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Single Addition of an Allyl Amine Monomer Enables Access to End-Functionalized RAFT Polymers for Native Chemical Ligation

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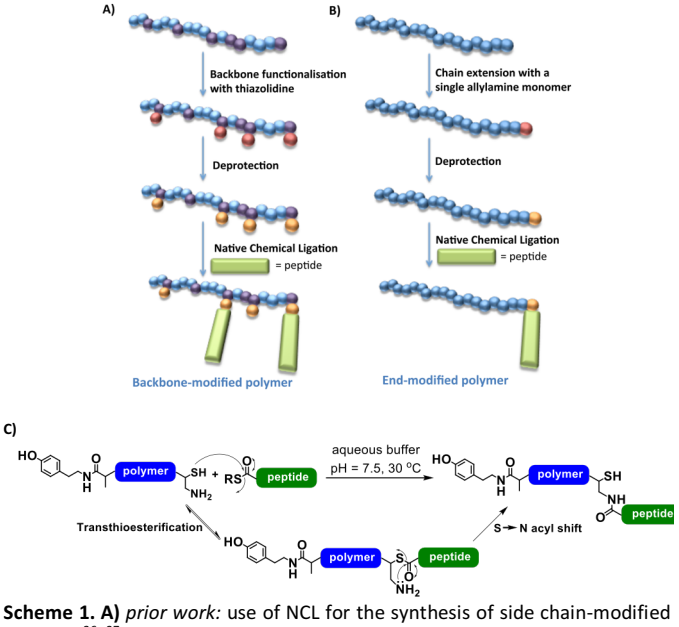
A novel method for the introduction of a single protected amine-functional monomer at the chain end of RAFT polymers has been developed. This monomer addition, in concert with native chemical ligation, facilitated the development of a simple and versatile method for the end-functionalization of polymers with peptides.

Integration of biomolecules such as peptides or proteins into the architecture of polymer scaffolds and nanoparticles affords biomaterials that display the functionality of both components. Such modifications of polymers enable researchers to enhance and optimize the existing properties of a biologically inert structure. For example, polymer-peptide conjugates[1] have been utilized to increase targeted cellular uptake of therapeutics[2] and improve cell adhesion[3] and growth in scaffold applications.[4-6] Equally, the modification of biological molecules with polymeric groups, e.g. polyethylene glycol (PEG), has been successfully employed to alter the physicochemical properties of numerous therapeutic proteins[2, 7] providing improved *in vivo* stability.

Access to polymer conjugates has been great expedited by advances in both synthetic polymer and bioconjugation chemistry. Specifically, developments in living radical polymerization (LRP) techniques, such as reversible addition fragment chain-transfer (RAFT) polymerization[8], make it possible to synthesize well-defined polymers with control over a number of variables, including composition, molecular shape, chain length and α- and ω-functionalities.[9-11] Furthermore, chemoselective bioconjugation methods have enabled a range of functionalities to be appended to polymers in high yield *via* a range of different chemical linkages.[12]

Three strategies are used to access polymer-peptide conjugates[13, 14]: (i) the *grafting from* method, where the polymer chain propagates (grows) from the pre-assembled peptide, (ii) the *grafting through* method which involves the copolymerization of peptide based macromonomers and (iii) the *grafting to* method, which involves the reaction between complementary reactive functional groups of a preformed polymer and a peptide. Among these approaches, the *grafting to* technique has been the most widely used, owing to the versatility and ease of performing the conjugation reactions in the final synthetic step to afford a given target. For polymers produced through RAFT, the thiocarbonylthio end group can serve as a latent thiol that can be liberated *via* aminolysis to unmask a new site of reactivity on the terminus of the polymer chain. The reactivity of this thiol has been exploited for conjugating a range of functionalities using efficient chemistries such as disulfide formation and thiol-ene and thiol-Michael reactions.[15, 16]

In this study we were interested in expanding the repertoire of chemistry that can be used to generate polymer-peptide conjugates through the use of native chemical ligation (NCL)[17] to functionalize the end group of RAFT polymers. NCL is the most robust and widely used ligation technology employed to assemble proteins from peptide fragments yet, surprisingly, has rarely been employed in the assembly of functional polymers. Traditionally, the NCL reaction uses two peptide substrates, one bearing an N-terminal Cys residue (or cysteine surrogate[18-20]) and the other a C-terminal thioester moiety and proceeds in aqueous solution at neutral pH without the



Scheme 1. A) *prior work:* use of NCL for the synthesis of side chain-modified polymers[26, 27] ,**B)** *this work:* synthesis of end-functionalized RAFT polymers and **C)** proposed general mechanism for the reaction of *pseudo*-cysteine end-functionalized RAFT polymers with peptide thioesters in NCL.

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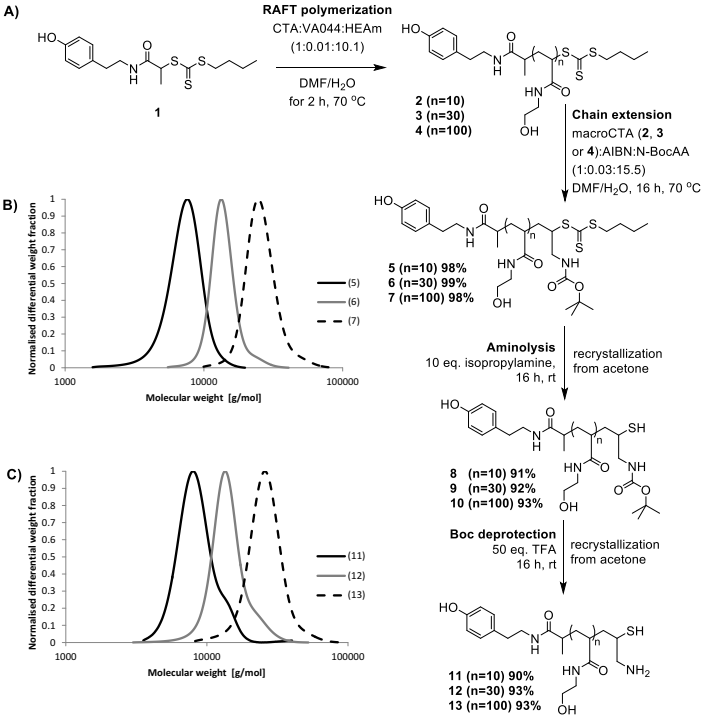
need for protecting groups on amino acid side chains.[21-25] To date NCL has only been reported on two occasions as a means to prepare functionalized polymers. [26, 27] In these examples, synthetic processing of monomeric units within the polymer (post-polymerization) was required to incorporate cysteine moieties into the side-chains of the polymers for subsequent NCL-based derivatization (see Scheme 1A for a schematic representation). While this approach provides a useful means for obtaining side-chain modified polymer-peptide conjugates, it is limited by the inherent heterogeneity of the products from incomplete modification of available sites. [26, 27] Herein, we report the use of NCL chemistry, in combination with RAFT polymerization technologies, to generate end-functionalized peptide-polymer conjugates in an efficient manner. Specifically, we describe a simple and reliable procedure to insert a single unit of a protected allylamine monomer[28, 29] at the chain end of RAFT polymers. This end group can be subsequently transformed into a *pseudo*-cysteine end-functionalized polymeric chain that can be used as a substrate for NCL reactions with thioesters (see Scheme 1B for schematic). Specifically, analogous to NCL on peptides, a thioesterification reaction between the thiol side chain on the polymer end group and the C-terminal peptide thioester would be followed by rapid rearrangement through a proximity-driven intramolecular S→N acyl shift to generate end-modified polymers (see Scheme 1C for schematic).

We began by polymerizing hydroxyethylacrylamide (HEAm), which was mediated by the RAFT agent tyramine-C4 **1**, to afford polymers **2**, **3** and **4** with degrees of polymerization of 10, 30 and 100, respectively (Scheme 2A). These polymers were subsequently extended, in a one-pot manner, with a single unit of a N-Boc allyl amine (N-BocAA) monomer by adopting a method that minimizes the amount of free radical initiator required for polymerization[30] to obtain excellent end group retention at full monomer conversion (see Supplementary Information for synthetic details). The resulting polymers (**5-7**) were analysed by size exclusion chromatography (Scheme 2B) and the addition of the single N-BocAA unit was confirmed *via* ^{1}H-NMR (See Supplementary Information). The addition of a single monomer unit can be rationalized by the deactivated nature of the N-BocAA monomer that, when added to the chain end of HEAm polymer (prepared from a much more activated HEAm monomer) dramatically decreases the chain transferability of the trithiocarbonate end group, thus ensuring that all terminal acrylamide units have the N-BocAA added. Once installed, the allylamine monomer unit cannot reinitiate[31, 32] thus resulting in a single monomer insertion to the end of the polymer.
Following insertion of the single N-BocAA unit, polymers **5-7** were next submitted to a two-step deprotection sequence involving aminolysis of the trithiocarbonate RAFT agent to give **8-10**, followed by acidolytic Boc-deprotection to afford **11-13** in excellent yield (Scheme 2A). It should be noted that the order of deprotection steps proved critical. Specifically, if Boc-deprotection preceded aminolysis, the amine facilitated intramolecular aminolysis of the trithiocarbonate to generate a stable dithiocarbamate moiety. Gratifyingly, the optimized deprotection sequence led to the quantitative generation of a 1,2-aminothiol functionality at the end of the polymeric chains, which we dubbed a "*pseudo*-cysteine" group, that served as the key functionality for NCL derivatization of the polymer end group. Molecular weight characterization of all polymers synthesized in this study were carried out using both size exclusion chromatography (Scheme 2B and 2C and Supporting Information) and MALDI-TOF mass spectrometry (see Supplementary Table 1).

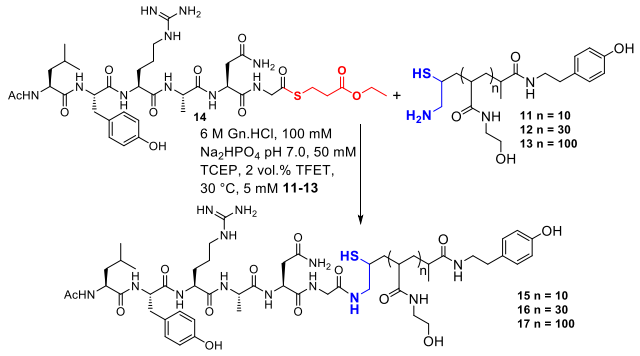


Scheme 2. A) Synthesis of *pseudo*-cysteine end-functionalized poly(hydroxyethylacrylamide) polymer, Size exclusion chromatography trace in DMAc solvent system analyzed with respect to PS standards for **B) 5-7** and **C) 11-13**. (CTA = chain transfer agent).

Polymers **11-13** bearing *pseudo*-cysteine end groups were next assessed as substrates for NCL reactions with model peptide thioester Ac-LYRANG-S$(CH_2)_2CO_2$Et **14** (synthesized by Fmoc-SPPS, see Supplementary Information) to generate end-functionalized polymer-peptide conjugates. All NCL reactions were conducted at pH 7.0 using *tris*-carboxyethylphosphine (TCEP) as a reductant and trifluoroethane thiol (TFET)[33] as an additive to convert the unreactive S-propionate ethyl ester of **14** into the corresponding reactive S-trifluoroethyl ester to accelerate the rate of the reactions (Table 1). We initially assessed the efficiency of NCL reactions between *pseudo*-cysteine end-functionalized polymer **11** and peptide thioester **14** in a number of different buffer/solvent mixtures. These included the use of phosphate buffer or HEPES, with or without the denaturant guanidine and HEPES buffer with and without the presence of the organic co-solvents NMP or DMF (see Supplementary Information for details). The reactions were performed using an excess of polymer (peptide/polymer = 0.8) and the yield of polymer-peptide conjugate was monitored by HPLC-MS by integrating the peaks corresponding to the starting polymer, peptide thioester and conjugate at λ = 280 nm. Interestingly the reactions proved to be tolerant to a range of conditions, with similar yields (67-70%) achieved regardless of the solvent system used to perform the reaction (see Supplementary Information).

Having established that NCL between thioester **14** and the *pseudo*-cysteine end group of polymer **11** was tolerant to a range of buffer/solvent conditions, we employed a single set of buffer conditions (6 M Gn•HCl, 100 mM Na_2HPO_4 pH 7.0), typically used for NCL reactions of peptide fragments, for the remainder of the experiments and focused on assessing the effect of stoichiometry of the polymer to peptide thioester reactants on the ligation (see Table 1). Towards this end, polymers **11-13** (at 5 mM concentration) were reacted with peptide thioester **14** at polymer:peptide ratios ranging from 0.3-3.0. Gratifyingly, the NCL reactions proceeded well under most conditions to afford polymer-peptide conjugates in moderate to excellent yield (42-78%) as judged by HPLC-MS analysis, with higher yields obtained when one of the reagents was used in large excess (e.g. three-fold excess of polymer or peptide). A general trend observed from these studies was that increasing the degree of polymerization (DP) of the polymer from 10-100 led to higher reaction yields, an observation which might be counterintuitive considering the increase in steric hindrance with larger polymeric chains. This trend could however be rationalized by an increase in hydrophilicity of the polymer with increasing degree of polymerization, thus leading to enhanced solubility and therefore a faster reaction with **14**.

Table 1. Yields for NCL reactions between *pseudo*-cysteine end-functionalized p(HEAm) polymers **11-13** and peptide thioester **14** to afford polymer-peptide conjugates **15-17**.



peptide thioester:polymer ratio	Yield of polymer-peptide conjugate **15**[a]	Yield of polymer-peptide conjugate **16**[a]	Yield of polymer-peptide conjugate **17**[a]
0.3	75	71	70
0.8	59	68	77
1.2	43	51	50
1.5	48	59	76
2.0	43	64	68
3.0	67	58	72

[a] Yields are based on HPLC-MS analysis by integration of the peak corresponding to polymer-peptide conjugates **15-17** with respect to the limiting reactant at λ = 280 nm and are the average of two independent experiments.

Conclusions

In summary, we have described an efficient method for the introduction of a single N-BocAA monomer into the chain end of RAFT polymers. Following deprotection the reactivity of the "*pseudo*-cysteine" end group was harnessed in the generation of polymer-peptide conjugates through the reaction with peptide thioesters. The simplicity and versatility of this methodology lays the foundation for its use as a ligation technique for the modification of polymeric chain ends with functional peptides or proteins for applications as biomaterials or therapeutic leads in the future.

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