Poly(L-Cysteine) Based Functional Polypeptides

A Thesis

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

by

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Certificate

This is to certify that this dissertation **Poly(L-Cysteine) Based Functional Polypeptides** towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents study/work carried out by **Rhujal Satyendra Mokal** at Indian Institute of Science Education and Research under the supervision of **Prof. Manickam Jayakannan**, Department of Chemistry, during the academic year 2023-2024.

Prof. Manickam Jayakannan

Signature of Supervisor

Date: 15 May 2024

Committee:

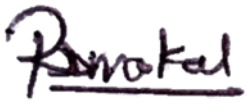
Supervisor: Prof. Manickam Jayakannan

TAC: Dr. Ashootosh V. Ambade

This thesis is dedicated to my Family.

Declaration

I hereby declare that the matter embodied in the report entitled **Poly(L-Cysteine) Based Functional Polypeptides** 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. Wherever others contribute, every effort is made to indicate this clearly, with due reference to the literature and acknowledgment of collaborative research and discussions.



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I consider myself extremely fortunate to have an amazing group of friends who have been by my side through all the highs and lows of the last five years, making my time at IISER full of joy and unforgettable moments.

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ABSTRACT

Stimuli-responsive polymers are promising materials that aim to mimic the adaptive responses of biological systems like proteins, which respond to changes in pH, oxidation, and other stimuli. Polypeptides stand out because of biocompatibility, biodegradability and their inherent nature of adopting ordered secondary structures that allow them to respond to stimuli specific to conformation. *tert*-butylbenzene functionalized poly(L-cysteine) based thioether polymers were synthesized via steric-hinderance assisted ring opening polymerization of N-carboxyanhydride monomer tailor-made via multi-step organic synthesis. The polymer was oxidized using various oxidants and the oxidation-responsive behaviours were investigated. The thioether functionality was oxidized to sulfoxide and sulfone groups in a controlled manner. These *tert*-butylbenzene functionalized poly(L-cysteine) adopted predominantly α-helical conformation which was maintained even after oxidation. Also, increase in the hydrophilicity was observed after oxidization. Further, thiol functionalized soluble random and block copolymers were synthesized using tBuBnCys and CbzCys and were subjected to oxidation. The deprotected block copolymer which initially adopted mixed conformation with predominant β-sheet, adopted an α-helical conformation thus switching the conformation on oxidation. Also, the thiol functional handle was used for metal-free thiol-ene click reaction during post-polymerization. The polymers were characterized by NMR, SEC, CD, TGA, DSC etc. Real-time FTIR kinetics was also done to ensure living nature of the polymers. DLS studies were also done to monitor the sizes before and after oxidation.

1. INTRODUCTION

Polymeric materials that are petroleum based are non-biodegradable and usually toxic. Proteins have long captivated scientists due to their complicated sequences and diverse chemical functionality, which may result in structurally defined folded chains with highly specific catalytic activities. Synthetic peptides and polypeptides are promising protein-mimetic materials that have received a lot of attention due to their biodegradability and biocompatibility in a variety of applications such as material applications, energy storage devices[1], drug delivery[2,3], biosensors[4,5], and so on. These materials have the same peptide bond backbone as proteins, allowing them to form stable secondary structures. As a result, there is growing interest in the controlled synthesis and investigation of synthetic peptide and polypeptide materials[6].

1.1. Synthesis Of Polypeptides

Synthetic peptide and polypeptide materials are typically synthesized through solid-phase peptide synthesis (SPPS), or ring-opening polymerization (ROP) of amino acid N-carboxyanhydride (NCA) monomers.

1.1.1. Solid-phase synthesis (SPPS)

Solid-phase synthesis was first introduced by B. Merrifield, for which he won Nobel Prize in 1984, entails one by one adding protected amino acids to a peptide chain that is attached to a polymeric support. The method allows us to synthesize peptides that are sequence-specific, length specific, and monodisperse. However, to guarantee sequence specificity, by-product elimination, and reaction completion, at least four steps are required for each monomer addition. Only oligopeptides with fewer than 50 residues can be synthesized using SPPS.[7] Its use in the synthesis of polypeptides is further limited by higher scalability costs and the numerous protection-deprotection steps required.

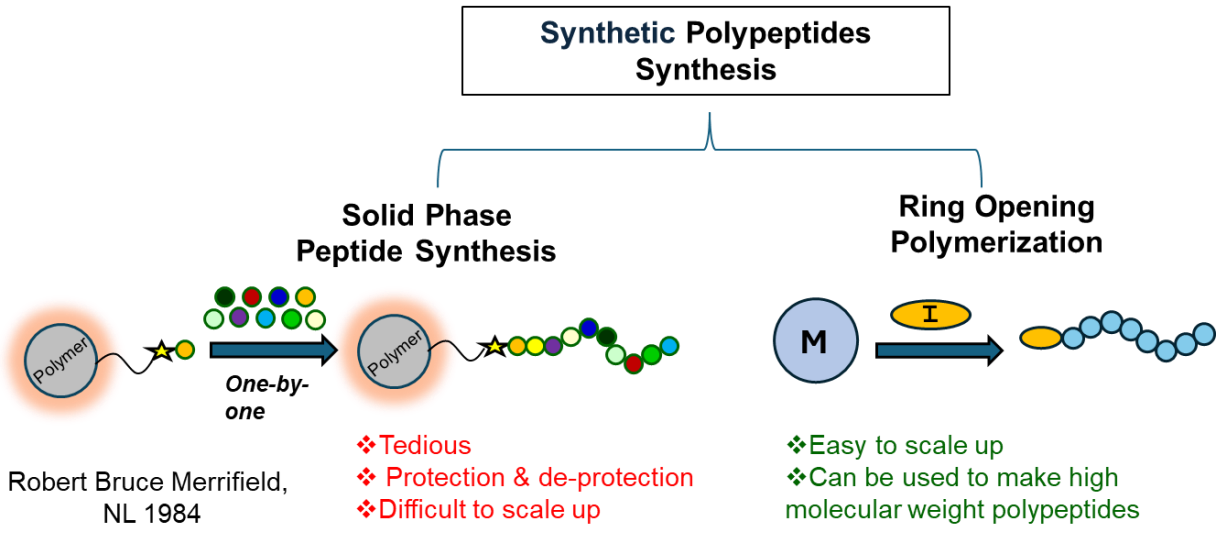


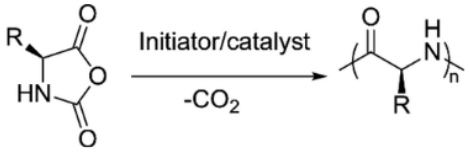
Figure 1: Synthetic Polypeptide Synthesis

1.1.2. Ring-opening polymerization (ROP) of amino acid N-carboxyanhydride (NCA) activated monomer

ROP of amino acid NCA monomers enables the synthesis of high molecular weight polypeptides without compromising the stereochemistry of the α-amino acid residues. These polypeptides are not sequence specific, but their synthetic benefits make them a powerful and relatively cheap method for the synthesis polypeptides in large scale. Furthermore, the ease of incorporating versatile side-chain groups during NCA synthesis creates countless opportunities for the synthesis of functional polypeptides.

NCA is as an activated monomer for polypeptide synthesis was first introduced by Leuch in 1906 while the Fuchs-Farthing method was first reported in 1922. The two approaches are shown in Figure 1. These approaches required anhydrous solvents, Schlenk line/glove box, and protection of side-chain functional groups. Although the Fuchs-Farthing method for making NCA appears to be fairly straightforward, it actually produces HCl as a byproduct that must be removed effectively in order to obtain pure NCA. This is because HCl can cause acid-catalyzed decomposition of NCA and termination of the polymerization under moist conditions which can lead to loss in control over polymerization.

Synthesis of polypeptides through ROP of NCAs



NCA Synthetic Methods

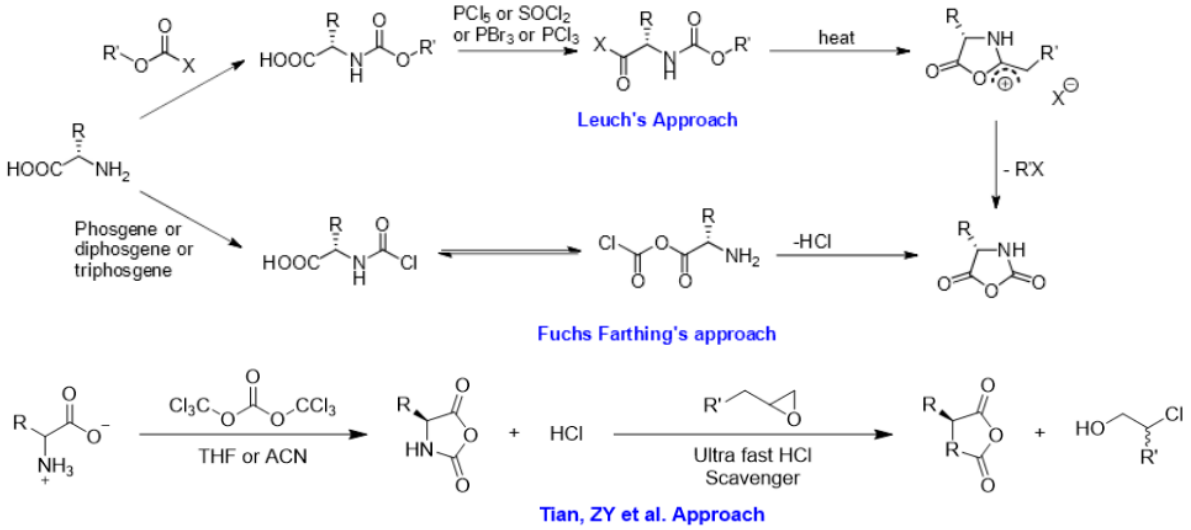


Figure 2: NCA synthetic methods

Later, in 2021, Tian, ZY et al. reported a robust method for preparing unprotected NCA monomers in air and under moisture.[8] The method utilized epoxy compounds as scavengers of HCl to facilitate assisted ring-closure and prevent NCA from acid-catalysed decomposition under moist conditions. This report was a remarkable contribution and almost eliminated all the hurdles.

The ROP of NCA has been initiated using a variety of nucleophiles and bases. These initiators include 1°, 2° and 3° amines, amine hydrochlorides, thiols, alkoxides, trimethylsilyl-amines, hexamethyldisilazane (HMDS), rare earth and transition metal complexes and organo-catalysts.[7] The NCA ROP mostly follows two common pathways, NAM (normal amine mechanism) and AMM (activated monomer mechanism) (see Figure 3). In traditional amine polymerizations, these can occur simultaneously and frequently compete with one another, reducing control over molecular weight and molecular weight distributions.

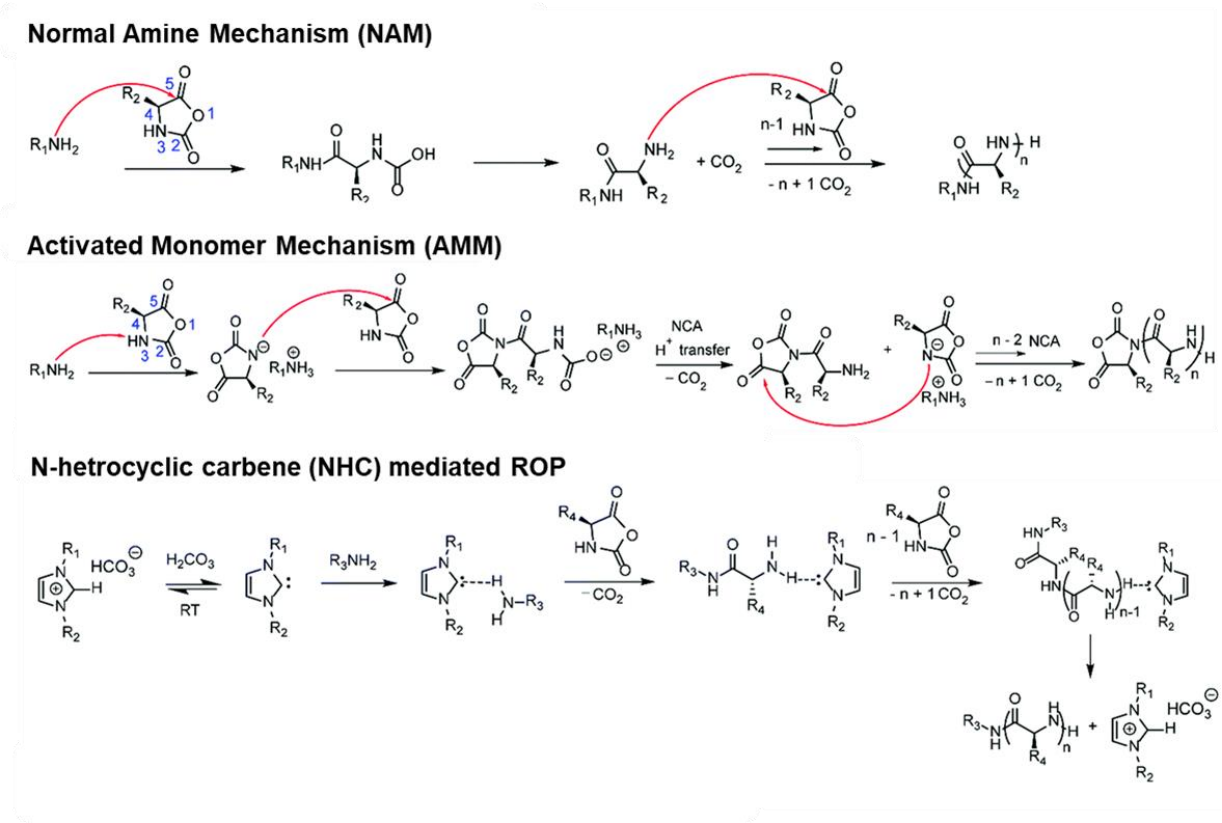


Figure 3: NCA ROP mechanism (adapted from Chem. Soc. Rev., 2020,49, 4737-4834)

N-heterocyclic Carbenes (NHC) catalysts have demonstrated considerable potential in the ROP of different NCA monomers. To facilitate a controlled ring-opening polymerization of NCAs, imidazolium hydrogen carbonate salts of NHC, which are air stable, function as a pre-catalyst by releasing the active NHC through the loss of H_2O and CO_2. By creating a hydrogen bond with the primary amine initiators, NHCs enable the nucleophilic attack on the NCAs, leading linear polypeptides (Figure 3).

1.2. Sulphur-Based Stimuli Responsive Polypeptides

Stimuli-responsive polymers can respond to specific stimuli such as pH, temperature, redox potential, light, enzymes, etc., and change their chemical or physical properties. Compared to conventional polymers, polypeptides are special because they have ordered secondary structures that allow them to respond to stimuli specific to conformation, besides being modified with various functional groups. Therefore, it is of great interest to design trigger-responsive, conformationally switchable polypeptides, which can be “activated” (coil-to-helix) or “deactivated” (helix-to-coil, β-

sheet-to-coil) or “transitioned” (α-helix-to-β-sheet) on demand.[9] Sulphur-containing amino acid residues, like methionine and cysteine, can undergo selective redox or alkylation processes, allowing peptides and proteins to change their structure and function through enzymatic action or reactive oxygen species (ROS) or reactive electrophile species.[10] These reactions can alter residue polarity and peptide chain conformations and have been applied to synthetic peptide materials. The utilization of sulfur switches has broadened the range of these switches, as they can include a diverse array of noncanonical synthetic amino acids that contain sulfur.

As shown in Figure 4, Redox processes drive many sulfur switches, with cystine disulfides and cysteine thiols being the most well-known. Thioether oxidation alters polarity and peptide chain conformations, making it a functional and reversible switch in peptide materials.

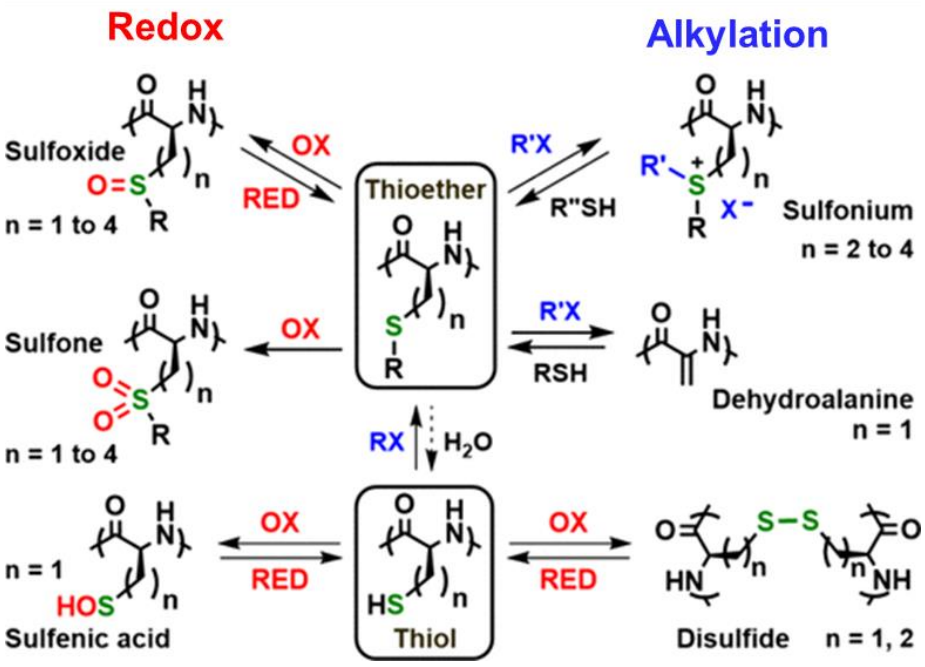


Figure 4: Sulphur Switches (Acc. Chem. Res. 2024, 57, 5, 661–669)

The versatile properties of thiolated polymers have been the subject of extensive research.[11–14] These polymers' ability to form disulfide bonds, which can cross-link within their own structure or be modified using thiol-ene, -Michael addition, and -yne, -epoxy chemistries in the polymer stage, is attributed to their thiol groups. The fact that thiol-ene chemistry reactions happen so quickly has sparked a renewed interest in the field because it offers new avenues for the fast photopolymerization of a wide range

of polymers for specialized uses, such as biomedical materials functionalized with a metal-free "click" polymer.

1.3. Steric-hindrance Polymerization Strategy for β-Sheet Polypeptides

Compared to the conventional synthetic polymer, which lacks any secondary structure, the behavior and functions of synthetic polypeptides can be tuned by controlling the conformation of polymers besides tuning their degree of polymerization (Xn), chemical compositions, or side-chain structures. In most advanced synthetic polypeptide systems, the interactions of one or two types of side chains, including hydrogen bonding interactions, hydrophobic interactions, and Coulombic interactions, have governed the development and stabilization of secondary structures.[9]

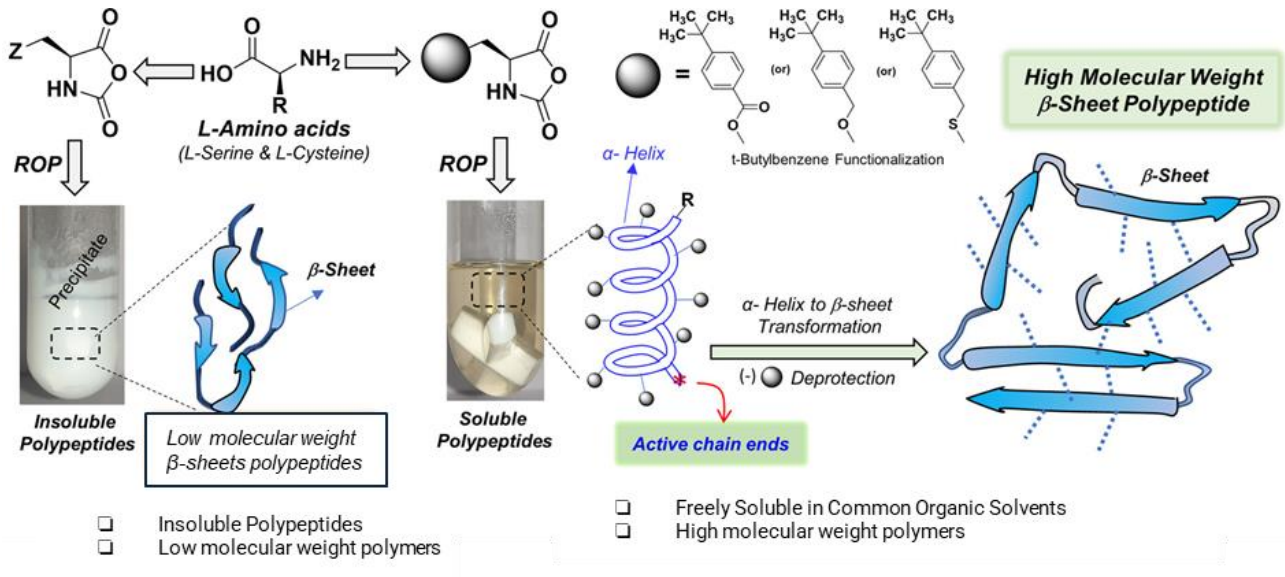


Figure 5: Steric-hindrance ROP strategy (adapted from Biomacromolecules 2022, 23, 6, 2667–2684)

High molecular weight can be achieved if the expanding polypeptide chain adopts homogeneous polymerization, which is possible if polypeptides adopt α-helical conformation. On the other hand, if a developing polypeptide chain maintains its β-sheet conformation during polymerization, it typically begins to precipitate, which causes heterogeneity in the polymerization process and produces low molecular

weight polymers. The polypeptide derived from amino acids like valine, isoleucine, glycine, serine, threonine, tyrosine, and cysteine tend to form β-sheet structures, and their polypeptides are highly insoluble.

Our research group devised a strategy based on the functionalization by *tert*-butylbenzene, a bulky-steric unit that serves as a solubilizing handle and a conformational controller to produce an in situ structural transition in the propagating chains and achieve total solubility in the polymerization medium. So, this strategy involves *tert*-butylbenzene functionalization of the amino acid, ROP of the NCA monomer of the protected amino acid, and then post-polymerization deprotection. The polypeptide of β-sheet favoring amino acid is forced to adopt an α-helical conformation. It hence could form high molecular weight polymers and later switch to β-sheet on post-polymerization deprotection. This method has been successfully applied to polymerize high molecular weight polypeptides based on serine, cysteine, and tyrosine. In the case of serine and tyrosine, we could switch the conformation from α-helix to β-sheet polypeptides in a post-polymerization step.

1.4. Ring-opening polymerization induced self-assembly (ROPISA)

The most economical and efficient polypeptide preparation process is the ring-opening polymerization of N-carboxyanhydride monomers, which uses simple reagents and yields good yields. However, this method has limitations like tedious purification steps, moisture sensitivity, and toxic solvents. To improve conditions, recent efforts focus on promoting polymerization rates without a catalyst, exploring reactive initiators, and using specific processes like emulsion polymerization. Recent advancements in polypeptide technology have led to synthesizing polypeptide block copolymer architectures, which can be self-assembled post-polymerization. Post-polymerization self-assembly usually requires a second step of nanoprecipitation, typically performed from toxic organic solvents under diluted conditions and is sensitive to scale. Ring-opening polymerization-induced self-assembly (ROPISA) is a strategy in which the moisture-sensitive NCA monomer is subjected to ROP in an aqueous medium with an amine initiator at basic pH.[15] This works because the initiator used is hydrophilic while the NCA monomer and the growing polypeptide chain are hydrophobic; hence, it self-

assembles from the start, and the NCA monomer is protected in the formed hydrophobic pocket. The basic pH ensures that the aminolysis is much faster than the hydrolysis of the NCA.

1.5. Aim of the Thesis

A significant amount of knowledge is known in the open literature for α-helical polypeptides. These polypeptides are readily soluble in common organic solvents for structural characterization and molecular weight determination. However, the β-sheet forming polypeptides such as poly(L-cysteine)s having thiol-functional group encounter uncontrolled precipitation during the ROP process and become inaccessible for structural analysis.

Our group developed a steric hindrance-assisted ROP method, which enabled the synthesis of high molecular weight poly(L-cysteine)s.[16] This was done using steric-hindrance assisted approach where *tert*-butyl benzyl functionalization of the cysteine thiol group with a thioether linkage, lead poly(L-cysteine) to adopt an α-helical conformation. As discussed earlier, thioethers can be an excellent stimuli-responsive unit that can respond to oxidation. So, we wanted to investigate further how our thioether-based polymer reacts to the oxidative stimuli in terms of secondary structures, water solubility, self-assembly, etc.

In the literature[17–19], there are many masking groups used for the polymerization of poly(L-cysteine), but later these masking agents are unable to deprotect and hence no free thiol units in the side chains. Some of the deprotectable groups like benzyloxycarbonyl (Cbz) can be used for getting free thiol side chain polymer after post-polymerization, but then we can grow only a few units and it crashes out and becomes insoluble due to the tendency of forming β-sheet secondary structure.[19]

So, we envisioned making a soluble polymer with a free thiol side chain and proposed synthesis of random and block copolymer based on S-CbzCys and a tBuBnCys group. Later, selectively deprotect only CbzCys and keep tBuBnCys to impart solubility. Thus, a soluble versatile polymer with a thiol functional handle can be synthesized and used for post-polymerization.

2. EXPERIMENTAL SECTION

2.1. Materials

4-(*tert*-butyl)benzaldehyde, phosphorus tribromide (PBr_3), anhydrous N, N-dimethyl formamide (DMF), anhydrous chloroform, MCPBA, Chloroform-d were purchased from Sigma Aldrich. L-cysteine hydrochloride monohydrate, trifluoracetic acid, diethyl ether, acetone, sodium hydroxide, ethanol, sodium sulfate (anhydrous), sodium bicarbonate, propylene oxide, triphosgene, ethyl acetate, 30% H_2O_2 aq. solution, 33% HBr/AcOH, benzyl alcohol, DTNB, and methanol were purchased from local sellers and used directly. Before use, dichloromethane was refluxed over P_2O_5 and then distilled. THF and hexane were dried over sodium and then distilled. To conduct ROPISA and thiol estimation experiments, milli-Q water, and HPLC grade DMF were utilized.

2.2. Methods

400 MHz Bruker spectrometers were used to record the ^{1}H and ^{13}C NMR spectra. ATR-FTIR spectra were recorded on Bruker alpha-II. GPC was carried out with a Viscotek VE 3580 RI detector, Viscotek VE 1122 pump, and two size exclusion columns using polystyrene standards. DLS experiments were carried out using the Malvern Nano ZS-90 system. The Shimadzu UV1900 spectrophotometer was used for absorbance measurements. Circular dichroism (CD) analysis was performed on a JASCO J-815 CD spectrometer at 25 °C. TGA was done on Perkin Elmer STA 6000. Water contact angle measurements were done on GBX Model (DIGIDROP instrument).

2.3. Synthesis

2.3.1. Monomer M1 Synthesis[16]

2.3.1.1. **(4-(*tert*-Butyl)phenyl) methanol (2)**

To an ice-cooled solution of (4-(tert-Butyl)benzaldehyde **1** (20 g, 123.28 mmol, 1 eq) in 250 mL methanol, sodium borohydride (6.99 g, 184.92 mmol, 1.5 eq) was added

slowly bit by bit. The reaction mixture was stirred at rt for 3 hours. After confirmation of completion of the reaction by TLC, the methanol was evaporated over rota, and then ~100 mL 2N HCl solution was added. Then, the workup was done using EtOAc (2 times), and then the combined organic layer was given a wash of aq. brine solution. Then the final organic layer was dried over Na_2SO_4 and concentrated over the rota to give a colorless liquid as the product. Yield = 17.63 g (87.84%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.40 (d, J = 8.4 Hz, 2H, Ar–**H**), 7.31 (d, J = 8.6 Hz, 2H, Ar–**H**), 4.66 (s, 2H, Ar-C**H$_2$**O), 1.81 (s, 1H, O**H**), 1.33 (s, 9H, C(C**H$_3$**)$_3$). ^{13}C NMR (101 MHz, $CDCl_3$): δ 150.9, 138.1, 127.0, 125.6, 65.3, 34.7, 31.5. ATR-FTIR (cm^{-1}): 3325, 2930, 2872, 1615, 1512, 1460, 1410, 1109, 1042, 1011. HRMS (ESI): $[M + Na]^+$ calcd for $C_{11}H_{16}NaO$, 187.1099; found, 187.1098

2.3.1.2. **1-(Bromomethyl)-4-(*tert*-butyl)benzene (3)**

To a solution of **2** (8 g, 48.78 mmol, 1 eq) dissolved in anhyd. dichloromethane (DCM) (80 mL) was added phosphorus tribromide (6.1 mL, 17.12 g, 63.41 mmol, 1.3 eq) dropwise at 0 °C under a nitrogen atmosphere. The reaction mixture was allowed to stir at 0 °C for 2 h and then at rt overnight. After confirming the completion of reaction by TLC, the reaction mixture was quenched by slowly adding water (25 mL) at 0 °C on ice bath. The DCM was evaporated over the rota, and then the aqueous layer was extracted using ethyl acetate (50 mL) twice. The combined organic layers were further extracted with aq. sat. $NaHCO_3$ solution (100 mL), and brine (100 mL) and then dried over anhyd. Na_2SO_4, gravity filtered and concentrated over rota to get 1-(bromomethyl)-4-(*tert*-butyl)benzene) as a pale yellow oil. Yield: 9.5 g (86%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.38 (d, J = 8.4 Hz, 2H, Ar–**H**), 7.34 (d, J = 8.5 Hz, 2H, Ar–**H**), 4.50 (s, 2H, Ar-C**H$_2$**Br), 1.32 (s, 9H, C(C**H$_3$**)$_3$). ^{13}C NMR (101 MHz, $CDCl_3$): δ 151.7, 134.9, 128.9, 125.9, 34.8, 33.8, 31.4. ATR-FTIR (cm^{-1}): 2962, 1612, 1515, 1462, 1440, 1411, 1267, 1229, 1202, 1108. HRMS (ESI): $[M + H]^+$ calcd for $C_{11}H_{15}Br$, 227.0364; found, 227.1762.

2.3.1.3. **S-(4-(*tert*-Butyl)benzyl)- L-cysteine (4)**

To an ice cooled solution of aq. 2 M NaOH solution (23 mL), was added L-cysteine hydrochloride monohydrate (3.4 g, 19.4 mmol). Then ethanol (46 mL) was added. To this reaction mixture was added 1-(bromomethyl)-4-(*tert*-butyl)benzene (4 g, 17.6

mmol). The reaction mixture was stirred at rt for 10 min, then became a thick white solid. So, it was stirred using a glass rod and sonicated for some time. Next, to precipitate the desired product, the reaction mixture was neutralized to pH 7 using 2 N HCl. The precipitate was filtered over the Buchner funnel and washed with water, ethanol, and diethyl ether to give a crude product. The crude product was resuspended in water (150 mL) and stirred at 60 °C for 15 min. The mixture was filtered over the Buchner funnel and was washed once with water (100 mL), acetone (10 mL), and ether (100 mL). Finally, it was lyophilized to obtain the pure product *S*-(4-(*tert*-butyl)benzyl)-L-cysteine as a white powder. Yield: 3.5 g (75%). ^{1}H NMR (400 MHz, $CDCl_3$-TFA) (0.7 mL, 6:1, v/v): δ 7.43 – 7.38 (m, 2H, Ar-**H**), 7.32 (s, 3H, $^{+}$N**H$_3$**), 7.25 – 7.19 (m, 2H, Ar-**H**), 4.17 (s, 1H, C**H**CH_2), 3.77 (s, 2H, Ar-C**H$_2$**-S), 3.16 (dd, *J* = 15.3, 4.1 Hz, 1H, CHC**H$_2$**), 3.04 (dd, *J* = 15.3, 8.6 Hz, 1H, CHC**H$_2$**), 1.30 (s, 9H, C(C**H$_3$**)$_3$). ^{13}C NMR (101 MHz, $CDCl_3$-TFA) (0.7 mL, 6:1, v/v): δ 171.99, 152.10, 133.10, 128.73, 126.49, 53.19, 36.11, 34.77, 31.16, 30.92. ATR-FTIR (cm^{-1}): 2960 (br), 1618, 1590, 1513, 1395, 1344, 1298, 1135, 1110. HRMS (ESI): $[M + H]^+$ calcd for $C_{14}H_{22}NO_2S$, 268.1371; found, 268.1372.

2.3.1.4. S-(4-(*tert*-Butyl)benzyl)-L-cysteine N-Carboxy Anhydride (S-tBuBnCys NCA) (M1)

To a suspension of S-(4-(*tert*-Butyl)benzyl)-L-cysteine (2.5 g, 9.36 mmol, 1 eq) in THF (50 mL) was added propylene oxide (2.17 g, 2.62 mL, 37.45 mmol, 4 eq), and triphosgene (1.39 g, 4.68 mmol, 0.5 eq) and the reaction mixture was left to stir at rt for 2 hours. The reaction mixture became clear after 15 minutes. After 2 hours, the reaction was quenched by adding cold water (25 mL) and stirring for a minute. Then, the aqueous layer was extracted twice using ethyl acetate (50 mL), and the combined organic layer was given a wash of aq. brine solution. The organic layer was dried over Na_2SO_4 and was concentrated to about ~10 mL, and then it was precipitated in excess hexane and kept at -20 °C overnight. The white precipitate was filtered and washed with more hexane and was again dissolved in THF, filtered, and precipitated in hexane at -20 °C for 12 hours. This was repeated once more. Finally, the precipitate was filtered and dried over vacuum to yield NCA. It is stored under the N_2 atmosphere at -20 °C. Yield = 2 g (74%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.37 (d, *J* = 8.3 Hz, 2H, Ar–**H**), 7.24 (d, *J* = 8.3 Hz, 2H, Ar–**H**), 6.15 (s, 1H, N**H**), 4.33 (dd, *J* = 3.6, 7.5 Hz, 1H,

C**H**CH_2), 3.75 (s, 2H, ArC**H**$_2$S), 2.97 (dd, *J* = 3.6, 14.5 Hz, 1H, SC**H**(H)CH), 2.77 (dd, *J* = 7.5, 14.5 Hz, 1H, SCH(**H**)CH), 1.31 (s, 9H, C(C**H**$_3$)$_3$). ^{13}C NMR (101 MHz, $CDCl_3$): δ 168.2, 151.8, 151.1, 134.1, 128.7, 126.0, 57.8, 36.8, 34.7, 32.9, 31.4. ATR-FTIR (cm^{-1}): 3310, 2959, 2870, 1857, 1777, 1515, 1464, 1397, 1362, 1287, 1180, 1109, 1085. HRMS (ESI): $[M + H]^+$ calcd for $C_{15}H_{19}NaO_3S$, 294.1164; found, 294.1166.

2.3.2. Monomer M2 Synthesis

2.3.2.1. **Benzyl Chloroformate (6)**

To a solution containing triphosgene (14.48 g, 48.82 mmol, 0.66 eq) in 40 mL dry THF, a solution of benzyl alcohol (8 g, 73.98 mmol, 1 eq) in 40 mL dry THF was added dropwise under ice-cold conditions at 0 °C in N_2 atmosphere. The reaction was allowed to stir for 2 hours at 0 °C and then slowly at rt overnight under N_2 atmosphere. Then, the reaction was quenched with 100 mL EtOAc followed by 100 mL water, and the workup was done with EtOAc two more times. Then, the combined organic layer was washed with brine, dried over sodium sulfate, and concentrated over a vacuum to yield a viscous liquid as the product. Yield = 12.24 g (97%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.40 (s, 5H, Ar-**H**), 5.30 (s, 2H, Ar-**CH$_2$**-O). ATR-FTIR (cm^{-1}): 1779, 1145

2.3.2.2. **S-carbobenzoxy- L-cysteine (7)**[20]

To an ice-cooled solution of L-cysteine hydrochloride monohydrate (4 g, 22.78 mmol,1 eq) in 1 N aq. sodium bicarbonate (36 mL), covered with diethyl ether (20 mL) was added benzyl chloroformate (3.88 g, 22.78 mmol, 1 eq) with vigorous stirring. After one hour of stirring at 0 °C, the temperature was allowed to rise to around 10 °C and maintained at this condition for another two hours. The product obtained was filtered over a Buchner funnel using 11 µm Whatman filter paper, washed with water, acetone, and ether successively, and dried over a vacuum to yield white solid S-protected L-cysteine as the product. Yield = 2.9 g (52%). ^{1}H NMR (400 MHz, $CDCl_3$-TFA) (0.7 mL, 6:1, v/v): δ 7.68 (s, 3H, $^+$N**H$_3$**), 7.48 – 7.28 (m, 5H, Ar-**H**), 5.37 – 5.18 (m, 2H, Ar-**CH$_2$**-O), 4.66 (s, 1H, C**H**CH_2), 3.71 – 3.39 (dd, 2H, CH**CH$_2$**). ^{13}C NMR (101 MHz, $CDCl_3$) δ 173.17, 171.05, 133.63, 129.66, 129.13, 128.90, 72.07, 54.94, 30.47. ATR-FTIR (cm^-

^{1}): 3002, 1701, 1609, 1588, 1522, 1387, 1140. MALDI-TOF: $[M + Na]^+$ calcd for $C_{11}H_{13}NO_4S$, 278.0462; found, 278.0756, $[M + K]^+$ calcd for $C_{11}H_{13}NO_4S$, 294.1548; found, 294.0438

2.3.2.3. **S-carbobenzoxy- L-cysteine N-Carboxy Anhydride (S-CbzCys NCA) [M2]** [20]

To a flame-dried reaction setup consisting of a two-neck RB, a magnetic bid and a condenser was added S-CbzCys **7** (2 g, 7.84 mmol, 1 eq) and was dried over vacuum for 2 hrs. After flushing with N_2, 20 mL anhydrous THF was added, and the white suspension was heated to 50 °C for 10 mins. A solution of triphosgene (1.53 g, 5.18 mmol, 0.66 eq) in 10 mL anhydrous THF was added slowly to the reaction mixture at 50 °C. The reaction mixture was stirred at 50 °C for 3 hours when it became a clear solution. The reaction mixture was filtered, added to hexane, and kept at -20 °C overnight. The white precipitate was filtered and washed with more hexane and was again dissolved in THF, filtered, and precipitated in hexane at -20 °C for 12 hours. This was repeated once more. Finally, the precipitate was filtered and dried over vacuum to yield NCA. It is stored under the N_2 atmosphere at -20 °C. Yield = 0.96 g (44%). 0^1H NMR (400 MHz, $CDCl_3$) δ 7.43 – 7.31 (m, 5H, Ar-**H**), 6.16 (s, 1H, N**H**), 5.29 (dd, 2H, Ar-**CH_2**-O), 4.64 (t, *J* = 4.6 Hz, 1H, C**H**CH_2), 3.35 (dd, 2H, CH**CH_2**). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.48, 167.88, 151.42, 134.40, 129.09, 128.93, 128.56, 70.55, 57.97, 31.92. ATR-FTIR (cm^{-1}): 3372, 2980, 2884, 1858, 1787, 1712, 1454, 1290, 1144, 927. HRMS (ESI): $[M + H]^+$ calcd for $C_{12}H_{11}NO_5S$ 282.0365; found, 282.0414.

2.3.3. Homopolymer Synthesis

General Procedure:

To a cylindrical polymerization tube was added initiator MeO-PEG_{5000}-NH_2 and 1,3-Bis(2,4,6-trimethylphenyl)imidazolium bicarbonate (Imes.HCO_3) and was dried over vacuum. Then it was flushed with nitrogen, and anhydrous solvent was added and stirred at rt. The corresponding NCA monomer solution in the anhydrous solvent was then pumped into the polymerization tube and stirred until completion. After confirming the complete conversion of monomer by using the disappearance of the anhydride peak in the FT-IR spectra, the excess solvent was evaporated using rota, and then

excess methanol was added to the residue to precipitate the polymer. It was transferred to a centrifuge tube and was centrifuged for 15 min at 4000 rpm. Then, the methanol supernatant was decanted, and the pellet was dried under a vacuum.

For polymer PEG-b-tBuBnCys **P1** with M/I = 100, initiator PEG-amine (50 mg,0.01 mmol), NCA monomer **M1** (293 mg, 1 mmol), and NHC catalyst (1.4 mg, 0.004 mmol) were polymerized in 10 mL $CHCl_3$.

For polymer PEG-b-CbzCys **P2** with M/I = 25, initiator PEG-amine (200 mg,0.04 mmol), NCA monomer **M2** (281 mg, 1 mmol), and NHC catalyst (1.4 mg, 0.016 mmol) were polymerized in 10 mL DMF-$CHCl_3$ (15:85).

2.3.4. Random copolymer synthesis

To a cylindrical polymerization tube was added initiator MeO-PEG_{5000}-NH_2 (100 mg, 0.02 mmol) and 1,3-Bis(2,4,6-trimethylphenyl)imidazolium bicarbonate (Imes.HCO_3) (2.92 mg, 0.008 mmol) and was dried over vacuum. Then, it was flushed with nitrogen, and anhydrous DMF-$CHCl_3$ (15:85) was added and stirred at rt. Then both the NCA monomers **M1** (234.4 mg, 0.8 mmol) and **M2** (56.2 mg, 0.2 mmol) solution in the anhydrous DMF-$CHCl_3$ (15:85) was then pumped into the polymerization tube in one go and was stirred until completion. After confirming the complete conversion of monomer by using the disappearance of the anhydride peak in the FT-IR spectra, the excess solvent was evaporated using rota, and then excess methanol was added to the residue to precipitate the polymer. It was then centrifuged for 15 min at 4000 rpm. Then, the methanol supernatant was decanted, and the pellet was dried under a vacuum.

2.3.5. Block copolymer Synthesis

To a cylindrical polymerization tube was added initiator MeO-PEG_{5000}-NH_2 (100 mg, 0.02 mmol) and 1,3-Bis(2,4,6-trimethylphenyl)imidazolium bicarbonate (Imes.HCO_3) (2.92 mg, 0.008 mmol) and was dried over vacuum. Then, it was flushed with nitrogen, and anhydrous DMF-$CHCl_3$ (15:85) was added and stirred at rt. Then, one of the NCA monomers M1 (234.4 mg, 0.8 mmol) or M2 (56.2 mg, 0.2 mmol) solution was made in the anhydrous DMF-CHCl3 (15:85) depending on the sequence of the desired block.

Then this solution was then pumped into the polymerization tube in one go and was stirred until completion. After confirming the complete conversion of the monomer by using the disappearance of the anhydride peak in the FT-IR spectra, the second monomer dissolved in the solvent was pumped and stirred until completion. After completion, excess solvent was evaporated using rota, and excess methanol was added to the residue to precipitate the polymer. It was then centrifuged for 15 min at 4000 rpm. Then, the methanol supernatant was decanted, and the pellet was dried under a vacuum.

2.3.6. Deprotection Of copolymers

To a polymerization tube, the polymer **P3**, **P4** or **P5** (100 mg) was added 1 mL $CHCl_3$:TFA (1:1) and dissolved. Then 1 mL 33% HBr/AcOH was added slowly and was allowed to stir at rt overnight. Then, it precipitated in excess diethyl ether three times and was transferred to a centrifuge tube, which was centrifuged for 10 min at 4000 rpm. Then, the diethyl ether supernatant was decanted, and the pellet was dried under vacuum. The product was stored in dark conditions and at -20 °C.

2.3.7. Procedures for post-polymerization oxidation

- For Oxidation using MCPBA: To an ice-cooled solution of polymer (50 mg) in chloroform (2 mL) in a Schlenk tube was added appropriate equivalents of MCPBA (For sulfoxide: 38 mg, 220.8 µmol, 1.1 eq and for sulfone: 103 mg, 602.4 µmol, 3 eq) solution in chloroform (1 mL). It was allowed to stir at rt overnight. Then, the excess peracid was quenched using 1 M sodium thiosulfate (2 mL), and the organic layer was washed with brine solution and dried using a few pints of sodium sulfate. The organic layer was concentrated and was precipitated in excess diethyl ether. It was transferred to a centrifuge tube and was centrifuged for 10 min at 4000 rpm. Then, the diethyl ether supernatant was decanted, and the pellet was dried under vacuum.

- For Oxidation using H_2O_2: To a solution of polymer (30 mg) in THF (2 mL) in a Schlenk tube was added slowly dropwise acetic acid (100 µL) followed by 500

µL 30% H_2O_2 aq. solution and was stirred at rt until completion of the reaction. The oxidized polymer was precipitated in excess methanol and was centrifuged for 10 min at 4000 rpm. Then, the methanol supernatant was decanted, and the pellet was dried under a vacuum.

- For Oxidation using $NaIO_4$: To a solution of polymer (30 mg) in THF (1 mL) in a Schlenk tube, was added slowly 1 mL solution of sodium periodate (83.2 mg, 481.9 µmol, 4 eq) in water under ice-cooled conditions and was allowed to stir at rt for two days. The excess THF was removed, and the aqueous layer was extracted using chloroform. The organic layer was then washed with brine and dried using a few pints of sodium sulfate. The excess solvent was removed over the rota and precipitated in diethyl ether. It was transferred to a centrifuge tube and was centrifuged for 10 min at 4000 rpm. Then, the diethyl ether supernatant was decanted, and the pellet was dried under a vacuum.

2.3.8. ROPISA-meditated polymerization

To a cylindrical polymerization tube containing MeO-PEG_{5000}-NH_2 (30 mg, 0.006 mmol, 1 eq) completely dissolved in 10 mL 50 mM aq. sodium bicarbonate solution stirred at 4 °C, was added the corresponding NCA monomer M1 (43.95 mg, 0.15 mmol, 25 eq) and was stirred at 4 °C reaction mixture was vigorously stirred in the dark at 4 °C for 8 hours and at 25 °C for 40 hours. The reaction was monitored using FT-IR by taking out a small aliquot (100 µL), extracting it with DCM (200 µL), and using the DCM layer for FT-IR measurements. After confirming the consumption of the monomer by monitoring the disappearance of anhydride peaks, the opalescent reaction mixture was dialyzed against MilliQ for 24 hours. 4 mL of the dialyzed solution was lyophilized and used for further characterization.

2.3.9. Oxidation Of ROPISA polymer

To the 2.5 mL of the ROPISA polymer aq. the solution, containing 35 mg polymer, was slowly added 125 µL acetic acid followed by 500 µL 30% H_2O_2 aq. solution. It was allowed to stir at rt until completion. For the FTIR monitor, 100 µL of the reaction mixture was taken, diluted to 200 µL using water, and extracted using 400 µL

chloroform. Then, the organic layer was precipitated in excess diethyl ether and again dissolved in chloroform, and FTIR was recorded.

2.3.10. Deprotection of tBuBnCys

To an ice-cooled solution of polymer **P1** (60 mg) dissolved in 2 mL $CHCl_3$:TFA (1:1 v/v), was added thioanisole (0.14 mL, 5 eq) was added dropwise trimethylsilyl iodide (1.2 mL, 25 eq) and was stirred at rt for 24 hours and then at 45 °C for 24 hours. It was then precipitated in diethyl ether and was dissolved in 9 mL aq. sat. Na_2CO_3 solution and then was extracted with diethyl ether twice. It was then dialyzed against MilliQ water for 24 hours and then was lyophilized to get yellowish white powder as the product.

3. RESULTS AND DISCUSSION

3.1. Monomer Synthesis and Characterization

The two S-protected L-cysteine N-carboxy anhydride (NCA) monomers were tailor-made by multi-step organic synthesis.

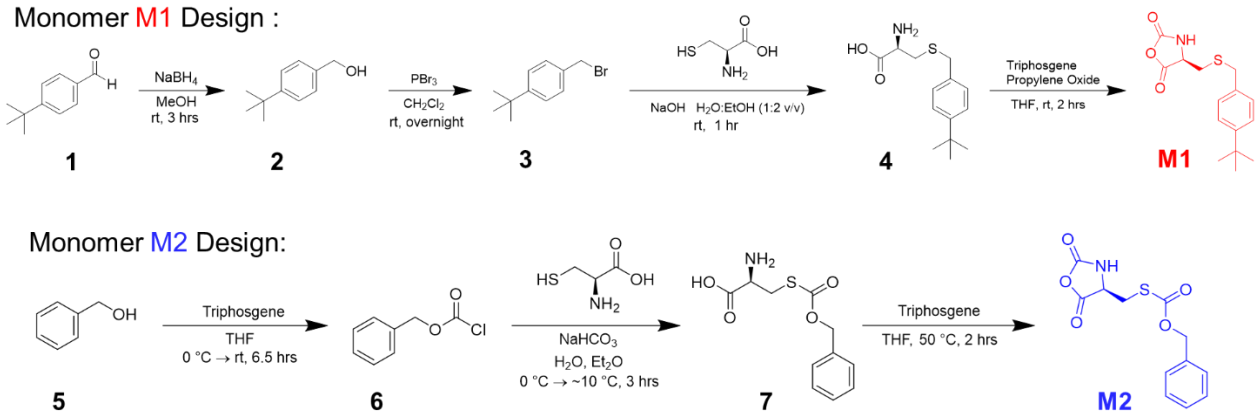


*Figure 6: Synthetic Scheme for NCA monomers **M1** and **M2***

For the synthesis of NCA monomer **M1** from the *tert*-butylbenzene protected amino acid tBuBnCys **4**, the moisture tolerant method of Tian, ZY et al. was used.[8] For the synthesis of tBuBnCys, we start from tBuBnOH **2**, which was synthesized by the reduction of tBuBnCHO (**1**), using $NaBH_4$. Then convert it to tBuBnBr **3** by function group transformation from alcohol to bromide using PBr_3. Then the tBuBnBr is coupled with cysteine by nucleophilic substitution by cysteine's thiol on bromine to get tBuBnCys **4**.

Monomer **M2** was synthesized from the S-Cbz protected L-cysteine (CbzCys) **7** using triphosgene under heating conditions at 50 °C. The S-Cbz-L-cysteine **7** was made from L-cysteine by selective S-Cbz protection by using biphasic heterogeneous conditions and maintaining pH 8.5 using 1M aq. $NaHCO_3$ solution. The benzyloxy carbonyl chloride (CbzCl) **6** was synthesized by converting benzyl alcohol to benzyl chloroformate using triphosgene. This method is not known in the literature and was done for the first time. The product formation was confirmed by the characteristic C=O stretching frequency at 1779 cm^{-1} and the 5.2 ppm benzylic CH_2 peak in the ^1H NMR.

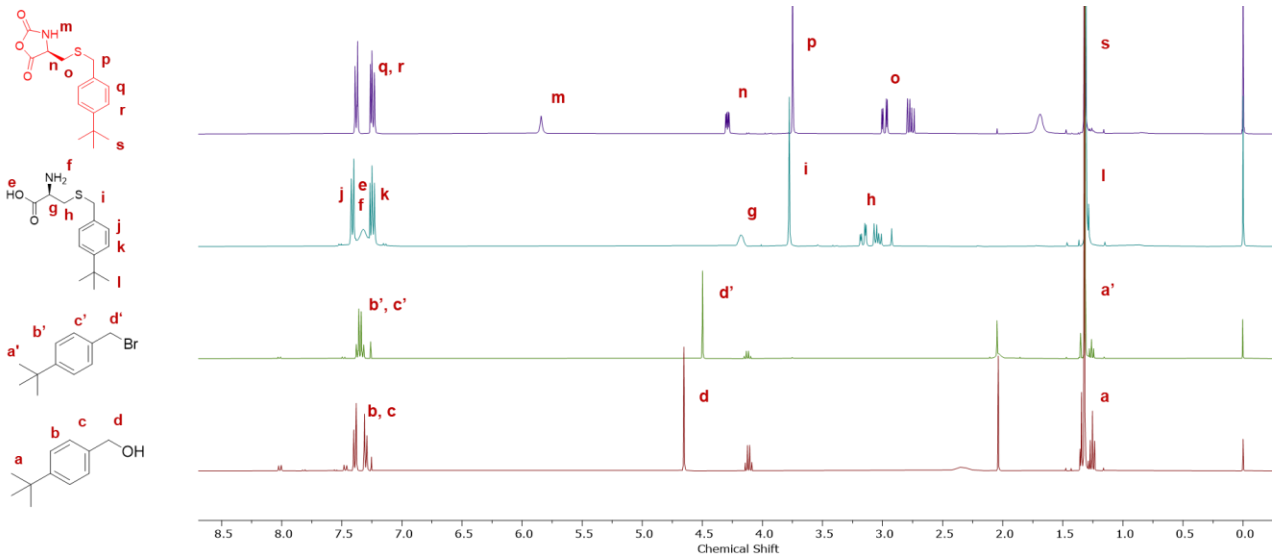


Figure 7: ^{1}H NMRs of NCA monomer M1 and its precursors.

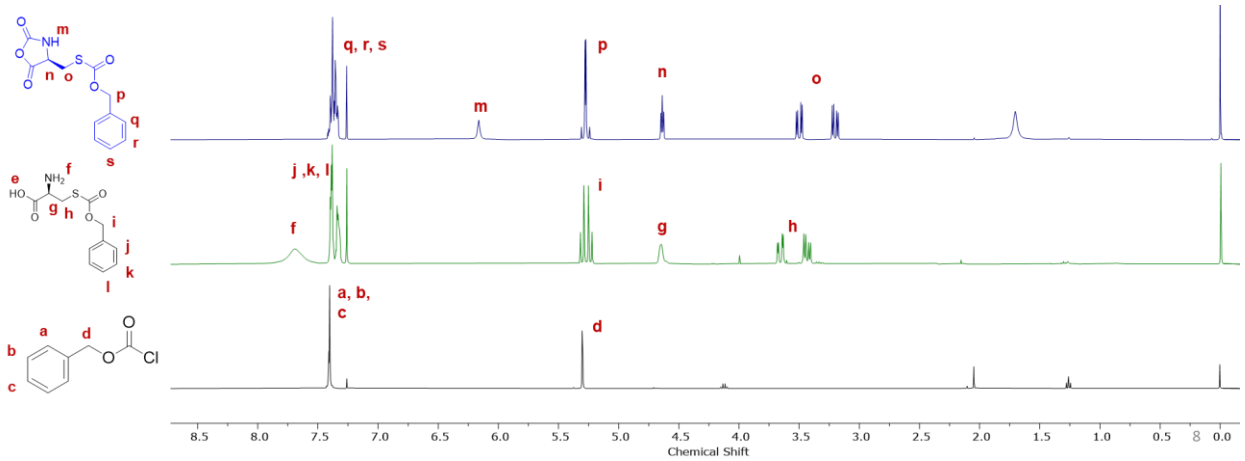


Figure 8: ^{1}H NMRs of NCA monomer M2 and its precursors.

The bromination of the alcohol **2** was confirmed by the benzylic CH_2 shifts from 4.6 ppm to 4.5 ppm (d → d') upfield because bromine is less electronegative than oxygen. A shift in NH proton is also observed when **4** is converted to **M1**. From Figure 7, the splitting is observed in the benzylic CH_2 because of chirality of the α-carbon of amino acid. Similar NH shift is observed for **M2**. Both the NCA monomers as well as their intermediate step products were characterized by ^{1}H, ^{13}C, FTIR and HRMS or MALDI-TOF. For both NCAs, appearance of the C=O stretching corresponding to anhydride were observed in the IR spectrum that confirms the NCA formation.

3.2. MeO-PEG$_{5000}$-NH$_2$ initiated Polymers Synthesis and Characterization

3.2.1. PEG-b-tBuBnCys

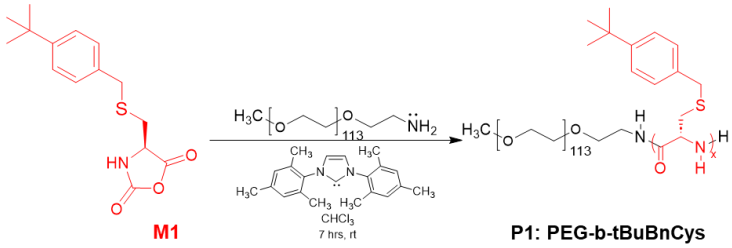
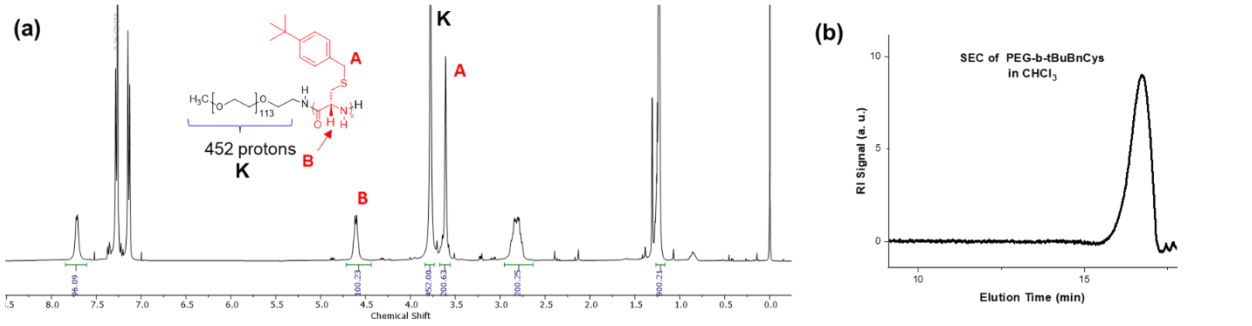


*Figure 9: Synthetic Scheme for polymerization of NCA monomer **M1***

The synthesized NCA monomer **M1** were subjected to ring opening polymerization using MeO-PEG$_{5000}$-NH$_2$ (PEG-amine) as the macroinitiator in presence Imes.HCO$_3$ as the NHC catalyst in CHCl$_3$ as the solvent. The characterization of polymer was done using ^{1}H NMR, ^{13}C NMR along with SEC. Also, FTIR kinetic study was done.



Polymer	M / I	n (^{1}H-NMR)	M_n NMR (g/mol	M_n SEC (g/mol)	M_w SEC (g/mol)	M_w/M_n
PEG-b-(tBuBnCys)	100	100	30000	18000	20000	1.1

Figure 10: (a) ^{1}H NMR, (b) SEC, (c) Characterization Table for PEGylated diblock PEG-b-tBuBnCys.

For the polymerization of NCA monomer **M1**, **P1** the feed ratio, M/I was 100. The degree of polymerization obtained was also found to be 100 as shown in ^{1}H NMR in Figure 10 (a). The degree of polymerization (X_n) was determined by integrating the PEG-chain protons (-O-C$_2$H$_4$-, peak “K” in Figure 10 (a)) was normalized to 452 and compared to the total integration of the chiral C**H** proton of the amino acid side chain

(C**H**CH_2, peak "B" in Figure 10 (a)). The polymer was further characterized by SEC (Figure 10 (b)) which indicated a very narrow dispersity with PDI (M_w/M_n) of 1.1. The results have been summarized in the characterization table in Figure 10 (c).

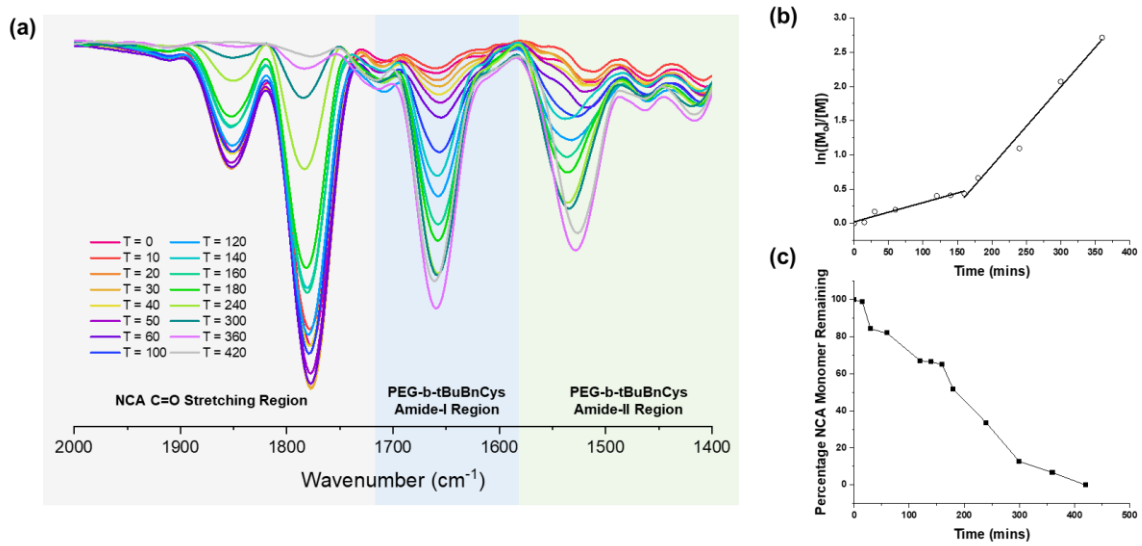


*Figure 11: (a) Real Time FTIR kinetics Plot for PEG-b-tBuBnCys **P1** (b) ln([M0]/[M]) vs time plot for PEG-b-tBuBnCys **P1** (c) Percentage NCA remaining at different time plot for PEG-b-tBuBnCys **P1.***

To learn more about the kinetics of the NCA monomer **M1**, real-time FTIR kinetics was conducted. The polymerization was monitored by periodically taking aliquots from the reaction mixture, using the carbonyl peaks in the monomer at 1857 cm^{-1} (HC-C═O stretching frequency) and at 1777 cm^{-1} (HN–C═O stretching frequency). The monomer consumption in the ROP was confirmed by the progressive drop in carbonyl peak intensities with increasing reaction time (Figure 11 (a)). The polymerization was completed in 6 hours. The amount of NCA remaining in the polymerization is calculated by comparing the initial area of peaks at 1780 cm^{-1} to the area at a specific time interval. The percentage of NCA monomer remaining is plotted against reaction time (Figure 11 (c)), revealing controlled ROP and complete consumption of monomer **M1** at the end of polymerization. The secondary structures were monitored using the amide-I peaks, which suggested that the polymerization goes from a β-sheet conformation in the initial stages and later adopts an α-helical conformation. The rate constant of polypeptide polymerization was determined by plotting $\ln([M]_0/[M])$ against time from unreacted NCA at different time points (Figure (11 (b)). Since there is a transition from initial β-sheet conformation to final α-helical conformation, a typical two-

stage kinetic was observed. Hence, the graph in Figure 11 (b) is fitted as two linear plots, one for the growth of α-helical chains at a rate constant of 11.54 × 10^{-3} min^{-1} and the other for the initial β-sheet oligomers at 2.8 × 10^{-3} min^{-1}.

3.2.2. PEG-b-CbzCys

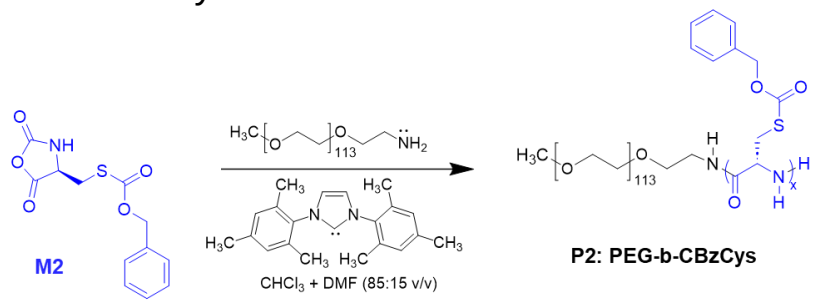
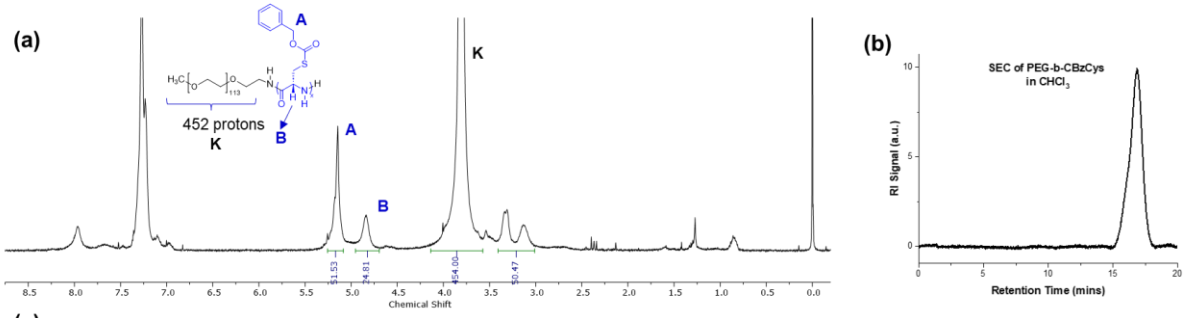


*Figure 12: Synthetic Scheme for polymerization of NCA monomer **M2.***

For polymerization of the NCA monomer **M2**, the polymerization was carried out in DMF-$CHCl_3$ (15:85 v/v) as the solvent due to its limited solubility in $CHCl_3$. The synthesized NCA monomer **M2** was also subjected to ring opening polymerization using MeO-PEG_{5000}-NH_2 (PEG-amine) as the macroinitiator in presence Imes.HCO_3 as the NHC catalyst in $CHCl_3$ as the solvent. The characterization of polymer was done using ^{1}H NMR, ^{13}C NMR along with SEC. Also, FTIR kinetic study was done.



(c)

Polymer	M / I	n (^{1}H-NMR)	M_n NMR (g/mol	M_n SEC (g/mol)	M_w SEC (g/mol)	M_w/M_n
PEG-b-(CBzCys)	25	25	11000	13800	19600	1.4

Figure 13: (a) ^{1}H NMR, (b) SEC, (c) Characterization Table for PEGylated diblock PEG-b-CbzCys.

For polymerization of NCA monomer **M2**, **P2** the feed ratio, M/I was 25 and the degree of polymerization obtained was found to be 25 as shown in ^{1}H NMR in Figure 13 (a).

The degree of polymerization (X_n) was determined by integrating the PEG-chain protons (-O-C_2H_4-, peak "K" in figure 13 (a)) was normalized to 452 and compared to the total integration of the chiral C**H** proton of the amino acid side chain (C**H**CH_2, peak "B" in Figure 13 (a)). The polymer was further characterized by SEC (Figure 13 (b)) which indicated a very narrow dispersity with PDI of 1.1. The results have been summarized in the characterization table in Figure 13 (c).

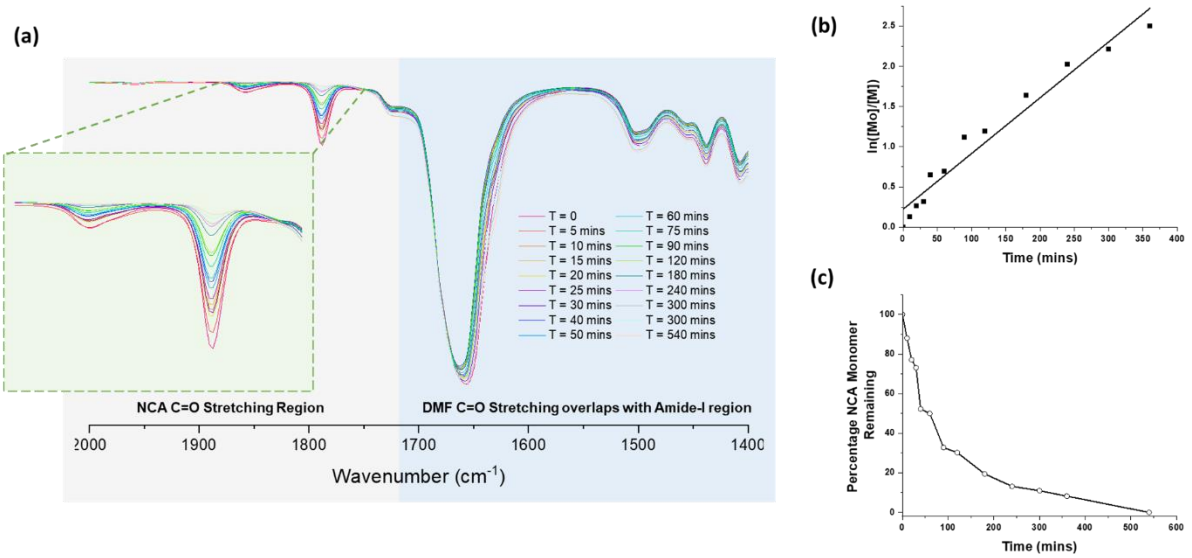


Figure 14: (a) Real Time FTIR kinetics Plot (b) ln([M_0]/[M]) vs time plot (c) Percentage NCA remaining at different time plot for PEG-b-CbzCys ***P2.***

Real time FTIR kinetics was performed for NCA monomer **M2** as well. The FTIR spectra of NCA monomer **M2** polymerization at various time intervals in DMF-$CHCl_3$ (15:85 v/v) are shown in Figure 14 (a). Because DMF is a non-volatile solvent, it is observed in every time point along with the monomer and the polymer formed. Because of the peaks of DMF overlapping with the amide region of polymer, the secondary structures were not able to monitor. We were able to observe only the consumption of NCA. The percentage of NCA monomer remaining is plotted against reaction time (Figure 14 (c)), revealing controlled ROP and complete consumption of monomer **M2** at the end of polymerization. The rate constant of polypeptide polymerization was determined by plotting ln([M]$_0$/[M]) against time from unreacted NCA at different time points (Figure (14 (b)). A typical first order kinetics for ROP was observed with rate constant of 6.9×10^{-3} min^{-1}.

3.2.3. Thermal Characterization of PEG-b-tBuBnCys and PEG-b-CbzCys

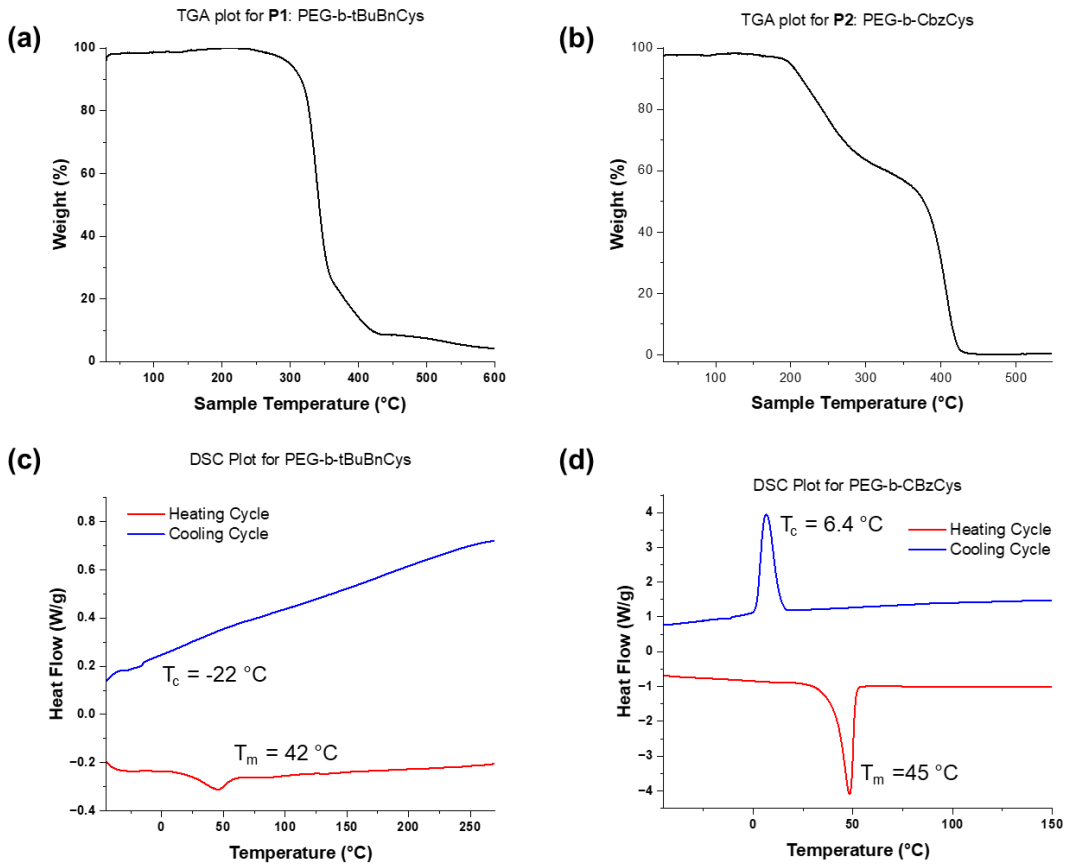


Figure 15: TGA and DSC plots for PEG-b-tBuBnCys and PEG-b-CbzCys

Thermal characterization of the polymers was done using Thermogravimetric Analysis (TGA) and Differential scanning calorimetry (DSC). The polymer P1 was found to be thermally stable till 338 °C above which a two-step degradation is observed. The first degradation step maybe due to the degradation of *tert*-butyl groups while the second degradation step maybe due to the degradation of the remaining polymer. The polymer P2 was found to be thermally stable till 238 °C above which it undergoes two-step degradation. The first degradation step is due to the degradation of the Cbz groups while the second degradation step is due to the degradation of the remaining polymer. The DSC plots for polymer P1 and P2 shows a melting and crystallization transitions corresponding to the crystalline PEG macroinitiator.

3.3. Post Polymerization Oxidation Of *tert*-butylbenzene functionalized based poly(L-Cysteine)

3.3.1. Oxidation Of PEG-b-tBuBnCys **P1**

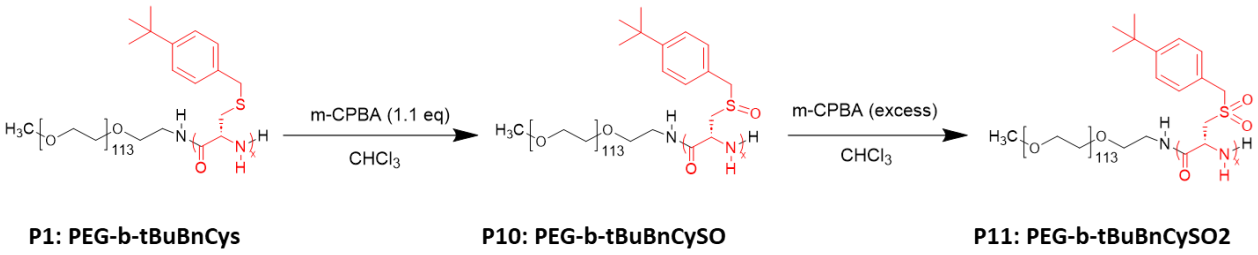


Figure 16: Scheme for oxidation of polymers.

Post polymerization oxidation was performed for PEG-b-tBuBnCys with three different oxidants – mCPBA, H_2O_2, and $NaIO_4$. mCPBA oxidation was performed in $CHCl_3$ and complete oxidation to sulfoxide or sulfone. The oxidation was controlled by the equivalents of mCPBA used. H_2O_2 oxidation was performed in THF and 30% solution of H_2O_2 in presence of acetic acid was used. In this case also the complete oxidation to sulfoxide was observed. But with $NaIO_4$, performed in THF:H_2O (1:1 v/v), only partial oxidation was observed even after 2 days.

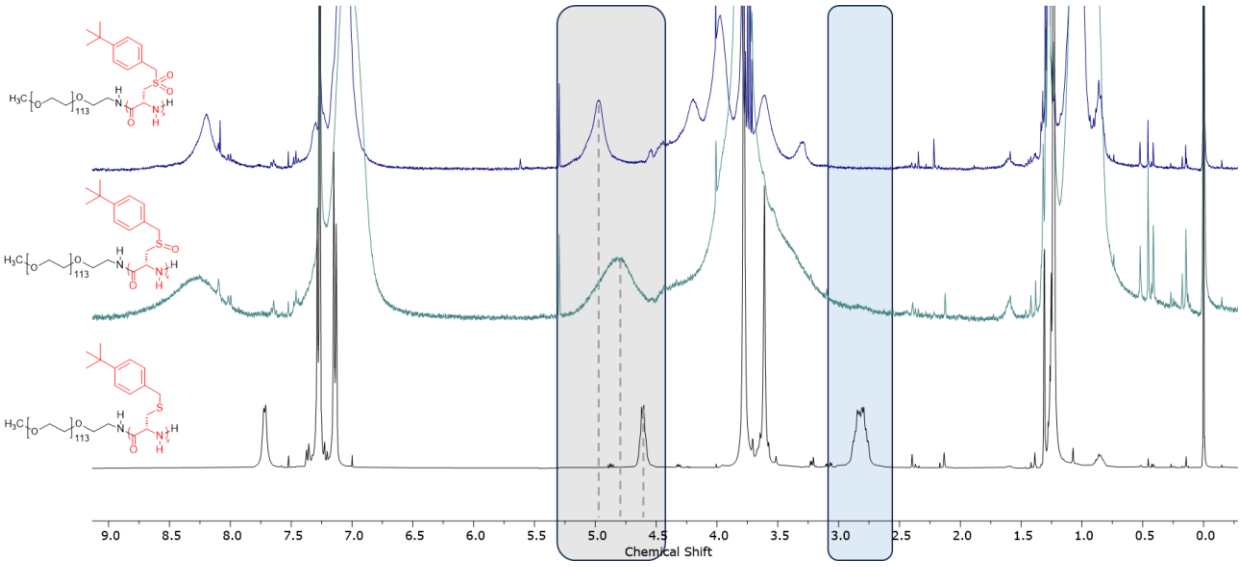
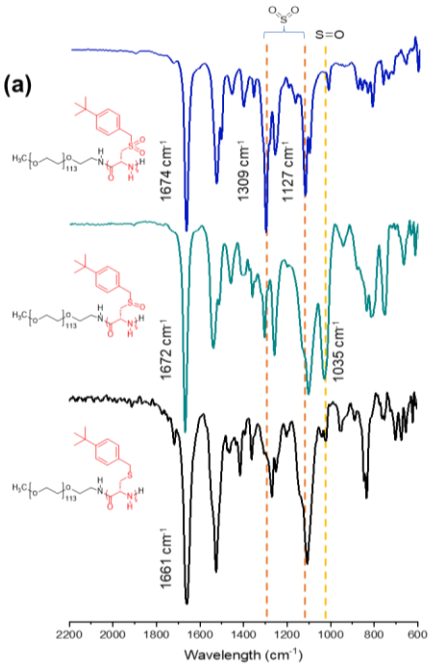
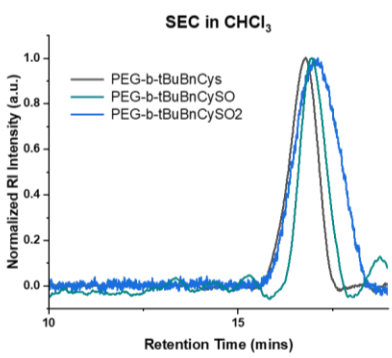


Figure 17: ^1H NMRs of the parent polymer and the oxidized polymers.

Figure 14 (b) shows ^{1}H NMR for the parent polymer **P1** and the oxidized polymer using mCPBA. The CH_2 peak at around 2.8 ppm has completely vanished from the parent polymer and shifted to downfield (shown in blue box in Figure 14(b)). We observed downfield shifts in the CH peaks as the polymer was oxidized (shown in grey box in Figure 14 (b)).





(c)

Polymer	M / I	M_n NMR (g/mol	M_n SEC (g/mol)	M_w SEC (g/mol)	M_w/M_n
PEG-b-tBuBnCySO	100	31500	10100	11400	1.1
PEG-b-tBuBnCySO2	100	33100	10900	15400	1.5

Figure 18: (a) FTIR plots (b) SEC plots for the parent polymer and the oxidized polymers (c) Charcterization Table for the oxidized polymers

The sulfoxide and sulfone formation can also be monitored using FTIR by observing for sulfoxide's S=O stretching frequency at around 1050 cm^{-1} or sulfone's O=S=O asymmetric stretching frequency at around 1325 cm^{-1} and symmetric stretching frequency at around 1140 cm^{-1} (Figure 14 (c)).

The CD spectra indicates that the oxidized polymers as well as the parent polymers adopts α-helical conformation. There is no change in the secondary structures. So, there is no disruption in the conformation due to oxidation of polymers in the backbone. Also, there is increase in hydrophilicity as we oxidize the polymer which is evident by decrease in the water contact angle as we oxidize the polymer (Figure 14 (d), (e), (f)).

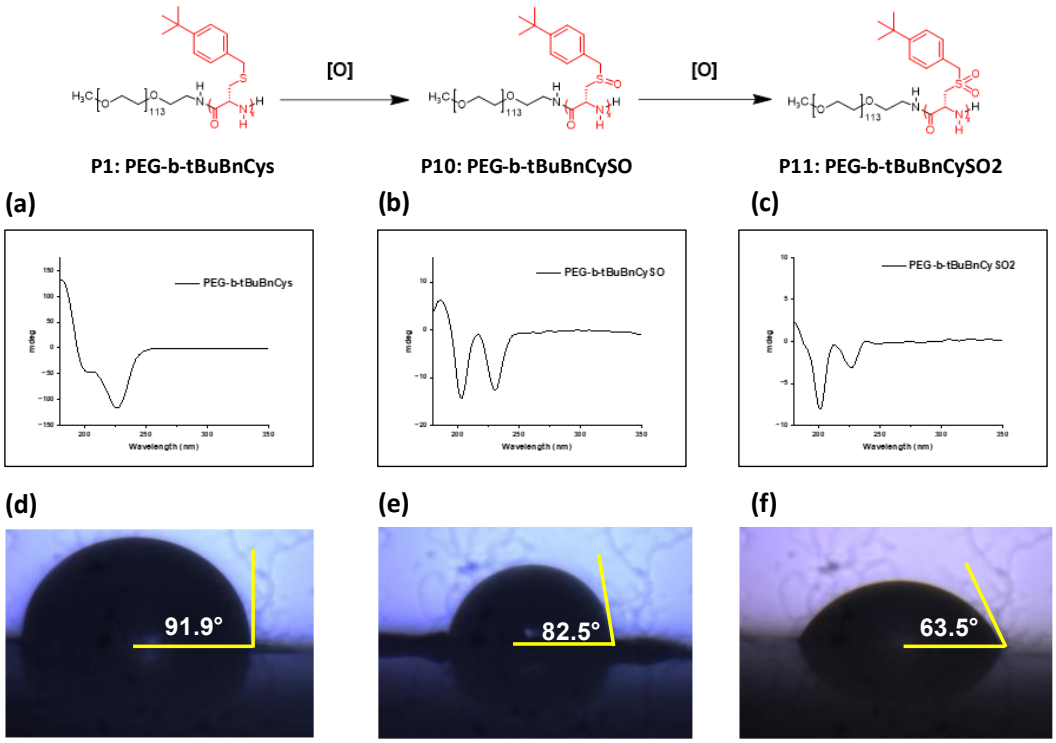


Figure 19: CD Spectra of (a) PEG-b-tBuBnCys recorded as a thin film made from 2 mg/mL solution in $CHCl_3$ (b) PEG-b-tBuBnCySO recorded in 0.1 mg/mL in HFIP (c) PEG-b-tBuBnCySO2 recorded in 0.01 mg/mL in HFIP ; Water Contact Angle measurements for (d) PEG-b-tBuBnCys (e) PEG-b-tBuBnCySO (f) PEG-b-tBuBnCySO2.

3.3.2. Oxidation of ROPISA (Ring Opening Polymerization Induced Self Assembly) polymer

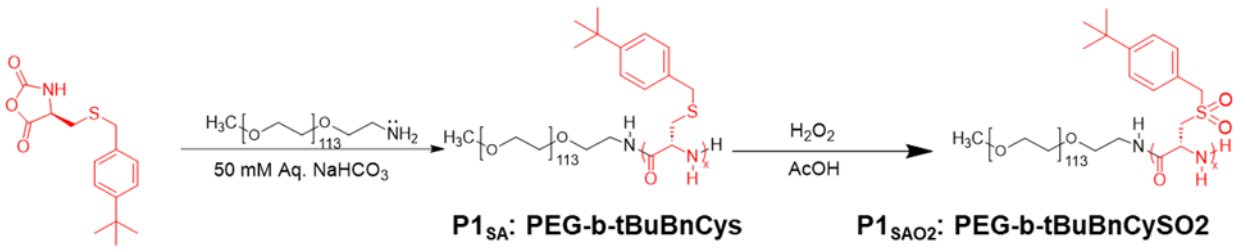


Figure 20: Synthetic Scheme for ROPISA and subsequent in-situ oxidation.

To study changes in the self-assembly, the polymer was made and self-assembled during polymerization using the ROPISA route in aqueous medium. The NCA monomer **M1** was subjected to polymerization in 50 mM aqueous $NaHCO_3$ (pH ~ 8.5) solvent using MeO-PEG_{5000}-NH_2 as a hydrophilic macroinitiator for a M/I ratio 25.

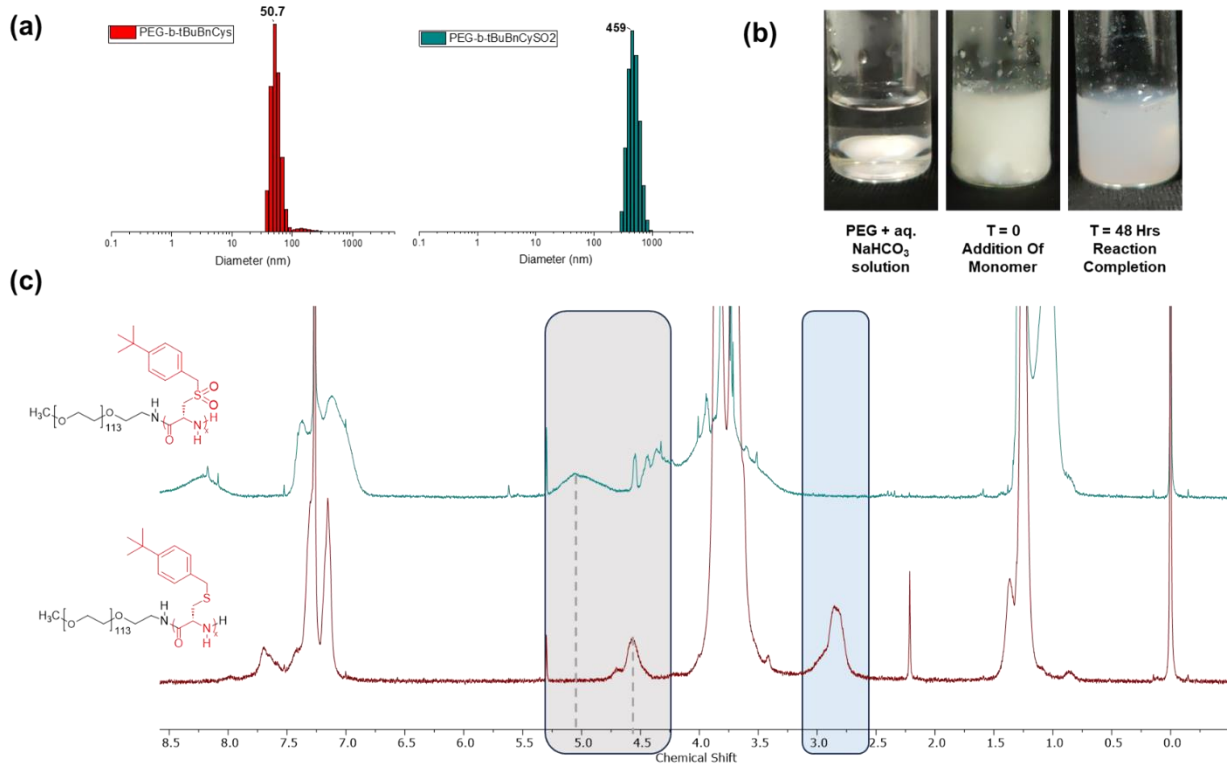


Figure 21: (a) DLS histograms before and after oxidation (b) Photos of reaction mixture at various stages (c) ^1H NMRs before and after oxidation.

Additionally, the amount of monomer and initiator used in the polymerization reaction was chosen in such a way that it produces an emulsion with a total solid content of 0.7 weight percent at the end of the reaction (i.e., 70 mg of polymer would be made in 10 mL of reaction, assuming total monomer consumption), while also maintaining the desired monomer to initiator ratio (M/I). The ROPISA was tried at 7 wt% as well but it precipitates at such high concentrations.

Then later the self-assembled polymer was subjected to in-situ oxidation using H_2O_2 to sulfone which was confirmed by the FT-IR. The NMR indicated complete conversion of thioether to sulfone as the **CH**CH_2 and CH**CH_2** peak of the amino acid shifted completely toward downfield region. The DLS showed increase in size from 50 nm to 459 nm. Thus, oxidation caused the change in the self-assembly.

3.3.3. Thermal Characterization of PEG-b-tBuBnCySO and PEG-b-tBuBnCySO2

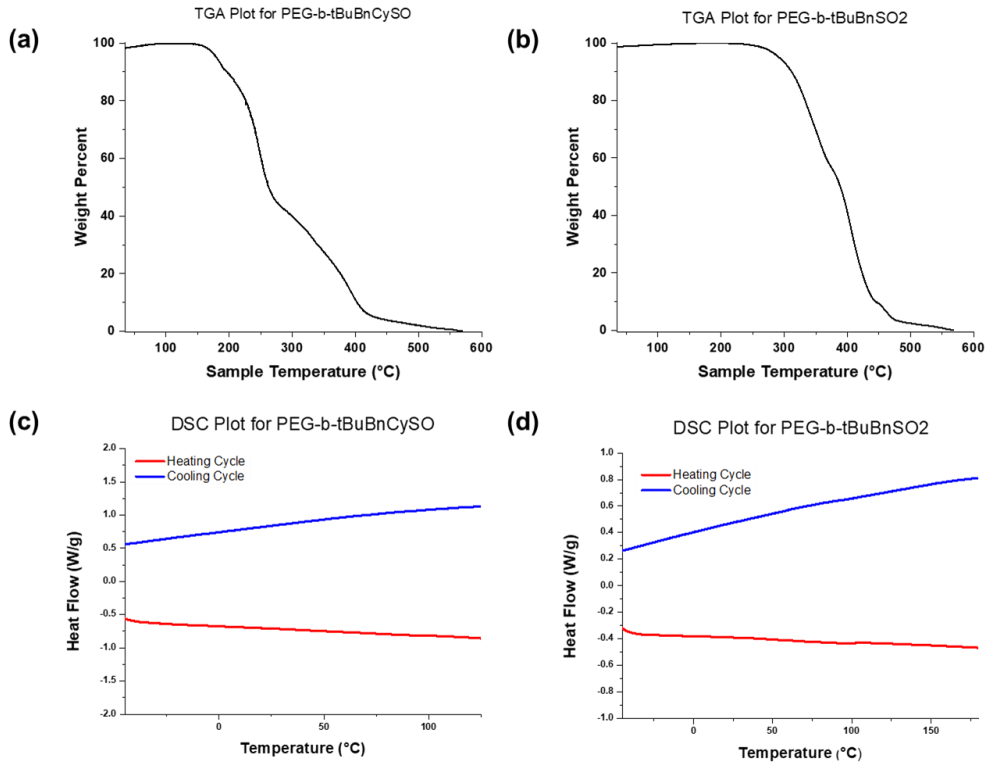


Figure 22: TGA and DSC plots for PEG-b-tBuBnCySO and PEG-b-tBuBnCySO2.

The polymer PEG-b-tBuBnCySO was found to be thermally stable till 156 °C above which a two-step degradation is observed. Similarly, the polymer P2 was found to be thermally stable till 254 °C and showed two-step degradation. The DSC plots for both the polymer shows absence of any transitions indicating amorphous nature.

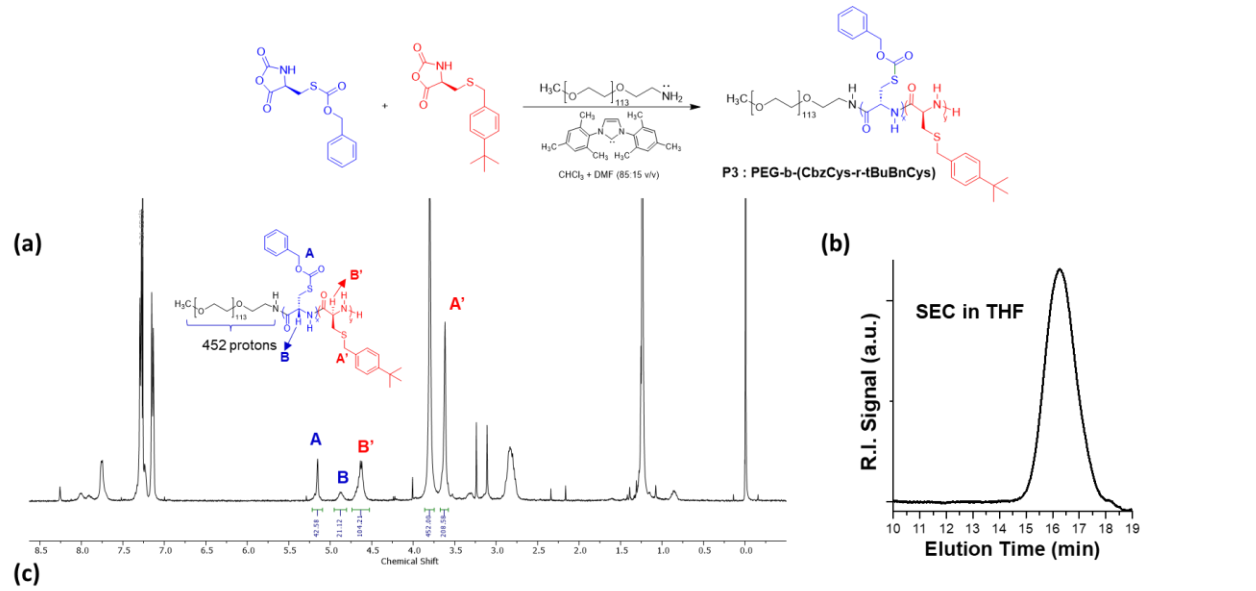
3.4. Thiol functionalized Copolymers

The *tert*-butylbenzene functionalized poly(L-cysteine) maintained α-helical conformation even after the oxidization to sulfoxide or sulfone. So, we thought that incorporating some units having propensity to adopt β-sheet conformation could lead to some conformational switch later due to oxidation. So, we decided to use the Cbz group which has the tendency to adopt β-sheet conformation. But it cannot form high molecular weight poly(L-cysteine) due to its growing β-sheet nature. Hence, we thought of making copolymers based on tBuBnCys and CbzCys so that the tBuBnCys units can act as a solubilizing unit. The Cbz group is deprotectable using HBr while the tBuBnCys is tolerant towards HBr. So, we can easily deprotect the Cbz units and hence can get actual β-sheet propensity units without affecting the α-helical tendency of tBuBnCys (which in turn would be useful for imparting solubility). So, we propose that the polymer may be predominantly α-helical in nature but after deprotection and subsequent oxidation may change its nature. Also, in this process we could be able to make polypeptide based thiolated polymers which are soluble and hence can be used in further applications. So, two birds in one stone.

To make soluble thiolated polymers, first random and block copolymers of protected NCA monomers M1 and M2 were synthesized and then later the Cbz units were subjected to post polymerization deprotection. The composition of CbzCys to tBuBnCys units and the chain length was fixed to 1:4 and 100 respectively.

3.4.1. Random Copolymer Synthesis

The random copolymer was made by taking both the monomers together and then subjecting them to ROP using PEG-amine as the initiator and Imes.HCO_3 as the NHC catalyst in DMF-$CHCl_3$ (15:85). As shown in Figure 24 (a), the composition obtained is almost similar to the feed ratio. The SEC has a monomodal distribution with polydispersity of 1.5. The characterization table in Figure 24 (c) summarized all the data.



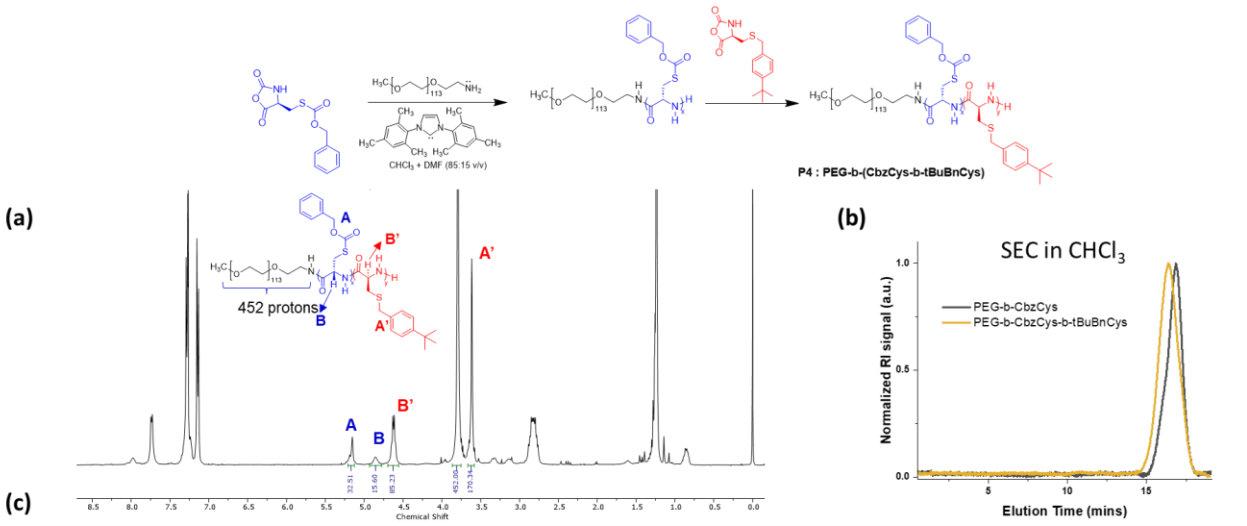
Polymer	Feed ratio (x : y)	M / I	Composition (x : y) obtained	n (^{1}H-NMR)	M_n NMR (g/mol)	M_n SEC (g/mol)	M_w SEC (g/mol)	M_w/M_n
PEG-b-(CbzCys-r-tBuBnCys)	1 : 4	100	21 : 104	125	35800	26300	38900	1.5

Figure 23: (a) ^{1}H NMR, (b) SEC, (c) Characterization Table for random copolymer PEG-b-((CbzCys-rtBuBnCys)).

3.4.2. Block Copolymer Synthesis

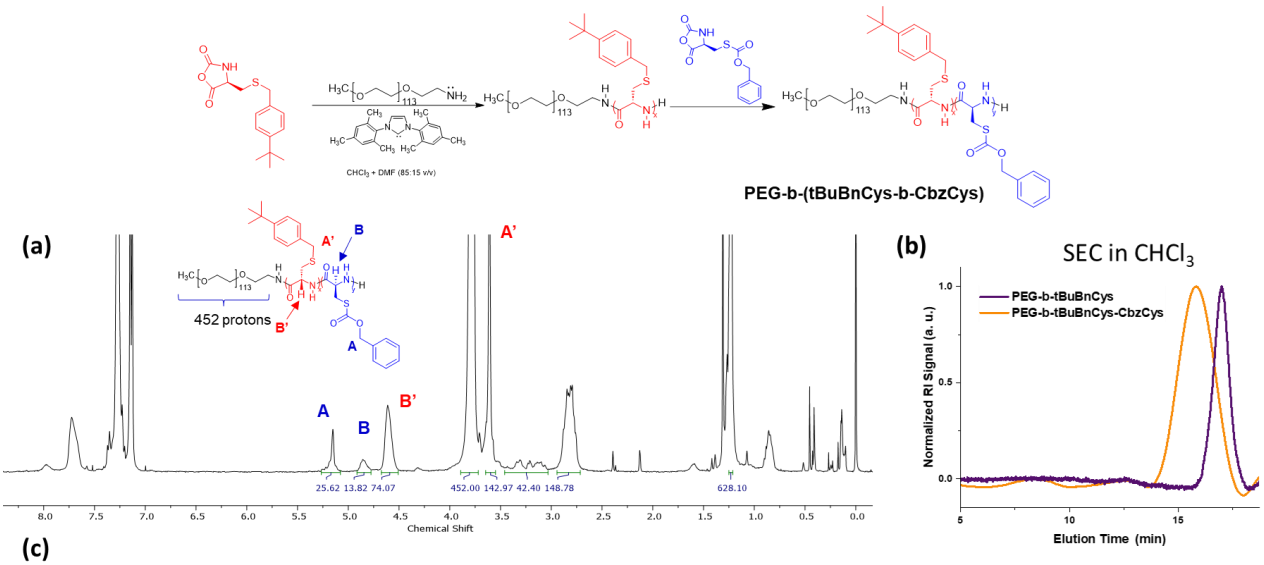
The block copolymers were made by subjecting the first monomer to ROP using PEG-amine as the initiator and Imes.HCO_3 as the NHC catalyst in DMF-$CHCl_3$ (15:85) and then subsequently growing the second monomer using the PEG diblock was the macroinitiator. The polymerization was monitored using FTIR by taking aliquots and observing the NCA's anhydride peaks.

For PEG-b-((CbzCys-b-tBuBnCys)), as shown in Figure 22 (a), the composition obtained is almost similar to the feed ratio. The SEC has a monomodal distribution with polydispersity of 1.5. The first block which was used as a macroinitiator for growing next block also has a monomodal distribution with polydispersity of 1.4. We could observe a shift in SEC plots between the first and the next block copolymers.



Polymer	Feed ratio (x : y)	M / I	Composition (x : y) obtained	n (^{1}H-NMR)	M_n NMR (g/mol	M_n SEC (g/mol)	M_w SEC (g/mol)	M_w/M_n
PEG-b-(CbzCys-b-tBuBnCys)	1 : 4	100	15 : 85	100	29700	23500	36300	1.5
PEG-b-(CBzCys)		25		15	8500	7800	11700	1.4

Figure 24: (a) ^{1}H NMR, (b) SEC, (c) Characterization Table for block copolymer PEG-b-((CbzCys-b-tBuBnCys)).



Polymer	Feed ratio (x : y)	M / I	Composition (x : y) obtained	n (^{1}H-NMR)	M_n NMR (g/mol)	M_n SEC (g/mol)	M_w SEC (g/mol)	M_w/M_n
PEG-b-(tBuBnCys-b-CbzCys)	1 : 4	100	25 : 74	99	29300	25000	37500	1.5
PEG-b-(tBuBnCys)		75		74	23400	14600	18700	1.3

Figure 25: (a) ^{1}H NMR, (b) SEC, (c) Characterization Table for block copolymer PEG-b-((tBuBnCys-b-CbzCys)).

For PEG-b-((tBuBnCys-b-CbzCys)), as shown in Figure 23 (a), the polymerization was in control and the composition obtained matches to the feed ratio. The SEC has a monomodal distribution with polydispersity of 1.5. The first block macroinitiator also had a narrow monomodal distribution with PDI of 1.3. The units were also confirmed using NMR.

So, a better control was observed for the PEG-b-((tBuBnCys-b-CbzCys)) because of its α-helical nature. The growing chain in case of PEG-b-((tBuBnCys-b-CbzCys)) is α-helical but in case of PEG-b-((CbzCys-b-tBuBnCys)), it is β-sheet. In case of random copolymer, it switches between the two.

3.4.3. Deprotection of Random and Block Copolymer

Now the Cbz protected units were selectively deprotected using 33% HBr/AcOH in 1:1 $CHCl_3$:TFA as the solvent. The deprotected polymers are prone to disulfide oxidation and hence they were stored in dark, and care was taken to avoid moisture exposure. The random copolymer, as seen from the FTIR and CD plots (Figure 25), it transitions from predominantly α-helical conformer to a random coil conformer which may be due to inherent randomness in the polymer composition.

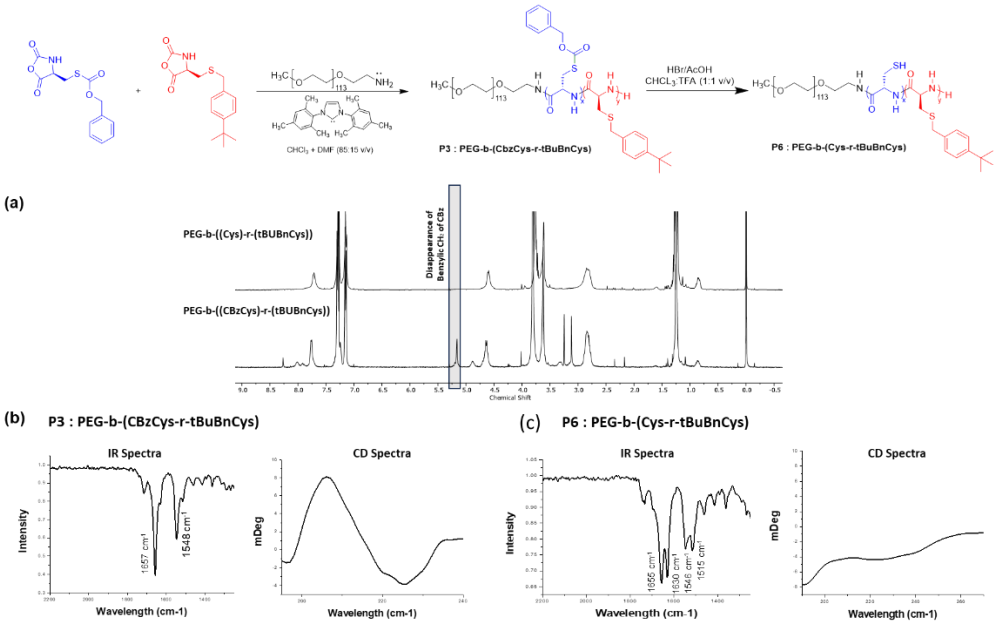


Figure 26: (a) ^{1}H NMR of Protected vs Deprotected Polymers (b) SEC of PEG-b-((CbzCys)-r-(tBuBnCys)) (c) FTIR and CD spectra of PEG-b-((CbzCys)-r-(tBuBnCys)) (d) FTIR and CD spectra of PEG-b-((Cys)-r-(tBuBnCys)).

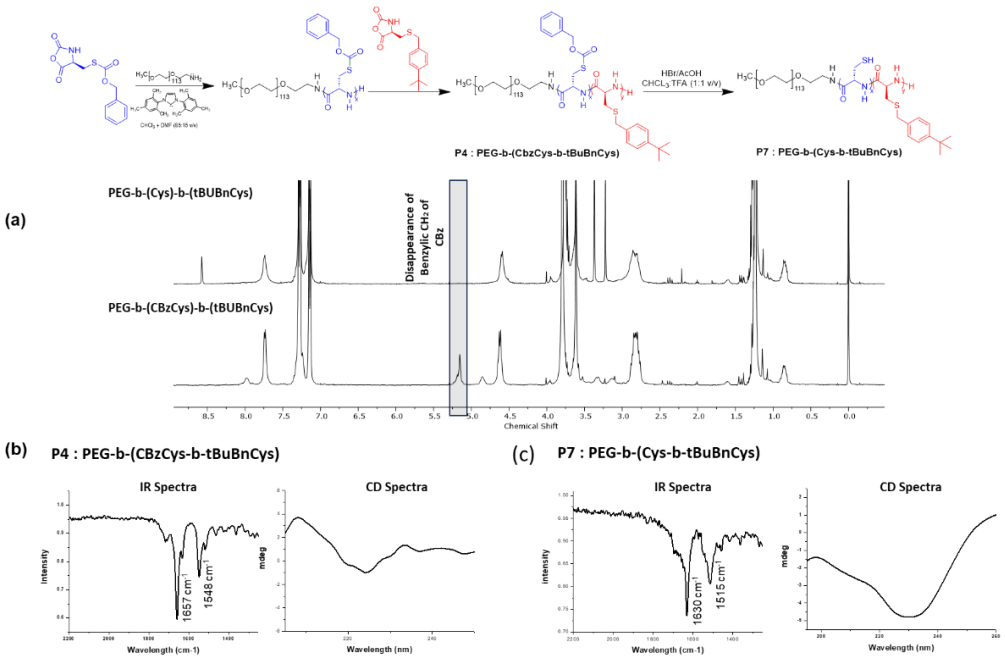


Figure 27: (a) ^{1}H NMR of Protected vs Deprotected Polymers (b) SEC of PEG-b-((CbzCys)-b-(tBuBnCys)) (c) FTIR and CD spectra of PEG-b-((CbzCys)-b-(tBuBnCys)) (d) FTIR and CD spectra of PEG-b-((Cys)-b-(tBuBnCys)).

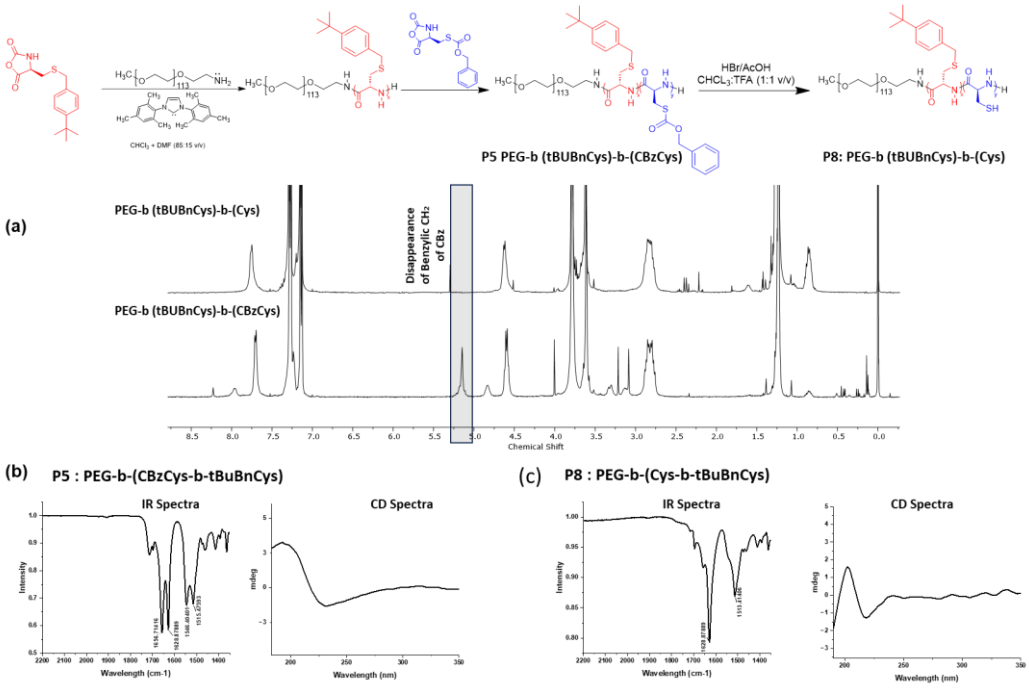


Figure 28: (a) ^{1}H NMR of Protected vs Deprotected Polymers (b) SEC of PEG-b-((tBuBnCys))-b-(CbzCys)) (c) FTIR and CD spectra of PEG-((tBuBnCys))-b-(CbzCys)) (d) FTIR and CD spectra of PEG-((tBuBnCys))-b-(CbzCys)).

In case of the PEG-b-((CbzCys-b-tBuBnCys)) block copolymer (Figure 26), it transitions from predominantly α-helix conformer to β-sheet conformer. While in the case of PEG-b-((tBuBnCys-b-CbzCys)) (Figure 27), it transitions from mixed conformation to β-sheet conformer.

3.4.4. Oxidation of deprotected polymer **P8**

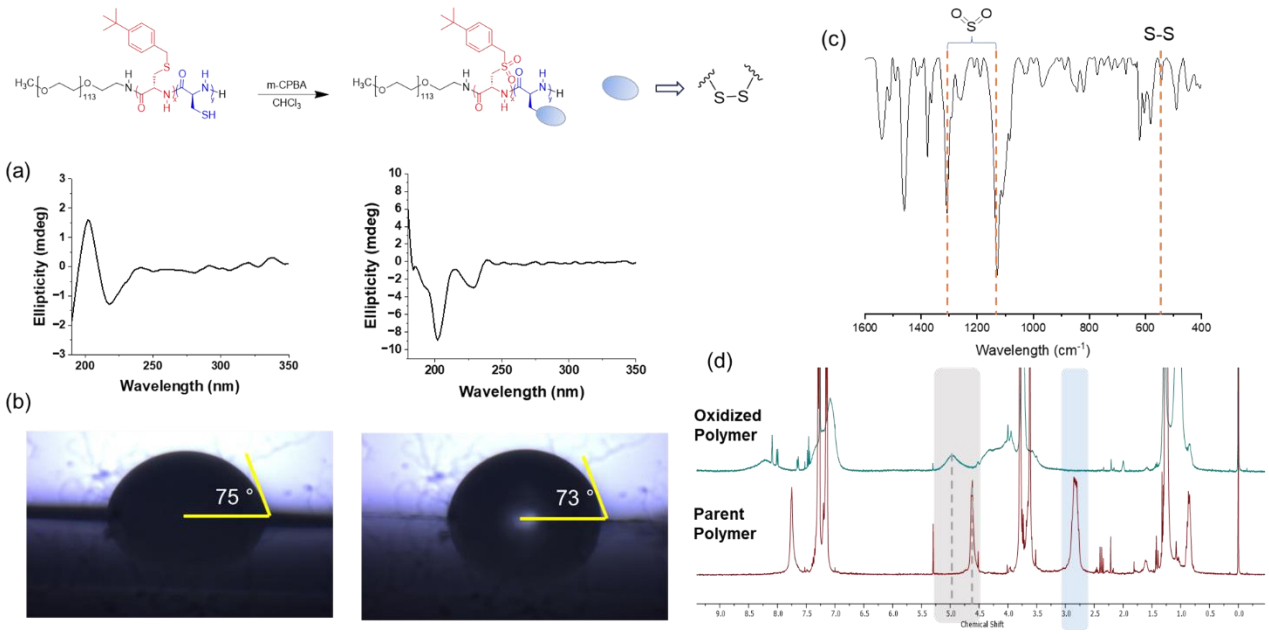


Figure 29: (a) CD Spectra for parent and oxidized polymers (b) Water contact angle measurements (c) FTIR spectra of oxidized polymer (b) ^{1}H NMR for parent and oxidized polymers.

Further, the deprotected polymer **P8** was oxidized using excess mCPBA to sulfone. The secondary structure changed from β-sheet to α-helical as seen in CD spectra. The disappearance of the CH_2 peak at 2.8 ppm as well as shift in the CH peak from 4.6 ppm to 4.9 ppm in the ^{1}H NMR indicates complete oxidation to sulfone. The peaks at 1129 cm^{-1} and 1307 cm^{-1} observed in the FTIR correspond to O=S=O sulfone peaks. The thiols usually can get oxidized to disulfide or sulfonates but since there is no peak in the IR in the sulfonate region, it might have formed disulfides instead as indicated by the peak at 541 cm^{-1} in FTIR spectra. The water contact angle measurements show an increase in hydrophilicity after oxidation.

3.4.5. Post polymerization Thiol-ene

To know the amount of free thiols, present in the deprotected thiolate polymers, DTNB, also known as Ellman's reagent, was used. So, we first tried performing the estimation in DMF; but the polymer does not solubilize quickly in DMF. So, a mixture of DMF-$CHCl_3$ (1:1 v/v) was tried and its absorbance at around 500 nm was observed. This use of mixture was done because the polymer was soluble in $CHCl_3$ but the DTNB itself was not. We were able to observe a small peak hump after 21 hours. It indicated that only some amount of free thiol was present. In the DMF-$CHCl_3$ (1:1 v/v) solvent system, only a limited amount of polymer was able to get dissolved. So, we wanted to investigate if the less prominent peak was due to less free thiols or due to low concentration of polymer dissolved and hence ineffectiveness of DTNB to detect such low concentrations. So, we measured free thiol content using DTNB for a standard molecule, L-cysteine at different concentrations in solvent DMF. As shown in the figure, we found that the DTNB was not effective to detect thiols below 3 uM concentrations for DMF as solvent. The thiol unit concentration in the polymer was around 1 uM and the solvent system was DMF-$CHCl_3$ (1:1 v/v). So, in such a solvent system the DTNB is even more less effective! So, an assay which is soluble in $CHCl_3$ (such as 4,4′-dipyridyl disulfide) and has a high molar extinction coefficient would have been a better choice.

Another way of confirming that if there were free thiols present was to perform the thiol-ene reaction directly with a reactive enes. So, thiol-ene reaction was performed with HEMA and **P7** in the same pot in which the deprotection was done. The thiol-ene clicking of HEMA was done using DBU as the catalyst with minimum amount of moisture exposure and dark conditions maintained throughout the reaction. The FTIR showed change in wavenumber in the amide-I region to 1648 cm^{-1} which indicates change in the conformation from β-sheet to α-helical nature.

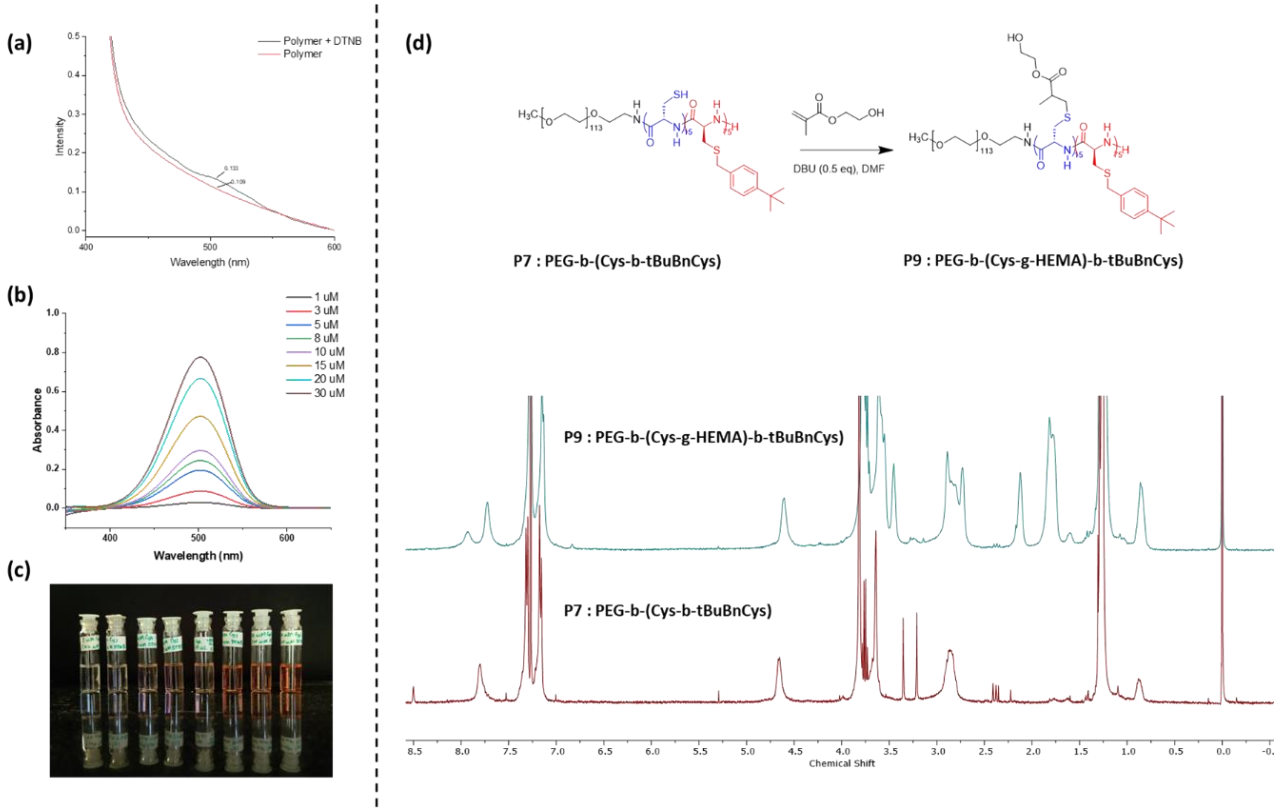


Figure 30: (a) Absorbance Spectra of P7 before and after treating with DTNB. (b) Absorbance Spectra for various concentrations of cysteine when treated with DTNB (c) Photos of the vials of different concentrations of Cysteine after treating with DTNB (d) ^{1}H NMR of clicked polymer.

3.5. Completely thiolated poly(L-cysteine)

For the first time, pure high molecular weight poly(L-cysteine) [without any substitution] was synthesized by completely deprotecting the *tert*-butylbenzene group. This was done is relatively less harsh conditions than using HBr for cleavage. The thioether was cleaved using TMSI in presence of a scavenger thioanisole in $CHCl_3$:TFA (1:1 v/v) as the solvent. ^1H NMR analysis shows complete disappearance of the protecting unit (Figure 30). There is some diethyl ether impurity which shows up in the NMR. But besides that, the CH, CH_2 and the $(CH_3)_3C$ protons have disappeared. We could observe a CH peak but the CH_2 peak was not observed which may be due to partial solubility of the polymer in $CDCl_3$-TFA. We might get a better NMR in TFA-d. CD analysis is still pending for the polymer.

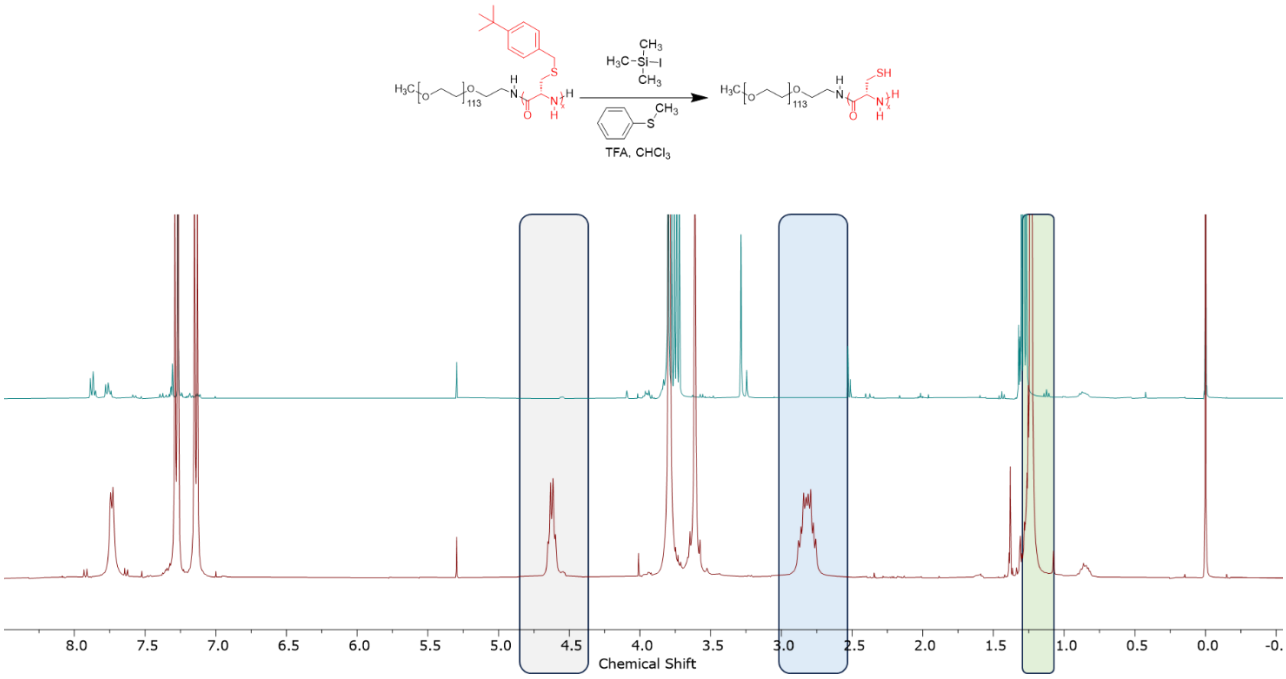


Figure 31: ^1H NMR Comparison between protected and deprotected polymer.

4. CONCLUSION

To study the oxidative response of the *tert*-butylbenzene functionalized poly(L-cysteine) **P1**, the polymer was synthesized from its NCA monomer **M1**. The monomer **M1** was tailor-made using multistep organic synthesis. Then it was subjected to ring opening polymerization using MeO-PEG$_{5000}$-NH$_2$ as the macroinitiator in presence of NHC catalyst. The NMR, SEC and the FTIR kinetic study was performed to ensure that the polymerization goes in a controlled manner and can be used for further block copolymer synthesis. The polymer was also found to be thermally stable till around 300 °C. The thioether polymer **P1** was oxidized to sulfoxide and sulfone using MCPBA in controlled manner during post-polymerization. Two other oxidants were also used such as biologically relevant H_2O_2 and $NaIO_4$. The PEG-b-tBuBnCys maintained α-helical conformation even when oxidized to sulfoxide or to sulfone. Self-assembly of PEG-b-tBuBnCys, done using ROPISA route, showed particle size of 50 nm which increased to 459 nm when oxidized to sulfone in-situ. So, an increase in size was observed when oxidized to sulfone even though there was no change in the secondary structures. Next to incorporate β-sheet favoring units, monomer **M2** was synthesized. First PEGylated homopolymer **P2** was to ensure that the polymerization goes in a controlled manner and can be used for further block copolymer synthesis. Then soluble thiolated poly(L-cysteine) base polypeptides were synthesized from the deprotection of the random and block copolymers. They showed transition in their secondary structures from α-helical to random for random copolymer, from α-helical or mixed structures to β-sheet conformations for the block copolymers. The control was better in the case of **P5** and hence its deprotected polymer **P8** was used for oxidation. A transition from β-sheet to α-helical was observed. Further, a thiol-ene click reaction with HEMA was done as a proof-of-concept for application of thiolated polymers. Instead of HEMA, any other fluorophore can also be clicked. The polymer **P1** was for the first time completely deprotected to get high molecular weight pure poly(L-cysteine).

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6. APPENDIX

- Supporting Data for M1, M2 and its precursors

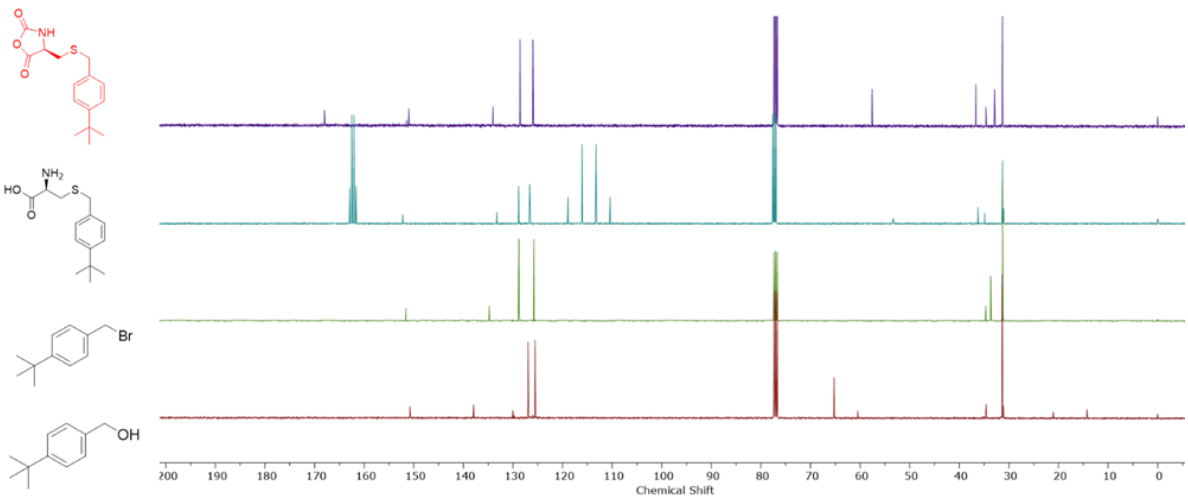

Figure 32: ^{13}C NMR of NCA monomer M1 and its precursors.

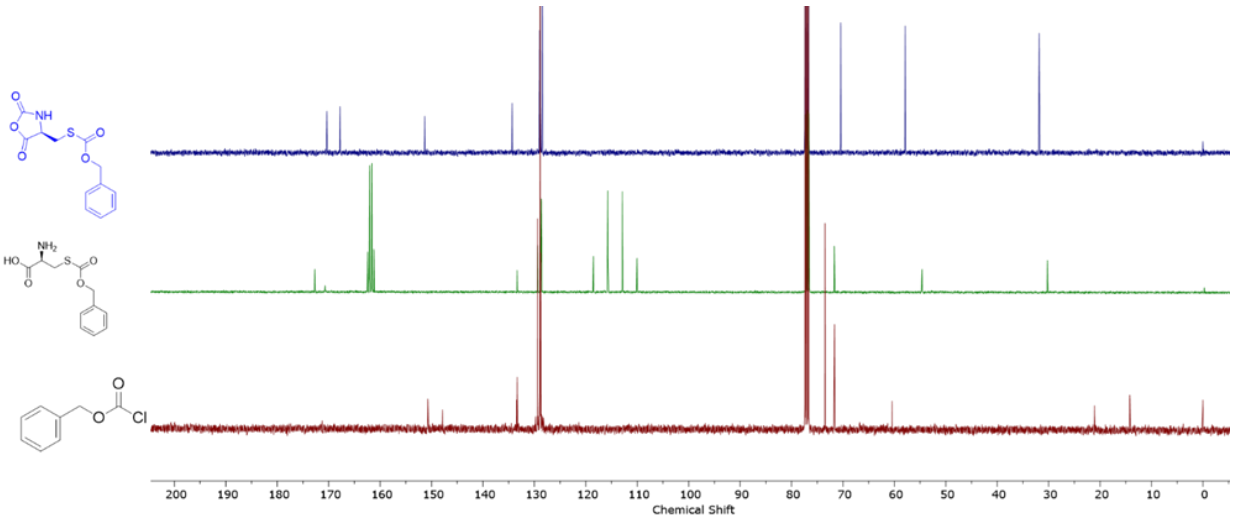

Figure 33: ^{13}C NMRs of NCA monomer M2 and its precursors.

- Block Copolymer Data

Polymer	Feed ratio (x : y)	M / I	Composition (x : y) obtained	n (^{1}H-NMR)	M_n NMR (g/mol)	M_n SEC (g/mol)	M_w SEC (g/mol)	M_w/M_n
ABC Triblock								
PEG-b-tBuBnCys-b-CbzCys	1 : 4	100	25 : 110	135	38300	40500	65000	1.6
PEG-b-tBuBnCys-b-CbzCys	1 : 4	100	25 : 74	99	29300	25000	37500	1.5
ACB Triblock								
PEG-b-CbzCys-b-tBuBnCys	1 : 4	50	15 : 85	100	29700	23500	36300	1.5
PEG-b-CbzCys-b-tBuBnCys	1 : 4	50	13 : 75	88	26700	33900	290326	1.8
PEG-b-CbzCys-b-tBuBnCys	3 : 2	50	22 : 7	29	12000	9700	11100	1.2
PEG-b-CBzCys-b-tBuBnCys	2 : 3	50	15 : 30	45	16000	7500	13500	1.8

Figure 34: Table summarizing data of block copolymers synthesized.

- S-Trityl as the protecting group data:

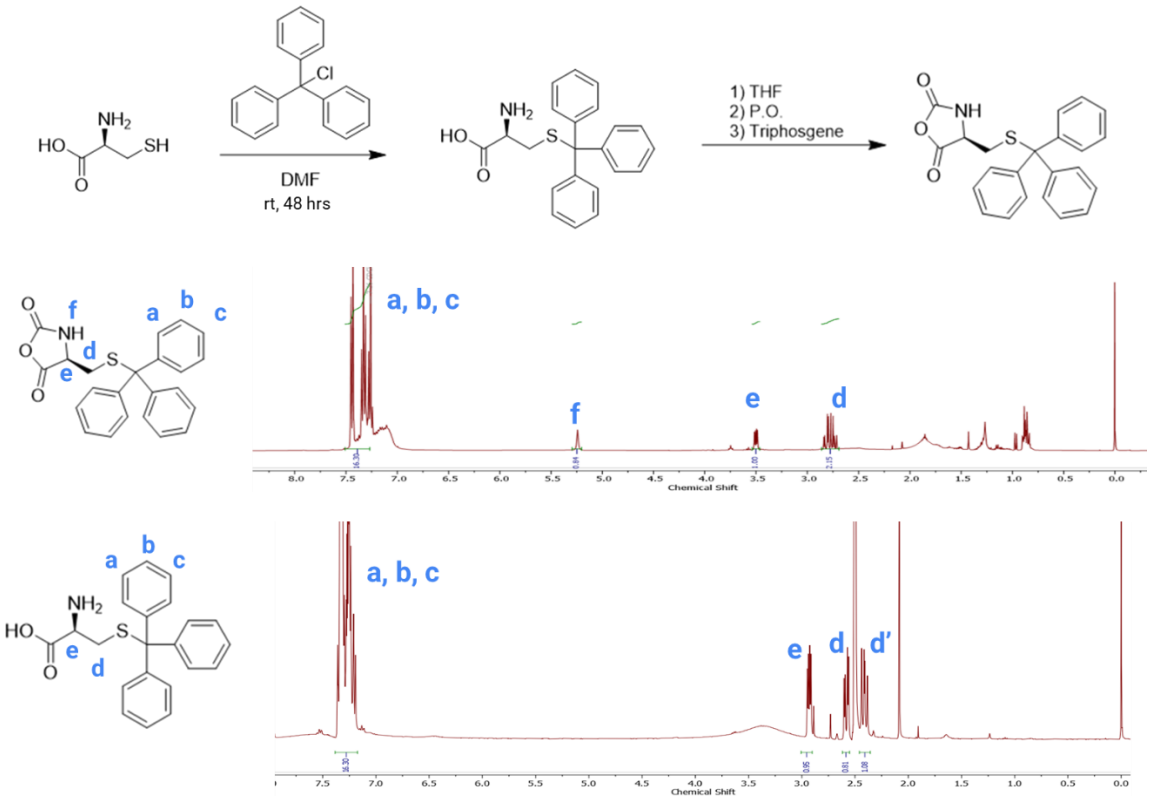

Figure 35: Synthetic scheme and ^{1}H NMRs of NCA monomer M3 and its precursor.

For the synthesis of S-TrtCys NCA monomer **M3** from the trityl protected amino acid S-TrtCys, the moisture tolerant method of Tian, ZY et al. was used. For the synthesis of S-TrtCys, the method was adapted from Cui et al. The NCA monomer was made after several attempts because the NCA is supposed to be solid, but some amount of solvent gets trapped and hence it forms a viscous liquid. The reaction conditions that worked the best was to quench the reaction in 45 mins (the reaction mixture becomes clear after ~10 secs) and do no workup and directly precipitate in dry hexane. Then triturate with THF-hexane system three times. Note that the degraded NCA still dissolves in the THF, EtOAc and hence check FTIR each time to confirm its presence.

The polymerization was done in $CDCl_3$ to do kinetic study and to get a better NMR.

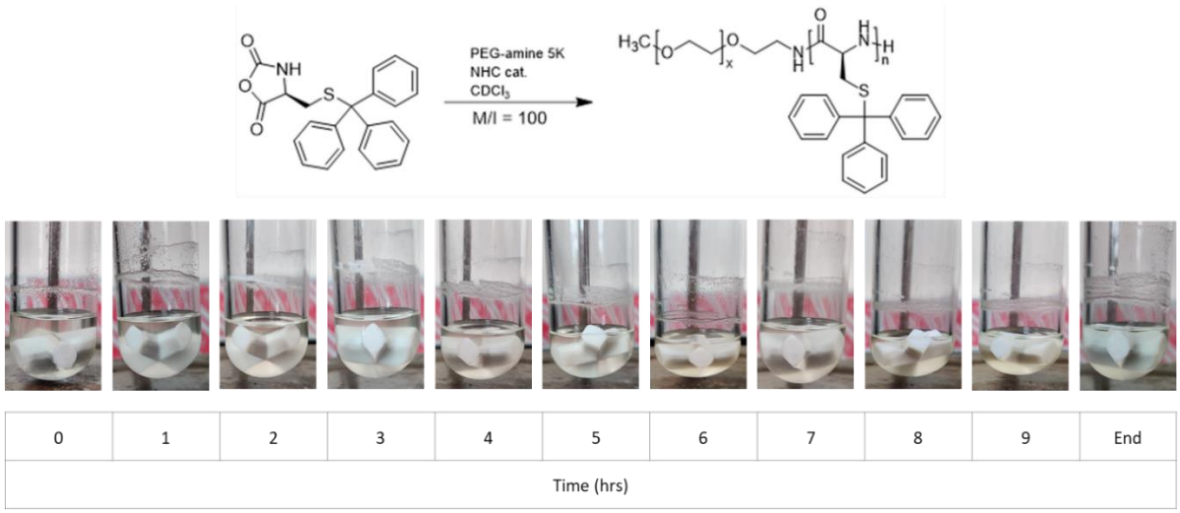

Figure 36: Polymerization of NCA monomer M3.

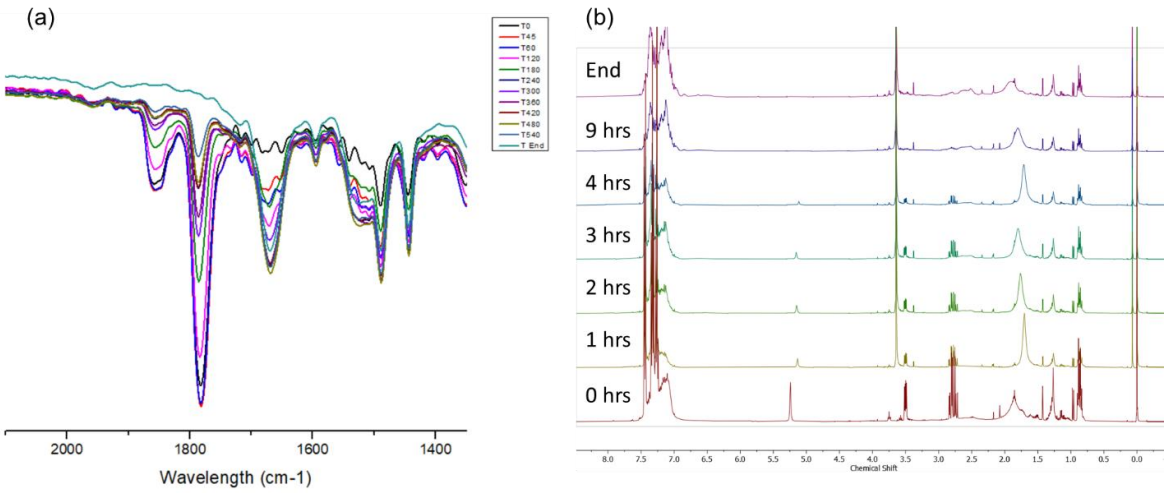

Figure 37: FTIR and NMR kinetics data of polymerization of NCA monomer M3.