Polyester-Polypeptide Hybrid Polymer Brushes

A Thesis

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Indian Institute of Science Education and Research Pune in partial fulfillment of the requirements for the BS-MS Dual Degree Programme

by

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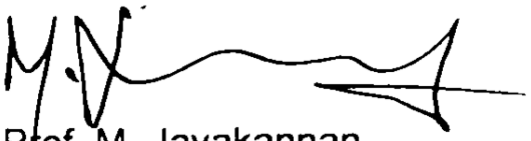
Certificate

This is to certify that this dissertation entitled **Polyester-Polypeptide Hybrid Polymer Brushes** towards the partial fulfillment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents study/work carried out by Ashutosh Ganpat Shirodkar at the Indian Institute of Science Education and Research under the supervision of Manickam Jayakannan, Professor, Department of Chemistry, during the academic year 2023-24.



Prof. M. Jayakannan

Date: 20/05/2024

Place: IISER Pune

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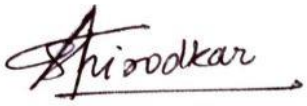
Dr. K. Shanmuganathan

Declaration

I hereby declare that the matter embodied in the report entitled **Polyester-Polypeptide Hybrid Polymer Brushes** are the results of the work carried out by me at the Department of Chemistry, Indian Institute of Science Education and Research, Pune, under the supervision of Prof. Manickam Jayakannan and the same has not been submitted elsewhere for any other degree.



Ashutosh Ganpat Shirodkar

Date: 27/03/2024

Place: IISER, Pune

This thesis is dedicated to my family

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ABSTRACT

Polypeptide and polyesters are well-known for their biocompatibility and bioresorbability. They have very contrasting but useful properties. Combining these polymers into a special architecture of Cylindrical Polymer Brushes (CPB) is the primary aim of this thesis. CPBs have grown as an important molecular architecture in material science and biology. In this project we have synthesized polycaprolactone grafted polymer brushes and studied its properties. To accomplish this, polycaprolactone and tertiary butyl ester containing polycaprolactone polymers containing azide functionality at the end-group were synthesized separately. Poly(γ-propargyl l-glutamate) was synthesized as the alpha-helical rigid scaffold. All the individual polymers were characterized by NMR, GPC, FT-IR, MALDI-TOF and TGA analysis. PPLG polymer was confirmed to have alpha-helical structure using circular dichroism and the polymer-azides were then stitched onto it. The versatile ligation tool – click chemistry was used in order to achieve maximum grafting efficiency. Finally, the synthesized graft polymers were properly characterized using NMR, GPC and TGA to analyse their structure and study the bulk properties.

1 INTRODUCTION

Biodegradable polymers for biomedical applications have been a hot research topic for the past thirty-forty years. Biodegradable polymers are extensively applied for drug delivery, gene delivery, tissue engineering, implants- bioimaging, etc. Amongst the best in this list are aliphatic polyesters and polyamides, specifically polypeptides[1].

1.1 ALIPHATIC POLYESTERS

Polycaprolactone (PCL) is a biodegradable and bioresorbable aliphatic polyester widely used in biomedical applications owing to its excellent biocompatibility and processibility. Its low melting transition (59-64°C), high solubility in low boiling organic solvents, and exceptional blend compatibility makes it a highly processable polymer[2]. PCL can be synthesized by ring- opening polymerization (ROP) of ε-caprolactone using various transition-metal catalysts, organo-catalysts, and other multivalent inorganic catalysts as well as via free radical ROP of 2-methylene-1- 3-dioxepane[3]. PCL although biodegradable has slow degradation compared to other aliphatic polyesters like polyglycolic acid (PGA) or Polylactic acid (PLA). This makes it unsuitable for fast drug delivery applications but makes it one of the best candidates for tissue engineering and in application where continuous release over a longer period of time is required[2].

There have been a lot of studies on substituted PCLs in past few years for developing nanoparticle for drug delivery. Many kinds of substituted polycaprolactones have been developed, among which carboxylic-PCL (CPCL), developed by our group, has been shown to undergo complete enzymatic degradation with higher rate of cleavage[4]. CPCL is amorphous, amphiphilic and anionic at physiological pH, allowing for its faster enzymatic degradation than normal PCL. The polymer chains can conform and fit into the enzymatic pockets to get cleaved by the lysosomal esterases. CPCL was demonstrated to work as a good delivery system for gastrointestinal targets due to its pH responsive carboxylic groups. In the acidic pH the backbone looses its hydrophobicity and the encapsulated drug is released. Although it has pH responsiveness only at high pH, which is only found in stomach it can be used in combination with other polymers to tune its properties. It can just serve as hydrophilic

unit or can be the anionic part of zwitterionic polymer micelle encompassing innumerable biological applications.

1.2 POLYPEPTIDES

Homo-poly(amino acid)s, or block co-polypeptides involving repeats of the same amino acid, are the closest we have been able to approach the natural proteins in terms of synthesis. Unlike polyesters, polypeptides are not melt-processable and have very limited solubility in many of the organic solvents. The reason for this is the regular extremely strong hydrogen bonding, which is as good as covalent bonds and doesn't allow the polymer chains to slide over each other decreasing its processibility. This property can be seen as a con, but rather it is one that renders polypeptides their peculiar property – complex secondary structures. Like proteins, they show a set of diverse secondary structures like alpha-helices, beta sheets, random coil, etc. that can be employed in various solution state applications as well as applications in bulk state.

Polypeptide synthesis has undergone tremendous development in last few decades. Moving on from solid-phase peptide synthesis for obtaining high molecular weight polypeptides, ROP of N-carboxy anhydride (NCA) has been the way forward. Synthesis of NCA has been one of the most challenging task in polypeptide synthesis. Due to some recent developments, some NCAs which initially required utmost care, and were only synthesizable in anhydrous organic solvents and get hydrolysed even in atmospheric moisture, can now be easily prepared using moisture tolerant route using propylene oxide as the HCl quencher in the system[5]. Although this works for many solid NCAs, liquid NCA synthesis is still lying under "intensive precautions" unit. Further polymerization of synthesized NCA is mostly done using a primary amine as the initiator. A wide scope of catalysts have been developed viz. N-heterocyclic carbenes, fluorinated alcohols, thiourea-based catalysts, bulky organic bases like DBU, or guanidine bases like TMG, etc. for synthesis of narrow dispersed polypeptides[6–8].

Although there exist a lot of synthetic polypeptides, poly(l-lysine) (PLL) and poly(l-glutamic acid) (PLG) are the most explored polypeptides due to their accessibility in terms of synthesis. As Cai et al. have elaborated in their review[9], poly(l-lysine) is a water-soluble, cationic polymer known for its antibacterial and antitumoral properties. Due to its biodegradability and biocompatibility with several tissues, easy

functionalization, and cell-adhesion properties, it has been explored in drug delivery, bioimaging, biosensing, and regenerative biodegradable implants. However, PLL has its limitations due to its hemolytic activity and cytotoxicity. Combining PLL with polymers that could reduce its downsides and improve specific anti-tumoral ability can afford utilization of the full potential of PLL as an anti-cancer biomaterial.

1.3 THE COMBINATION

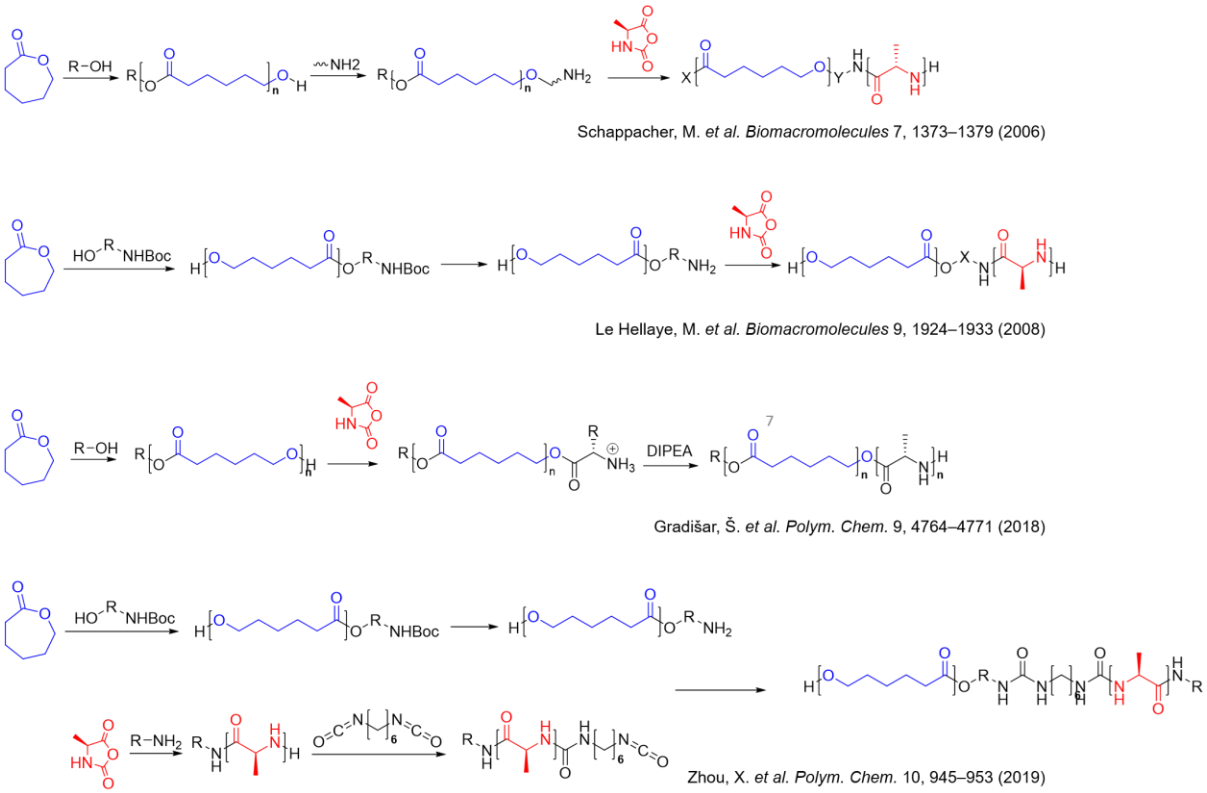

Figure 1 Methods used to construct block copolymers of polycaprolactone and polypeptides

Polycaprolactones and polypeptides are both synthesized using ring opening polymerization. The primary difference is in one case the growing chain is an alcohol and in the other case its an amine. This difference is aggravated by their solubility differences. Polypeptides are mostly soluble and polymerizable in polar aprotic solvent like DMF, and less so in chloroform or dichloromethane. On the other hand, polymerization of caprolactones can work either in melt condition, or in dichloromethane or toluene in solution route. As most polypeptides do not melt the first option is inconsiderable. It is also insoluble in toluene. Thus, we are left with copolymerization in chloroform or dichloromethane. Previous works in synthesis of

block copolymers involving polycaprolactones and polypeptides in these halogenated solvents have been made through different approaches such as bifunctional initiator, post-polymerization end-functionalization, etc[10–12]. But many of these techniques involve some or other challenges. Deprotection of amine group after making homopolymer of PCL may attack any ester bond in the pool causing dead-end of the chain[12]. Sequential polymerization of caprolactone and NCA using methanesulfonic acid (MSA) as a catalyst in chloroform or dichloromethane as solvent has been shown to provide well-defined block copolymers but this chemistry has only been optimised for aspartic acid NCAs[13].

To get beyond these obstacles of bringing these synthetically immiscible monomers together, we have used the click chemistry which is tried and verified approach for macromolecular conjunction[14].

1.4 CLICK REACTION

Copper-catalyzed azide-alkyne cycloaddition (CuAAC) is considered as the hallmark of click chemistry. It was discovered by Sharpless and Mendal in 2002[15]. CuAAC is orthogonal to wide variety functional groups and can be performed at dilute concentrations of reactants and in mild conditions. Recent studies and extensive investigations of the mechanism for click reaction has suggested formation of dinuclear copper intermediates during CuAAC[16].

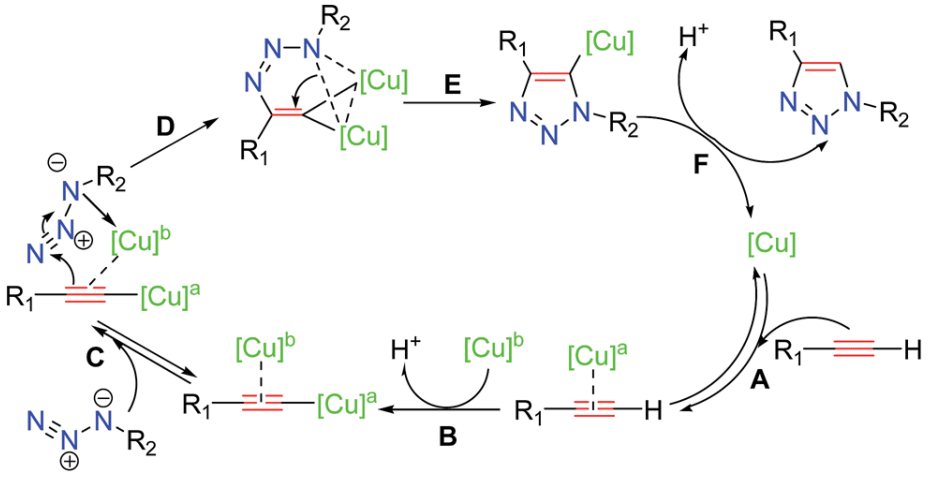

Figure 2 Catalytic cycle of CuAAC **|** *adapted from: Chem. Sci.,2016, 7,6298*

Click reaction has emerged as a versatile, and orthogonal tool for polymer synthesis and functionalization. It has made possible to combine polymers having incomparable solubilities or incompatible methods of polymerization. It has also provided access to

higher order structures which are often difficult to synthesized using traditional coupling reactions. Linear, graft, star, branched, cyclic structures or polymer brushes can be constructed using click reactions[16].

1.5 CYLINDRICAL POLYMER BRUSHES

Cylindrical polymer brushes (CPB) comprise of a rod-like backbone grafted by numerous linear polymers of low polydispersity to create uniform bottle-brush like structures. As compared to linear polymers having similar molecular weights, CPB afford lower viscosity due to their limited chain-entanglement and show a higher local concentration of polymer chain ends over its surface. This makes CPBs attractive candidate for drug delivery vehicles, stimuli-responsive materials, lubricants, molecular nano-templates, supersoft materials, etc. There are three known approaches to synthesizing cylindrical polymer brushes (CPBs)[17].

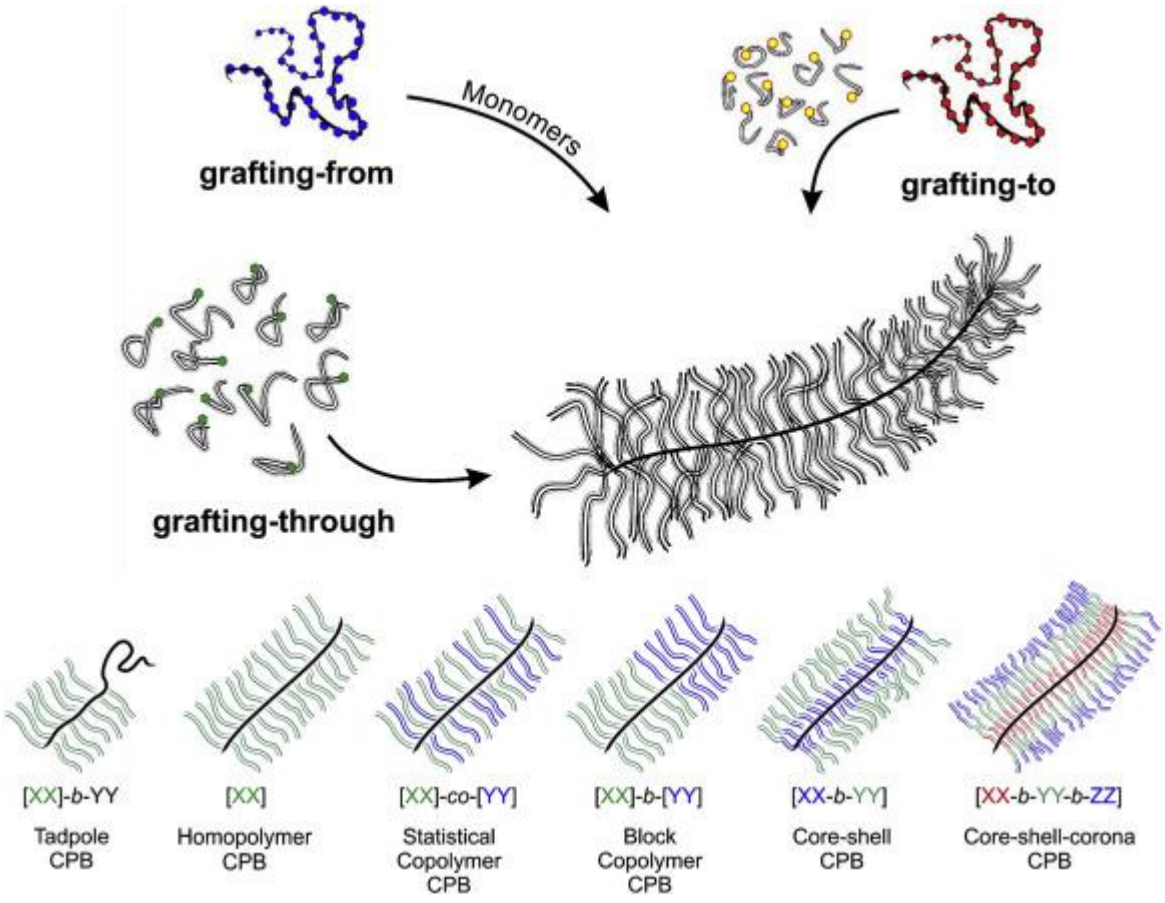

Figure 3 Schematic describing types of methods for synthesizing CPBs | *adapted from: Polymer 98 (2016) 389-401*

Grafting-through: This approach involves polymerization of macromonomer. Although narrow dispersed bristles can be obtained through this method, high viscosity build-up and steric hinderance restrict the length of CPB that can be achieved. Highly efficient polymerization methods like ring opening metathesis polymerization (ROMP) and atom radical transfer polymerization (ATRP) under specific conditions can only achieve CPB formation by grafting-through pathway.

Grafting from: This method uses polymer sidechains as the initiator for growing new polymer as the bristles. ROP can be easily used for this kind of methods, but as the chains grow, steric repulsion can cause some initiators to get lost below others and less grafting density would be achieved.

Grafting-to: It involves separately prepared side-chains and very powerful coupling reactions like click chemistry. Steric hindrance can limit the grafting efficiency. Nevertheless, it allows complete characterization of the backbone brush as well as the side-chains independently.

Although grafting-to approach has been talked about as a very low grafting-efficiency yielding technique, Hammond et al. (2009)[18], have reported almost complete grafting efficiency for poly(γ-propargyl l-glutamate) (PPLG) owing to its alpha helical nature and efficient click reaction. The regular turns in the helix increase the distance between the consecutive reactive groups. Moreover, the rigid polypeptide backbone avoids entanglement of chains which can be a potential hindrance for grafting.

Grafting onto PPLG backbone opens several avenues for merging polyesters with polypeptides. Individually synthesized, well-characterized azide end group polymer chains, can be clicked altogether onto PPLG in polar solvents like DMSO or DMF, which can dissolve all the components necessary for click reaction to create statistical copolymer CPB. A priori synthesized block copolymers containing azide end group in the hydrophobic side could also be clicked onto PPLG to create unimolecular rod-shaped micelle.

1.6 AIM OF THE THESIS

To combine polycaprolactone and polypeptide two well-known bio-resorbable polymers into polymer brush architecture using click chemistry as a ligation tool. Firstly, poly(γ-propargyl l-glutamate) polymer would be synthesized and characterized.

Individual azide end group containing polymers viz. PCL-N_3, BPCL-N_3, poly(l-lysine)-N_3 would be synthesized and characterized. These polymers would be then clicked onto PPLG's alpha-helical backbone to produce cylindrical polymer brushes. The final polymer would be characterized using, NMR, GPC(SEC), and TGA. All the synthesized polymers will be studied for their thermal properties, and further deprotected and its self-assembly would also be studied.

2 EXPERIMENTAL SECTION

2.1 MATERIALS

1,4-Cyclohexane diol, potassium t-butoxide, t-butyl acrylate, metachloroperbenzoic acid (MCPBA), pyridinium chlorochromate (PCC), tin(II) 2-ethylhexanoate ($Sn(Oct)_2$), ε-caprolactone, copper sulfate, copper (I) bromide, N,N,N',N",N"-Pentamethyldiethylenetriamine (PMDETA), methanesulfonic acid (MSA) were purchased from Aldrich chemicals. ε-Caprolactone was distilled under vacuum and stored at 4°C. All other solvents like tetrahydrofuran (THF), hexane, dichloromethane (DCM), propargyl alcohol, etc. were purchased locally and dried, distilled and kept under inert atmosphere prior to use. Ammonia aq. solution (25%), HCl (32 % aq. solution), sodium sulfate (anhydrous), acetic acid, triphosgene, sodium carbonate, trifluoroacetic acid, propylene oxide, glutamic acid, 98% H_2SO_4, benzene-1,4-dimethanol, sodium azide, toluene, anhydrous potassium carbonate, dry ethyl acetate, dry DMF, HPLC grade DMSO were purchased locally and used directly. Monomethyl (Polyethylene glycol) amine (PEG-5k-NH_2) (MW = 5000 g/mol) was already synthesized in lab by using ammonia as the aminating reagent.

2.2 METHODS

Bruker 400-MHz NMR spectrophotometer was used for recording NMR spectra. $CDCl_3$, DMSO-d_6, and D_2O containing TMS as an internal standard were used as solvents for recording NMR spectra. $CDCl_3$ with 1:6 amount of TFA was used wherever denoted in the document. Gel permeation chromatographic (GPC) analysis was performed using Viscotek VE 1122 pump, and Viscotek VE 3580 RI in chloroform ($CHCl_3$), and dimethyl formamide (DMF) using polystyrene as standards. Perkin-Elmer thermal analyzer STA 6000 model at a heating rate of 10 °C/min in nitrogen atmosphere was used for thermogravimetric analysis of polymer samples. Molecular weight of small molecules was recorded on HRMS-ESI-Q-Time of Flight LC/MS (Synapt G2, Waters). 4800 Plus MALDI TOF-TOF instrument from AB Sciex was used for recording MALDI-TOF mass spectra. Bruker Alpha-II was used to record FT-IR spectra.

2.3 SYNTHESIS

2.3.1 γ-propargyl l-glutamate (PLG) (1)

Glutamic acid (10.0 g, 68 mmol) was suspended in propargyl alcohol (15 ml, used as the solvent) and mixture was cooled with ice bath at 0°C and concentrated H_2SO_4 (4 ml, 72 mmol) was added dropwise and very slowly using a constant pressure funnel *(Caution: Propargyl alcohol and H_2SO_4 (Bronsted acid) can be potential explosive combination, never heat the mixture and perform slow addition at cold conditions only)*. The solution becomes clear after a few hours. Reaction progress was monitored using TLC (MeOH: DCM: AcOH = 1:1:0.1). After complete consumption of glutamic acid (3 days) the solution was neutralized using 8 wt.% solution of sodium carbonate (72 mmol) till the pH of the solution was close to 7, and stored at 4°C for 12 hr. The precipitate was filtered and the filtrate was again stored at 4°C, repeating the procedure till no more precipitate is observed. The precipitate containing γ-propargyl glutamate and sodium sulfate (generated by neutralization) was purified using reprecipitation in ethanol: water (1:1). The product was stirred in THF to remove access ethanol and water, and then freeze-dried to get white solid (6 g, Yield: 46%). ^{1}H NMR (400 MHz, D_2O) δ 4.68 (d, J = 2.5 Hz, 2H), 3.70 (t, J = 6.4 Hz, 1H), 2.84 (t, J = 2.4 Hz, 1H), 2.54 (td, J = 7.4, 4.0 Hz, 2H), 2.19 – 2.00 (m, 2H).

2.3.2 γ-propargyl l-glutamate N-carboxy anhydride (PLG-NCA) (2)

An oven-dried round-bottom flask was charged with **1** (3.5 g, 18.9 mmol), and 105 ml of anhydrous ethyl acetate was added to it in a nitrogen atmosphere. The reaction was kept on reflux, and triphosgene (1.63 g, 5.7 mmol) was added. After refluxing for 4 hr, the reaction solution was cooled to -20°C under a nitrogen atmosphere. Then, the flask was opened, and the solution was immediately washed with cold water and 5% sodium bicarbonate solution, both at 4°C. Anhydrous sodium sulfate was added to the collected organic layer. The transparent solution was filtered and concentrated to get yellow viscous oil as the product (2.0 g, Yield: 55%, contains ethyl acetate). ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.65 (s, 1H), 4.72 (d, J = 2.4 Hz, 2H), 4.44 (t, 1H), 2.62 (t, J = 6.9 Hz, 2H), 2.53 (t, J = 2.5 Hz, 1H), 2.35 – 2.12 (m, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 171.77, 169.29, 151.69, 75.55, 67.98, 56.85, 52.66, 29.61, 26.86. HRMS (ESI): expected for $[C_9H_{11}NO_5]^+$, calculated 212.0559, found 212.0911

2.3.3 PEG-b-poly (γ-propargyl l-glutamate) (PEG5k-b-PPLG) (3)

Two Schlenk tubes were charged with **2** (105 mg, 0.50 mmol) and PEG5k-NH_2 (100 mg, 0.02 mmol), respectively, and flushed with nitrogen gas. 3 ml of anhydrous chloroform was added to the PLG-NCA and 2 ml was added to the PEG5k-NH_2. Acetic acid (54 µl, 0.50 mmol) was added to the stirring solution of PLG-NCA, and the PEG5k-NH_2 solution was immediately transferred into that. After completion of the polymerization, as monitored by FT-IR Spectroscopy, the chloroform was removed using reduced pressure, and the polymer was precipitated in diethyl ether: methanol (5:1). The precipitated polymer was centrifuged and dried under a high vacuum to get white powder (150 mg, Yield: 82%).

2.3.4 (4-(Azidomethyl)phenyl)methanol (4)

1,4-Benzenedimethanol (4 g) was stirred in 150 ml toluene and 10 ml concentrated HCl was added and stirred for 12 hr. The TLC of two phases showed the presence of product only in the toluene layer and the presence of starting material in the aqueous layer. The aqueous HCl layer was then removed using a separatory funnel. 50 ml ethyl acetate was added, and two washes of 100 ml saturated sodium bicarbonate were given to the organic layer. The organic layer was then dried over sodium sulfate and concentrated to yield a yellowish-white crystalline compound. Without any subsequent purification, it was dissolved in 10 ml DMF, and sodium azide was added and stirred for 24 hrs at room temperature. 50 ml water was added and extracted thrice with 10 ml ethyl acetate. The combined organic layer was washed five times with 10 ml distilled water to remove excess DMF. The organic layer was then dried over sodium sulfate and concentrated to obtain a yellowish liquid. The obtained product was purified by column chromatography by ethyl acetate/pet ether (1:10) to yield clear liquid (yield) 1H NMR (400 MHz, $CDCl_3$) δ 7.39 (d, J = 8.0 Hz, 2H), 7.31 (d, J = 8.0 Hz, 2H), 4.70 (s, 2H), 4.33 (s, 2H).

2.3.5 Tert-butyl 3-(((1r,4r)-4-hydroxycyclohexyl)oxy)propanoate (5)

1,4-Cyclohexanediol (35.0 g, 301.0 mmol) and potassium t-butoxide (350 mg, 6.23 mmol) were suspended in freshly prepared anhydrous THF (400 mL) and stirred for 30 min under nitrogen atmosphere at 40°C. t-Butyl acrylate (30.8 g, 241 mmol) dissolved in anhyd. THF (100 mL) was added dropwise using a constant pressure funnel, and the reaction mixture was refluxed for 24 h. The insoluble solid was filtered using a Buchner funnel. THF was removed under reduced pressure and mild heating

and DCM was added to precipitate unreacted diol. The residue was again filtered over Buchner funnel and DCM was rota-evaporated to get viscous liquid. This was then purified by column chromatography using silica column and ethyl acetate and pet-ether (1:10) as the eluting phase. Yield: 16.8 g (23%). ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 3.64 (m, 3H), 3.29−3.39 (m, 1H), 2.4 (t, 2H), 1.96−1.81 (m, 4H,), 1.64−1.32 (m, 4H), 1.45 (s, 9H). ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm: 171.08, 80.42, 69.50, 63.93, 63.53, 32.55, 30.33, 29.18, and 27.43. FT-IR (cm−1): 3421, 2978, 2935, 2863, 1728, 1455, 1393, 1366, 1255, 1155, 1107, and 1034.

2.3.6 Tert-butyl 3-((4-oxocyclohexyl)oxy)propanoate (6)

5 (9.5 g, 38.9 mmol) was dissolved in dry DCM (100 mL) and activated molecular sieves were added, and the solution was maintained under a nitrogen atmosphere. PCC (16.7 g, 77.9 mmol) was added to the reaction mixture and the color of the reaction turned dark maroon. The solution was stirred at 25 °C for 4 h. The sticky reaction mixture was filtered over Whatman filter paper, by multiple rinsing of DCM. The filtrate was concentrated, and the resultant liquid was purified by column chromatography. passing through silica gel column by eluting with pet ether/ ethyl acetate (1:4) was used to elute the compound through the column to obtain **6** as a colorless liquid. Yield: 8.7 g (94%). ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 3.56 (m, 3H), 2.58 (t, 2H), 2.64 (m, 2H), 2.26 (m, 2H), 2.09 (m, 2H), 1.90 (m, 2H), 1.45 (s, 9H). ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm: 211.40, 170.98, 80.53, 72.74, 64.01, 37.03, 36.57, 30.39, and 20.04. FT-IR (cm−1): 2974, 2874, 2361, 1716, 1455, 1419, 1393, 1367, 1306, 1249, 1211, and 1101.

2.3.7 Tert-butyl 3-((7-oxooxepan-4-yl)oxy)propanoate (7)

6 (5 g, 21 mmol) was dissolved and stirred in dry DCM (20 mL) under nitrogen atmosphere and m-Chloroperbenzoic acid (7.1 g, 42 mmol) was slowly added to it. Anhyd. NaHCO3 was added to the reaction mixture (5.2 g, 62 mmol), and the reaction was stirred for 12 h at 25°C. The reaction mixture was filtered and the filtrate was rota-evaporated. The resultant liquid was dissolved in ethyl acetate, washed with water, saturated $NaHCO_3$ and brine. The organic layer was dried over anhydrous Na_2SO_4. After rota-evaporation of the solvent, the crude product was purified by column chromatography using pet ether/ ethyl acetate (4:6) to obtain **7** as a slightly viscous liquid. Yield = 4.0 g (76%). The product 3 was obtained as colorless liquid. ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 4.40 (dd, 1H), 4.06 (dd, 1H), 3.60 (m, 4H), 2.98 (dd, 1H),

2.48 (t, 2H), 2.42−1.81 (m, 4H), 1.46 (s, 9H). ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm: 176.55, 171.23, 80.99, 74.23, 64.24, 63.63, 36.77, 34.14, 28.42, 27.82, and 27.59. FT-IR (cm−1): 2926, 1725, 1455, 1393, 1367, 1253, 1156, 1100, and 1057.

2.3.8 Polycaprolactone-azide (8)

- *Catalyst: $Sn(oct)_2$*

To an oven-dried Schlenk tube, cooled in presence of nitrogen, 1.0 g (M/I = 20) ε-caprolactone was weighed. 4-azidomethyl benzyl alcohol (71 mg, 1 eq.) and catalyst $Sn(oct)_2$ (45 mg, 0.5 eq.) was added as the initiator. The Schlenk tube was connected to high vacuum to remove any residual amount of moisture. The Schlenk tube was put in an oil bath at 110°C. After 2 hr, the reaction was cooled down and dissolved in 1 ml THF and precipitated in 50 ml cold methanol. The precipitate was filtered and dried to obtain the final polymer.

- *Catalyst: MSA*

To an oven-dried Schlenk tube, cooled in presence of nitrogen, 1.0 g (M/I = 20) ε-caprolactone was weighed. 4-azidomethyl benzyl alcohol (71 mg, 1 eq.) was added as the initiator. The Schlenk tube was connected to high vacuum to remove any residual amount of moisture, and flushed with nitrogen gas. Dry DCM (10 ml) was added to it using a needle via a septum. The reaction mixture was heated to 30°C and methanesulfonic acid (85 µl, 3 eq.) was added to start the polymerization reaction. The reaction was quenched after 2 hr by giving a brine wash to the DCM layer, which was then dried using sodium sulfate, concentrated and precipitated in cold methanol.

2.3.9 Substituted Polycaprolactone-azide (9)

- *Catalyst: $Sn(oct)_2$*

To an oven-dried Schlenk tube, cooled in presence of nitrogen, 1.0 g (M/I = 20) substituted-caprolactone was weighed. 4-azidomethyl benzyl alcohol (71 mg, 1 eq.) and catalyst $Sn(oct)_2$ (45 mg, 0.5 eq.) was added as the initiator. The Schlenk tube was connected to high vacuum to remove any residual amount of moisture. The Schlenk tube was put in an oil bath at 130°C. After 3 hr, the reaction was cooled down and dissolved in 1 ml THF and precipitated in 50 ml cold diethyl ether: hexane (3:2) and stored at -20°C overnight. The solvent was

decanted and the viscous liquid was dried using high vacuum to obtain the final polymer.

- *Catalyst: MSA*

To an oven-dried Schlenk tube, cooled in presence of nitrogen, 1.0 g (M/I = 20) substituted-caprolactone was weighed. 4-azidomethyl benzyl alcohol (32 mg, 1 eq.) was added as the initiator. The Schlenk tube was connected to high vacuum to remove any residual amount of moisture, and flushed with nitrogen gas and dry DCM (10 ml) was added. The reaction mixture was heated to 30°C and methanesulfonic acid (12.6 µl, 1 eq.) was added to start the polymerization reaction. The reaction was quenched after 2 hr by giving a brine wash to the DCM layer, which was then dried using sodium sulfate, concentrated and precipitated in 50 ml cold diethyl ether: hexane (3:2) and stored at -20°C overnight. The solvent was decanted and the viscous liquid was dried using high vacuum to obtain the final polymer.

2.3.10 PCL-g-(PEG5k-b-PPLG)) 10

1. PEG-b-PPLG (10 mg, 27 eq. propargyl groups), PCL (89 mg, 26*27=702 eq. azide groups) and 1 ml DMSO were taken in a Schlenk tube, and $CuSO_4$ (3.5 mg, 0.5 eq.) was added, and the reaction was kept for stirring at 60°C. After the complete dissolution of all the contents, sodium ascorbate (6.6 mg, 1.2 eq.) was added. The solution was stirred for 4 hr. Then the solution was cooled and 5 ml dichloromethane (DCM) was added and the solution was washed with water 3x5 ml and brine 2x5 ml and dried using sodium sulfate, concentrated using reduced pressure, and then precipitated in cold methanol and stored at -20°C for better precipitation and then filtered and dried to obtain the final polymer.

2. PEG-b-PPLG (10 mg, 27 eq. propargyl groups), PCL (89 mg, 26*27=702 eq. azide groups) and 1 ml DMF were taken in a Schlenk tube, and the solution was purged with nitrogen gas for 30 min to deoxygenate the solution. PMDETA (2.0 µl, 9.5 µmol) followed by CuBr (1.36 mg, 9.5 µmol) was added to the solution. As soon as CuBr was added the solution turned from colorless to intense blue. The reaction mixture was kept for stirring at rt for 24 hrs. Then the solution was cooled and 5 ml dichloromethane (DCM) was added to it and the solution was washed with water 3x5 ml and brine 2x5 ml and dried using sodium sulfate, concentrated using reduced pressure, and then

precipitated in cold methanol and stored at -20°C for better precipitation and then filtered and dried to obtain the final polymer.

2.3.11 BPCL-g-(PEG5k-b-PPLG) 11

1. PEG-b-PPLG (10 mg, 27 eq. propargyl groups), BPCL (158 mg, 27*21=567 eq. azide groups) and 1 ml DMSO were taken in a Schlenk tube, and $CuSO_4$ (3.5 mg, 0.5 eq.) was added, and the reaction was kept for stirring at 60°C. After the complete dissolution of all the contents, sodium ascorbate (6.6 mg, 1.2 eq.) was added. The solution was stirred for 4 hr. Then the solution was cooled and 5 ml dichloromethane (DCM) was added and the solution was washed with water 3x5 ml and brine 2x5 ml and dried using sodium sulfate, concentrated using reduced pressure, and then precipitated in cold methanol and stored at -20°C for better precipitation and then filtered and dried to obtain the final polymer.

2. PEG-b-PPLG (10 mg, 27 eq. propargyl groups), BPCL (158 mg, 27*21=567 eq. azide groups) and 1 ml DMF were taken in a Schlenk tube, and the solution was purged with nitrogen gas for 30 min to deoxygenate the solution. PMDETA (2.0 µl, 9.5 µmol) followed by CuBr (1.36 mg, 9.5 µmol) was added to the solution. As soon as CuBr was added the solution turned from colorless to intense blue. The reaction mixture was kept for stirring at rt for 24 hrs. Then the solution was cooled and 5 ml dichloromethane (DCM) was added to it and the solution was washed with water 3x5 ml and brine 2x5 ml and dried using sodium sulfate, concentrated using reduced pressure, and then precipitated in diethyl ether: hexane (3: 2) and stored at -20°C for better precipitation and then the solvent wad decanted and the settled viscous liquid was dried using high vacuum to obtain the final polymer.

3 RESULTS AND DISCUSSION

3.1 POLY (PROPARGYL L-GLUTAMATE)

Poly (propargyl l-glutamate) PPLG was first reported by Hammond et al. (2009)[18] as a polypeptide that can undergo highly efficient grafting of PEG polymers of different chain length owing to its α-helix structure which reduces the steric hinderance. Following this, PPLG has been used extensively to create grafts of various polymers and small molecules[19–22].

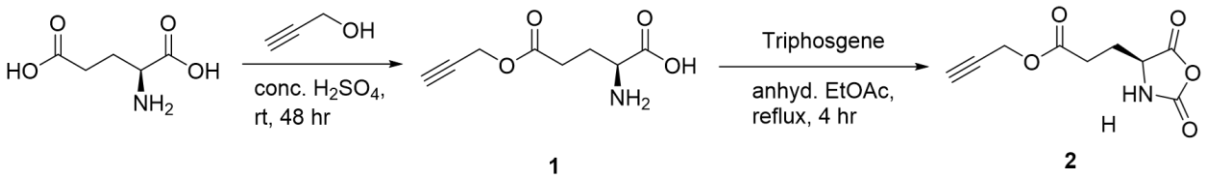

Figure 4 Synthetic scheme of PLG-NCA

γ-Propargyl l-glutamate is synthesized starting from l-glutamic acid and propargyl alcohol using Fischer esterification with catalytic concentrated sulfuric acid according to the literature reports[6]. The reaction carried out at room temperature and yielded selective esterification at γ position. The synthesized amino acid is then purified by reprecipitation in 3:2 ethanol: water to remove residual propargyl alcohol and sodium sulphate which is generated during neutralization. The formation of pure amino acid was confirmed by ^{1}H NMR (Figure S1.).

Table 1 Procedures attempted for the synthesis of PLG-NCA and the results

Reaction Solvent	Workup	Precipitation	Result	Polymerization
THF	cold water, NaHCO3	Yes	Cloudy solution + white solid	-
THF	No work up	Yes	Viscous oil + cloudy precipitate	No, NCA was consumed after 7 days
Ethyl acetate	cold water, NaHCO3	No	Clear viscous oil	Polymerized in chloroform

Although alkyl glutamates are easy to synthesize the N-Carboxy Anhydrides are prone to easy hydrolysis upon exposure to moisture. Alkyl glutamate NCAs are isolated as

a viscous liquid and handling it becomes a difficult task. There are many different procedures available but only a few are practical under even mild ambient conditions. PLG-NCA when synthesized in THF using triphosgene and precipitated using THF/hexane combination forms viscous liquid containing cloudy solid precipitate of hydrolyzed NCA. This occurs even though both solvents are used in their anhydrous form. Resorting to the synthesis in ethyl acetate with less than 0.33 equivalents of triphosgene, avoids the need of reprecipitation[23]. But this is not possible with most types of amino acid as they are not as highly souble in water as alkyl glutamates. In this procedure of synthesizing NCA, PLG-NCA hydrolyzed back to the amino acid can be removed during water washes to the organic layer. Moreover, oligomerized NCA is insoluble in ethyl acetate and can be filtered using a 0.22μ filter to get PLG-NCA as clear viscous oil.

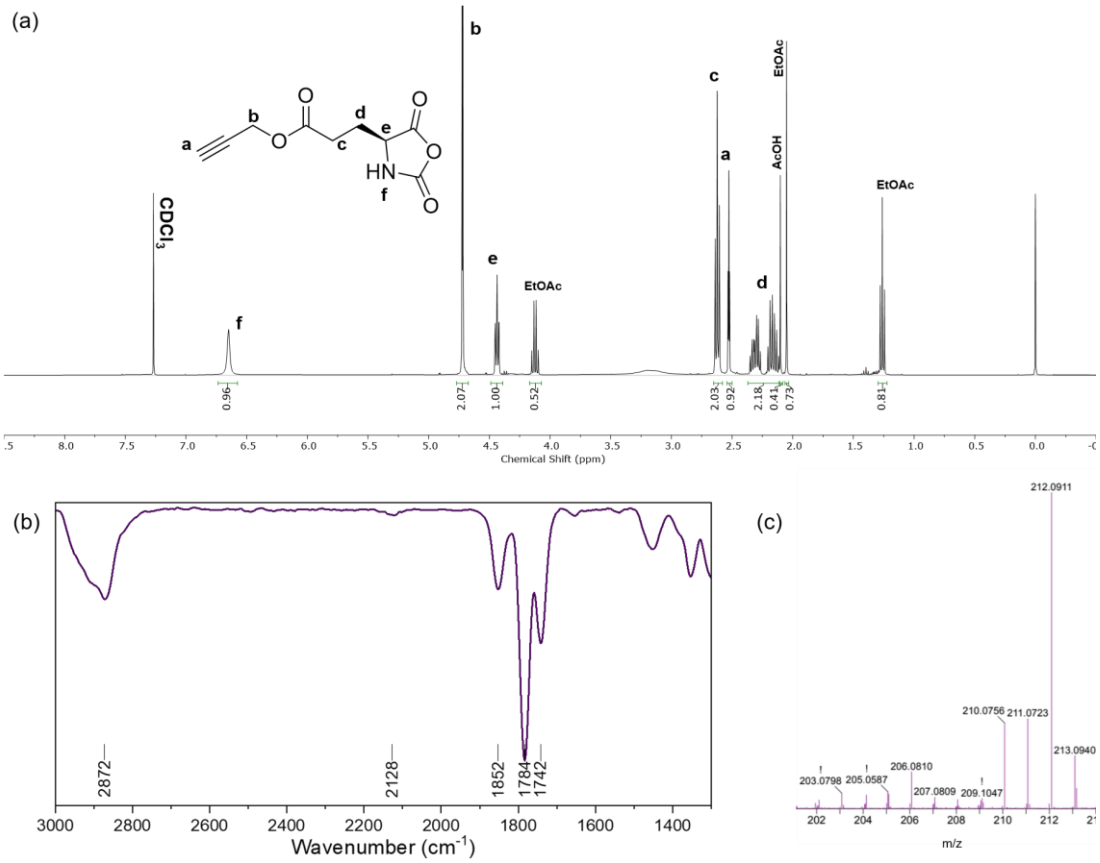

Figure 5 Characterization of PLG-NCA

(a) NMR of finally obtained polymerizable monomer (b) FT-IR spectrum showing characteristic peaks (c) HR-MS spectrum

It was observed that in presence of ethyl acetate in small amount this NCA can tolerate some exposure to normal conditions. Even lyophilized samples of the NCA tend to

become cloudy, an indication of hydrolysed NCA. The NCA obtained even after synthesis in ethyl acetate contained some impurities which are difficult to separate viz. ethyl chloroformate and acetic acid. This NCA could not be polymerized in highly polar solvent like DMF or even in chloroform-DMF combination. The origin of ethyl chloroformate is ethanol which, along with acetic acid, can come from impure ethyl acetate. Freshly purified ethyl acetate was used to finally eliminate the impurity of ethyl chloroformate. The polymerization of this NCA could be successfully completed in chloroform or DCM as a solvent. The final polymerizable NCA was characterized by NMR, FTIR and HRMS. NMR of the NCA shows peak at 6.6 ppm corresponding to N-H in the anhydride ring (Figure 5a). All other protons show expected intensities when C-H, the chiral proton of amino acid is assigned the value 1.00. The alkynyl C-H shows proper integration value although it showed integration of 0.7 in the NMR of amino acid in D_2O as compared to other peaks (Appendix). The FT-IR spectrum of PLG-NCA shows clear peaks at 1852 cm^{-1} and 1784 cm^{-1} which correspond to the symmetric and asymmetric stretching frequencies of the anhydride carbonyls (ref?) (Figure 5b). The propargyl ester peak at 1742 cm^{-1} and alkynyl C-H peak at 2128 cm^{-1} is also observed. The HR-MS data also confirmed the formation of PLG-NCA showing peak at 212.0911 which matches with the calculated value of 211.0481 (Figure 5c).

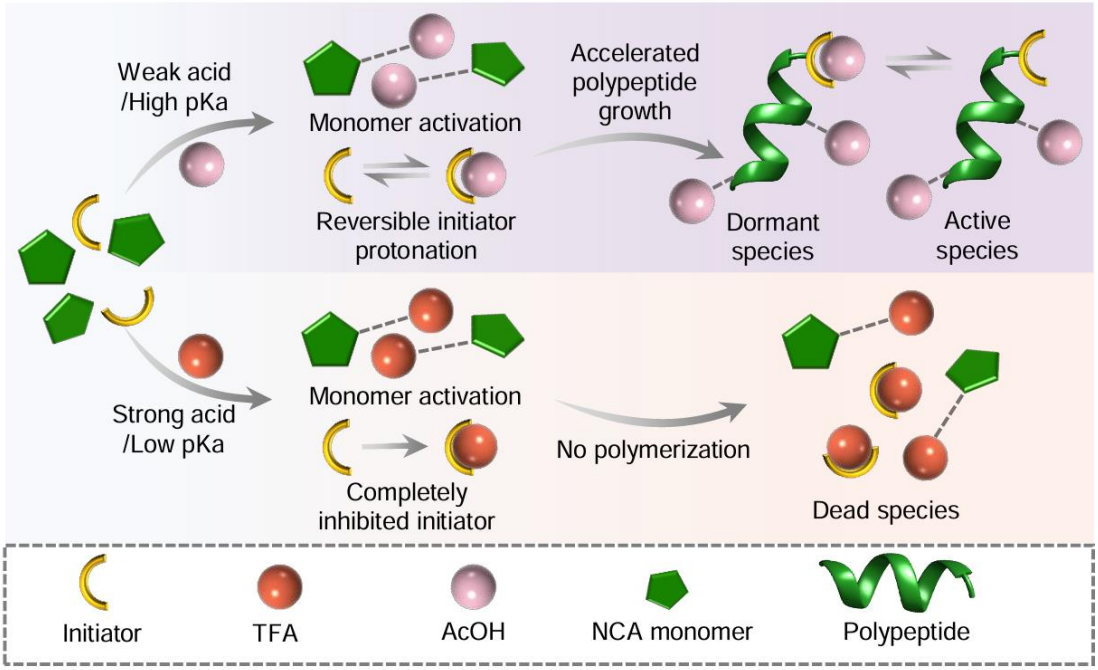

Figure 6 Effect of the strength of acid on acid-catalysed NCA polymerization **|** *adapted from: ChemRxiv,* ***Liu, X.*** **et al.** ***(2023)***

A recent paper yet from ChemRxiv by Cheng et al. proposes accelerated NCA-polymerization using acid catalyst in less polar solvents like chloroform and dichloromethane[24]. According to the authors, the polymerization is driven by activated monomer mechanism. After screening for multiple acids for their catalytic ability they have found acetic acid as the best candidate. In contrast to strong acids like trifluoroacetic acid, which protonate the initiators and hinder the polymerization reactions, acetic acid which is a weaker acid, activates the monomer while protonating the initiators reversibly to produce narrowly dispersed polymers (Figure 6).

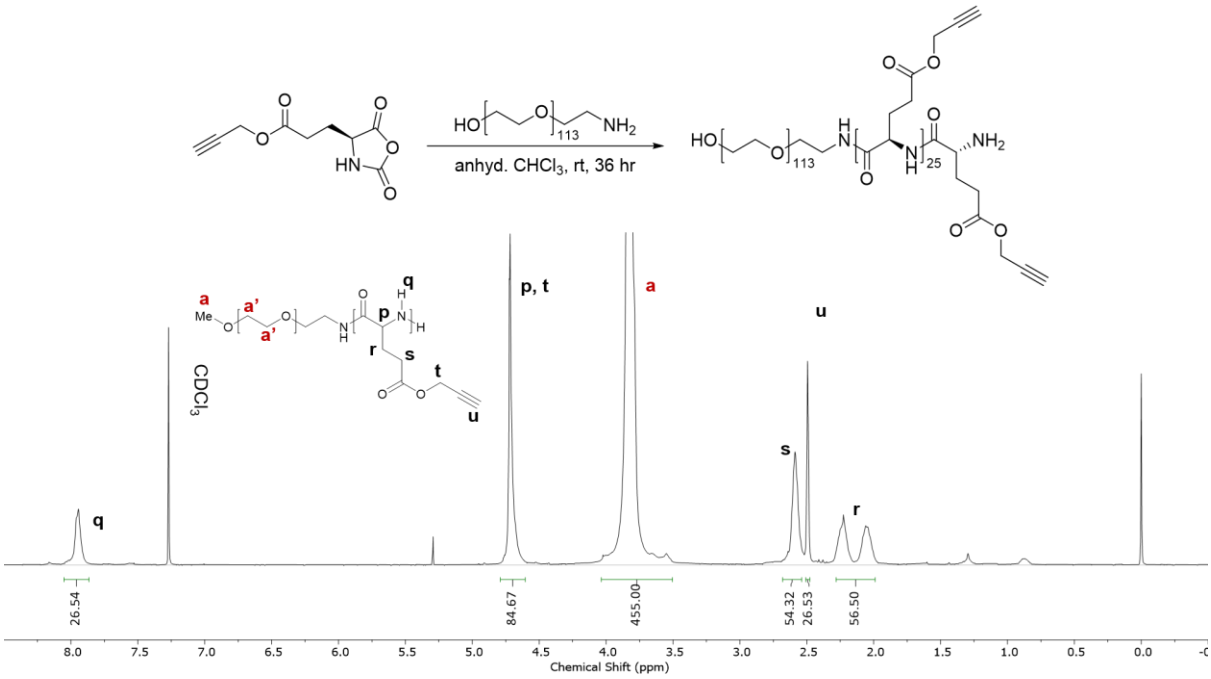

Figure 7 Synthetic scheme and NMR analysis of PEG-b-PPLG25

The number of units can be calculated by integrating peg protons to be 113 units*4H = 455H, and the peak **u** corresponding to the alkyne CC*H* can be found to be 26.53 which can be approximated to 27

Table 2 Acetic acid catalyzed polymerization of PLG-NCA

Sr. No.	Polymer	M/I	M (theor.)	M_n- NMR	M_n- GPC	M_w- GPC	Đ
1	PEG5k-PPLG50	50:1	13300	12700	11700	14700	1.26
2	PEG5k-PPLG25	25:1	9200	9300	10300	13300	1.29

Typically, Cheng et al. have used 100/1 acetic acid to initiator ratio to obtain well defined, narrow dispersed polymers. Therefore, using acetic acid with the same ratio has made it possible to polymerize PLG-NCA in less amount of time. The polymers synthesized through this procedure have moderate polydispersity index (Table 2). A good control in aimed and obtained number of units was observed in this polymerization. The NMR spectra of PEG-b-PPLG25 submitted in $CDCl_3$-TFA (5:1) showed shift of N-H from 6.6 ppm to 7.9 ppm confirming conversion of anhydride to amide (Figure 7). After integrating protons of PEG which in case of PEG-5k are 455, each proton belonging to the peptide shows integration value of 27. The formation of well-defined polymers was further confirmed by Gel-permeation Chromatography. The gel-permeation chromatograms of PEG-PPLG deblocks recorded using RI detectors with dimethylformamide as the mobile phase showed monomodal and moderately disperse peaks. For instance, PEG-b-PPLG 25 shows clear shift in elution time of the polymer as compared to the first block which is PEG5k-NH_2 (Figure 8). Similarly, the MALDI-TOF analysis of the polymer shows the merged peaks of di-block copolymer at mass greater than 7000 g/mol along with the lysed PEG peak.

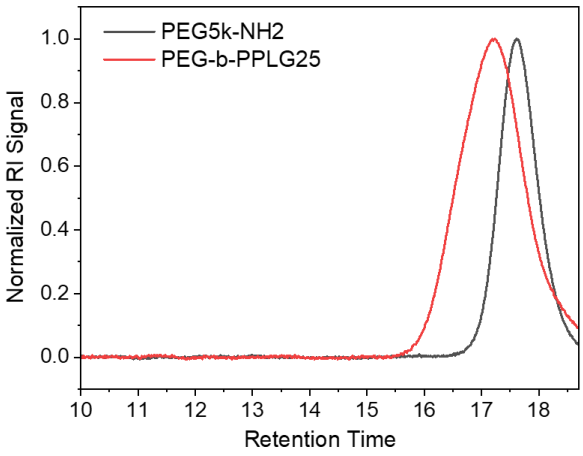

Figure 8 Gel-Permeation chromatogram of PEG and PEG-b-PPLG25
GPC of these polymers was recorded with polystyrene as the standard and DMF as the eluting solvent.

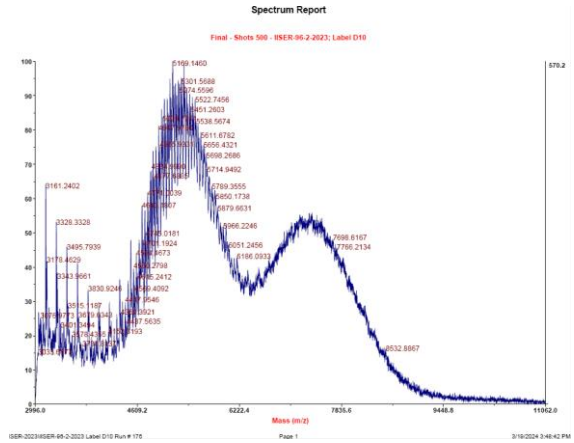

Figure 9 MALDI-TOP spectrum of PEG-b-PPLG25
MALDI-TOF shown here was recorded using mixed matrix in linear mode

The FT-IR spectra shows major peaks at 1654 cm^{-1} and 1549 cm^{-1} which correspond to the stretching frequency of amide I and amide II respectively for an alpha-helical polypeptide (Figure 10b). The CD spectra of synthesized polymer also shows characteristic alpha helix minima at 210 nm and 222 nm (Figure 10a). Thus, the secondary structure of the polymer was confirmed and found to be in accordance with the literature reports[18].

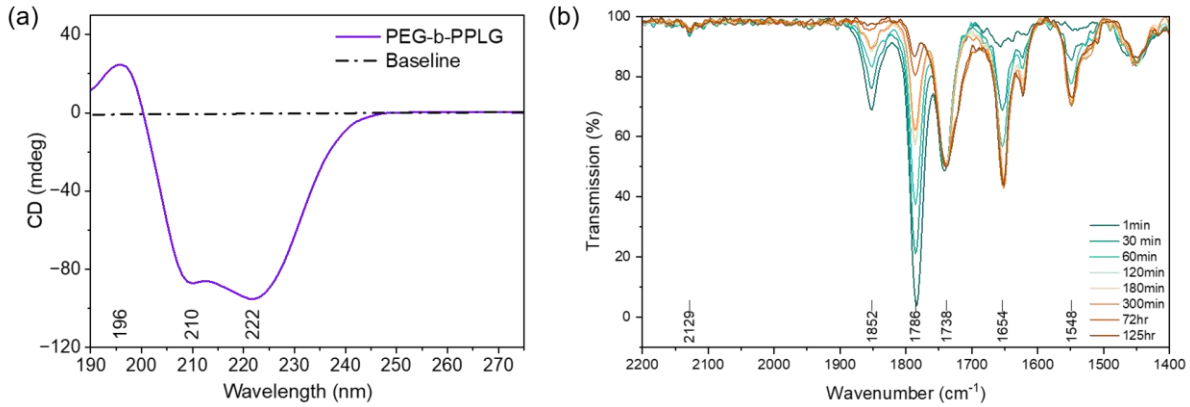

Figure 10 Secondary structural analysis of PEG-b-PPLG25
(a) CD spectrum of PEG-b-PPLG25. Measurement shown here is done in HFIP solvent. (b) FT-IR spectrum kinetics plot for PLG-NCA polymerization done in chloroform.

3.2 POLYCAPROLACTONE

3.2.1 The Monomers

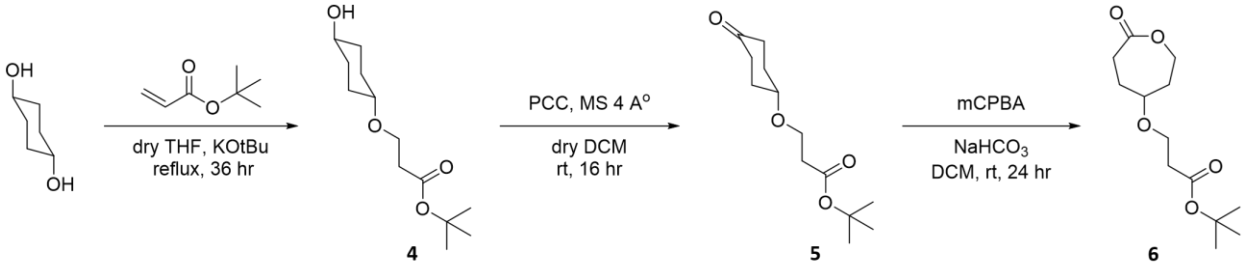

Figure 11 Synthetic scheme for tertiary butyl ε-caprolactone monomer

The ε-caprolactone monomer was distilled, the purity was confirmed using ^{1}H NMR, and directly used for polymerization. t-Butyloxycarbonyl substituted ε-caprolactone monomer was first reported by our lab[4] and was shown to be polymerizable to yield the substituted polycaprolactone (BPCL) as a biodegradable, biocompatible and amorphous polymer which has been shown to have application in drug delivery. The monomer was synthesized starting from Michael addition of tertiary butyl acrylate to one of the alcohols of cyclohexane-1,4-diol. This was followed by oxidation of the other alcohol to ketone and subsequent Baeyer-Villiger oxidation to obtain the final monomer as a viscous liquid. The formation of the monomer was confirmed by NMR spectroscopy and HR-MS. The protons a and a` corresponding to the $-CH_2-$ next to the cyclic ester in its two conformations can be found in the range of 4.0-4.5 ppm (Figure 12).

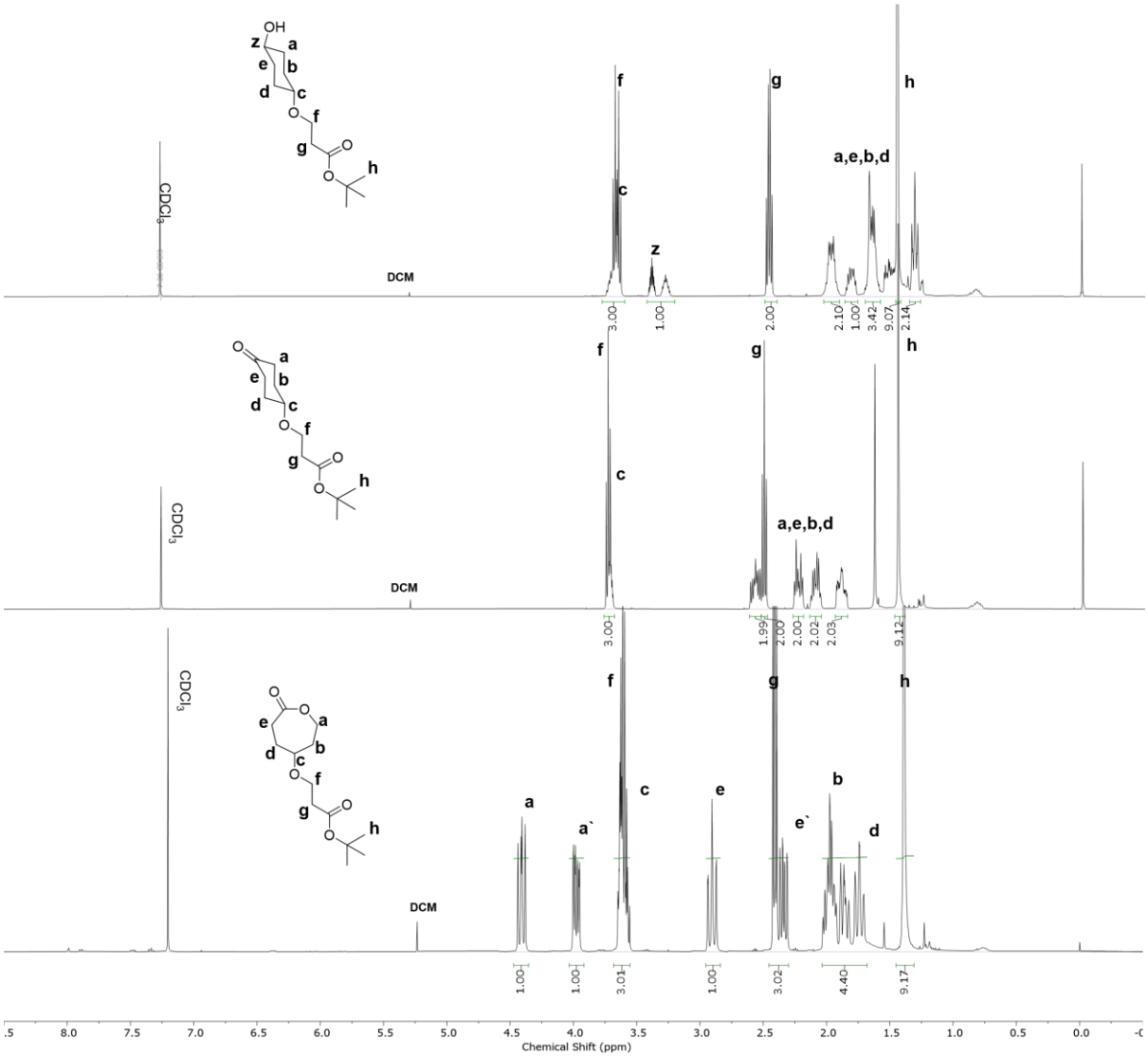

Figure 12 ^{1}H NMR of step-wise synthesis of substituted-caprolactone monomer

3.2.2 The Initiator

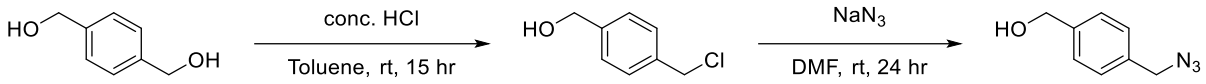

Figure 13 Synthetic scheme for azide-containing initiator

To click the substituted polycaprolactone on the PPLG, the monomer was initiated with an azide containing initiator. (a-azidomethylphenyl)methanol was used as the azide initiator since all the peaks can be observed distinctly from that of the polymer backbone in ^{1}H NMR for easy quantification. Post-polymerization conversion of chloride to azide is though possible, does not guarantee 100% conversion. Therefore, I have used azide containing initiator for the polymerization. The initiator was easily synthesized by monochlorination of 1,4-benzene dimethanol using concentrated HCl and toluene which is a biphasic reaction. 1,4-Benzene dimethanol is soluble in conc. HCl only and the monochlorinated product in the toluene layer alone. As soon as monochlorination happens the product migrates to the organic layer facilitating easy purification. This reaction is followed by simple nucleophilic substitution reaction using sodium azide with DMF as the solvent.

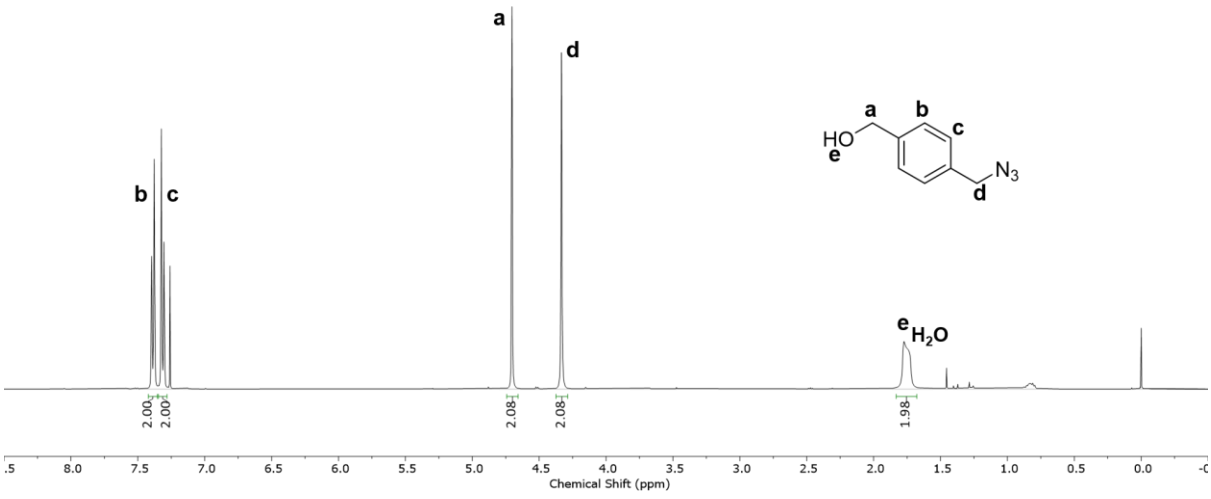

Figure 14 ^{1}H NMR of (4-azidomethylphenyl)methanol

3.2.3 Polymerization

The polymerization reaction was done using methanesulfonic acid (MSA) as the catalyst according to the reported procedure[25]. Generally, caprolactone polymerization is performed using $Sn(oct)_2$ as catalyst in melt condition[26] which has been followed in our lab in all previous reports. But addition of the tin catalyst into the polymer tube containing azide initiator and the monomer shows a slowly progressing bubbles of agas. To confirm the reaction of initiator with the catalyst 1: 1 ratio of both was mixed and submitted for NMR which showed minor but additional peaks of benzylic $-CH_2-$ and the intensity of $-CH_2-$ azide is reduced (Figure 15).

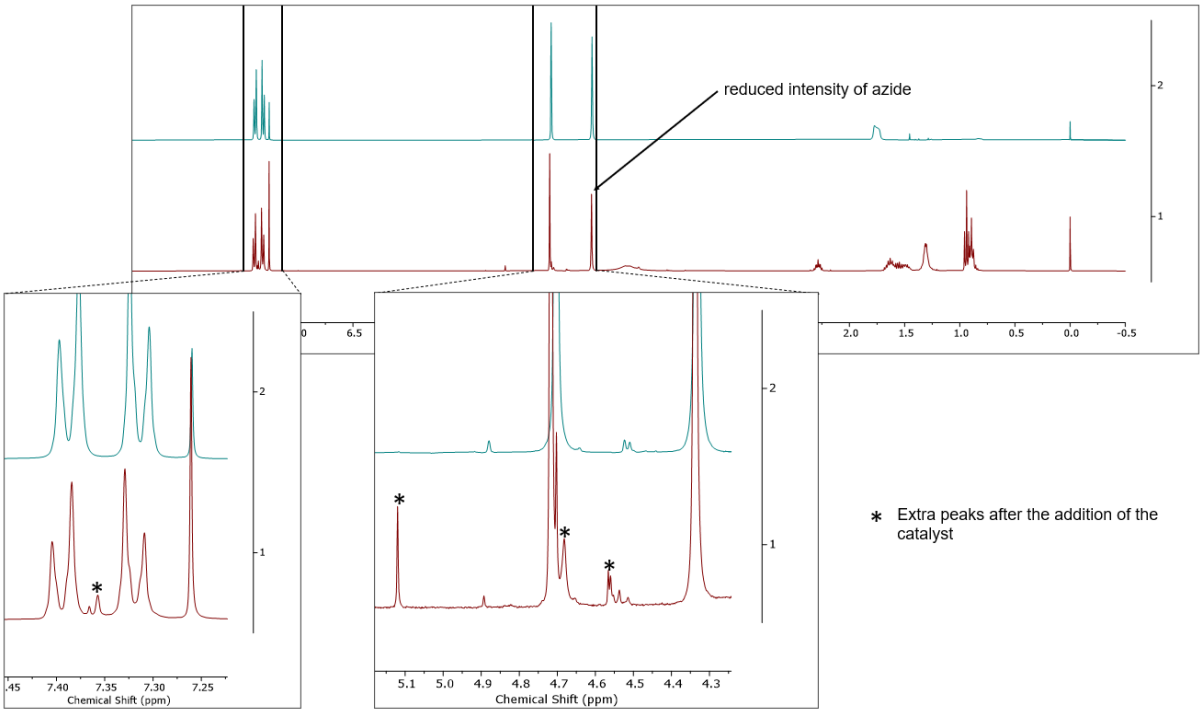

Figure 15 Confirmation of side-reactivity of initiator and the catalyst
^1H NMR in $CDCl_3$ of mixture (1:1) of catalyst Sn(oct)2 and (4azidomethylphenyl)methanol)

In NMR spectra of polymer synthesized by melt route, two peaks corresponding to benzylic ester and reduced -CH2- azide intensity is observed (

Figure S3.). Therefore, the polymerization of ε-caprolactone was carried out using MSA (3 eq.) as a catalyst at 30°C in dry DCM. M/I =20 was aimed, and DP =26 was obtained. In case of BPCL, the same reaction condition (3 eq., 30°C, 4 hr) yielded DP close to 20 but half the tertiary butyl groups were cleaved as confirmed by NMR (

Figure S4.). The original report of using MSA as a catalyst has shown that increasing the amount of MSA till 3 eq. increases the rate of reaction. But according to the mechanistic perspective, the

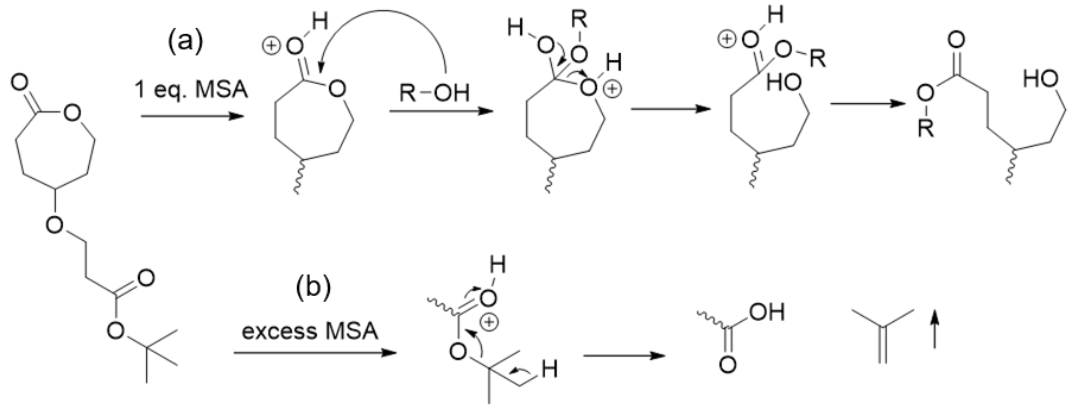

Figure 16 Reaction of MSA with the substituted caprolactone monomer
(a) Mechanism of acid-catalyzed polymerization of caprolactone monomer (b) Mechanism of deprotection of tertiary butyl ester.

requirement of catalyst, ideally, is one proton per progressing chain end. So, the excess acid can involve in deprotection of Boc- group, releasing isobutylene gas (Figure 16). shows two processes in the polymerization flask thet determine the structure of obtained polymer. By changing the reaction condition (1 eq., 30°C, 2 hr), well defined polymer with DP =21 with almost all tertiary butyl groups intact were obtained.

Table 3 PCL-N3 and BPCL-N3 characterization data by NMR and GPC

Sr. No.	Polymer	M/I (feed ratio)	DP-NMR	M_n- NMR	M_n- GPC	M_w- GPC	Đ
1	PCL-N3	20:1	26	3100	2800	5300	1.8
2	BPCL-N3	20:1	21	5600	3700	5600	1.4

The synthesized polymers were characterized by NMR, GPC and MALDI. Integrating benzylic ester -CH_2- at 5.2 ppm to be 2.0 gives DP= 26 for PCL-N3 and DP = 21 for BPCL-N3 with azide moiety intact showing integration value of 2.0 (Figure 18). GPC of both the polymers are moderately disperse (Figure 17). MALDI-TOF spectra show repeating unit mass difference and also indicate cleavage of N_2 as observed by vailue of each mass peak. This can happen during ionization of the molecules (Figure S5., Figure S6.).

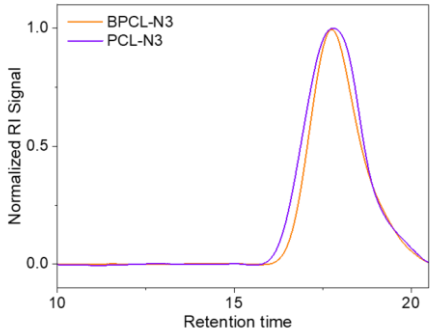

Figure 17 GPC of PCL and BPCL polymers.
GPC of these polymers was recorded with polystyrene as the standard and chloroform as the eluting solvent.

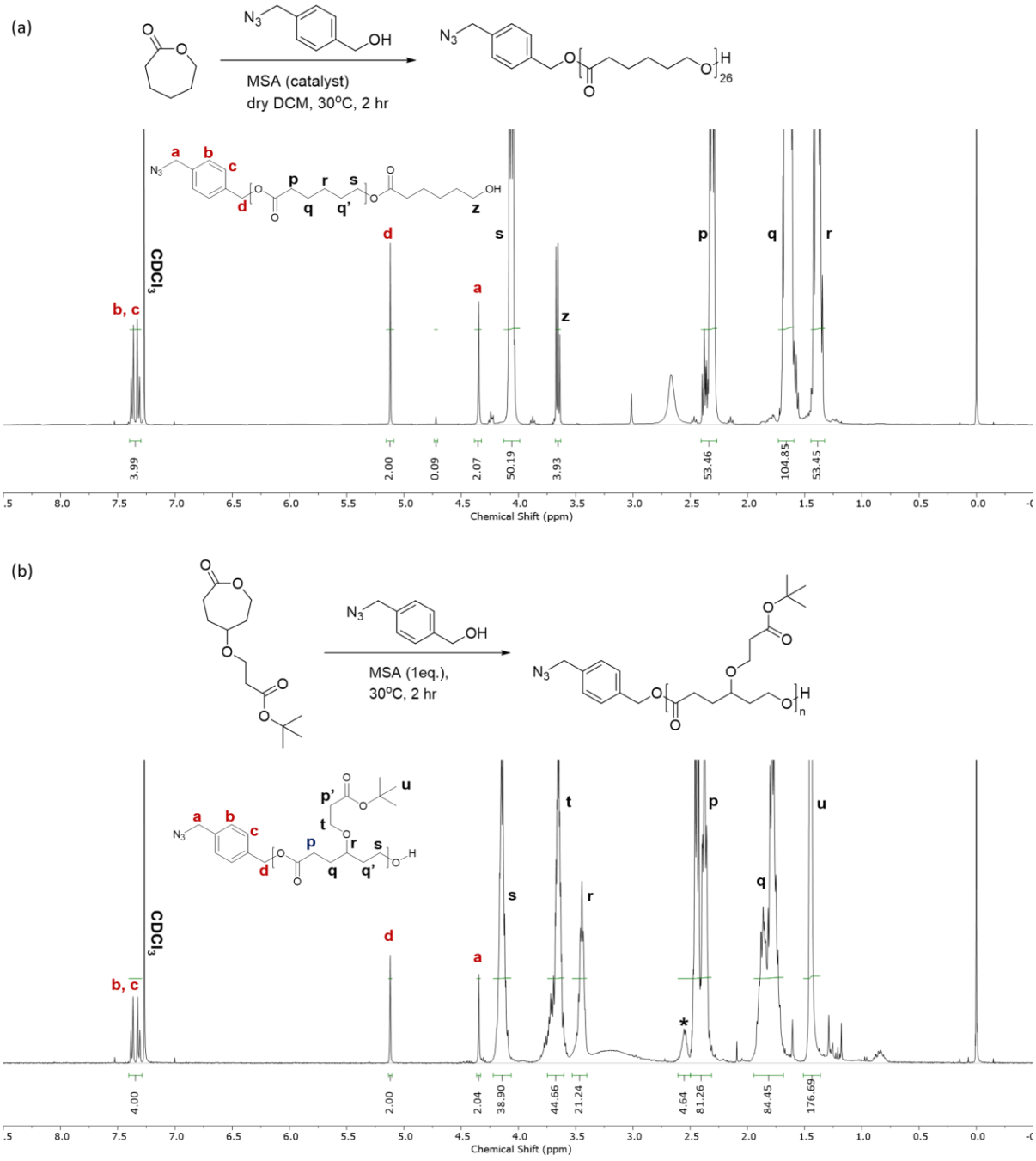

Figure 18 Synthetic and NMR characterization of polycaprolactone-azides

(a) NMR of polycaprolactone showing **d** in the initiator assigned 2H and 50H value obtained for other esters in polymer backbone indicating DP = 26. (b) NMR of substituted polycaprolactone showing **d** in the initiator assigned 2H and 39H value obtained for other esters in polymer backbone indicating DP = 20.5 approximated to 21.

3.3 CLICK REACTION

Click reaction was tried using two routes: CuBr/PMDETA/DMF/rt and $CuSO_4$/Na-ascorbate/DMSO/60°C (Figure 19). Both reaction routes showed similar results. All the click reactions were carried out with 1:1 alkyne to azide ration. Polycaprolactones cannot be removed by dialysis using water as it is insoluble in it. Also, the reconstructed-cellulose dialysis tubes can show wear and tear in high concentrations of organic polar solvents like DMSO or DMF in which unreacted monomer removal was possible. Therefore, we have not done grafting for a higher ratio of polymer azides and the alkyne groups on PPLG. According to the NMR of PCL-g-(PEG-b-PPLG25), it is apparent that almost hundred percent grafting has been achieved (Figure 21). The peak around 5.5 ppm corresponds to the -CH_2- between triazole and benzene ring of the initiator. When these protons are assigned integration of 2, PEG protons match to be around 452 (theoretical) indicating presence of backbone polymer, though the other protons of repeating unit are not visible which may be due to their overlap with bigger PCL peaks or complete shielding due to heavy side-chains. Integration of the proton in -*CH_2*-O-(C=O)- at 4.1 ppm for complete grafting should come to be 1304. The observed integration: 1296 which indicates almost 100% grafting of PCL over PPLG. Though this is the case, GPC shows unreacted polymer-azide peak (Figure 20). An increase in molecular weight in case of both BPCL-N3 and PCL-N3, is observed, although the overall chromatogram is bimodal. The values of molecular weight shown by GPCs are less reliable compared to NMR because the standards which we use for recording GPC are for linear polymers (Table 4). This standard when used for a short and wide brush like this would highly under-estimate the molecular weight of the polymer brush. Therefore, most likely, actual molecular grafting efficiency is more than what GPC shows.

Table 4 Graft polymer characterization

Sr. No.	Polymer	Feed ratio	M_n- NMR	M_n- GPC (New Peak)	M_w- GPC (New Peak)	Đ
1	PCL-g-(PEG-b-PPLG25)	1:1	93000	31200	38900	1.25
2	BPCL-g-(PEG-b-PPLG25)	1:1	294000	50100	67300	1.34

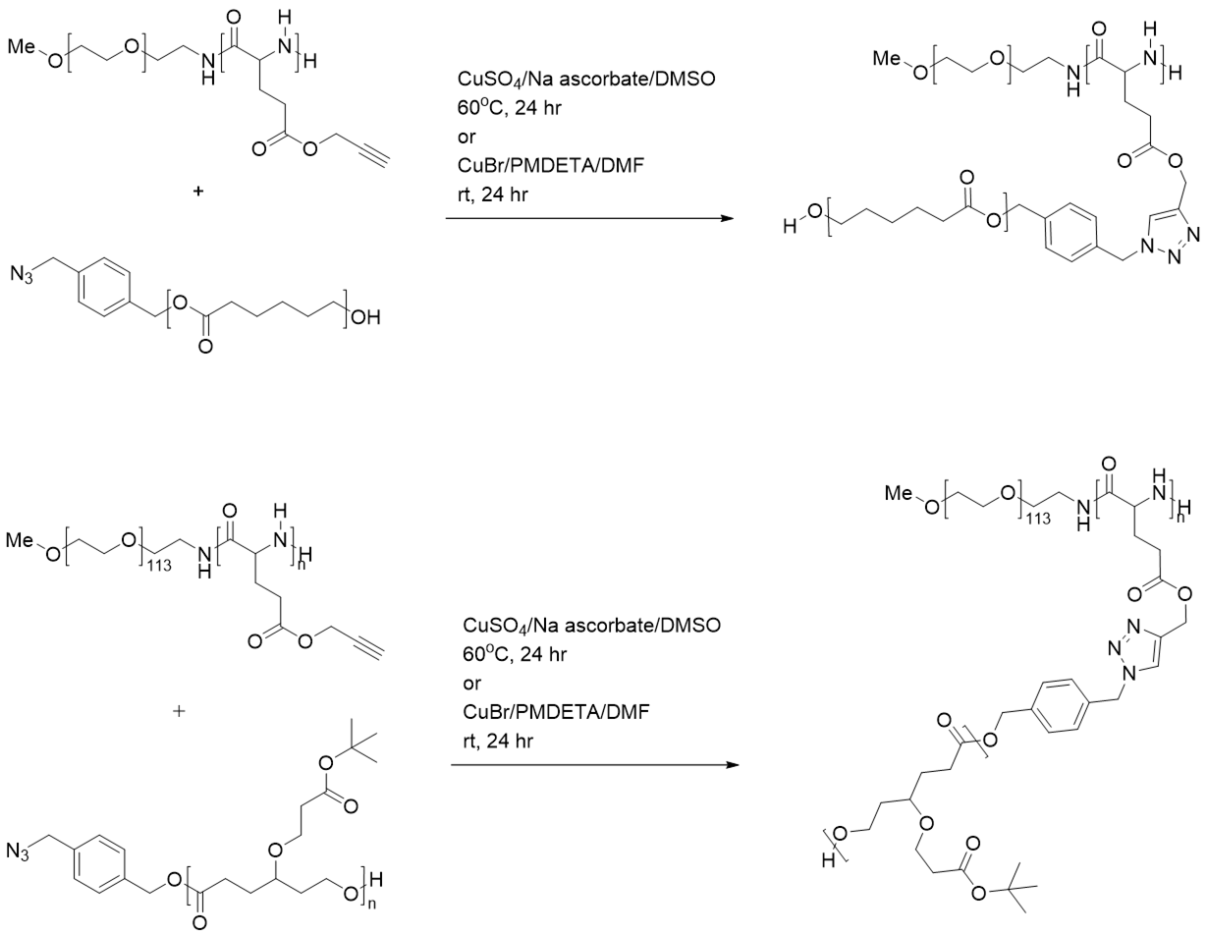

Figure 19 Synthetic scheme of click reaction of polycaprolactone azides and PPLG

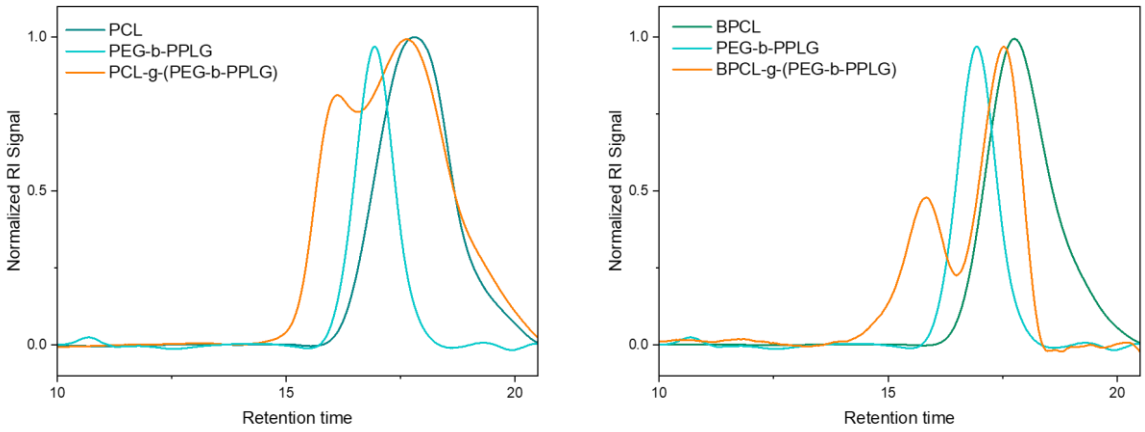

Figure 20 GPC comparison of before and after click reaction

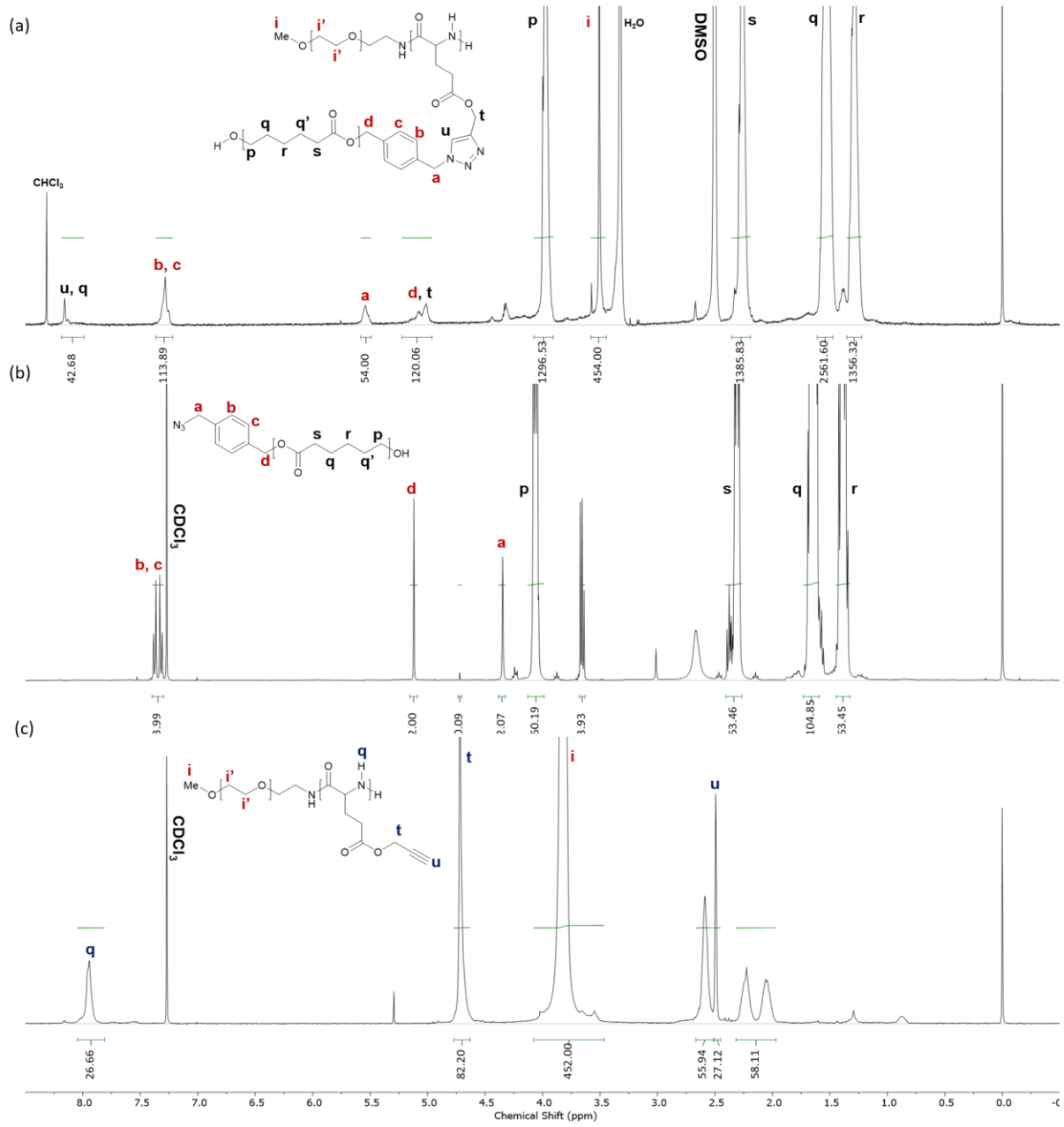

Figure 21 ^{1}H NMR of PEG-b-PPLG, PCL and PCL-g-(PEG-b-PPLG)
(a) NMR of PEG-b-(PCL-g-PPLG) in DMSO-d6 showing almost 100

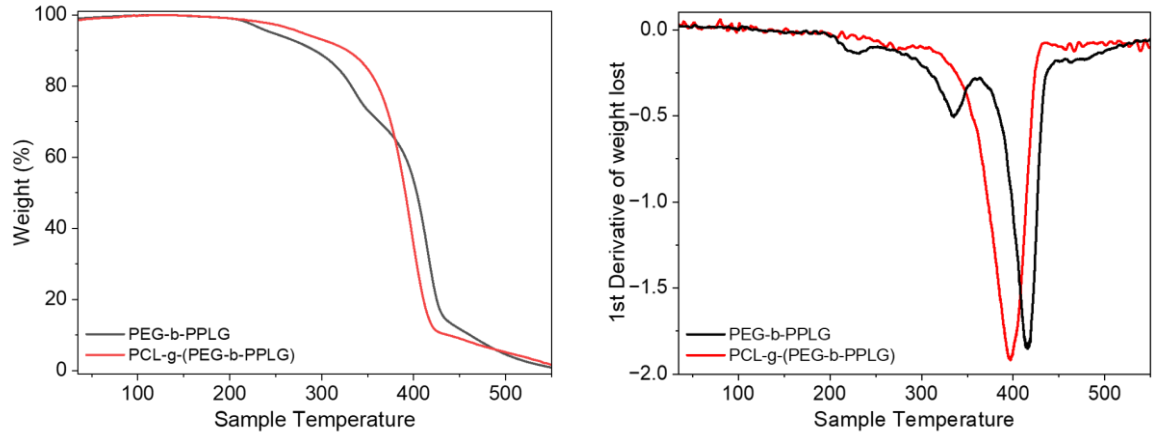

Figure 22 TGA plot and 1st derivative plot of polymer degradation before and after grafting

The thermogravimetric analysis of PEG-b-PPLG shows a three-step degradation process corresponding sequential degradation of propargyl ester, polypeptide bonds and polyethylene glycol at 231°C, 333°C and 414°C respectively. The PCL grafted polymer shows degradation steps at 274°C and 396°C, respectively. The lower degradation at 274°C is most likely due to the polypeptide backbone and the benzyl ester which connects PCL to the backbone. The later degradation step is of PCL backbone and some contribution due to the PEG chain which is around 5 % in weight.

4 CONCLUSION

In this study, poly (γ-propargyl glutamate) (PPLG) was successfully synthesized after meaningful optimization of existing protocols for synthesizing PLG-NCA and the subsequent polymer. Tertiary butyl substituted caprolactone monomer was synthesized according to procedure developed in our lab. A highly characterization-efficient, easy-to-synthesize, azide-containing initiator was used to polymeriza polycaprolactones. After confirmation of reaction of azide in the intiator with catalyst in melt-route, a polymerization route well-documented for PCL but new to tertiary butyl caprolactone was explored viz. methanesulfonic acid-catalysed ROP. The challenges in retaining the polymer structure avoiding unwanted deprotection of tertiary butyl ester was overcome by optimizing catalyst-amount and reaction time. Complete characterization of PPLG which was to be used as the backbone and PCL-N_3 and BPCL-N_3 which are to be grafted on this backbone. Click reaction between these polymers was done using two well-established routes viz. using CuBr/PMDETA/DMF and $CuSO_4$/Na(ascorbate)/DMSO. Grafting of PCL and BPCL onto the PPLG polymer was achieved successfully, although these polymers contain some unreacted polymer azides. The PCL grafted polymer was studied for its thermal properties. The BPCL grafted polymer will be further deprotected and purified using dialysis to get rid of unreacted BPCL-N_3 and self-assembly aspects will be studied.

5 REFERENCES

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APPENDIX

Figure S1. ^{1}H NMR in D_2O of γ-propargyl l-glutamate

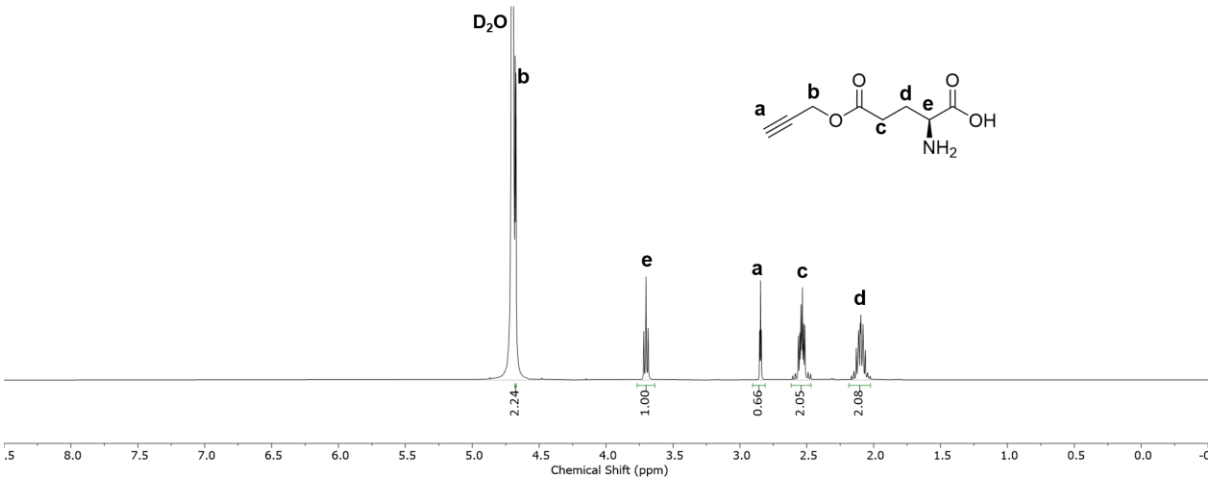

Figure S2. ^{1}H NMR in $CDCl_3$/TFA of PEG-b-PPLG50

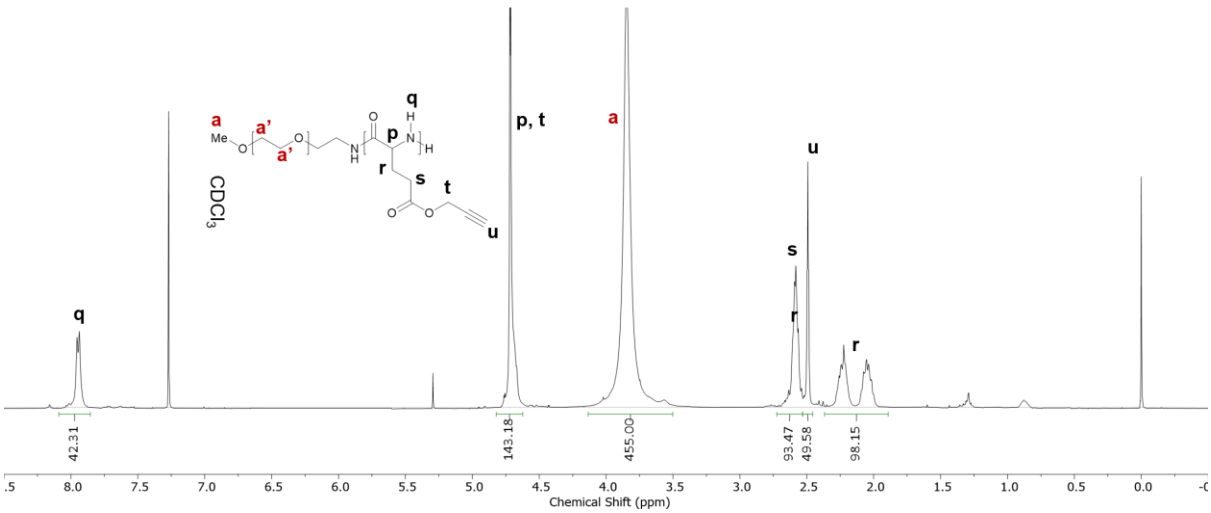

Figure S3. ^{1}H NMR in $CDCl_3$ of PCL synthesized with the initiator in melt route, using $Sn(oct)_2$ as the catalyst

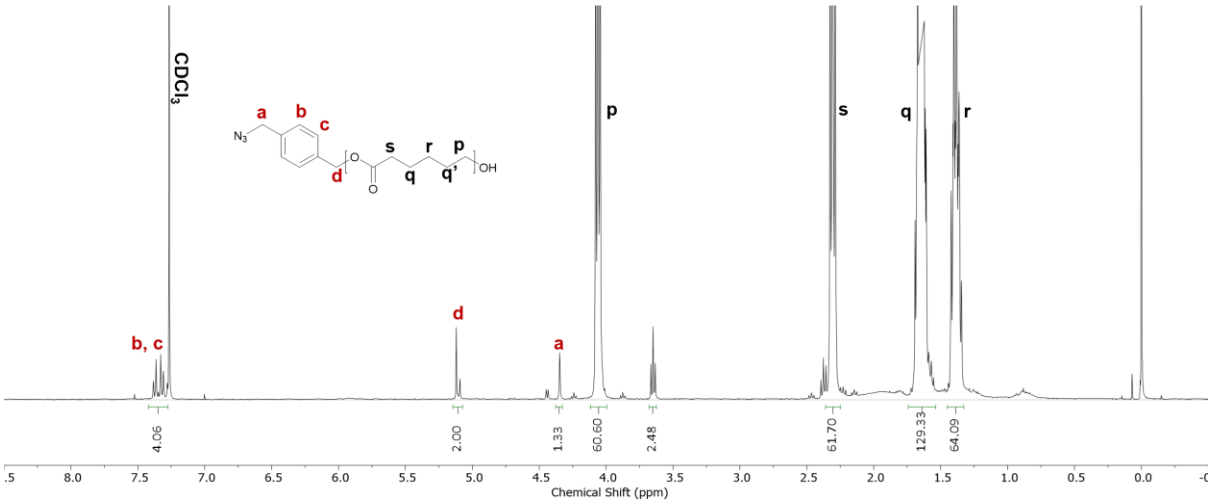

Figure S4. ^{1}H NMR in $CDCl_3$ of BPCL synthesized by 3 eq. of MSA

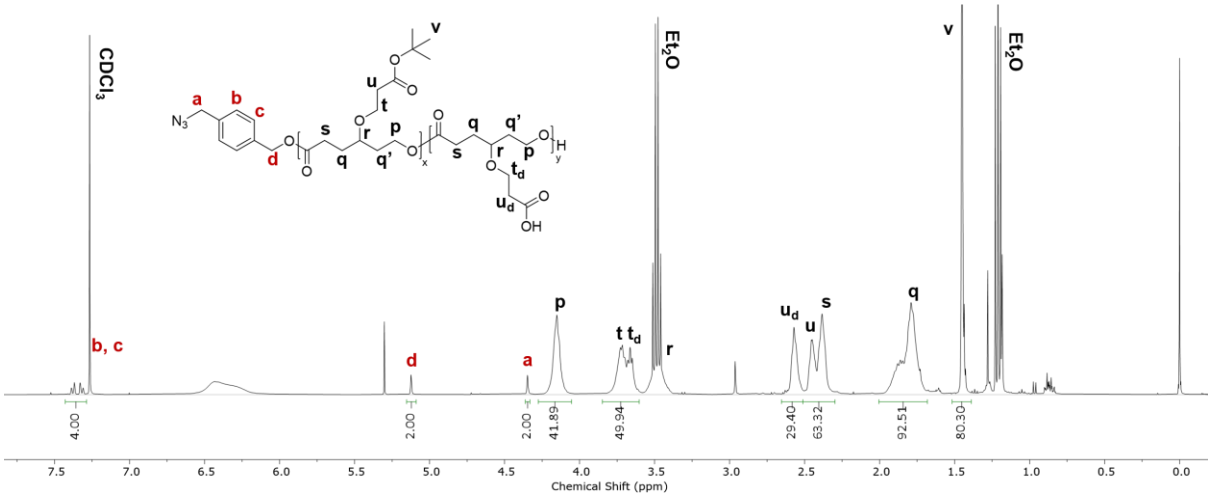

Figure S5. MALDI-TOF spectrum of BPCL-N3 recorded on CD matrix

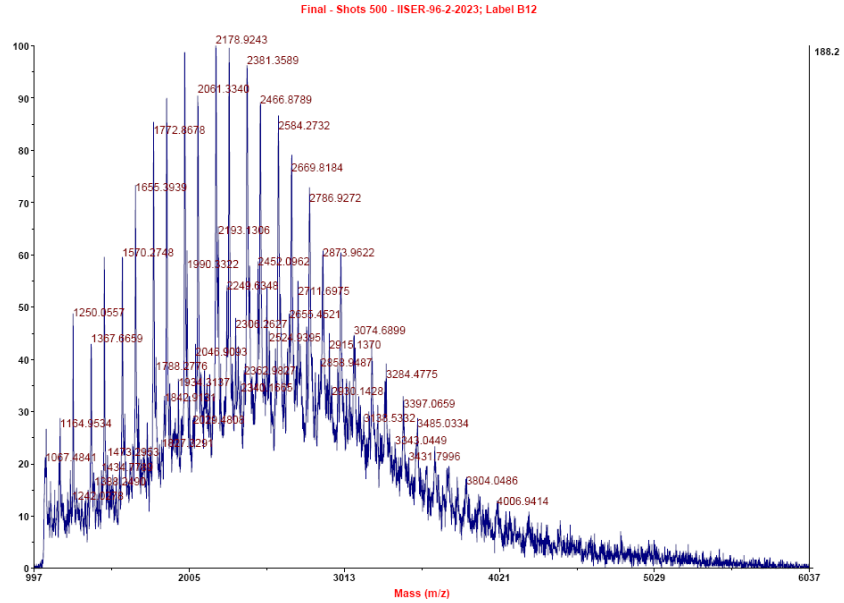

Figure S6. MALDI-TOF spectrum of PCL-N3 recorded on CD matrix