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Synthesis of Polypeptides by Ring-Opening Polymerization of α-Amino Acid *N*-Carboxyanhydrides

Jianjun Cheng and Timothy J. Deming

Abstract This chapter summarizes methods for the synthesis of polypeptides by ring-opening polymerization. Traditional and recently improved methods used to polymerize α-amino acid *N*-carboxyanhydrides (NCAs) for the synthesis of homopolypeptides are described. Use of these methods and strategies for the preparation of block copolypeptides and side-chain-functionalized polypeptides are also presented, as well as an analysis of the synthetic scope of different approaches. Finally, issues relating to obtaining highly functional polypeptides in pure form are detailed.

Keywords Amino acid · Block copolymer · *N*-Carboxyanhydride · Polymerization · Polypeptide

Contents

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Abbreviations

AM	Activated monomer
Bn-Glu	γ-Benzyl-L-glutamate
bpy	2,2′-Bipyridyl
CD	Circular dichroism
COD	1,4-Cyclooctadiene
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediamine tetraacetic acid
EG	Ethylene glycol
FTIR	Fourier transform infrared spectroscopy
GPC	Gel permeation chromatography
GTP	Group transfer polymerization
HMDS	Hexamethyldisilazane
MS	Mass spectroscopy
NACE	Non-aqueous capillary electrophoresis
NCA	α-Amino acid-*N*-carboxyanhydride
PBLG	Poly(γ-benzyl L-glutamate)
PDMS	Poly(dimethylsiloxane)
PEG	Poly(ethylene glycol)
TFA-Lys	ε-Trifluoroacetyl-L-lysine
THF	Tetrahydrofuran
TMS	Trimethylsilyl
TMSDC	Trimethylsilyl dimethylcarbamate
Z-Lys	ε-Carbobenzyloxy-L-lysine

1 Introduction

Biological systems produce proteins that possess the ability to self-assemble into complex, yet highly ordered structures [1]. These remarkable materials are polypeptide copolymers that derive their properties from precisely controlled sequences and compositions of their constituent amino acid monomers. There has been recent interest in developing synthetic routes for preparation of these natural polymers as well as de novo designed polypeptide sequences to make products for applications in biotechnology (e.g., artificial tissues and implants), biomineralization (e.g., resilient, lightweight and ordered inorganic composites), and analysis (e.g., biosensors and medical diagnostics) [2, 3].

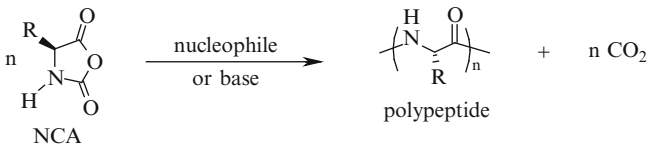
To be successful in these applications, it is important that materials can self-assemble into precisely defined structures. Peptide-based polymers have many advantages over conventional synthetic polymers since they are able to hierarchically assemble into stable, ordered conformations [4]. Depending on the substituents of the amino acid side chain, polypeptides are able to adopt a multitude of

conformationally stable, regular secondary structures (e.g., helices, sheets, and turns), tertiary structures (e.g., the β-strand-helix–β-strand unit found in β-barrels), and quaternary assemblies (e.g., collagen microfibrils) [4]. The synthesis of polypeptides that can assemble into non-natural structures is an attractive challenge for polymer chemists.

Synthetic peptide-based polymers are not new materials: homopolymers of polypeptides have been available for many decades but have only seen limited use as structural materials [5, 6]. However, new methods in chemical synthesis have made possible the preparation of increasingly complex polypeptide sequences of controlled molecular weight that display properties far superior to ill-defined homopolypeptides [7]. These polymers are well suited for applications where polymer assembly and functional domains need to be at length scales ranging from nanometers to micrometers. These block copolymers are homogeneous on a macroscopic scale, but dissimilarity between the block segments typically results in microphase heterogeneity yielding materials useful as surfactants, micelles, membranes, and elastomers [8]. Synthesis of simple hydrophilic/hydrophobic hybrid diblock copolymers, when dispersed in water, allows formation of peptide-based micelles and vesicles potentially useful in drug and gene delivery applications [9, 10]. The regular secondary structures obtainable with the polypeptide blocks provide opportunities for hierarchical self-assembly that are unobtainable with typical block copolymers or small-molecule surfactants.

Upon examining the different methods for polypeptide synthesis, the limitations of these techniques for preparation of well-defined copolymers readily become apparent. Conventional solid-phase peptide synthesis is neither useful nor practical for direct preparation of large polypeptides (> 100 residues) due to unavoidable deletions and truncations that result from incomplete deprotection and coupling steps. The most economical and expedient process for synthesis of long polypeptide chains is the polymerization of α-amino acid-*N*-carboxyanhydrides (NCAs) (Scheme 1) [11, 12]. This method involves the simplest reagents, and high molecular weight polymers can be prepared in both good yield and large quantity with no detectable racemization at the chiral centers. The considerable variety of NCAs that have been synthesized (> 200) allows exceptional diversity in the types of polypeptides that can be prepared [11, 12].

Since the late 1940s, NCA polymerizations have been the most common technique used for large scale preparation of high molecular weight polypeptides [13]. However, these materials have primarily been homopolymers, random copolymers, or graft copolymers that lack the sequence specificity and monodispersity of natural



Scheme 1 Polymerization of α-amino acid-*N*-carboxyanhydrides (*NCA*)

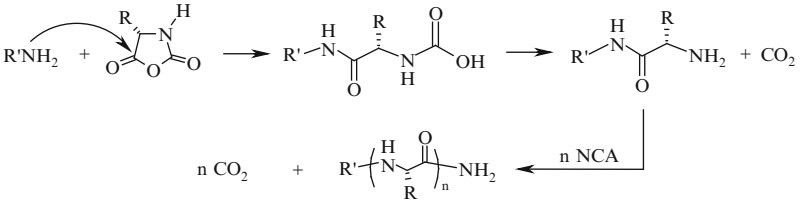
proteins. Until recently, the level of control in NCA polymerizations has not been able to rival that attained in other synthetic polymerizations (e.g., vinyl addition polymerizations) where sophisticated polymer architectures have been prepared (e.g., stereospecific polymers and block copolymers) [14]. Attempts to prepare block copolypeptides and hybrid block copolymers using conventional NCA polymerization has usually resulted in polymers whose compositions did not match monomer feed compositions and that contained significant homopolymer contaminants [15–17]. Block copolymers could only be obtained in pure form by extensive fractionation steps, which significantly lowered the yield and efficiency of this method. The limitation of NCA polymerizations has been the presence of side reactions (chain termination and chain transfer) that restrict control over molecular weight, give broad molecular weight distributions, and prohibit formation of well-defined block copolymers [18]. Recent progress in elimination of these side reactions has been a major breakthrough for the polypeptide materials field. A variety of metal- and organo-catalysts have been developed and utilized in recent years for the formation of multiblock polypeptides or polypeptide-containing hybrid materials with well-defined structures via controlled polymerization of NCAs [19–22].

2 Polypeptide Synthesis Using NCAs

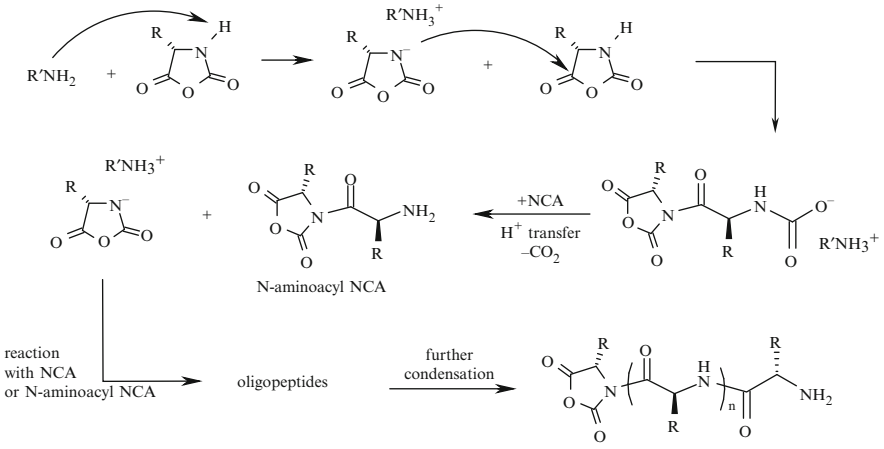
2.1 Conventional Methods

NCA polymerizations are traditionally initiated using many different nucleophiles and bases, the most common being primary amines and alkoxide anions [11, 12]. Primary amines, being more nucleophilic than basic, are good general initiators for polymerization of NCA monomers. Tertiary amines, alkoxides, and other initiators that are more basic than nucleophilic have found use since they are in some cases able to prepare polymers of very high molecular weights where primary amine initiators cannot. Optimal polymerization conditions have often been determined empirically for each NCA and thus there have been no universal initiators or conditions by which to prepare high molecular weight polymers from any monomer. This is in part due to the different properties of individual NCAs and their polymers (e.g., solubility and reactivity) but is also strongly related to the side reactions that occur during polymerization.

The most likely pathways of NCA polymerization are the so-called “amine” and the “activated monomer” (AM) mechanisms [11, 12]. The amine mechanism is a nucleophilic ring-opening chain growth process where the polymer could grow linearly with monomer conversion if side reactions were absent (Scheme 2). On the other hand, the AM mechanism is initiated by deprotonation of an NCA, which then becomes the nucleophile that initiates chain growth (Scheme 3). It is important to note that a given system can switch back and forth between the amine and AM mechanisms many times during a polymerization: a propagation step for one



Scheme 2 Proposed mechanism for NCA polymerization initiated by nucleophilic amines



Scheme 3 Proposed mechanism for NCA polymerization initiated by activated monomers

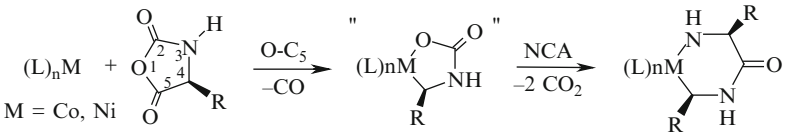
mechanism is a side reaction for the other, and vice versa. It is because of these side reactions that block copolypeptides and hybrid block copolymers prepared from NCAs using amine initiators often have structures different to those predicted by monomer feed compositions and most likely have considerable homopolymer contamination. These side reactions also prevent control of chain-end functionality, which is crucial for preparation of hybrid copolymers.

One inherent problem in conventional NCA polymerizations is that there is no control over the reactivity of the growing polymer chain-end during the course of the polymerization. Once an initiator reacts with a NCA monomer, it is no longer active in the polymerization and the resulting primary amine, carbamate, or NCA anion end group is free to undergo a variety of undesired side reactions. Another problem is associated with the purity of NCA monomers. Although most NCAs are crystalline compounds, they typically contain minute traces of acid, acid chlorides, or isocyanates that can quench propagating chains. The presence of other adventitious impurities, such as water, can cause problems by acting as chain-transfer agents or even as catalysts for side reactions. Overall, the sheer abundance of potential reactions present in reaction media make it difficult to achieve a living polymerization system where only chain propagation occurs.

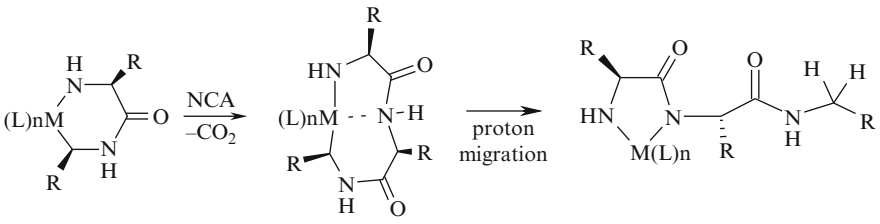
2.2 Transition Metal Initiators

One strategy for eliminating side reactions in NCA polymerizations is the use of transition metal complexes as active species to control addition of NCA monomers to polymer chain-ends. The use of transition metals to control reactivity has been proven in organic and polymer synthesis as a means to increase both reaction selectivity and efficiency [23]. Using this approach, substantial advances in controlled NCA polymerization have been realized in recent years. Highly effective zerovalent nickel and cobalt initiators (i.e., $(PMe_3)_4Co$ [24], and bpyNi(COD), where bpy = 2,2′-bipyridine and COD = 1,5-cyclooctadiene [19]) were developed by Deming that allow the living polymerization of NCAs into high molecular weight polypeptides via an unprecedented activation of the NCAs into covalent propagating species. The metal ions can be conveniently removed from the polymers by simple precipitation or dialysis of the samples after polymerization.

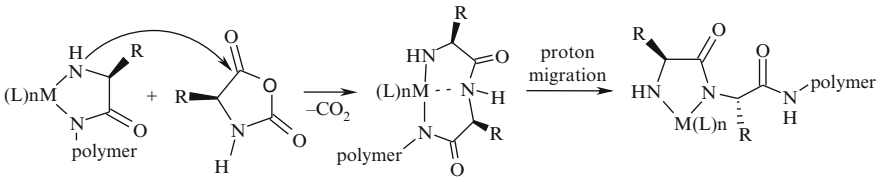
Mechanistic studies on the initiation process showed that both these metals react identically with NCA monomers to form metallacyclic complexes by oxidative addition across the anhydride bonds of NCAs [19, 24, 25]. These oxidative-addition reactions were followed by addition of a second NCA monomer to yield complexes identified as six-membered amido-alkyl metallacycles (Scheme 4). These intermediates were found to further contract to five-membered amido-amidate metallacycles upon reaction with additional NCA monomers. This ring contraction is thought to occur via migration of an amide proton to the metal-bound carbon, which liberates the chain-end from the metal (Scheme 5) [26]. The resulting amido-amidate complexes were thus proposed as the active polymerization intermediates. Propagation through the amido-amidate metallacycle was envisioned to occur by initial attack of the nucleophilic amido group on the electrophilic C_5 carbonyl of an NCA monomer (Scheme 6). This reaction would result in a large metallacycle that



Scheme 4 Oxidative-addition of NCAs to zerovalent cobalt and nickel complexes



Scheme 5 Metallacycle ring contraction mediated by NCA addition

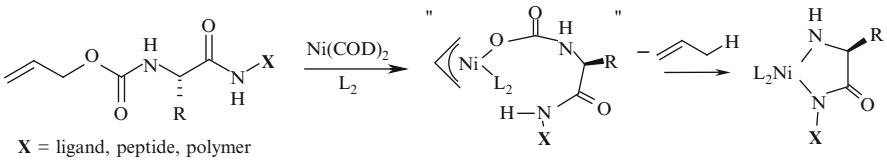


Scheme 6 Chain propagation in transition-metal-mediated NCA polymerization

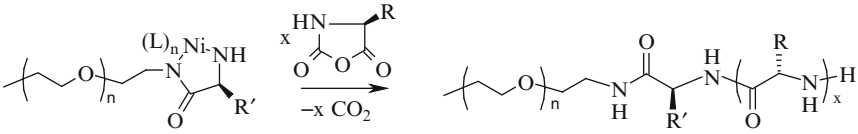
could contract by elimination of CO_2. Proton transfer from the free amide to the tethered amidate group would further contract the ring to give the amido-amidate propagating species, while in turn liberating the end of the polymer chain and becoming available for reaction with the next incoming NCA molecule.

In this manner, the metal is able to migrate along the growing polymer chain, while being held by a robust chelate at the active end. The formation of these chelating metallacyclic intermediates appears to be a general requirement for obtaining living NCA polymerizations using transition metal initiators. These cobalt and nickel complexes are able to produce polypeptides with narrow chain length distributions (given by the polydispersivity index, i.e., the weight-average molecular weight divided by the number-average molecular weight, M_w/M_n, which in this case is < 1.2) and controlled molecular weights ($500 < M_n < 500{,}000$ g/mol) [26]. This polymerization system is very general, and gives controlled polymerization of a wide range of NCA monomers as pure enantiomers (D- or L-configuration) or as racemic mixtures. By addition of different NCA monomers, the preparation of block copolypeptides of defined sequence and composition is feasible [7, 27].

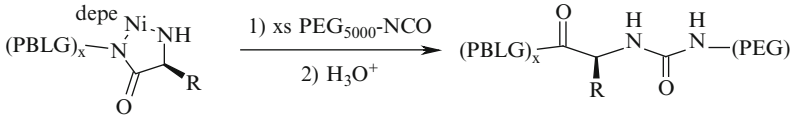
The transition metal initiators for NCA polymerization described above should provide a means for controlled synthesis of polypeptide hybrid block copolymers. However, a limitation of this methodology when using zerovalent metal complexes as initiators is that the active propagating species are generated in situ, where the C-terminal end of the polypeptide is derived from the first NCA monomer. Consequently, this method does not allow attachment of functionality (e.g., polymer or small molecule) to the carboxyl chain-end of the polypeptides. To facilitate control over the C-terminal chain-end, Deming and coworkers pursued alternative methods for direct synthesis of the amido-amidate metallacycle propagating species and developed allyloxycarbonylaminoamides as universal precursors to amido-amidate nickelacycles. These simple amino acid derivatives undergo tandem oxidative additions to nickel(0) to give active NCA polymerization initiators (Scheme 7) [28]. These complexes were found to initiate polymerization of NCAs yielding polypeptides with defined molecular weights, narrow molecular weight distributions, and with quantitative incorporation of the initiating ligand as a C-terminal end-group. This chemistry provides a very facile way to incorporate diverse molecules such as polymers, peptides, oligosaccharides, or other ligands onto the carboxyl chain-ends of polypeptides via a robust amide linkage (Scheme 8), and was further elaborated by Menzel's group to grow polypeptides off polystyrene particles [29].



Scheme 7 Formation of chain-end functionalized nickelacycle initiators



Scheme 8 Preparation of PEG-polypeptide diblock copolymers by macroinitiation



Scheme 9 Preparation of PEG-polypeptide diblock copolymers by isocyanate end-capping

As an extension of this work, Deming also developed a means to end-cap living polypeptide chains with electrophilic reagents. When a macromolecular electrophile is used, the resulting product is a polypeptide hybrid block copolymer. It is well known that in NCA polymerizations the electrophiles, such as isocyanates, act as chain-terminating agents by reaction with the propagating amine chain-ends [11, 12]. Deming and coworkers reported that the reactive living nickelacycle polypeptide chain-ends could be quantitatively capped by reaction with excess isocyanate, isothiocyanate, or acid chloride [30]. Using this chemistry, they prepared isocyanate end-capped poly(ethylene glycol) (PEG) and reacted this, in excess, with living poly(γ-benzyl L-glutamate) (PBLG) to obtain PBLG-PEG diblock copolymers (Scheme 9). Reaction with living ABA triblock copolymers (vide infra) gave the corresponding PEG-capped CABAC hybrid pentablock copolymers, where A was PBLG; B was polyoctenemer, PEG or PDMS; and C was PEG. Since excess PEG was used to end-cap the living polypeptide chains, the pentablock copolymers required purification, which was achieved by repeated precipitation from THF into methanol. Overall, it can be seen that the use of controlled NCA polymerization allows formation of very complex hybrid block copolymer architectures that rival those prepared using any polymerization system.

2.3 Recent Developments

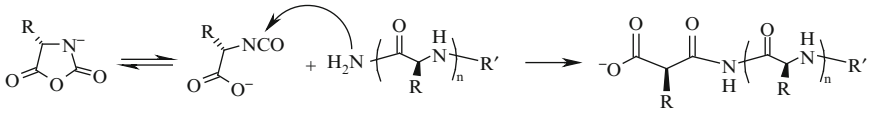
In recent years, a number of new approaches have been reported for obtaining controlled NCA polymerizations. These approaches share a common theme in that

they are all improvements on the use of classical primary amine polymerization initiators. This approach is attractive since primary amines are readily available and because the initiator does not need to be removed from the reaction after polymerization. In fact, if the polymerization proceeds without any chain-breaking reactions, the amine initiator becomes the C-terminal polypeptide end-group. In this manner, there is potential to form chain-end-functionalized polypeptides or even hybrid block copolymers if the amine is a macroinitiator. The challenge in this approach is to overcome the numerous side reactions of these systems without the luxury of a large number of experimental parameters to adjust.

In 2004, the group of Hadjichristidis reported the primary-amine-initiated polymerization of NCAs under high vacuum conditions [21]. The strategy here was to determine if a reduced level of impurities in the reaction mixture would lead to fewer polymerization side reactions. Unlike the vinyl monomers usually polymerized under high vacuum conditions, NCAs cannot be purified by distillation. Consequently, it is doubtful whether the NCAs themselves can be obtained in higher purity under high vacuum recrystallization than by recrystallization under a rigorous inert atmosphere. However, the high vacuum method does allow for better purification of polymerization solvents and the *n*-hexylamine initiator. It was found that polymerizations of γ-benzyl-L-glutamate NCA (Bn-Glu NCA) and ε-carbobenzyloxy-L-lysine NCA (Z-Lys NCA) under high vacuum in DMF solvent displayed all the characteristics of a living polymerization system [21]. Polypeptides could be prepared with control over chain length, chain length distributions were narrow, and block copolypeptides were prepared. Controlled polymerization of NCAs under high vacuum was later confirmed by Messman and coworkers [31].

The authors concluded that the side reactions normally observed in amine-initiated NCA polymerizations are simply a consequence of impurities. Since the main side reactions in these polymerizations do not involve reaction with adventitious impurities such as water, but instead reactions with monomer, solvent, or polymer (i.e., termination by reaction of the amine-end with an ester side chain, attack of DMF by the amine-end, or chain transfer to monomer) [11, 12], this conclusion does not seem to be well justified. It is likely that the role of impurities (e.g., water) in these polymerizations is very complex. A possible explanation for the polymerization control observed under high vacuum is that the impurities act to catalyze side reactions with monomer, polymer, or solvent. In this scenario, it is reasonable to speculate that polar species such as water can bind to monomers or the propagating chain-end and thus influence their reactivity.

Further insights into amine-initiated NCA polymerizations were also reported in 2004 by the group of Giani and coworkers [32]. This group studied the polymerization of ε-trifluoroacetyl-L-lysine NCA (TFA-Lys NCA) in DMF using *n*-hexylamine initiator as a function of temperature. In contrast to the high vacuum work, the solvent and initiator were purified using conventional methods and the polymerizations were conducted under a nitrogen atmosphere on a Schlenk line. After complete consumption of NCA monomer, the crude polymerization mixtures were analyzed by gel permeation chromatography (GPC) and non-aqueous capillary electrophoresis (NACE). A unique feature of this work was the use of NACE to



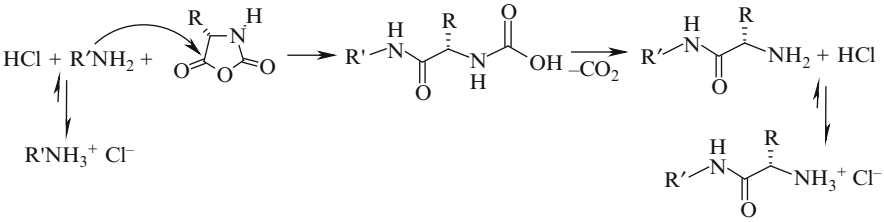
Scheme 10 Polypeptide chain termination by reaction with NCA anions

Scheme 11 Polypeptide chain termination by reaction with DMF solvent

separate and quantify the amount of polymers with different chain-ends, which corresponded to living chains (amine end-groups) and "dead" chains (carboxylate and formyl end-groups from reaction with NCA anions and DMF solvent, respectively; see Schemes 10 and 11). Not surprisingly, at 20 °C, the polymer products consisted of 78% dead chains and only 22% living chains, which illustrates the abundance of side reactions in these polymerizations under normal conditions.

An intriguing result was found for polymerizations conducted at 0 °C where 99% of the chains had living amine chain-ends, and only 1% were found to be dead chains. To verify that these were truly living polymerizations, additional NCA monomer was added to these chains at 0 °C, resulting in increased molecular weight and no increase in the amount of dead chains. Although this was only a preliminary study and further studies need to be conducted to explore the scope of this method, this work clearly shows that the common NCA polymerization side reactions can also be eliminated by lowering the temperature. The effect of temperature is not unusual, as similar trends can be found in cationic and anionic vinyl polymerizations [33]. At elevated temperature, the side reactions have activation barriers similar to chain propagation. When the temperature is lowered, it appears that the activation barrier for chain propagation becomes lower than that of the side reactions and chain propagation dominates kinetically. A remarkable feature of this system is that the elevated levels of impurities, as compared to the high vacuum method, do not seem to cause side reactions at low temperature. This result further substantiates the idea that the growing chains do not react with the adventitious impurities, but that they mainly affect these polymerizations by altering the rates of discrete reaction steps. The same group reported recently that addition of urea in the polymerization solution could also improve the polymerization and minimize the tendency of formation of bimodal molecular weight distribution [34].

Another innovative approach to controlling amine-initiated NCA polymerizations was reported in 2003 by Schlaad and coworkers [20]. Their strategy was to avoid formation of NCA anions, which cause significant chain termination after rearranging to isocyanocarboxylates [11, 12], through use of primary amine hydrochloride salts as initiators. The reactivity of amine hydrochlorides with NCAs was first explored by the group of Knobler, who found that they could react



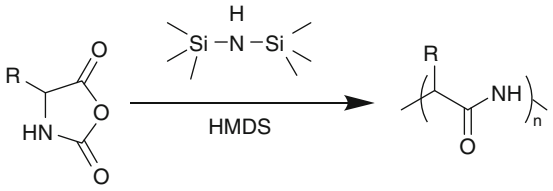
Scheme 12 Polypeptide synthesis using amine-hydrochloride initiators

hydrochlorides with NCAs to give single NCA addition products [35, 36]. Use of the hydrochloride salt takes advantage of its diminished reactivity as a nucleophile compared to the parent amine, which effectively halts the reaction after a single NCA insertion by formation of an inert amine hydrochloride in the product. The reactivity of the hydrochloride presumably arises from formation of a small amount of free amine by reversible dissociation of HCl (Scheme 12). This equilibrium, which lies heavily toward the dormant amine hydrochloride species, allows for only a very short lifetime of reactive amine species. Consequently, as soon as a free amine reacts with an NCA, the resulting amine end-group on the product is immediately protonated and is prevented from further reaction. The acidic conditions also assist elimination of CO_2 from the reactive intermediate and, more importantly, suppress formation of unwanted NCA anions.

To obtain controlled polymerization, and not just single NCA addition reactions, Schlaad's group increased the reaction temperature (from 40 to 80 °C), which was known from Knobler's work to increase the equilibrium concentration of free amine, as well as increase the exchange rate between amine and amine hydrochloride [35, 36]. Using primary amine hydrochloride end-capped polystyrene macroinitiators to polymerize Z-Lys NCA in DMF, Schlaad's group obtained polypeptide hybrid copolymers in 70–80% yield after 3 days at elevated temperature. Although these polymerizations are slow compared to amine-initiated polymerizations, the resulting polypeptide segments were well defined with very narrow chain length distributions ($M_w/M_n < 1.03$). These distributions were much narrower than those obtained using the free amine macroinitiator, which argues for diminished side reactions in the polypeptide synthesis. The molecular weights of the resulting polypeptide segments were found to be approximately 20–30% higher than would be expected from the monomer-to-initiator ratios. This result was attributed to termination of some fraction of initiator species by traces of impurities in the NCA monomers, although the presence of unreacted polystyrene chains was not reported.

Although more studies need to be performed to study the scope and generality of this system, the use of amine hydrochloride salts as initiators for controlled NCA polymerizations shows tremendous promise. Fast, reversible deactivation of a reactive species to obtain controlled polymerization is a proven concept in polymer chemistry, and this system can be compared to the persistent radical effect employed in all controlled radical polymerization strategies [37]. Like those systems, success of this method requires a carefully controlled matching of the

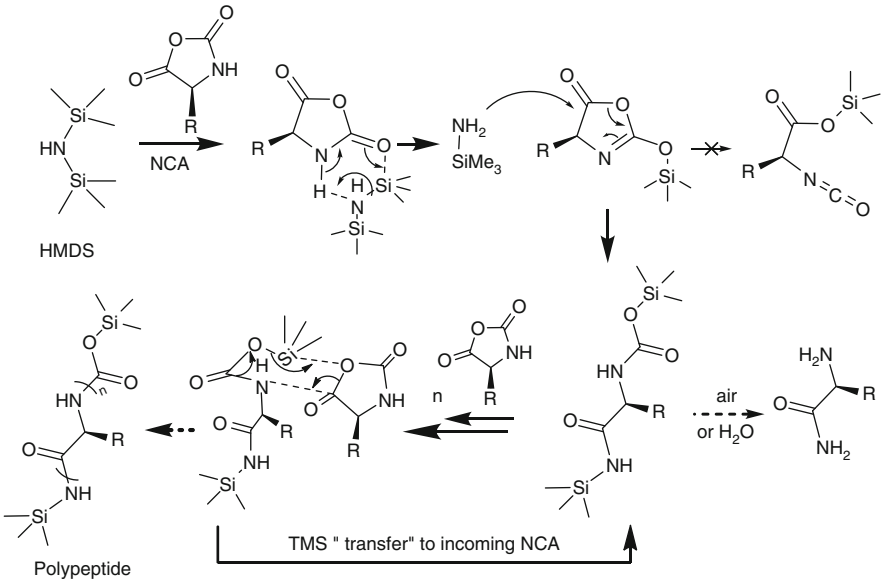
Scheme 13 Polypeptide synthesis using HMDS initiator



polymer chain propagation rate constant, the amine/amine hydrochloride equilibrium constant, and the forward and reverse exchange rate constants between amine and amine hydrochloride salt. This means that it is likely that reaction conditions (e.g., temperature, halide counterion, solvent) will need to be optimized to obtain controlled polymerization for each different NCA monomer, as is the case for most vinyl monomers in controlled radical polymerizations. Within these constraints, it is possible that controlled NCA polymerizations utilizing simple amine hydrochloride initiators can be obtained.

A new approach of controlling NCA polymerization was reported by the Cheng group in 2007 [22]. In a screen of amine initiators for the polymerization of Bn-Glu NCA, they found that hexamethyldisilazane (HMDS) showed remarkable control of polymerizations and led to formation of PBLG with excellent chain length control, with less than 22% deviation from the expected molecular weights, and narrow molecular weight distributions ($M_w/M_n < 1.2$) (Scheme 13). The NCA polymerizations initiated with HMDS differed greatly from those initiated with conventional secondary amine initiators, e.g., diethylamine, in which elevated PBLG molecular weights (three to four times higher than the expected molecular weights) and broad molecular weight distributions were observed.

The controlled NCA polymerizations observed with HMDS suggested that their initiation and chain propagation mechanisms differ from either the "amine" (Scheme 2) or the "AM" mechanism (Scheme 3), typically observed with amine initiators. As a secondary amine, HMDS can either function as nucleophile to open the NCA ring at C_5, or act as a base to deprotonate the NH group [11]. Previous studies showed that secondary amines with bulky alkyl groups (e.g., diisopropylamine) exclusively deprotonated NCAs [38]. Therefore, it is unlikely that HMDS, a secondary amine containing two bulky TMS groups, attacks the C_5 of Bn-Glu NCA. If the first step involves the deprotonation of the NCA NH group by HMDS, an N-TMS NCA would form that should undergo rapid rearrangement to form an α-isocyanatocarboxylic acid [39]. However, no isocyanate stretch (~2,230–2,270 cm^{-1}) was observed when a mixture of equimolar Bn-Glu NCA and HMDS was analyzed by FTIR. Interestingly, it was observed that the Si–N absorption band of HMDS at 932 cm^{-1} in FTIR disappeared, indicating the cleavage of an Si–N bond during initiation. It therefore seems likely that a TMS group is transferred to C_2 from HMDS in a coordinated manner (Scheme 14). Instead of forming an isocyanate, the NCA-TMS intermediate instead undergoes rapid ring-opening by the in-situ-generated TMS-amine to form a TMS-carbamate. Cheng and coworkers confirmed the formation of TMS-carbamates through a combination of ^{13}C NMR and mass spectroscopy (MS) analysis of an equimolar

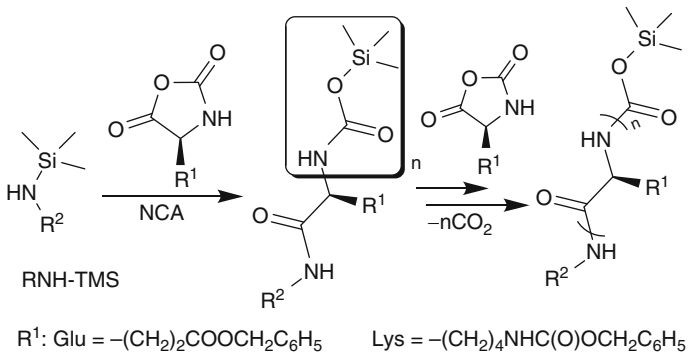


Scheme 14 Proposed mechanism for polypeptide synthesis using HMDS initiator

mixture of Bn-Glu NCA and HMDS in DMSO-d_6. The MS analysis of a mixture of Bn-Glu NCA and HMDS (at a 5:1 molar ratio) further showed peaks corresponding to the molecular weights of the dimer through the pentamer containing the TMS-carbamate end-group. This suggests that polypeptide chains were indeed propagated through the transfer of the TMS group from the terminal TMS-carbamate to the incoming monomer to form a new TMS-carbamate terminal propagating group (Scheme 14). To demonstrate that controlled NCA polymerizations can be mediated by the TMS-carbamate group, Cheng synthesized trimethylsilyl dimethylcarbamate (TMSDC), a TMS-carbamate compound, and used it as the initiator for Bn-Glu NCA polymerization. Polymerizations using this initiator yielded PBLG chains with controllable molecular weights and narrow molecular weight distributions, as in the HMDS-mediated polymerizations.

These TMS-carbamate-mediated NCA polymerizations resemble to some extent the group-transfer polymerization (GTP) of acrylic monomers initiated by organosilicon compounds [40]. Unlike GTPs that typically require Lewis acid activators or nucelophilic catalysts to facilitate the polymerization [41], TMS-carbamate-mediated NCA polymerizations do not appear to require any additional catalysts or activators. However, it is still unclear whether the TMS transfer proceeds through an anionic process as in GTP [41] or through a concerted process as illustrated in Scheme 14.

As the polypeptide chains are propagated only at the amine-end through the transfer of the terminal TMS-carbamate to the incoming monomer to form a new TMS-carbamate terminal propagating group, Cheng and his team reasoned that use of a N-TMS amine as the initiator will generate an amine and a TMS group



Scheme 15 Polypeptide synthesis using TMS-amine initiators

(Scheme 15) that can subsequently form a corresponding amide at the C-terminus and a TMS-carbamate at the N-terminus after NCA ring opening. Thus, chain propagation should proceed in the same manner as HMDS-mediated polymerization. Because a large variety of N-TMS amines are readily available, this method should allow facile functionalization at the C-terminal ends of polypeptides. It has been demonstrated that a variety of primary amines, ranging from small molecules to polymers or containing a variety of functional groups (e.g., norbornene, alkyne, azide, PEG, etc.), could be readily introduced to the C-terminal ends of polypeptides via N-TMS amine-mediated, controlled NCA polymerization [42].

The polymerizations initiated by HMDS and N-TMS amines usually complete within 24 h at ambient temperature with quantitative monomer consumption. These polymerizations in general are slower than those mediated by Deming's Ni(0) or Co (0) initiators (about 30–60 min at ambient temperature) [19, 24, 25], but are much faster than those initiated by amines at low temperature or using amine hydrochloride initiators [20]. These HMDS and N-TMS amine-mediated NCA polymerizations can also be applied to the preparation of block copolypeptides of defined sequence and composition [22]. This organosilicon-mediated NCA polymerization, which was also shown by Zhang and coworkers to be useful for controlled polymerization of γ-3-chloropropanyl-L-Glu NCA [43], offers an advantage for the preparation of polypeptides with defined C-terminal end-groups.

3 Copolypeptide and Functional Polypeptide Synthesis via NCA Polymerization

3.1 Block Copolypeptides

For the examination of model protein–protein interactions and the assembly of novel three-dimensional structures, block copolypeptides are required that have

structural domains (i.e., amino acid sequences) whose size and composition can be precisely adjusted. Such materials have proven elusive using conventional techniques. NCA polymerizations initiated by a strong base are very fast. These polymerizations are poorly understood and block copolymers generally cannot be prepared. NCA polymerizations initiated by primary amines are also not free of side reactions. Even after fractionation of the crude preparations, the resulting polypeptides are relatively ill-defined, which may complicate unequivocal evaluation of their properties and potential applications. Nevertheless, there are many reports on the preparation of block copolypeptides using conventional primary amine initiators [44]. Examples include many hydrophilic–hydrophobic and hydrophilic–hydrophobic–hydrophilic di- and triblock copolypeptides (in which hydrophilic residues were glutamate and lysine, and hydrophobic residues were leucine [28, 29], valine [45], isoleucine [46], phenylalanine [30], and alanine [47]) prepared to study conformations of the hydrophobic domain in aqueous solution. These conformational preferences of different amino acid residues were used as the basis for early models for predicting protein conformations from sequence. Consequently, these copolypeptides were studied under conditions favoring isolated single chains (i.e., high dilution), and self-assembly of the polymers was not investigated. These copolymers were often subjected to only limited characterization (e.g., analysis of amino acid composition) and, as such, their structures, and the presence of homopolymer contaminants, were not conclusively determined. Some copolymers, which had been subjected to chromatography, showed polymodal molecular weight distributions containing substantial high and low molecular weight fractions [30]. The compositions of these copolymers were found to be very different from the initial monomer feed compositions and varied widely for different molecular weight fractions. It appears that most, if not all, block copolypeptides prepared using amine initiators have structures different to those predicted by monomer feed compositions and probably have considerable homopolymer contamination due to the side reactions described above.

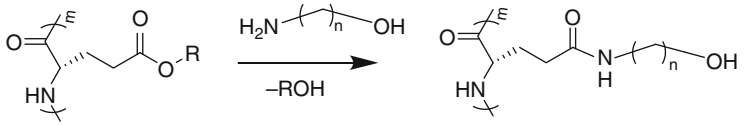
Polypeptide block copolymers prepared via transition-metal-mediated NCA polymerization are well-defined, with the sequence and composition of block segments controlled by the order and quantity of monomer, respectively, added to initiating species. These block copolypeptides can be prepared with the same level of control found in anionic and controlled radical polymerizations of vinyl monomers, which greatly expands the potential of polypeptide materials. The unique chemistry of these initiators and NCA monomers also allows NCA monomers to be polymerized in any order, which is a challenge in most vinyl copolymerizations, and the robust chain-ends allow the preparation of copolypeptides with many block domains (e.g., more than four). The self-assembly of these block copolypeptides has also been investigated (e.g., to direct the biomimetic synthesis of ordered silica structures [48]) for formation of polymeric vesicular membranes [49–51], or for preparation of self-assembled polypeptide hydrogels [52] and nanoscale emulsion droplets [53]. Furthermore, poly(L-lysine)-*block*-poly(L-cysteine) block copolypeptides have been used to generate hollow, organic–inorganic hybrid microspheres composed of a thin inner layer of gold nanoparticles surrounded by a thick layer of

silica nanoparticles [54]. Using the same procedure, hollow spheres could also be prepared, which consisted of a thick inner layer of core–shell CdSe/CdS nanoparticles and thicker silica nanoparticle outer layer [55]. The latter spheres are of interest because they allow for microcavity lasing without the use of additional mirrors, substrate spheres, or gratings.

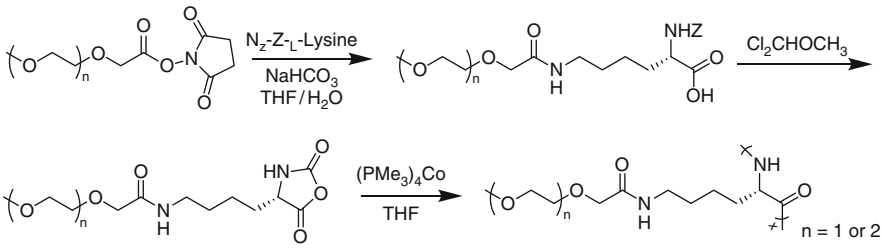
3.2 Side-Chain-Functionalized Polypeptides

There have been many examples in which polypeptides were chemically modified to improve their properties for delivery applications. Typically, this strategy involves the hydrophobic modification of poly(lysine) or poly(glutamate/aspartate) side chains by covalent attachment of lipophilic groups [5, 6]. These modifications are akin to polymer grafting reactions and thus result in random placement of these hydrophobic substituents (typically long alkyl chains) along the polypeptide chains. These modifications were performed in order to increase the polypeptide's ability to bind hydrophobic drugs, aggregate in aqueous solution, and/or penetrate the lipid bilayers of cell walls. The random placement of the hydrophobes along the chain means that they cannot act as a distinct domain in supramolecular assembly, as in a block copolymer, thus limiting their ability to organize.

Other types of chemical polypeptide modification include addition of sugars, or sugar-binding groups [27, 43, 56–58], to increase functionality, and the addition of nonionic, polar groups to increase solubility and blood circulation lifetime [8]. Increasing bioavailability is a major concern for drug delivery using synthetic polypeptides. The amino acid functionalities that provide water solubility (e.g., the amino group of lysine, or the carboxylate groups of glutamate and aspartate) are also detrimental in that their polymers behave as polyelectrolytes. As such, they bind strongly to oppositely charged biomolecules (i.e., proteins, polynucleic acids, polysaccharides, lipids) resulting in aggregation and either rapid removal from the bloodstream or rapid digestion within cells [59]. To circumvent this problem, nonionic, water-solubilizing polypeptide residues have been sought. Following the discovery that optically pure polyserine is not water-soluble at chain lengths greater than 20 residues [21], there have been many attempts to prepare chemically modified residues that would impart these desired features. The simplest of these approaches is the grafting of PEG chains ($1{,}000 < M_n < 5{,}000$) onto side-chain functionalities, as described above, which results in highly heterogeneous materials that retain considerable charge [9]. A more sophisticated solution was the development of hydroxyalkylglutamine polymers, prepared by the reaction of poly(alkylglutamate) esters with α,ω-amino alcohols (Scheme 16). These polypeptides, particularly poly(hydroxypropylglutamine) and poly(hydroxybutylglutamine), were found to be nonionic and soluble in water over a wide pH range [32]. As such, these polymers should be useful as solubilizing domains in polypeptide-based drug delivery systems. The major detriments of these materials, however, are that they are recognized as foreign entities and rapidly degraded in vivo, they are



Scheme 16 Synthesis of water-soluble poly(hydroxyalkyl glutamines)



Scheme 17 Synthesis of water-soluble EG-grafted poly(L-lysine)s

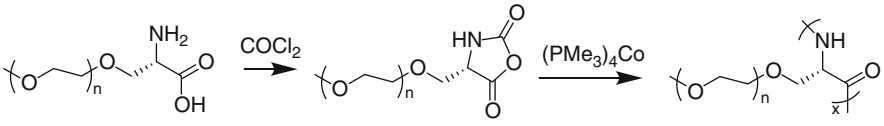
difficult to prepare without significantly degrading the polypeptide chains, and they lack ordered secondary structure in solution [32]. The last property is important since one of the main reasons for using polypeptide scaffolds in biomedical applications is to take advantage of their ordered chain conformations to mimic protein structures.

Deming and, more recently, Klok have taken a different approach toward the development of nonionic, water-soluble polypeptides. They incorporated the solubilizing and protective properties of PEG into polypeptides by conjugation of short ethylene glycol (EG) repeats onto amino acid monomers, as opposed to the well-documented approach of grafting PEG to the ends or side chains of polypeptides [60]. Deming has also recently extended this work by attaching nonionic monosaccharides to amino acid monomers to give nonionic, sugar-functionalized polypeptides [57] (Scheme 17). The functionalized NCA approach avoids the need for expensive amino- or carboxylato-functionalized PEG molecules necessary for coupling, and avoids difficulties associated with derivatization of polymers that are usually associated with polypeptide chain cleavage and broadening of molecular weight distributions. In particular, the presence of short EG repeats or saccharides on every residue resulted in a high density of hydrophilic moieties around the polymer chain. In effect, the polypeptides are surrounded by an EG or saccharide sheath that should mimic the physical properties of PEG or polysaccharides [33], respectively, yet not deleteriously affect the secondary structure of the polypeptide core. For instance, circular dichroism (CD) analysis revealed that EG-grafted poly (L-lysine) is essentially 100% α-helical in pH 7 water at 25 °C. This conformation was unaffected by many environmental factors. Its helical structure was stable in water over an examined pH range of 2–12. EG-grafted poly(L-lysine) was also stable in solutions containing various denaturing agents, such as up to 3 M NaCl, 1 M urea, or 1 M guanidinium–HCl. The thermal stability of the helical

conformation of EG-grafted poly(L-lysine) was also very high, and as much as 75% of its helicity is retained at 85 °C. This polymer is also soluble and helical in many organic solvents (e.g., THF, MeOH, and $CHCl_3$), and was not digested by hydrolytic enzymes that readily digest poly(L-lysine) (e.g., papain, trypsin) [7], indicative of the PEG-like properties imparted by the EG sheath. The polymer has surface properties similar to unstructured PEG, but also possesses a rod-like backbone due to its α-helical conformation. Similar monomers and polymers were prepared by Klok using succinate linkages between the EG segments and lysine [61]. In these polymers, the ester linkages to the EG segments are potentially degradable in water, and the polymers were found to prevent nonspecific protein adsorption when used to coat surfaces.

Recently, Deming and coworkers also reported glycopolypeptides via living polymerization of glycosylated-L-lysine NCAs [57], demonstrating the feasibility of synthesizing water-soluble, highly helical (ca. 88% helicity) polypeptides via monosaccharide-functionalized NCA monomers. Measurement of CD spectra from 4 to 90 °C revealed that the α-helical conformation of poly(galactosyl-L-lysine) was gradually disrupted as temperature was increased, with roughly 40% helicity being retained at 90 °C. This behavior is probably due to disruption of amide H-bonding by interactions with water molecules [6]. These disordered polypeptides remained water-soluble, and their α-helical conformations were completely regained upon cooling, showing that this process is reversible. The molecular weights of these rod-like "PEG-mimic" [60] or "polysaccharide-mimic" [57] polymers could also be easily adjusted by varying the NCA–to-Co(0) initiator ratios.

Similar oligo-EG modifications to the β-sheet preferring amino acids L-serine and L-cysteine were found to allow facile aqueous processing of their corresponding β-forming polymers. The EG side chains were found to provide good water solubility to the polymers, which could then form β-sheet structures upon solvent evaporation or by controlled addition of a solvent that stabilizes the β-conformation. The synthesis of EG-modified polyserine is shown in Scheme 18, where the EG repeats were coupled onto the amino acid using an ether linkage [62]. The modified amino acids were then converted to their corresponding NCA monomers to allow subsequent polymerization. CD analysis of the water-soluble polymer in deionized water at pH 7 revealed that it was in a disordered chain conformation [35, 36]. The CD spectra of this polymer were also invariant with solution pH and buffer strength, consistent with this result. However, films cast from aqueous solutions of this polymer from a variety of buffers all gave CD spectra indicative of the β-sheet conformation. The transformation from disordered conformation to β-sheet was also achieved in water by addition of increased percentages of methanol or



Scheme 18 Synthesis of water-soluble EG-grafted poly(L -serine)s

acetonitrile. Wide-angle X-ray scattering data from films of oligo-EG-grafted poly (L-serine) revealed reflections that were also commensurate with the antiparallel β-sheet structure. Overall, these EG-modified polypeptides provide "PEG-like" α-helix and β-sheet forming segments that can be incorporated into block copolypeptide delivery agents. Such domains provide not only improved solubility and bioavailability, but allow incorporation of secondary structure to control self-assembly of the drug complexes.

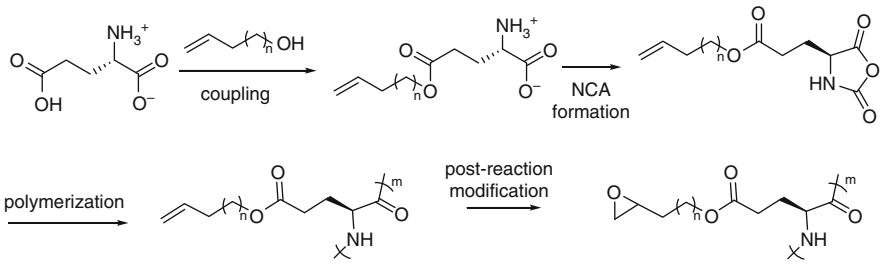
4 Polypeptide Deprotection and Purification

Although quite complex copolypeptide architectures can now be synthesized, obtaining these materials in a state of high purity typically requires additional measures. As discussed above, many of the copolypeptides synthesized using conventional methods contain homopolymer impurities, which must be removed by selective solvent extractions or fractional precipitation, when possible. Since conventional NCA polymerizations also usually give polypeptide segments with large chain length distributions, these samples are ideally also fractionated to give samples of well-defined composition. An additional purification issue arises from the amphiphilic nature of many of these copolymers, e.g., PEG-PBLG. Such polymers tend to associate in most solvents, leading to trapped solvents or solutes in the copolymer sample, which can complicate analytical studies. In the case of polymerizations initiated by a transition metal, removal of the metal from the sample is also important for most applications. For rigorous purification of these amphiphilic copolymers, Deming's group has found that exhaustive dialysis of the samples against deionized water is very effective at removing small molecule contaminants. In cases where a polymer segment can bind strongly to metals such as Co^{2+} and Ni^{2+}, the addition of a potent metal chelator, such as EDTA, in the early stages of dialysis was found to be sufficient to remove all traces of the metal ions.

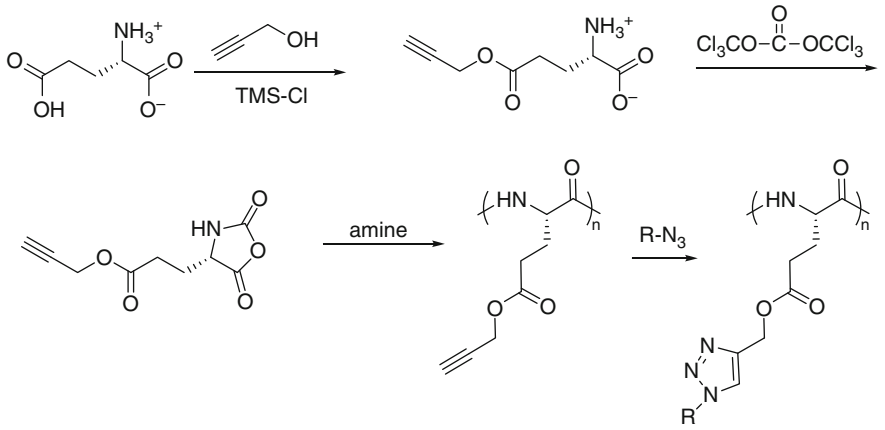
A highly useful feature of copolypeptide materials is their functionality. The common naturally occurring amino acids contain numerous acidic and basic functional groups that provide interesting pH-responsive character to these materials. These functional groups are masked by protecting groups before synthesis of the NCA monomers, since they will typically interfere with polypeptide synthesis or NCA stability [11, 12]. Consequently, these protecting groups must be removed after polymerization if the functional group chemistry is to be used. The first concern with polypeptide deprotection is whether or not all the protecting groups have been removed. Small amounts of residual protecting groups can significantly influence the resulting polypeptide properties, especially since the protecting groups are typically hydrophobic and the deprotected chain is typically hydrophilic. Fortunately, most of the common protecting groups are removed without difficulty, and deprotection levels greater than 97% are readily attained. The second, and more serious, consequence of deprotection is cleavage of the peptide chain, or racemization of the optically pure amino acid residues.

Basic polypeptides, such as polylysine or polyarginine, are readily deprotected [11, 12, 48]. Acidic polypeptides, such as polyglutamic acid or polyaspartic acid, require more care in deprotection reactions due to an abundance of potential side reactions. PBLG, for example, can be debenzylated using strong acid, aqueous base, or catalytic hydrogenation. Use of strong acid (e.g., gaseous HBr or HBr in acetic acid) avoids any racemization, but is known to lead to significant chain cleavage arising from protonation of side-chain ester groups that react with the amide backbone [49]. Basic conditions avoid this reaction, but can lead to significant racemization unless the amount of base is carefully controlled [50, 51]. Hydrogenation would appear to be the most attractive method, however, it is only effective for chains less than 10 kDa in mass. Larger PBLG chains adopt stable helical conformations that prevent access of the hydrogenation catalyst to the ester groups [50, 51]. Ester cleavage using trimethylsilyl iodide was found to give clean conversion to the readily hydrolyzed trimethylsilyl ester, without any racemization or chain cleavage [52]. The major drawbacks of this reagent are its expense as well as its reactivity with most other functional groups, such as the ether linkages in PEG. The deprotection of poly(β-benzyl-L-aspartate) shows less side reactions under acidic conditions compared to PBLG. However, it has been reported that the polymer backbone undergoes partial rearrangement to β-peptide linkages under basic conditions, presumably through an imide intermediate [54]. The degree of racemization in these samples was not discussed.

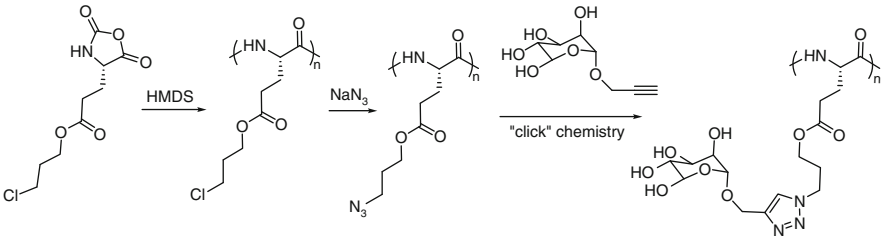
To avoid the issues of deprotection chemistry and to allow facile side-chain functionalization, an emerging field is focused on the development of new NCA monomers bearing side-chain functional groups that stay intact during polymerization and can be used for highly efficient conjugation of functional moieties after polymerization. The synthesis of γ-alkenyl-L-glutamate NCAs was reported by Poché et al. in 1997, and these were utilized for preparing poly(L-glutamate)s containing pendant alkene functional groups that were modified by a variety of reactions (Scheme 19) [63]. In the past few years, a number of other NCAs bearing conjugation-amenable side-chain functional groups, as well as their polymerizations, have been reported. These include γ-propargyl-L-glutamate NCA by Hammond [64] and Chen [56] (Scheme 20), γ-3-chloropropyl-L-glutamate NCA by Zhang (Scheme 21) [43], propargyl-DL-glycine NCA by Heise (Scheme 22) [65],



Scheme 19 Synthesis and derivatization of alkene-terminated poly(glutamates)



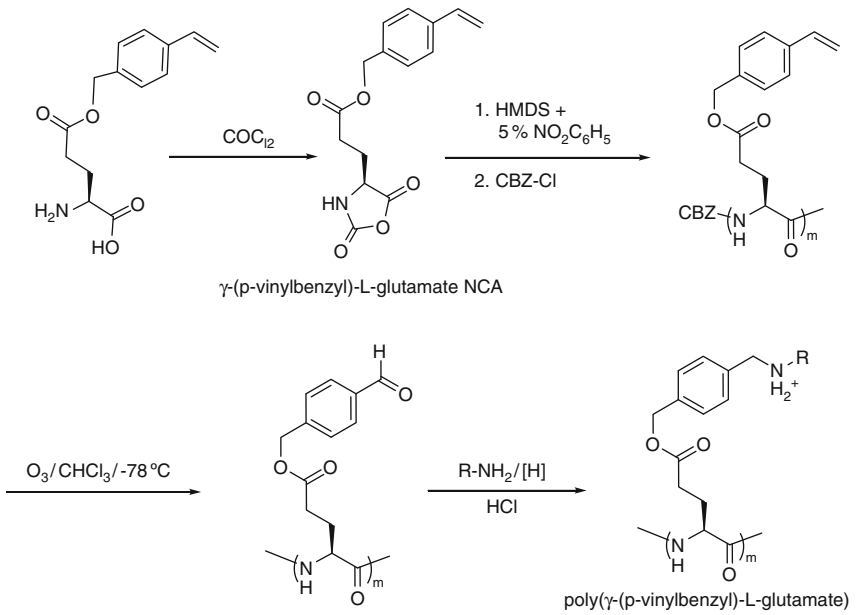
Scheme 20 Synthesis and derivatization of alkyne-terminated poly(glutamates)



Scheme 21 Synthesis and derivatization of azido-terminated poly(glutamates)

Scheme 22 Alkene and alkyne bearing NCAs

allyl-DL-glycine NCA by Schlaad (Scheme 22) [58] and γ-(*p*-vinylbenzyl)-L-glutamate NCA by Cheng (Scheme 23) [66]. Through a variety of azide-alkyne, thiol-ene and other chemistries, these functionalized polypeptides were modified with a variety of functional moieties including monosaccharides and PEG chains. These reactive polypeptides have the potential for generation of a large library of functional polymers from a single precursor. For instance, conversion of alkene groups in poly(γ-(*p*-vinylbenzyl)-L-glutamate) to aldehydes, followed by reductive amination with primary amines was used to generate poly(L-glutamate) analogs with charged groups distally situated on their side chains (Scheme 23) [66]. These



Scheme 23 Synthesis and derivatization of aldehyde-terminated poly(glutamates)

side-chain charged poly(L-glutamate) analogs were found to be water-soluble and able to adopt stable α-helical conformations over a wide range of pH (pH 1–12), in contrast to poly(L-lysine) or poly(L-glutamic acid) that are known for their pH-dependent helix–coil transitions.

5 Conclusions and Future Prospects

The synthesis of polypeptides by ring-opening polymerization of NCAs is an area that has been under study for more than six decades. Initially, this field suffered from limitations in the synthesis of the polypeptide components, which required excessive sample purification and fractionation to obtain well-defined copolymers. In recent years, vast improvements in NCA polymerizations, either using metal initiators [19, 67] or improved conventional initiators [21, 22, 68] now allow the synthesis of hybrid and block copolymers of controlled dimensions (molecular weight, sequence, composition, and molecular weight distribution). Such materials will greatly assist in the development and identification of new self-assembled structures made possible with ordered polypeptide segments, as well as yield new materials with a wide range of tunable properties.

There are still many challenging issues that remain to be addressed in the field of synthetic polypeptides. NCA purification has been one of the bottlenecks limiting the availability and scale-up of NCA monomers. Recrystallization has long been the

only practical method for obtaining NCA monomers with satisfactory purity for polymerization. Recently, Deming and coworkers reported the purification of NCA monomers using flash chromatography, which may substantially improve the purity of NCAs and potentially make it possible to purify NCA monomers that do not crystallize [69]. Polymerizations of NCAs are usually conducted in anhydrous organic solvents, which are not environmentally friendly. As an alternative approach, Mori and coworkers recently attempted the use of ionic liquids for the polymerization of Bn-Glu NCA, underscoring an effort to integrate green chemistry with the synthesis of polypeptides [70].

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