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Reversible chemoselective tagging and functionalization of methionine containing peptides†

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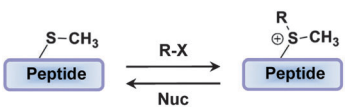
Reagents were developed to allow chemoselective tagging of methionine residues in peptides and polypeptides, subsequent bioorthogonal functionalization of the tags, and cleavage of the tags when desired. This methodology can be used for triggered release of therapeutic peptides, or release of tagged protein digests from affinity columns.

There is considerable interest in the site specific conjugation of molecules, *i.e.* "tags", to peptides and proteins.[1] These tags may be used for attachment of probes for imaging, for selective purification or detection in complex mixtures, for enhancement of therapeutic properties, or as labels to assist in proteomic analysis.[2] Such modifications typically rely on chemoselective reactions with natural amino acid functional groups, *e.g.* cysteine thiols,[1] or biosynthetic incorporation of unnatural amino acids that present functionality for bioorthogonal reactivity, *e.g.* azide groups.[2] While many approaches exist for selective tagging of peptides and proteins, few of these, aside from labile disulfides, are reversible modifications that allow triggered regeneration of unmodified samples.[1–5] Tag removal would be advantageous for some applications, such as release of therapeutic peptides from a carrier, or recovery of affinity purified, tagged peptide fractions from protein digests for downstream proteomic analysis.[6–8] Here, we report the development of reagents for completely reversible, chemoselective alkylation of natural methionine (Met) residues in peptides and polypeptides (eqn (1)). These reagents have been optimized to give stable sulfonium products, allow secondary modifications, and allow selective tag removal under mild conditions.



(1)

Our lab recently reported the use of Met alkylation as a facile means to irreversibly introduce useful functionality and chemically reactive groups onto polypeptides.[9] This work was based on the pioneering studies of Met alkylation in proteins, which were mainly focused on use of non-functional alkylating reagents to probe inhibition of enzyme active sites.[10–14] While many of these alkylations were reported to be irreversible,[10–12] some studies,[13,14] as well as subsequent experiments with peptides,[15–20] found that Met alkylation can be reversed under certain conditions. Since each study typically employed a different substrate (amino acid, peptide or protein), different alkylating reagents, and different nucleophilic cleavage reagents,[10–20] comparison of reactivity and properties of the various alkylated Met sulfonium groups that have been reported is challenging. This is especially true since Met alkylations with some reagents (*e.g.* benzylic halides)[11,20] have been reported to be reversible in some cases and irreversible in others.[14,18,21] As a further complication, reaction of Met sulfoniums with nucleophiles can give other products, aside from regenerating Met, depending on where nucleophilic attack occurs.[15] In light of these uncertainties, we sought to evaluate Met sulfonium stability as functions of both alkylating reagent and added nucleophile using a model copolypeptide substrate. Since the chemical modification of removable Met alkylating tags has not been reported, we also sought to develop tags with this unprecedented feature. We sought to identify optimized reagents and conditions for stable sulfonium formation, introduction of bioorthogonal reactive groups for tag modification, and triggered removal of the functional tags when desired.

To study the stability of different Met sulfonium species, we prepared a statistical copolymer of Met and lysine (**KM**, **1**) and reacted this model substrate with a variety of alkylating reagents (Fig. 1). Under the acidic conditions employed, all the Met residues in **KM** were chemoselectively alkylated in near quantitative yields, similar to previous results.[9] Note that since peptides and polypeptides are routinely handled and manipulated in strongly acidic media, the acidic alkylation conditions are compatible with these molecules.[4,5,21] The resulting sulfonium containing copolymers were each reacted with four common sulfur nucleophiles as shown in Fig. 1. We chose alkylating agents to cover a range of properties. The methyl (**2a**) and carboxymethyl (**2b**) groups

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† Electronic supplementary information (ESI) available: Experimental procedures and spectral data for all new compounds, ESI mass spectra, GPC-LS data, and additional dealkylation and stability studies. See DOI: 10.1039/c3cc42214c

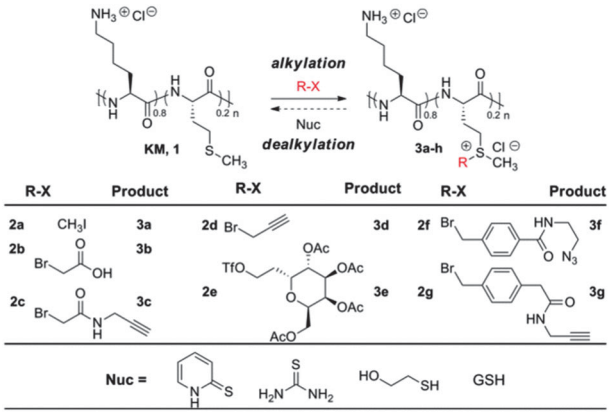

Fig. 1 Structures of **KM** substrate, alkylating reagents (**R-X**), and nucleophiles (**Nuc**) used in this study. GSH = glutathione. Reagent **2e** was reacted with a Met homopolymer instead of **KM**.

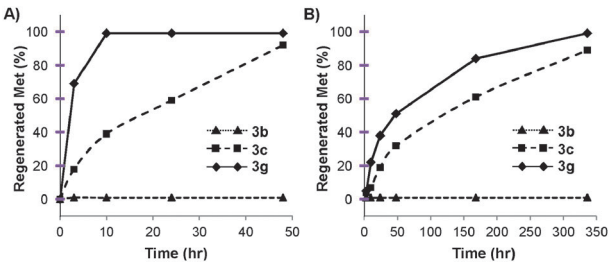

Fig. 2 Dealkylation of polymers **3b**, **3c**, and **3g** over time using different **Nuc** (0.1 M in PBS, 37 °C). (A) PyS. (B) GSH.

were chosen as controls with non-reactive side-chains, and their sulfoniums **3a** and **3b** were found to be stable to all four nucleophiles, as well as strong base (pH 10) and heat (80 °C) in water (Fig. 2, see ESI†). Although some reports state that these sulfoniums can dealkylate,[14,18] our results are consistent with many studies that show these groups to be inert under similar conditions.[10–12] The reagents **2c**, **2d**, and **2g** were chosen to introduce desirable alkyne functionality that is useful for subsequent modification of the tagged copolypeptides under bioorthogonal conditions.[2] An azide containing analog (**2f**) was also used to showcase the ability to incorporate different reactive groups, and finally a galactose containing reagent (**2e**) was used to introduce a model biofunctional side-chain.

Upon treatment with sulfur nucleophiles, the copolypeptides **3c**, **3d**, **3f**, and **3g** all showed some dealkylation back to parent **KM** as the sole product, while glycopolymer **3e** was found to be stable under these conditions (Fig. 2, see ESI,† Fig. S1). The stability of **3e**, like **3a**, is likely due to the lack of an electron withdrawing substituent on the alkylating carbon, resulting in the sulfonium being less electrophilic. The alkylating carbons of samples **3c**, **3d**, **3f**, and **3g** all have an activating substituent (carbonyl, alkyne or phenyl), which greatly increases the electrophilicity of these sulfoniums. The least reactive nucleophile was found to be glutathione (GSH), but it did give high yields of dealkylated **KM** over time (Fig. 2), which is relevant for applications *in vivo*. 2-Mercaptoethanol, thiourea,[22] and 2-mercaptopyridine (PyS) were all effective for quantitative dealkylation of sulfonium groups to regenerate **KM** (Fig. 2, see ESI,† Fig. S1), and PyS was chosen as the optimal reagent since it provides rapid sulfonium dealkylation, and also shows low reactivity with disulfides (see ESI,† Fig. S2). While excess nucleophile was used in these studies, stoichiometric PyS was also found to effect quantitative sulfonium dealkylation with longer reaction times (see ESI†).

To identify optimal alkylating reagents, we focused on the alkyne containing polymers **3c**, **3d**, and **3g**, which differ in linkage structure. While all of these copolypeptides were quantitatively dealkylated by PyS back to **KM** as the sole product, it was found that **3c** was less desirable since it reacted much slower compared to **3d** and **3g** (Fig. 2, see ESI,† Fig. S1). The propargyl sulfonium **3d** also had drawbacks since it was found to be unstable in basic aqueous media and upon prolonged storage as a solid (see ESI†). Consequently, the benzylic sulfonium derivatives **3f** and **3g**, were chosen since they provide an excellent combination of facile formation, stability against hydrolysis (pH 10), and rapid, facile dealkylation back to **KM** when treated with PyS. It is also worth noting that **3g** was found to be completely stable in PBS buffer at 20 °C for 2 weeks, and that no peptide chain cleavage was detected after alkylation and dealkylation reactions (see ESI,† Fig. S8).

To showcase the potential of this optimized system, we performed proof of concept tag, modify, and release studies using the copolypeptide **3g** (Fig. 3). A sample of **3g** was prepared from **KM** as described above, and its alkyne tags were then modified *via* copper catalyzed cycloadditions using a variety of functionalized azides.[2] Polyethylene glycol (PEG) chains and glucose units, which may be useful for improving biological lifetimes of peptide based therapeutics,[23] were quantitatively attached to all alkylated Met residues in **3g** (Fig. 3). As a model probe, 5-azidoacetamido-fluorescein was also attached to **3g** (*ca.* 1 per polypeptide chain), to give the fluorescent derivative **5c** (see ESI,† Fig. S3). Treatment of each of these derivatives **5a**, **5b**, or **5c** with PyS resulted in their quantitative conversion back to parent **KM**, confirming the facile release of the modified tags. We envision that a wide variety of azide, alkyne, or cycloalkyne containing molecules or substrates could be used to modify methionine containing peptide samples that have been tagged with one of the alkylating reagents **2f** or **2g**, making this chemistry attractive for applications when eventual release of the modified tag is desired.

For broad utility in tagging of peptides, Met alkylation needs to be a chemoselective process that is compatible and doesn't interfere with other peptide functional groups. In peptides and proteins, there are many nucleophilic functional groups that can react with alkylating reagents.[24] Of these, all except Met exist in protonated forms at low pH, which greatly decreases their reactivity.[25] While alkylations of proteinaceous functional groups, such as thiols, are common practice at high pH,[26] Met is the only functional group in proteins able to react with alkylating reagents at low pH.[11,13,16,27]

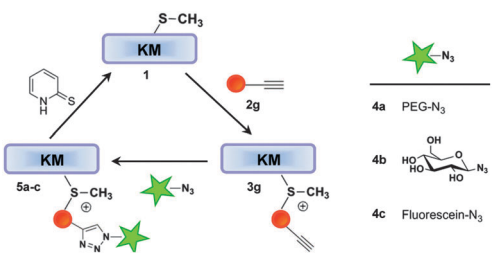

Fig. 3 Schematic showing tag, modify, and release studies on **KM** copolypeptide.

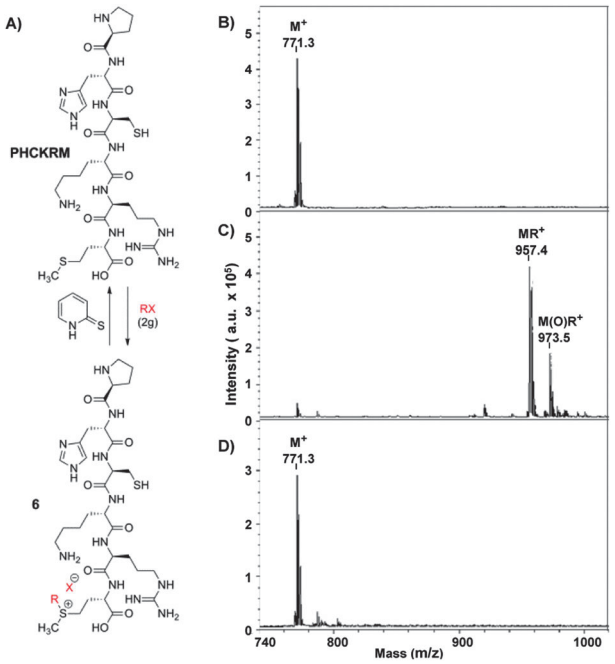

Fig. 4 Reversible alkylation of **PHCRKM** with **2g**. (A) Reaction scheme. MALDI-MS spectra of (B) **PHCRKM** (M^+), (C) **PHCKRM** alkylated with **2g** to give **6** (MR^+), and (D) **6** after treatment with PyS to regenerate **PHCRKM**. $M(O)R^+$ is some **6** that had oxidized during MS ionization.

To demonstrate this selectivity, we attempted to alkylate only the Met residues in the antioxidant peptide **PHCKRM**, which also contains highly nucleophilic histidine, cysteine and lysine residues (Fig. 4A). Treatment of **PHCKRM** with alkylating agent **2g** in 0.2 M aqueous formic acid (pH 2.4) gave a single product (**6**) in 92% isolated yield, where only the Met residue was alkylated. The composition of **6** was confirmed using MALDI MS (Fig. 4B and C), as well as ^{1}H NMR and ESI-MS analysis (see ESI,† Fig. S4–S6), where the addition of a single 186 Da **2g** tag in MS, and shift of the Met methyl resonance in NMR, were observed. Control experiments where N_α-*Z*-histidine, N_α-*Z*-lysine, and N_α-*Z*-cysteine were each reacted with benzyl bromide at pH 2.4 also showed no alkylation under these conditions (see ESI†). For contrast, reaction of **PHCKRM** with **2g** (2 eq.) in carbonate buffer (pH 8.3) showed the formation of a complex mixture of multiply alkylated peptides (see ESI,† Fig. S7). These results demonstrate that peptides containing a variety of nucleophilic natural amino acid side-chains can be chemoselectively, and near quantitatively, modified at Met residues at low pH.

The alkylated peptide **6** was also readily dealkylated by addition of PyS to give unmodified **PHCKRM** as the sole product along with the alkylated PyS byproduct (Fig. 4D). This tag removal reaction is also selective, as we have found that Met sulfoniums can be dealkylated using concentrations of PyS that do not react with the disulfide bond in cystine under identical conditions (see ESI†), which is an advantage in using PyS instead of 2-mercaptoethanol.

Overall, we have developed functionalized alkylating reagents that were optimized for high yield, chemoselective tagging of Met residues in peptides and polypeptides. Since the synthesis of these reagents is modular and straightforward, we envision that related compounds can be readily prepared to introduce other desirable features, such as isotopic labels to assist MS analysis.[7] Once installed, the tags can be further modified using bioorthogonal reactions to introduce additional functionalities, such as affinity tags, or for attachment to substrates for purification.[6–8] Finally, facile removal of these modified tags provides a unique advantage of this tag and modify method, where tag release may be useful for peptide concentration and purification from solid supports, as well as for release of unmodified peptide therapeutics from carriers.

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