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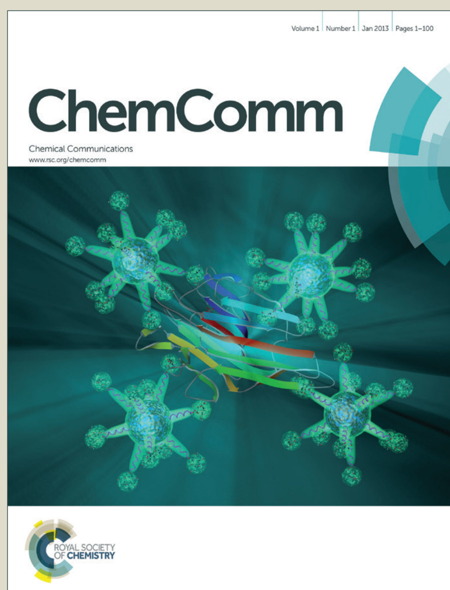

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The Best of Both Worlds: Active Enzymes by Grafting-To followed by Grafting-From a Protein

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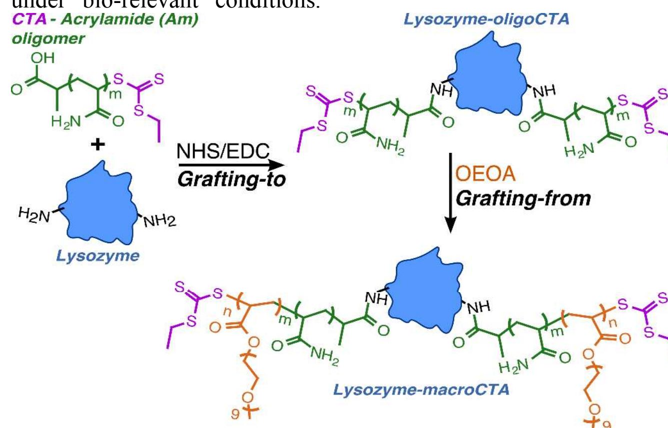
Hydrophilic polymers were attached to lysozyme by a combination of grafting-to and grafting-from approaches using RAFT polymerization. A hydrophilic oligomer was synthesized, and attached to the protein. The protein-oligomer hybrid contained the RAFT end group, enabling chain extension in solution. Lysozyme maintained activity throughout this process.

Protein-polymer conjugates are important and fascinating biomaterials. Tethering polymers from the protein surface or the covalent incorporation of proteins into polymeric materials can improve the stability of proteins,[1] enhance or shift the pH/temperature optimum of enzymes,[2] or synthesize responsive and functional materials.[3-6] There are two commonly used strategies for the synthesis of protein-polymer conjugates, the "grafting-from" and the "grafting-to" methods.[7] In grafting-from, an initiator or chain transfer agent (CTA) is attached to the protein, followed by the growth of the polymer from the protein-initiator/CTA in an aqueous medium.[7, 8] In the grafting-to approach, a polymer is synthesized in a separate reaction, characterized and subsequently attached to the protein using an efficient organic reaction.[7] The advantages of grafting-from include facile purification of the protein-polymer conjugate, and in many cases a high graft density.[7] When grafting-from, it can be a challenge to design reaction conditions that lead to well controlled polymers while maintaining protein structure.[7] The advantages of the grafting-to approach are facile characterization of the protein and polymer before conjugation, and that the polymerization conditions do not affect protein stability.[7, 9, 10] The disadvantages of grafting-to are the relatively low graft densities, especially with longer polymers, and difficulties in the purification process.[8, 11] Recently, reversible deactivation radical polymerization (RDRP) methods,[12] have revolutionized the field of protein-polymer conjugates.[3, 9] RDRP methods allow well-defined protein-polymer to be synthesized by either grafting-to or grafting-from a protein.[3]

The three most commonly used RDRP methods are reversible addition-fragmentation chain transfer polymerization (RAFT),[13] atom transfer radical polymerization (ATRP)[14, 15] and nitroxide mediated polymerization (NMP).[16] RDRP methods, including RAFT, offer several advantages including compatibility with various functional groups, and the ability to chain extend the polymer after it is attached to the protein. Protein-polymer conjugates have been synthesized by each RDRP method,[2, 10, 17-25] however, there still remain significant challenges. The two main challenges are: ensuring that all of the native protein is conjugated with well defined polymers, and using polymerization conditions that preserve protein structure and function.[7, 8, 11] This leads to the concept of reactions under "bio-relevant" conditions.[11]



Scheme 1. The hybrid grafting-to and grafting-from method for protein-polymer conjugate synthesis. A preformed oligoCTA is attached to lysozyme, followed by chain extension by RAFT.

In our preliminary work, the small molecule CTA, 2-(((ethylthio)carbonothioyl)thio)propanoic acid (PAETC), was attached to the protein lysozyme. However, as shown in Figure S1, even though PAETC is water soluble, the formed protein-CTA

conjugate was unstable, and a significant portion of this enzyme-CTA precipitated out of solution. UV-Vis indicated that 2 PAETC molecules attached to lysozyme (Figure S2). This challenge inspired a new synthetic strategy, which combines the strengths of RAFT polymerization with the best of the grafting-to and grafting-from techniques. In this communication lysozyme was selected as a model protein due to its well-characterized structure and function, which can be used to confirm that the protein-polymer conjugate retains function and has not lead to degradation of the protein. The hybrid-grafting-to and grafting-from approach is shown in Scheme 1.

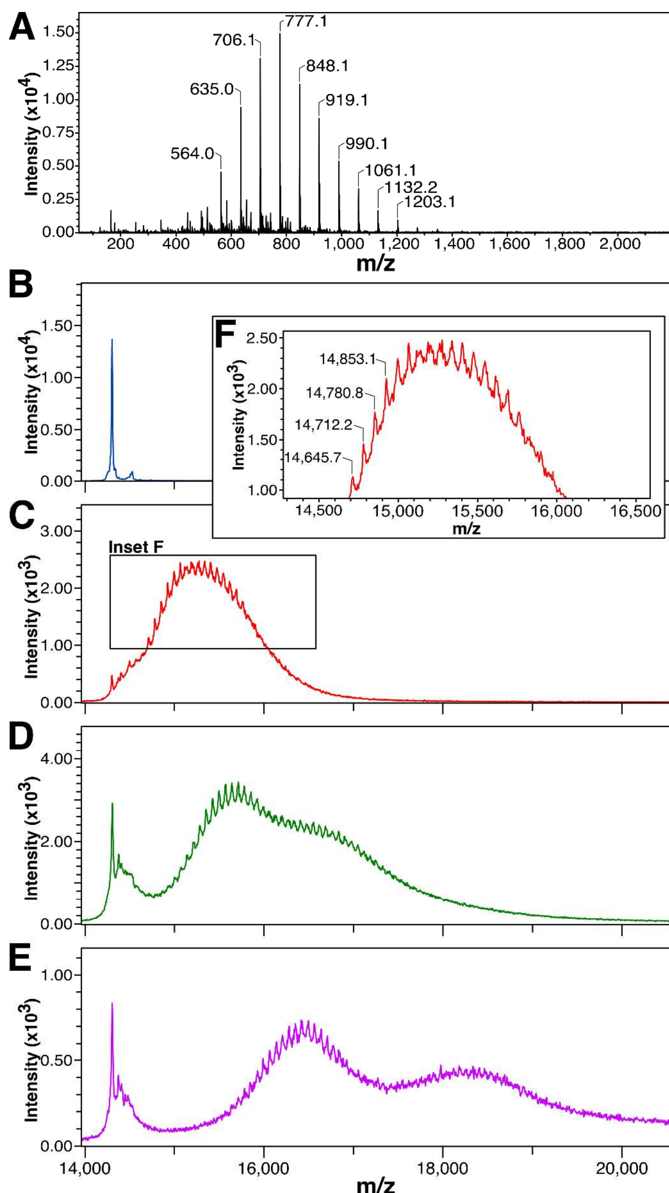


Figure 1. A) ESI-MS of the Am oligoCTA centered at approximately 7-8 units of Am. B) MALDI-MS of native lysozyme. C) MALDI mass spectrum of the lysozyme conjugated with the Am oligoCTA with average DP≈5. D) MALDI-MS of the lysozyme conjugated with the Am oligoCTA with average DP≈10. E) MALDI-MS of the lysozyme conjugated with the Am oligoCTA with average DP≈20. F) Inset of the peak MALDI-MS of the lysozyme conjugated with the Am oligoCTA with average DP≈5, showing that the spacing of the peak is consistent with the molar mass of Am.

The combined grafting-to and grafting-from approach utilizes a short oligoCTA in the grafting-to step to ensure solubility and stability of the resultant biohybrid. The oligoCTA, in this case an oligomer of acrylamide, is less subject to the limitations of low grafting density that can affect longer polymers. Subsequently, the protein-oligomer conjugate can be extended using RAFT polymerization.

Initially a series of 3 acrylamide (Am) oligomers were synthesized by RAFT using PAETC as the CTA, since RAFT is an excellent tool for the synthesis of oligomers.[26] Three oligomers of Am targeted degrees of polymerization (DP) of 5, 10 and 20. Figure S3 and Figure 1A show the electrospray ionization-mass spectrometry (ESI-MS) data for all three oligomers, indicating that the polymers are well defined and narrowly distributed. Table S1 confirms that the polymers were well controlled with acceptable agreement between the theoretical and experimentally determined molecular weights. Fractional precipitation of high molecular weight polymers could explain the larger measured molecular weight compared to theory.

Once synthesized, the oligomers were conjugated to lysozyme using an *in-situ* N-Hydroxysuccinimide/ 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (NHS/EDC) coupling procedure. The effectiveness of the NHS coupling was assessed using matrix assisted laser desorption/ionization mass-spectrometry (MALDI-MS). Figure 1B presents the MALDI-MS data for native (unmodified) lysozyme. The major peak is at an m/z 14300 and there are no high molecular weight peaks. Figures 1 C, D, and E show the MALDI-MS data for lysozyme conjugated with the oligoCTAs with approximately 5, 10 and 20 Am units in the backbone. In this conjugation protocol, 60 units of carboxylic acid on the oligoCTA were used for every amine on lysozyme. Figures 1 C, D, and E indicate that as the length of the oligomer increases, the conjugation efficiency decreases dramatically, even though the concentrations of the carboxylic acid, amine group on lysozyme, etc. were the same in all cases. In particular, the short DP≈5 oligomer lead to almost complete coupling efficiency, with virtually no unmodified lysozyme. Figure 1F illustrates the saw tooth pattern in the protein polymer conjugate, with each peak being separated by approximately 70 Da, which is the molar mass of acrylamide. Careful examination of the MALDI-MS data for the DP≈5 oligomer indicate that on average two oligoCTAs were added per lysozyme on average, as described in the supporting information. This attachment of two DP≈5 oligoCTAs per lysozyme is consistent with literature data attaching small molecule CTAs to lysozyme.[19] When attaching poly(ethylene glycol) chains to amine residues on lysozyme through NHS coupling, lysines 33 and 97 have been found to be the most reactive.[27] This implies that lysines 33 and 97 are expected to be the two residues to which the oligoCTA attaches. However, Figure 1 D and E indicate that when the larger oligoCTAs of DP≈10 and DP≈20 were attached to lysozyme there is also a significant peak from unmodified lysozyme. This suggests that steric hindrance caused by the longer chain reduces the conjugation efficiency of longer polymers. Furthermore, the protein-polymer conjugated peaks in Figure 1 D and E are distinctly bimodal, with the lower molecular weight peak consistent with one oligomer attached to lysozyme, and the higher molecular weight peak, consistent with two oligomers attached to lysozyme. Similar conjugation protocols were applied with the ratio of 120 oligoCTA to amine on lysozyme. Figure S4 shows the MALDI data for the protein-polymer conjugates synthesized with the ratio of 120 oligoCTA to amine in lysozyme. As seen in Figure S4, the larger ratio of oligoCTA to amine gives more complete conjugation, although the use of higher polymer concentrations is less desirable, since a larger fraction of polymer is unused in the conjugation step.

As indicated in Figure 1, protein-polymer conjugates can be formed by reacting lysozyme with oligoCTAs of acrylamide, using

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NHS/EDC coupling. However, it is also important to determine if the enzyme is active after the conjugation with a polymer. This can be achieved by two independent assays. The first assay examines lysis of *Micrococcus lysodeikticus* due to hydrolysis of glycosidic bonds within the cell wall. The other assay is based on the enzymatic hydrolysis of a glycosidic bonds in 4-methylumbelliferyl β-D-N,N',N"-triacetylchitotrioside ($(NAG)_3$-MUF), which leads to a fluorescent product. Figure 2 gives the activities of the native enzyme compared to the conjugate with the DP≈5,10 and 20 length oligoCTA. For each length of oligomer, the activity was determined for the conjugate synthesized under a molar ratio of 60 or 120 units of oligoCTA to amine on lysozyme (denoted by 60 and 120). The key conclusion of Figure 2 is that each lysozyme-oligoCTA conjugate retains activity with the larger *Micrococcus lysodeikticus* and the smaller ($(NAG)_3$-MUF) substrates. The lower activity with the *Micrococcus lysodeikticus* substrate is presumably due to steric effects, which are more significant for the larger *Micrococcus* substrate than the small ($(NAG)_3$-MUF) substrate.

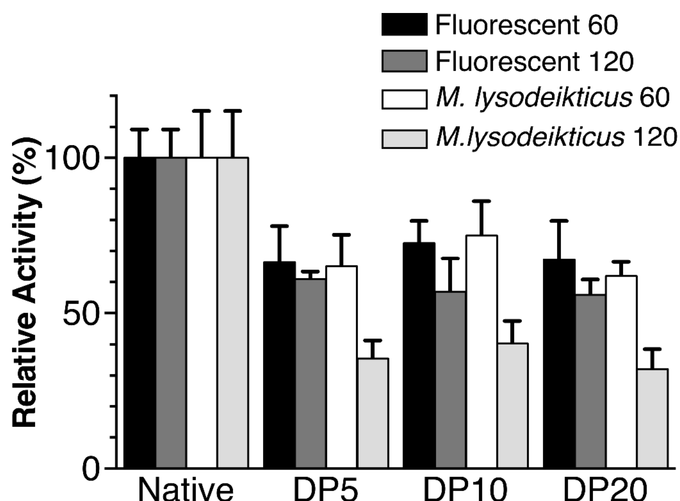

Figure 2. Activity of native lysozyme compared to the lysozyme-acrylamide oligoCTA conjugates. The conjugates involving oligomers of length DP=5, 10, 20 conjugated with either a 60 or 120 molar excess of polymer to lysine on lysozyme are evaluated for Micrococcus lysodeikticus and small molecule Fluorescent assay.

Given the absence of native lysozyme in the conjugate based on the DP≈5 oligoCTA of Am, this conjugate was used for chain extension. with oligo(ethylene oxide) methyl ether acrylate of average molecular weight =480 (OEOA). The polymerization was conducted at 35 °C. The target degree of polymerization was 200 units and the final monomer conversion was over 90%. This chain extension is an attractive feature of the RAFT mechanism, and central to the combined grafting-from and grafting-to method.
Figure 3A shows results from polyacrylamide gel electrophoresis (PAGE) for the native lysozyme (well 2), lysozyme-oligoCTA conjugate (well 3), and the OEOA extended conjugate (well 4). Figure 3 clearly shows an increase in molecular weight from the native lysozyme to the lysozyme-oligoCTA conjugate, and an even larger increase in molecular weight after chain extension with OEOA. The macromonomer, OEOA, leads to a particularly large increase in the molecular weight of the conjugate as expected. A lower intensity lower molecular weight band could arise from lysozyme functionalized with only one oligoCTA. It is important to note that negligible native protein remains in all protein polymer conjugates.

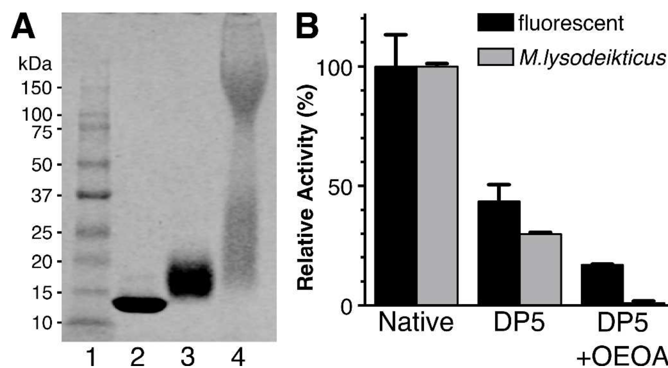

Figure 3: A) PAGE data for various conjugates and modified lysozyme structures. Well 1 is a series of standards, Well 2 is native lysozyme, Well 3 is the lysozyme-oligoCTA (DP=5) conjugate, and Well 4 is the lysozyme-oligoCTA conjugate chain extended with OEOA. B) Plot of relative activities for native lysozyme, DP5 lysozyme-oligoCTA and OEOA chain extended lysozyme conjugate against the fluorescent $(NAG)_3$-MUF or *M. lysodeikticus* substrates.

Finally, the activity of the chain extended conjugate was assessed using both the *Micrococcus* and ($(NAG)_3$-MUF) substrates. As shown in Figure 3B, the chain extended conjugate showed negligible activity with the *Micrococcus* substrate, however, the enzyme retained 25% activity with the small fluorescent ($(NAG)_3$-MUF) substrate. This indicates that steric effects are preventing lysozyme interaction with the larger *Micrococcus* substrate since the enzyme retained activity with the smaller fluorescent substrate.

Conclusions

This paper outlines a new approach to the synthesis of protein-polymer conjugates, where a short hydrophilic oligoCTA was first grafted-to lysozyme followed by grafting-from the protein with a second monomer. This approach combines the advantages of grafting-from the biomolecule such as simple purification and a relatively high graft density even with moderately low concentrations of (macro)CTA or (macro)initiator during the conjugation step, with the advantage of grafting-to, such as characterization of the conjugate, improved solubility and stability of the initially formed conjugate. The activity of the lysozyme enzyme was assessed at each stage, and it was shown that the protein retains its enzymatic activity after each stage. The outlined strategy results in negligible quantities of unmodified protein, and does not lead to precipitation of the CTA or initiator-protein conjugate. This makes the outlined procedure an attractive and useful strategy that combines the strengths of grafting-from and grafting-to a protein.

Notes and references

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Electronic Supplementary Information (ESI) available: [Experimental Details, Photographs of precipitated protein-CTA conjugate, UV-Vis data, characterization data for oligoCTAs, additional MALDI-MS data for the protein-oligoCTA conjugates]. See DOI: 10.1039/c000000x/

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