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Synthesis of Functional Polypeptides and Development of New Synthetic Strategies toward Polypeptides

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SYNTHESIS OF FUNCTIONAL POLYPEPTIDES AND DEVELOPMENT OF NEW SYNTHETIC STRATEGIES TOWARD POLYPEPTIDES

A Dissertation

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Louisiana State University and
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I need to give my special thanks to my family. I couldn't have completed my Ph.D studies without their resolute support. My parents set great examples of inside qualities for me to achieve so much. I would also like to thank my wife and my son for their love, support and laughter.

I want to thank my advisor Prof. Donghui Zhang for her mentorship through my graduate career. With her encouragement, I stepped into the exciting yet challenging field of polypeptides. With her guidance, I learned how to succeed in science by addressing one key challenge in the field of polypeptides. I would also like to thank all my committee members for their insightful suggestions, and great willingness to help when needed.

Lastly, my collaborators deserve many thanks for all inspiring discussion and their excellent work.

I am truly grateful. Thank you all.

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LIST OF ACRONYMS

AAMM……………….………..Accelerated amine mechanism through monomer activation

AMM…………………………………………...………...Activated monomer mechanism

AIBN…………………………………………….……2,2-azobis(2-methylpropionitrile)

BLG NCA………………………………………γ-benzyl-L-glutamate N-carboxyanhydride

CD……………………….…..Circular dichroism

CuAAC……………………………………………Copper catalyzed alkyne-azide cycloaddition

DBU……………………………………………………...…..1,8-diazabicycloundec-7-ene

DCM…………………………………………………….………..Dichloromethane

DLS……………………………………………………………Dynamic light scattering

DMF………………………………………………………………..Dimethylformamide

DMEDA……………………………………………….…………N,N-dimethylethylenediamine

DP…………………………………………………….. Degree of polymerization

DRI…………………………….......................................Differential refractive index

ee…………………………………………………………………….. Enantiomeric excess

ESI MS………………………………………………… Electrospray ionization mass spectroscopy

FTIR...……………………………………………………Fourier transform infrared radiation

GRGDS……………………………….…Pentapeptide (glycine-arginine-glycine-aspartic acid-serine)

HMDS…………………………………………………………………Hexamethyldisilazane

H-Glu(OBz)-OH………………………………………….……… L-glutamic acid γ-benzyl ester

H-Lys(Z)-OH…………………………………………..ε-N-carbobenzyloxy-L-lysine

MALDI-TOF MS…..Matrix assisted light desorption ionization time of flight mass spectroscopy

MALS……………………………………………………………Multi-angle light scattering

M_n……………………………………………………..Number average molecular weight

ABSTRACT

The objective of this work is to develop a new synthetic strategy for synthesizing advanced functional polypeptides or polypeptides in general. Polypeptides are amino acids based polymers with appealing properties for different applications. One of the key challenges in polypeptide research is the synthesis of functional polypeptides under mild conditions. We developed a system based on ring-opening polymerization (ROP) of *N*-thiocarboxyanhydrosulfides (NTAs) to synthesize polypeptides with wide range of molecular weights under mild conditions. Owing to NTAs' good stability, our system serves as an excellent alternative to the traditional ROP of *N*-carboxyanhydrides (NCA). In Chapter 1, the fundamental knowledge and the cutting-edge research of polypeptides will be reviewed.

The focus of the work in Chapter 2 is to develop a new class of functional polypeptide in traditional method via ROP of NCAs. This class of polypeptides combines several desired attributes for biomedical applications, which include: clickable pendant side chains for further functionalization, good water solubility, non-ionic nature to avoid unspecific interactions in biological systems, and unique secondary conformations (e.g. α-helix, β-sheet).

In Chapter 3, I developed the first system to prepare polypeptides with controlled molecular weight via primary amine initiated solid-phase ring-opening polymerization (sROP) of NTAs under mild conditions in open air. Model NTA (e.g. BLG NTA, LYS NTA) monomers were synthesized for the first time, and were found to possess better thermal and moisture stability as compared to NCA analogs. The sROP proceeds by a normal amine mechanism as evidenced by matrix assisted light desorption ionization time of flight mass spectroscopy (MALDI-TOF MS). The controlled polymerization behavior of sROP is the direct result of high local monomer concentration in the solid phase, thus allowing for faster polymerization under relatively mild conditions.

The work in Chapter 4 focuses on the development of solution phase polymerization of NTAs with TMG/benzoic acid as co-initiation system. PBLG with high molecular weights (33.6 kg/mol - 66.7 kg/mol) and narrow molecular weight distribution (PDI < 1.12) can be readily prepared with this system. The mechanism of the TMG/benzoic acid mediated ROP of NTAs is proposed to be activated monomer mechanism (AMM).

CHAPTER 1. INTRODUCTION OF POLYPEPTIDES

1.1 Background and Significance

Proteins derived from twenty amino acids fold into highly ordered three-dimensional structures, which give rise to their complex functions in biological systems. Polypeptides are synthetic mimics of proteins, which also can self-assemble into secondary structures (e.g. α-helix, β-sheet) stabilized by inter or intra-molecular hydrogen-bonding from the amide bond and carbonyl in each repeat unit.[1] Such secondary structure self-assembly character is rarely seen in other traditional polymers (e.g. vinyl polymers) and accounts for the unique properties in certain applications. For example, Cheng et al investigated the application of positively charged polypeptides in gene delivery.[2] The results indicated that the positively charged polypeptides were able to mediate enhanced gene transfer efficiency as compared to standard polymer such as polyethylenimine (PEI). On the other hand, the random coil analog was not able to mediate effective transfection. This example showcased the significance of secondary structures in the polymer's performance for targeted applications. Polypeptides can be synthesized from abundant naturally existing or synthetic amino acids, contributing to the structure and functionality diversities of polypeptides. Other advantages of polypeptides over conventional synthetic polymers for in *vivo* applications include enzymatic degradability and low cytotoxicity, which are critical factors to consider in the design of functional materials for in *vivo* applications.[3,4] Owing to these appealing features, they have been extensively investigated to be used in the fields of biomedical applications (e.g. drug delivery, tissue engineering, antimicrobial and etc.).[4,5]

Functions of proteins associate closely to their structures. Sequence defined peptides are usually synthesized through solid phase peptide synthesis (SPPS).[6] This strategy involves step-wise reactions to link amino acids onto solid support resins through repeated cycles of

deprotection-coupling for each amino acid to be attached. This method produces mono-disperse sequence defined peptides. Scheme 1.1 shows the synthesis of   peptide (GRGDS) via SPPS, which will be studied in Chapter 2. The major limitation of this strategy is the synthesis of long peptides (>50 repeat units) in high purity and large scale, due to incomplete deprotection or coupling during the cycles.[7] Other drawbacks include excess reagents consumption and time consuming tedious operation.

Scheme 1.1. Synthesis of peptide GRGDS via SPPS.
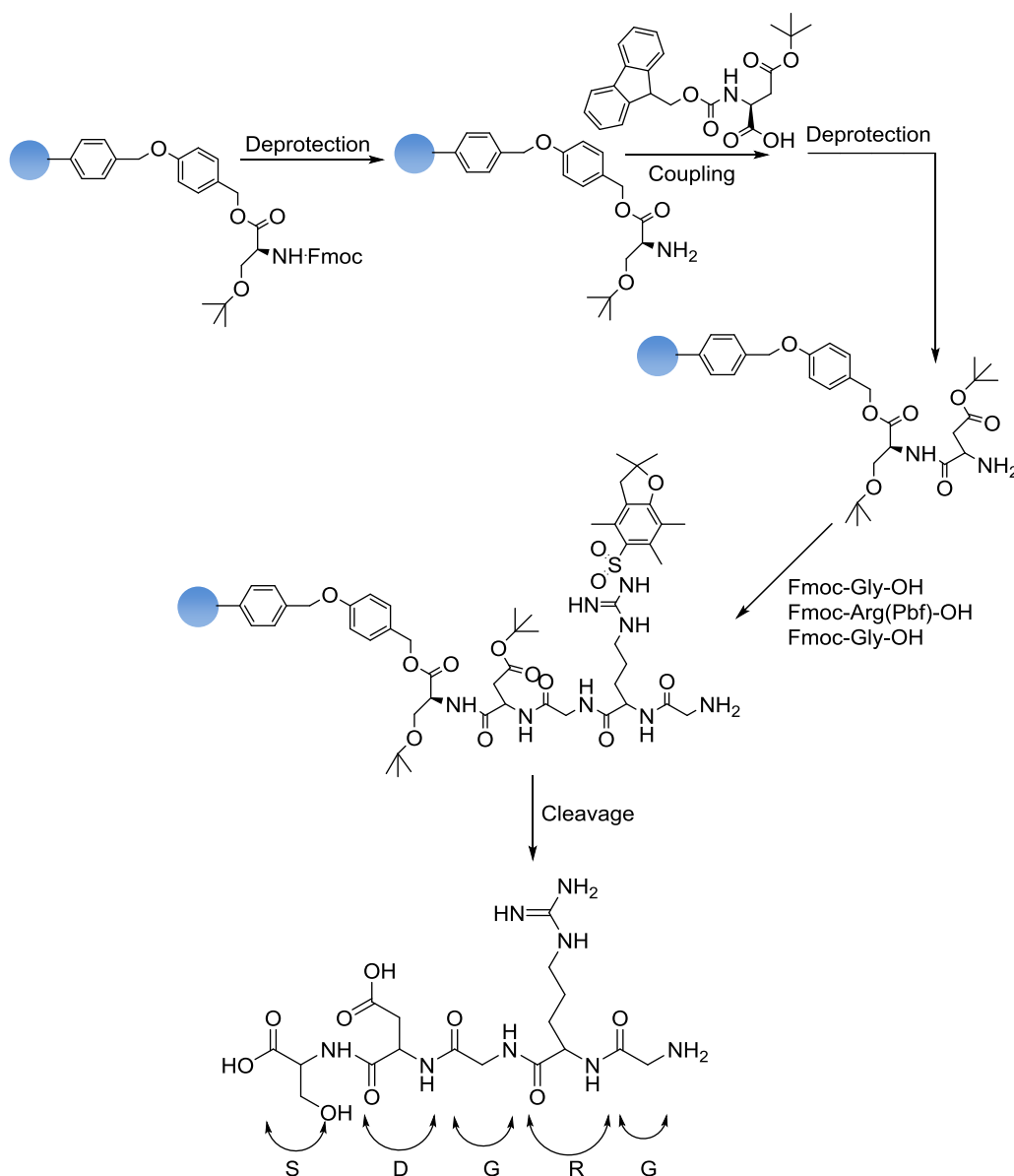
1.2 Preparation of polypeptides through ROP of NCAs

Polypeptides with controlled molecular weights are desired for certain applications, as molecular weights affect polypeptides' properties. For instance, the secondary structure of poly(L-lysine(Z)) will transit from coil to helix when the degree of polymerization (DP) is increased over 15.[8] Controlled ROP of NCAs enables access to well-defined polypeptides with tailorable composition, architecture, functionality, and size.

NCAs, initially named Leuchs' anhydrides, were discovered more than 100 years ago by Hermann Leuchs et al. when they tried to purify *N*-ethoxycarbonyl and *N*-methoxycarbonyl amino acid chlorides by distillation[9-11] and their applications in polypeptide synthesis has been found decades later. Nowadays, there are generally two methods in synthesizing NCAs: Leuchs method[9-11] and Fuchs-Farthing method[12,13] (Scheme 1.2). The Fuchs-Farthing method treats amino acids directly with phosgene or phosgene derived reagents (e.g. diphosgene and triphosgene). The Leuchs method treats *N*-alkyloxycarbonyl amino acids with halogenating agents (e.g. PBr_3, PCl_3, PCl_5, $SOCl_2$, α,α-dichloromethylmethyl ether).

Scheme 1.2. Synthesis of NCAs.
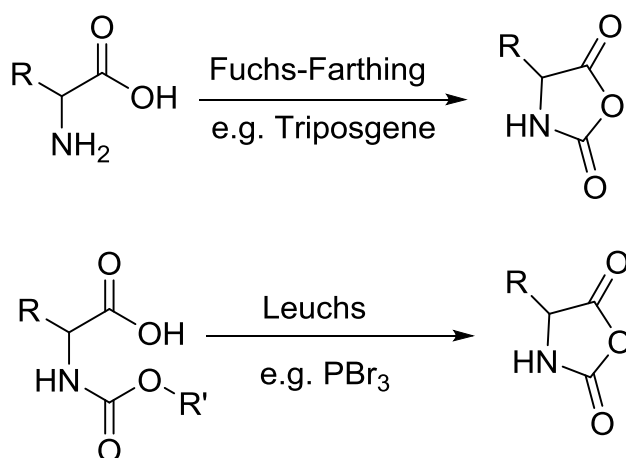
Despite the appealing high reactivity of NCAs in the polymerization, they share certain drawbacks that need to be addressed. The primary issue associated with NCAs is their stability.

NCAs are moisture and heat susceptible, which complicate handling conditions and shortens their shelf lives. We have chosen γ-benzyl-L-glutamate *N*-carboxyanhydride (BLG NCA) as a model to test NCAs' general stability. The results indicate BLG NCA will start to degrade in less than 11 days in air. The thermogravimetric analysis (TGA) test indicates it will start to degrade at 122 °C under nitrogen. Such moisture and thermal instability issue makes inert atmosphere and low temperature standard conditions for the preparation and storage of NCAs. Secondly, high NCA monomer purity is required for the controlled ROP. To achieve high purity, multiple recrystallizations or flash chromatography purification under anhydrous condition is required. The reason for this is to avoid potential side reactions that arise from contaminations due to impurities from cyclization step. For instance, HCl is a common impurity for both methods, which can quench and inhibit chain growth.[14] Chloride ion contamination from both methods is shown to be able to initiate polymerization in DMF.[15]   Another issue associated with NCA preparation is the use of highly toxic phosgene derived reagents in the Fuchs-Farthing method. Due to these issues associated with polypeptides synthesis via NCAs, researchers have been trying to find alternative in polypeptide preparation without much success. It will be the focus of my third chapter to address aforementioned problems associated with polypeptides synthesis via ROP of NCAs. We have found an alternative of NCA, NTAs that can be synthesized without the need to rigorously exclude moisture. The NTAs can be polymerized efficiently in open air to prepare well-defined polypeptides.

1.2.1 Different ROP systems

ROP of NCAs can be initiated with different initiation systems. Traditionally, nucleophiles (e.g. primary amine) and base (e.g. triethylamine) have been employed to prepare polypeptides of a wide range of molecular weights.[16] However they each share certain limitations.

For instance, primary amines can mediate controlled ROP of NCA to prepare low to medium molecular weight polypeptides. However, multiple side reactions can occur during the course of polymerization. Base catalyzed ROP of NCA can be used to prepare high molecular weight polypeptides. On the other hand, the M_n control by this method is not sufficient, and the strategy is rarely used nowadays. Well defined polypeptides with controlled molecular weights, narrow molecular weight distribution, tailored composition, and architectures can readily prepared by new initiation systems (transition metal catalyst, organosilicon amine and etc.).[17-28] There are also reports where well-controlled polymerization can be achieved under optimized polymerization conditions for the primary amine initiated ROP of NCAs (high vacuum,[29-31] low temperature,[30,32,33] nitrogen flow[34]). The respective polymerization systems will be discussed separately below in detail.

1.2.1.1 Nucleophiles initiated ROP of NCA

Primary amine is the most commonly used simple nucleophile to initiate the ROP of NCAs, which undergoes NAM. The initiation step of the NAM is a nucleophilic attack of primary amine at the C5 position carbonyl to open the reactive five-membered ring containing anhydride group. The following proton transfer generates a terminal carbamic acid. Due the thermal instability of the carbamic acid, it subsequently undergoes a decarboxylation step to form a new terminal primary amine to complete the initiation step. The newly formed primary amine attacks another monomer in a similar manner until all monomers are consumed or chain termination occurs, which constitutes the propagation event (Scheme 1.3).[16]

Despite the simplicity and wide application of the primary amine initiation system in the ROP of NCA, the method has inherent drawbacks. Primary amine can work either as a good nucleophile or base. When primary amine works as base, it deprotonates the NCA to generate

negatively charged NCA as a nucleophile. Then new initiation and propagation events can happen with the negatively charged NCA as initiator. The base facilitated ROP of NCA is generally known as AMM, which will be discussed in following section. The NAM or AMM pathways are determined by the nucleophilicity or basicity nature of the amines used. Strong neucleophilic primary amine generally initiates the ROP of NCAs via NAM pathway. However less nucleophilic primary amine and bulky secondary amines may mediate the ROP of NCAs via the AMM pathway.[16] In addition, AMM and NAM can switch back and forth in the primary amine initiated ROP, which will affect the polymerization control.[31]

Scheme 1.3. Primary amine initiated ROP of NCA mechanism (NAM).

Initiation

Propagation
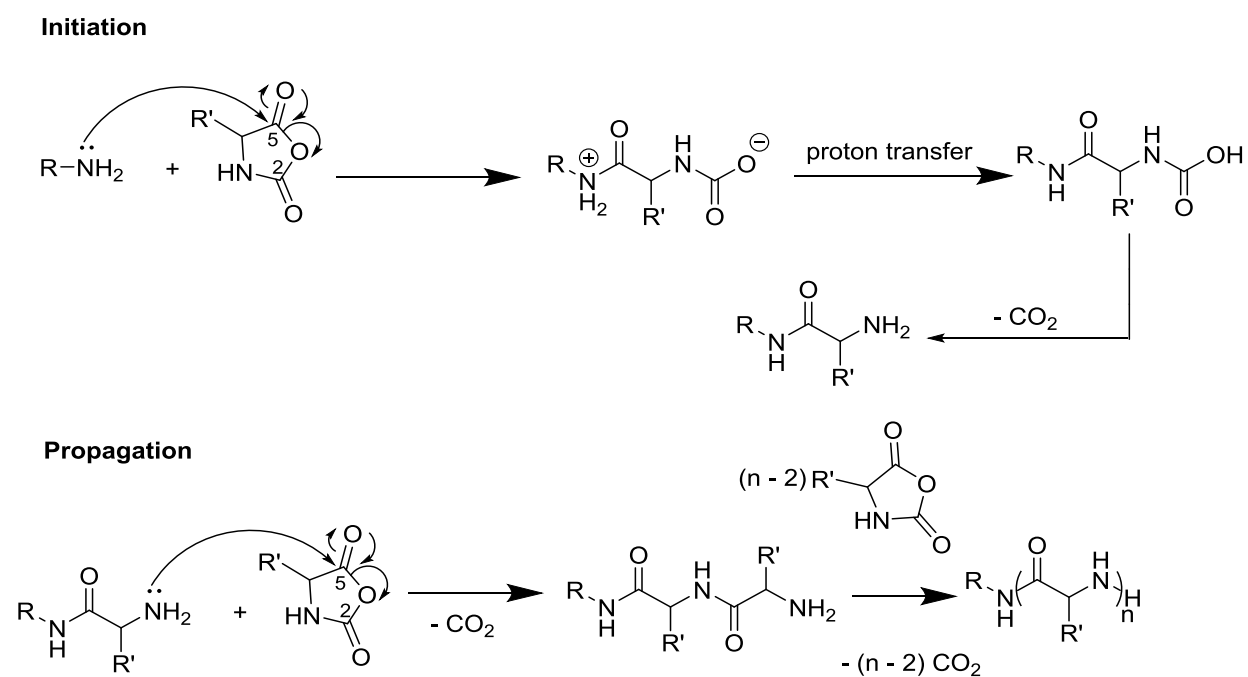
In the course of ROP through NAM, there are other side reactions, which will affect the control of polymerization. Usually the active primary amine chain end accounts for these side reactions. The nucleophilic primary amine can undergo reactions with any electrophile in the system other than the C5 carbonyl. For instance, NCAs without rigorous purification usually contain electrophiles like acid, acid chlorides or isocyanates.[14] These electrophiles can quench

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the propagating chain when they react with the terminal amine. These side reactions will affect

the control of ROP by decreasing the number of available propagating chains.

There are two electrophilic carbonyl centers in the NCA five membered-ring: the C2 and

C5 position carbonyls. In an ideal scenario, the primary amine initiator should attack the NCA at

the C5 position during the initiation and propagation events for a living polymerization. However,

studies show attack at the C2 carbonyl of NCA can occur to form stable hydantoic acid (Scheme

1.4).[16,35,36] Both carboxylate and urea are inert towards the polymerization, which makes the

chain terminated.

Scheme 1.4. Primary amine attack C2 carbonyl to terminate polymerization.
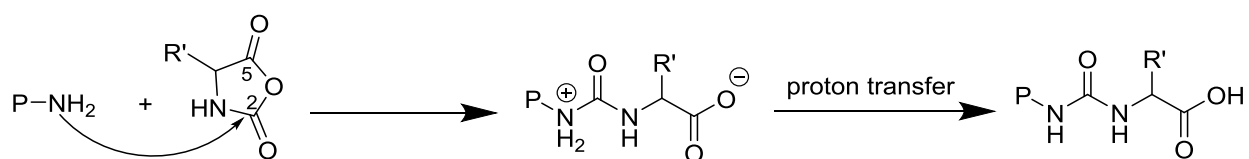
Another common termination reaction observed in the primary amine initiated ROP of

selected NCAs is well-known as intramolecular transamidation by a back-biting mechanisam in

the case of the glutamic acid derived NCAs (e.g. BLG NCA).[30,32,37] The back-biting happens

when the active terminal amine undergoes an intramolecular aminolysis at the ester position on

the side chain resulting in a cyclic pyroglutamate (Scheme 1.5). This pyroglutamate formation is

Scheme 1.5. General scheme of terminal primary amine back-biting to from pyroglutamate in glutamic acid derived NCAs ROP.
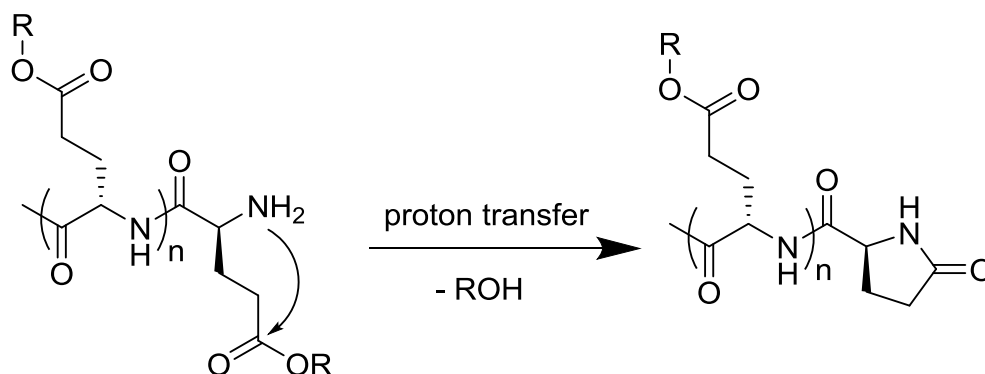
responsible for the chain termination. Decreasing the polymerization temperature to 0 ℃ was shown to minimize the side reaction, which contributes to obtaining amine terminated polypeptides.[32]

Polypeptides usually possess poor solubility in most organic solvents due inter or intra-molecular association via hydrogen bonding. DMF is generally a good solvent for polypeptides and often used for the polymerization of NCAs. However, there are issues associated with DMF in the polymerization of NCAs where it can interfere with the propagating polymer chains.[31,38] DMF affects the polymerization in two ways (Scheme 1.6). First, the propagating chain end amino group can affect the polymerization in two ways. First, the propagating chain end amino group can react with DMF resulting in a formyl end group, which will terminate the propagating chain. This will occur even if DMF is freshly distilled.[31] In addition, during the reaction of the terminal amine with DMF, a secondary amine, dimethyl amine, is released to the polymerization medium. The dimethyl amine can work as a new initiator to mediate new chain propagating event, which will affect the M_n control. In addition, the primary amine or secondary amine in the polymerization solution may also deprotonate the NCA for polymerization via the AMM pathway (AMM will be discussed in section 1.2.1.2). The polypeptides formed via the AMM pathway also possess terminal amines that can potentially undergo coupling with DMF again. Hence, the end-groups can be complicated when DMF is used as solvent in the primary amine initiated ROP of NCAs, which was demonstrated by Pickel et al (Scheme 1.7).[31] According to Vayaboury and coworkers,[33] 78% of all polymer chains are terminated either by DMF or NCA itself when nucleophilic attack happens at C2 position, which leaves only about 22% living chains with amine as termination group under standard ROP condition in DMF at room temperature.

Scheme 1.6. A)Terminal reaction in primary amine initiated ROP of NCA via reaction with DMF; B) re-initiation by dimethylamine released form A).
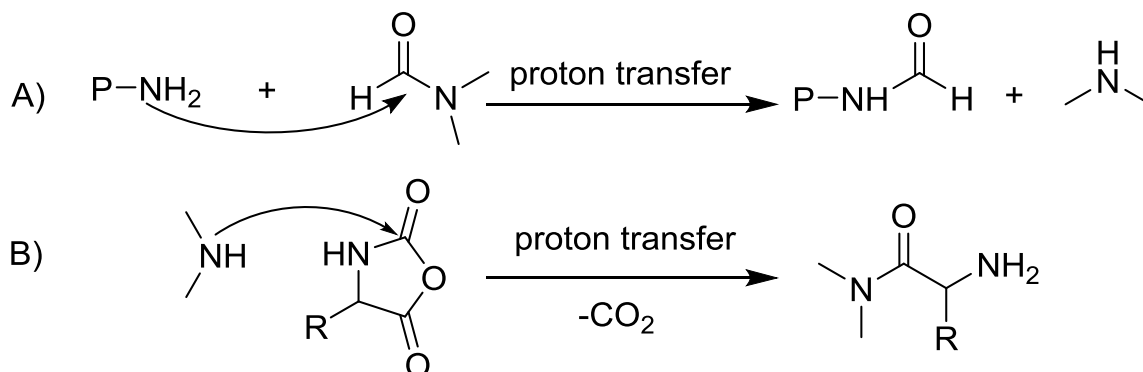
Scheme 1.7. Possible termination products in the polymerization of O-benzyl-L-tyrosine NCA with 1,6-diaminohexane as initiator (reprinted with permission from reference,[31] copyright (2009) American Chemical Society).
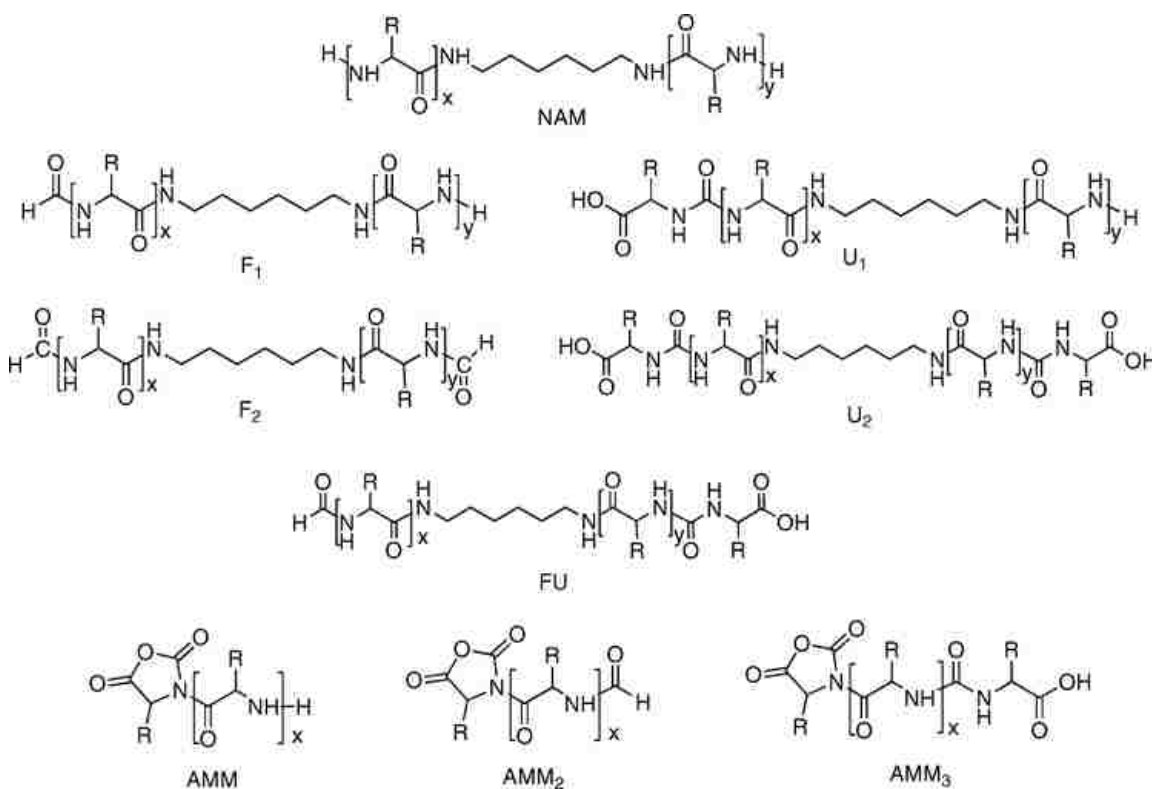
1.2.1.2 Base catalyzed ROP of NCAs

AMM is a method traditionally employed to generate polypeptides with high molecular weights. It requires base (e.g. triethylamine, sodium methoxide) as a catalyst.[16,39-41] There are

multiple mechanisms proposed for the base catalyzed ROP of NCAs.[42] The popular mentioned

one is introduced as follows. The base works as co-initiator/catalyst to deprotonate the NCA first

forming NCA anion. The NCA anion subsequently undergoes nucleophilic attack at another

neutral NCA, which constitutes the initiation step. During the propagation steps, newly formed

NCA anion will attack the terminal cyclic N-substituted NCA at the C5 position to add onto the

propagating chain until all monomers are consumed (Scheme 1.8).[16] The existence of the

terminal cyclic N-substituted NCA was verified by Scoffone and coworkers.[43] One character of

AMM is the fact that the propagation rate is higher than the initiation rate, contributing to high

molecular weight polypeptide.[16] The higher propagation rate originates from C5 carbonyl's

better electrophilicity due to N-acylated terminated cyclic NCA. The poor M_n control of AMM

makes the technique less popular in polypeptide preparation, especially with the development of

transition metal based catalysts which enable access to high molecular weight polypeptide with

good control. Yin and coworkers have investigated the effect of $[\text{BLG NCA}]_0/[\text{TEA}]_0$ on the M_n

of the afforded PBLG in TEA mediated ROP of BLG NCA.[44] It can be seen that only high

molecular weight PBLG can be prepared regardless of the $[\text{BLG NCA}]_0/[\text{TEA}]_0$ (Figure 1.1).

While the above discussed base catalyzed mechanism ROP of NCA is generally accepted, there

still exists much criticism against it.[42]

The main counter-argument against AMM is the continuous formation of activated

monomer during polymerization via deprotonation of NCA by carbamate ion. This is unlikely to

happen considering the acidity difference of NCA and carbamic acid (ΔpKa ~ 6). The second

issue lies in the cause of high molecular weight afforded via the AMM. Based on Harwood's

paper, the C5 carbonyl electrophilicity difference between N-acylated terminated cyclic NCA

and NCA is not significant enough to account for a huge k_p/k_i (~ 10^5) that is required for

affording high molecular weight polypeptide (DP > 300). On the other hand, Kricheldorf added

better electrophiles as compared to NCAs to the triethylamine mediated ROP of NCAs.[45] The

polymerization was accelerated in the presence of the good electrophile, and the DP of the

resulting polypeptide was also low, similar to the ratio of NCA to electrophile. In addition, the

electrophile additives were also detectable in the NMR of the polypeptide. If worse electrophiles

were used, then no acceleration was observed. These results are consistent with the proposed

AMM as the neutral NCA is replaced with a better electrophile in the initiation step, which will

contribute to a faster initiation step. Consequently, the molecular weight will decrease, and the

polymerization rate will increase. Nevertheless, more work is necessary to elucidate the

complicate mechanism of AMM.

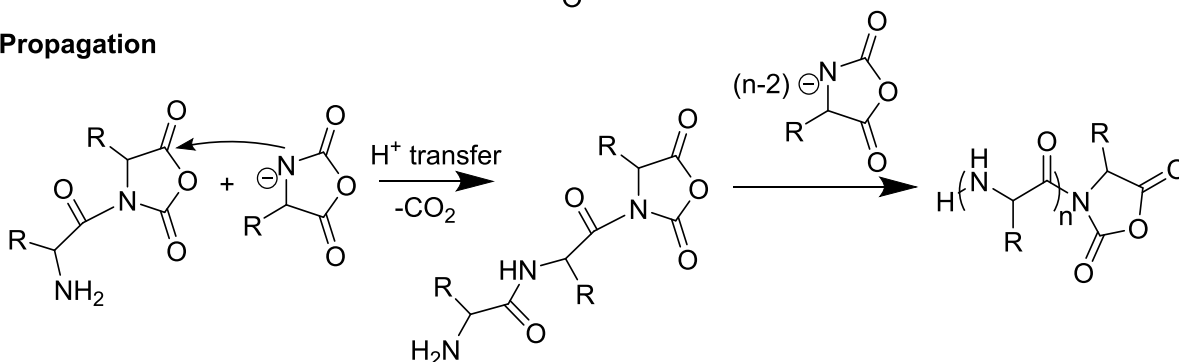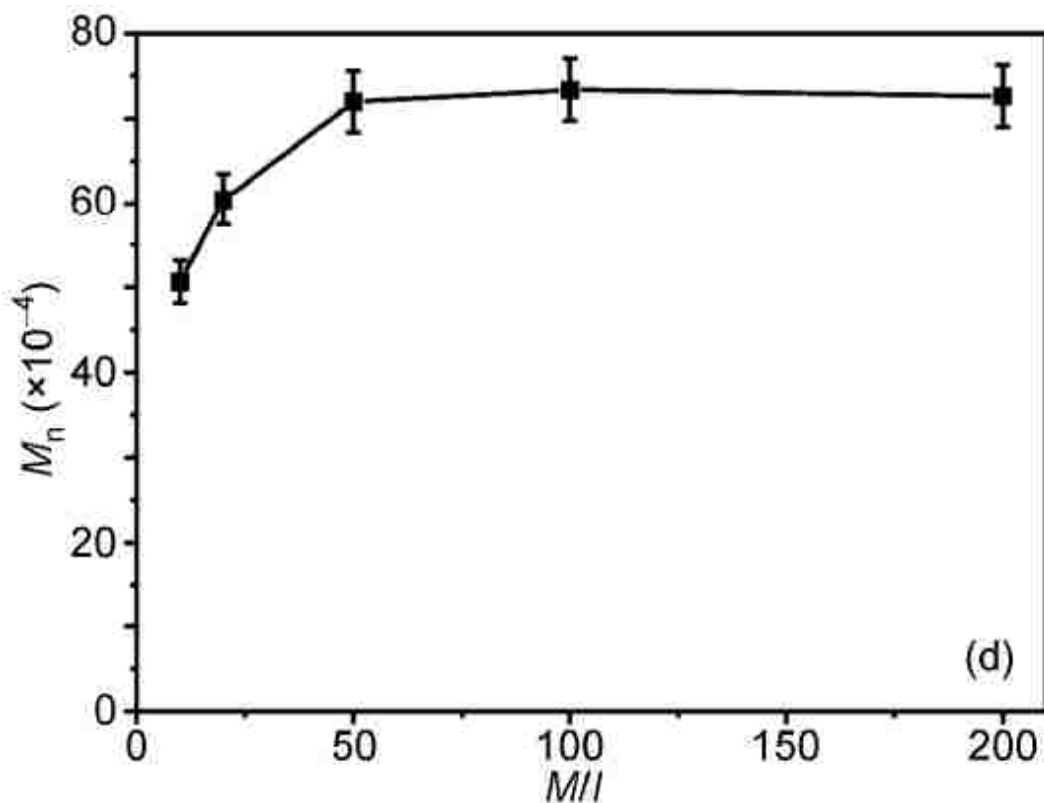Scheme 1.8. Base catalyzed ROP of NCA via AMM.
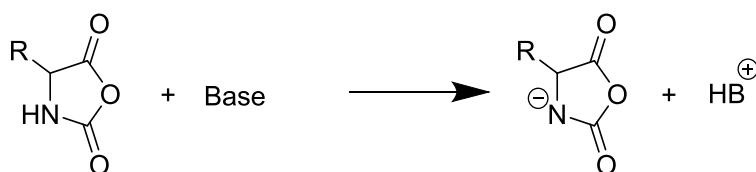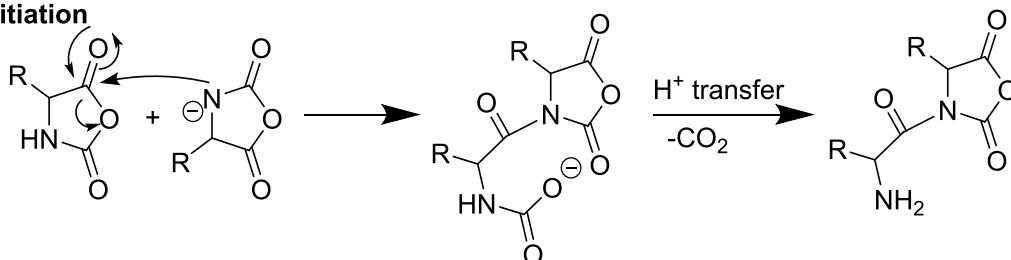
Figure 1.1. The relationship between M_n of PBLG and M/I ([BLG NCA]$_0$/[TEA]$_0$) in TEA mediated ROP of BLG NCA (reprinted with permission from reference[44], copyright (2013) Science China Press and Springer-Verlag Berlin Heidelberg).

1.2.1.3 Transition metal catalysts initiated ROP of NCAs

Traditional initiation systems (e.g. primary amine) for ROP of NCA suffer from many possibilities of side reactions as discussed above. The living polymerization of NCAs with transition metal catalysts (e.g. Ni(bipy)(COD), (PMe$_3$)$_4$Co) was first established by Deming et al to enable access to high molecular weight polypeptide in a controlled manner.[18,46] Transition metal nickel based initiator (Ni(bipy)(COD)) was first introduced twenty years ago.[18] Polymerization of BLG NCA with Ni(bipy)(COD) in DMF indicates molecular weights of PBLG agree well with target monomer to initiator ratios up to 500 with narrow PDI (<1.2).[18] Diblock and triblock polypeptides can also be prepared with the Ni(bipy)(COD) initiation system with narrow PDI (<1.2), indicating characteristics of living polymerizations.[18] The transition

metal catalyst initiation systems mediate the ROP of NCAs in a different mechanism (Scheme 1.9), thus avoids some of the common side reactions seen in AMM or NAM. This is the first

Scheme 1.9. General mechanism of transition metal catalysts initiated ROP of NCAs.

Initiation
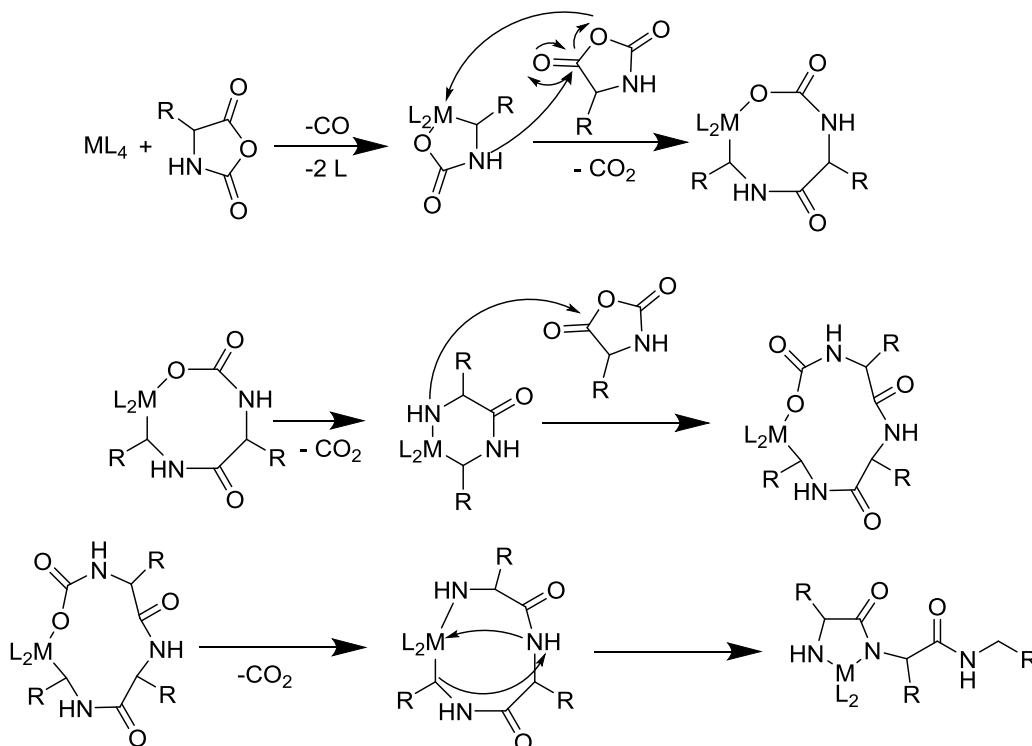
Propagation
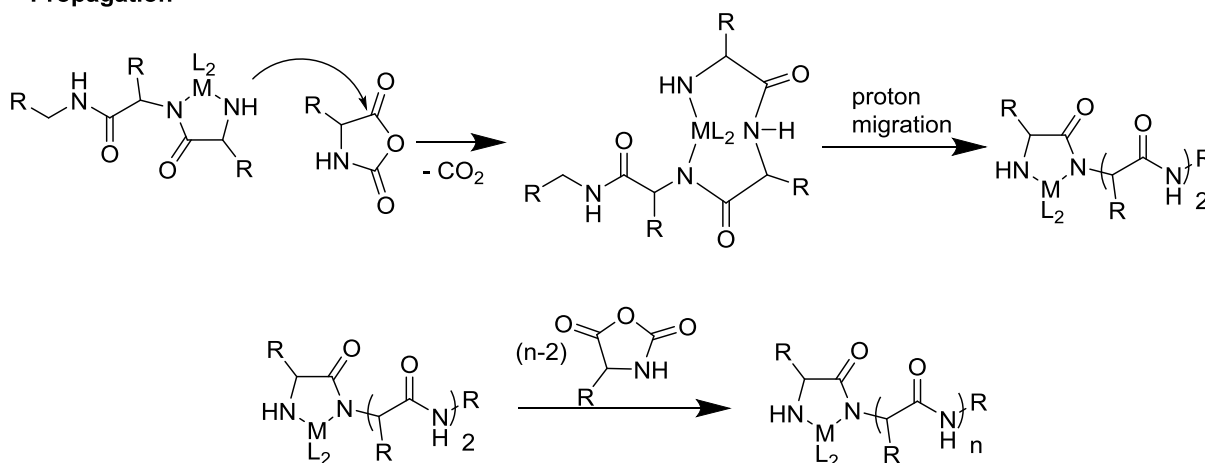
example of living polymerization of NCA in the preparation of polypeptides. Later on, Deming introduced another system based on cobalt ($(PMe_3)_4Co$), which also enables living

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polymerization of NCA.[46] However, there is an increase in the reactivity of $(PMe_3)_4Co$ during the initiation step, which makes the preparation of short peptide sequence possible with $(PMe_3)_4Co$. In contrast, Ni(bipy)(COD) is only able to produce high molecular weight polypeptides.[46] The authors studied the mechanism of transition metal catalysts initiated ROP of NCAs. The catalysts ML_4 first selectively and oxidatively insert into O-C5 bond as evidenced by isotropic labeling. The newly formed five membered metallacycle will subsequently react with additional NCAs to form the propagating active five-membered amino-amidate group. The five-membered amino-amidate reacts with another NCA at the C5 position via nucleophilic attack to form a new five-membered amino-amidate upon release of CO_2, proton migration, and ring contraction, which constitute the propagation step. Despite the advantages of the transition metal catalysts, one potential problem is the trace metal residue left in the polypeptides, which can be troublesome if the materials are designed for biological applications.

1.2.1.4 Organosilicon amine

In 2007, Lu and Cheng found that silicon amines (e.g. hexamethyldisilazane (HMDS)) can efficiently polymerize NCAs (e.g. BLG NCA, LYS NCA) with good molecular weight and narrow molecular weight distribution (~1.2).[19] Block copolymers can also be readily prepared by sequential addition of NCA monomers (e.g. PBLG-*b*-PZLL).[19] Considering their inexpensive and commercially availability nature, the HMDS initiated living polymerization of NCA represents a good alternative to transition metal catalysts. Additionally, the HMDS initiator provides a new mechanism to control the polymerization of NCAs. Cheng et al. initially proposed that a trimethylsilane (TMS) of HMDS transferred to carbonyl of NCA first, followed by the anhydride ring break by the in situ formed TMS-amine. The newly formed terminal TMS-carbamate interacts with another incoming NCA via a six-membered ring transition state,

followed by release of CO_2 and TMS migration to chain end for next propagation event (Scheme 1.10a). However, further investigation indicates HMDS can polymerize N-methyl glycine NCA via the same TMS-carbamate end group. This is in contradiction with the originally proposed mechanism which required hydrogen on the nitrogen to go through tautomerization (Scheme 1.10b). In addition, HMDS can react with succinic anhydride at 1:1 to afford the product 3 in Scheme 1.10c, which indicates HMDS can open the anhydride rings without assistance from nitrogen on the NCA ring. Hence, they proposed a new route where TMS transfer and amide bond formation happen simultaneously as the initiation step (Scheme 1.10d). The propagation still proceeds the same way as proposed previously.

Scheme 1.10. Proposed mechanism for the HMDS mediated NCA polymerization (Reproduced from reference[47] with permission from The Royal Society of Chemistry).
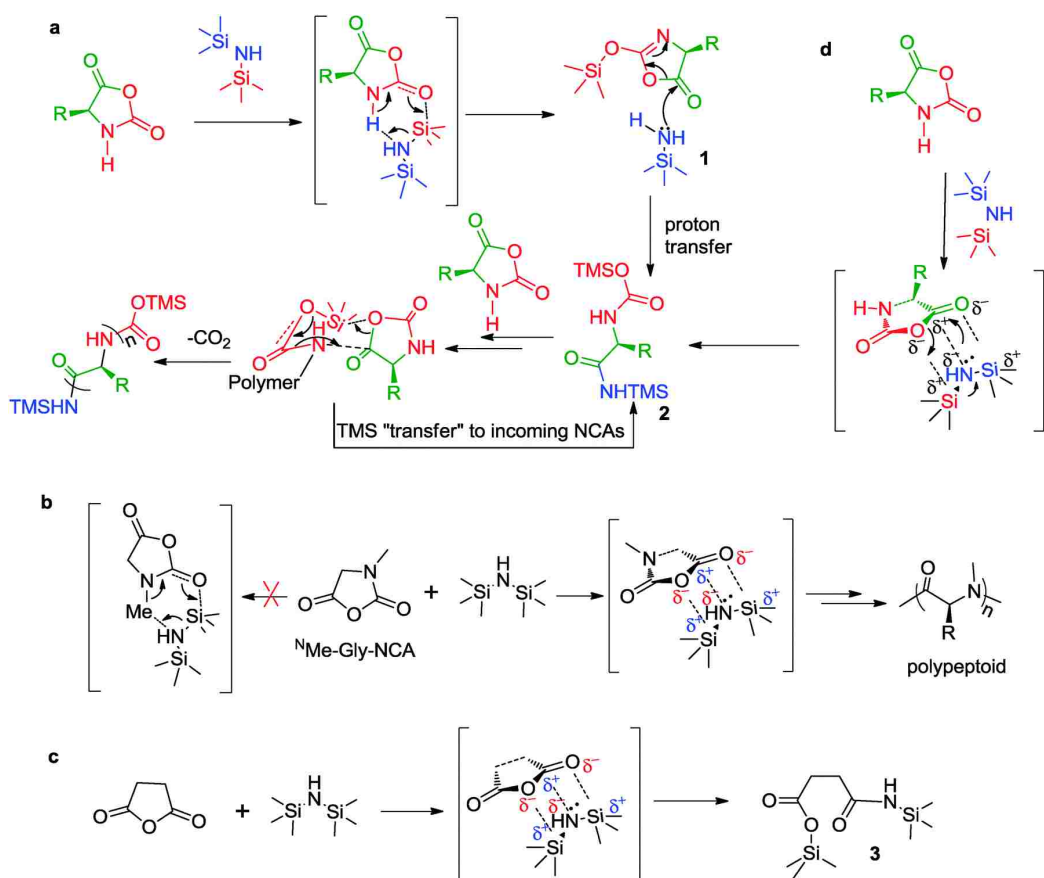
The scope of the organosilicon amine initiation system was expanded upon with *N*-trimethylsilyl amine (*N*-TMS), which shares TMS group for the living polymerization of NCA.[48-50] Meanwhile it also possesses another functional group on the other side of the nitrogen (e.g. alkyne, alkene, norbornene, *etc*.), which enables post-polymerization modification through effective chemistries for different applications. For example, ring-opening metathesis polymerization (ROMP) was combined with *N*-NMS mediated ROP of NCA to prepare brush-like copolymer and linear hybrid block copolymers.[49,50] Norbornenes containing *N*-TMS was first polymerized through ROMP to afford poly(norbornene)s bearing pendant *N*-TMS groups. The macroinitiators were then used directly to polymerize NCAs via the terminal N-TMS on the side chains to afford brush-like copolymers with polypeptide chains as "bristles". With ROMP and NCA monomers are widely available; such combination of polymerization techniques allows easy access to abundant hybrid materials with controlled size, functionalities, grafting densities, *etc*.[49]

Despite the simplicity, these organosilicon amines initiation systems share one major drawback which is their low reactivity as compared to transition metal catalysts. For instance, HMDS alone cannot polymerize γ-(4-vinylbenzyl)-L-glutamate *N*-carboxyanhydride (VB Glu NCA) to afford high molecular weight polypeptide (< 30 kg/mol).[51] To address the above issue, a co-initiation system with added trace amount of 1,5,7-triazabicyclo[4.4.0]dec-5-ene (TBD) was developed. With the HMDS/TBD initiation system, VB Glu NCA can be effectively polymerized with good molecular weight control (up to 47 kg/mol) and narrow PDI. They propose that the TBD might accelerate the polymerization by opening the NCA ring to form certain active intermediates for faster chain propagation.

Recently, Lu et al developed a similar new initiation system based on trimethylsilyl sulfide (*S*-TMS) species, which also provides controlled ROP of NCAs with narrow PDI.[17] As compared to *N*-TMS based initiation systems, *S*-TMS enables faster initiation step, which favors the control of polymerization. The enhanced initiation rate is proposed to originate from the increased nucleophilicity of sulfur and increased reactivity of the S-Si bond. The mechanism of *S*-TMS initiated ROP is similar to that of *N*-TMS initiated ROP. Various monomers screened can be successfully polymerized with the system. However, one major drawback of the system is the highest molecular weight limitation. Only medium molecular weight (DP<150) polypeptide can be prepared by the above system, which leaves the field to be investigated more in the future.

1.2.1.5 Tertiary or secondary amine assisted primary amine initiated ROP of NCA

Hadjichristidis et al. developed a new initiation system combining primary amine and secondary or tertiary amine to afford fast yet well-controlled polymerization of NCA.[21,28] The motivation of the work was to combine features of AMM and NAM to afford fast living polymerization of NCA. The NAM is well known to mediate controlled ROP of NCA to prepare low to medium molecular weight polypeptides (DP<150), yet the polymerization is slow as compared to AMM mediated fast ROP of NCA for high molecular weight polypeptides preparation. They initially screened several initiators containing both primary amines and tertiary amines. Triethylaminetriamine (TREN) was found to bring a fast and remarkably controlled polymerization of NCAs. For example, TREN initiated polymerization of BLG NCA with a target DP of 200 reached 100% conversion in 2 hours with good molecular weight control and narrow molecular weight distribution (PDI=1.17). The mechanism was proposed to be monomer activation through hydrogen bonding, where the NCA's amido proton acts as a hydrogen bonding donor and TREN's tertiary amine acts as hydrogen bonding acceptor. The primary

amine of TREN then initiates the polymerization of activated monomer to yield fast and living polymerization of NCA. However, the effect of accelerated amine mechanism through monomer activation (AAMMA) is only limited to certain initiators. For instance, *N,N*-dimethylethylenediamine (DMEDA) is unable to mediate similar well controlled polymerization of NCA, which also contains one primary amine and one tertiary amine. The authors attribute the reason to different electron densities on the tertiary nitrogen. Regular tertiary amines (e.g. tetramethylethylenediamine) have higher electron densities (higher basicity) than TREN (verified via ^{15}N NMR), which enables NCA deprotonation for AMM polymerization route.

They later expanded the scope of AAMMA to initiators combining primary and secondary amines.[21] Triethylenetetramine (TETA) is one example of those initiators that is able to mediate controlled polymerization of NCA with fast kinetics. The proposed mechanism is similar to the one discussed above, where secondary amine nitrogen act as hydrogen bonding acceptor to activate NCA monomer by forming hydrogen bonding with NCA amido proton (Scheme 1.11). Additionally, the primary amine and secondary amine system is not limited to only one initiator. Several amines containing both primary and secondary amines can efficiently mediate controlled ROP of NCA with almost identical results (Figure 1.2). More importantly, individual primary and secondary amines can be combined to achieve similar controlled ROP of NCA if the ratio of secondary amine to primary amine is 1. When secondary amine is used in excess (e.g. 2 ° amine/1 ° amine = 2), then polymerization with poor control is observed due to the dominance of AMM.

In summary, the simple and effective alliance of primary amine with secondary or tertiary amines can function as good initiators for living polymerization of NCAs with fast kinetics.

Linear block copolymer and star copolymer can be readily prepared with this strategy. A new mechanism AAMMA was proposed for the metal-free ROP of NCA.

Scheme 1.11. Proposed accelerated amine mechanism through monomer activation (AAMMA) (reprinted with permission from reference[21], copyright (2015) American Chemical Society).
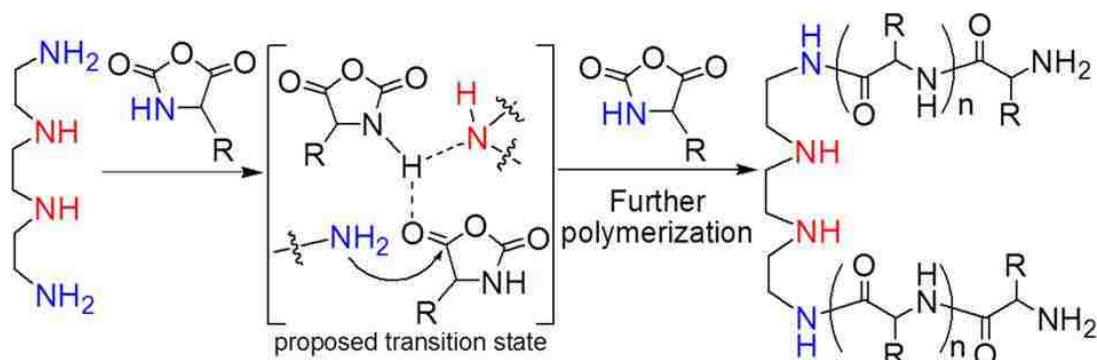
Accelerated Amine Mechanism through Monomer Activation (AAMMA)
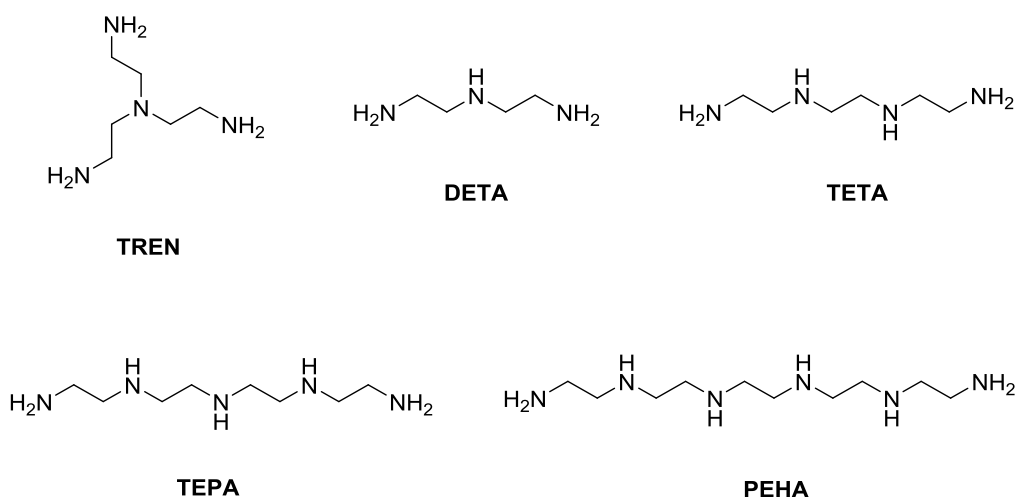
Figure 1.2. Structures of amines that can mediate ROP of NCAs via AAMMA.

1.3 Polypeptide preparation via NTAs

Polypeptides of high molecular weight are primarily prepared from ROP of NCAs via various initiation systems as discussed previously. However, there is one major drawback associated with this route. NCAs monomers are moisture sensitive and thermally unstable. Low temperature and anhydrous conditions are required during the synthesis, purification, and storage of NCA monomers. Certain NCA monomers with high melting point (e.g. BLG NCA) can be

purified by multiple recrystallizations from anhydrous THF/hexanes under nitrogen atmosphere.[52] However, some newly developed NCAs bearing functional side chains could not be recrystallized.[53-55] Hence, flash chromatography with anhydrous silica and anhydrous solvents in glovebox was developed to provide a general purification strategy for NCAs.[14] Based on our experience, the operation of anhydrous flash chromatography is tedious as it was proposed to be done in glovebox. The drying process of silica gel could be potentially dangerous due to the fact that silica particles will "fly" everywhere in the sealed system when being heated under vacuum at over 100 ℃. Finding an alternative to NCA is of great interest to the polypeptide community, and the searching has been ongoing for decades.

Endo et al have reported the synthesis of polypeptides using activated amino acids urethane derivatives which was proposed to form NCAs in situ which polymerize in a chain growth fashion.[56] However, the polypeptides are mostly limited to low to moderate molecular weight (DP<100). NCA's analogs NTAs also have been investigated for their polymerization activities to prepare polypeptides. NTAs were well-known for more than fifty years; they were studied mostly for the step-wise peptide synthesis, arising from the fact that their less likely to polymerize and hydrolyze in aqueous solution as compared to NCAs.[57-62] The glycine NTA also has been investigated for wool surface modification for shrink proofing purpose.[63,64] These early reports indicate NTAs have better stability as compared to NCAs.[58,60] Kricheldorf et al first systematically investigated the ROP of amino-acid derived NTA (e.g. Gly NTA, DL-Phe NTA, DL-Leu NTA) in solution using primary amine initiator.[65-67] All polymerizations resulted in low conversion, yielding oligopeptide products. Even with the unsuccessful trial of NTAs polymerization, they still represent promising alternatives for NCAs for following reasons. First, NTAs are generally more stable as compared to their NCA analogs. Secondly, primary amines

are still capable of opening the NTA five membered ring at the beginning of polymerization in Kricheldorf's initial study, which also follows the traditional NAM based MALDI-TOF MS analysis, though the termination mechanism remains unclear at this point. Thirdly, the NTAs were found to undergo alcoholysis and aminolysis exclusively at the C5 position[16], which is favorable in potential nucleophile initiated ROP of NTAs. The reason is that the termination via reaction between propagating terminal amine and NTA at C2 carbonyl can be avoided. Recently, Ling and coworkers demonstrate the polymerization of *N*-substituted NTA with primary amine and rare earth borohydride initiators.[68,69] Polysarcosine of controlled molecular weight can be obtained via above systems. However, there is no other reports on the polymerization of amino acids derived NTAs.

Systematic polymerization study of NTA was not available prior to Kricheldorf's pioneering work. With the unsatisfied polymerization results from Kricheldorf, the field of NTA polymerization has been left unexplored ever since. Nevertheless, it is still of great importance and interest to develop initiation system that can effectively polymerize NTAs to afford polypeptides, based on the fact that they can potentially replace NCAs owing to their merits like good stability. For NTAs to be utilized in the preparation of polypeptides, either new initiator or optimized condition based on primary amine initiation system should be developed to afford controlled polymerization. This will be the focus of Chapter 3 and Chapter 4.
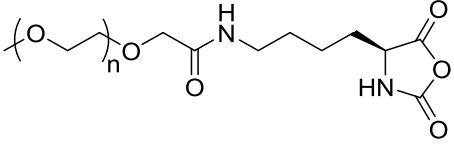
1.4 Functional Polypeptides

Functional polypeptides can be treated as side chain modified poly(amino acids), which originate from naturally existing amino acids. The purpose of such side chain modification is to expand the structural and properties diversity of polypeptides for different applications.[55,70] There are two strategies available to synthesize functional polypeptides: the post-polymerization modification method and functional NCA monomer route. With the post-polymerization

modification method, different functional groups are chemically linked to the polypeptide backbone.[2,71] For the functional NCA monomer route, NCA bearing functional side chain is polymerized via ROP to afford functional polypeptide.[55,72] The post-polymerization method is more popular due to several factors based on my experience. First, functional NCA monomer synthesis is no trivial task. It involves design of a new synthetic route, optimization of purification, and polymerization conditions, and also the handling of NCAs with poor stability. Secondly, the post-polymerization method is well established with various highly efficient platforms. For instance, copper catalyzed alkyne-azide cycloaddition (CuAAC) type "click" chemistry makes the post-polymerization method really robust, where various selected functionalities can be "clicked" onto well studied polypeptide platform.[53,73] Hence, I will focus on the post-polymerization method, where different polypeptide platforms for further functionalization will be introduced separately. The functional NCA monomer method will be briefly summarized, and corresponding NCA structures will be included in Table 1.1.
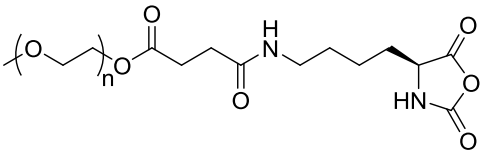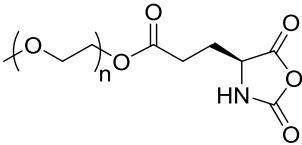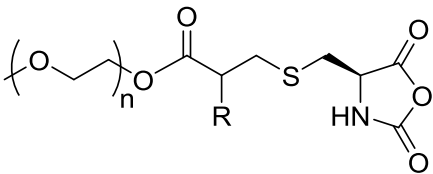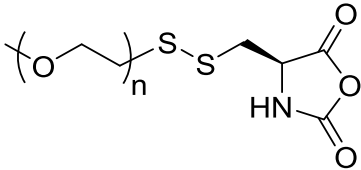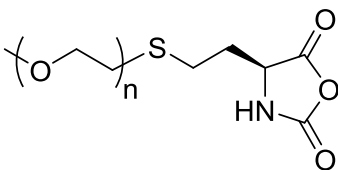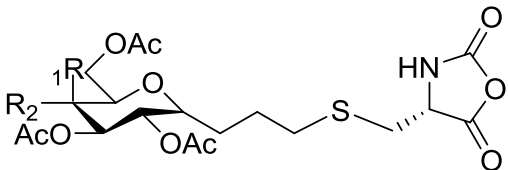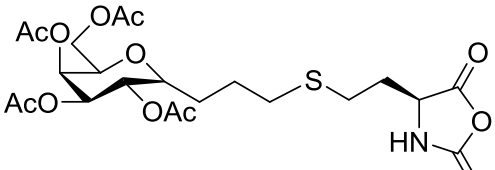
1.4.1 Functional polypeptides prepared via direct ROP of functional NCA monomers

Functional NCA monomer based on modified amino acids has been known for a long time. There are many examples of NCAs that bear non-natural amino acids side chains. Hence, I will only summarize functional NCAs that can afford polypeptides with certain properties (thermal responsive, bioactive, photo reactive, etc.). These NCAs will be summarized in Table 1.1 with their structures and description of corresponding polypeptides properties.

Table 1.1. List of functional NCAs that can afford polypeptides with certain properties.
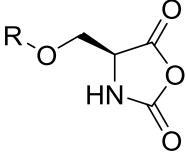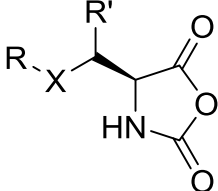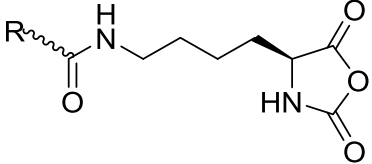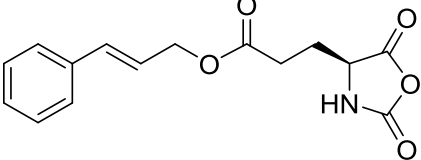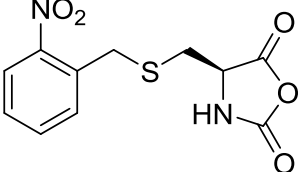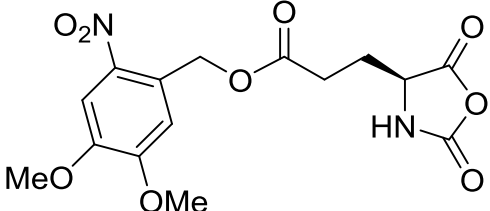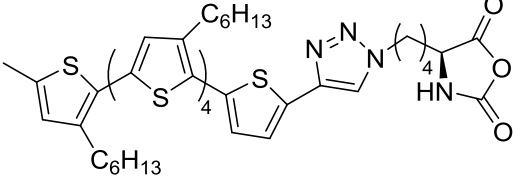
NCA	Structure	Properties
EG$_n$ LYS NCA[74]	 n = 1, 2	Thermal responsive

(Table 1.1 continued)
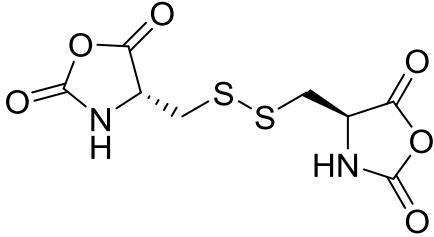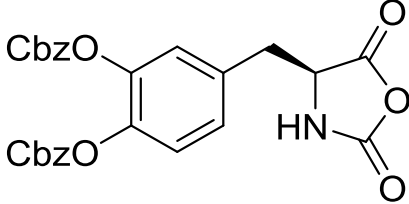
NCA	Structure	Properties
PEG$_n$ LYS NCA[75]	 n = 3, 8	Non-fouling polypeptide
EG$_n$ Glu NCA[55]	 n = 1,2,3	Thermal responsive
EG$_n$(M)A-C NCA[76]	 n = 1~9, R = H, CH$_3$	Thermal responsive
EG$_n$-SS-Cys NCA[77]	 n = 2~4	Thermal responsive, redox responsive
EG$_n$-C^H NCA[78]	 n = 2, 4	Thermal responsive, reversible oxidation and alkylation, reversible helix-coil conformation switch
Glyco-C NCA[79]		Oxidation responsive, irreversible helix-coil transition
α-gal-C^H NCA[78,79]		Reversible oxidation and alkylation upon deprotection, reversible helix-coil conformation switch

(Table 1.1 continued)

NCA	Structure	Properties
Glyco NCA[80]	R = Ac$_4$Glc	Synthetic glycosylated polypeptides
Glyco NCA[81]	X = O, S R' = H, CH$_3$ R = Ac$_4$Glc, Ac$_4$Gal, Ac$_7$Lac	Synthetic glycosylated polypeptides
Glyco-Lys NCA[72,82-84]	R = carbohydrates, ∿∿ various linker	Synthetic glycosylated polypeptides
CLG NCA[85]		Photo responsive
NBC NCA[86]		Photo responsive
DMNB Glu NCA[87]		Photo responsive
Hexithiophene Lys NCA[88]		Organic photovoltaic and organic field effect transistor devices

(Table 1.1 continued)

NCA	Structure	Properties
L-Cys NCA[89]		Redox responsive
DOPA NCA[90,91]		Bio-adhesive upon deprotection

1.4.2 Functional polypeptides prepared via post-polymerization method

Post-polymerization modification is attracting much attention in preparation of functional polypeptides due to the development of highly efficient "click" chemistry. Prior to the development of click type modification reaction, inefficient reactions (e.g. transesterification) were utilized to modify polypeptides with certain functionalities for different purposes. For instance, ester exchange was applied to introduce various functionalities (e.g. halide, azido, alkene, alkyne) to PBLG.[92] Excess alcohol (5 molar equivalents relative to that of the repeat unit of PBLG) containing the desired functionality is required for high degree of functionalization, which is still below 56%. The development of the "clickable" type polypeptide addressed such problems by enhancing the grafting efficiency to almost quantitative under mild conditions.[54]

"Click" type reaction refers to those reactions that share following characters: high yield, easy purified byproducts, stereospecific, high chemoselectivity, etc.[93] For instance, CuAAC and thiol-ene reaction are two examples of "click" chemistry. In the following section, clickable type polypeptides will be the focus of my discussion. All the discussed NCAs will be summarized in Figure 1.3.

1.4.2.1 Alkyne containing polypeptides

Hammond et al developed the first "clickable" polypeptide poly(γ-propargyl-L-glutamate) (PPLG) containing pendant alkyne side chains.[54] The alkyne groups can be modified with various azido containing compounds (e.g. PEG-azide, amine-azide) in nearly 100% grafting

PLG NCA   **POB-L-Glu NCA**   **DLPG NCA**

Anv NCA   **Anl NCA**   **CP NCA**

DLAG NCA   **ALG NCA**   **AOB-L-Glu NCA**

PE-L-Ser NCA   **AOB-L-Glu NCA**
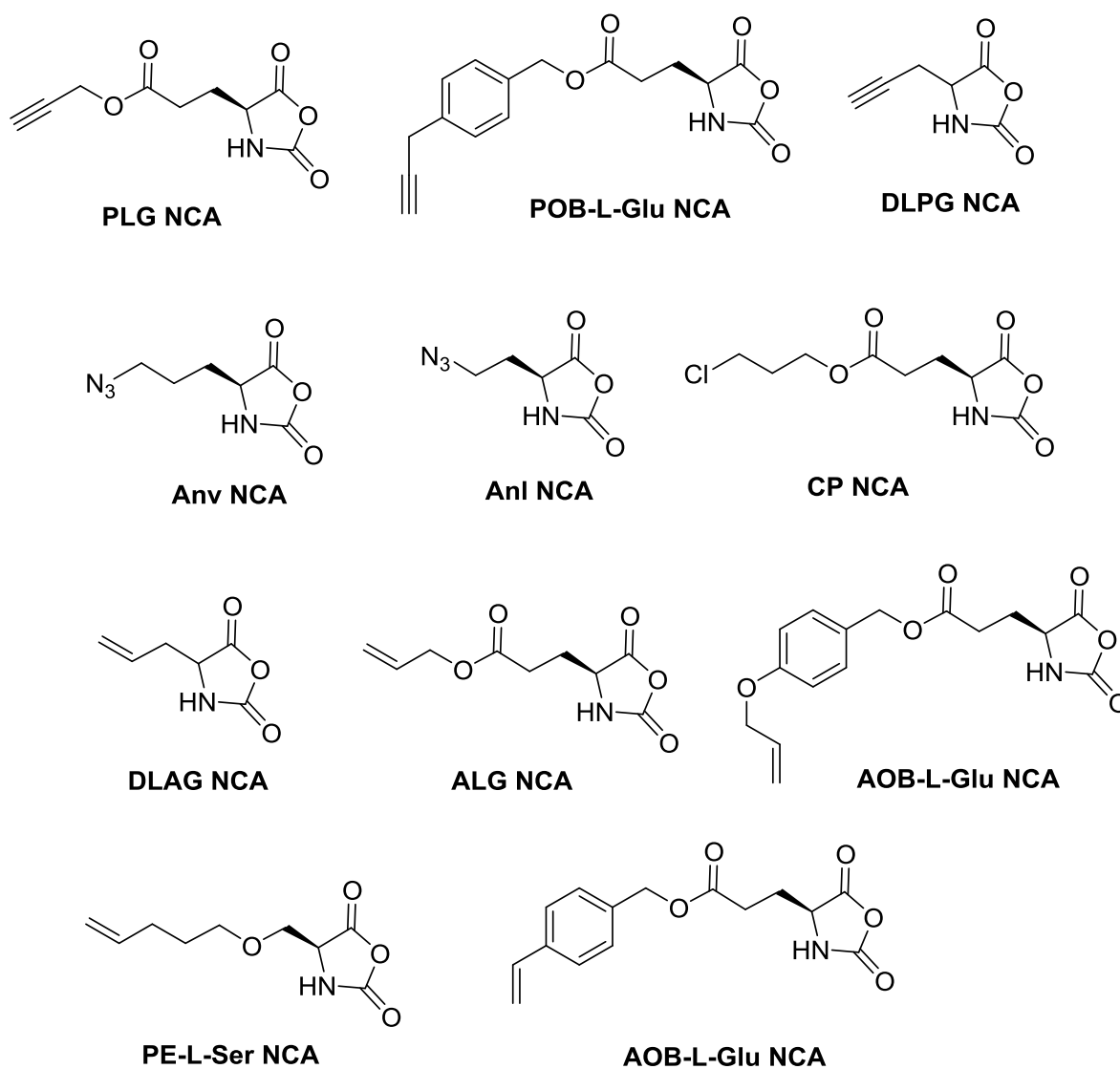
Figure 1.3. Structures of NCAs bearing clickable side chains.

efficiency via CuAAC.[54,71,94] PPLG functionalized with azido containing amines (primary, secondary, tertiary and quaternary) via CuAAC exhibited good antimicrobial properties (Scheme 1.12).[71] Later on, they also developed the amphiphilic block copolymer PEG-b-PPLG, where the

PPLG block is modified with different side chains. Such amphiphilic block copolymer can self-assemble to form stable micelle structures, and the side chains can be tuned systematically to control the size and CMC of the micelles.[95]

Scheme 1.12. Functionalization of PPLG with various amines via CuAAC (reprinted with permission from reference[71], copyright (2011) American Chemical Society).
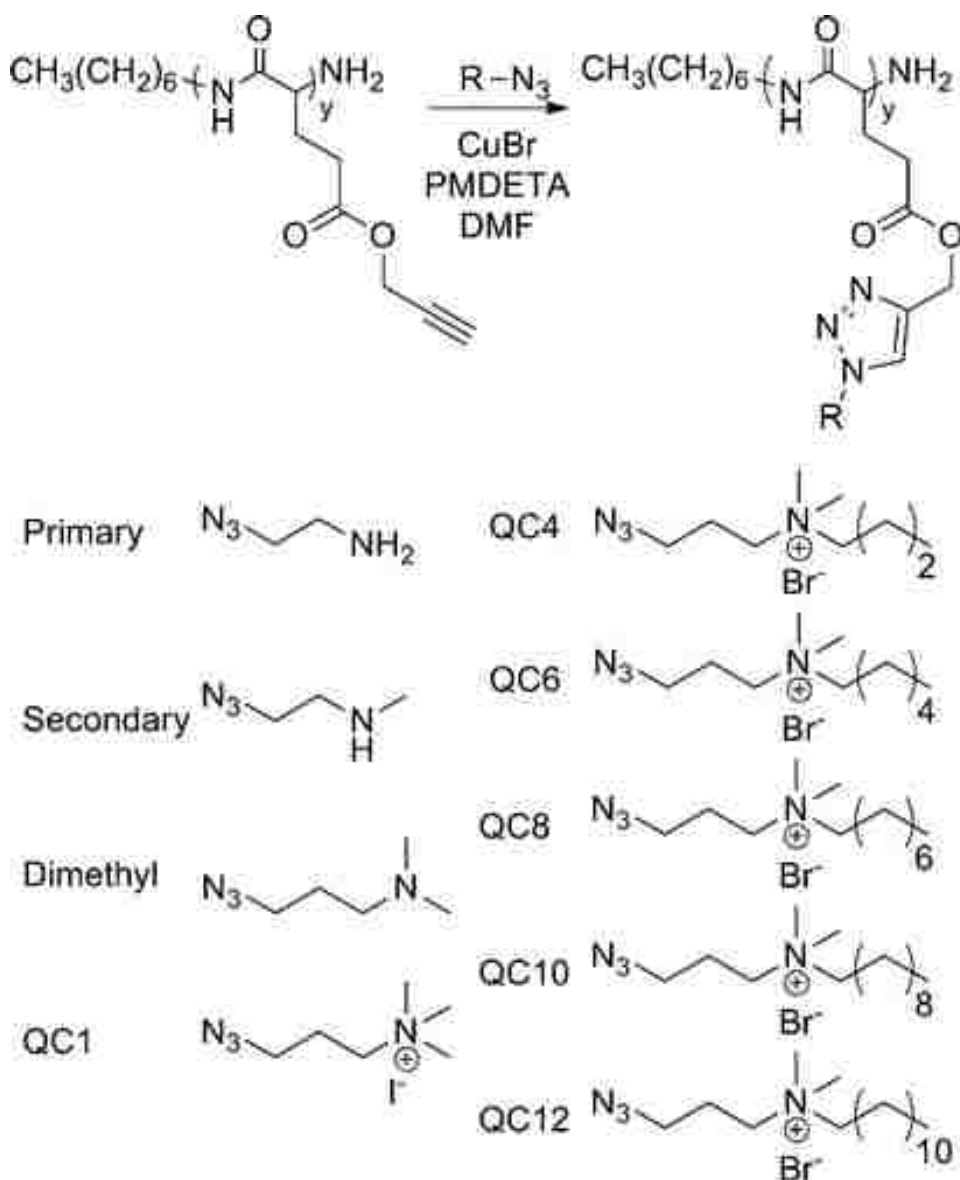
Chen et al also established several of their systems based on the same platform later on. In their first publication, three azide modified saccharides were conjugated onto PPLG with high efficiency via CuAAC to afford synthetic glycopolypeptides.[53] The afforded glycopolypeptides

were found to be water-soluble while maintaining α-helical conformation. Subsequently, short azido-PEGs were grafted onto PPLG via the same method to afford thermal responsive polypeptides.[96] The PEG grafted PPLGs exhibited no detectable cytotoxicity and can be enzymatically degraded via proteinase K. Later on, various functionalities (e.g. DOX, tertiary amines, PEG) have been clicked onto PPLG for different applications via CuAAC.[97-99]

Another alkyne containing NCA was later developed by Cheng and coworkers.[100] The NCA γ-(4-propargyloxybenzyl)-L-glutamic acid N-carboxyanhydride (POB-L-Glu NCA) with longer side chain is also based on glutamic acid. The POB-L-Glu NCA was polymerized via HMDS, and followed by efficient derivatization with azido containing amines and guanidine compounds for gene delivery study.

In addition to the advantages (e.g. high grafting efficiency) of PPLG based platform for further modification, it also shares an inherent limitation. The stability of the ester linkage on the PPLG side chain can be an issue. Usually, dialysis purification of PPLG based conjugates is conducted under pH controlled condition to avoid hydrolysis of the ester linkage.[71,73,97] Hence, an alternative polypeptide was reported to eliminate the labile ester link. Heise et al investigated a "clickable" polypeptide platform based on a non-natural amino acid D,L-propargylglycine.[101] The DLPG NCA monomer was first developed and polymerized back in 1960.[102] And this is actually the first example of NCA monomer that bears alkyne side chain. Heise et al. first investigated the post-polymerization modification via CuAAC. Benzylamine initiated ROP of the DLPG NCA yielded low molecular weight poly(D,L-propargylglycine) (M_n = 2250 g/mol), which resulted from poor solubility of the polymer due to strong intermolecular interaction. Copolymerization with BLG NCA helped increase the molecular weight M_n to 5800 g/mol. Azido containing saccharides can be efficiently conjugated onto the polypeptide via CuAAC to

prepare bioactive glycopolypeptides. However, as mentioned previously, the solubility and low molecular weight are two major limitations for this system. Another alternative to the above mentioned limitations is to switch the role of alkyne and azido, which will be elaborated in the following section.

1.4.2.2 Azido containing polypeptides

Deming and coworkers recently synthesized two azido containing NCAs: L-azidonorvaline-*N*-carboxyanhydride (Anv NCA) and L-azidonorleucine (Anl NCA).[103] They both can be polymerized via $(PMe_3)_4Co$ to prepare well-defined polymers. Following derivatization studies indicate the azido pendant side chain can be converted quantitatively via CuAAC, and different functionalities (e.g. carboxylic acid, amino acid and monosaccharide) can be incorporated with high yields. Though this is the first example of azido containing polypeptides prepared directly from azido functionalized NCAs, previous work done in our lab already demonstrated examples of azido containing polypeptides prepared via nucleophilic substitution of poly (γ-3-chloropropyl-L-glutamate) (Scheme 1.13).[104-106] Poly (γ-3-chloropropyl-L-glutamate) of controlled molecular weight (MW = 5 ~ 28 kg/mol) was prepared with HMDS used as initiator. Alkyne containing mannose was quantitatively grafted onto the polymer via CuAAC. Bottle-brush copolymer was also prepared in a similar manner via grafting alkyne terminated polylactide-*b*-poly(ethylene glycol) copolymer onto side chains of polypeptide containing azido functionalities.

1.4.2.3 Alkene containing polypeptides

Alkene containing NCA will be the last class of functionalized monomer covered in this section. The preparation of alkyne containing NCA has a long history, which originates back to 1954.[107] Many NCAs bearing alkenes have been developed recently, presumably due to the

Scheme 1.13. Synthesis of azido containing polypeptide via post-polymerization modification. (Reprinted with permission from reference[104], copyright (2010) American Chemical Society).
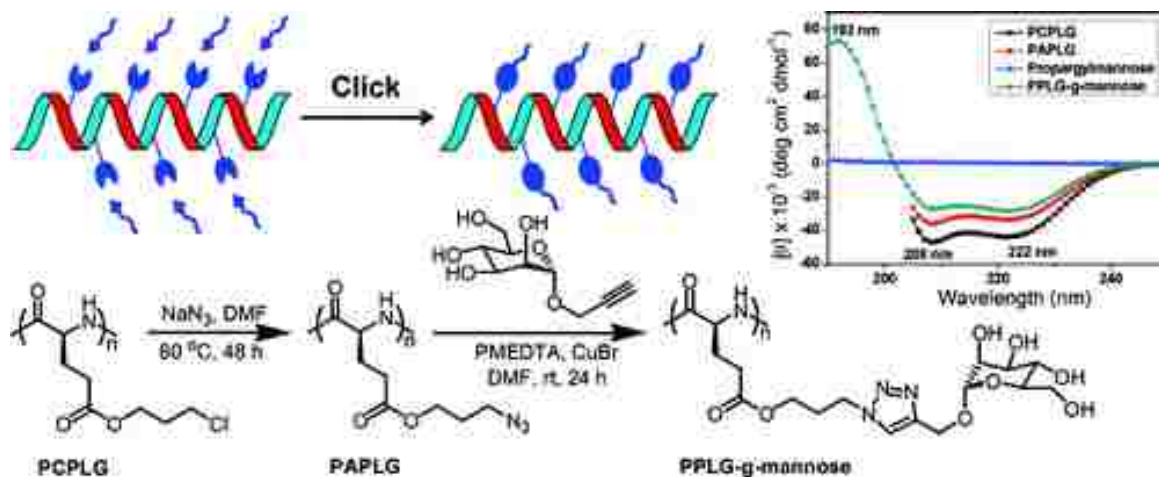
versatile radical thiol-ene click chemistry that can be employed for polypeptide derivatization. Schlaad et al first demonstrate the thiol-ene click reaction to be used in the post-polymerization modification of derivatizing alkene.[108] DL-allylglycine NCA (DLAG NCA) was first polymerized with hexylamine or PEG-NH$_2$ as initiator to prepare well-defined poly(DL-allylglycine), though with low molecular weight (< 2000 g/mol) due to polymer solubility issues (Scheme 1.14). Monosaccharide and 3-mercaptopropionate containing thiol were screened first to demonstrate the efficiency of the post-polymerization modification. The thiol-ene radical addition was done under different conditions (e.g. heated, UV light) with 2,2-azobis(2-methylpropionitrile) (AIBN) as the radial initiator. The grafting efficiency ranges from 10% to 100% depending on the reaction condition. The authors attributed the low efficiency to bad solubility of homopolymer, as the efficiency reached 100% when PEG-b-poly(DL-allylglycine) was used in the glucosylation reaction. Later on, they increased the solubility of poly(DL-allylglycine) by copolymerizing DLAG NCA with BLG NCA followed by debenzylation to introduce carboxylic acid on the side chains in the copolymer. The subsequent glucosylation can be achieved in 100% grafting efficiency under mild condition.[109]

Scheme 1.14. Synthetic pathway of synthesis (A) and polymerization (B) of allylglycine NCA and subsequent radical thiol addition (C). (Reprinted with permission from reference[108], copyright (2011) American Chemical Society).
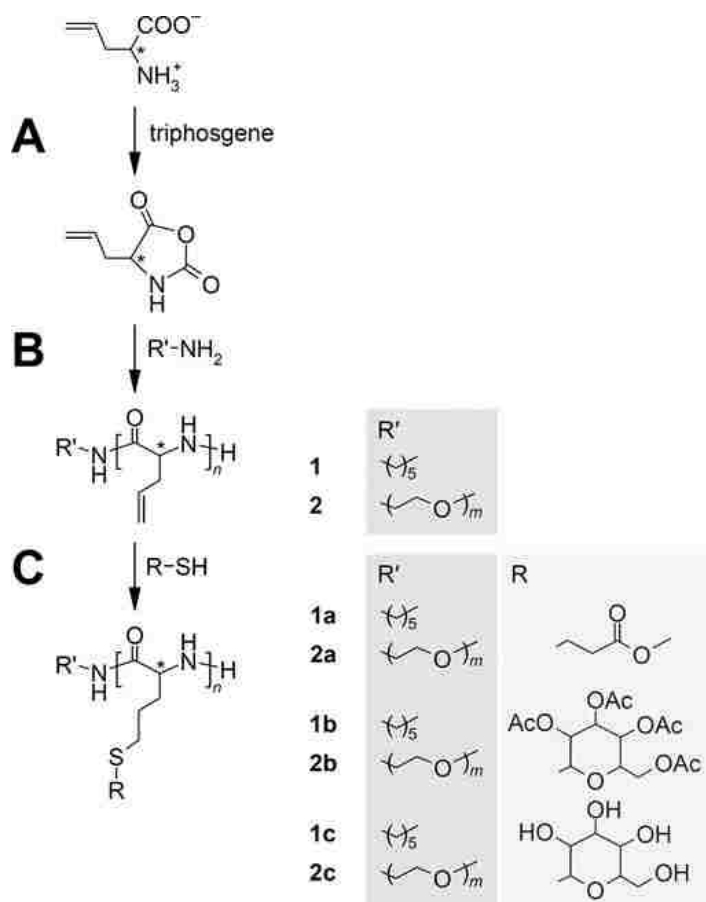
Zhang et al. later studied the thiol-ene derivatization on copolypeptide containing alkene with 3-mercaptopropionic acid under UV light with high efficiency. The alkene functionality was introduced via a NCA monomer γ-allyl-L-glutamate-*N*-carboxyanhydride (ALG NCA), which was synthesized previously.[110]

Cheng et al. also reported several works related to alkene containing polypeptides modification via thiol-ene chemistry. γ-(4-Allyloxylbenzyl)-L-glutamate *N*-carboxyanhydride (AOB-L-Glu NCA) with a long hydrophobic side chain based on L-glutamic acid was prepared for the first time.[111] The AOB-L-Glu NCA can be readily polymerized with HMDS as initiator to prepare to afford well-defined polypeptide PAOBLG. Charged compounds 2-aminoethanethiol

chloride and 3-mercaptopropionic acid can be grafted onto the PAOBLG polypeptide with nearly quantitative grafting efficiency. The resulting conjugates maintain helical conformation in aqueous solution regardless of the charge on the side chains, owing to the sufficiently long side chains. Similarly, poly(L-serine) bearing terminal alkene on the side chain was synthesized from O-pentenyl-L-serine based N-carboxyanhydride (PE-L-Ser NCA) with PEG-NH$_2$ as initiator.[70] Expectedly, the resulting polypeptide can be efficiently modified through thiol-ene reaction.

Even though the thiol-ene click reaction is the most commonly used chemistry to derivatize alkene containing polypeptides; there are also other chemistries available in literature for the modification. One excellent example is reported by Cheng et al (Scheme 1.15).[51,112] The example is based on γ-(4-vinylbenzyl)-L-glutamate N-carboxyanhydride (VB-Glu NCA), which was first synthesized by Schouten and coworkers in 2007 for surface functionalization study.[113] The intensive polymerization and post-polymerization functionalization were conducted by Chen's group later on. Poly(γ-(4-vinylbenzyl)-L-glutamate) (PVBLG) was prepared via HMDS mediated ROP of VB-Glu NCA to prepare well-defined polymer with molecular weight up to 47000 g/mol. The afforded PVBLG can subsequently undergo various reactions with at least 90% grafting efficiency and isolation yield in the range of 60% - 90%. Various functionalities can be introduced via the reaction with the terminal alkene, which are summarized in Scheme 1.15. For instance, alcohol, aldehyde, carboxylic acid can be introduced via ozonolysis or osmium tetroxide oxidation. New carbon-carbon bond formation can be achieved through Suzuki coupling, photo crosslinking and metathesis. Numerous different kinds of functionalities (e.g. alcohol, amine, crown ether, cyclic alkene, cyclic alkane and etc.) have been successfully incorporated into the PVBLG platform for different applications (e.g. gene delivery[2], stable cationic helix[112]).

Scheme 1.15. Synthesis and modification of PVBLG (Reprinted with permission from reference[51], copyright (2011) American Chemical Society).
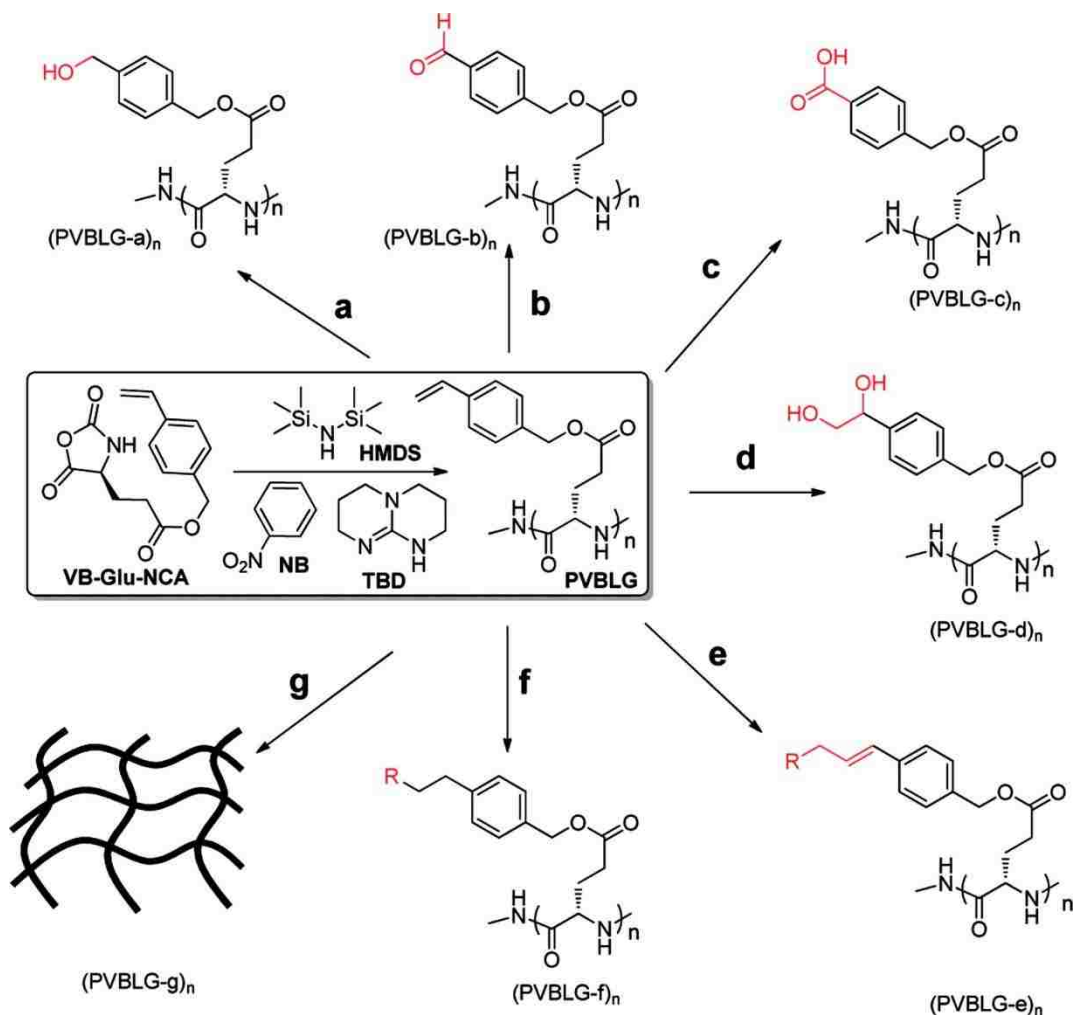
Reagents and conditions: (a) i. O_3, −78 ℃, 1–5 min; ii. $NaBH_4$, rt, 16 h; (b) i. O_3, −78 °C, 1–5 min; ii. PPh_3, rt, 2–3 h; (c) OsO4, oxone, rt, 48 h; (d) OsO_4, NMO, rt, 20 h; (e) second-generation Grubbs catalysts, *cis*-RCH=CHR, rt, 24 h; (f) i. 9-BBN, rt, 16 h; ii. Ar–Br, Pd(PPh_3)_4, $NaHCO_3$(aq), N_2, 70 ℃, 20 h; (g) UV.

1.4.2.4 Methionine alkylation

The last class of post-polymerization modification discussed here is alkylation based on poly(L-methionine). The efficient alkylation derivatization of poly(L-methionine) was first investigated more than 50 years ago.[114] Only methyl and carboxymethyl sulfonium derivatives were demonstrated back then. Deming et al recently expanded the poly(L-methionine) alkylation

to introduce wide range of functional groups (e.g. alkyne, amine, carboxylic acid and etc.)

(Scheme 1.16).[115,116] The poly(L-methionine) with up to 400 repeat units was synthesized via

Scheme 1.16. Post-polymerization modification of poly(L-methionine) via alkylation (reprinted with permission from reference[117], copyright (2016) American Chemical Society).
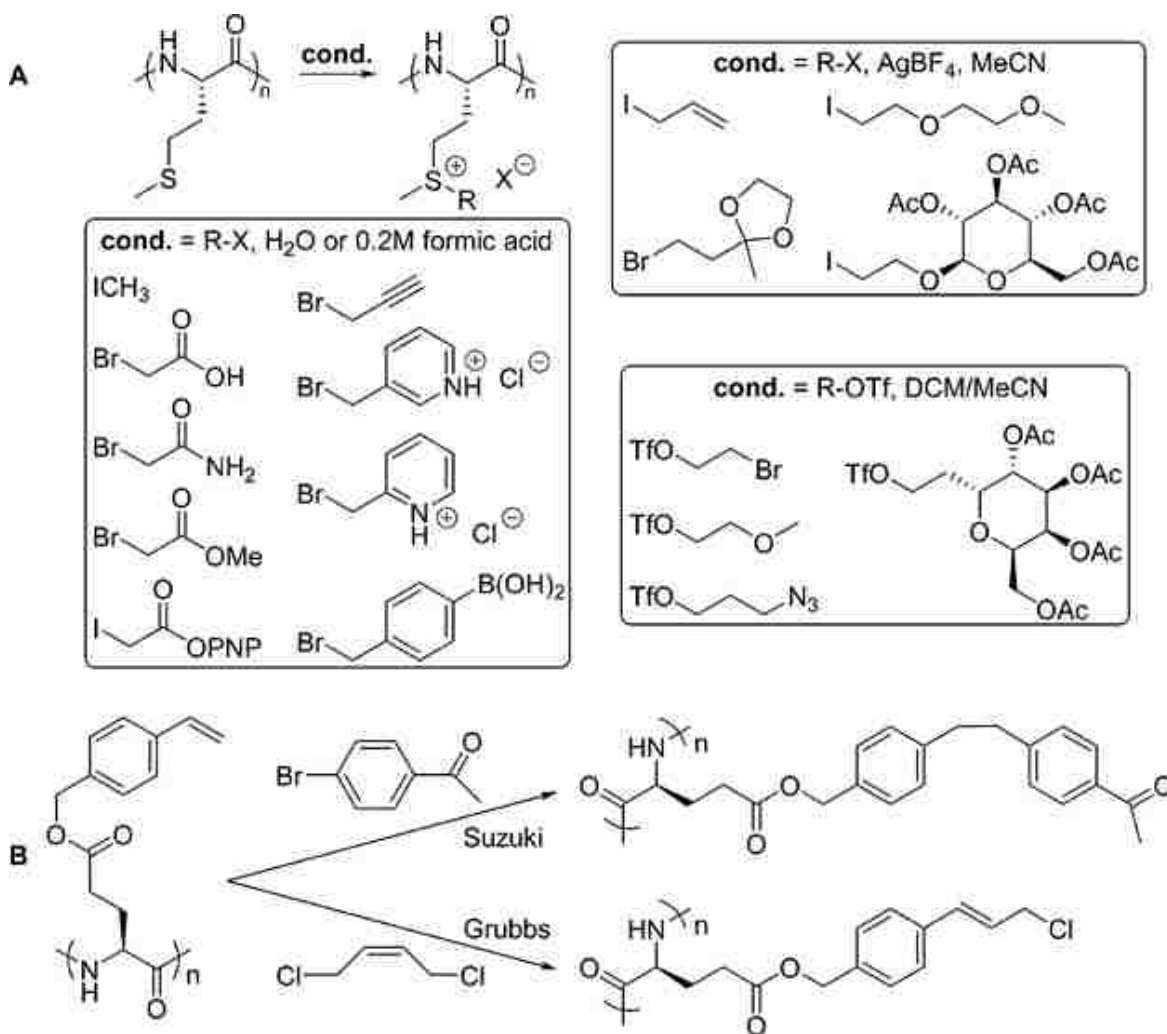
ROP of L-methionine NCA with Co(PMe₃)₄ as initiator. PEG was attached to the poly(L-methionine) after polymerization for the purpose of molecular weight characterization due to the poor solubility of poly(L-methionine). As shown in Scheme 1.16, a wide range of functionalities can be grafted onto poly(L-methionine) under different conditions. And all the alkylation can be done in almost quantitatively conversion (94% ~ 99%). Importantly, the alkylation is also reversible in the presence of sulfur nucleophile (e.g. 2-mercaptoethanol). Alternatively,

irreversible alkylation was also introduced later via reaction with epoxides containing different functionalities (alkyne, alkene, azido, etc.).[118] The derivatization is also efficient as 100% grafting efficiency can be achieved in most cases. The efficient alkylation and dealkylation makes the thioether containing polypeptides interesting platforms for stimuli responsive biomaterials.[78] One inherent limitation of poly(L-methionine) is the solubility in common solvents, which makes the characterization of homo poly(L-methionine) to be troublesome.

The fundamental knowledge and the cutting-edge research of polypeptides have been reviewed in this chapter. In the following Chapter 2, the development of a new class of functional polypeptide will be introduced. In Chapter 3, the preparation of polypeptides via ROP of NTAs in solid phase will be discussed. The solution phase polymerization of NTAs mediated by TMG/benzoic acid will be elaborated in Chapter 4. The Chapter 5 concludes the dissertation, and the future direction of further investigation will be discussed.

CHAPTER 2. NON-IONIC WATER-SOLUBLE "CLICKABLE" POLYPEPTIDES

2.1 Introduction

Polypeptides are α-amino acid based polymers capable of adopting basic secondary structures such as α-helix or β-sheets reminiscent of those observed for proteins.[70,74] Recent development in the controlled ROP has enabled access to well-defined polypeptides with tailorable composition, architecture, functionality and size.[1] In addition, polypeptides are proteolytically degradable and exhibit low cytotoxicity.[96] As a result, synthetic polypeptides have been increasingly investigated as a platform for biomedical applications such as gene therapy[2,70,87], drug delivery[22,119-121] and tissue engineering scaffolds.[122,123]

The solubility and conformation of polypeptides are strongly dependent on the side chain structures. Water-soluble polypeptides often bear charges on the side chains (*e.g.*, poly(L-glutamic acid), polysulfonium based on poly(L-homocystine)[78]). The electrostatic repulsive interactions between the charges on the side chains cause the polymer backbones to adopt random coil conformations. Moving the charges further away from the backbone has successfully yielded water-soluble ionic polypeptides adopting helical conformations.[112] In spite of their high water solubility, ionic water-soluble polypeptides (*e.g.*, poly(L-lysine)) tend to bind to oppositely charged biomolecules *in vivo*, resulting in cytotoxicity.[124] As a result, non-ionic water-soluble polypeptides are highly sought after to complement the charged polymers for certain biomedical applications (*e.g.*, delivery carrier for hydrophobic therapeutics).

Several non-ionic water-soluble polypeptides that adopt stable secondary structures have been reported. They are synthesized either through post-polymerization modification of

polypeptides bearing reactive side chains with appropriate hydrophilic moieties such as monosaccharides and PEG[53,54,96,99,104-106,108,125,126] or by direct ROP of discrete NCA monomers bearing hydrophilic side groups (*e.g.*, glycosylated-L-lysine NCA).[55,72,74,78,79,127] Most of the above mentioned polypeptides having hydrophilic side chains lack functional sites for further modifications, limiting their potential uses for bio-conjugation.

Synthetic polymers have been actively investigated as multivalent ligand scaffolds. Conjugation of bioactive ligands onto the polymers enables control of the size, shape and density of the ligand ensembles.[128] While a majority of the polymers that have been investigated have random coil conformation, we reason that the polypeptides with helical conformations offer several advantages over the random coil counterparts as multivalent ligand scaffolds. First of all, they provide more efficient display of the ligands attached on the side chains relative to the random coil counterparts where some of the side chain moieties are buried in the polymer interior. Secondly, multivalent binding to the random coil scaffold causes uncoiling of the scaffold, resulting in free energy penalty. By contrast, the free energy penalty is expected to be minimal in the multivalent binding to the helical scaffold, as the polymers adopt more extended conformation than the random coil counterparts. Before the helical polypeptides can be fully investigated for multivalent scaffold applications, efficient synthetic methods toward non-ionic water-soluble helical polypeptides, which also bear functional side chains to enable conjugation of bioactive moieties, should be developed.

Herein, we report the design and synthesis of non-ionic water-soluble polypeptides that can be modified with hydrophobic or hydrophilic moieties on the side chains by CuAAC. In addition, the polypeptides may self-assembly into different secondary conformations based on the nature of corresponding amino acids. Two strategies were designed to achieve the goal.

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The first method involves random copolymerization of γ-propargyl L-glutamic acid *N*-carboxyanhydride (PLG NCA) (M1)[54] and *N*-ε-2-[2-(2-methoxyethoxy)ethoxy]acetyl-L-lysine *N*-carboxyanhydride (EG$_2$-LYS NCA) (M2) (Scheme 2.1 and Scheme 2.2).[74] The propargyl groups on the side chains are expected to enable facile and efficient conjugation of various ligands using click chemistry. The oligomeric ethylene glycol units are expected to confer water solubility and reduce non-specific interactions in cellular environment. The copolymerization method allows for control over the relative solubility in water and the density of "clickable" sites by tuning the feed ratio of the two monomers.

Scheme 2.1. A schematic depicting the synthesis of non-ionic water-soluble helical polypeptides bearing "clickable" side chains.
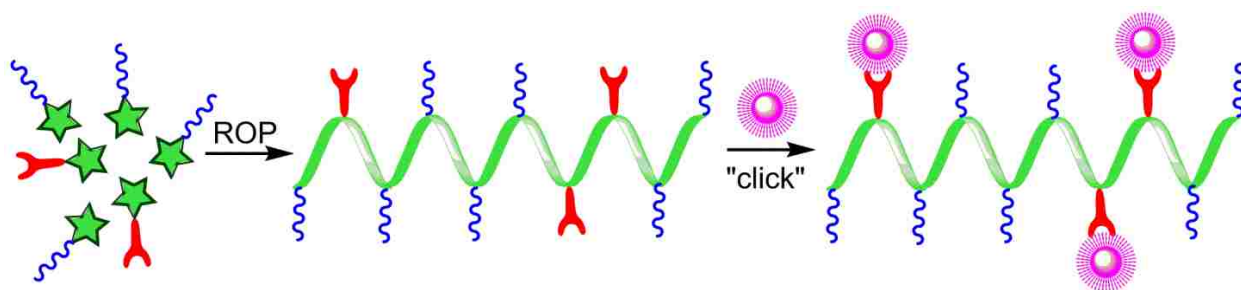
The second strategy involves designing of a new NCA (**5** in Scheme 2.3) based on L-serine, which contains oligomeric ethylene glycol unit as well as azido on the side chain. Upon polymerization, the polypeptide is expected to possess designed features such as water solubility, clickable pendant side chains and ability to self-assemble into unique secondary conformation. The pendant azido is expected to provide "clickable" site to introduce functionalities via CuAAC, and the oligomeric ethylene glycol units are included to increase water solubility.

It was found that the copolypeptides [denoted as P(PLG$_m$-r-PLL$_n$)] with less than 50 mol% hydrophobic PPLG segment are readily dissolved in water. Post-polymerization grafting with hydrophobic or hydrophilic moieties can be achieved quantitatively by CuAAC chemistry,

Scheme 2.2. The synthesis of polypeptide copolymers and post-polymerization conjugation.
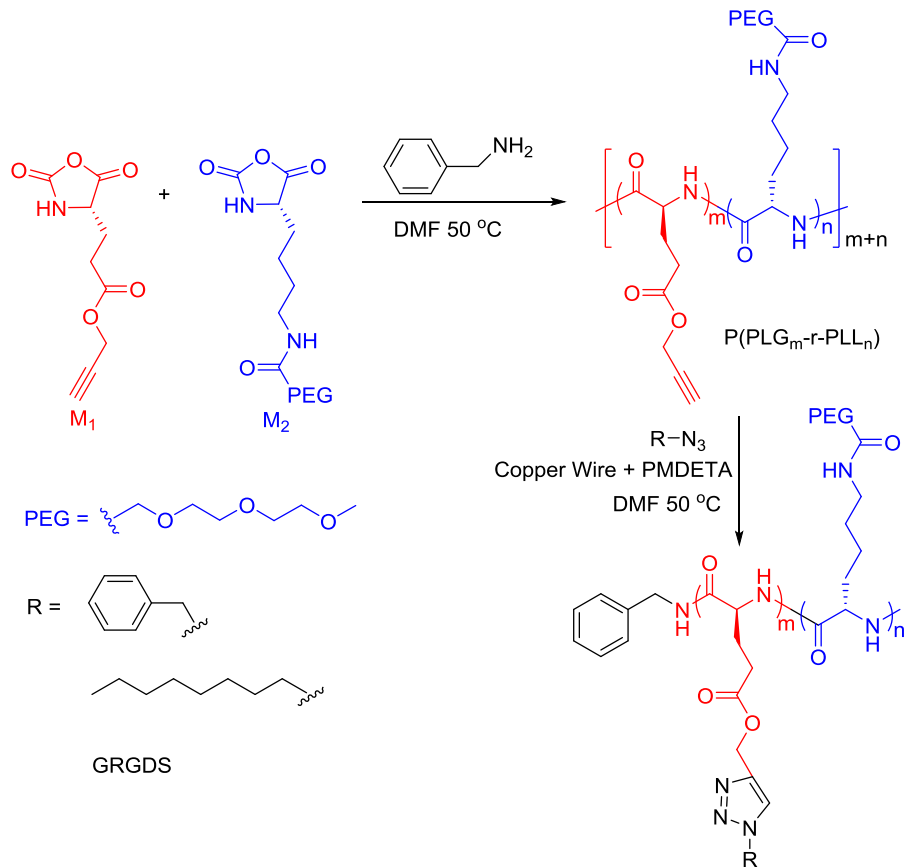
Scheme 2.3. Synthetic route towards AEG$_2$ Ser NCA.
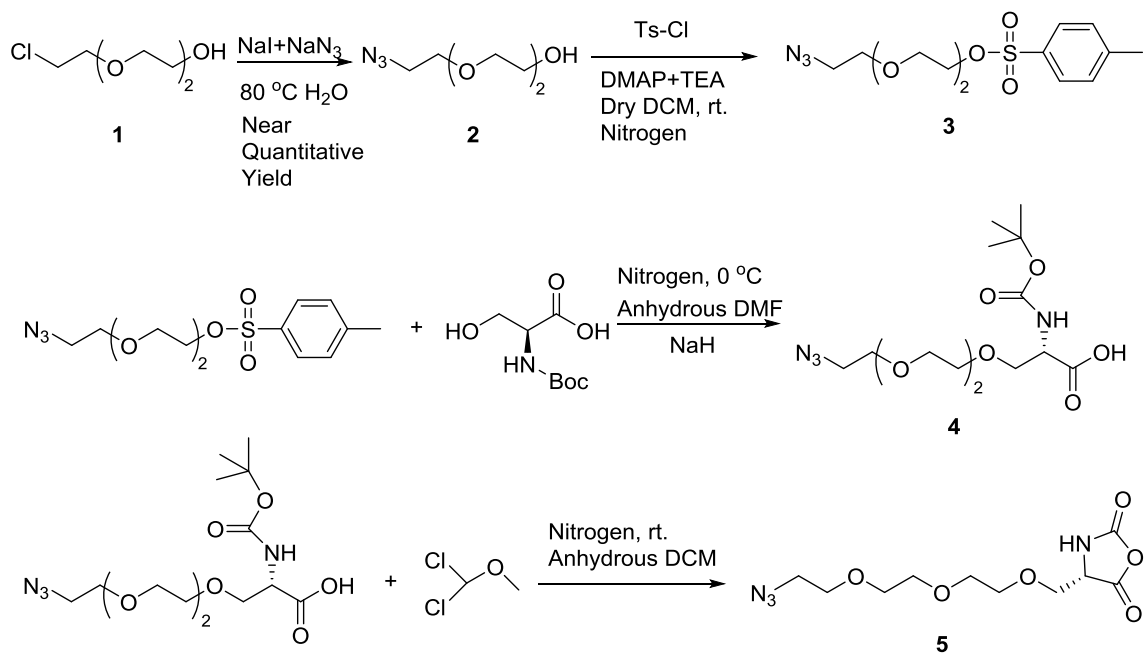
yielding non-ionic polypeptide conjugates that retain good water solubility and high percentage of α-helical content. To the best of our knowledge, this is the first study on the synthesis of non-ionic water-soluble helical polypeptides that bear "clickable" side chains through direct ROP of discrete NCA monomers. Preliminary cell culture study reveals that the polypeptide conjugates bearing a integrin binding peptide GRGDS (Gly-Arg-Gly-Asp-Ser) display positive effects in inducing integrin-mediated cell adhesion.

The synthesis of AEG$_2$ Ser NCA has been explored. Condition optimization is still needed to synthesis AEG$_2$ Ser NCA in high purity and yield. Preliminary polymerization study was conducted with benzylamine as initiator. Polymerization with poor M_n control was obtained. Hence, further investigation into the polymerization activity is required before well-defined polypeptide to be prepared with desired features.

2.2 Materials and Methods

2.2.1 General

All the chemicals and Dowex® Marathon® C resin were all purchased from Sigma-Aldrich and used as received unless specified. Benzylamine was first stirred with calcium hydride overnight, and then distilled under vacuum. Anhydrous solvents were purified by passing through alumina columns under argon. NCA monomers precursors γ-propargyl L-glutamate hydrochloride and N-ε-2-[2-(2-Methoxyethoxy)ethoxy]acetyl-*N-α*-Z-L-Lysine were synthesized according to reported procedures.[54,74] Azido terminated GRGDS, benzyl azide and octyl azide were synthesized according to a reported procedure.[129,130] Compound 2 and 3 were synthesized according to a reported procedure.[131]

^{1}H NMR spectra were recorded on a Bruker AV-400 or AV-500 spectrometer. Chemical shifts in parts per million (ppm) were referenced relative to proton impurities or ^{13}C isotope of deuterated solvents (e.g., CDCl$_3$). SEC-DRI analyses were performed with an Agilent 1200

system equipped with three Phenomenex 5 μm, 300 × 7.8 mm columns [100 Å, 1000 Å and Linear(2)], Wyatt DAWN EOS multi-angle light scattering (MALS) detector (GaAs 30 mW laser at λ=690 nm) and Wyatt Optilab rEX differential refractive index (DRI) detector with a 690 nm light source. DMF containing 0.1 M LiBr was used as the eluent at a flow rate of 0.5 mL min^{-1}. The temperature of the column and detector was 25 ℃. Polymer molecular weight (M_n) and molecular weight distribution (PDI) were obtained by conventional SEC analysis with a calibration curve built using polystyrene standards. Circular dichroism (CD) data were collected on a Jasco J810 CD spectrometer (Japan Spectroscopic Corporation) with a path length of 0.1 cm and a band width of 1 nm at 20 ℃. Three scans were collected and averaged between 190 nm and 250 nm at a scanning rate of 50 nm min^{-1} with a resolution of 1 nm. The content of various secondary structures were calculated by DICHROWEB using Contin-LL. Fourier transform infrared radiation (FTIR) spectra were collected with a Bruker Alpha FTIR spectrometer. Dynamic light scattering (DLS) analysis was conducted on a Malvern Zetasizer Nano-ZS instrument using zetasizer software 6.12.

Cell adhesion on immobilized human fibrinogen and GRGDS conjugated polymers was assessed by the measurement of cellular lactate dehydrogenase (LDH) activity. Briefly, Chinese hamster ovary cells (CHO cells) stably expressing integrin $\alpha_{IIb}\beta_3$ were maintained in MEM-α medium (Life Technologies, Grand Island, NY) supplemented with 10% FBS, 1% None-Essential Amino Acids, 1% L-Glutamine, 1% Sodium Pyruvate and 1% Penicillin-Streptomycin (All from Life Technologies, Grand Island, NY). Before the assay, cells were first detached by trypsin-EDTA and suspended in HBS supplemented with 5.5 mM glucose and 1% bovine serum albumin and 1 mM Ca^{2+} were seeded on flat bottom 96-well plates (~5000 cells/well) pre-coated with GRGDS conjugated polymers or fibrinogen at the concentrations ranged from 1.5 μg/mL to

50 μg/mL and blocked with 1% bovine serum albumin. After incubation at 37 ℃ for 30 min, wells were washed three times with ice-cold PBS. Remaining adherent cells were lysed with 1% Triton X-100, and LDH activity was assayed using the Cytotoxicity LDH Detection Kit (CloneTech Laboratories Inc., CA) according to the manufacturer's instructions. The 492 nm absorbance of formazan was measured by a BIO-RAD microplate reader (Model 680, Life Science Research, CA). Cell adhesion was expressed as a percentage of bound cells relative to total input cells.

2.2.2 NCA monomers synthesis

2.2.2.1 Synthesis of PLG NCA (M1)

PLG NCA was synthesized by adapting a published procedure.[54] Briefly, γ-propargyl L-glutamatic acid hydrochloride (3.00 g, 13.5 mmol) was suspended in anhydrous THF (100 mL) at 50 ℃ under nitrogen, triphosgene (1.64 g, 5.52 mmol) was added to the above suspension under nitrogen flow. After the heterogeneous solution turned clear within 3 h, THF was removed under vacuum to afford a clear oil product. The oily product was purified by flash chromatography first with anhydrous hexanes and then gradient anhydrous THF/hexanes (9:1 ~ 1:1 v/v) mixtures (R_f = 0.23 in 1 : 1 THF/hexane). Fractions containing the desired product were combined and concentrated to afford a clear oil which solidified upon storage at - 20 ℃ inside glovebox (1.40 g, 49% yield). ^{1}H NMR (400 MHz, CDCl$_3$) δ (ppm): 6.69 (s, 1H), 4.73 (d, 2H), 4.46 (t, 1H), 2.63 (t, 2H), 2.54 (t, 1H), 2.31-2.18 (m, 2H).

2.2.2.2 Synthesis of EG$_2$-LYS NCA (M2).

EG$_2$-LYS NCA was synthesized by adapting a published procedure.[74] Briefly, N-ε-2-[2-(2-Methoxyethoxy)ethoxy]acetyl-N-α-Z-L-Lysine (6.02 g, 13.7 mmol) was dissolved in anhydrous CH$_2$Cl$_2$ (200 mL) in an oven dried round bottom flask equipped with a stir bar under nitrogen flow. 1,1-Dichloromethylmethyl ether (6.80 g, 59.2 mmol) was added to the above

solution. The reaction mixture was heated to at 45 ℃ and allowed to reflux under nitrogen for 12 h during which time a 90% conversion was reached. The reaction mixture was then concentrated under vacuum using a Schlenk line to afford a clear oil. The crude product was first purified by recrystallization in anhydrous THF/hexanes in glovebox at - 20 ℃ to afford a white solid. The white solid was further purified by dry flash chromatography under nitrogen. Column was eluted with anhydrous THF/hexanes (2:1 ~ 1:0 v/v). Fractions containing the product were combined and the volatiles were removed to yield a white solid as the final product (1.60 g, 35% yield) (R_f = 0.34 in THF). ^{1}H NMR (400 MHz, CDCl$_3$) δ (ppm): 7.68 (s, 1H), 7.33 (s, 1H), 4.30 (m, 1H), 4.00 (m, 2H), 3.70-3.59 (m, 8H), 3.39-3.31 (m, 5H), 2.02-1.81 (m, 2H), 1.64-1.49 (m, 4H).

2.2.2.3 Synthesis of *O*-(2-(2-(2-azidoethoxy)ethoxy)ethyl)-*N*-(tert-butoxycarbonyl)-L-serine (**4**)

Boc-Ser-OH (31.5 g, 0.154 mol) and NaH (7.38 g, 0.308 mol) were first suspended in anhydrous DMF (800 mL) in an ice bath under nitrogen atmosphere. After stirring for 2h, then **3** (51.53 g, 28.1 mmol) was added to the above mixture. 3d later, solvent DMF was removed by vacuum distillation at 45 C. The left over off-white solid was then dissolved in 400 ml DI water, followed by diethyl ether wash (2 × 150 mL). The aqueous solution was then acidified with 1 M HCl to pH ~ 3. The above acidic aqueous cloudy solution was then extracted with ethyl acetate for 3 times (600 mL in total). The combined organic layer was then dried over MgSO$_4$, followed by filtration and concentration to afford 40.97 g light yellow oil as crude product, which was used directly for next step. Caution! The handling of large scale NaH can be dangerous in air. Smaller scale is thus recommended for this reaction.

2.2.2.4 Synthesis of *O*-(2-(2-(2-azidoethoxy)ethoxy)ethyl)-L-serine *N*-carboxyanhydride (AEG$_2$ Ser NCA **5**)

4 (3.67 g, 10.1 mmol) was first dissolved in anhydrous DCM (100 mL) in nitrogen atmosphere. Then dichloromethyl methyl ether (4.0 mL, 44 mmol) was added to the above clear

solution while stirring. 20 h later, solvent DCM was removed under vacuum to afford clear oil. The residue clear oil was subject to anhydrous column chromatography purification. The elution solvent consists of 1:1 anhydrous THF/hexanes (v:v). The R_f of AEG_2 Ser NCA is 0.2. 0.70 g clear oil was collected after the column chromatography purification with 24% yield.

2.2.3 General procedure for polymerization of AEG$_2$ Ser NCA

AEG_2 Ser NCA (73.0 mg, 0.253 mmol) was dissolved in anhydrous DMF (0.41 mL) inside a glovebox. A measured volume of DMF stock solution of benzylamine (94.1 μL, 50.6 μmol, 53.8 mM) was added to the above solution. The reaction was stirred at room temperature inside a glovebox for 2.5 day. Aliquot was taken to track conversion by ^{1}H NMR. Polymerization solution was also taken for SEC characterization.

2.2.4 General procedure for synthesis of polypeptide P(PLG$_m$-r-PLL$_n$)

PLG NCA (150.7 mg, 0.714mmol) and EG$_2$-LYS NCA (0.9548 g, 2.88 mmol) were dissolved in anhydrous DMF (8.3 mL) inside a glovebox. A measured volume of DMF stock solution of benzylamine (668 μL, 0.0359 mmol, 53.8 mM) was added to the above solution. The reaction was stirred at 50 ℃ inside a glovebox for 12 h. Diethyl ether was added to the above solution to yield an oily precipitate. The crude oily product was then re-dissolved in DI water (~10 mL) and transferred into a centrifugal dialysis tube (10 kDa MWCO). The centrifugal tube was spun at 3000 rpm till all of the water went through the filter membrane. The residue polymer sample was then re-dissolved in DI water for repeated dialysis (3 times). Aqueous solution obtained after dialysis was lyophilized to give a clear sticky solid (0.68 g, 72% yield).

2.2.5 General procedure for the grafting of P(PLG$_m$-r-PLL$_n$) with hydrophobic moieties through CuAAC

P(PLG$_{17}$-r-PLL$_{69}$) (91.2 mg, 0.00402 mmol) and octyl azide (13.5 mg, 0.0871 mmol) were dissolved in anhydrous DMF (1.34 mL). PMDETA (14.2 mg, 0.0821 mmol) were added to

the above solution together with a half inch length of freshly filed copper wire. The reaction was stirred at 50 ℃ under nitrogen for 24 h. The copper wire was removed by filtration. The remaining clear light blue solution was diluted with THF (~ 10 mL), added with Dowex[®] Marathon[®] C resin (0.7 g) and stirred for 24 h to remove residual Cu (II) ion. Removal of the resin by filtration yielded a clear solution which was further concentrated under a flow of nitrogen. Addition of diethyl ether to the concentrate produced a white precipitate, which was collected by centrifugation and dried under vacuum (35.8 mg, 35% yield).

2.2.6 Representative procedure for the grafting of P(PLG$_m$-r-PLL$_n$) with N$_3$-GRGDS by CuAAC

P(PLG$_{14}$-r-PLL$_{72}$) (65.1 mg, 0.00282 mmol) and N$_3$-GRGDS (25.7 mg, 0.0409 mmol) were dissolved in anhydrous DMF (1 mL). PMDETA (3.3 mg, 0.019 mmol) were added to the above solution together with a half inch length of freshly filed copper wire. The reaction was stirred at 50 ℃ under nitrogen for 24 h. The copper wire was removed by filtration. DMF was removed by vacuum distillation. The residue solid was then re-dissolved in an edetate disodium (EDTA) (0.4g, 1mmol)/DI water (30 mL in total) solution, transferred into a centrifugal dialysis tube (10 kDa MWCO). The centrifugal tube was spun at 3000 rpm till all of the aqueous solution went through the filter membrane. The residue polymer sample was then re-dissolved in 3 mL DI water to repeat the centrifugal dialysis 3 times. The aqueous solution obtained after dialysis was lyophilized to give a white solid (28.2 mg, 31% yield).

2.3 Results and Discussion

2.3.1 NCA synthesis

The monomers (PLG NCA and EG$_2$-LYS NCA) were synthesized by adapting published procedures[54,74] and purified by column chromatography under anhydrous conditions[14] prior to polymerization. It is worth mentioning the condition for anhydrous flash chromatography. In

Deming's report, the flash chromatography was conducted inside a glovebox.[14] As an easier alternative, it was found that the anhydrous flash chromatography can also be conducted in fume hood with house nitrogen as pressure source. Anhydrous elution solvent, oven baked silica gel and glassware are still required. NCAs with good purity level can be prepared by this simplified operation as evidenced by ^{1}H NMR (Figure S2.1 and Figure S2.2).

The preparation of AEG$_2$ Ser NCA was first developed by the author as shown in Scheme 2.3. Both NMR and FTIR indicate success synthesis of the NCA (Figure 2.1, 2.2 and 2.3). However, the yield is still low around 24%. And the preparation condition should be further optimized to purify the NCA, as tiny impurities signals are present in the ^{1}H NMR. For instance, the precursor **4** should be purified prior to cyclization. The interrupted continuous investigation of Ser NCA is a result of the work in Chapter 3 and 4, the success of which will have significant scientific impact.
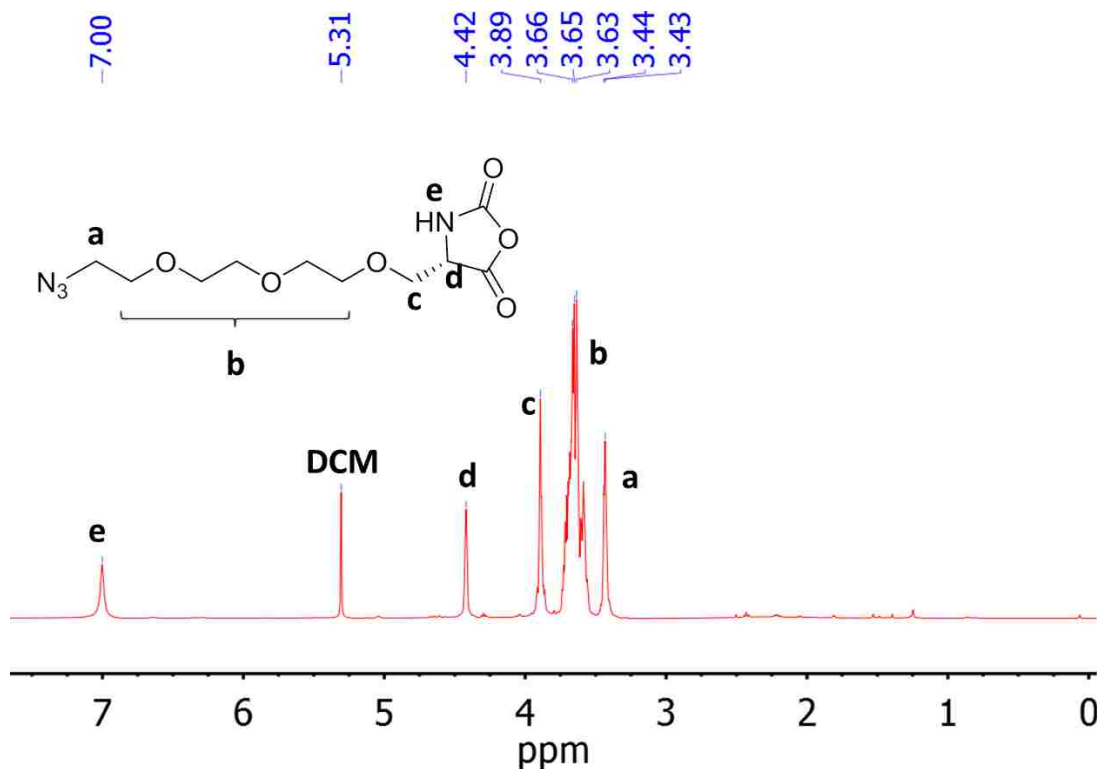
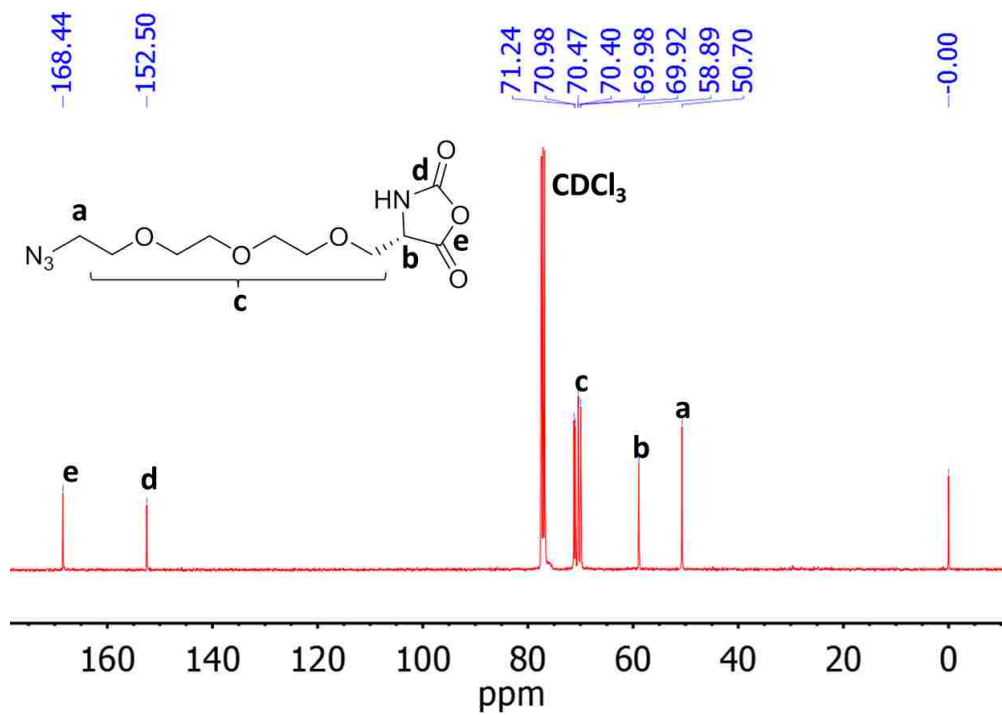

Figure 2.1. ^{1}H NMR spectrum of AEG$_2$ Ser NCA in DCM-d$_2$.

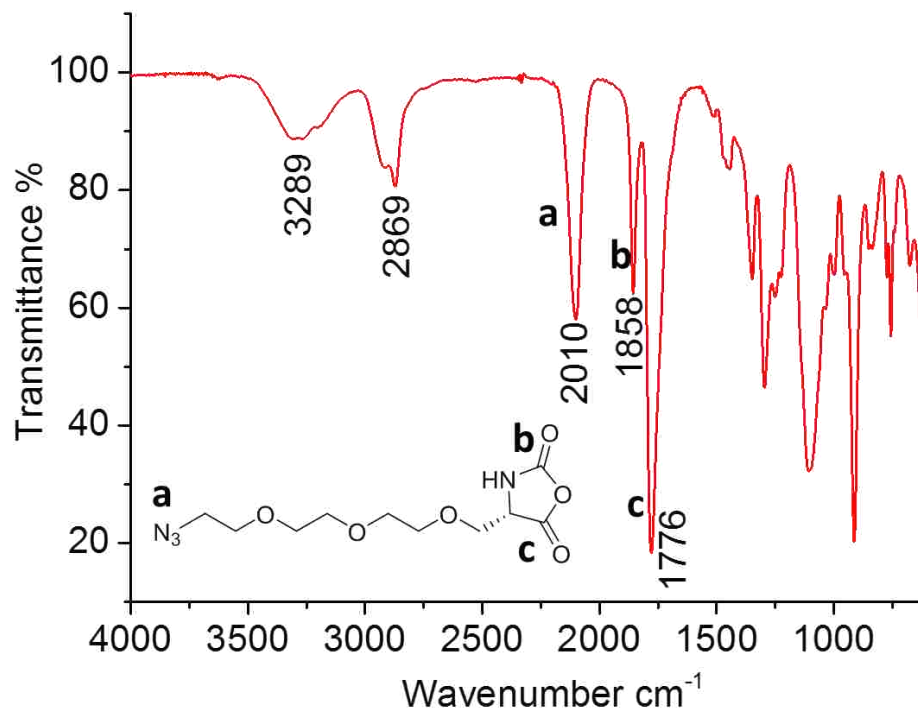Figure 2.2. $^{13}C\{H\}$ NMR spectrum of AEG_2 Ser NCA in $CDCl_3$.

Figure 2.3. FTIR spectrum of AEG_2 Ser NCA

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2.3.2 Synthesis of polypeptide P(PLG$_m$-r-PLL$_n$)

Polypeptide copolymers P(PLG$_m$-r-PLL$_n$) with a targeted DP of 100 and varying PPLG and PPLL content were prepared by ROP of PLG NCA (M$_1$) and EG$_2$-LYS NCA (M$_2$) in different feed ratios using benzylamine initiators (Scheme 2.2). All reactions ([M$_1$] + [M$_2$] = 0.4 M, ([M$_1$]+[M$_2$]) : [BnNH$_2$] = 100 : 1) were allowed to proceed at 50 °C in DMF under nitrogen and reached nearly quantitative conversion in 12 h. The resulting polymers were isolated by precipitation with diethyl ether and further purified by dissolution in distilled water and centrifugal dialysis. The polypeptide copolymer compositions were analysed by ^{1}H NMR and ^{13}C{H} NMR (Figure S2.3) spectroscopy (Table 2.1). Specifically, the methylene protons (e and j, Figure 2.4) due to the respective PPLG and PPLL segments were integrated relative to the aromatic protons of the benzyl end-groups (a, Figure 2.4) to give the polymer composition. The experimentally determined polymer compositions are in agreement with the theoretical values calculated from the monomer feed ratio and conversion. The copolymer molecular weight (M_n)
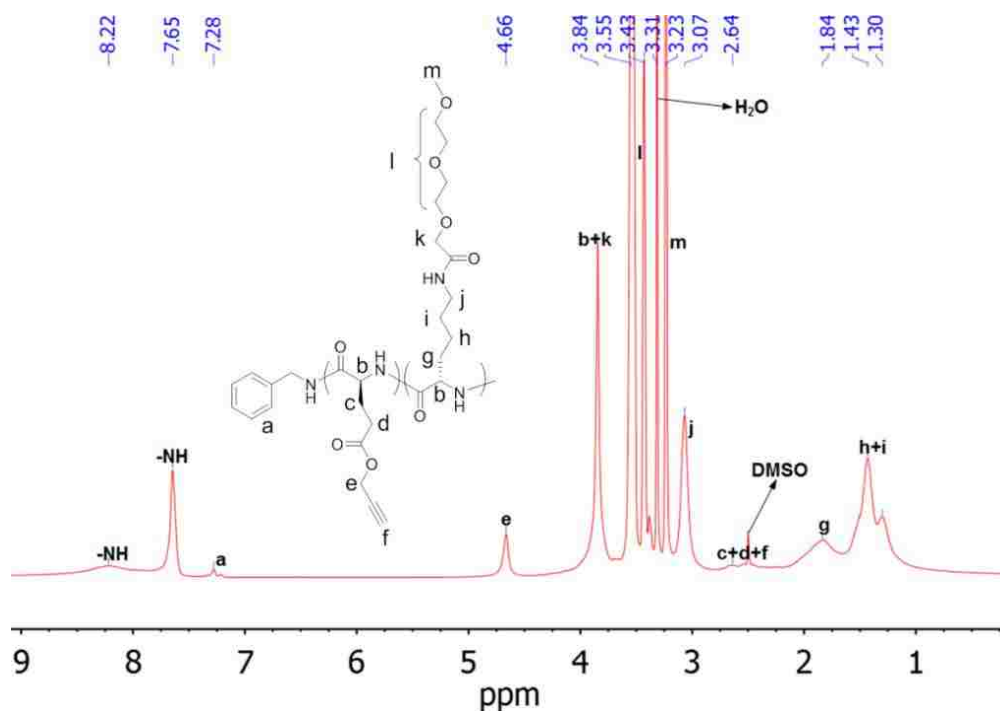

Figure 2.4. ^{1}H NMR spectrum of the P(PLG$_{17}$-r-PLL$_{69}$) copolymer in DMSO-d$_6$.

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Table 2.1. Benzylamine-initiated ROP of PLG NCA (M_1) and EG$_2$-LYS NCA (M_2) in different feed ratios to yield the P(PLG$_m$-r-PLL$_n$) polypeptides.

	Polymer composition [a]	$[M_1]_0/[M_2]_0$ $/[I]_0$	M_n (theo.) [b] (kg·mol^{-1})	M_n (NMR) [a] (kg·mol^{-1})	M_n (SEC) [c] (kg·mol^{-1})	PDI [c]	conv. (%)
1	P(PLG$_{14}$-r-PLL$_{72}$)	19/81/1	26.5	23.1	30.7	1.23	100[d], 100[e]
2	P(PLG$_{17}$-r-PLL$_{69}$)	20/80/1	26.4	22.7	35.4	1.39	100[d], 100[e]
3	P(PLG$_{45}$-r-PLL$_{47}$)	49/51/1	22.9	21.1	34.4	1.32	100[d], 100[e]
4	P(PLG$_{70}$-r-PLL$_{18}$)	79/21/1	18.0	16.9	28.4	1.24	98[d], 84[e]

[a.] determined by ^{1}H NMR spectroscopy; [b.] theoretical molecular weight based on single-site initiation; [c.] determined by SEC-DRI in 0.1M LiBr/DMF using polystyrene standards; [d.] conversion for [M_1] determined by ^{1}H NMR spectroscopy; [e.] conversion for [M_2] determined by ^{1}H NMR spectroscopy.

and molecular weight distribution (PDI) were determined by size exclusion chromatography (SEC-DRI) technique. All samples regardless of the polymer composition exhibited mono-modal SEC chromatograms and eluted out at approximately the same elution time, indicating similar hydrodynamic sizes of the polypeptide copolymers. The molecular weight distributions (PDI) are modest in the 1.2 - 1.4 range. There is a small peak at the long elution time, suggesting the presence of low molecular weight species (Figure 2.5) whose origin is presently unclear. The low molecular weight species can be removed after purification, as evidenced in SEC-DRI (Figure 2.6). In addition, a shoulder peak appearing at high molecular weight region in the SEC chromatogram of the purified polymer was not observed in the SEC trace of the polymerization reaction mixture. It is attributed to partial solubilisation of polymers after they are completed dried during the workup.

The water solubility of the polypeptides is controlled by the copolymer composition; higher molar fraction of PPLL enhances water solubility, while higher PPLG content lowers the copolymer solubility in water. The P(PLG$_m$-r-PLL$_n$) copolymers with up to 50 mol% PPLG are found to readily dissolve in water (up to around 5.0 mg/mL at 25 C). A further increase of PPLG
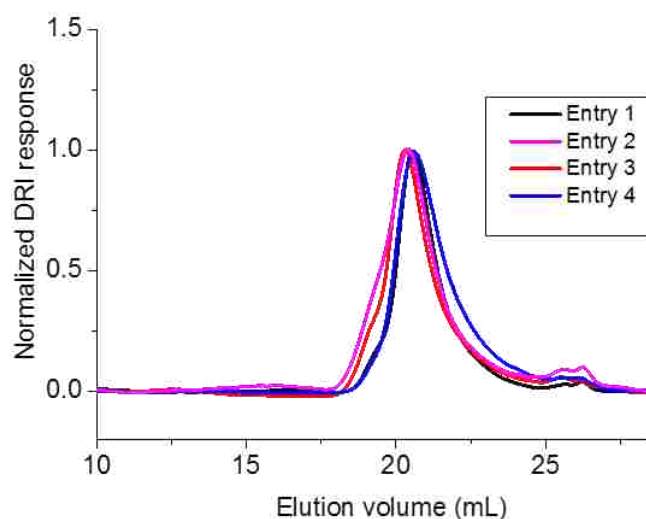
Figure 2.5. SEC-DRI chromatograms of P(PLG$_m$-r-PLL$_n$) polymers (Entries 1-4, Table 2.1) in 0.1 M LiBr/DMF. (Note: the polymerization reaction mixtures were analyzed directly by SEC-DRI after high conversions were reached).
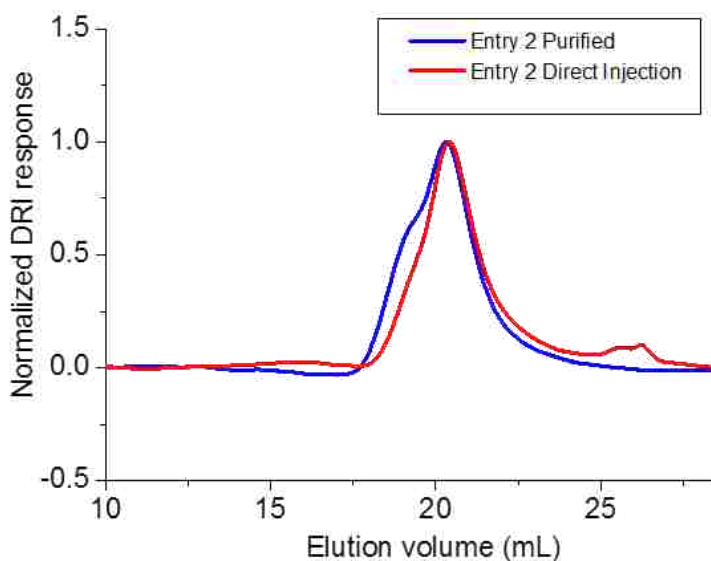
Figure 2.6. SEC-DRI chromatograms of the P(PLG$_{17}$-r-PLL$_{69}$) copolymers (Entry 2, Table 2.1) in 0.1 M LiBr/DMF.

content to 80 mol% results in polypeptides with negligible water solubility (Entry 4, Table 2.1).

The polymers were also characterized by CD and FTIR spectroscopy to verify the secondary

structures in aqueous solution and solid state, respectively. CD spectra of all polypeptide samples

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at varying concentrations (0.2-1.0 mg/mL) in water revealed two negative minima at 209 and 222 nm as well as a positive maximum at 190 nm, indicative of primarily *α*-helical conformations for the polypeptide backbones (Figure 2.7, 2.8 and 2.9). Further spectral analysis
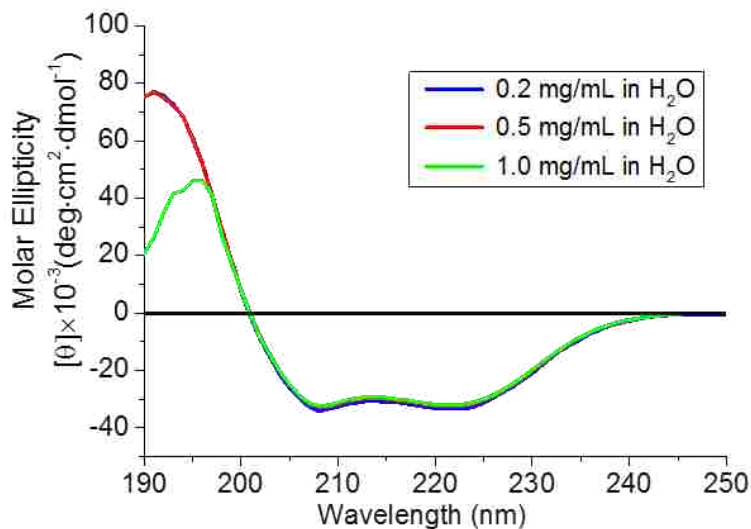
Figure 2.7. CD spectra of P(PLG$_{14}$-r-PPL$_{72}$) copolymer at different concentrations in H$_2$O.
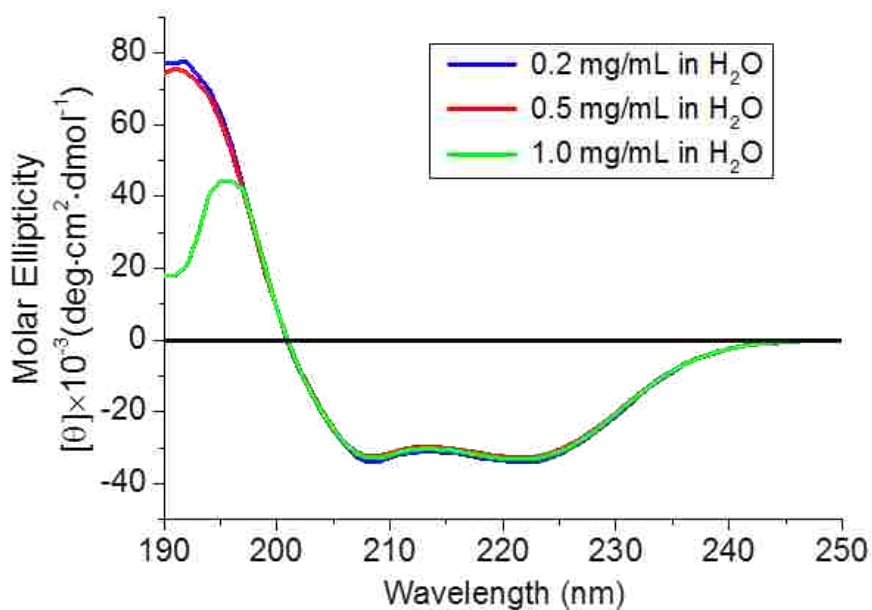
Figure 2.8. CD spectra of the P(PLG$_{17}$-r-PPL$_{69}$) copolymer at different concentrations in H$_2$O.
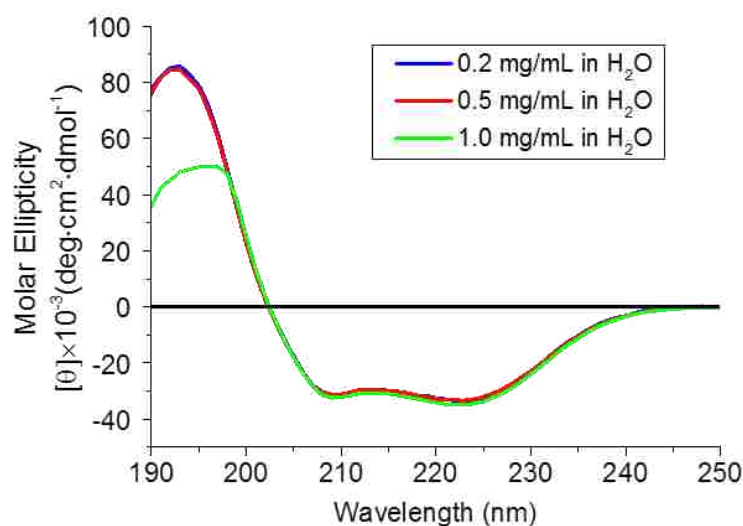
Figure 2.9. CD spectra of the P(PLG$_{45}$-r-PPL$_{47}$) copolymer at different concentrations in H$_2$O.

by DICHROWEB using Contin-LL revealed that the all polypeptides have significant level of helical conformations in the 84-95% range (Table 2.2).[132,133] In the solid state, the polymers also retain α-helical conformations, evidenced by the strong characteristic amide I (1650 cm^{-1}) and amide II (1536 cm^{-1}) peaks in the FTIR spectrum (Figure 2.10).[134]
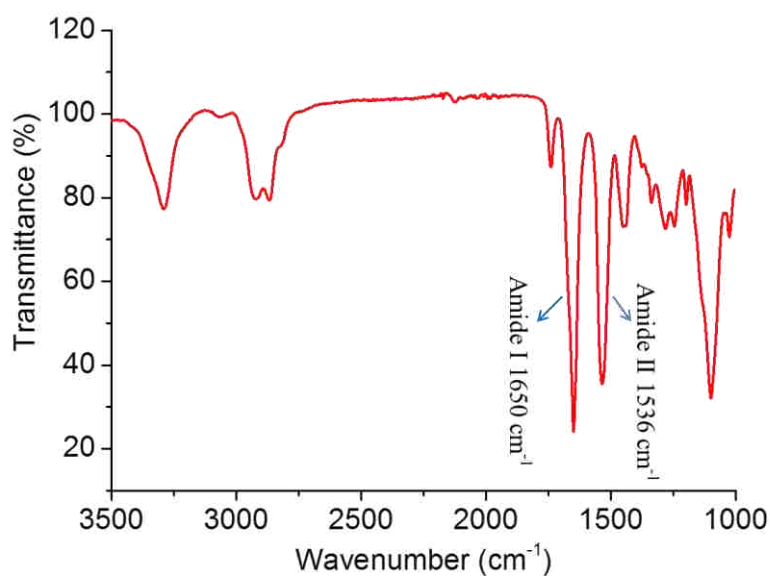
Figure 2.10. FTIR spectrum of P(PLG$_{17}$-r-PPL$_{69}$)

Table 2.2. Secondary conformation analysis of polypeptides using Dichroweb.[133,135]

Polymer composition	α-helix	β-strand	turn
P(PLG$_{14}$-r-PPL$_{72}$)	89.0%	3.7%	7.3%
P(PLG$_{17}$-r-PPL$_{69}$)	84.5%	4.1%	11.4%
P(PLG$_{45}$-r-PPL$_{47}$)	94.2%	1%	4.7%
P((PLG$_{17}$-g-Oct)-r-PPL$_{69}$)	92.6%	1.8%	5.6%
P((PLG$_{17}$-g-Bn)-r-PPL$_{69}$)	83.7%	3.1%	13.1%
P((PLG$_{14}$-g-GRGDS$_8$)-r-PPL$_{72}$)	99.1%	0.8%	0%

2.3.3 P(PLG$_m$-r-PLL$_n$) derivatization through CuAAC

To demonstrate that the water-soluble P(PLG$_m$-r-PLL$_n$) polypeptides can be further modified with hydrophobic moieties by CuAAC chemistry to afford water-soluble conjugates, P(PLG$_m$-r-PLL$_n$) was treated with 1.3 equivalents of octyl azide or benzyl azide in the presence of copper wire[135] and 1.3 eqv. of PMDETA ligand at 50 °C in DMF for 24 h. $^1$H NMR spectra of the resulting polymers [denoted as P((PLG$_m$-g-Oct)-r-PPL$_n$) and P((PLG$_m$-g-Bn)-r-PPL$_n$)] revealed a new peak at 8.1 ppm (d', Figure 2.11), which is characteristic of the triazole proton formed from the CuAAC reaction. The grafting of the hydrophobic moieties is quantitative (Figure 2.11), as evidenced by the complete disapperance of the methylene protons due to non-modified PPLG segment (c, Figure 2.11) at 4.8 ppm and the appearance of the same protons at 5.1 ppm (c', Figure 2.11) resulted from the successful formation of the triazole group. The ^{13}C{H} NMR of the conjugate is shown in Figure S2.4. These reactions suggests that P(PLG$_m$-r-PLL$_n$) copolymers can be grafted with different hydrophobic molecules for targeted applications.

For hydrophobically modified polypeptides, it is important to assess the water solubility of the resulting conjugates. It was found that both P((PLG$_{17}$-g-Oct)-r-PPL$_{69}$) and P((PLG$_{17}$-g-Bn)-r-PPL$_{69}$) can be dissolved in water at concentrations up to 2.0 mg·mL^{-1}. This indicates that
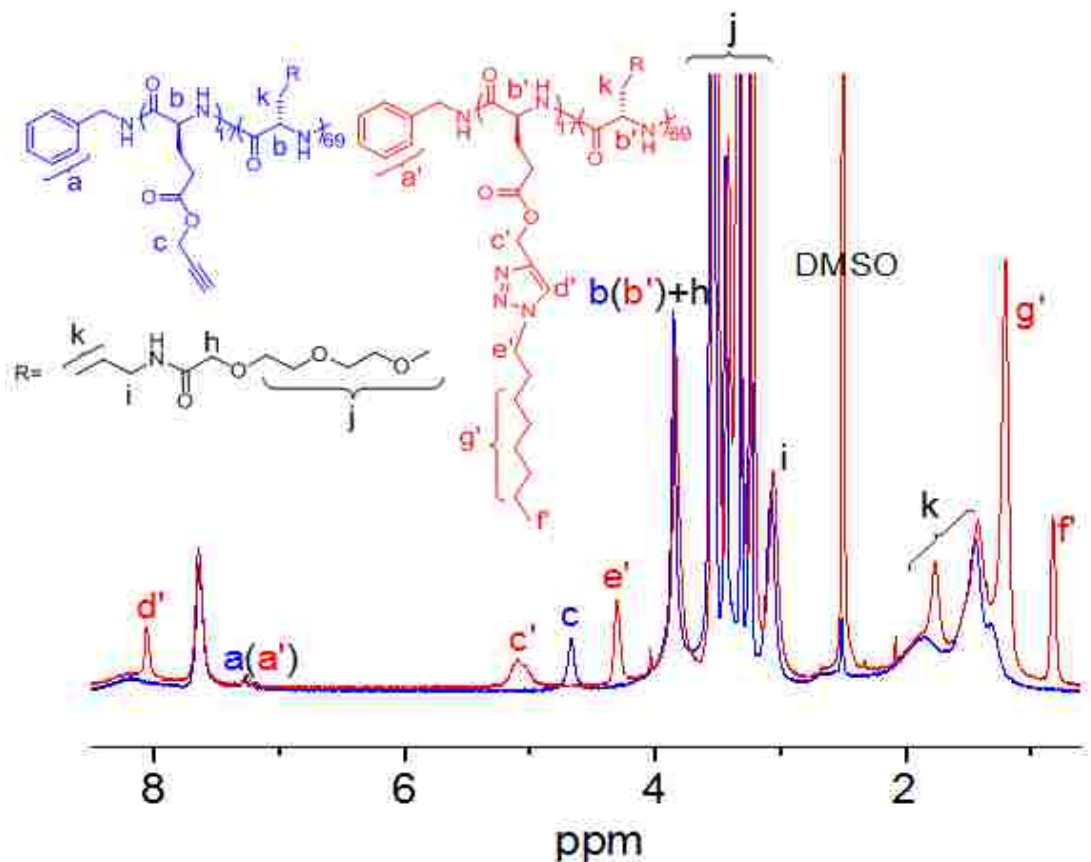
Figure 2.11. ^{1}H NMR spectra of P(PLG$_{17}$-r-PPL$_{69}$) and P((PLG$_{17}$-g-Oct)-r-PPL$_{69}$) in DMSO-d$_6$.

conjugation to P(PLG$_m$-r-PLL$_n$) polymer is effective in enhancing the water solubility of small hydrophobic molecules to a significant concentration. Dynamic light scattering analysis of a P((PLG$_{17}$-g-Oct)-r-PPL$_{69}$) solution in water revealed a mono-modal particle size distribution with a hydrodynamic diameter of 18.8 nm (PDI = 0.205) for the freshly prepared sample (Figure 2.12). It is consistent with the calculated end-to-end for P((PLG$_{17}$-g-Oct)-r-PPL$_{69}$) polymer (12.9 nm), assuming the polymer adopting a helical rod conformation. After the sample was left to stand for 1 d, the hydrodynamic diameter is 19.60 nm (PDI = 0.173). This indicates the particle size is fairly stable. CD analysis of the resulting conjugates revealed that both P((PLG$_{17}$-g-Oct)-r-PPL$_{69}$) CD analysis of the resulting conjugates revealed that both P((PLG$_{17}$-g-Oct)-r-PPL$_{69}$) and P((PLG$_{17}$-g-Bn)-r-PPL$_{69}$) retain α-helical conformations with residual molar ellipticities

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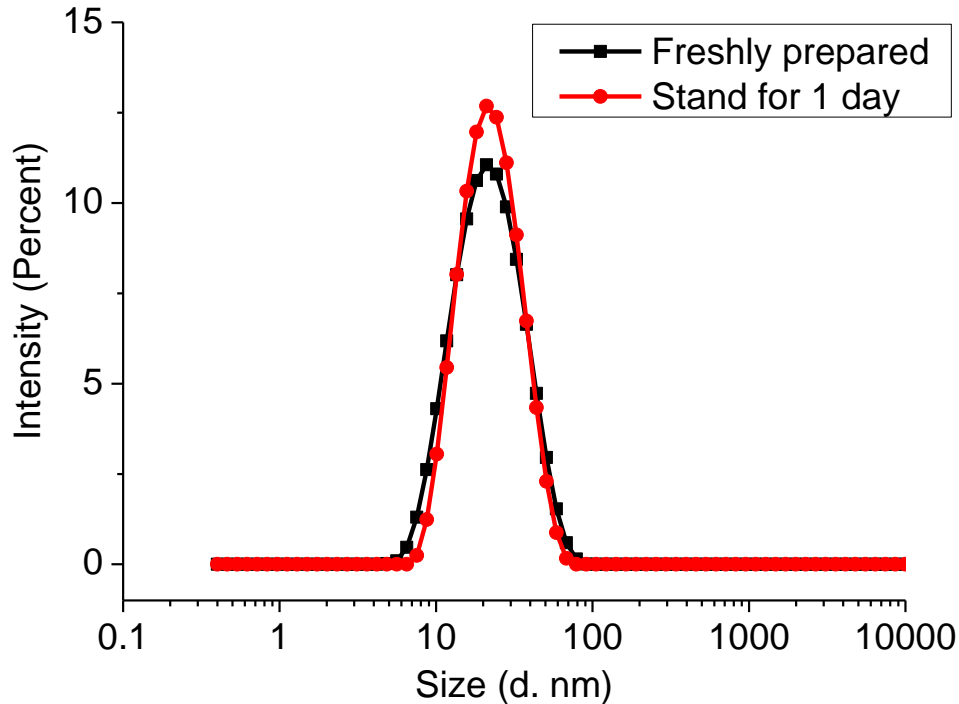
Figure 2.12. Size distribution of P((PLG$_{17}$-g-Oct)-r-PPL$_{69}$) in Milli-Q water (0.5 mg/mL) measured by DLS.

comparable to that of the parent polypeptide in water in the 0.2–1.0 mg·mL^{-1} concentration range (Figures 2.13 and 2.14). The noisy signal below 200 nm for 1.0 mg/mL samples are due to the detection limit of the instrument. Grafting of octyl and benzyl side groups do not appear to disrupt the backbone conformations, as the helical content of the hydrophobically modified polypeptides remains high (93% and 84% respectively) as compared to that of the parent polypeptide (85%) (Table 2.2). The non-disturbed helical conformations are desirable for many biomedical applications such as multivalent ligand platform, where the grafted hydrophobic moieties on the helical surface can be more readily accessed than the random-coil counterpart.

To further demonstrate that the sidechain grafted polypeptides are effective carriers for biologically active moities, an azido-terminated GRGDS pentapeptide which is known to induce integrin-mediated cell adhesion[136] was conjugated to the polypeptide P(PLG$_{14}$-r-PLL$_{72}$) by
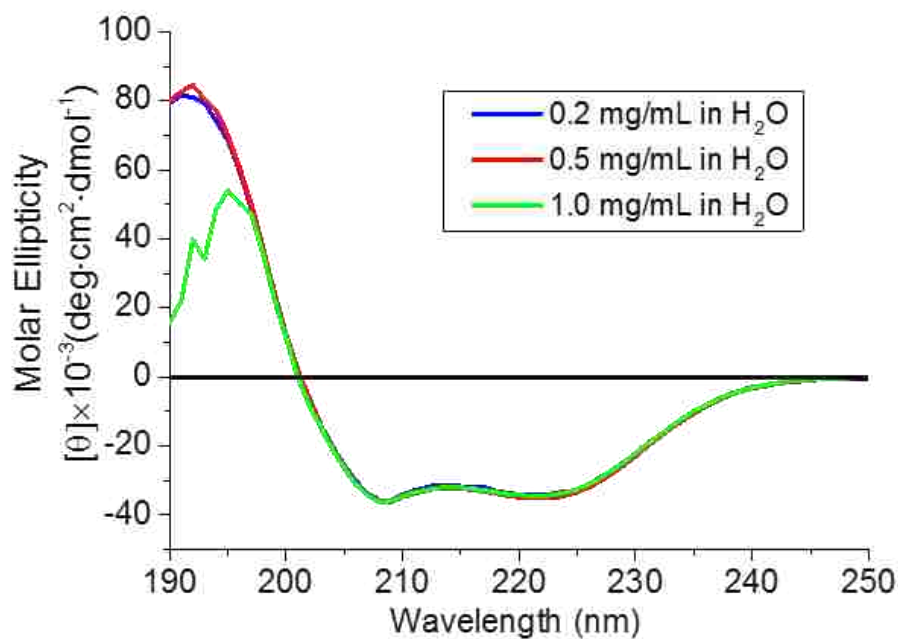
Figure 2.13. CD spectra of the P((PLG$_{17}$-g-Oct)-r-PPL$_{69}$) copolymer at different concentrations in H$_2$O.
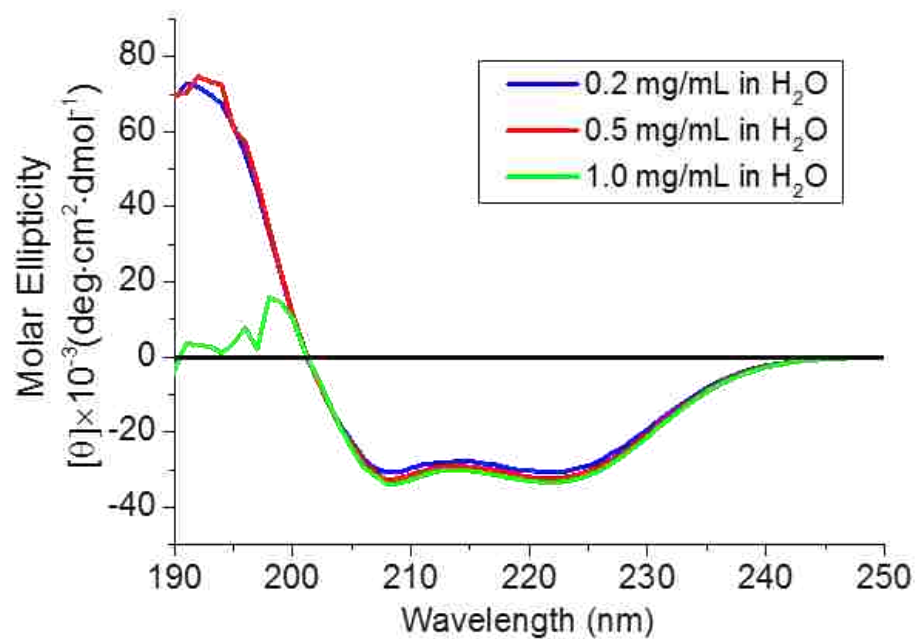
Figure 2.14. CD spectra of the P((PLG$_{17}$-g-Bn)-r-PPL$_{69}$) copolymer at different concentrations in H$_2$O.

CuAAC chemistry. The conjugation yielded a GRGDS-polypeptide conjugate P((PLG$_{14}$-GRGDS$_8$)-r-PLL$_{72}$) with 9 mol% GRGDS sites along the helical backbone (Figure 2.15). CD analysis indicates the conjugate retains high level of helical conformation (99%) in aqueous solution (Figure 2.16 and Table 2.2). Preliminary cell adhsion studies (Figure 2.17) revealed that GRGDS-polypeptide conjugate can promote the adhesion of Chinese hamster ovary (CHO) cells,
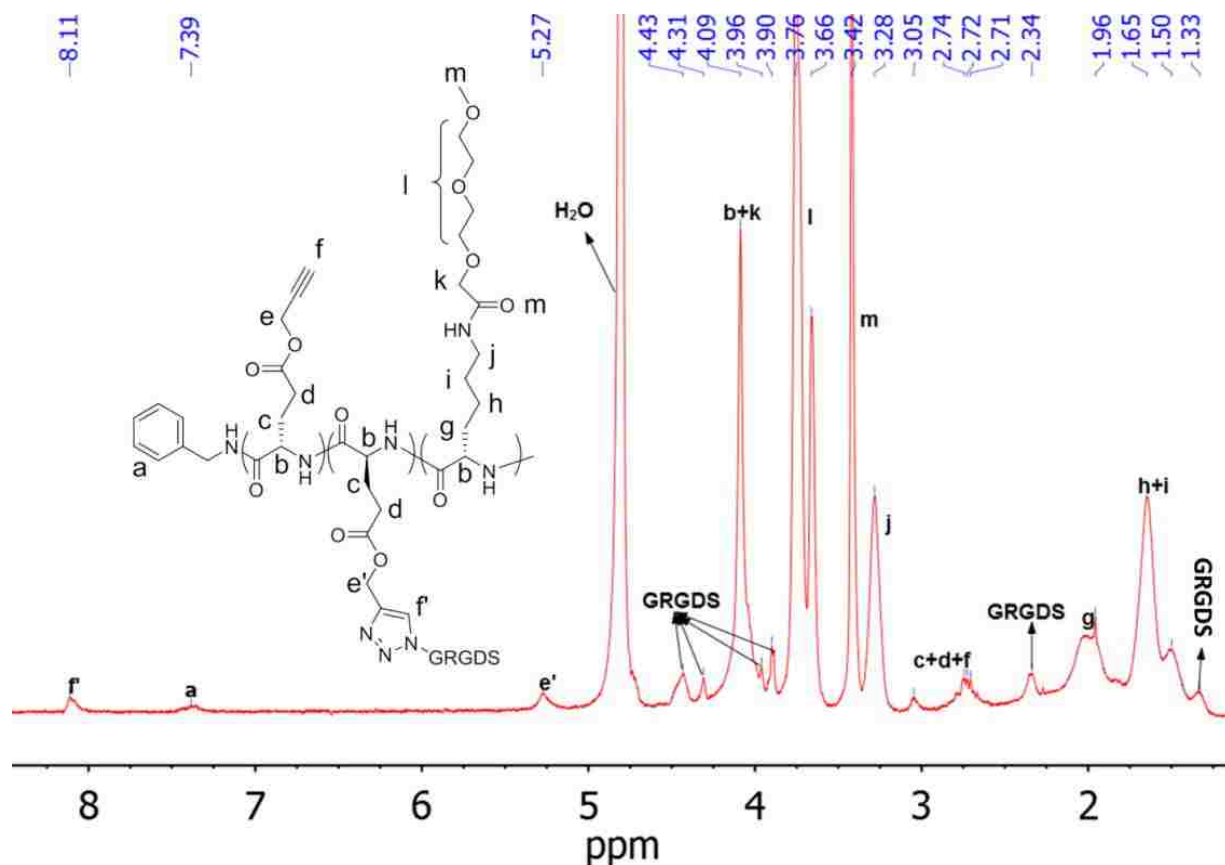
Figure 2.15. ^{1}H NMR spectrum of P((PLG$_{14}$-g-GRGDS$_8$)-r-PPL$_{72}$) in D$_2$O. (Note: the GRGDS composition in the conjugate is calculated based on the ratio of the triazole proton **f'** relative methylene protons of PPLL segment **j**)

though less effective than the natural ligand fibrinogen. By contrast, the parent polypeptide without GRGDS was unable to promote any cell adhesion. This result suggests that GRGDS moieties on the helical polypeptide side chains are not cloaked by the oligo(ethylene glycol) side chains and can be accessed by the cell membrane-bound receptors, resulting in cell adhesion.
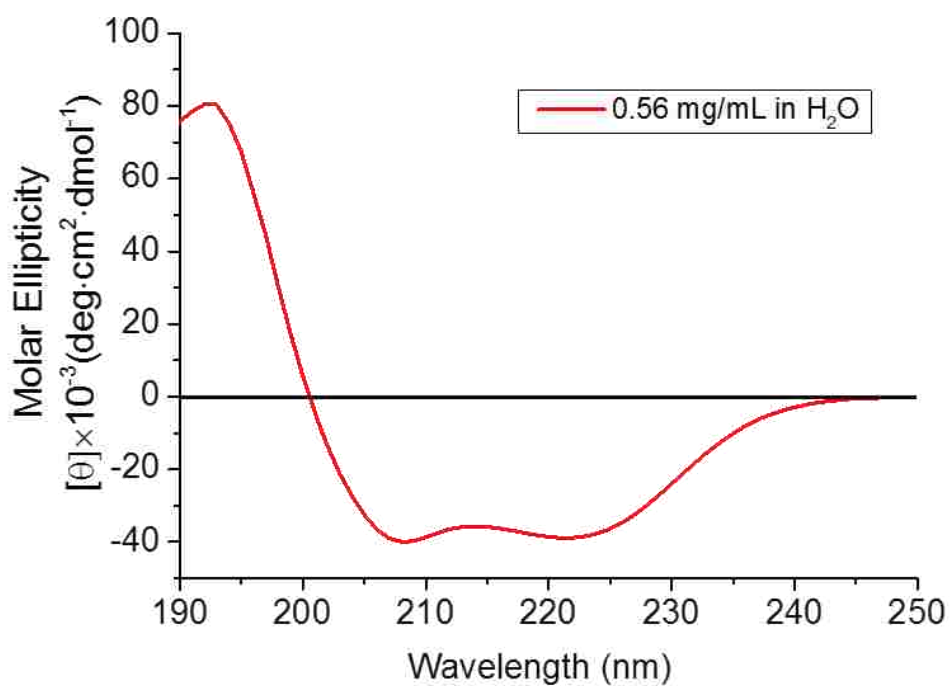
Figure 2.16. CD spectrum of the P((PLG$_{14}$-g-GRGDS$_8$)-r-PPL$_{72}$) copolymer in H$_2$O.
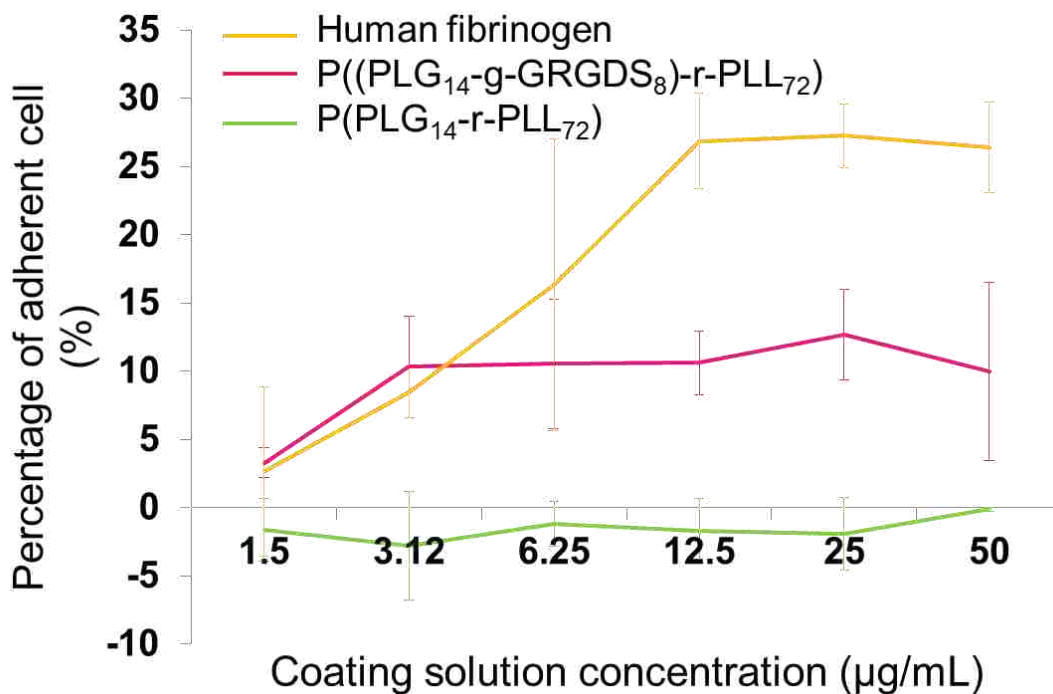
Figure 2.17. Number percentage of adhered cells versus the concentration of P((PLG$_{14}$-g-GRGDS$_8$)-r-PPL$_{72}$), fibrinogen and P(PLG$_{14}$-r-PPL$_{72}$) used in the coating of the plates.

2.3.4 Preliminary polymerization study of AEG$_2$ Ser NCA

The AEG$_2$ Ser NCA was initially polymerized in DMF with benzylamine as initiator (Table 2.3). Different initial monomer to initiator ratios were set up (e.g. 50, 100, 150). 100% NCA monomer conversions were reached for all polymerizations within 2.5 days. The polymerization solutions were subjected to SEC characterization. SEC indicates formation of polypeptides (Figure S2.5). However, the polypeptides prepared from different initial monomer to initiator ratios have similar molecular weights as evidenced by similar elution times. Even though the M_n control of benzylamine initiated ROP of AEG$_2$ Ser NCA remains poor at this point, the polypeptide preparation from AEG$_2$ Ser NCA has been successfully demonstrated. The control may be improved once the NCA preparation condition is optimized, which is the future direction of this topic.

Table 2.3. Polymerization results of Benzylamine initiated ROP of AEG$_2$ Ser NCA in DMF at room temperature.

| $[M]_0/[I]_0$	$[M]_0$	Relative M_n	PDI	Conversion
50	0.5 M	4.2 kg/mol	1.41	100%
100	0.5 M	3.9 kg/mol	1.40	100%
150	0.5 M	4.2 kg/mol	1.29	100%

[a.]determined by SEC-DRI in 0.1M LiBr/DMF using polystyrene standards; [b.]determined by ^{1}H NMR.

2.4 Conclusions

In conclusion, we have demonstrated a successful synthetic route towards a new class of non-ionic water-soluble "clickable" helical polypeptides by organo-mediated copolymerization of discrete NCA monomers. The polypeptides maintain stable α-helical conformations in aqueous solution and in solid state. The copolymerization strategy produces polypeptides having tunable densities of "clickable" sites which allow for further conjugation of hydrophobic or hydrophilic moieties. It was shown that the hydrophobically and hydrophilically-modified polypeptide conjugates retain high level of α-helical conformations. The water solubility of the

conjugates is strongly dependent on the relative hydrophobic and hydrophilic content in the conjugates. We envision the potential use of the helical polypeptides as multivalent ligand scaffolds. The helical polypeptides are expected to offer more efficient ligand display and reduced entropy penalty upon multivalent binding relative to the random coil counterparts.

In addition, the synthesis of a new NCA monomer AEG_2 Ser NCA was also explored. The AEG_2 Ser NCA combines oligomeric ethylene glycol unit as well as azido on the side chain, which can afford clickable water-soluble polypeptide upon polymerization. Further investigation is required to optimize the monomer preparation and subsequent polymerization study.

2.5 Supplemental data

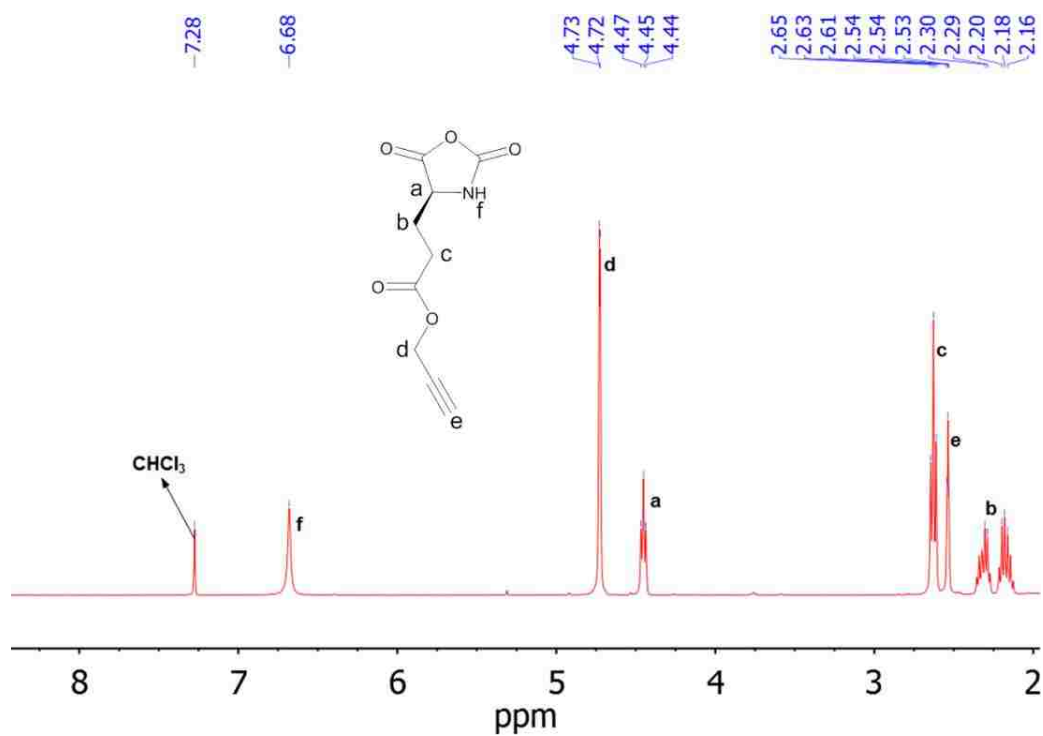Figure S2.1. ^{1}H NMR spectrum of PLG NCA in CDCl$_3$.
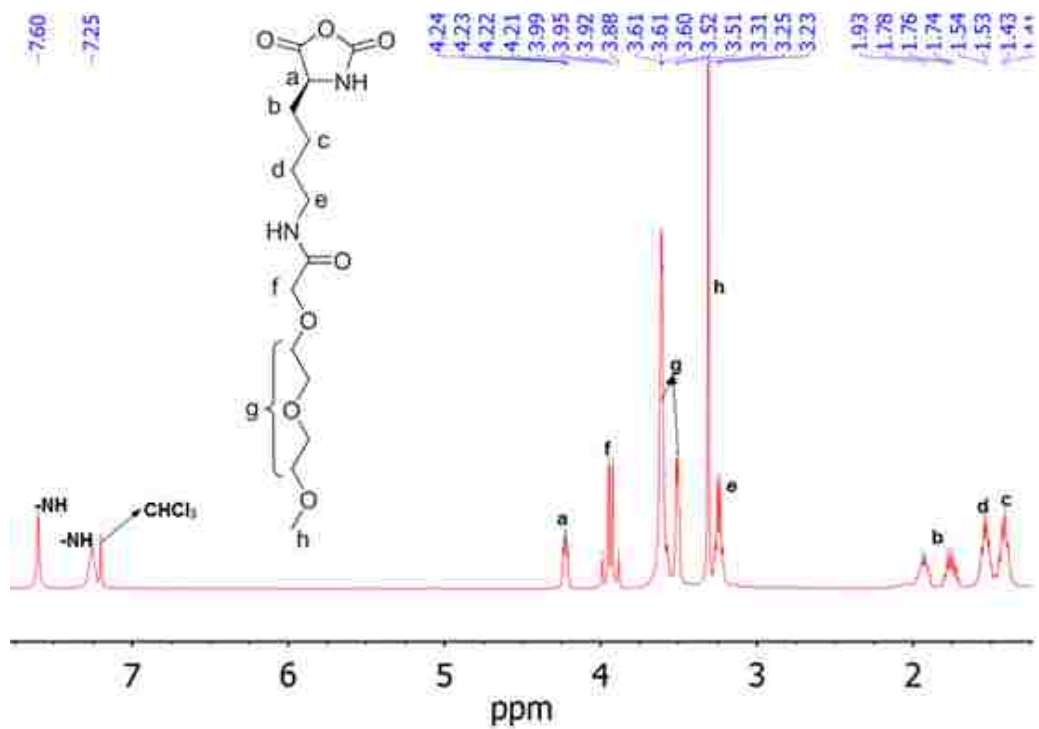
Figure S2.2. ^{1}H NMR spectrum of EG$_2$ LYS NCA in CDCl$_3$.
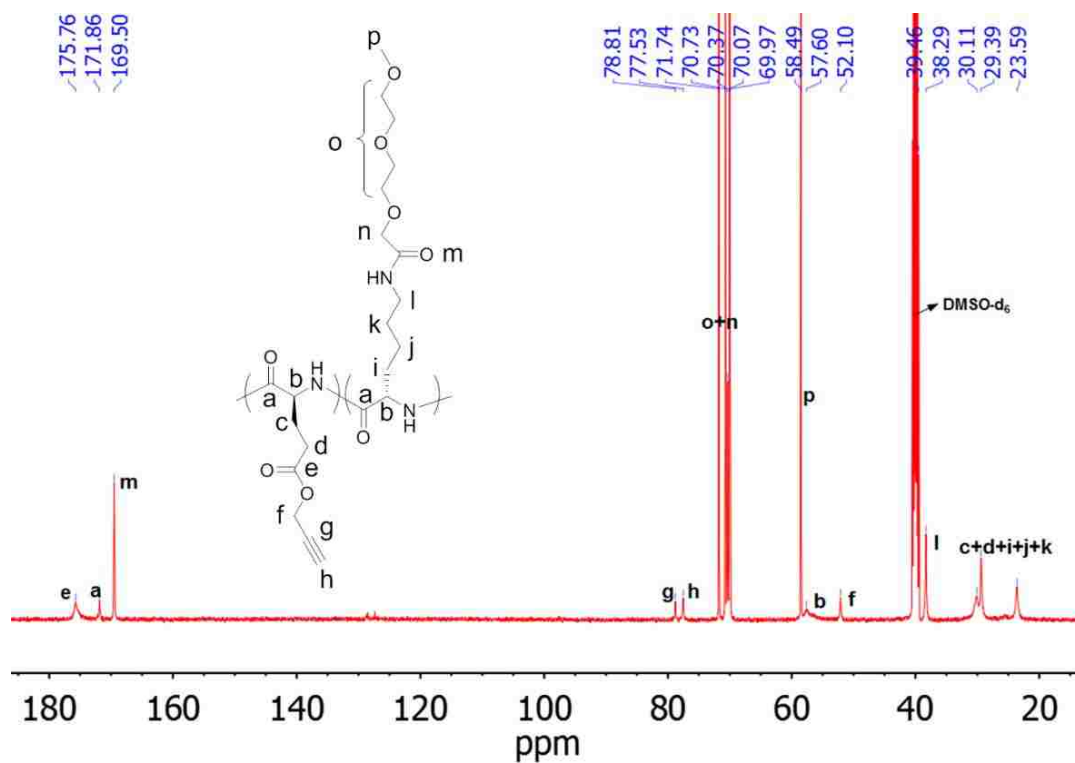
Figure S2.3. ^{13}C{H} NMR spectrum of the P(PLG$_{17}$-r-PLL$_{69}$) copolymer in DMSO-d$_6$.
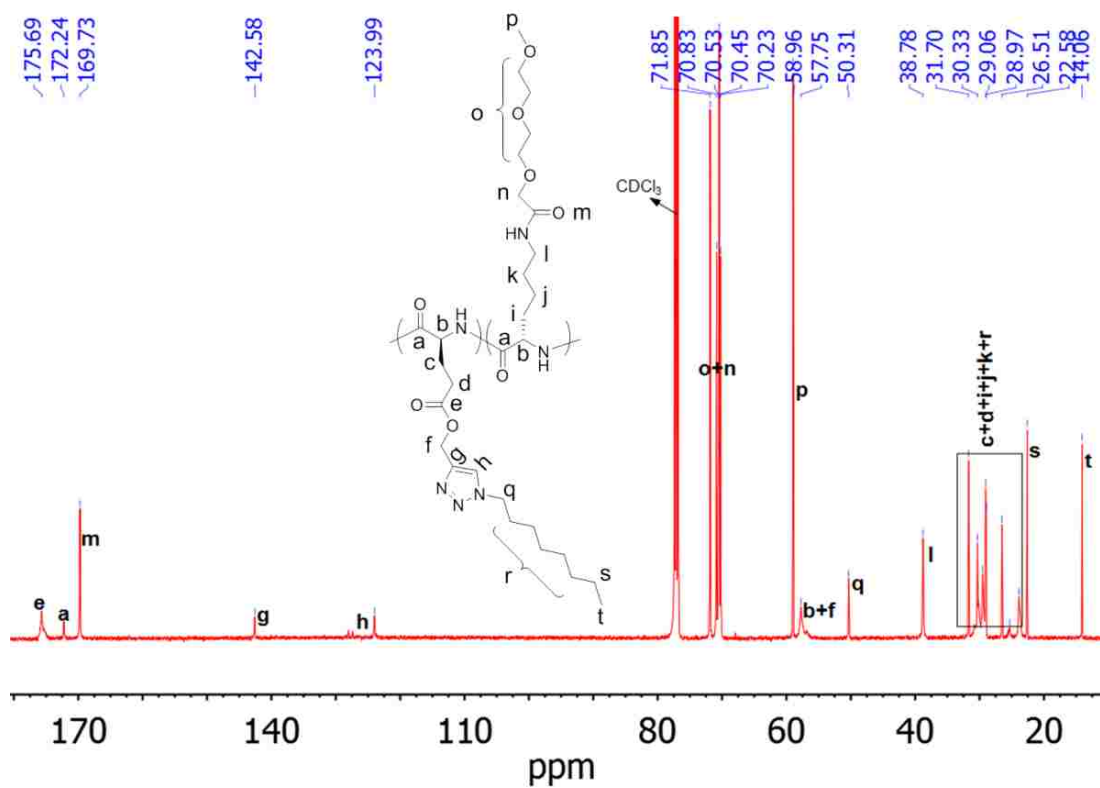
Figure S2.4. ^{13}C{H} NMR spectrum of the P((PLG$_m$-g-Oct)-r-PPL$_n$) copolymer in CDCl$_3$.
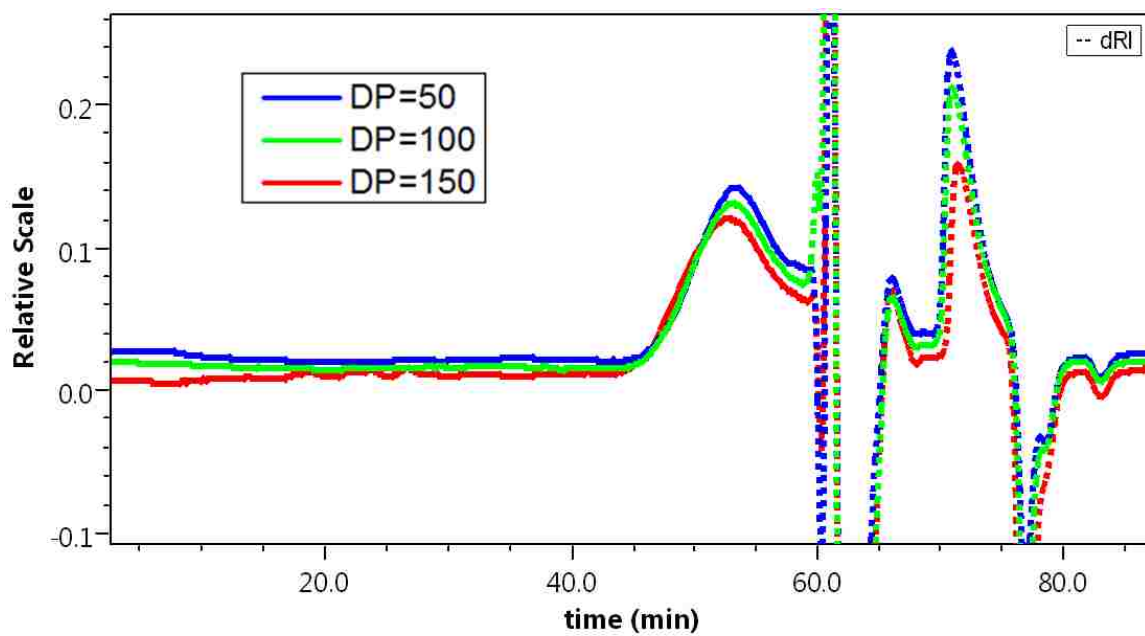
Figure S2.5. SEC-DRI chromatograms of the poly(AEG$_2$ Ser) in 0.1 M LiBr/DMF.

CHAPTER 3. SYNTHESIS OF POLYPEPTIDES THROUGH SOLID-PHASE RING-OPENING POLYMERIZATION OF *N*-THIOCARBOXYANHYDROSULFIDES

3.1 Introduction

Polypeptides have been increasingly investigated for various biomedical and biotechnological applications (e.g., gene delivery, drug delivery, tissue engineering, etc.) due to a combination of favorable attributes such as structural tunability, backbone degradability, and low cytotoxicity.[4,5,134] Due to the availability of a large variety of natural and synthetic amino acids, the molecular characteristics and backbone conformations of polypeptides can be readily adjusted by controlling the sidechains to meet the needs of various technical applications.

Polypeptides are typically prepared by the ROP of amino-acid derived NCAs in solution phase[17-21,29,30,33,34,73] and occasionally in the solid phase.[137-144] A variety of initiating systems have been shown to mediate the controlled polymerization of various NCAs to produce well-defined polypeptides having diverse structures.[17-21,29,30,33,34] However, NCA monomers are moisture sensitive[143] and thermally unstable[145] resulting in poor shelf-life. Anhydrous conditions or low temperature is required during the synthesis, purification, and storage of NCA monomers. For example, dry flash chromatography in the glovebox using anhydrous silica gel and solvents was developed to purify NCA monomers due to their moisture instability.[14] In addition, highly toxic phosgene or phosgene-derived reagents are required in the synthesis of NCA monomers. Endo et al have reported the synthesis of polypeptides using activated amino acids urethane derivatives which was proposed to form NCAs in situ and polymerize in a chain growth fashion.[56] However, the polypeptides are mostly limited to low to moderate molecular weight (DP<100). Furthermore, all polymerizations of pre-formed or in situ generated NCA monomers require anhydrous conditions, limiting the broad use of polypeptides by researchers who are not

equipped with advanced synthetic facilities. There is a clear need to further develop alternative and robust synthetic approach to access well-defined polypeptides with diverse structures.

Amino-acid derived NTAs, thio-analogues of the corresponding NCAs, were reported to be more stable and less reactive towards polymerization than the corresponding NCAs.[16,67] Kricheldorf et al first investigated the polymerization of Gly NTA, D,L-Phe NTA, D,L-Leu NTA, in solution using primary amine initiators.[66,67] All reactions resulted in low conversions even after 2 days at 20 ℃ and 60 ℃, yielding oligopeptide products. The origin of the low conversions remains puzzling particularly in view of the predominant primary amine chain ends revealed by end-group analysis. We speculate that the low conversions may result from the restricted access of chain ends due to the polymer aggregation in solution, thus hindering further chain growth. Ling et al recently reported that ROPs of sarcosine-derived NTAs (i.e., N-methyl NTA) using rare earth borohydride or primary amine initiators in solution can produce polysarcosine in a controlled manner.[68,69] But no other amino acid-derived NTAs bearing N-proton were investigated.

In this work, we report a study on the solid-phase polymerization of γ-benzyl-L-glutamate (BLG), ε-Cbz-lysine (LYS) and D,L-Methionine derived NTAs (BLG NTA, LYS NTA and MET NTA) (Scheme 3.1). Solid-phase polymerization of these NTAs using primary amine initiator produce well-defined polypeptides with controlled molecular weight (M_n) and low-to-moderate molecular weight distribution (PDI), whereas the solution phase polymerization is slow and resulted in poor conversions. The enhanced polymerization activity in the solid phase is attributed to the high local monomer concentration. The sROP was shown to operate by the NAM as evidenced by end-group analysis. In addition, sROP of NTAs can be conducted in open

air without significant change of the M_n and PDI control from those conducted under air-free conditions.

Scheme 3.1. Synthetic route towards polypeptides in sROP and representative picture of sROP.
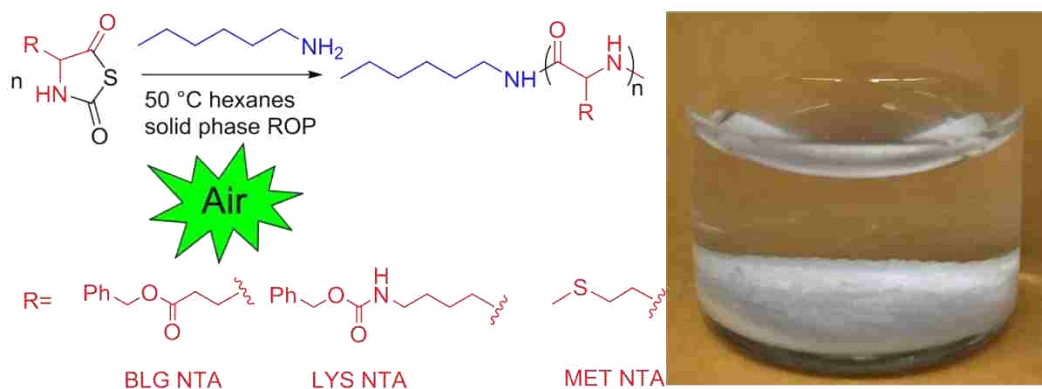
3.2 Materials and Methods

3.2.1 General

All chemicals were purchased from Sigma-Aldrich and used as received unless specified. L-glutamic acid γ-benzyl ester (H-Glu(OBz)-OH) and ε-*N*-carbobenzyloxy-L-lysine (H-Lys(Z)-OH) were purchased from AAPPTec, LLC and used as received. BLG NCA was synthesized by using a published procedure.[52] D,L-Methionine NTA was prepared similarly to LYS NTA's PCl$_3$ route by collaborator Brandon A. Chan. All solvents are regular ACS grade solvents and used directly in the reactions without any special drying or purification step unless specified. All reactions are conducted in open air unless otherwise noted.

^{1}H and ^{13}C{H} NMR spectra were recorded on a Bruker AV-400 or AV-500 spectrometer. Chemical shifts in ppm were referenced relative to proton impurities or ^{13}C isotope of deuterated solvents (e.g., CDCl$_3$). SEC-DRI analyses were performed with an Agilent 1200 system equipped with three Phenomenex 5 μm, 300 × 7.8 mm columns [100 Å, 1000 Å and Linear(2)], Wyatt DAWN EOS MALS detector (GaAs 30 mW laser at λ=690 nm) and Wyatt Optilab rEX DRI detector with a 690 nm light source. DMF containing 0.1 M LiBr was used as

the eluent at a flow rate of 0.5 mL min^{-1}. The temperature of the column and detector was 25 ℃. FTIR spectra were collected on a Bruker Alpha FTIR spectrometer. Electrospray ionization mass spectroscopy (ESI MS) was conducted on an ESI TOF 6210 (Electrospray Time-of-Flight) mass spectrometer (Agilent Technologies). Samples were prepared by dissolving 5 mg sample in 0.5 mL chloroform. The experiments were carried out in positive mode ionization. MLADI-TOF MS experiments was conducted on a Bruker UltrafleXtreme tandem time-of-flight (TOF) mass spectrometer. The instrument was calibrated with Peptide Calibration Standard II consisting of standard peptides Angiotensin I, Angiotensin II, Substance P, Bombesin, ACTH clip 1-17, ACTH clip 18-39, and Somatoratin 28 (Bruker Daltonics, Billerica, MA) prior to experiment. A saturated methanol solution of α-cyano-4-hydroxycinnamic acid was used as matrix. Samples were prepared by mixing 5 mg/ml THF solution of polymers with matrix at 1:1 volume ratio, which were then deposited onto a 384-well ground-steel sample plate using the dry droplet method. Experiments were done in positive reflector mode. The data analysis was performed with flexAnalysis software. Structures of BLG NTA were determined from data collected at T= 90 K with MoKα radiation on a Bruker Kappa Apex-II diffractometer equipped with a Triumph focusing monochromator. The racemic form of BLG NTA, space group P2$_1$/c, exhibited a disorder with the two enantiomeric molecules overlapped with 0.9509:0.0491(14) populations. CIFs have been deposited at the Cambridge Crystallographic Data Centre, CCDC 1479403. The structure of the S form of LSY NTA, space group P2$_1$, was determined from data collected at T= 200 K with MoKα radiation on a Nonius KappaCCD diffractometer. A phase change with twinning occurs below about 180K, so data were collected from a single crystal at a higher temperature. The asymmetric unit has two independent molecules, and no indication of the presence of the R enantiomer was evident. The absolute configuration was confirmed, with Flack

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parameter x=0.12(9). CIFs have been deposited at the Cambridge Crystallographic Data Centre, CCDC 1479404. The structure of the S form of Leu NTA, space group $P2_12_12$, was determined from data collected at T= 90 K with MoKα radiation on a Bruker Kappa Apex-II diffractometer. The asymmetric unit has two independent molecules, which form a hydrogen-bonded dimer. The absolute configuration was confirmed, with Flack parameter x=0.13(7) and no indication of the presence of the R enantiomer was evident. The CIF has been deposited at the Cambridge Crystallographic Data Centre, CCDC 1487624. Single crystals of the NTA monomers for the X-ray structure determination was prepared by slow solvent evaporation of solvent from a chloroform solution of the monomers. TGA experiments were carried on TA 2950 TGA under nitrogen atmosphere with a heating rate of 10 °C/min. The scanned temperature range was rt.~600 °C. Data was analyzed with Thermal Advantage Software. The X-ray powder diffraction (XRD) measurements were performed on in-house PANanalytical Empyrean instrument, using the characteristic X-ray of Cu target. The range of scattering angle covers from 4 degree up to 90 degree. The correction to a fixed slit was done before the analysis of spectrum. The samples were ground to powder and uniformly distributed on a zero-background silicon wafer for the measurements. The background of the silicon wafer was also measured and subtracted from the spectra before further analysis to obtain the crystallinity. After subtracting the background, in a spectrum, the scattering contribution of amorphous structure is determined by fitting the selected data points connecting the bottom of peaks to describe the overall shape of the amorphous region, using spline interpolation. The crystallinity value is calculated as the ratio of peak area above the baseline, which corresponds to the crystalline contribution, to the total area, which is the sum of the peak area and the area below the baseline for amorphous region.[146] The corresponding spacing for 2θ is calculated based on Bragg's law with n = 1. The wavelength

of the X-ray is 1.54 Å. HPLC analyses were conducted on Dionex Ultimate 3000 system equipped with Chiralcel column (OD-H 0.46 cm $\times$ 25 cm). The elution solvent consists of 85% hexanes+15% isopropanol. The NTA samples for HPLC were prepared by dissolving NTA in 1:1 isopropanol/hexanes to make 2 mg/mL concentration. Prior to eject into HPLC, the samples were filtered with 0.22 μm PTFE filters. The *ee* was determined by subtract the two peaks' area in HPLC.

3.2.2 NTA monomers synthesis

3.2.2.1 Synthesis of *S*-Ethoxythiocarbonyl Mercaptoacetic Acid (XAA)

The synthesis route is modified from a published procedure.[67] NaOH (9.31 g, 23.3 mmol) was first dissolved in chilled DI water (233 mL), followed by addition of chloroacetic acid (22.02 g, 23.30 mmol) to afford a clear solution. Potassium ethyl xanthogenate (37.36 g, 23.31 mmol) was then added to the above solution. The mixture was allowed to stir at room temperature for one day, followed by acidification with 4 M HCl to pH ~ 1. The resulting cloudy mixture was then extracted with chloroform (3 $\times$ 150 mL). The combined organic extract was dried over $MgSO_4$ and concentrated under vacuum. Hexanes (500 mL) was then added to the oily residue with vigorous stirring to afford an off-white solid, which was collected by filtration and washed with hexanes and dried under vacuum to give the final product (38.06 g, 91% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 1.43 (t, 3H, CH_3), 3.97 (s, 2H, CH_2), 4.65 (q, 2H, CH_2).

3.2.2.2 Synthesis of γ-benzyl-L-glutamic acid *N*-thiocarboxyanhydrosulfide (BLG NTA) via the PCl_3 method

H-Glu(OBz)-OH (4.95 g, 20.9 mmol) and XAA (3.76 g, 20.9 mmol) were suspended in a saturated $NaHCO_3$ aqueous solution (70 mL). The suspension was stirred vigorously for 2 days at room temperature to afford a clear solution. The clear solution was then acidified with concentrated HCl to pH ~ 3, followed by extraction with ethyl acetate (3 $\times$ 60 mL). The

combined organic extract was dried with $MgSO_4$ and concentrated under vacuum. The oily residue was then re-dissolved in ethyl acetate (70 mL) under nitrogen, followed by the addition of PCl_3 (2.2 mL, 25 mmol) at room temperature. The reaction mixture was stirred at room temperature for 20 h and then sequentially washed with a saturated $NaHCO_{3 (aq)}$ solution (100 mL), DI water (100 mL) and brine solution (100 mL). The organic phase was separated and dried over anhydrous $MgSO_4$ and concentrated under vacuum to afford light yellow oil. The oil was dissolved in a minimum amount of THF and precipitated into excess hexanes with vigorous stirring to afford an off-white solid (3.24 g). The crude solid product was further purified by flash chromatography with ethyl acetate/hexanes (2:3, v/v) (R_f = 0.33) off a silica gel column. A white solid (2.84 g, 49% yield) was collected after the chromatography purification. HRMS-ESI (m/z): $[M + H]^+$ calculated for $C_{13}H_{14}NO_4S$ 280.0638, found 280.0640. Melting point: 71.6 °C – 72.5 °C. ^1H NMR (400 MHz, $CDCl_3$) δ (ppm): 7.42 (s, 1H, NH), 7.34 (m, 5H, C_6H_5), 5.12 (s, 2H, CH_2), 4.37 (t, 1H, CH), 2.55 (t, 2H, CH_2), 2.10 – 2.27 (m, 2H, CH_2) $^{13}C\{H\}$ NMR (400 MHz, $CDCl_3$) δ (ppm): 198.33, 172.43, 167.10, 135.42, 128.74, 128.57, 128.37, 67.02, 66.09, 29.46, 27.82.

3.2.2.3 Synthesis of ε-*N*-Carbobenzyloxy-L-lysine *N*-thiocarboxyanhydrosulfide (LYS NTA) via the PCl_3 method

NaOH (2.87 g, 71.8 mmol) was dissolved in chilled DI H_2O (120 mL). H-Lys(Z)-OH (10.04 g, 35.8 mmol) and XAA (6.47 g, 35.9 mmol) were sequentially added to the above clear solution to afford a cloudy mixture. The reaction mixture was stirred vigorously for 3 days at room temperature followed by acidification with 4 M HCl to pH ~ 3 and extraction with ethyl acetate (3 × 200 mL). The combined organic extract was dried over anhydrous $MgSO_4$ and then concentrated under vacuum to ~120 mL, to which PCl_3 (3.7 mL, 42 mmol) was added under nitrogen. The reaction was allowed to stir at room temperature for 20 h and then sequentially

washed with a saturated NaHCO$_{3(aq)}$ solution (200 mL), DI water (100 mL) and brine solution (100 mL). The organic phase was separated, dried over anhydrous MgSO$_4$ and concentrated under vacuum to afford a light yellow oil. The oil was re-dissolved in minimum THF and precipitated into excess hexanes under vigorous stirring to afford a white solid (8.26 g, 25.7 mmol, 72% crude yield). A fraction of the crude solid (2.50 g, 7.76 mmol) was further purified by flash chromatography using gradient elution solvent diethyl ether/hexanes (from 4:1 to 10:1, v/v) (R$_f$ = 0.22 in 10:1 diethyl ether/hexanes) off a silica gel column. The final purified product was collected as a white solid (2.08 g, 83% yield) after the chromatography purification. HRMS-ESI (m/z): [M + H]$^+$ calculated for C$_{15}$H$_{19}$N$_2$O$_4$S 323.1060, found 323.1063. Melting point: 104.0 C – 105.1 C. ^{1}H NMR (400 MHz, DMSO-d6) δ (ppm): 9.32 (s, 1H, NH), 7.33 (m, 5H, C$_6$H$_5$), 7.24 (t, 1H, NH), 5.01 (s, 2H, CH$_2$), 4.55 (t, 1H, CH), 3.00 (m, 2H, CH$_2$), 1.72 (m, 2H, CH$_2$), 1.40 (m, 4H, CH$_2$CH$_2$). ^{13}C{H} NMR (500 MHz, CDCl$_3$) δ (ppm): 198.88, 167.38, 156.96, 136.55, 128.69, 128.34, 128.26, 67.03, 66.87, 40.23, 31.94, 29.39, 21.23.

3.2.2.4 Synthesis of BLG NTA via the PBr$_3$ method

The H-Glu(OBz)-OH (9.89 g, 36.2 mmol) and XAA (6.52 g, 36.2 mmol) were used for the coupling reaction following the procedure described in 3.3.3.2. 9.55 g crude product BLG-XAA (compound **1** in Scheme 3.2) was afforded. For the cyclization step, BLG-XAA (4.96g, 15.2 mmol) and imidazole (1.04 g, 15.3 mmol) were first dissolved in THF (31 mL) in ice bath. Nitrogen was purged through the solution for 10 mins before PBr$_3$ (1.71 mL, 18.2 mmol) was dropwise added to the above solution. Ice cold mixture of 50 mL ethyl acetate and 50 mL saturated NaHCO$_3$ (aq) was poured into the reaction after 10 mins stirring. The organic layer was separated from the basic aqueous solution, which was then successively washed with 50 mL cold 1M HCl (aq), 50 mL saturated NaHCO$_3$ (aq) and 50 mL brine. The organic layer was

concentrated down to afford clear oil after being dried over MgSO$_4$ and filtration. The crude oil was then precipitated into excess 400 mL hexanes while stirring to afford white solid after filtration. The white solid was further purified by column chromatography with ethyl acetate/hexanes (2:3, v/v) off a silica gel column. White solid (1.81 g, 6.49 mmol) was collected after the chromatography purification.

Scheme 3.2. Synthetic route towards NTAs.
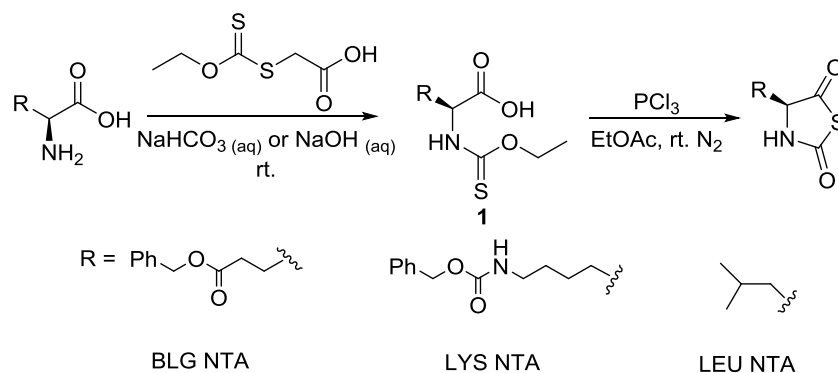
3.2.2.5 Synthesis of LYS NTA via the PBr$_3$ method

H-Lys(Z)-OH (14.44 g, 51.5 mmol), XAA (9.28 g, 51.5 mmol) and NaOH (4.13 g, 103 mmol) were used for the coupling reaction following the procedure described in 3.3.3.3. 16.01 g crude oil product LYS-XAA (compound **1** in Scheme 3.2) was afforded. For the cyclization step, LYS-XAA (6.33 g, 17.2 mmol) and imidazole (1.17 g, 17.2 mmol) were first dissolved in THF (35 mL) in ice bath. Nitrogen was purged through the solution for 10 mins before PBr$_3$ (1.94 mL, 20.6 mmol) was dropwise added to the above solution. Ice cold mixture of 100 mL ethyl acetate and 100 mL saturated sodium bicarbonate aqueous solution was poured into the reaction after 10 mins stirring. The organic layer was separated from the basic aqueous solution, which was then successively washed with 50 mL cold 1M HCl (aq), 50 mL saturated NaHCO$_3$ (aq) and 50 mL brine. The organic layer was concentrated down to afford clear oil after being dried over MgSO$_4$ and filtration. The crude oil was then precipitated into excess 400 mL hexanes while stirring to

afford white solid after filtration. The white solid was further purified by column chromatography with ethyl ether/hexanes (10:1, v/v) off a silica gel column. White solid (2.96 g, 9.19 mmol) was collected after the chromatography purification.

3.2.2.6 Synthesis of L-leucine N-thiocarboxyanhydrosulfide (LEU NTA) via PBr$_3$ method

NaOH (1.72 g, 43.0 mmol) was first dissolved in 72 mL DI water in ice bath. L-Leucine (2.82 g, 21.5 mmol) and XAA (3.87 g, 21.5 mmol) were then added to the clear aqueous solution. Two days later, the clear solution was worked up by acidification with 4 M HCl (aq) to pH ~ 3. Then ethyl acetate (3×200 mL) was used to extract the aqueous solution. The combined organic solution was then washed with brine, dried over MgSO$_4$. Clear oil was afforded after the organic solution was filtered and concentrated under vacuum. The clear oil was used directly for the cyclization step by dissolving in 43 mL THF together with imidazole (1.46 g, 21.5 mmol) in ice bath. Nitrogen was purged through the solution for 10 mins before PBr$_3$ (2.42 mL, 25.8 mmol) was dropwise added to the above solution. Ice cold mixture of 100 mL ethyl acetate and 100 mL saturated sodium bicarbonate aqueous solution was poured into the reaction after 10 mins stirring. The organic layer was separated from the basic aqueous solution, which was then successively washed with 50 mL cold 1M HCl (aq), 50 mL saturated NaHCO$_3$ (aq) and 50 mL brine. The organic layer was concentrated down to afford clear oil after being dried over MgSO$_4$ and filtration. The crude oil was then recrystallized form ethyl acetate/hexanes to afford needle-like solid (2.41 g, 65%).

3.2.3 sROP of NTAs

A representative polymerization was conducted as followed. BLG NTA (52.8 mg, 0.189 mmol) was suspended in hexanes (0.87 mL) in open air. A measured volume of a stock solution of hexylamine in hexanes (74.7 μL, 1.58 μmol, 133 mM) was added to the above mixture. The polymerization was stirred at 50 °C for 2 days to allow a near quantitative monomer conversion.

The solvent was then removed under vacuum to afford the reaction mixture which was re-dissolved in DMF (TFA in the case of D,L-MET NTA sROP). The final polymer product was precipitated by adding excess diethyl ether into the polymer solution, separated by filtration and dried under vacuum to afford a white solid (36.2 mg, 91% yield).

3.2.4 Solution-Phase ROP of NTAs

A representative polymerization was conducted as followed. BLG NTA (59.9 mg, 0.215 mmol) was dissolved in anhydrous dioxane (0.40 mL) under nitrogen atmosphere in glovebox. A measured volume of a stock solution of hexylamine in hexanes (31.1 μL, 1.79 μmol, 57.5 mM) was added to the above solution. The polymerization was stirred at 50 °C for 2 days before sampling a reaction aliquot for conversion analysis. The polymer was isolated by precipitation into diethyl ether, followed by filtration, diethyl ether wash and vacuum dry.

3.3 Results and Discussion

3.3.1 NTA monomers synthesis

The NTA synthesis precursor XAA was prepared in one step from commercially available potassium ethyl xanthogenate with chloroacetic acid in NaOH aqueous solution (Scheme 3.3). Compared to Kricheldorf's two step method, the new method avoids use of toxic carbon disulfide. And XAA in large scale (100 g) can be made in two days with high purity and yield under mild condition with easy work up. The ^{1}H NMR of XAA is shown in Figure S3.1.

Scheme 3.3. Synthetic route towards XAA.
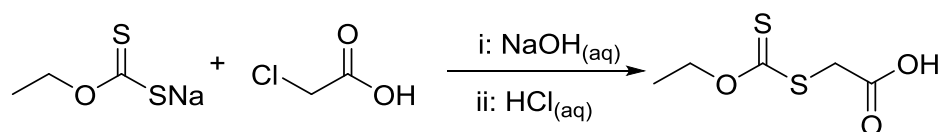
The amino acid-derived NTA monomers were synthesized in two steps from the corresponding amino acids by adapting a previously reported procedure.[67] NTAs with simple

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aliphatic side chains have been previously synthesized by coupling amino acids and XAA in aqueous solution containing 2 equivalences of NaOH, followed with cyclization. However, when the same condition was applied to H-Glu(OBz)-OH and XAA coupling, H-Glu(OBz)-OH ester hydrolysis was observed in the first coupling step due to too basic pH. Hence, the NaOH aqueous solution was switched to saturated $NaHCO_3$ aqueous solution to ensure mild condition for ester during coupling. The second step cyclization with PCl_3 is the only operation done under nitrogen atmosphere during NTA synthesis for the purpose of avoiding side reaction between PCl_3 and moisture. The resulting NTAs with good yield (50-60%) were readily obtained by flash column chromatography in open air. The NTA structures have been unambiguously verified by X-ray crystallography (Figure 3.1 and S3.2), MS and NMR (Figure S3.3, S3.4, S3.5, S3.6). The overall synthetic route for the first time made BLG NTA and LYS NTA is shown in Scheme 3.2.

NTAs are generally more likely to undergo racemization during synthesis.[16,59] It was found that NTA monomers prepared via our method undergoes large extent of racemization, as evidenced by crystal structure (Figure 3.1), chiral HPLC (Figure S3.7 and S3.8), small specific optical rotation (e.g., α_D^{25} = - 0.51 for BLG NTA in $CHCl_3$ c = 0.924; α_D^{25} = - 10.35 for LYS NTA
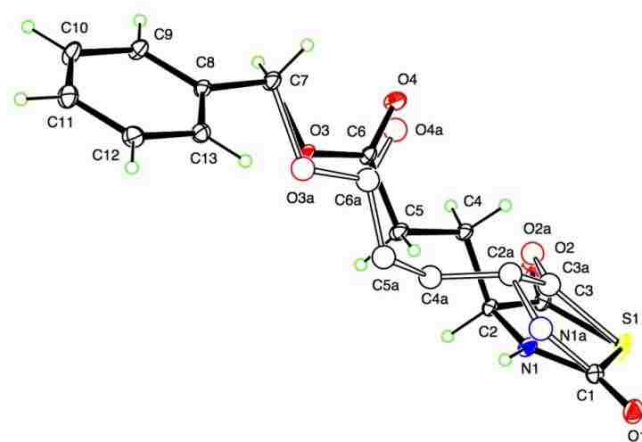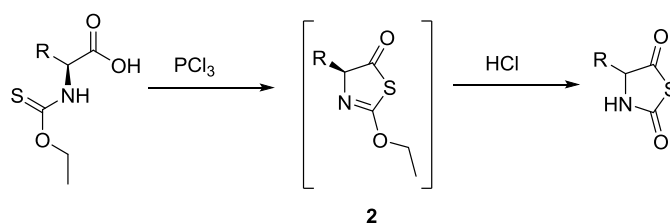

Figure 3.1. Crystal structure of BLG NTA (crystal structure parameters summarized in Table S3.1)

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in CHCl$_3$ c = 0.994). The enantiomeric excess (*ee*) values of BLG NTA and LYS NTA based on chiral HPLC are both 2%. Based on Hirschmann's study,[59] the racemization happens to the intermediate (**2**) during the cyclization step under acidic condition (Scheme 3.4). And *N*-ethoxythiocarbonyl amino acids are recommended to be cyclized with phosphorus tribromide for 5-10 mins at 0 ℃. However, when the same recommended cyclization condition was applied to series of *N*-ethoxythiocarbonyl amino acids (BLG, LYS, LEU), significant racemization was still for BLG NTA and LYS NTA as evidenced by chiral HPLC (Figure S3.9 and S3.10). The *ee*

Scheme 3.4. Cyclization of *N*-ethoxythiocarbonyl amino acids

values of BLG NTA and LYS NTA based on chiral HPLC are 10%, 40% respectively. On the other hand, crystal structure (Figure 3.2), chiral HPLC (Figure S3.11) and specific optical rotation analysis (e.g., α_D^{25} = - 57.55 for LEU NTA in CHCl$_3$ c = 1.07) indicate no racemization occurred during LEU NTA synthesis under identical condition. Hence, the racemization during the cyclization depends strongly on the nature of amino acids. Nevertheless, the positive effect of the PBr$_3$ cyclization agent has been demonstrated, which improved the *ee* of LYS NTA from 2% to 40% as compared to the PCl$_3$ method.
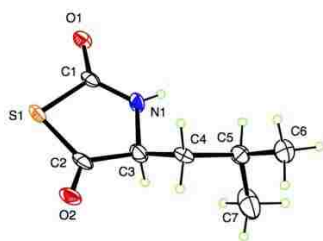
Figure 3.2. Crystal structure of LEU NTA (crystal structure parameters summarized in Table S3.1)

The effect of PBr$_3$ over PCl$_3$ shed light on the future direction to further improve the optical purity of NTA. Hirschmann proposed that the racemization for NTA synthesis occurred at intermediate **2** in Scheme 3.4 and 3.5.[59] The intermediate **2** can directly undergo normal dealkylation to form NTA. It also can go through enolization to afford racemized NTA after dealkylation (Scheme 3.5). The above two pathways are competitive during the cyclization. Their relative rate can be affected by temperature, pH, nucleophilicity of anions (e.g. Cl or Br anion) and etc. Apparently, Br anion will facilitate the normal dealkylation pathway, as evidenced by higher *ee*. This makes sense as Br anion has better nucleophilicity as compared to

Scheme 3.5. Cyclization of *N*-ethoxythiocarbonyl amino acids.

A: Normal dealkylation pathway from **2** to form NTA
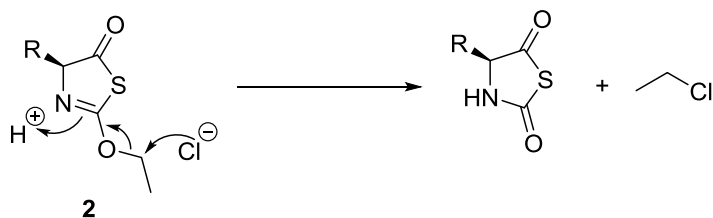
B: Acid-catalyzed racemization pathway from **2** to form NTA
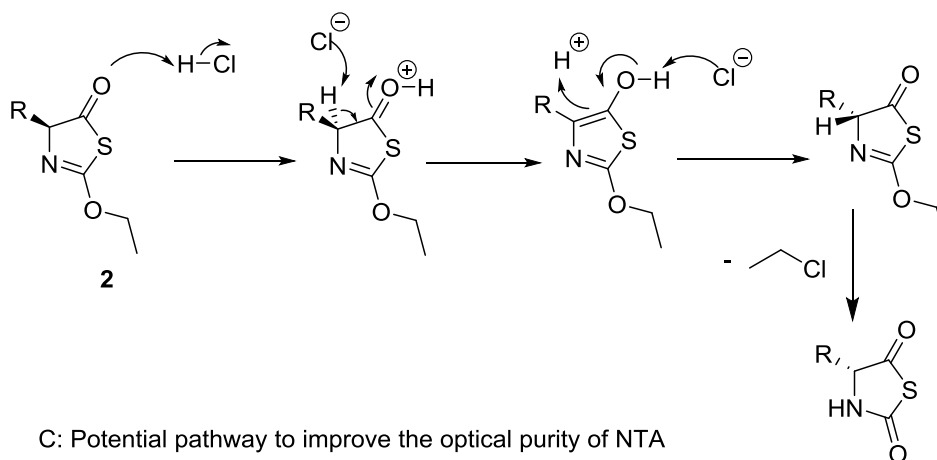
C: Potential pathway to improve the optical purity of NTA
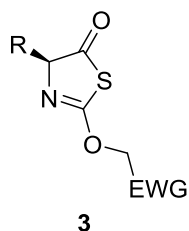
Cl anion. The better nucleophilicity facilitates the dealkylation, which results in better optical purity of NTA. Alternatively, it was also reported that replacing the ethoxy side chain with methoxy would may slightly improve the optical impurity, as the steric hindrance is reduced in the methoxy case.[58] The strategy was reproduced in our lab for BLG-NTA synthesis to improve the *ee* from 10% to 20%, which is still not satisfactory. Nevertheless, the above proposed racemization pathway can guide the direction for future investigation to improve the optical purity of NTA. For instance, the effect of neucleophilic additives to the cyclization step should be investigated. Alternatively, electron withdrawing group (EWG) can be attached to the terminal of the ethoxy group of the intermediate (**3**) to speed up the nucleophilic attack (Scheme 3.5). This can be conveniently done by modifying the route of XAA synthesis with different alcohol.[67] The temperature effect is worth investigation as well. In summary, it remains a challenge to develop a synthetic route to prepare optically pure NTAs for all amino acids, and it will be the focus of future work for this topic. The motivation behind is that optically pure NTA is critical for secondary structure of polypeptide.

The NTAs are found to be significantly more stable against moisture and heat than the NCA analogs (Table 3.1). For example, when stored as solids in open air or in a desiccator (with Drierite) at room temperature, BLG NTA has a shelf-life of 2 months or a minimum of 5 months respectively, whereas BLG NCA started to become hydrolyzed in less than 11 days or 1 month period (Figure 3.3). The high stability of NTAs against moisture is presumably due to the less electrophilic nature of the carbonyl [O=C(5)] of NTAs relative to that of NCAs.[16] This is consistent with the observed lower carbonyl stretch frequency of BLG NTA (1717 cm^{-1}) (Figure S3.12) relative to that of BLG NCA (1842 cm^{-1}). In addition, TGA revealed that BLG NTA is thermally much more stable than the NCA analog, evidenced by a significant higher onset of

thermal degradation temperature at 225 °C than that of the NCA (122 °C) under nitrogen (Figure

S3.13). The phosgene free synthesis, the enhanced thermal stability and long shelf-life of NTAs

relative to the NCA analogs significantly enhance the appeal of the former as substrate for

polypeptide synthesis.

Table 3.1. BLG NTA and BLG NCA stability comparison based on FTIR monitoring.

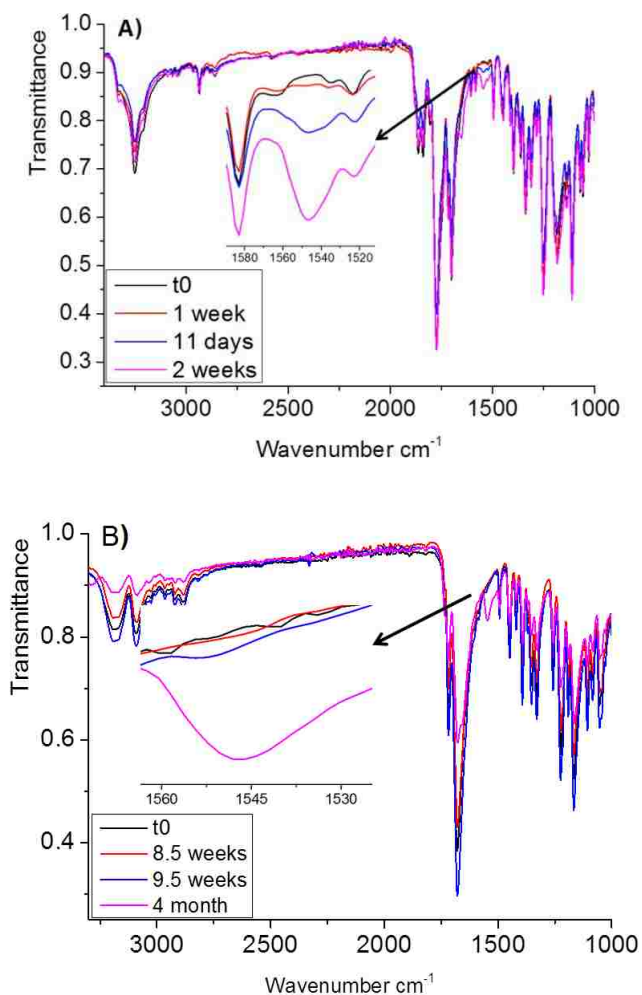|	Stored in air	Stored in desiccator	Degradation T (°C)
BLG NCA	<11d	~ 1 m	122
BLG NTA	~ 2 m	>5 m	225

Figure 3.3. (A) FTIR spectra of BLG NCA and (B) BLG NTA solids that have been stored in open air for varying period of time to determine their relative shelf-life. (Both monomers show new peak around 1545 cm^{-1} upon storage, which correspond to amide II bands indicating formation of polymers).

3.3.2 Polypeptide synthesis from NTAs

We initially investigated the solution phase polymerization of BLG NTA using several initiators (e.g., hexylamine, triethylamine, HMDS, NiBiPyCOD) that are well-known to mediate ROP of the corresponding BLG NCA with high activities (Entry 11-14, Table 3.2).[16,18,19] Hexylamine is the only initiator to afford any polymerization activity (5% conversion, DP=120) after 2 d at room temperature under nitrogen or in open air. The polymerization product was analyzed by MALDI-TOF MS. The MS indicates the formation of oligomeric PBLG. Based on end group analysis from MS, the PBLG is exclusively initiated by hexylamine. And the terminal groups are either active amine (major) or back-biting terminated PBLG (Figure S3.15). The cause for the polymerization to stop remains unclear. Increasing temperature to 50 °C still resulted in low conversions (5-12%) for all initiators (Entry 11-14, Table 3.2). High temperature is known to promote undesired side reactions (e.g., transamidation to form pyroglutamate) that can terminate polymerization prematurely. This is also verified in our study based on the end group analysis from MALDI-TOF MS. The hexylamine initiated BLG NTA solution polymerization at 50 °C afforded PBLG with mainly back-biting terminated end group (Figure S3.16).

The low reactivity of BLG NTA limits its polymerization activity with all common initiation systems. To further enhance the polymerization under mild conditions, we set to investigate the sROP of BLG NTA. Kanazawa *et al* have previously demonstrated that primary amines can initiate the ROPs of several amino-acid derived NCA crystals in the solid state under mild conditions (20 - 50 °C) with faster polymerization rate as compared to solution state polymerization.[139-144] The specific layer crystal packing of these NCAs in the solid state was suggested to be responsible for the accelerated chain growth.

Table 3.2. Solid or solution phase polymerization of amino-acid derived NTAs using various initiators[a].

Entry	Polym. State	Initiator	NTA	$[M]_0$:$[I]_0$	M_n (Theo.) (g/mol)	M_n (Exp.)[c] (g/mol)	PDI[c]	conv[d] (%)
1	solid (in air)	HA[b]	BLG	40:1	8700	9500	1.25	98
2	solid (in air)	HA[b]	BLG	80:1	17400	19000	1.21	99
3	solid (in N$_2$)	HA[b]	BLG	80:1	17100	18700	1.24	97
4	solid (in air)	HA[b]	BLG	120:1	25300	26800	1.21	96
5	solid (in air)	HA[b]	BLG	150:1	31600	29700	1.26	96
6	solid (in air)	HA[b]	LYS	80:1	20600	19300	1.31	97
7	solid (in air)	HA[b]	LYS	150:1	38700	37600	1.29	97
8	solid (in air)	HA[b]	LYS	200:1	51600	50300	1.28	97
9	solid (in air)	HA[b]	MET	40:1	5300	5500[e]	NA	100
10	solid (in air)	HA[b]	MET	80:1	10600	10300[e]	NA	100
11	solution (in N2)	HA[b]	BLG	120:1	NA	NA	NA	12
12	solution (in N2)	TEA[b]	BLG	50:1	NA	NA	NA	<5
13	solution (in N2)	HMDS[b]	BLG	120:1	NA	NA	NA	6
14	solution (in N2)	Ni(BiPy) (COD)[b]	BLG	120:1	NA	NA	NA	5

[a.] All reactions were allowed to proceed for 2 days. Solid-phase ROP conditions: BLG NTA, MET NTA $[M]_0$ = 0.2 M, 50 °C, in hexanes; LYS NTA, $[M]_0$ = 0.2 M, 80 °C, in heptane. Solution-phase ROP conditions: BLG NTA, $[M]_0$ = 0.5 M, 50 °C, dioxane. [b.] HA = hexylamine, TEA = triethylamine, HMDS = hexamethyldisilazane, Ni(BiPy)(COD) = Ni(2,2'-bipyridyl)(1, 5-cyclooctadiene); [c.] determined by SEC-DRI-MALS analysis (dn/dc = 0.1292 mL/g for PBLG, 0.123 mL/g for PZLL in 0.1M LiBr/DMF at 25 °C). [d.] Determined by ^{1}H NMR analysis of reaction aliquots; [e.] Determined by end-group analysis from ^{1}H NMR in TFA-d$_1$(Figure S3.14).

We found that solid-state polymerization of BLG NTA in a hexanes suspension of the monomer in the presence of soluble primary amine initiators produced the corresponding PBLG with high conversion at 50 °C after 2 d, in stark contrast to the low activity of the solution phase polymerization. The polymer molecular weight (M_n) can be readily controlled by adjusting the initial monomer to initiator ratio and agrees well with the theoretical value for controlled polymerization (Entry 1-5, Table 3.2). The molecular weight distribution is modest in the 1.2-1.3 range. The M_n was also shown to track linearly with the conversion with low PDIs (<1.2)

throughout the reaction course (Figure 3.4), indicating controlled polymerization characteristic. The monomer conversion versus time plot indicates the polymerization rate remains fast during
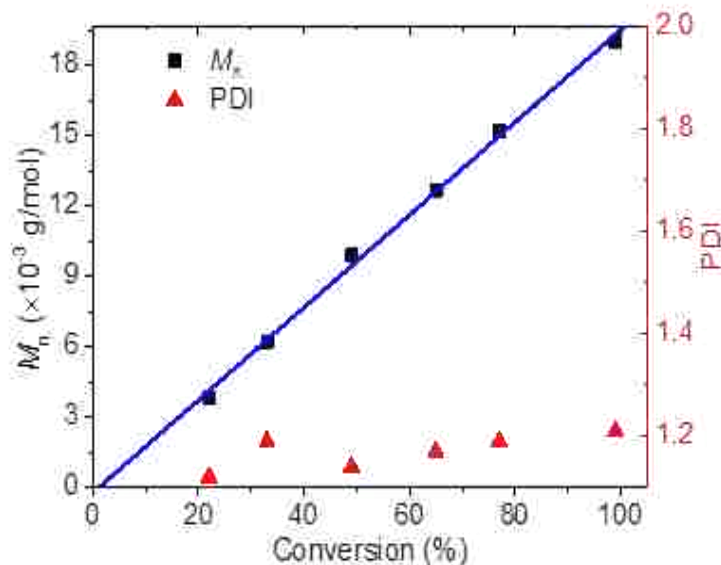

Figure 3.4. Plot of M_n (■) and PDI (▲) versus conversion for the sROP of BLG NTA in hexanes suspension at 50 ℃ ([M]$_0$:[I]$_0$=80:1, [M]$_0$=0.2 M) together with the linear fit (—) of the M_n versus conversion plot.

the polymerization period due to a nearly constant local monomer concentration in the solid phase (Figure S3.17). MS analysis of a low molecular polymer sample revealed that sROP of BLG NTA occurs by a normal amine mechanism where the primary amine initiates the solid-phase polymerization of BLG NTA by regio-selective addition to the C5-carbonyl position. A small fraction of polymer chain was also found to be terminated with the pyroglutamate chain end, consistent with the undesired transamidation of the growing chain ends resulting in termination of chain growth (Figure S3.18).

To further demonstrate that the sROP can be applied to other NTAs for polypeptides preparation, several other NTA monomers (e.g., LYS NTA and MET NTA) were screened (Entry 6-10, Table 3.2). LYS NTA was also initially polymerized under standard condition. However, PZLL only low M_n can be afforded (<13,000 g/mol) under standard condition. This is

presumably due to the low reactivity of LYS NYA. Hence, the polymerization temperature was increased to 80 °C. Under the modified condition, LYS NTA can be efficiently polymerized in the solid state to afford PZLL with M_n over 50,000 g/mol. MET NTA was found to polymerize well under the same standard condition. The end group analysis through ^{1}H NMR (Figure S3.14) indicates formation of poly(methionine) with good molecular weight control.

To further investigate whether crystal packing of NTAs is important for the controlled polymerization behavior, we prepared several batches of the BLG NTA monomers by recrystallization under various conditions. While X-ray crystallographic analysis of BLG NTA single-crystals revealed the formation of lamellar packing of BLG NTA (Figure S3.19 and Figure S3.20), which is similar to the NCAs crystal packing with enhanced solid state polymerization reactivity in Kanazawa's study. The in plane distance between the –NH to C5 carbonyl is in the range of 4.074 Å ~ 4.454 Å for two adjacent BLG NTA monomers. The distance range is close to the one in L-Leu NCA (e.g. 4.407 Å ~ 6.76 Å)[142], which can be efficiently polymerized in the solid state with primary butylamine as initiator. The out of plane distance between the –NH to C5 carbonyl of two BLG NTA monomers is larger than 13.622 Å. It seems that the layer-by-layer crystal packing is responsible for the enhanced polymerization activity of NTAs in the solid state. However, XRD analysis of the polycrystalline BLG NTA revealed only about 40-50 % degree of crystallinity (Figure S3.21 and Figure S3.22).The experimental XRD pattern of the racemic BLG NTA resembles the predicted one based on racemic BLG NTA single crystal structure (Figure S3.23). The major peaks in the XRD spectra are listed in Figure S3.24, and their corresponding spacing are summarized in Table S3.2. XRD patterns also suggest the presence of different polymorphism among various batches of the BLG NTA monomers. BLG NTA prepared from recrystallization in THF/Hexanes has different XRD

pattern as compared to the ones purified by other methods, which indicates different molecule packing. However, polymerization of the monomer prepared from THF/Hexanes produced PBLG polymers with nearly identical M_n and PDI as compared to polymerization results from other batches of BLG NTAs. These results together suggest that observed controlled polymerization characteristics is mainly the result of enhanced local monomer concentration in the solid state which accelerates the polymerization under mild conditions without promoting undesired side reactions.

For characterization purpose, representative SEC chromatograms and ^{1}H NMR of polypeptides are shown in the supplemental data section (Figure S3.25, S3.26, S3.27, S3.28).

3.4 Conclusion

In this study, we demonstrated for the first time that polypeptides with controlled M_n and PDI can be obtained from the solid state ROPs of NTA monomers using soluble primary amine initiators under mild conditions in open air. The sROP reactions proceed by a normal amine mechanism. The controlled polymerization behavior of sROP is the direct result of high local monomer concentration in the solid phase, thus allowing for faster polymerization under relatively mild conditions. In view of the facile synthesis of various amino-acid derived NTA monomers, their significantly enhanced moisture and thermal stability and long shelf-life relative to the analogous NCAs, sROP of NTAs represent an attractive alternative synthetic method to access well-defined polypeptides with controlled molecular characteristics and greatly reduced operational complexity. The future direction for this work is to develop optimized NTA preparation condition for optically pure NTA monomers.
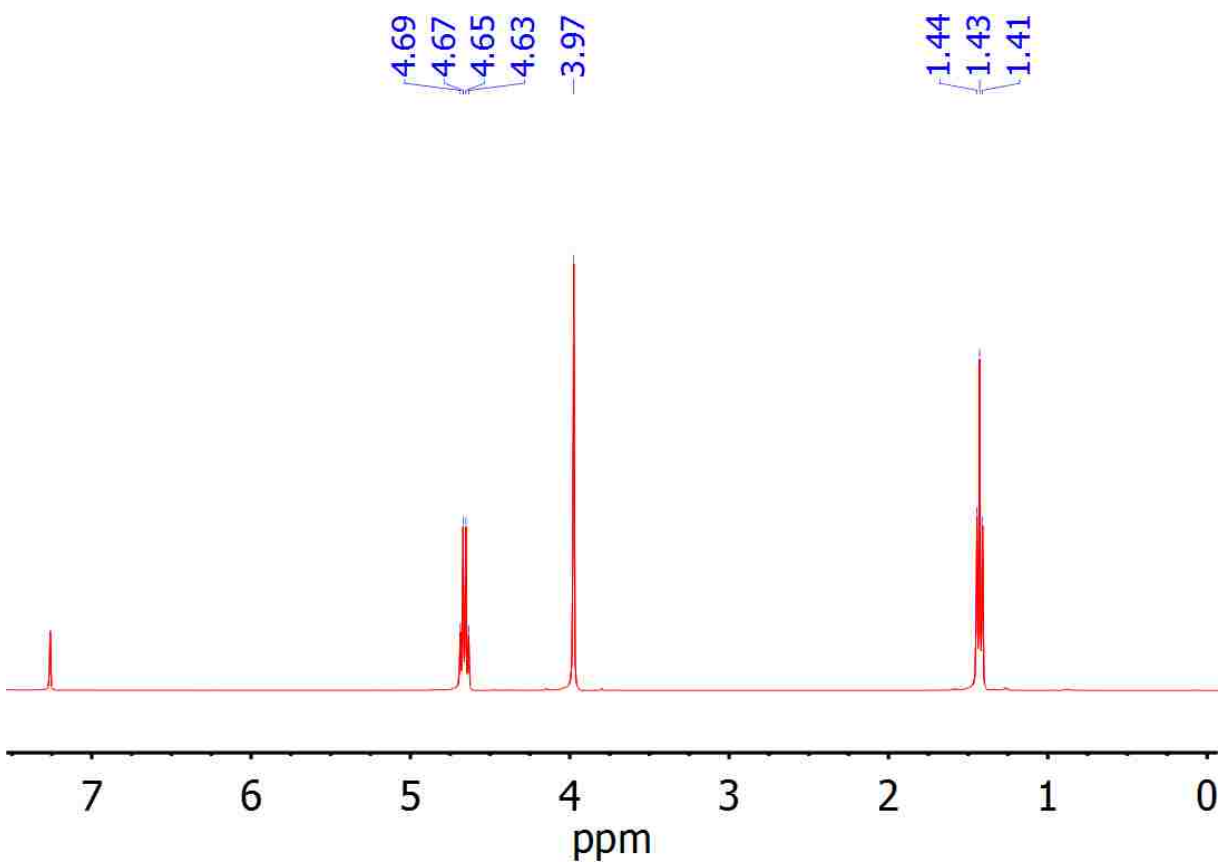
3.5 Supplemental data

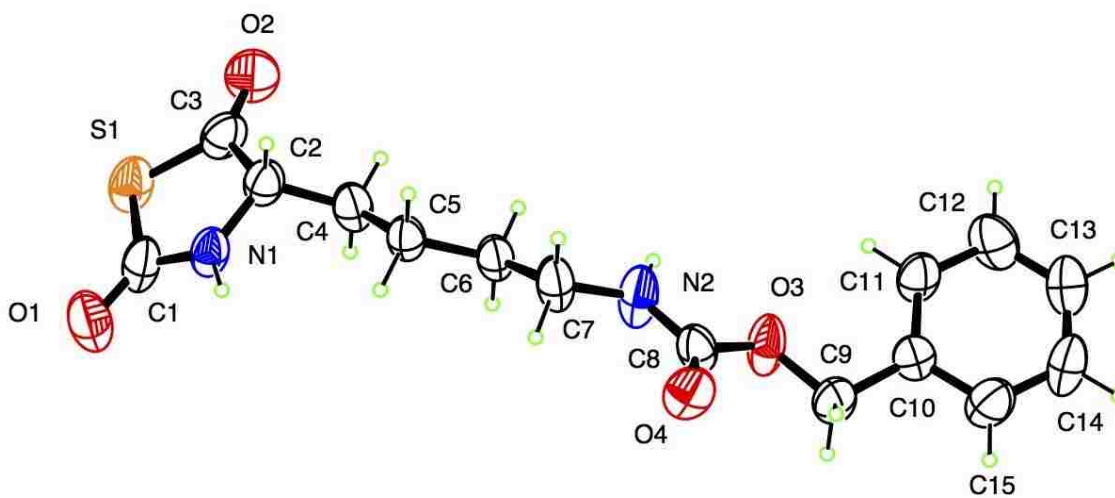Figure S3.1. ^{1}H NMR spectrum of XAA in CDCl$_{3.}$

Figure S3.2. Crystal structure of LYS NTA.

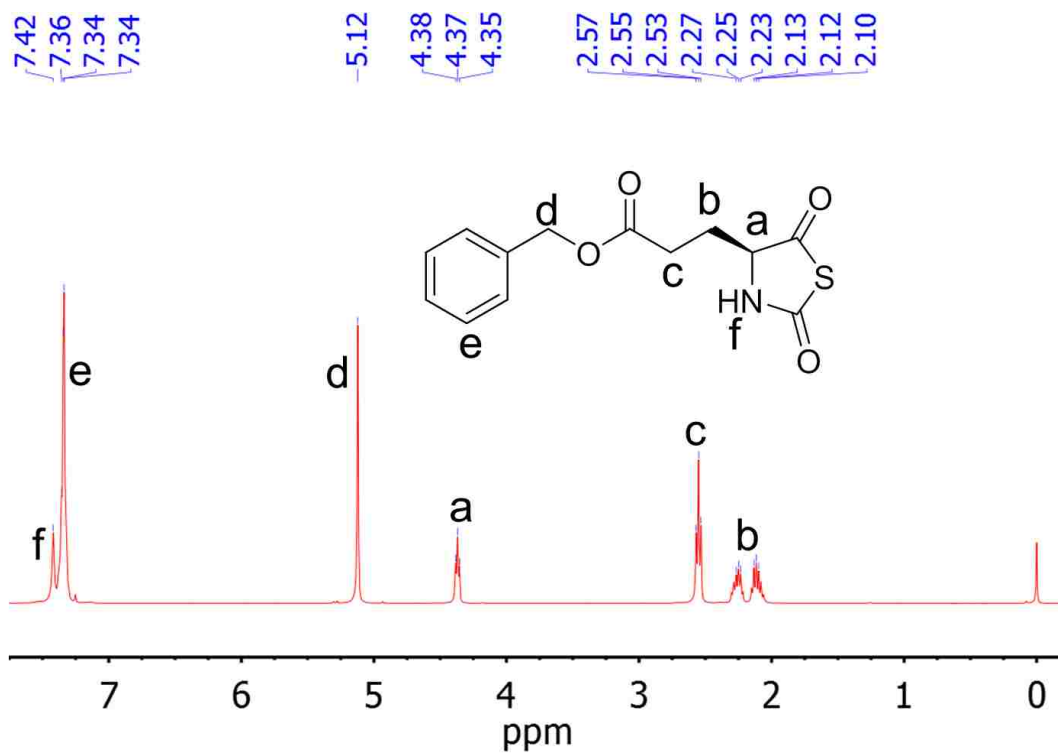Figure S3.3. ^{1}H NMR spectrum of BLG NTA in CDCl$_3$.
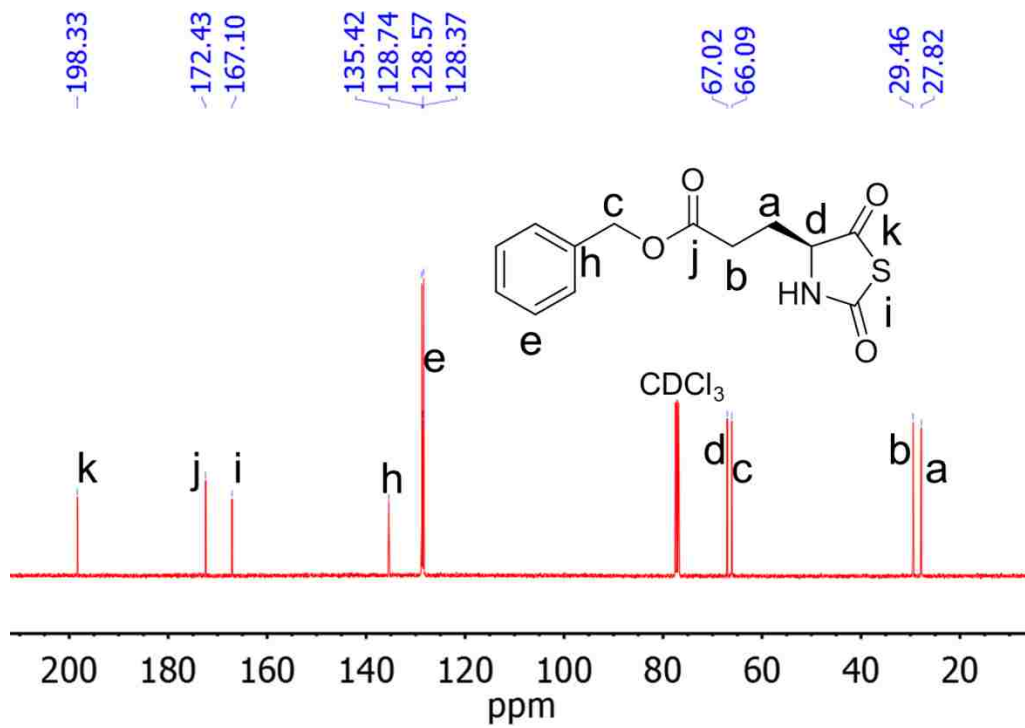
Figure S3.4. ^{13}C NMR spectrum of BLG NTA in CDCl$_3$.
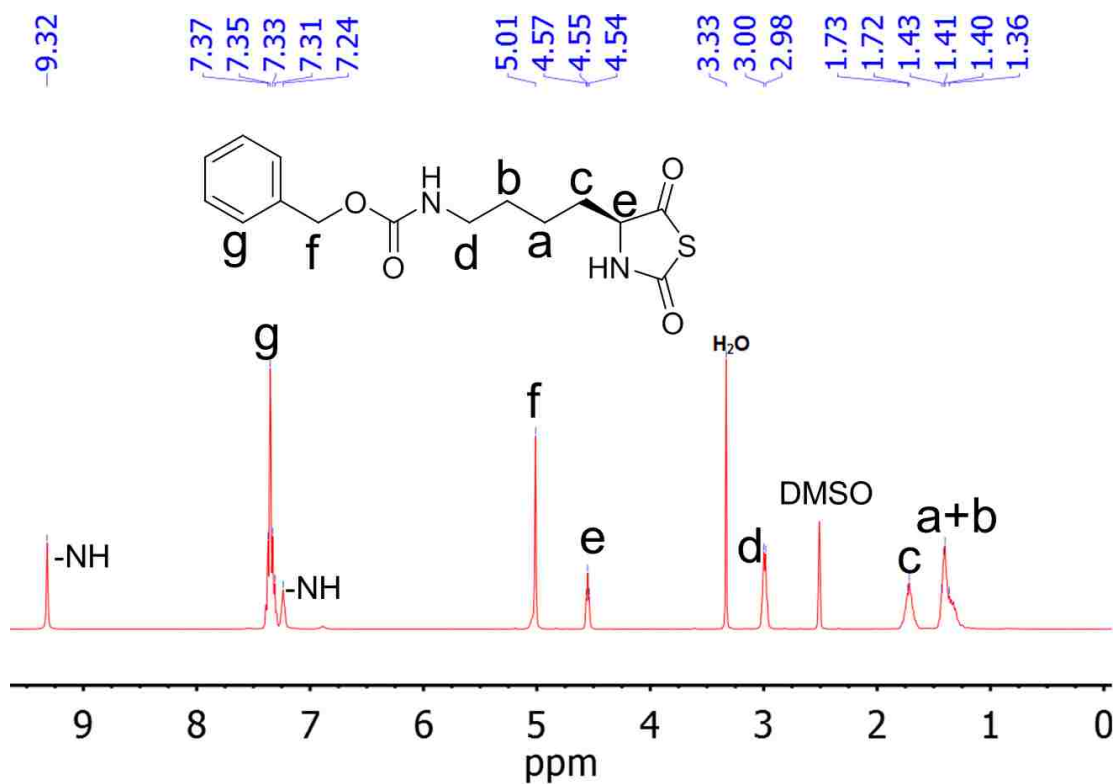
Figure S3.5. ^{1}H NMR spectrum of LYS NTA in DMSO-d$_{6}$.
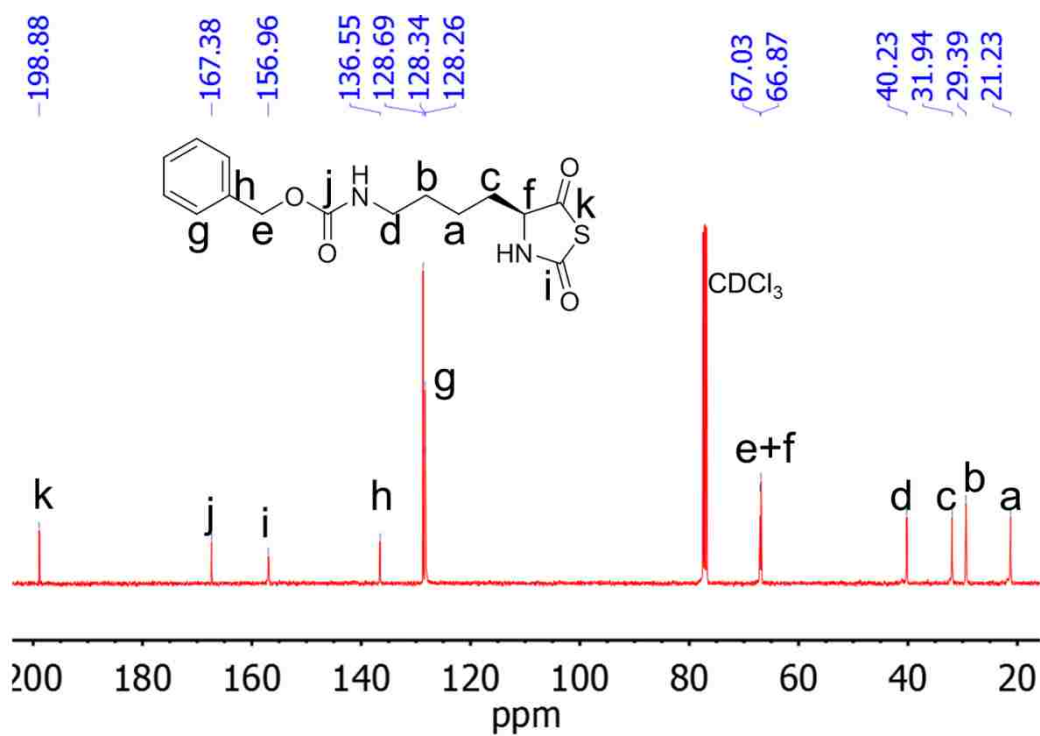
Figure S3.6. ^{13}C{H} NMR spectrum of LYS NTA in DMSO-d$_{6}$.

87
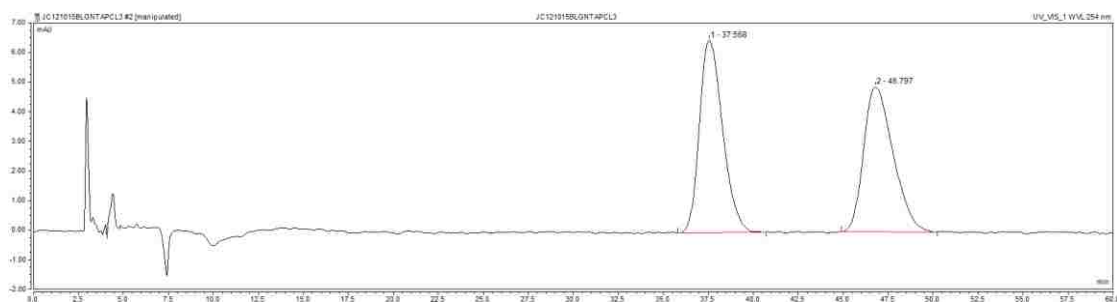
Figure S3.7. HPLC trace of BLG NTA prepared with PCl$_3$ as cyclization agent at rt. (peak area percentage 51% (left), 49% (right)).
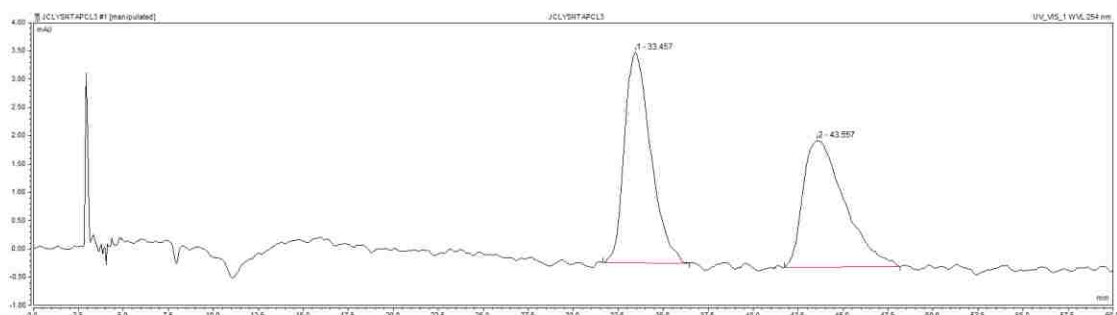
Figure S3.8. HPLC trace of LYS NTA prepared with PCl$_3$ as cyclization agent at rt. (peak area percentage 51% (left), 49% (right)).
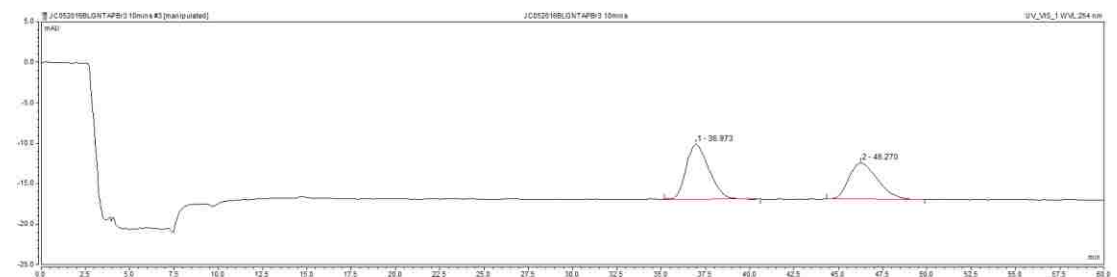
Figure S3.9. HPLC trace of BLG NTA prepared with PBr$_3$ as cyclization agent at 0 ℃ (peak area percentage 55% (left), 45% (right)).
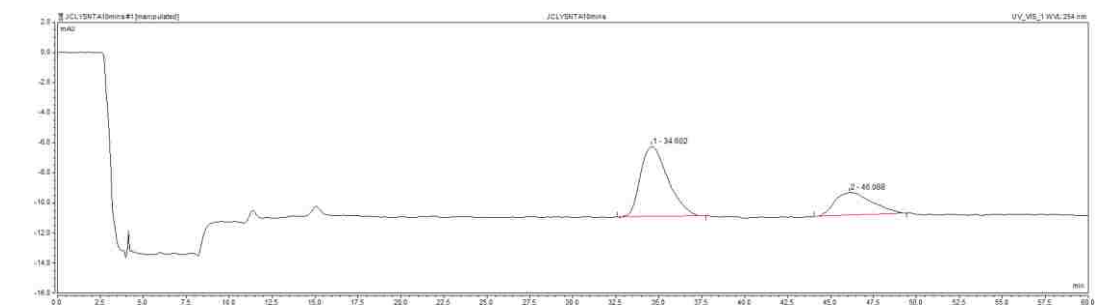
Figure S3.10. HPLC trace of LYS NTA prepared with PBr$_3$ as cyclization agent at 0 ℃ (peak area percentage 70% (left), 30% (right)).
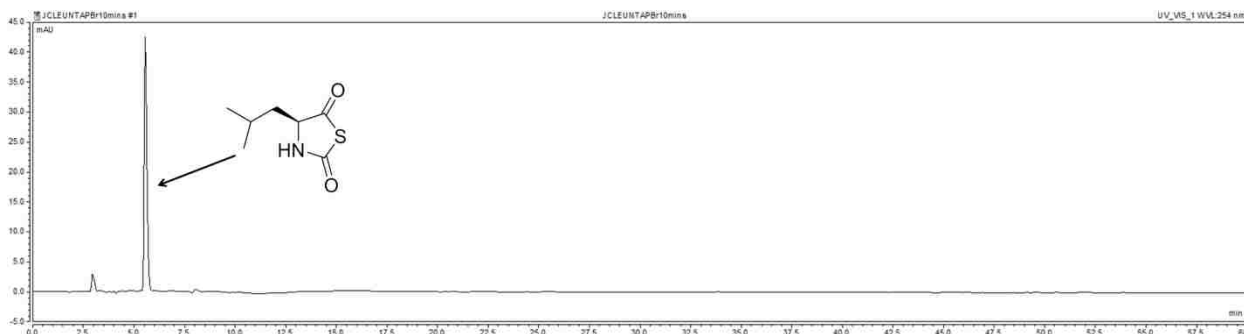
Figure S3.11. HPLC trace of Leu NTA prepared with PBr$_3$ as cyclization agent at 0 °C.

Table S3.1. Crystal structure parameters of BLG NTA, LYS NTA and LEU NTA.
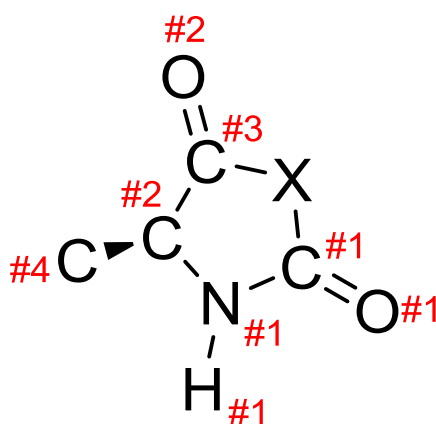
(General structure of NCA or NTA with side chains removed)

|		BLG NTA (X=S)	LYS NTA (X=S)	Leu NTA (X=S)	BLG NCA[147] (X=O)
Distance (A)	X-C3	1.779	1.765	1.776	1.368
	X-C1	1.797	1.804	1.781	1.404
	C3-O2	1.207	1.183	1.193	1.186
	C1-O1	1.230	1.226	1.229	1.193
	C2-C3	1.520	1.519	1.532	1.512
	C1-N1	1.339	1.324	1.327	1.333
	C2-N1	1.448	1.438	1.456	1.447
	C2-C4	1.535	1.520	1.514	1.522
Angle (degree)	C1-X-C3	91.19	91.81	91.89	109.0
	X-C3-C2	111.24	110.78	110.27	108.9
	C3-C2-N1	106.54	107.08	105.83	100.5
	C2-N1-C1	119.03	119.94	119.34	112.8
	N1-C1-X	111.26	110.37	111.27	108.7
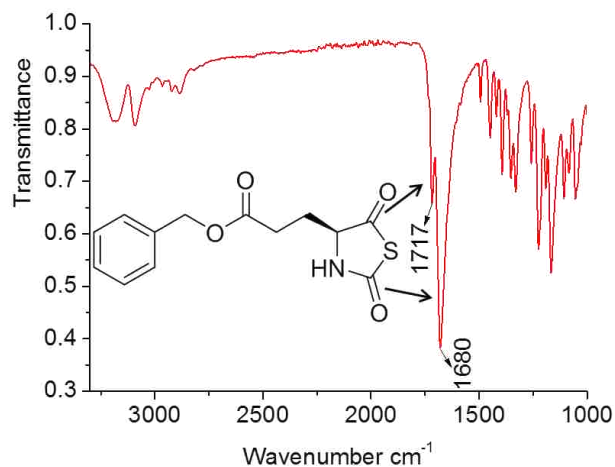
Figure S3.12. FTIR spectrum of BLG NTA in solid state (1717 cm^{-1} peak corresponds to stretch of O=C(5), 1680 cm^{-1} peak corresponds to stretch of O=C(2), the ester carbonyl stretch (1729 cm^{-1}) is merged into the big carbonyl stretch region, which will show up in the polymer).
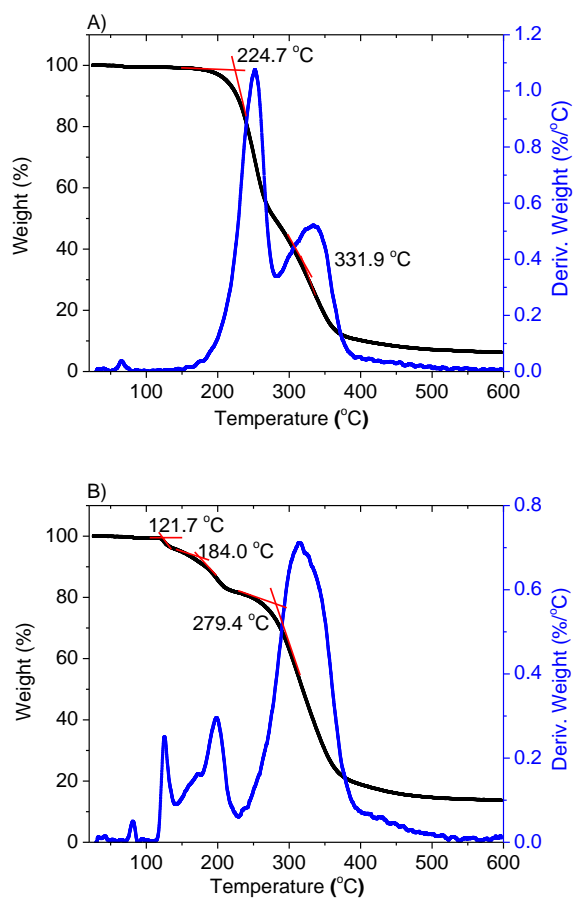
Figure S3.13. TGA (black curve) and the first derivative (blue curve) of TGA plot for BLG NTA (A) and BLG NCA (B).
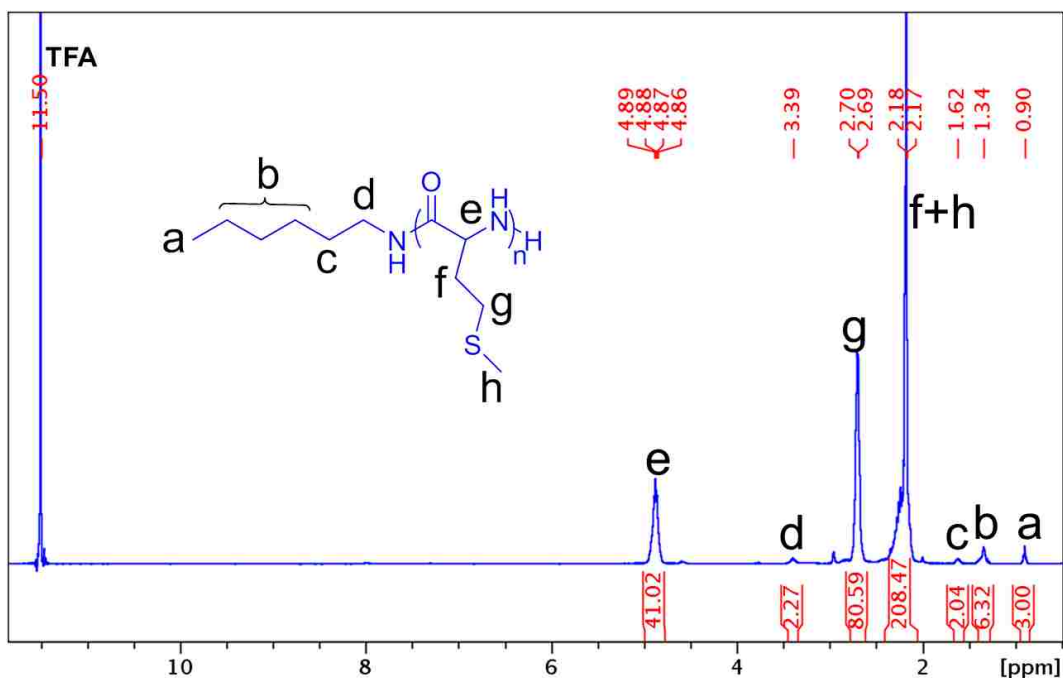
Figure S3.14. ^{1}H NMR spectrum of poly(methionine)$_{41}$ polymer obtained from solid-phase polymerization of MET NTA (TFA-d$_1$). The DP was determined by the end-group analysis through the equation e/(a/3).
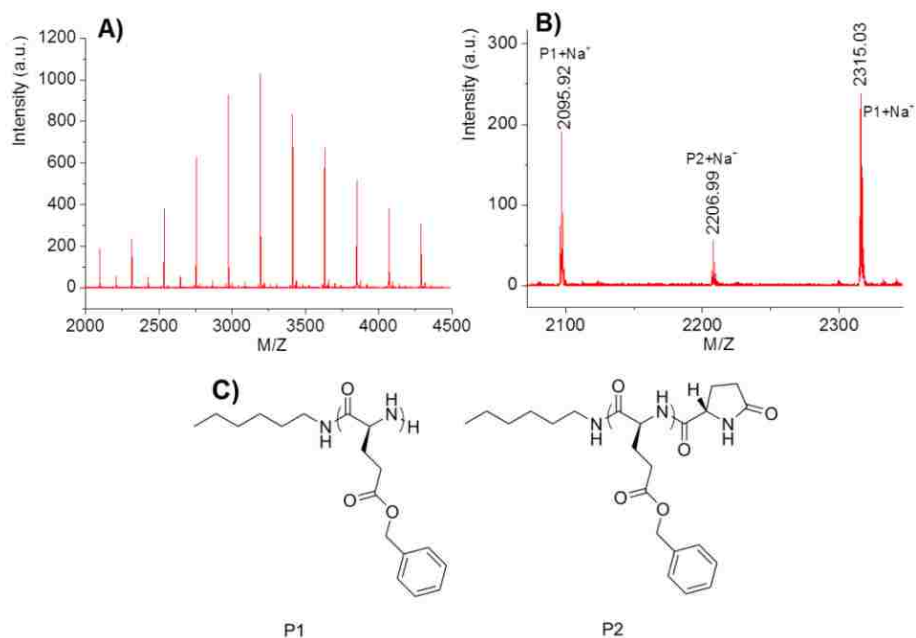
Figure S3.15. (A) Full and (B) expanded MALDI-TOF MS spectra of a low molecular weight PBLG polymer obtained by solution-phase polymerization of BLG-NTA in dioxane at rt. together with (C) the PBLG chemical structures where the end-groups are determined by the MS analysis.
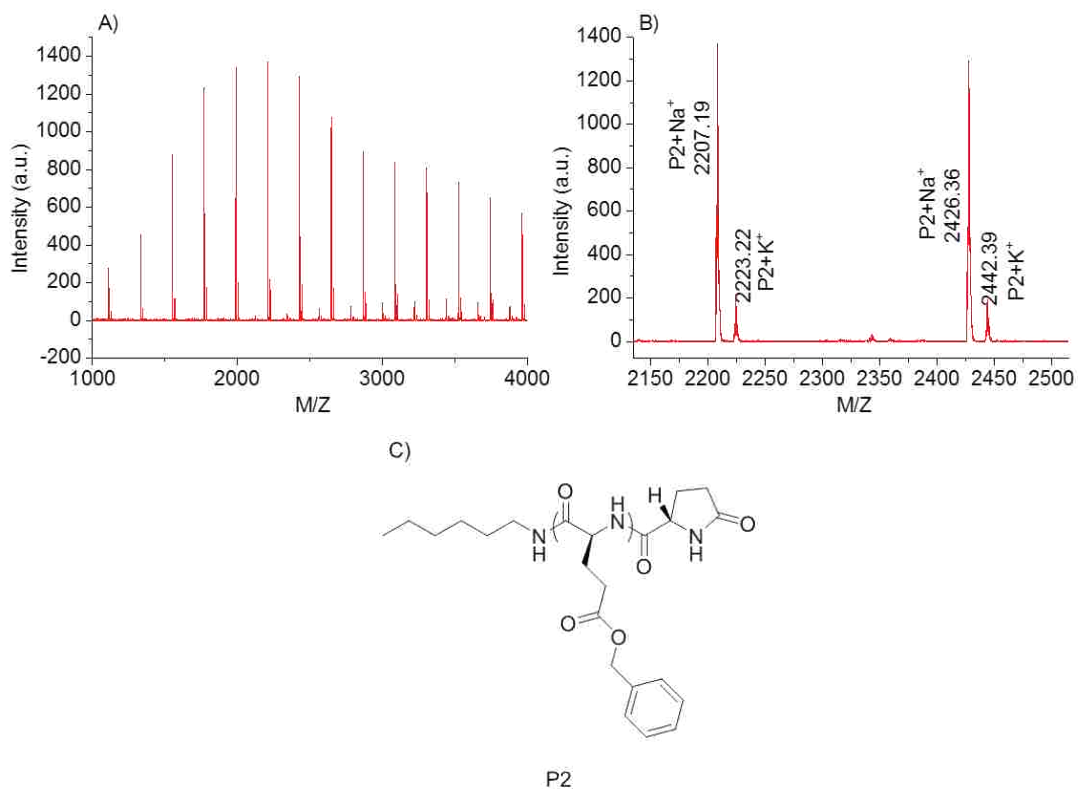
Figure S3.16. (A) Full and (B) expanded MALDI-TOF MS spectra of a low molecular weight PBLG polymer obtained by solution-phase polymerization of BLG-NTA in dioxane at 50 ℃ together with (C) the PBLG chemical structures where the end-groups are determined by the MS analysis.
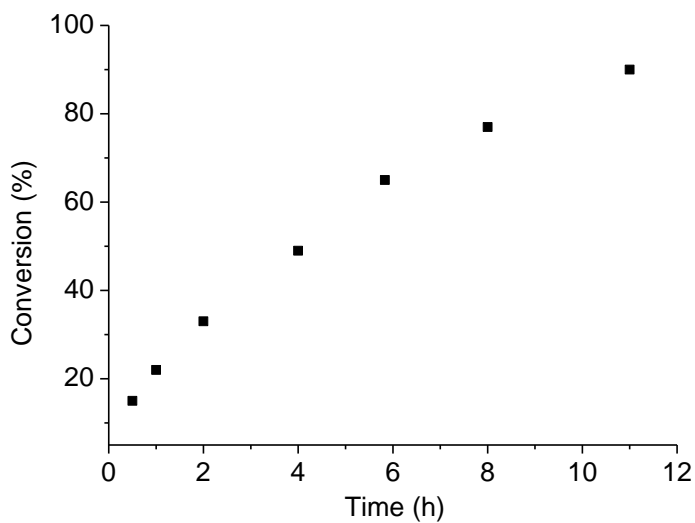
Figure. S3.17. Plot of conversion versus time for the solid-phase polymerization of BLG NTA (Conditions: $[M]_0:[I]_0 = 80 :1$, 50 ℃, hexanes suspension).
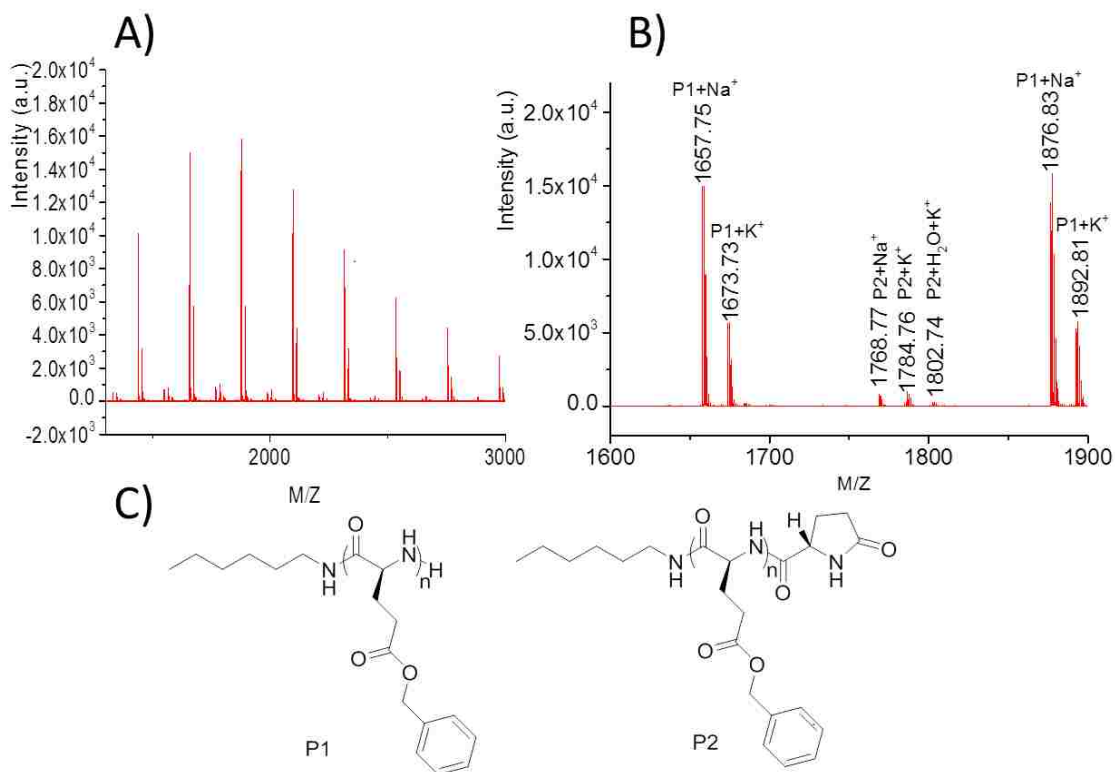
Figure S3.18. (A) Full and (B) expanded MALDI-TOF MS spectra of a low molecular weight PBLG polymer obtained by solid-phase polymerization of BLG-NTA in hexanes suspension at 50 °C together with (C) the PBLG chemical structures where the end-groups are determined by the MS analysis.
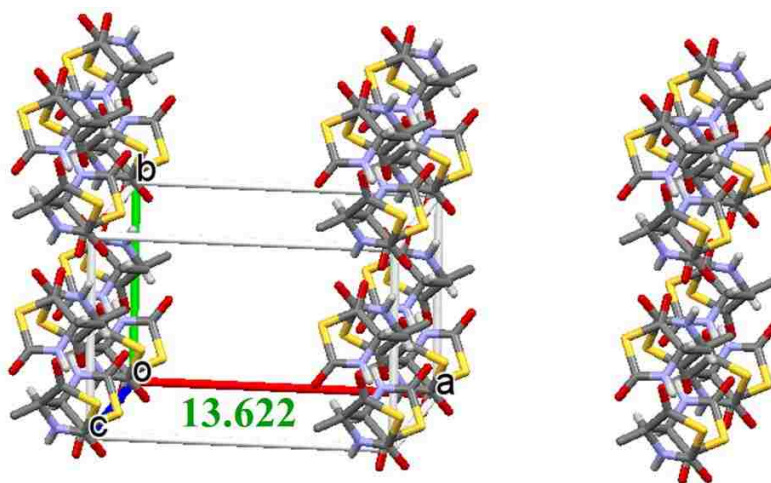
Figure S3.19. Lamellar packing of BLG NTA monomers as revealed by the X-ray crystallographic analysis of the single crystal of the monomer. (Note: BLG NTA side chains and the S enantiomer were not shown for an unobstructed view of the packing structure, 13.622 Å is the distance between layers as well as the cell length along a axe).
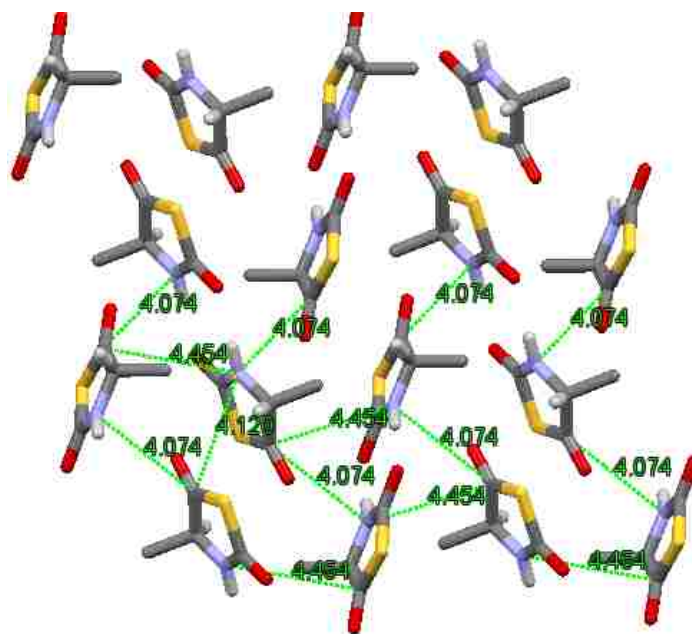
Figure S3.20. In plane (bc plane) packing of BLG NTA monomers as revealed by the X-ray crystallographic analysis of the single crystal of the monomer. (Note: BLG NTA side chains and the S enantiomer were not shown for an unobstructed view of the packing structure. The green dash lines represent connection between –NH and C5 carbonyl. Unit for distance is Å).
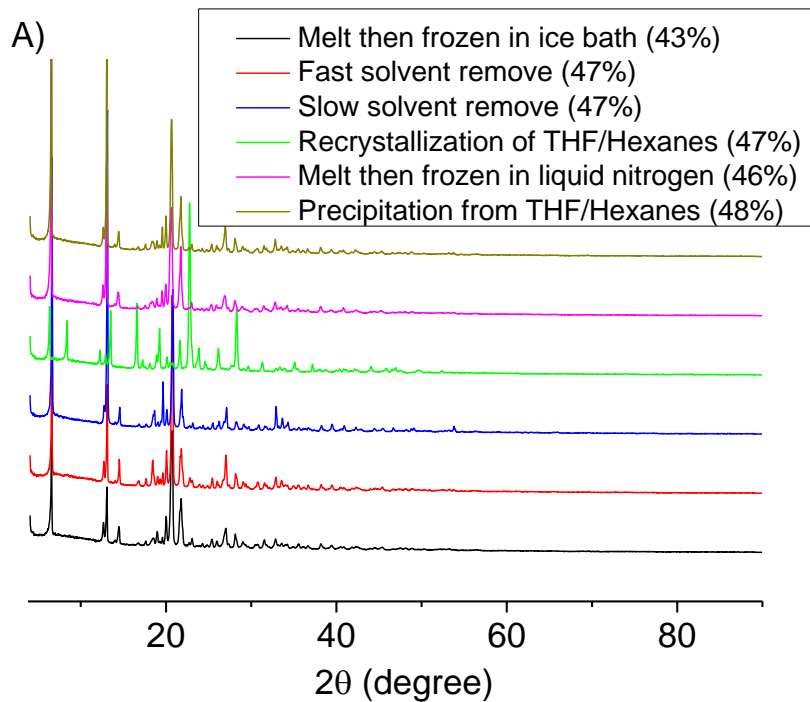
Figure S3.21. Stacked powder XRD spectra of BLG NTA crystals obtained by different recrystallization procedures.
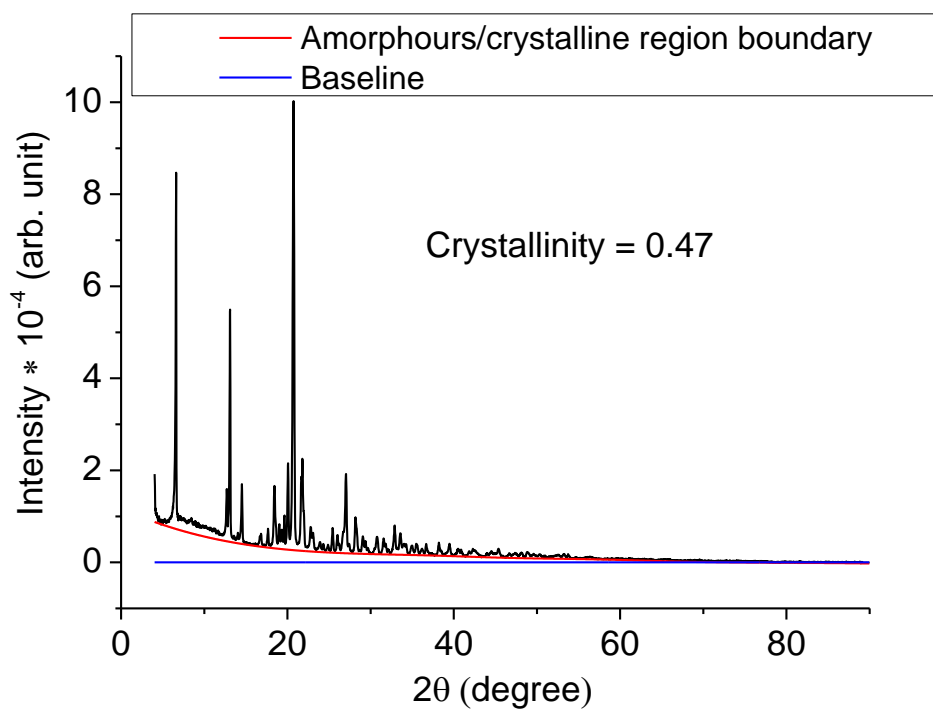
Figure S3.22. Representative XRD spectrum showing the determination of the baseline and the percentage crystallinity of the monomer.
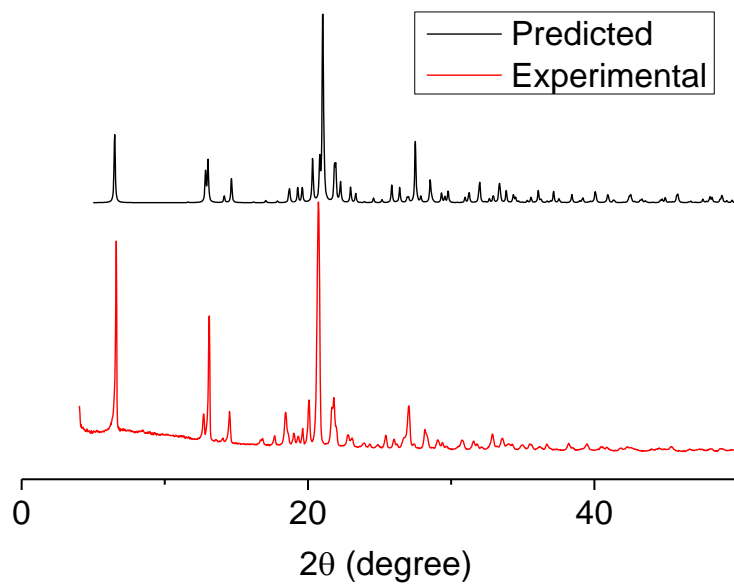
Figure S3.23. Stacked predicted and experimental XRD spectra of racemic BLG NTA.
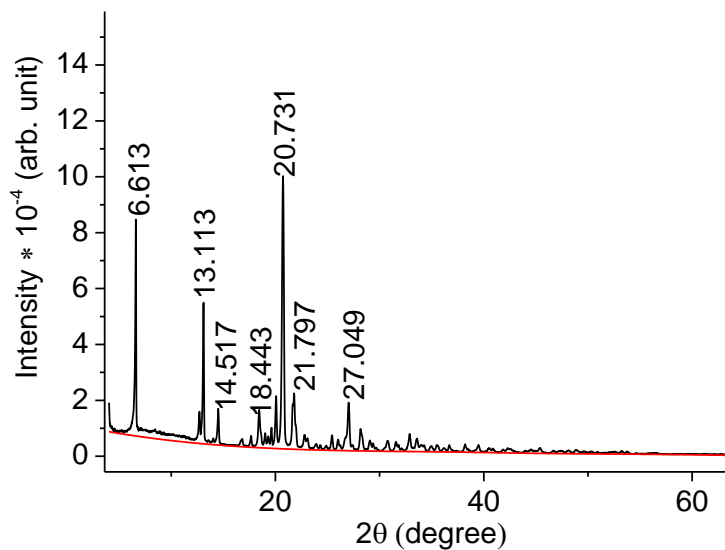
Figure S3.24. Representative XRD spectrum of BLG NTA with 2θ values.

Table S3.2. Summary of 2θ values of major peaks in Figure S3.24 and their corresponding spacing (d).

2θ (degree)	6.613	13.113	14.517	18.443	20.081	20.731	21.797	27.049
d (Å)	13.4	6.74	6.09	4.80	4.42	4.28	4.07	3.29
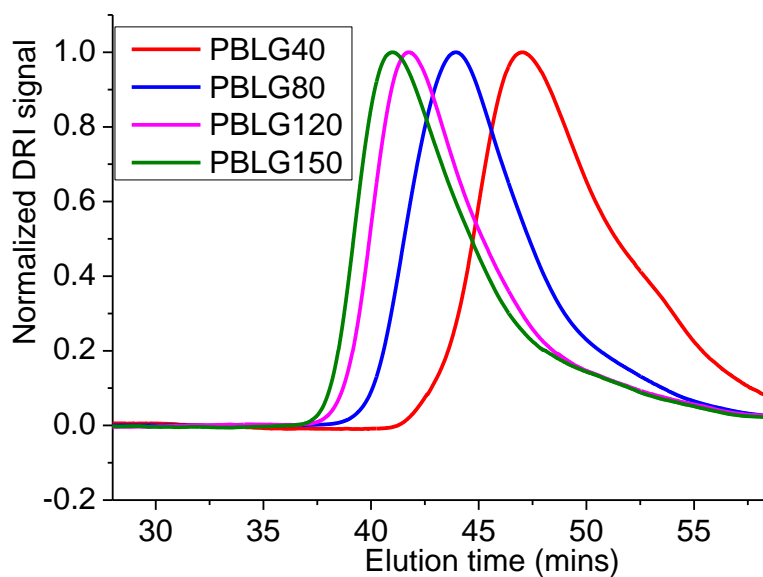
Figure S3.25. Representative SEC chromatograms of PBLG polymers obtained by sROP at 50 °C in hexanes suspension.
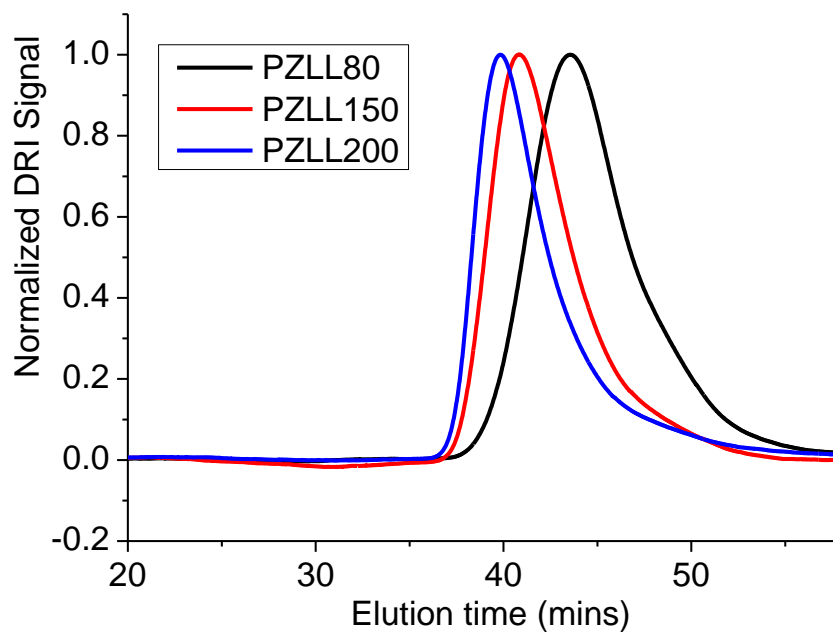
Figure S3.26. Representative SEC chromatograms of PZLL polymers obtained by sROP at 80 ℃ in heptanes suspension.
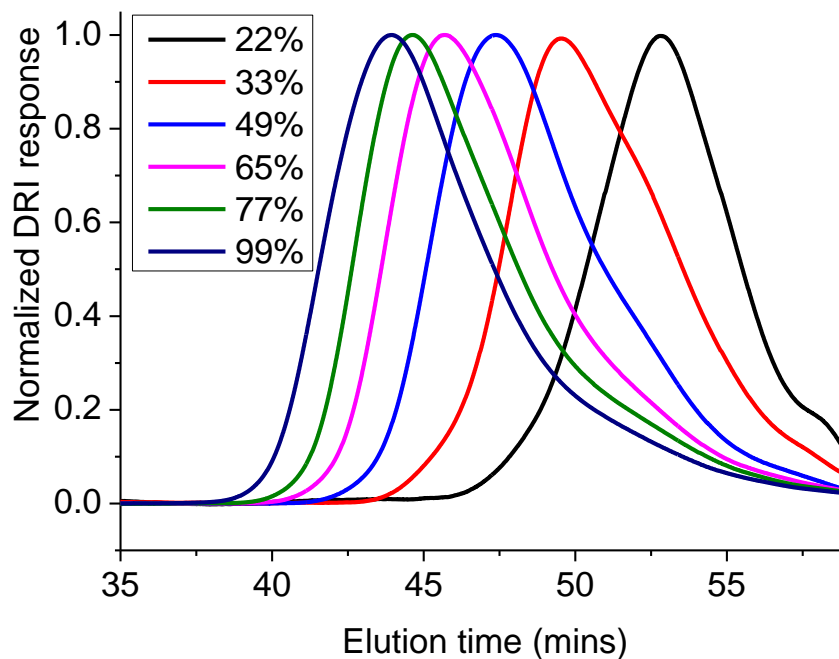
Figure S3.27. Representative SEC chromatograms of PBLG polymers obtained at different conversions of the solid-phase polymerization of BLG NTA (Conditions: $[M]_0:[I]_0 = 80:1$, 50 ℃, hexanes suspension).
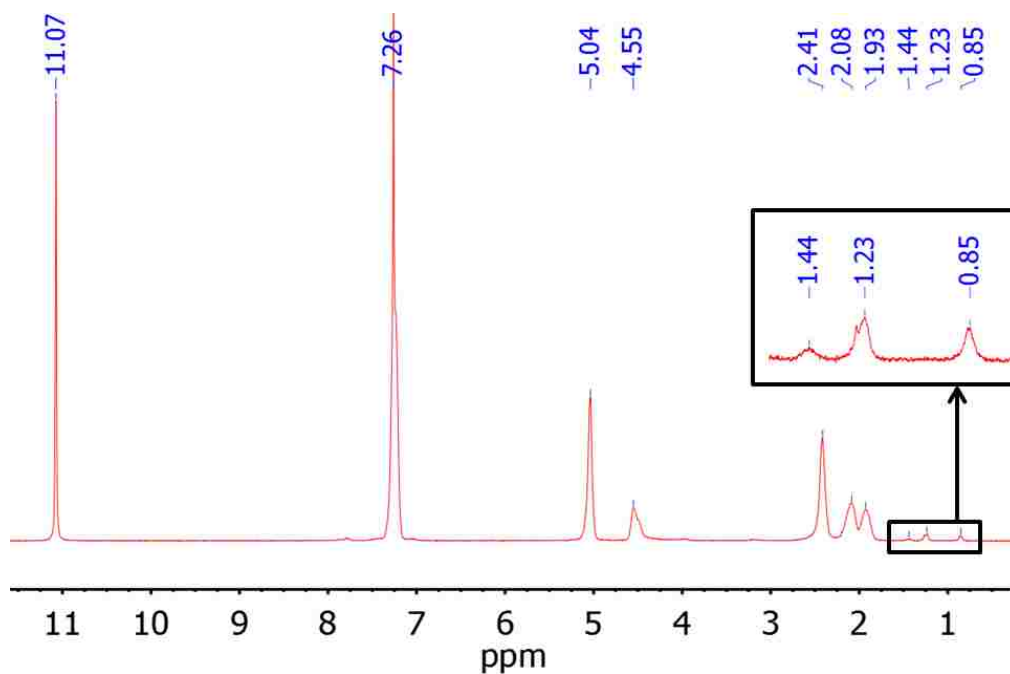
Figure S3.28. Representative ^{1}H NMR spectrum of PBLG polymer obtained from solid-phase polymerization of BLG NTA (CDCl$_3$:TFA-d$_1$=95:5, v/v).

CHAPTER 4. SOLUTION-PHASE RING-OPENING POLYMERIZATION OF *N*-THIOCARBOXYANHYDROSULFIDES

4.1 Background and introduction

Polypeptides have been intensively investigated to be used in different applications due to their combined merits such as unique secondary structures (e.g. α-helix, β-sheet), structure diversity, backbone degradability, etc.[3,96,148] Polypeptides can self-assemble into different secondary conformations based on the nature of the corresponding amino acids. For instance, L-glutamic acid based polypeptides tend to form α-helical conformations,[112] while L-serine based polypeptides tend to form β-sheet conformations[70]. These unique secondary structures are of vital importance for their applications.[2] For example, they have been studied for their potential applications in gene delivery[2] and antimicrobial,[71] antifouling[75]. Well-defined high molecular weight polypeptides are generally prepared through ROP of NCA with different initiation systems.[17-28] However, one major limitation of polypeptide synthesis that the community faces is the stability of the NCA monomers. Their moisture and thermal instability issue limits facile preparation and large scale industrial production.

Previous researchers have proposed alternative methods to prepare polypeptides in order to avoid the afore mentioned problems. Endo et al have reported the synthesis of polypeptides using activated amino acid urethane derivatives which was proposed to form NCAs in situ and polymerize in a chain growth fashion in presence of primary amine initiator.[56] However, the polypeptides are mostly limited to low to moderate molecular weight (DP<100). Kricheldorf investigated the polymerization activity of some NTAs (Gly NTA, L-Phe NTA, L-Leu NTA, Sar NTA) through ROP. However only low conversion can be achieved even for targeted M/I = 60, and the resulting product is only oligomer. In Chapter 3, I demonstrated the preparation of polypeptides via solid-phase ROP of NTAs initiated by hexylamine. This is the first example of

polypeptides preparation under mild conditions via controlled ROP of NTAs. Merits of the solid-phase ROP of NTAs include: facile NTA monomer preparation, long shelf life of NTAs, air-friendly ROP conditions and the accessibility of well-defined polypeptides. However, there are also some drawbacks associated with our systems. For instance, sophisticated architectures (e.g. block copolypeptides) can be challenging to prepare, which are important for the applications of polypeptides materials. Block copolypeptides can self-assemble to form various micro structures (e.g. micelles,[149] vesicles[150]), which contribute to their biomedical applications such as drug delivery.[4] In addition, the solid-phase ROP of NTAs can only be applied to certain NTAs, where a proper poor solvent is available. For instance, Leu NTA cannot be polymerized due to the fact that it is soluble in all common organic solvents. We are motivated by these challenges to explore alternative initiating systems for solution phase polymerization of NTAs in a controlled manner.

As demonstrated in the Chapter 2, all traditional initiators (e.g. primary amine, triethylamine, HMDS, NiBiPyCOD) are not able to mediate efficient polymerization of NTA. Another class of molecule we screened is strong organo-bases, which have been intensively investigated as efficient catalysts for ROP of cyclic monomers (e.g. cyclic esters).[151] Our group has investigated the effect of several organo-bases (*N*-heterocyclic carbene (NHC), 1,8-diazabicycloundec-7-ene (DBU), 1,1,3,3-tetramethylguanidine (TMG)) for the ROP of *N*-substituted NCAs.[152-155] They can either initiate the zwitterionic ROP of *N*-substituted NCA in a controlled manner (Scheme 4.1),[152,154,155] or they can activate the alcohol based initiators via hydrogen bonding to mediate the ROP (Scheme 4.2)[153]. We are motivated by theses previous findings to investigate the effect of organo-bases for the ROP of NTAs. Hence, it will be the focus of this chapter to discuss organo-base mediated ROP of NTAs.

Scheme 4.1. NHC mediated ROP of *N*-substituted NCA (reprinted with permission from reference[152], copyright (2009) American Chemical Society).
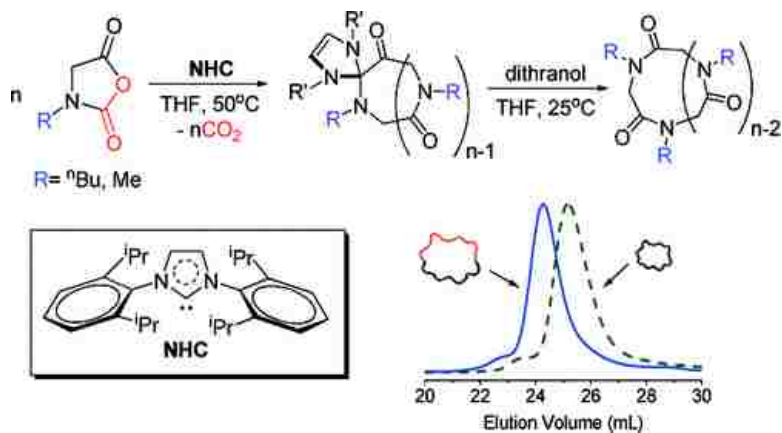
Scheme 4.2. TMG promoted ROP of Bu NCA using alcohol initiators (reprinted with permission from reference[153], copyright (2016) American Chemical Society).
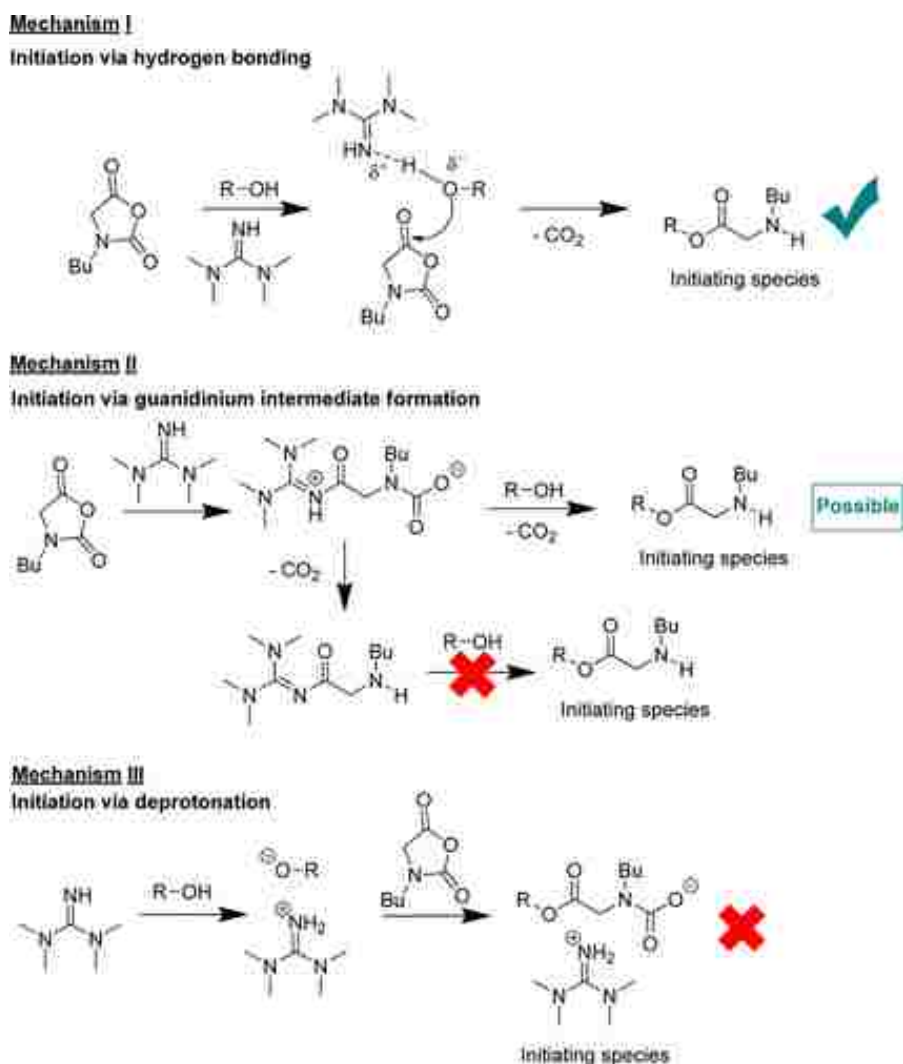
4.2 Materials and Methods

4.2.1 General

All chemicals were purchased from Sigma-Aldrich and used as received unless specified. H-Glu(OBz)-OH and H-Lys(Z)-OH were purchased from AAPPTec, LLC and used as received. All solvents are regular ACS grade solvents and used directly in the reactions without any special drying or purification step unless specified. The BLG NTA and LYS NTA were synthesized according to the procedures described in Chapter 3.

^{1}H and ^{13}C{H} NMR spectra were recorded on a Bruker AV-400 or AV-500 spectrometer. Chemical shifts in ppm were referenced relative to proton impurities or ^{13}C isotope of deuterated solvents (e.g., CDCl$_3$). SEC-DRI analyses were performed with an Agilent 1200 system equipped with three Phenomenex 5 μm, 300 × 7.8 mm columns [100 Å, 1000 Å and Linear(2)], Wyatt DAWN EOS MALS detector (GaAs 30 mW laser at λ=690 nm) and Wyatt Optilab rEX DRI detector with a 690 nm light source. DMF containing 0.1 M LiBr was used as the eluent at a flow rate of 0.5 mL min^{-1}. The temperature of the column and detector was 25 ℃. ESI MS was conducted on an ESI TOF 6210 (Electrospray Time-of-Flight) mass spectrometer (Agilent Technologies). Samples were prepared by dissolving 5 mg sample in 0.5 mL chloroform. The experiments were carried out in positive mode ionization. MLADI-TOF MS experiments was conducted on a Bruker UltrafleXtreme tandem time-of-flight (TOF) mass spectrometer. The instrument was calibrated with Peptide Calibration Standard II consisting of standard peptides Angiotensin I, Angiotensin II, Substance P, Bombesin, ACTH clip 1-17, ACTH clip 18-39, and Somatoratin 28 (Bruker Daltonics, Billerica, MA) prior to experiment. A saturated methanol solution of α-cyano-4-hydroxycinnamic acid was used as matrix. Samples were prepared by mixing 5 mg/ml THF solution of polymers with matrix at 1:1 volume ratio, which were then deposited onto a 384-well ground-steel sample plate using the dry droplet

method. Experiments were done in positive reflector mode. The data analysis was performed with flexAnalysis software.

4.2.2 Representative procedure for polymerization of NTA in solution

A representative polymerization was conducted as followed. BLG NTA (53.6 mg, 0.192 mmol) was dissolved in anhydrous dioxane (0.21 mL) under nitrogen atmosphere in glovebox. A measured volume of a stock solution of benzoic acid in dioxane (95.4 μL, 19.2 μmol, 201.3 mM) was added to the above solution. The mixture remained as a clear solution. Then a measured volume of a stock solution of TMG in dioxane (75.2 μL, 3.84 μmol, 51.1 mM) was added to the above clear solution. The polymerization was stirred at room temperature until 100% conversion has reached. The conversion was tracked via ^{1}H NMR via sampling a reaction aliquot. The white solid polymer was isolated by precipitation into diethyl ether, followed by filtration, diethyl ether wash and vacuum dry (30.6 mg, yield: 73%).

4.2.3 Representative procedure for the polymerization chain extension experiment

A representative chain extension experiment was conducted as followed. BLG NTA (59.5 mg, 0.213 mmol) was dissolved in anhydrous dioxane (0.24 mL) under nitrogen atmosphere in glovebox. A measured volume of a stock solution of benzoic acid in dioxane (106 μL, 21.3 μmol, 201.3 mM) was added to the above solution. The mixture remains as clear solution. Then measured volume of a stock solution of TMG in dioxane (83.5 μL, 4.27 μmol, 51.1 mM) was added to the above clear solution. The polymerization was stirred at room temperature for 2.5 hours. 10 μL reaction mixture was sampled for conversion check via ^{1}H NMR, which indicates 100% conversion of BLG NTA. Then 50 μL reaction mixture was taken for GPC analysis directly by dissolving into 0.95 mL DMF containing 0.1 LiBr. Additional BLG NTA (105.5 mg, 0.378mmol) was then added to the above reaction mixture, followed by addition of 0.39 mL anhydrous dioxane. The ratio of the second batch of BLG NTA over TMG

is 103, and the starting concentration of the second batch of BLG NTA is 0.5 M. 20 hours later, ^{1}H NMR indicated nearly quantitative conversion was reached. The white solid polymer was isolated by precipitation into diethyl ether, followed by filtration, diethyl ether wash and vacuum dry (87.9 mg, yield, 68%).

4.3 Results and Discussion

4.3.1 Early study on organo-bases catalyzed ROP of BLG NTA

The initial study of organo base mediated polymerization of BLG NTA was conducted in presence of primary amine. The base used in the co-initiator is TBD. TBD can activate NTA either by hydrogen bonding or by ring opening addition to form an activated monomer intermediate. The polymerization results were promising as full conversion can be reached unlike primary amine initiator alone resulting in partial conversion. However, the M_n control was poor. The obtained MW of PBLG was much higher than the theoretical MW calculated from the ratio of BLG NTA over primary amine. In a control study, high molecular weight PBLG (45.4 kg/mol) was obtained when TBD alone was used as the initiator when [BLG NTA]$_0$:[TBD]$_0$ = 50. It encouraged me to screen the effect of similar organo-bases such as NHC and TMG. Similarly, both NHC and TMG are able to mediate the ROP of BLG NTA to afford high molecular weight PBLG. Interestingly, by varying the loading of organo-bases, the molecular weight of the final PBLG can be manipulated in the range of 13.9 kg/mol - 52.6 kg/mol with a narrow PDI (<1.2). There is one thing worth pointing out for the early study. The BLG NTA monomer used for the early polymerization study was purified by recrystallization from THF/hexanes. With rigorous purification of the BLG NTA via column chromatography later on, the above results cannot be reproduced. TMG mediated polymerization of the rigorously purified BLG NTA resulted in broad MW distribution of PBLG, and a low rate of polymerization. I speculated there might be some impurities in the previous prepared BLG NTA samples that enable the enhanced

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polymerization activity of BLG NTA. Hence, I introduced several additives (e.g. benzoic acid, benzyl alcohol, sodium bicarbonate, etc.) into TMG mediated ROP of BLG. Surprisingly, the addition of benzoic acid greatly increased the polymerization rate (Table 4.1). When 1 mol % of benzoic acid was added to the TMG mediated polymerization of BLG NTA ($[M]_0:[I]_0 = 50$) in dioxane at room temperature under nitrogen atmosphere, over 95% conversion was observed in 12h. In comparison, only 27% conversion was reached without benzoic acid in 12h. Benzoic acid alone does not initiate polymerization of BLG NTA. This finding inspired me to further investigate the TMG/BnCOOH co-initiation system mediated ROP of NTAs.

Table 4.1. Effect of benzoic acid in TMG mediated ROP of BLG NTA.

$[BLG\ NTA]_0$	Loading	Conversion[a.]	Time
0.5 M	$[BLG\ NTA]_0 : [TMG]_0 = 50 : 1$	27%	12h
0.5 M	$[BLG\ NTA]_0 : [BnCOOH]_0 = 50 : 1$	0%	12h
0.5 M	$[BLG\ NTA]_0 : [BnCOOH]_0 : [TMG]_0 = 50 : 0.5 : 1$	95%	12h

[a.] determined through ^{1}H NMR

4.3.2 TMG/BnCOOH mediated ROP of NTAs

The TMG/BnCOOH co-initiation system was demonstrated to mediate ROP of NTAs. The effect benzoic acid loading on polymerization was then investigated. It was found that the loading of benzoic acid affects the MW as well as the distribution of MW. In the study, ratio of BLG NTA over TMG was kept constant at 50, the ratio of benzoic acid over TMG ranged from 0 to 5. The GPC traces of the PBLGs are shown in Figure 4.1. From the GPC traces, it can be seen that there is small molecular weight species at low loading of benzoic acid. The low molecular weight species disappears when the ratio of BnCOOH/TMG is kept at 5. Hence, a BnCOOH /TMG ratio is kept at 5 in the following investigations as standard condition with an initial 0.5 M monomer concentration in dioxane at room temperature.
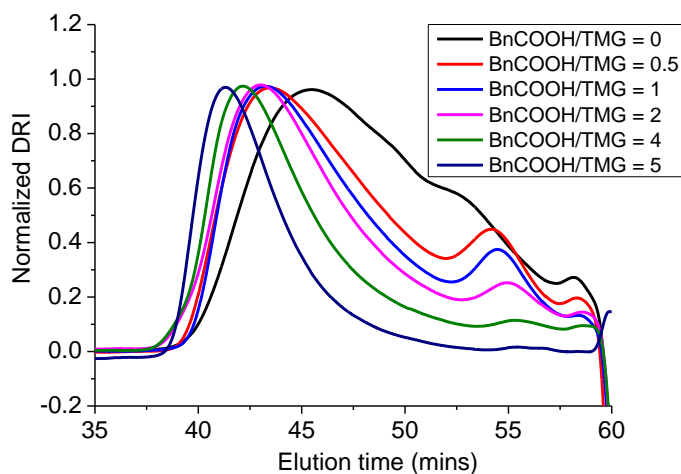
Figure 4.1. SEC chromatograms of PBLG polymers obtained by TMG/BnCOOH mediated ROP of BLG NTA with different loadings of BnCOOH (BLG NTA/TMG = 50)

Under the standard condition, a series of polymerizations with different BLG NTA to TMG ratios were set up. The polymerization results (i.e. M_n, PDI) are shown in Table 4.2 and Figure 4.2. It can be seen that molecular weight of PBLG increases with the increase of BLG NTA to TMG loading. However, it is not a simple linearly relationship (Figure 4.3); the polymerization is clearly not controlled ROP. The molecular weight distribution is narrow indicated by the small PDI (<1.12). The polymerization rate is also fast, as PBLG of high molecular weight (67 kg/mol) can be prepared in less than one day under mild condition. Though the polymerization proceeds in an uncontrolled manner, narrowly distributed polypeptides of various MW can still be obtained by this method.

Table 4.2. TMG mediated ROP of BLG NTA with benzoic acid catalyst in dioxane at room temperature.

Entry	$[M]_0$	$[M]_0:[I]_0$	M_n[a.] kg/mol	PDI[a.]	Conversion[b.]	Time
1	0.5 M	20	33.6	1.12	100%	2.5 h
2	0.5 M	50	35.6	1.05	100%	2.5 h
3	0.5 M	100	42.7	1.08	100%	15 h
4	0.5 M	150	66.7	1.11	100%	15 h

[a.] Obtained through SEC with dn/dc = 0.1292 mL/g; [b.] obtained via ^{1}H NMR

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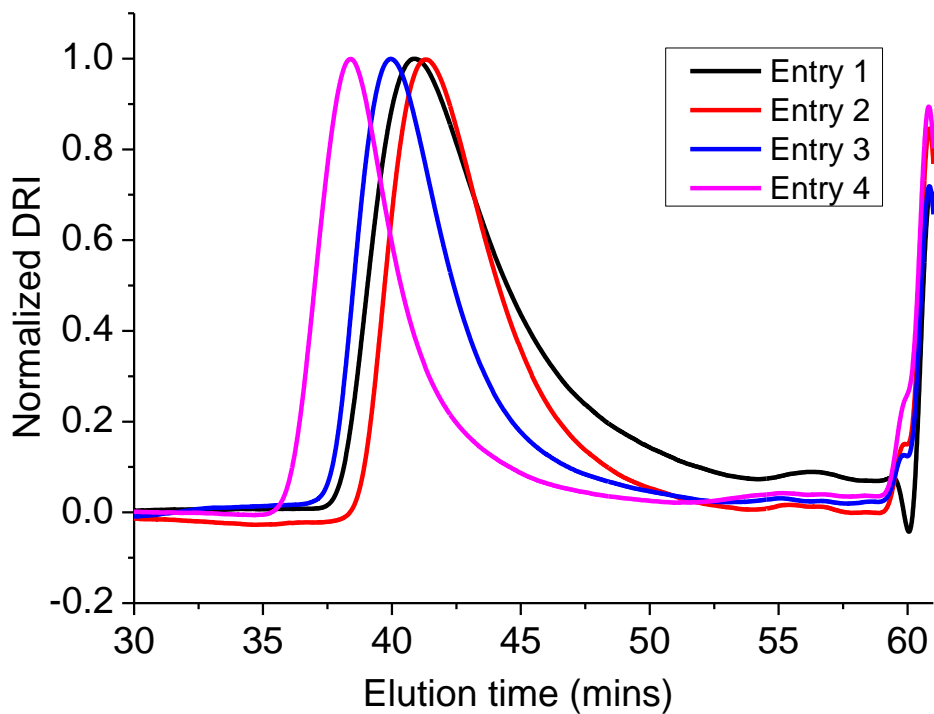
Figure 4.2. Representative SEC chromatograms of PBLG (corresponds to Table 4.1) polymers obtained by TMG/BnCOOH mediated ROP of BLG NTA.
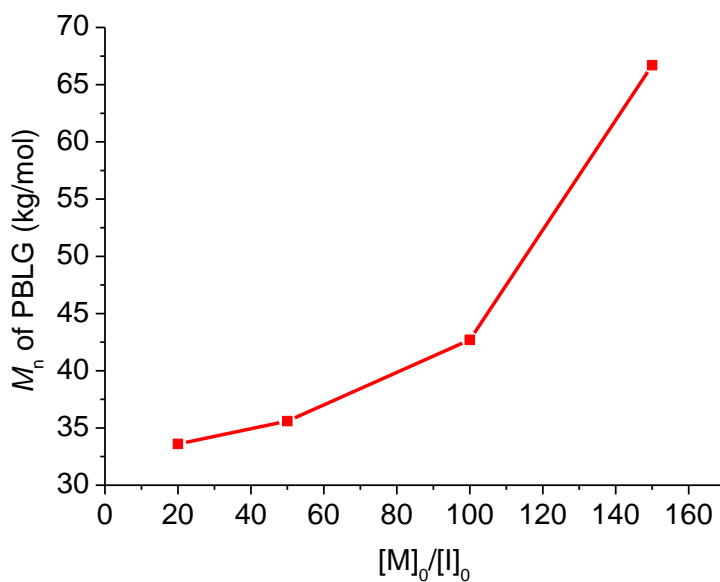
Figure 4.3. Plot of M_n versus $[M]_0:[I]_0$ for the TMG/BnCOOH mediated ROP of BLG NTA in dioxane at room temperature.

Further chain extension studies reveal that block copolymers also can be made via this system. In the chain extension study, another batch of BLG NTA was added to the polymerization solution of BLG NTA when conversion reached 100%. The GPC traces clearly show an increase in the hydrodynamic volume of PBLG, which indicates the second batch of BLG NTA monomers were added to the existing polymer chains instead of forming new chains (Figure 4.4). The molecular weights characterized via GPC are shown in Table 4.3. Similarly, PBLG-b-PZLL also can be readily prepared via the chain extension (Figure 4.5). Thus, this method overcomes the limitation of solid phase ROP of NTAs, where block copolymers can not be readily accessed.

Table 4.3. TMG mediated chain extension study of BLG NTA with benzoic acid catalyst in dioxane at room temperature.

Entry	$[M]_0$	$[M]_0$:$[C]_0$[a]:$[I]_0$	M_n[b] (kg/mol)	PDI[b]	Conv.[c]
1st batch BLG NTA	0.5	50:5:1	36.5	1.06	100% in 2.5 h
2nd batch BLG NTA	0.5	103:5:1	70.4	1.24	>99% in 20 h

[a.] $[M]_0$ represetnts initial concentration of BLG NTA, $[C]_0$ represents initial concentration of benzoic acid, $[I]_0$ represents initial concentration of TMG. [b.] Obtained through SEC with dn/dc = 0.1292 mL/g; [c.] obtained via ^{1}H NMR
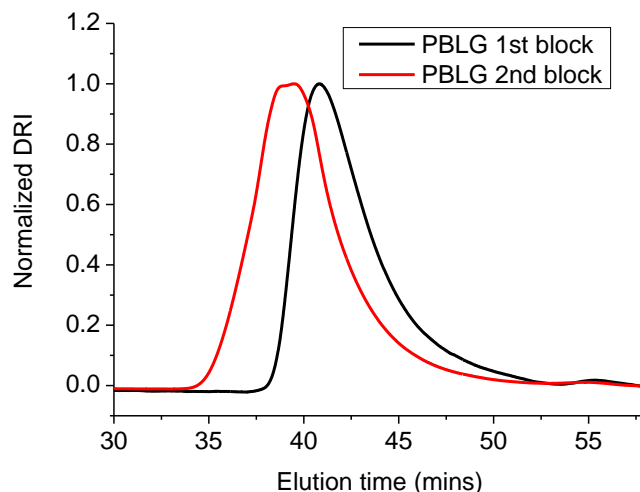
Figure 4.4. Representative SEC chromatograms of PBLG (corresponds to Table 4.3) polymers obtained by TMG/BnCOOH mediated ROP of BLG NTA in dioxane at room temperature for chain extension study.
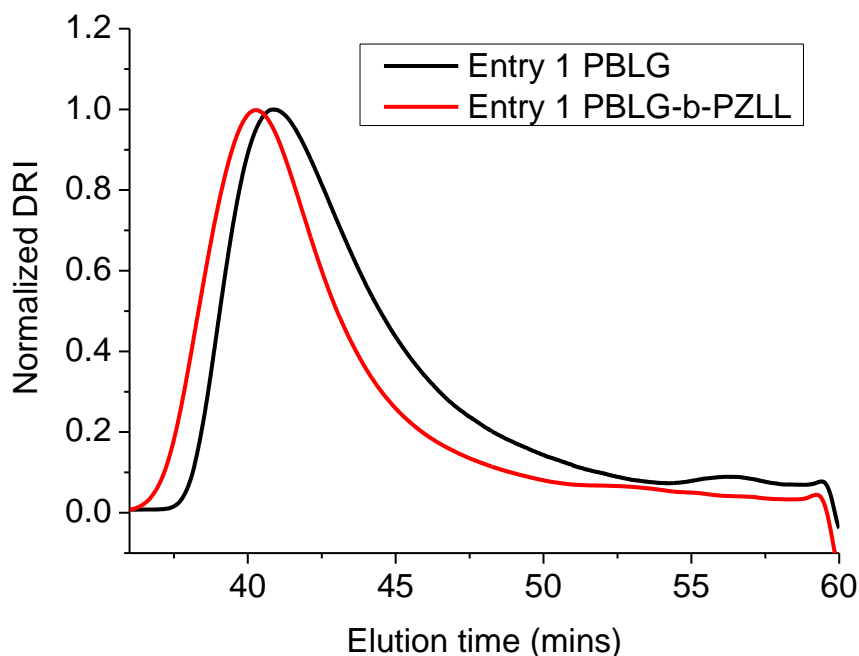
Figure 4.5. Representative SEC chromatograms of PBLG and PBLG-*b*-PZLL polymers obtained by TMG/BnCOOH mediated ROP of BLG NTA and subsequent LYS NTA in dioxane at room temperature for chain extension study.

4.3.3 Mechanism investigation of TMG/BnCOOH mediated ROP of NTAs

To further investigate the mechanism of the TMG/benzoic acid mediated polymerization of NTAs. MALDI-TOF MS, ESI MS and ^{1}H NMR were employed to characterize the PBLG end group structures prepared by the method. Both MALDI-TOF MS and ^{1}H NMR verify the formation of PBLG from TMG/benzoic acid mediated polymerization of BLG NTA. The mechanism of the polymerization was proposed to be AMM initially, as the polymerization shares characteristics of base catalyzed ROP of NCAs. Specifically, only high molecular weight PBLG can be obtained via the TMG/benzoic acid mediated polymerization. In addition, the polymerization propagation is fast as high molecular weight PBLG can be prepared in relatively short period of time.

The MALDI-TOF MS spectrum indicates the polymer has a repeat unit of 219.11 g/mol, which confirms the formation of PBLG (repeat unit MW = 219.09 g/mol) (Figure 4.6). The molecular weights of potential end groups plus dopants are 179.14 g/mol, 68.02 g/mol, 195.11 g/mol based on MALDI-TOF MS analysis. The corresponding structures of the above MW are listed in Figure 4.6C. Surprisingly, dimethylamine is attached to the PBLG after losing one proton. This structure is further verified via ^{1}H NMR (Figure 4.7) with a singlet signal at 3.02 ppm, which corresponds to dimethylamine. The source of the dimethylamine can be elucidated by equimolar BLG NTA and TMG reaction (Scheme 4.1). The reaction mixture was characterized by ESI MS (Figure 4.8). Compounds with M/Z of 116.1186 and 290.1506 are two major species in the ESI MS, which correspond to protonated TMG and protonated compound **1**. Similar result was also observed in equimolar mixtures of TMG and *N*-substituted NCA.[153]

D P1+Na$^+$(1820.90 experimental): 219.09×8+44.08+1+22.99=1820.79 (theo)
P2+Na$^+$(1712.91 experimental): 219.09×7+44.08+112.04+22.99=1712.74 (theo)
P2+K$^+$(1728.88 experimental): 219.09×7+44.08+112.04+38.96=1728.71 (theo)
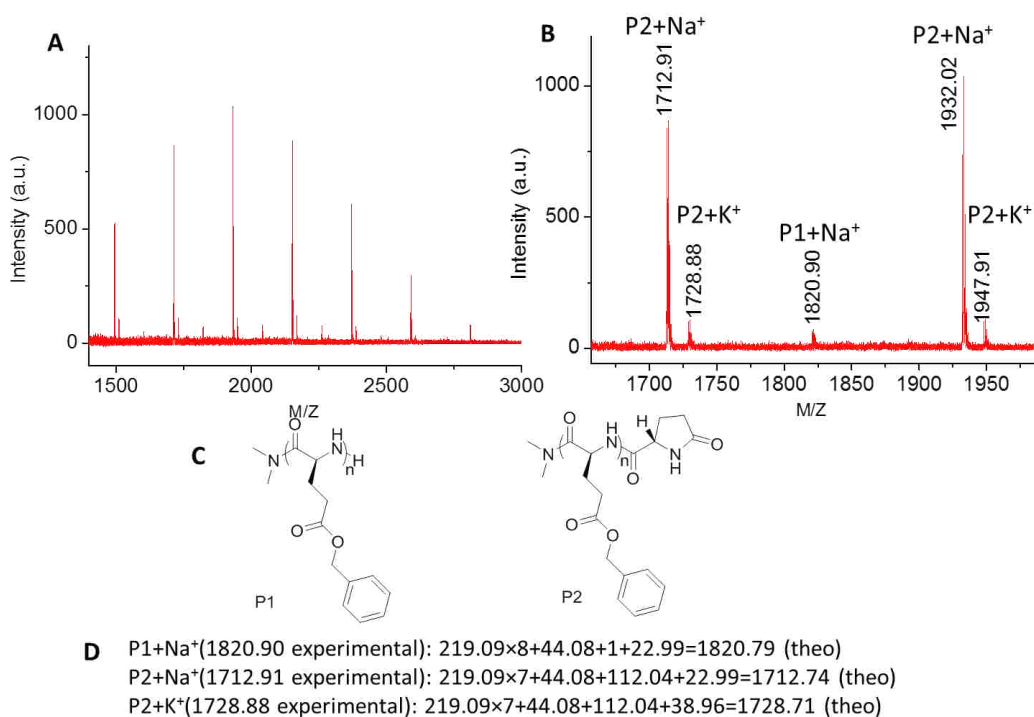
Figure 4.6. MALDI-TOF MS spectra of PBLG prepared via TMG/BnCOOH mediated ROP of BLG NTA in dioxane at room temperature
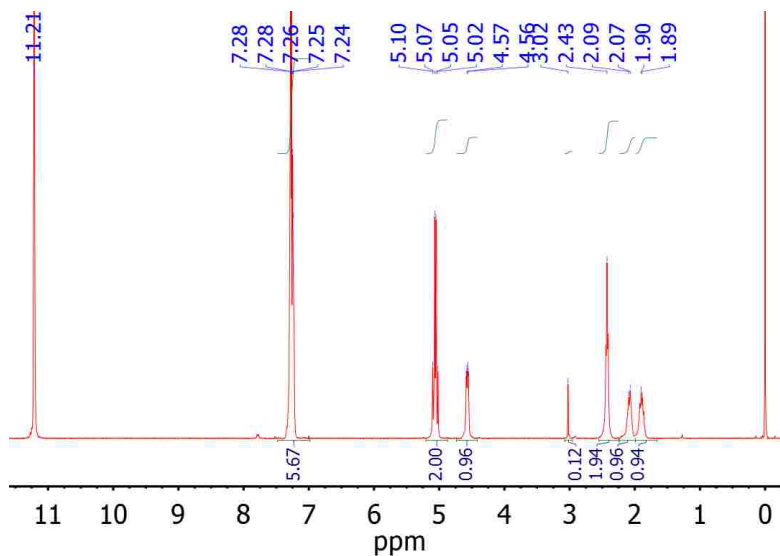
Figure 4.7. Representative ^{1}H NMR spectrum of PBLG polymer obtained from TMG/BnCOOH mediated ROP of BLG NTA in dioxane at room temperature (CDCl$_3$:TFA-d$_1$~70:30, v/v).

Scheme 4.1. Equimolar reaction between BLG NTA and TMG.
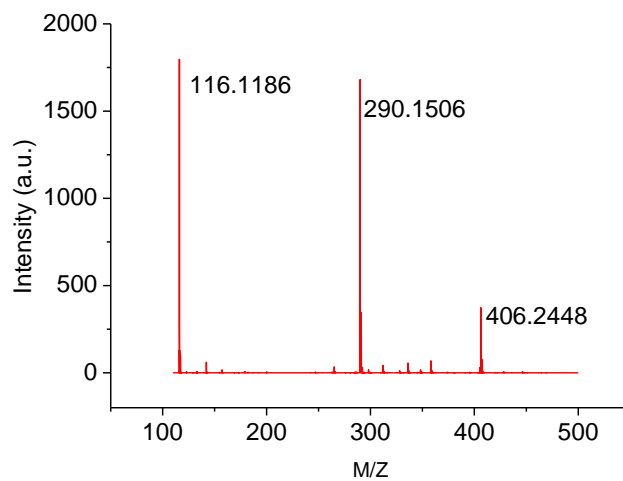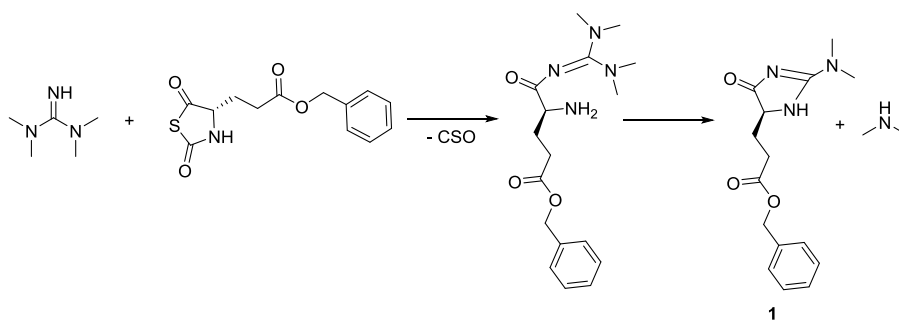
Figure 4.8. ESI MS spectrum of TMG and BLG NTA (1:1) reaction mixture.

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With the source of the dimethylamine elucidated, the next important question to ask is how the dimethylamine attaches to the polymer chain end. There are two possible pathways: NAM and AMM. In the NAM pathway, the dimethylamine acts as nucleophile to initiate chain propagation via NAM. In the AMM pathway, dimethylamine reacts with the terminal *N*-acylated cyclic NTA when the polymerization is finished. The NAM is less likely to account for the polymerization results for following reasons. First, the other end-group of PBLG is mainly pyroglutamate formed via back-biting aminolysis, which is unable to mediate propagation during polymerization to afford high MW PBLG. Secondly, high molecular weight polypeptide (DP>150) is hard to achieve in NAM pathway generally. In addition, benzylamine/BnCOOH co-initiation system was also screened to mediate the ROP of BLG NTA, which was unable to mediate efficient polymerization to afford high MW PBLG.

Considering the high MW polypeptide and relative short polymerization time, the AMM pathway is more likely to be responsible for TMG/BnCOOH mediated ROP. Importantly, all the structures detected from MALDI-TOF can be explained by the AMM pathway, which will be elaborated below. PBLG with structure (**2**) shown in Scheme 4.2 will form first during polymerization via the AMM pathway. Following the completion of the chain propagation, dimethylamine will react with the terminal *N*-acylated cyclic NTA via nucleophilic attack at C5 carbonyl to form dimethylamine terminated PBLG (**3**). The PBLG with pyroglutamate (P2 in Figure 4.6) as the other terminal group is formed by transamidation, which has been discussed in Chapter 1. It is worth mentioning that hydroxyl terminal group was also detected in some PBLG samples. The formation pathway is also similar to the dimethylamine one. During the polymerization purification or MALDI-TOF sample preparation, moisture in air or solvent may react with the terminal *N*-acylated cyclic NTA to form hydroxyl terminal groups. The existence

of both dimethylamine and hydroxyl groups as terminal groups can only be explained via the AMM pathway at this point. The terminal dimethylamine and hydroxyl groups serve as indirect evidence for the presence of *N*-acylated cyclic NTA during polymerization.

Scheme 4.2. Formation of dimethylamine terminated PBLG in TMG/benzoic acid mediated ROP of BLG NTA.
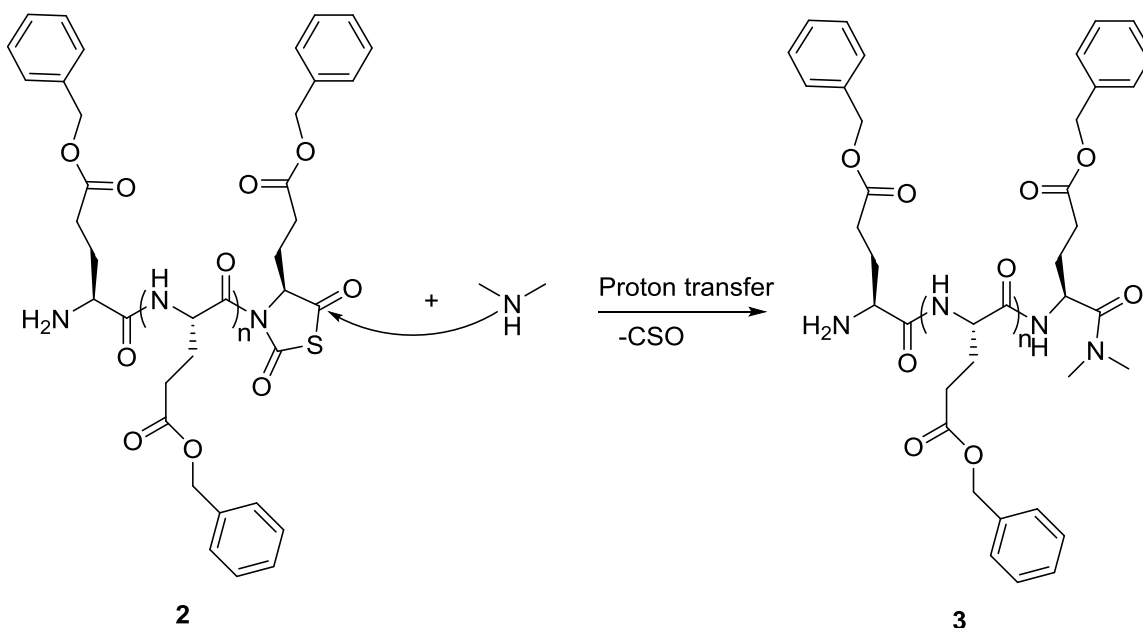
Another critical question to ask is the source of base used to deprotonate the NTA during polymerization, as acid benzoic acid is also present in addition to base TMG. To answer this question, it is important to know the pKa values. In organic solvent acetonitrile, benzoic acid has a pKa of 21.5[156] while conjugate acid of TMG has a pKa of 23.3[157]. The pKa difference is less than 2 orders of magnitude, which indicates free TMG is present at the beginning of the polymerization to deprotonate NTA to undergo polymerization via AMM. If benzoic acid is replaced by a stronger acid chloroacetic acid (pKa = 18.8[158]), then high MW polypeptide can not be accessed and the polymerization can only reach partial monomer conversion. Hence, the stronger acid may protonate the TMG completely, which will inhibit the polymerization via AMM pathway.

Lastly, the effect of benzoic acid in the system remains unclear at this point. However, one possible role of benzoic acid is hydrogen bonding donor which can activate the *N*-acylated cyclic NTA through hydrogen bonding. As discussed in Chapter 2, NTAs are generally more stable than NCA analogs. In other words, NTAs are less electrophilic. It is also possible for the *N*-acylated cyclic NTA to be not electrophilic enough for the negatively charged NTA to attack. Once, the hydrogen bonding is formed between benzoic acid and terminal *N*-acylated cyclic NTA, the activated *N*-acylated cyclic NTA will subsequently react with negatively charged NTA. The hydrogen bonding formation is modeled via benzoic acid and BLG NTA. The chemical shift of hydroxyl group in benzoic acid will shift from 12.5 ppm to 9.9 ppm when mixed with BLG NTA in 1:1 ratio in $CDCl_3$. This upfield shift is clearly a result of hydrogen bonding. However, whether the hydrogen bonding formation is solely responsible for the enhanced polymerization rate with benzoic acid remains to be explored further. For instance, various hydrogen bonding donor compounds can be added to the TMG mediated ROP of NTA to screen their effects.

4.4 Conclusion

In summary, polypeptides of high molecular weight can be accessed in TMG/benzoic acid mediated polymerization of NTAs. The loading of benzoic acid in the polymerization will affect both the molecular weight and molecular weight distribution. Higher loading of benzoic acid affords high molecular polypeptides with narrow molecular weight distribution. The polypeptide molecular weight will also be affected by the ratio of NTA monomer over TMG. Higher ratio of BLG NTA over TMG will contribute to polypeptide with higher molecular weight. PBLG with different molecular weights (33.6 kg/mol - 66.7 kg/mol) and narrow molecular weight distribution (PDI < 1.12) can be readily prepared under mild condition. In addition, block copolypeptides also can be prepared via sequential addition of NTA monomers. The polymerization mechanism was proposed to be similar to AMM observed in base catalyzed

ROP of NCA, which was verified by MALDI-TOF MS and ^{1}H NMR. Considering the facile preparation and long shelf life of NTA monomers, commercial availability of the initiators, this solution phase polymerization of NTAs mediated by TMG/benzoic co-initiation system represents a promising system to be widely employed both in large industrial scale preparation as well lab scale synthesis.

CHAPTER 5. CONCLUSIONS AND FUTURE WORK

The main objective of this work is to develop new synthetic strategies toward advanced functional polypeptides or polypeptides in general. The main motivation originates from the appealing properties of polypeptides and their broad applications in different fields (e.g. gene delivery,[2,87] drug delivery,[98] antimicrobial[71] and etc.). The individual research topic is directed by currently existing issues in the discipline. Chapter 1 highlighted the recent progress in the polypeptide based functional materials as well as fundamental aspects about polypeptides. In addition, it also shines light on the issues that should be addressed to further facilitate the development of polypeptides.

In Chapter 2, the focus is to develop a new class of functional polypeptide which combines several desired attributes for biomedical applications into one synthetic platform. These features include: clickable pendant side chains for further functionalization, good water solubility, non-ionic nature to avoid unspecific interactions in biological systems, and unique secondary conformations (e.g. α-helix, β-sheet). Two strategies were designed to achieve the goal. The first method is straight forward, which involves copolymerizing two NCA monomers (PLG NCA and EG$_2$-LYS NCA) via benzyl amine initiated ROP. The afforded polypeptides maintain stable α-helical conformations in aqueous solution (up to 50% hydrophobic segment PPLG) and in solid state. The copolymerization strategy produces polypeptides having tunable densities of "clickable" sites which allow for further conjugation of hydrophobic or hydrophilic moieties. It was shown that the hydrophobically and hydrophilically-modified polypeptide conjugates retain high level of α-helical conformations. The second method is to develop a new L-serine based NCA (Ser NCA) that contains a water-soluble ethylene glycol unit as well as a clickable azido pedant side chains. Upon polymerization, the corresponding polymer is expected to maintain aforementioned features. The secondary structure is expected to be β-sheet based on

previous study.[70] The Ser NCA monomer synthesis route has been established. The purification and following polymerization activity of the Ser NCA should be the topic of future direction. The interrupted continuous investigation of Ser NCA is a result of the work in Chapter 3 and 4, the success of which will have significant scientific impact.

The focus of the Chapter 3 is to develop the first controlled polymerization of amino acid derived NTAs under mild condition to prepare polypeptides. Several model NTA monomers (e.g. BLG NTA, LYS NTA) were synthesized for the first time. They were found to possess better thermal and moisture stability as compared to NCA analogs, which results in a long shelf-life for NTAs. In the polymerization study, polypeptides with controlled M_n and PDI can be obtained from the solid phase ROPs of NTA monomers using soluble primary amine initiators under mild conditions. This stands as the first example of polypeptide preparation through ROP of NTAs with regular reagents in open air. The sROP reactions proceed by a normal amine mechanism. The controlled polymerization behavior of sROP is the direct result of high local monomer concentration in the solid phase, thus allowing for faster polymerization under relatively mild conditions. The future direction of this topic is optimization of NTA synthesis to obtain optically pure monomer. It was found that enantiomerically pure L-Leu NTA can be synthesized, while racemization occurred to a large extent during BLG NTA and LYS NTA synthesis even though the conditions are identical. As optically pure NTAs are vital for the self-assembly of polypeptides into secondary structures, it would be of great interest to optimize the NTA synthesis conditions to prepare optically pure NTAs.

The work in Chapter 4 expands the solid phase ROP of NTA to solution phase, which may have general applicability towards block copolypeptides synthesis. In summary, PBLG with different molecular weights (33.6 kg/mol - 66.7 kg/mol) and narrow molecular weight (PDI <

1.12) can be readily prepared with TMG/benzoic acid as co-initiation system under mild conditions. The polypeptide molecular weight can be adjusted by controlling the ratio of NTA monomer over TMG. Higher ratio of BLG NTA over TMG will contribute to polypeptide with higher molecular weight, however there is no linearly relationship found between the NTA/TMG ratio and final polypeptide molecular weight. In addition, block copolypeptides also can be prepared via sequential adding NTA monomers. The mechanism of the TMG/benzoic acid mediated ROP of NTAs is proposed to be AMM. However, further investigation is necessary to elucidate the mechanism. For instance, what is the role of benzoic acid? What are the effects of other bases (e.g. triethylamine, sodium methoxide) traditionally used in ROP of NCA? Nevertheless, considering the stability and facile preparation of NTA monomers, the TMG/benzoic acid mediated solution phase ROP of NTA is a promising alternative of NCAs for high molecular weight polypeptide preparations.

Another attractive research direction based on NTAs is the preparation of functional NTAs that can afford polypeptides with various functionalities via ROP. The ROP of functional NCA is one of the two strategies toward functional polypeptides as discussed in the Chapter 1. The application of this method is limited by several factors with one of them being the poor stability of NCAs that requires rigorously anhydrous conditions for synthesis, purification and polymerization. The development of NTA polymerization in this work can potentially drive the further development of functional polypeptides preparation via direct ROP of functional NTAs. The good stability of NTAs allows direct derivatizations to introduce different functionalities, which can be subsequently polymerized via the two ROP systems developed in this work to prepare functional polypeptides. The biggest advantage of this strategy is that it allows one to prepare polypeptides with 100% functionalized side chains. In addition, it also enables facile

controlled incorporation of various functionalities into polypeptides via copolymerization strategies. Various copolypeptides systems (e.g. random copolypeptide, block copolypeptide) can be manipulated by copolymerization techniques such as one-pot copolymerization or sequential adding monomer copolymerization.

In summary, one type of functional polypeptide has been successfully prepared via direct ROP of NCAs. Two polymerization systems have been developed to prepare polypeptides of different molecular weights and narrow molecular weight distribution via the ROP of NTAs. The commercial availability of initiators and mild polymerization conditions make the two polymerization systems promising for large scale polypeptide preparation if desired.

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Author: Jing Sun, Helmut Schlaad

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Author: Hua Lu, Yugang Bai, Jing Wang, et al

Publication: Macromolecules

Publisher: American Chemical Society

Date: Aug 1, 2011

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VITA

Jinbao Cao was born and raised in Zizhou, Shaanxi, China. Jinbao received his Bachelor of Science degree in polymer science and engineering from University of Science and Technology of China in 2010. Jinbao joined Professor Donghui Zhang's group in 2011 to start his graduate research at Louisiana State University. His research interests focus on the development of new synthetic strategies towards polypeptides. A novel polypeptides preparation method via NTAs was discovered by Jinbao. He anticipates graduating with his Ph.D. degree in August 2016.