



Self-Immolative RAFT-Polymer End Group Modification

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Dedicated to Professor Horst Kunz on the occasion of his 80th birthday

Reversible modifications of reversible addition–fragmentation chain transfer (RAFT)-polymerization derived end groups are usually limited to reductive degradable disulfide conjugates. However, self-immolative linkers can promote ligation and traceless release of primary and secondary amines as well as alcohols via carbonates or carbamates in β -position to disulfides. In this study, these two strategies are combined and the concept of self-immolative RAFT-polymer end group modifications is introduced: As model compounds, benzylamine, dibenzylamine, and benzyl alcohol are first attached as carbamates or carbonates to a symmetrical disulfide, and in a straightforward one-pot reaction these groups are reversibly attached to aminolyzed trithiocarbonate end groups of RAFT-polymerized poly(*N,N*-dimethylacrylamide). Quantitative end group modification is confirmed by ^1H NMR spectroscopy, size exclusion chromatography, and mass spectrometry, while reversible release of attached compounds under physiological reductive conditions is successfully monitored by diffusion ordered NMR spectroscopy and thin layer chromatography. Additionally, this concept is further expanded to protein-reactive, self-immolative carbonate species that enable reversible bioconjugation of lysozyme and α -macrophage mannose receptor (MMR) nanobodies as model proteins. Altogether, self-immolative RAFT end group modifications can form the new basis for reversible introduction of various functionalities to polymer chain ends including protein bioconjugates and, thus, opening novel opportunities for stimuli-responsive polymer hybrids.

transfer (RAFT) polymerization has been most frequently used for various purposes.^[2–5] To control regular polymer growth, small molecular thiocarbonylthio compounds act as chain transfer agents which result in heterotelechelic polymers carrying thiocarbonylthio groups at the propagating chain end.^[6,7] For several applications, however, including commercialization^[8] or biomedical applications^[9,10] the presence of dithioesters or trithiocarbonates is often considered as less beneficial, e.g., due to strong UV-absorbance or reactivity toward nucleophiles. Thus, multiple attempts have been established to remove or modify them after polymerization quantitatively.^[11,12]

For instance, by treatment with excess radical initiator, Perrier et al. could replace dithiobenzoates with AIBN-derived cyanoisopropyl groups and other analogues,^[13] while Chong et al. introduced hydrogens via radical-induced reduction by silanes, stannanes, or hypophosphite salts.^[14] More lately, Alagi et al. could use trialkylboranes in the presence of oxygen for that purpose as well.^[15] Instead of hydrogens also hydroxyl groups could be introduced, either by hydrogen peroxide^[16] or in the presence of oxygen.^[17] Similarly, irradiation^[18] or thermolysis^[19] could remove or convert thiocarbonylthio end groups, too. Alternatively, the thiocarbonyl double bond was utilized as reversible anchoring point for dienes under hetero-Diels Alder reaction conditions.^[20]

Moreover, under nucleophilic attack, the thiocarbonylthio end groups can as well release thiol terminated polymers. Because of their selective reaction profile, these thiols can subsequently be converted in a post-polymerization modification process^[21] either irreversibly into thioethers or reversibly into disulfides.^[22] For instance, Lima et al. or Qui et al. demonstrated aminolysis of trithiocarbonates and dithiobenzoates followed by Michael addition to α,β -unsaturated esters,^[23,24] while Boyer et al. included maleimides for the introduction of biotins for polymer–protein conjugation.^[25] In analogy, Grover et al. reacted aminolyzed thiols with excess divinyl sulfones and the affording vinyl sulfone group could be utilized for further Michael-type bioconjugation to the cysteine of bovine serum albumin.^[26] This approach was also applied by us and colleagues to RAFT block copolymer based nanogels affording core/shell protein-reactive nanoparticles. However, due to the

1. Introduction

For about 25 years, controlled radical polymerization techniques have revolutionized polymer synthesis providing access to narrowly dispersed homo- and block copolymers with well-defined end groups.^[1] Because of its broad applicability to various types of functional monomers, solvents and reaction conditions, the reversible addition–fragmentation chain

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basic reaction conditions favoring gradual thiol oxidation it was necessary to first trap the aminolyzed dithiobenzoate and trithiocarbonate end groups as dithiopyridines, followed by reduction and conversion into vinylsulfones.^[27]

In general, converting thiol end groups into reactive dithiopyridines is highly beneficial, as it provides immediate access to reversible conjugation with other functional thiols as bioreducible disulfides.^[28] Furthermore, also Ellman's reagent (5,5'-dithiobis(2-nitrobenzoic acid) has already been used for that purpose trapping RAFT-derived polymers into thiol reactive dithionitrobenzoic acids.^[29,30] Alternatively, introduction of functional asymmetric disulfides can be successfully obtained in a one pot procedure by aminolysis in the presence of functional methanethiosulfonates, as reported by multiple examples from Roth et al.^[31–35]

Although reversible end group modification into bioreducible disulfides seems highly attractive, especially for biomedical purposes guaranteeing reductive triggered release of the conjugated functional group,^[36,37] it is only limited to either single thiol-bearing active molecules or requires the introduction of thiols onto the desired entity which, however, may affect the molecule's bioactivity.

To circumvent this problem, reductive responsive self-immolative linkers have been investigated making use of carbonates or carbamates in β -position to the disulfide.^[38,39] Upon disulfide cleavage these molecules undergo an intramolecular 5-exo-trig cyclization (or 3-exo-tet cyclization)^[40] and during 1,3-oxathiolan-2-one formation the adjacent alcohol, primary or secondary amine is released.^[41] This approach has successfully

been applied by the Zelikin group to various RAFT-polymerizable monomers carrying different types of drugs via this self-immolative linker strategy.^[42–50]

Additionally, self-immolative systems have also been developed in recent years to enable the reversible attachment of polymer chains to proteins. Besides reversible PEGylation by thiol-ester exchange with self-immolation by cyclization,^[51] there are also approaches that use 1,6-elimination linkers, exploiting esterases,^[52] thiol-disulfide exchange,^[53] or azoreductases^[54] as trigger mechanisms.

In this study, we now aim to combine RAFT end group modification and reductive responsive self-immolative linkers in a one pot fashion. By liberating the RAFT end group's thiol via aminolysis, it is subsequently converted into a disulfide with a carbamate or carbonate in β -position. Under these circumstances, one can access self-immolative RAFT end groups that upon disulfide cleavage release the conjugated primary and secondary amine or alcohol, respectively. This broadens the applicability of reversible RAFT end group conjugations to a variety of functional active groups including reversible protein bioconjugates.

2. Results and Discussion

To convert the RAFT-polymerization derived thiocarbonylthio end group into a self-immolative disulfide species, we established the reaction process summarized in **Figure 1**: A reactive 4-nitrophenyl carbonate is first generated on both

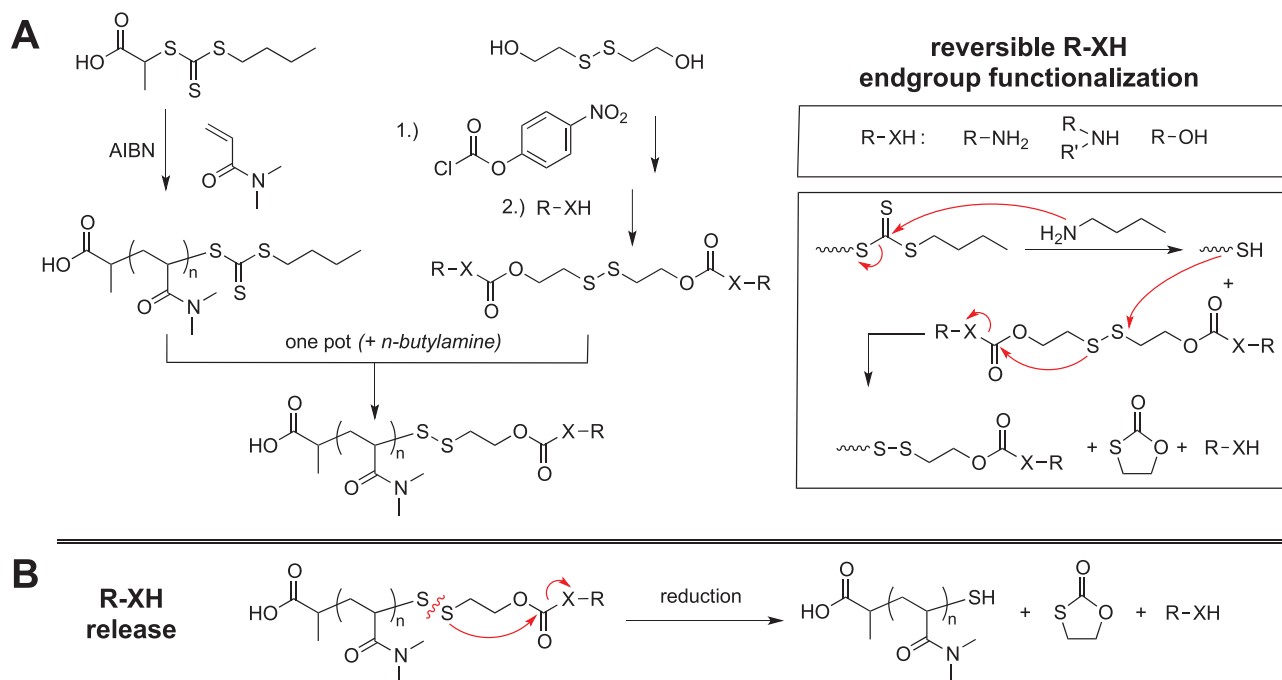


Figure 1. Synthetic scheme for reversible end modification of RAFT-polymerized poly(*N,N*-dimethylacrylamide) (pDMA). A) *N,N*-Dimethylacrylamide (DMA) is polymerized by RAFT-polymerization with azobis(isobutyronitril) (AIBN) and 2-(butylthiocarbonothioylthio)propanoic acid as chain transfer agent (CTA) (left). 4-nitrophenyl chloroformate is introduced to 2,2'-disulfanediyldiethanol as symmetric disulfide that allows subsequent conjugation of component R with variable functionality X (= primary/secondary amine, alcohol). This compound can be coupled to the polymer end group in a one-pot reaction after in situ aminolysis of the trithiocarbonate by *n*-butylamine (right). B) Self-immolation mechanism after disulfide reduction followed by, e.g., 5-exo-trig cyclization and release of component R-XH.

sides of a symmetric 2,2'-disulfaneyldiethanol, which can easily be substituted by desired functional groups of choice, including alcohols as well as primary amines and secondary amines. This molecule can subsequently be applied during a one-pot RAFT-polymer trithiocarbonate group aminolysis. The released thiol reacts in situ with the symmetrical component during a disulfide reshuffling process liberating one of the attached functional groups under entropically favorable intramolecular cyclization conditions (Figure 1A). At the same time, the affording polymer is equipped with a self-immolative end group that is responsive to disulfide reduction as external trigger. Thereby, the cleaved disulfide can undergo again, e.g., a 5-exo-trig cyclization releasing the originally attached alcohol and primary or secondary amine, respectively, in a fully unmodified fashion (Figure 1B). The experimental results of this concept are outlined in the following chapters by different model compounds.

2.1. Introduction of the Self-Immolative RAFT-Polymer End Group

Under RAFT-polymerization conditions using 2-(butylthiocarbonothioylthio)propanoic acid as chain transfer agent (CTA) (Figure S1, Supporting Information), narrow distributed poly(*N,N*-dimethylacrylamide) (pDMA) was synthesized from *N,N*-dimethylacrylamide (DMA) and carefully analyzed by ^1H NMR spectroscopy, size exclusion chromatography (SEC), and

matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-ToF MS) (Figure 2 and Figure S2, Supporting Information). pDMA was chosen as a suitable platform, since it meets all requirements necessary for this study: It is both water-soluble and biocompatible making it, therefore, appropriate for biological applications including conjugation to drugs and proteins.^[55–57] In addition, the chosen system exhibits excellent properties for MALDI characterization with high end-group fidelity^[58] (in our hands, dithiobenzoates as alternative CTAs usually provided degradation and backbiting processes during MALDI measurement, thus, did not allow to trace back successful end group modifications quantitatively).

In order to successfully convert the RAFT-derived trithiocarbonate group on each polymer into a self-immolative conjugate, a reagent was required which would always lead to the formation of a well-defined product under usually random disulfide-exchange reactions by the end group released thiol. For that purpose, our strategy was restricted to symmetric disulfide carbonates or carbamates. Starting from 2,2'-disulfaneyldiethanol, we first introduced reactive nitrophenyl carbonate units which allows to conjugate afterwards various functionalities of choice as carbonates and carbamates. Altogether, by this simple two-step synthesis, benzylamine (H_2NBz), dibenzylamine (HNDiBz) and benzyl alcohol (HOBz) were attached as model compounds for primary and secondary amines as well as alcohols, respectively, demonstrating the versatility of this platform (for more experimental details compare Figures S3–S18, Supporting Information).

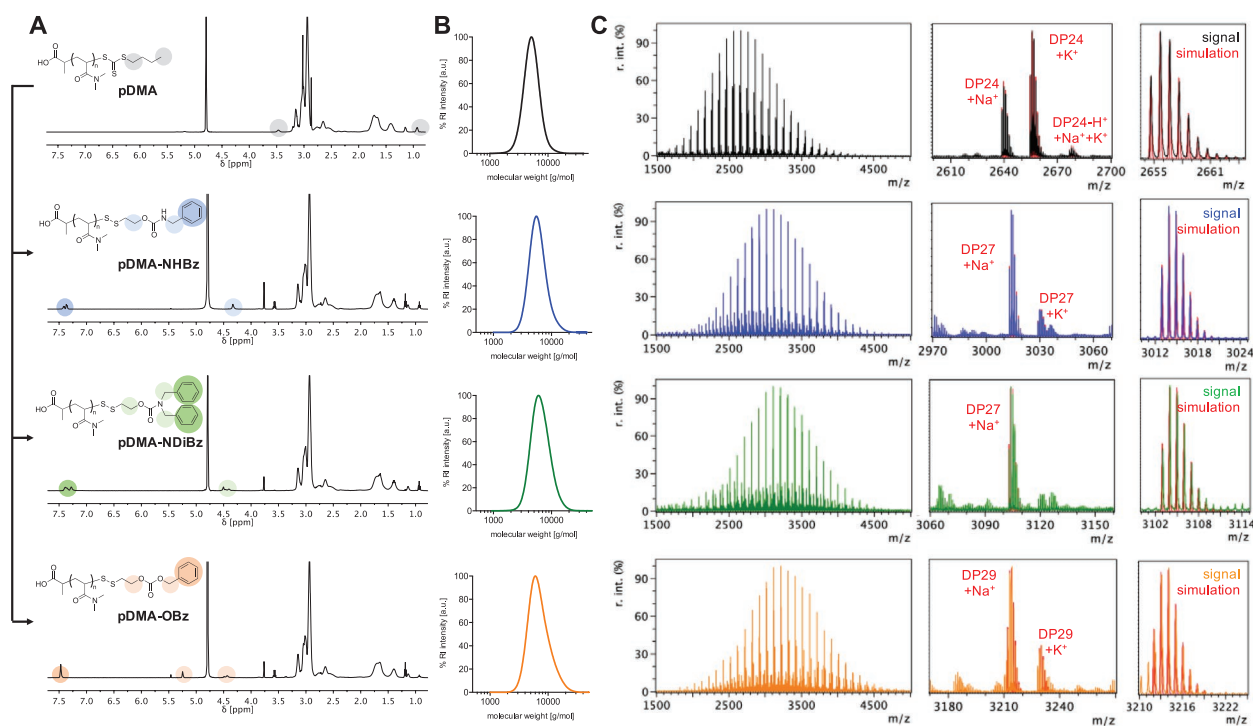


Figure 2. Characterization of end group modified RAFT-polymers (pDMA, black; pDMA-NHBz, blue; pDMA-NDiBz, green; pDMA-OBz, orange). A) ^1H NMR spectra of pDMA and end group modified pDMAs. The butyl trithiocarbonate signals as well as the introduced respective aromatic and benzylic proton signals are highlighted. B) SEC traces of pDMA and end group modified pDMAs. C) MALDI-ToF MS data of pDMA and end group modified pDMAs: full polymer mass range (left); zoomed mass range of DP with highest relative intensity (middle); overlay of DP with highest relative intensity and its corresponding simulated isotope pattern in red (right).

In a straightforward postpolymerization reaction, these symmetrical disulfide compounds could be attached during a one pot reaction to the polymer. The free thiol end group was initially generated in situ by aminolysis with five equivalents of *n*-butylamine. At the same time, five equivalents of the symmetric disulfide compound were added. Due to their symmetrical structure, regardless of the reaction site of the thiol toward the disulfide, only the formation of one specific product was possible during the disulfide reshuffling reaction (under sacrifice of the other half of the symmetric disulfide, compare Figure 1A).

The obtained pDMA-NHBz, pDMA-NDiBz, and pDMA-OBz polymers were isolated by three precipitation cycles into diethyl ether and subsequently characterized by various techniques to verify the polymers' new end group carefully. While SEC revealed a preservation of the narrow molecular weight distribution during the modification reaction (Figure 2B), the introduced end groups could all be found by ¹H NMR spectroscopy (Figure 2A—a detailed characterization is summarized in the Supporting Information in Figures S19, S21, and S23, Supporting Information). More precisely, a comprehensive investigation of which species was actually generated could be performed by MALDI-ToF MS: For all end group modifications, successful and quasi-quantitative conversion of the whole polymer distribution was found, as provided by Figure 2C. Within the distributions, each mass peak could be annotated to the corresponding degree of polymerization together with the appropriate self-immolative disulfide end group. Having a closer look, detailed end group modification could also be confirmed by the recorded isotope pattern which is in perfect agreement with its simulated isotope pattern (Figure 2C—note that there was a slight shift of the molecular weight distribution to higher molecular weights as a result of additional polymer precipitation cycles for reaction work-up). At this point one should further highlight that neither SEC nor MALDI-ToF MS revealed any traces of disulfide-based polymer dimerization that would theoretically be conceivable through a second disulfide exchange from a polymer end to an already modified one.

Altogether, the applied reaction conditions (five equivalents of symmetric disulfide compound and five equivalents of *n*-butylamine) were all fully sufficient to convert all trithiocarbonate end groups of the RAFT-derived pDMA into self-immolative disulfide units for reductive responsive release of alcohols, primary and secondary amines, respectively.

2.2. Self-Immolative End Group Release Upon Reductive Trigger

In order to demonstrate the reversibility of this modification, a controllable release of the attached components should only occur under reductive conditions. For disulfides, this is commonly achieved by an excess of free thiols in form of, e.g., intracellular glutathione in a biological surrounding.^[59,60] In order to mimic those physiological conditions and simultaneously ensure a reasonable traceability, release experiments were carried out in a deuterated, aqueous environment in the presence of 10×10^{-3} M DTT as a source of free thiols. Thereby, the re-establishment of the originally introduced compounds could

be observed instantaneously by signals shifting from benzyl carbonates/ carbamates to benzyl alcohols/amines appearing by ¹H NMR spectroscopy (Figures S20, S22, and S24, Supporting Information). In order to get a better insight into those processes, diffusion ordered spectroscopy (DOSY) experiments were additionally recorded of these samples before and after addition of DTT (Figure 3A): While the reversibly attached aromatic end group protons provided similar diffusion characteristics like the macromolecular pDMA protons, these signals disappeared after disulfide reduction and instead low molecular weight aromatic components were recorded. This behavior could be found for all three end group modified polymers (pDMA-NHBz, pDMA-NDiBz, and pDMA-OBz) underlining full release and separation of benzylamine (H₂NBz), dibenzylamine (HNDiBz), and benzyl alcohol (HOBz) from the macromolecular species (Figure 3A).

In addition, the restoration of the unmodified, original functionalities was further confirmed by thin layer chromatography (TLC) with reference substances and corresponding staining agents (Figure 3B). A ninhydrin solution served as a suitable proof of tracelessly released amines (benzylamine and dibenzylamine), whereas a vanillin staining was used for detecting the released, unmodified benzyl alcohol.^[61] In each of those cases, while exhibiting identical retardation factors, moreover the same characteristic coloration could be observed compared to the reference substances, indicating successful and residue-free release (Figure 3B).

Altogether, these observations confirm that via our approach a reversible conjugation and reductive-triggered release of alcohols, primary and secondary amines to RAFT-derived end groups is possible. Interestingly, instead of introducing alcohols, primary and secondary amines first via the reactive nitrophenyl carbonate species to the symmetrical disulfide, it would also be feasible to directly attach the reactive nitrophenyl carbonates to the polymer end group and then perform a ligation via another post-polymerization modification reaction. This approach would especially be highly beneficial for bioconjugations, e.g., of proteins where one does not want to sacrifice half of the amount of the molecule of interest.

2.3. Self-Immolative RAFT–Polymer Protein Conjugation

By applying excess symmetrical nitrophenyl carbonate disulfides to the RAFT end groups, protein-reactive end groups can be made accessible for reversible polymer bioconjugations to lysine residues. However, since the in situ release of thiol is achieved by aminolysis with 5 equivalents of *n*-butylamine, some of them would also react with the introduced reactive carbonate end group. A prior isolation of the free thiol after aminolysis is, indeed, not possible without triggering partial dimerization of two chain ends by oxidation. To circumvent these possibilities, we decided to use a large excess of symmetrical nitrophenyl carbonate (50 equivalents) that can react with remaining amine units but still leaves a fair amount of end group reactive symmetrical nitrophenyl carbonate disulfides available for successful modification (Figure 4A). As a result, the whole procedure could still be carried out as a one-pot reaction affording only minor polymer dimerization (Figure S25,

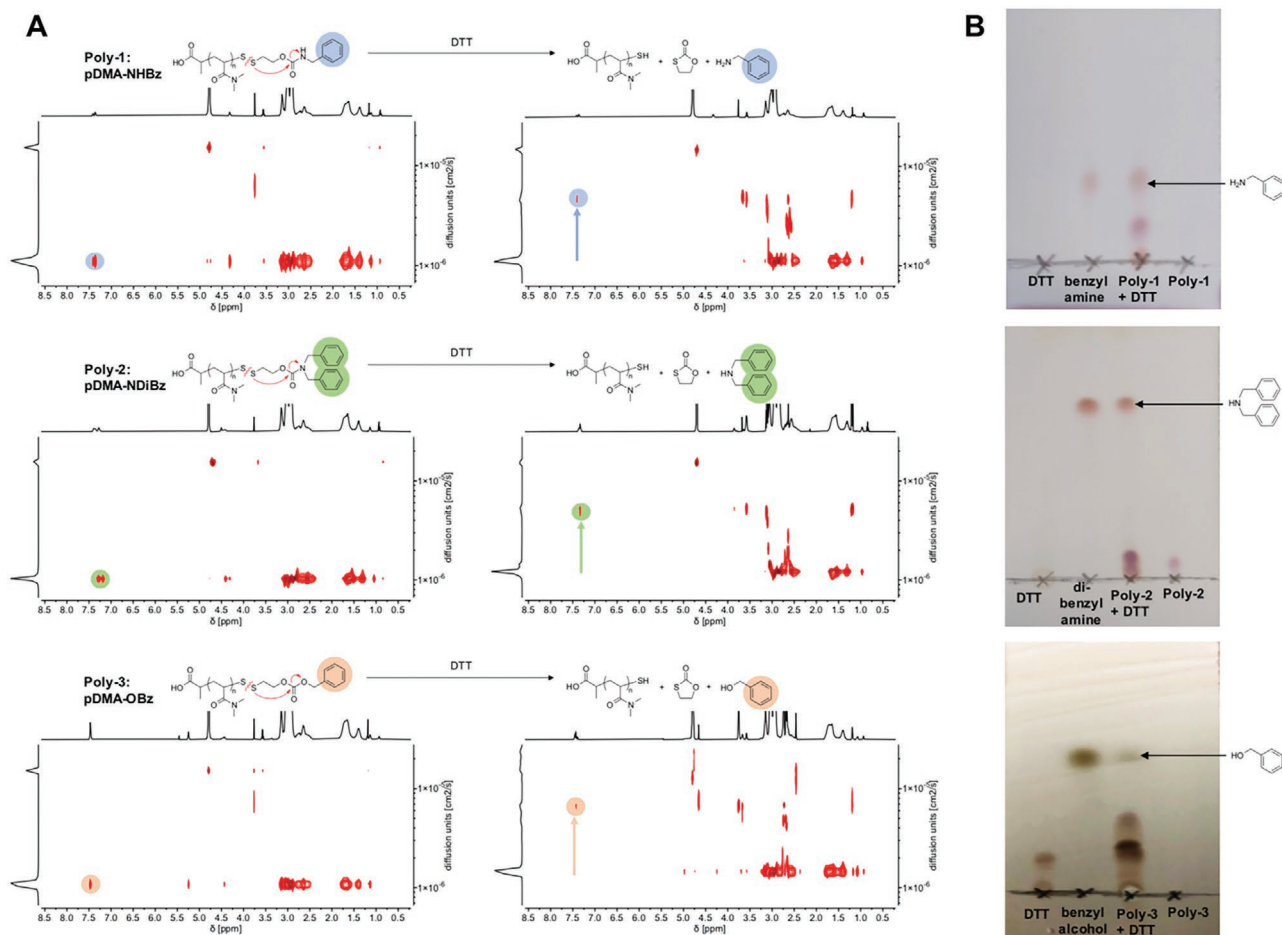


Figure 3. Residue-free release of benzylamine (blue, top), dibenzylamine (green, middle) and benzyl alcohol (orange, bottom) by self-immolation of the introduced RAFT-derived end group. A) DOSY experiments showing the attachment of aromatic compounds (H_2NBz , HNDiBz , and HOBz) to the polymer (left) and the successful release of aromatic components as lower molecular weight species (right) after treatment with 10×10^{-3} M DTT in D_2O . B) TLC of the polymers before and after reduction with 10×10^{-3} M DTT next to respective references. Ninhydrin staining (top, middle) and vanillin staining (bottom) indicating the re-establishment of initial functionalities (H_2NBz , HNDiBz , and HOBz).

Supporting Information). After purification the reactive nitrophenyl species ligated to the polymer end group could be detected by ^1H NMR spectroscopy successfully (Figure S26, Supporting Information).

In a next step, lysozyme was reversibly modified with the reactive end group polymer as a model protein (Figure 4—left). Here, a straightforward approach was again chosen in which the protein dissolved in PBS was simply incubated with a 75 equivalent excess of reactive polymer in PBS. After reversible polymer protein conjugation, the release of native protein was carried out under physiological concentrations of free thiols by addition of mercaptoethanol at a millimolar range. The individual steps of covalent conjugation and release were followed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Figure 4B). For the conjugate the sharp lysozyme band (lane 2) blurred due to the binding of multiple polymer chains (lane 3). Fortunately, it can reversibly be released under controlled reduction conditions (lane 4) emphasizing successful conjugation and release of the unmodified protein under the proposed self-immolative reaction conditions.

In addition to this model protein, we selected a more relevant protein in the same molecular weight regime: a single chain antigen-binding antibody fragment called nanobody.^[62,63] In general, such types of proteins are considered as promising replacement for cumbersome produced antibodies used for diagnostic or therapeutic purposes.^[64,65] Some nanobodies have even been tested to prevent infection by the SARS-COV2 virus.^[66–68] In nanomedicine, they furthermore function as targeting moiety for precise delivery of, e.g., pH-responsive nanogels.^[69,70] One disadvantage compared to antibodies, however, is their weaker pharmacokinetic profile with a relatively short circulation time in the blood stream.^[71]

Conceptually, this might be enhanced by reversible polymer conjugation, e.g., of pDMA as polymer of significantly lower antibody-mediated accelerated blood clearance compared to the widespread PEGylation.^[72] Representatively, here, a nanobody against the macrophage mannose receptor ($\alpha\text{-MMR Nb}$)^[73] was modified in analogy to lysozyme (Figure 4—right). Again, by SDS-PAGE (Figure 4B) an almost complete conversion of the native protein (which partially occurred as dimer—lane 2) could

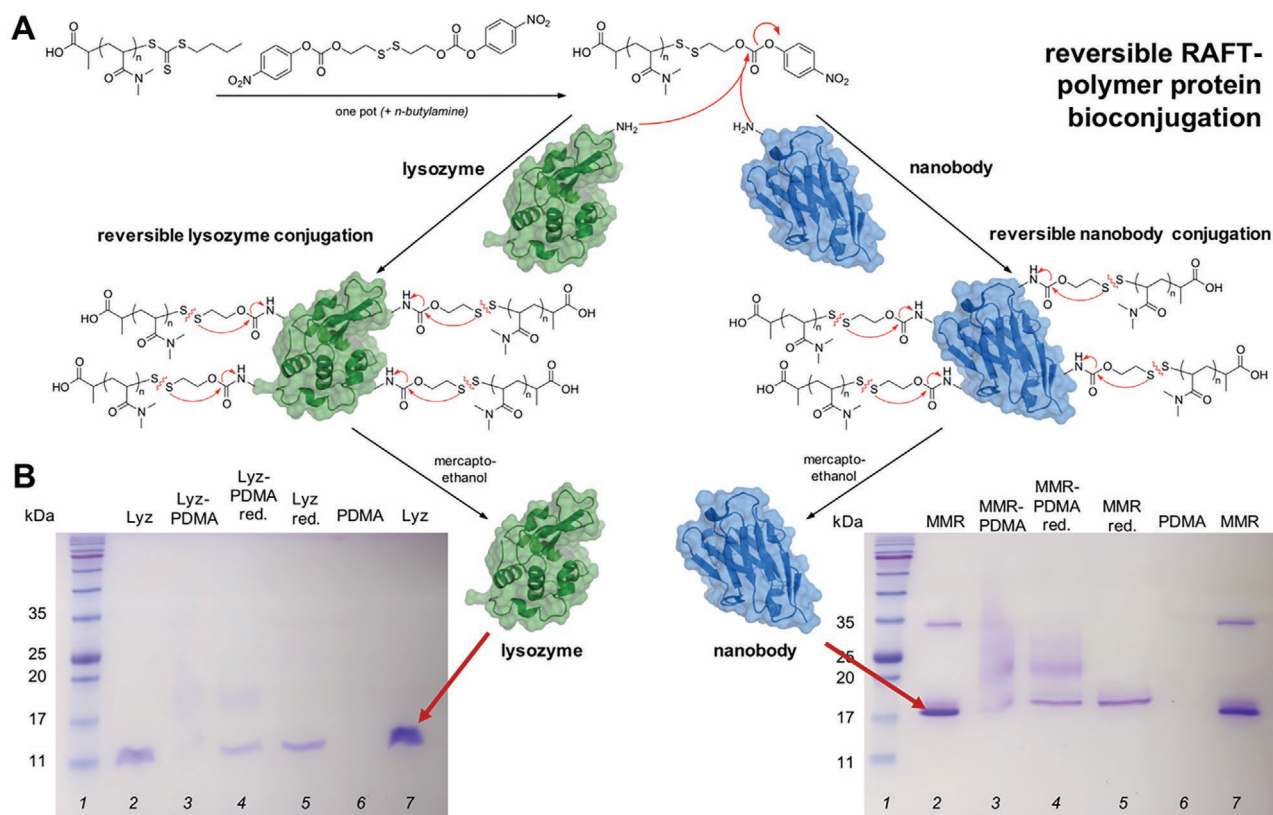


Figure 4. Reversible bioconjugation of lysozyme and α -MMR Nb via self-immolative end group modified RAFT-polymer. A) Synthetic scheme for reversible polymer modification of lysozyme (green, left) and α -MMR nanobody (blue, right). The RAFT-derived trithiocarbonate end group of pDMA is cleaved by aminolysis and the resulting thiol group undergoes a disulfide exchange reaction with a symmetrical disulfide bearing reactive carbonate. It results in the presence of an amine sensitive polymer end group which is used for the coupling to lysine residues onto proteins. Reductive treatment with mercaptoethanol will cause disulfide reduction followed by subsequent self-immolation and release of native protein. B) Corresponding SDS-PAGE results for the reversible polymer conjugation of lysozyme (left) and α -MMR nanobody (right). In each gel, a protein standard is used in the first lane. The native proteins are shown by lanes 2 and 7. After polymer conjugation a broad blur without distinct molecular weight is observed (lanes 3), but after treatment with mercaptoethanol the distinct protein band reoccurs (lanes 4). For comparison, mercaptoethanol-treated native protein (lanes 5) and blank polymer (lanes 6) were loaded onto the gel, too. Note that the second band for native α -MMR Nb at doubled molecular weight (lanes 2 and 7, right) is due to dimerization of monomeric protein during storage.

initially be observed (lane 3). It was again successfully restored by reduction (lane 4) providing the reduced nanobody protein as single band (lane 5).

In conclusion, this approach might inaugurate a new method for reversibly modifying various types of proteins and other functional biomolecules with water-soluble, RAFT-polymerization derived functional polymers following the reductive cleavable self-immolative linker concept.

3. Conclusion

In this work, the combination of postpolymerization modification of RAFT-polymer derived end groups and self-immolation strategies could be accomplished. For this purpose, model compounds of primary and secondary amines and alcohols were reversibly attached to a symmetrical disulfide equipped with reactive carbonates in β -position to the disulfide. In a straightforward one-pot reaction, it was possible to attach these conjugates to the aminolyzed trithiocarbonate end group of

RAFT-polymerized pDMA and confirm successful end group modification by ^1H NMR spectroscopy, size exclusion chromatography, and MALDI-ToF mass spectrometry. The reversibility of this modification by reduction with physiological concentrations of free thiols could be demonstrated in the presence of 10×10^{-3} M DTT by means of DOSY experiments showing full release of the primary and secondary amine and the alcohol as low molecular compound. In addition, the re-establishment of their original functionalities could be confirmed by thin layer chromatography (TLC) and comparison with corresponding reference substances and respective staining solutions underlined successful release in a self-immolative manner.

In addition, a protein-reactive, self-immolative carbonate species could further be introduced onto the pDMA end group and utilized for the bioconjugation of lysine residues of lysozyme and the α -MMR nanobody. When treated with free thiols, these conjugates were able to release the native protein again, as monitored by SDS-PAGE.

To conclude, this approach forms the basis for a new opportunity of reversible end-group modifications of different

RAFT-derived polymers with various types of primary, secondary amines or alcohols. By further introducing protein reactive groups, this versatile concept can also be applied to polymer–protein bioconjugates and, thus, opens to opportunities for various reductive-responsive polymer–protein hybrids.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

Data available in article supplementary material or on request from the authors.

Keywords

biodegradable polymers, disulfide, end group modification, RAFT polymerization, self-immolative linkers

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