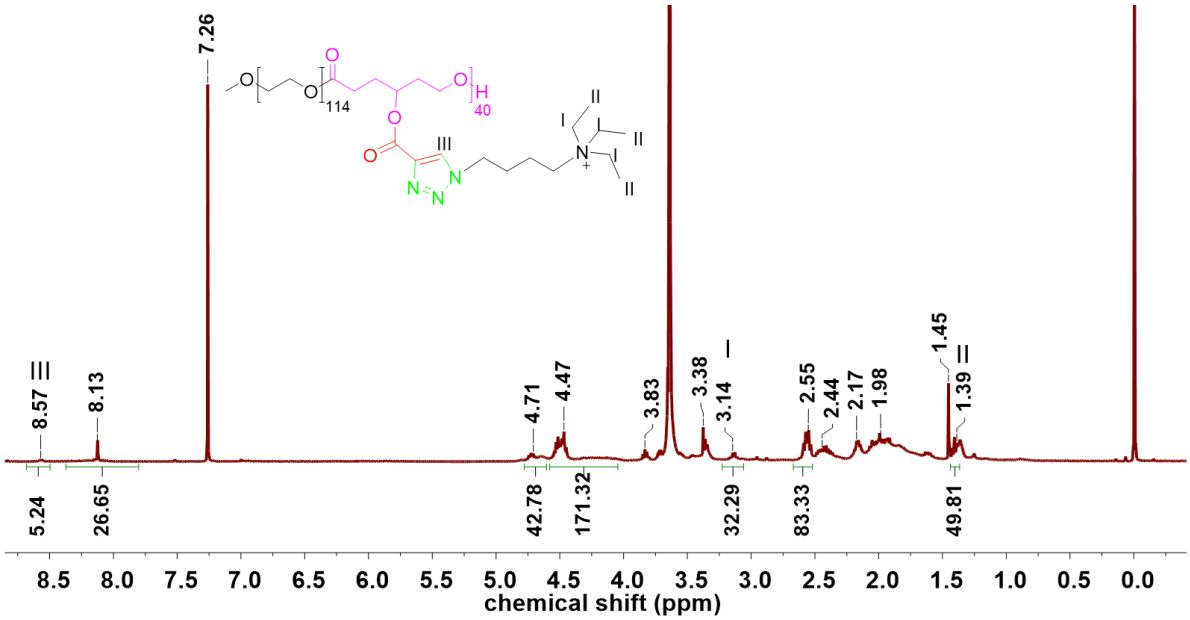


Figure 3.12. ^{1}H NMR of Quat polymer 1 and 2

3.4.1 Self-assembly of polymers:

DLS (dynamic light scattering) experiment was done to check the self-assembly of polymers in water. Both homo and block quaternized polymers (quat 1 and quat 2) formed clear solutions in water. Zeta potential and size measurements were done for these polymers. DLS size for homo polymer was determined to be 127nm at 0.1 mg/ml concentration and its zeta potential was found to be +4.98. Similarly, the size of the quaternized block copolymer was found to be 70 nm at 0.1 mg/ml concentration and zeta potential was measured to be +4.78 (see figure 3.13). The block quaternized polymer showed a smaller size compared to homo quaternized polymer, this could be because of the aggregated structure it is forming due to the influence of the PEG block in the system. Since both the polymers showed a positive zeta potential, they were taken forward for MIC determination.

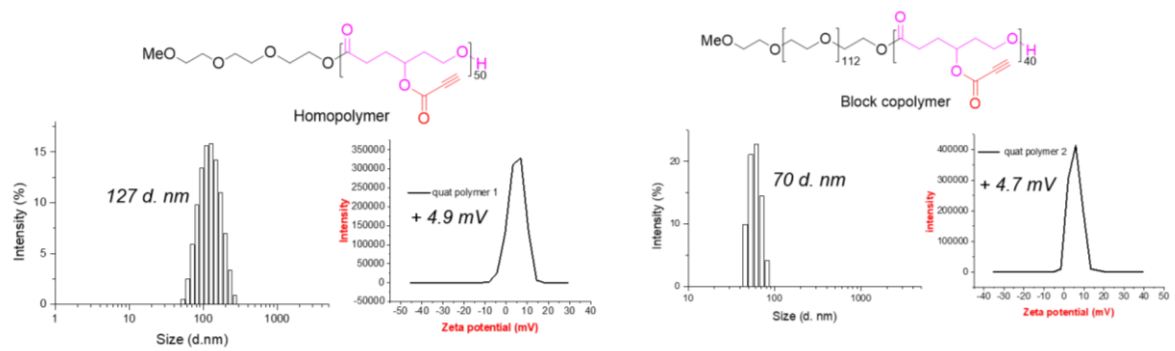


Figure 3.13. a) Schematic representation of dls size and zeta potential of homopolymer and block copolymer

3.5. MIC determination of quaternised homopolymer and block copolymer

In order to check the antimicrobial activity of triethylamine functionalised quaternary amine polymers, colony suspension method was utilised. The experiment was carried out with the help of Ruma (senior member of the group). A fixed O.D of bacterial suspension was incubated with serially diluted polymeric samples and scattering of *E. coli* suspension was measured as a function of time. A positive control was also used where only *E. coli* growth was monitored as a function of time, along with a negative control where bacterial cells were incubated with a known antibiotic (ampicillin) at its MIC concentration (16 µg/mL). The plots are shown in figure 3.14.

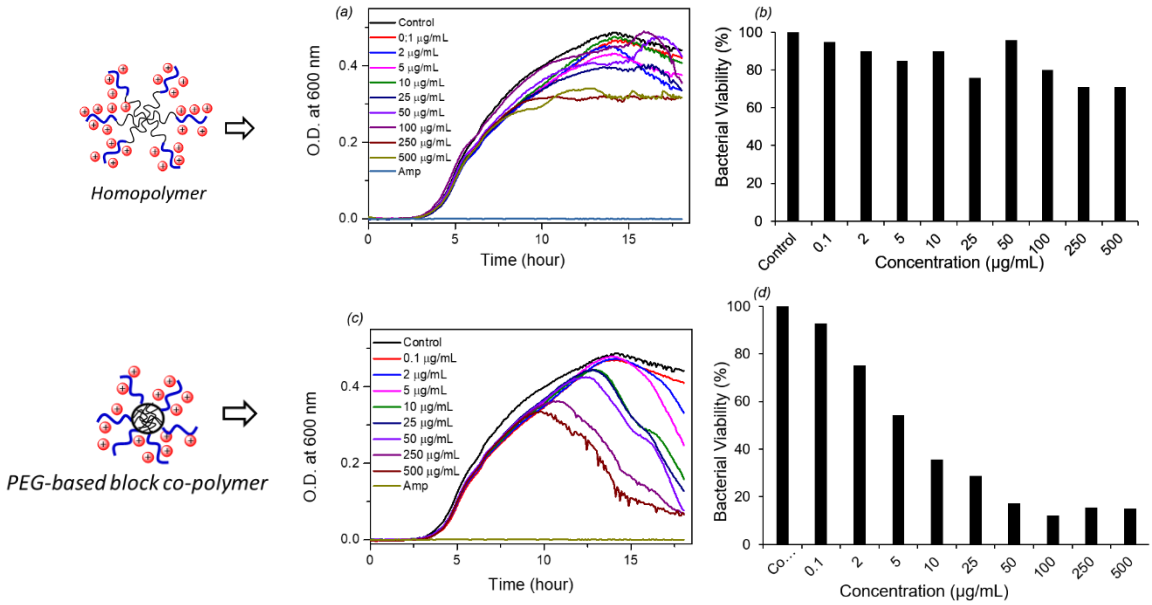


Figure 3.14. a) Colony suspension method for the determination of Minimum inhibitory concentration for homopolymer after 18 h incubation (b) Plot of bacterial viability for various polymeric concentrations at 18 h. (c) Determination of MIC concentration for quaternised block copolymer. (d) Change in bacterial viability for polymeric concentrations at 18 h .

In case of quaternised homopolymer (see figure 3.14 a), it was observed that there was no effect of polymer on bacterial growth. At 500 ug/mL, only 30 % inhibition in growth was observed. This shows that these TEG-initiated polymers are unable to inhibit the growth of bacteria. This could be due to the imperfect hydrophobic and hydrophillic balance. This can also be reinstated through DLS size, where the size of homo polymer is larger than block copolymer. It states that the compactness of

the molecule is enhanced in the presence of PEG-5k macroinitiator. For block copolymer, at 50 ug/mL 80% bacterial inhibition is observed which confirms that quaternised PEGylated block copolymer showed antibacterial activity agaist *E. coli*. In order to further understand the activity of polymer on the surface of bacteria, an azide functionalised fluorescein derivative was synthesized.

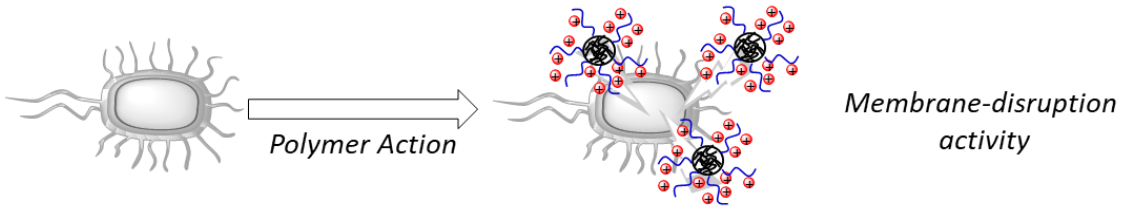


Figure 3.15. Cationic Block co-polymer action on E-coli cells

3.6 Synthesis and characterization of fluorophore conjugate:

For making the direct visualization of the antimicrobial activity of the polymer possible, we also synthesized an azide functionalized fluorophore, i.e; fluorescein azide. The reaction was carried out in DMF at room temperature for 2 days and we were able to synthesize the molecule successfully. Product was characterized using ^{1}H NMR, FTIR and HRMS. The ^{1}H NMR of the compound is given in figure 3.15.

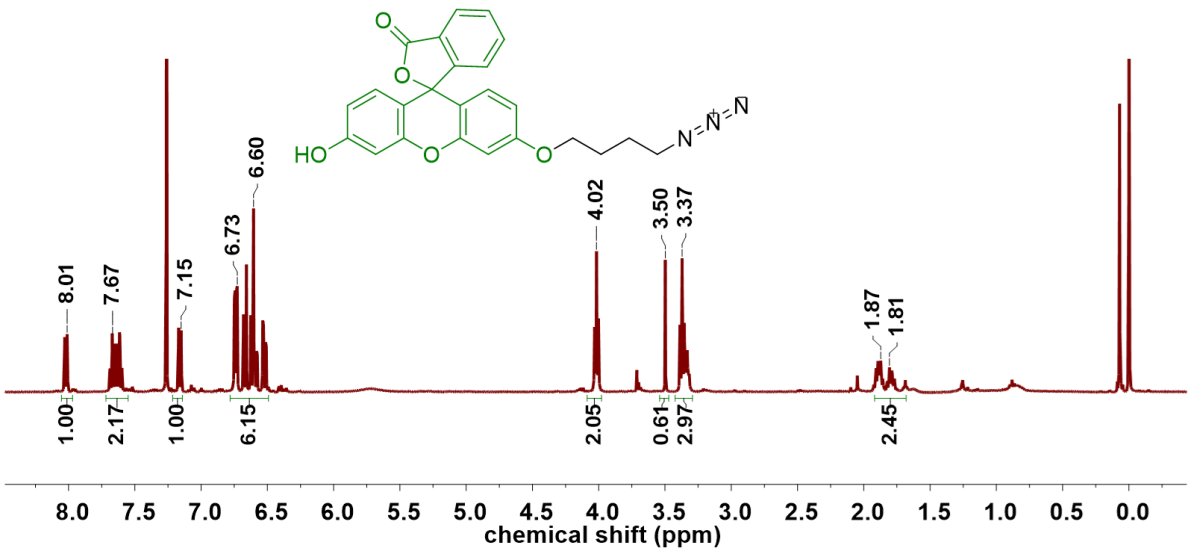


Figure 3.16. ^{1}H NMR of fluorescien azide

In order to check the stability of the fluorophore after attaching the azide unit, absorbance and fluorescence of the fluorescein derivative was determined (see figure 3.17). It was observed that fluorescein azide had λ_{ab} at 440 nm and 490 nm

as similar to fluorescein molecule. It confirms that the ground state structure and chemical nature of the molecule did not alter. Fluorescence of the molecule also remained unchanged as λ_{em} was found out to be 550 nm. This molecule can be used for synthesizing fluorescently labelled polymers through click chemistry. The fluorescently labelled polymers would be highly efficient in labelling *E. coli* cell membrane upon polymeric action on its surface. These polymers are under preparation and their antimicrobial activity through confocal imaging will also be done and it will be presented in the MS thesis presentation.

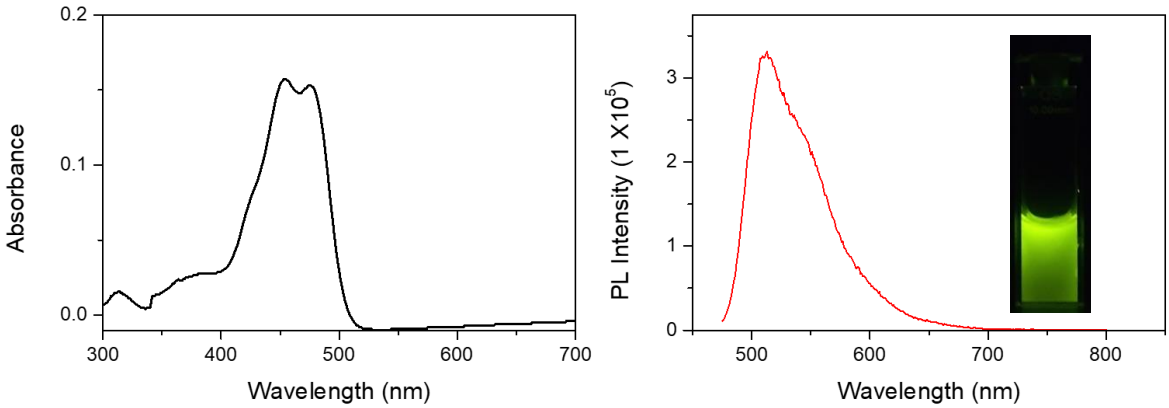


Figure 3.17. a)Absorbance spectra of fluorescien azide b)fluorescence of fluorescien azide.

4. Conclusion:

Alkyne substituted polycaprolactone based biodegradable polymers were synthesized using two different initiators through metal catalysed, solvent free ring opening polymerization technique (melt route) and polymer formation was confirmed using ^{1}H NMR spectroscopy. We aimed for 50 units for both polymers and we were able to obtain 50 units for polymer 1 and 40 units for polymers 2 respectively. The absolute molecular weights of polymer 1 and 2 were found to be 9000 and 12200 respectively using NMR spectroscopy. The molecular weights determined through GPC using polystyrene standards were highly under estimated. So, the absolute molecular weight of the block copolymer was determined by the triple detector absolute molecular weight determination method using PEG-99k as standard. The dn/dc value of PEG-5K in $CHCL_3$ was determined to be **0.047889.** Using this value, the dn/dc value of the block copolymer was determined (**0.09735**) and the absolute molecular weight of the block copolymer was found to be 12700(Mn), 19000 (Mw) and polydispersity index as 1.5 respectively. These values were in a good match with the molecular weight determined from NMR. TGA and DSC were done to obtain the decomposition and glass transition temperature values of the polymers.

The second part of the project was the synthesis of various clickable units and characterisation was done using NMR, HRMS and FTIR. Both cationic as well as neutral small molecular azide containing groups were synthesized. We tried stitching these molecules to the polymer using click chemistry approach. But clicking the small molecule cationic unit we synthesized, i.e; the compound 5 to the polymeric chains, did not work as shown in figure 3.9. Various conditions were also tried towards solving the problem but the influence of quaternary unit on click reaction could not be substantiated. Further analysis is needed to be put in this aspect to understand the poblem and solving the same. So we decided to move forward through another route,i.e; by clicking the azide functionalized neutral molecules to the polymer chain and then quaternize them.

The neutral molecules were stitched to the polymers and these polymers were then quaternized using triethyl amine to obtain positively charged polymers. Zeta potential and size measurements were done for these polymers. The block quaternized polymer showed a smaller size compared to homo quaternized polymer, this could be because of the aggregated structure it is forming due to the influence of the PEG

block in the system. Since both the polymers showed a positive zeta potential, they were taken forward for MIC determination.

It was observed that quat-polymer 1 had no effect on bacterial growth. At 500 ug/mL, only 30 % inhibition in growth was observed. This shows that these TEG-initiated polymers are unable to inhibit the growth of bacteria. This could be due to the imperfect hydrophobic and hydrophillic balance. This can also be reinstated through DLS size, where the size of homo polymer is larger than block copolymer. It states that the compactness of the molecule is enhanced in the presence of PEG-5k macroinitiator. For block copolymer, at 50 ug/mL 80% bacterial inhibition is observed which confirms that quaternised PEGylated block copolymer showed antibacterial activity agaist *E. coli*.

An azide functionalised fluorescein derivative was also prepared. We are planning to utilize this molecule for the synthesis of fluorescently labelled polymers through click chemistry which would be highly efficient in labelling *E. coli* cell membrane upon polymeric action on its surface. These polymers are under preparation and their antimicrobial activity through confocal imaging will also be done and it will be presented in the MS thesis presentation.

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