Native Chemical Ligation: Ultrafast Synthesis of Block Copolymers

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ABSTRACT: Native chemical ligation (NCL) allows the chemical synthesis of proteins and peptides with excellent efficiency, starting from a mixture of unprotected short peptide fragments. The chemo and regioselectivity of NCL provide access to functional biomacromolecules such as peptides without protection-deprotection strategies under mild conditions. In contrast, less progress has been made in non-natural polymer conjugation. Metal contamination, synthetic difficulties, laborious purifications or lack of functional group tolerance limit complex functional polymer design. Here, we have studied the potential of NCL for non-natural polymer conjugation. Diblock and ABA triblock copolymers were synthesized via NCL with high efficiency within one hour at room temperature. An A-alt-B block copolymer was also synthesized with a remarkable degree of polymerization from bifunctional thioester and cysteine end functional polymers. The SEC and NMR spectroscopy analyses of the synthesized polymers confirmed the block structure of the polymers and the excellent efficiency of NCL for non-natural polymer conjugation.

Introduction

Proteins and peptides are biopolymers conducting various functions in living organisms. Nature has masterminded the synthesis of such complex macromolecules via ribosomal synthesis. Besides biological synthesis, exceptional advancements have been made in the chemical synthesis of proteins and peptides. The development of ligation techniques allows the convergent chemical synthesis of such biomacromolecules. [1,2] A typical chemical preparation of proteins involves the solid-supported synthesis of short peptides followed by chemical ligation of the unprotected peptides in aqueous buffer solutions.[3]

In a chemical ligation process, an equivalent mixture of two entirely unprotected peptides is reacted at room temperature under mild reaction conditions. This allows the synthesis of unprotected peptides without the requirement of purification. The outstanding chemo and regioselectivity with ultrafast reaction kinetics allows protein synthesis with excellent efficiency.

In the early 1990, Kent and coworkers established the principle of chemical ligations, and thenceforth several ligation techniques have been developed.[4,5] Amongst these, native chemical ligation (NCL) is considered to be the most efficient to obtain native peptides under mild conditions and it is extensively used in seminal protein syntheses.[1,2] In addition, NCL proved to be equally effective in polar aprotic organic solvents like DMF and allowed the synthesis of hydrophobic peptides with excellent yields and selectivity.[6,7]

Beside monodispersed natural peptides synthesis, NCL has also been exploited for block copolypeptide and copolypeptoids synthesis.[8,9] Although NCL is often used for the synthesis of highly functional biomacromolecules such as peptides, the use of such ligation techniques for non-natural functional polymer synthesis is limited. Nonetheless, the NCL technique has also been used for non-natural block copolymer, hydrogel, star and degradable star copolymer syntheses.[10-13] However, a detailed analysis of the NCL coupling efficiency with diverse coupling partners is scarce. We, therefore, believe that the NCL technique has the potential for a more widespread use in polymer and materials chemistry.

The click chemistry introduced by Sharpless and coworkers is mainly used for non-natural polymer conjugation. Progress has been made to develop high yielding and modular post-polymerization modification strategies via click chemistry for functional polymer design or bioconjugation.[14-17] Among these strategies, Cu catalyzed and metal free strain-induced azide-alkyne click reactions are extensively used in polymer synthesis.[18-19] Although these click reactions offer efficient functionalization, they are limited in terms of metal contamination and difficulties associated with the synthesis of the azide and highly strained alkyne functional polymers.[20,21]

Diels-Alder (DA) cycloadditions provide an alternative metal free click platform for polymer synthesis.[22,23] Recently, Hawker and coworkers reported an innovative DA click strategy for polymer conjugation via in-situ generation of the highly reactive cyclopentadiene (Cp) from dormant norbornadiene derivatives (NBD).[24,25] However, the use of highly reactive substrates like dipyridyl-tetrazine and maleimide derivatives are limited, especially when unsaturated and highly nucleophilic polymer backbones are concerned.**Error! Bookmark not defined.**,[26,27] Retro-DA reactions at elevated temperatures also limit the use of such polymer conjugates for high-temperature applications.[28]
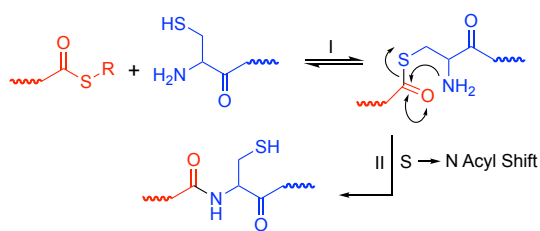
Figure 1. General representation of native chemical ligation (NCL).

The synthesis of functional polymers with controlled monomer arrangements is one of the central challenges in polymer chemistry.[29-30] The study of structure-property relationship of functional polymers facilitates the development of smart materials for modern technologies. Amongst these, block copolymers (BCP) have been studied the most.[31,32] The macroscopic properties of a block copolymer are the consequence of the microscopic arrangements of the individual blocks in bulk.[33,34] BCP undergo spontaneous phase separation and offer a vast variety of application from nanolithography to drug delivery.[35-47]

Living polymerizations are the state-of-the-art methods for well-defined, narrowly dispersed, high molecular weight block copolymer synthesis.[48-57] Block copolymers are generally synthesized via sequential addition of monomers in a living chain growth process.[58] However, living polymerizations find their limitations especially when the synthesis of BCP prepared by two non-complimentary polymerization techniques is concerned. Furthermore, limited access of the chain end in high molecular weight polymers and the presence of additional functional groups along the polymer backbone are challenging constraints in BCP synthesis via classical organic coupling reactions between two homopolymers. Using an excess of one of the coupling partners is often necessary to achieve higher conversions. Nonetheless, click strategies overcome these limitations to some extent with the drawback discussed above.

In contrast, much progress has been made for biopolymer conjugations. Ligation techniques allow for the ultrafast synthesis of highly functional biopolymers without laborious purifications under mild conditions. In fact, the ligation techniques can also provide an alternative platform for non-natural functional polymer synthesis especially when macro initiation is out of reach.[10-12]

For an example, an efficient method for macro initiation in metathesis polymerization is still out of reach. Hence, here, we have studied the potential of NCL for block copolymer synthesis involving one polymer block prepared by ring-opening metathesis polymerization. Under native chemical ligation conditions, AB diblock, ABA triblock, and AB alternating copolymers were synthesized with high efficiency.

Results and Discussion

In NCL, a *C*-terminal peptide thioester reacts with cysteine *N*-terminal peptide to yield a native amide bond between two peptide fragments. The first step involves the reversible *trans*-thioesterfication via a nucleophilic

attack on the *C*-terminal peptide thioester by the *N*-terminal cysteine thiol group (Figure 1I). Thereafter, the intermediate thioester undergoes a rapid *S-N* acyl shift forming a native peptide bond at the ligation site (Figure 1II). The reversible first step and selective intramolecular *S-N* acyl shift make the NCL process exceptionally regio and chemoselective. Consequently, a very high yield of ligation products is achieved even in the presence of internal cysteine moieties in one or both fragments.

As a proof of principle that NCL can be used for conjugation of olefin metathesis polymers, a set of thioester and cysteine end functional polymers were synthesized. Ring-opening metathesis polymerization was used to synthesize thioester end functional polymers, whereas commercial polyethylene glycol (PEG), polylactide (PLA), and polydimethylsiloxane (PDMS) were functionalized with cysteine end groups via ester linkages (See Scheme 1 and SI). The thioester mono end functional ROMP polymer **PA1** (Mn= 4.5 kDa.; Đ= 1.09, See SI, Table S1) was synthesized via initiating *exo-N*-methylnorborneneimide (**M1**) with 5mol% Grubbs' third-generation catalyst (**G3**) followed by functional quenching with a thioester functionalized symmetrical chain transfer agent (**CTA1**) (See SI). The cysteine mono end functional polymer (**PB1**) was obtained by DCC, DMAP coupling of polyethylene glycol monomethyl ether (Mn= 2.2 kDa; Đ= 1.08; See SI, Table S1) with *N*-Boc and *S*-Trt protected cysteine. Acidic deprotection yielded the corresponding polymer with free thiol and amine end groups (**PB1D**, see SI).
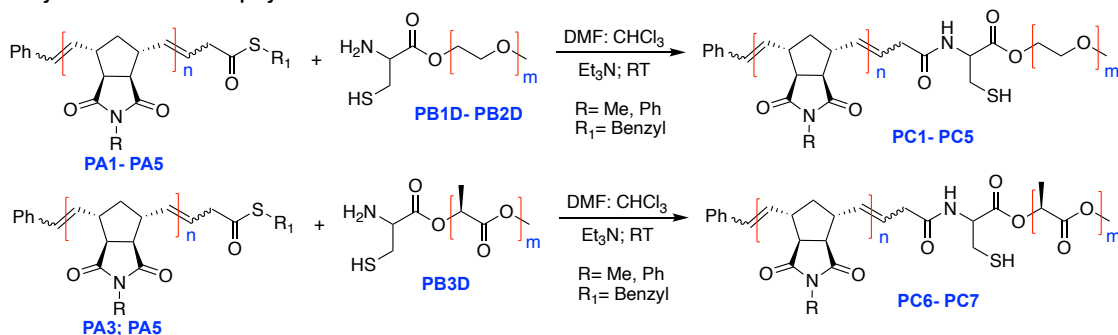
Next, the cysteine mono end functional **PB1D** was reacted with exactly one equivalent of thioester mono end functional ROMP polymer **PA1** in the presence of catalytic amounts of triethylamine in a DMF, CHCl$_3$ mixture (3:1) (Scheme 1A). Surprisingly, within one hour, a SEC measurement of the crude reaction mixture showed a monomodal distribution (**PC1**; Mn= 4.7kDa.; Đ= 1.12; Fig S27, Table S3), indicating complete conjugation between two polymers, whereas a comparable SEC analysis of the mixture of the protected homopolymers showed two distinct distributions corresponding to **PA1** and **PB1** (Fig S36).

To confirm the block copolymer nature of **PC1**, a series of NMR analyses were performed (See SI). ^{1}H and ^{13}C NMR analysis confirmed the presence of characteristic signals from both homopolymers. Moreover, a DOSY NMR analysis showed one diffusion coefficient for the chemical signatures of both polymer blocks confirming the excellent conjugation efficiency between the two homopolymers (Fig S1). In contrast, a comparable DOSY NMR analysis of an equivalent mixture of protected **PB1D** and **PA1** showed two distinct diffusion coefficients, each belonging to an individual homopolymer (Fig S2).

Furthermore, **PC1** was analyzed by MALDI-ToF mass spectrometry (Figure 2B, Fig S86). The molecular mass of the repeating unit of the metathesis polymer (**PA1, M(C$_{10}$H$_{11}$NO$_2$)= 177,08**) happened to be close to an integer multiple of the molecular mass of ethylene oxide, the repeating unit of PEG (**PB1, M(C2H4O)= 44.03**). This coincidence dramatically reduced the number of peaks in the mass spectrum for the block copolymer leading to more intense peaks. Careful analysis of each distribution confirmed the formation of the block copolymer and proved the excellent efficiency of NCL for non-natural polymer conjugation (Figure 2B, Fig S86). To test the

Scheme 1. General representation of block copolymer synthesis via native chemical ligation.
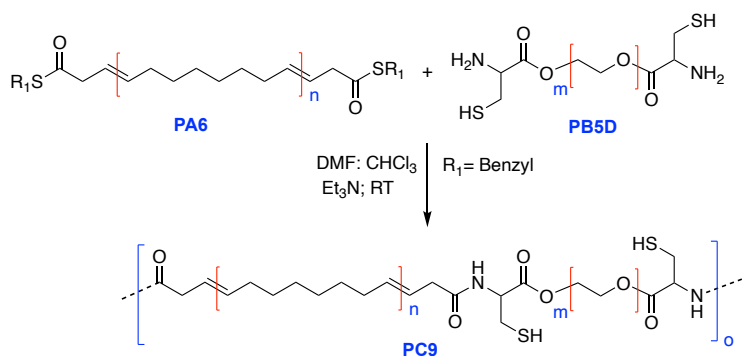
A. Synthesis of Diblock Copolymer

B. Synthesis of ABA Triblock Copolymer
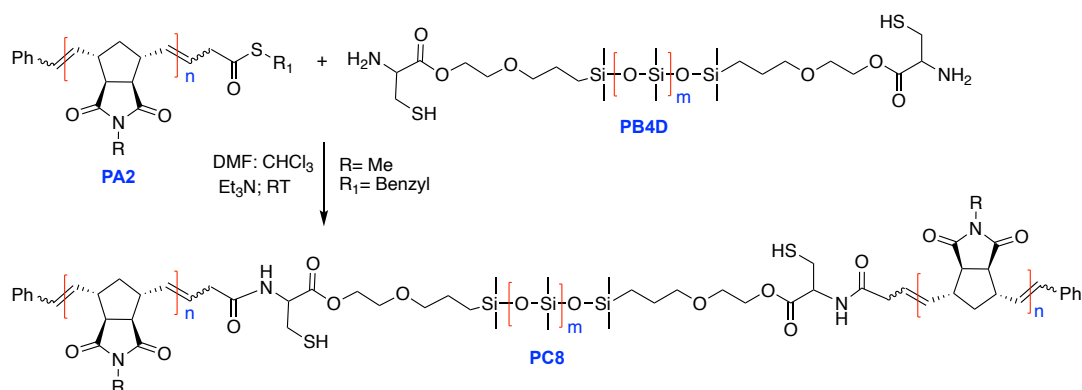
C. Synthesis of A-*alt*-B Block Copolymer

efficacy of NCL for higher molecular weight non-natural polymers, two thioester mono end functional ROMP polymers (**PA2**, **PA3**) and a cysteine mono end functional PEG polymer (**PB2D**) of higher molecular weight were synthesized (See SI). Next, an equivalent mixture of **PA2** (Mn= 6.3kDa.; Đ= 1.08; See SI, Table S1) and **PB2D** (Mn= 20.4kDa.; Đ= 1.11; Table S2) was dissolved in DMF, chloroform mixture (3:1) in the presence of catalytic amounts of triethylamine (Scheme 1A). Interestingly, within one hour the SEC analysis of the crude reaction mixture showed a single monomodal distribution indicating the completion of the conjugation reaction (Fig S28, S36). The obtained number average molecular weight of the block copolymer did not match the sum of the individual homopolymer molecular weights. This phenomenon is currently under investigation. However, the DOSY NMR analysis of the conjugated polymer also confirmed the high extent of coupling efficiency and the formation of block copolymer **PC2** (Mn= 13.1kDa.; Đ= 1.11; Table S3; Fig S3, S4). A remarkably fast conjugation reaction was also observed when a block copolymer **PC3** (Mn= 32.2kDa.; Đ= 1.14; Table S3)

was synthesized from two high molecular weight thioester and cysteine mono end functional polymers **PA3** (Mn= 33.5kDa.; Đ= 1.1; Table S1), and **PB2D** (Mn= 20.4kDa.; Đ= 1.11; Table S2). The SEC and DOSY analysis of **PC3** confirmed once more the excellent efficiency of NCL for non-natural high molecular weight polymer conjugation (Fig S5, S6, S18, S38). The bulk self-assembly of the diblock copolymer **PC3** was studied via small- and wide-angle X-ray scattering (S/WAXS) and atomic force microscopy (AFM) which further supported the formation of a diblock copolymer (Figure S88).
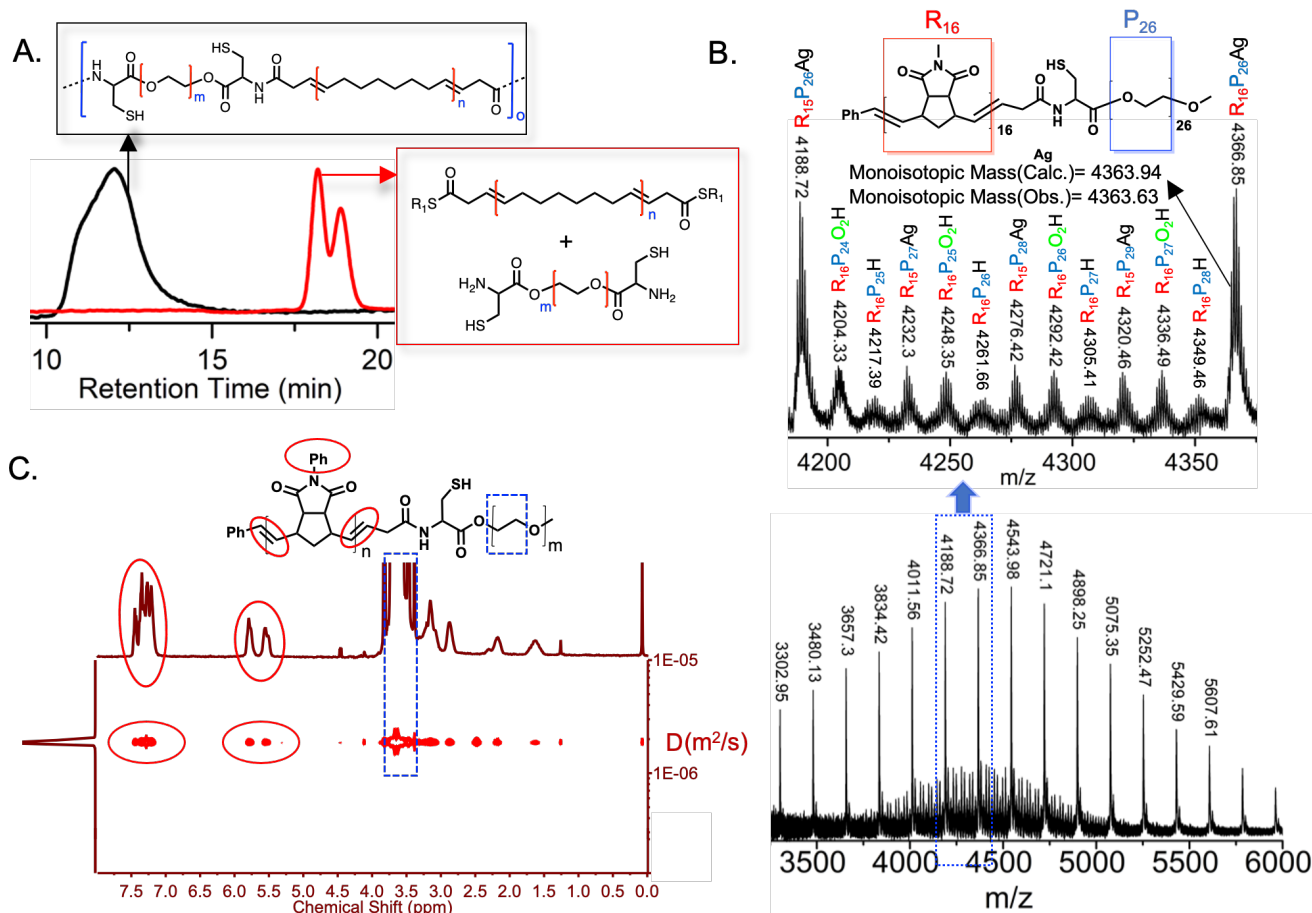
Figure 2. A. SEC trace before (red) after coupling (black) of **PA6** and **PB5D**; B. MALDI-ToF mass distribution of **PC1**. **R** and **P** represent the ROMP and PEG repeat units respectively. The counter ion Ag^+ and H^+ are highlighted in black. Ag(I) in the presence of water causes the oxidation of thiols to sulfoxide and are represented in green; C. DOSY NMR spectra (400MHz, CDCl$_3$) of **PC5**

Thereafter, a set of sterically hindered thioester mono end functional ROMP polymers (**PA4, PA5**) were synthesized to study the effect of steric bulk on the NCL. A comparable conjugation reactivity was observed when a block copolymer **PC4** (Mn= 4.9kDa.; Đ= 1.09; Table S3) was synthesized from **PA4** (Mn= 4.1kDa.; Đ= 1.12; Table S1) and **PB1D** (Mn= 2.2kDa.; Đ= 1.08; Table S2). The block copolymer nature of **PC4** was confirmed by SEC and NMR analysis (Fig S30, S72).

Next, another conjugation reaction was performed with sterically hindered and higher molecular weight **PA5** (Mn= 14.1kDa.; Đ= 1.11; Table S1) and **PB2D** in a DMF-d7, CDCl$_3$ mixture (3:1) and the progress of the reaction was followed by DOSY NMR at 15, 30, 45 and 60 min. (Fig 2C, Fig S15). Complete conversion was observed within one hour and was also confirmed by SEC analysis of the crude reaction mixture (**PC5**; Mn= 18.8kDa.; Đ= 1.07; Fig S31). This also showed the excellent efficiency of NCL even with sterically hindered high molecular weight polymers.

To further illustrate the versatility of this method, another high molecular weight cysteine mono end functional PLA (**PB3D**; Mn= 20.6kDa.; Đ= 1.09; Table S2) was synthesized via DCC, DMAP coupling (See SI). Thereafter, **PB3D** was reacted with an equivalent amount of high molecular weight **PA3** to obtain **PC6** (Mn= 31.3kDa.; Đ= 1.15; Table S3), whereas **PC7** (Mn= 17.7kDa.; Đ= 1.05; Table S3) was obtained when high molecular weight sterically hindered **PA5** was reacted with **PB3D**. SEC and NMR spectroscopy analysis confirmed an excellent degree of

conjugation and formation of block copolymer **PC6** and **PC7** (Fig S9, S10, S32, S33, S39).

Next, the potential of this method was tested in ABA-type triblock copolymer synthesis. A commercially available bis(hydroxyalkyl) terminated poly(dimethylsiloxane) (PDMS) was end-functionalized with cysteine to obtain the cysteine bifunctional PDMS (**PB4D**; Mn= 5.1kDa.; Đ= 1.79) middle block. Thereafter, **PB4D** was reacted with two equivalents of thioester mono end functional **PA2** in the presence of a catalytic amount of triethylamine in DMF, CHCl$_3$ mixture (3:1) to obtain ABA type triblock copolymer **PC8**. Within one hour, a SEC analysis of the crude reaction mixture showed a single monomodal distribution (Mn= 14.3kDa.; Đ= 1.67) along with a complete shift in the SEC trace which confirmed the formation of the triblock copolymer (Fig S11, S12, S34). The excellent degree of conjugation and ABA-type triblock copolymer formation was also confirmed by DOSY NMR spectroscopy analysis of **PC8** (See SI).

The efficiency of NCL techniques was also tested in A-*alt*-B block copolymer synthesis in a step-growth fashion. At first, a thioester bifunctional polycyclooctene (**PA6**; Mn= 6.9kDa.; Đ= 1.72) was synthesized via ROMP in the presence of **CTA1** with a catalytic amount of Grubbs' 2nd generation catalyst (**G2**) under thermodynamic equilibration conditions (See SI). Subsequently, a cysteine bifunctional PEG (**PB5D**; Mn= 10.6kDa.; Đ= 1.06) was synthesized from commercially available PEG diol. Afterwards, an equivalent mixture of **PA6** and **PB5D** was reacted in the presence of catalytic amounts of triethylamine in DMF, CHCl$_3$

mixture (3:1), and the progress of the reaction was followed by SEC analysis. Within 3 hours, the reaction reached the final conversion indicated by a single distribution in the SEC trace (**PC9**; Mn= 1318kDa.; Đ= 1.78, Fig 2A, Fig S40). To rule out any possible crosslinking within **PC9**, the polymer sample was treated with the disulfide reducing agent DTT and reanalyzed in SEC. No significant change was observed in the SEC elugram before and after treatment with DTT (Fig S40c). Thus, the conjugated product must be a strict **PA6**-*alt*-**PB5D** block copolymer out of mechanistic necessity. The NMR spectroscopy and SEC analyses of the **PC9** have confirmed the formation of the alternating block copolymer with excellent efficacy and established the importance of NCL in non-natural complex polymer synthesis (Fig S13, S14).

Conclusion

In conclusion, we have demonstrated that NCL provided an alternative platform for complex non-natural macromolecular architecture design under mild reaction conditions with excellent efficiencies. The cysteine end functional polymers were synthesized from commercially available polymer, whereas thioester end functional polymers were synthesized via ROMP. Under NCL conditions, diblock copolymers were synthesized from mono end functional homopolymer within a short reaction time. Simultaneously, ABA-type triblock copolymer was also obtained from a bifunctional and mono end functional polymer with excellent efficiency. A complex A-*alt*-B alternating block copolymer was also obtained from a cysteine and a thioester bifunctional polymer within a short interval with a high degree of polymerization. NCL offers a unique platform to synthesize complex polymeric architecture from commercially available polymers. Simultaneously, excellent regio and chemoselectivity, high functional group tolerance, ultrafast coupling kinetics, mild reaction conditions make NCL superior over previously reported non-natural polymer coupling techniques. The pendant thiol group located at the center of the diblock copolymers was stable and did not cause any undesired disulfide side reactions. However, it could provide a reactive chemical handle for further post polymerization reaction such as thiol-ene reactions, nucleophilic aromatic substitutions and others. This is currently being investigated. We believe that the NCL approach to coupling synthetic polymers has not yet received the importance it deserves. It clearly has the potential to provide the route to synthesize complex polymeric architecture for smart material and technology developments.

Experimental Section

An equivalent mixture of cysteine end-functional polymer (**PB1D** to **PB5D**, see table S2) and thioester end-functional polymer (**PA1** to **PA6,** see table S1) was dissolved in 3:1 dry DMF and chloroform mixture. Thereafter, catalytic amount of triethylamine was to the polymer mixture and stirred for 1 to 4 hours under an argon atmosphere. After completion of the reaction (SEC monitoring) the solvent was evaporated in vacuum and the solid residue redissolved in DCM. Then, the concentrated polymer solution was precipitated into cold methanol to obtain the corresponding conjugated polymer (**PC1** to **PC9**, see table S3)

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Instruments data, Experimental methods, NMR data, MALDI-ToF data, SEC data and HRMS data. (PDF)

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Notes

There are no financial conflict to declare.

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Thioester end-functional ROMP

Native Chemical Ligation

Cysteine end-functional PEG/ PLA/ PDMS

Block Copolymer