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Author
Deming, Timothy J

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Functional modification of thioether groups in peptides, polypeptides, and proteins

Timothy J. Deming[*]

5121 Engineering 5, Department of Bioengineering, University of California, Los Angeles, California 90095, USA. Tel: +1-310-267-4450 e-mail: demingt@seas.ucla.edu

Department of Chemistry and Biochemistry, University of California, Los Angeles, California 90095, USA.

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Abstract

Recent developments in the modification of methionine and other thioether containing residues in peptides, polypeptides and proteins are reviewed. Properties and potential applications of the resulting functionalized products are also discussed. While much of this work is focused on natural Met residues, modifications at other side-chain residues have also emerged as new thioether containing amino acids have been incorporated into peptidic materials. Functional modification of thioether containing amino acids has many advantages, and is complimentary methodology to the widely utilized methods for modification at cysteine residues.

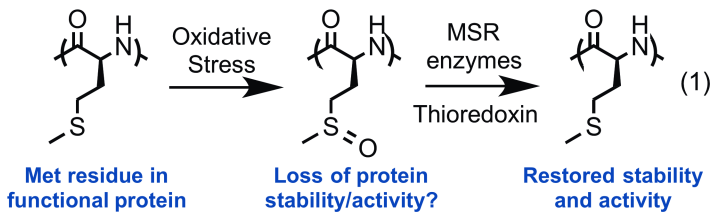
Introduction

In areas including chemical biology,[1,2] materials science,[3,4] and therapeutics,[5,6] there are needs for practical methods to selectively functionalize amino acid residues in peptides, polypeptides and proteins. Introduced functionality is useful since it can allow attachment of probes for imaging,[1,2,7] provide mimics of desirable post-translational modifications,[1,2] or be used as a means to precisely adjust biological and physical properties of biomacromolecules.[3,4] Numerous strategies, including biological[8] and chemical synthesis,[1,2] have been developed to either react with or modify natural residues, or replace them entirely, using highly selective processes. Many of these methods have focused on reaction at or replacement of L-cysteine (Cys),[9] L-methionine (Met),[8,10,11] and N-terminal residues[12,13] since these are often present in low abundance and thus can provide unique sites for functional modification. Most chemical strategies have focused on reactions at highly nucleophilic Cys thiol groups, where a variety of different types of modification are possible.[9] In recent years, however, a number of studies have emerged showing that the unique chemical reactivity of thioether (aka sulfide) functional groups, e.g. present in natural Met residues, has significant potential for use in functional modification and bioconjugation reactions. These methods build upon the rich biochemistry of Met, where its reversible alkylation and oxidation reactions are well known for their importance in enzyme catalyzed methyl transfer processes[14] and protection against oxidative protein damage,[15] respectively. This review article describes recent advances in selective chemical modification reactions on thioether groups in peptides, polypeptides and proteins, and the properties and potential applications of the resulting functionalized products. While much of this work is focused on natural Met residues, modifications at other side-chain residues have also emerged as new thioether containing amino acids have been incorporated into peptidic materials. In addition,

the methods developed for functionalization of thioether containing polypeptides have recently been applied to other thioether containing polymer systems.

Oxidation of thioether groups

It is well-known that Met thioether groups in peptides and proteins can be readily oxidized, either chemically or biologically, into methionine sulfoxide, MetO, residues.[15] In biological systems, MetO residues are believed to be generated by reaction of Met residues with reactive oxidative species (ROS) in stressed environments (Eq. 1), such as in areas of trauma or hypoxia, e.g. tumors.[16] In response, organisms can produce intracellular methionine sulfoxide reductase A and B (MSR) enzymes, which are able to reduce peptidic MetO back to Met, and thus regenerate the unmodified peptide or protein.[17,18] The two MSR enzymes reduce different diastereomers of MetO that arise from sulfoxide group chirality.[17,18] It is widely believed that Met plays a critical role in biology by acting as a sacrificial reductant of ROS in cells, which helps prevent oxidation of other critical protein functionalities (e.g. active-site Cys) or DNA.[19,20] In conjunction with MSR enzymes, which are ubiquitous in cells of plants and animals, Met residues are able to catalytically remove ROS and thus may themselves be considered for potential therapies.[21]



The oxidation of Met residues in proteins has been studied for some time, with earliest results on chemical oxidation being reported in the 1960s.[22] MetO formation in enzymes was

found to result in conformation changes as well as changes in enzymatic activity that varied with the specific protein under study. In some cases, changes in conformation and enzymatic activity were found to be reversible upon chemical reduction of MetO residues back to Met, leading to a hypothesis that Met oxidation may be used to regulate protein activity.[22] Met oxidation in peptides was found to exert similar effects. In a detailed study, Gellman and coworkers found that Met-rich peptides, designed to form amphiphilic α-helices, switched to amphiphilic β-strand conformations upon oxidation of Met residues to more polar MetO, or methionine sulfone, MetO2 groups, where the change in ordering of polar and nonpolar residues drove the observed changes.[23,24] Garcia-Echeverria also reported that coiled-coil peptide dimers in water could be dissociated by oxidation of single Met residues to more polar MetO groups, as long as these residues reside in the hydrophobic faces of the helices.[25] Further, a recombinant silk-like protein that incorporated Met residues in the repeat sequences was reported by Kaplan and coworkers, where an oxidation "trigger" converted Met residues to MetO and led to disruption of β-sheet crystallites.[26]

In the synthetic polypeptide field, homopolymers and copolymers of Met, prepared from α-amino acid N-carboxyanhydride (NCA) monomers, have been known since the late 1950s.[27,28] The first studies on oxidation of poly(L-methionine), **M**, were reported in the late 1970s by Minoura and coworkers, who examined properties of thin films of **M** that were subsequently oxidized with hydrogen peroxide.[29] Oxidation of essentially water insoluble **M** to poly(L-methionine sulfoxide), $\mathbf{M^O}$, resulted in increased hydrophilicity and good water solubility. Follow up studies on aqueous solutions of $\mathbf{M^O}$ by Aiba and coworkers revealed that these chains possess disordered conformations in water, which contrasts to the α-helical conformation of **M** found in organic solvents and in the solid-state (Figure 1).[30] Circular dichroism (CD) analysis of

dried films of $\mathbf{M^O}$ showed that this polymer primarily adopts an α-helical conformation in the solid-state, which is converted to a disordered conformation upon film hydration.[30] These results suggest that the disordered conformation of $\mathbf{M^O}$ in water is likely due to solvation of the sulfoxide groups by water rather than any disorder introduced by the racemic configurations of the sulfoxides themselves.[31,32] More recently, Deming and coworkers prepared poly(L-methionine sulfone), $\mathbf{M^{O2}}$, by oxidation of **M** using elevated peroxide concentrations and temperature (Figure 1).[33] Unlike disordered $\mathbf{M^O}$, $\mathbf{M^{O2}}$ is highly α-helical in water, yet this rod-like conformation also leads to inter-chain aggregation that limits water solubility.[11] The observed restoration of helicity in $\mathbf{M^{O2}}$ may be due to strong dipolar sulfone-sulfone side-chain interactions that can stabilize the α-helical conformation.[34] Although sulfone groups have greater dipole moments than sulfoxides, giving them higher polarity, these dipoles tend to interact strongly, which can alter solvation by water and effectively make sulfone containing polypeptides less hydrophilic.[34,35]

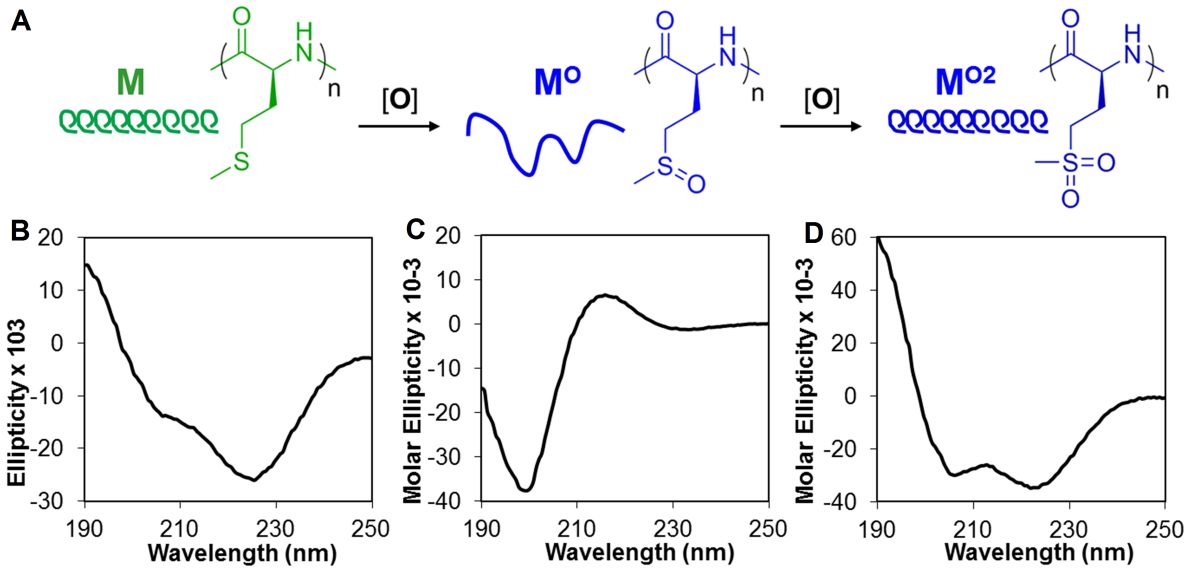


Figure 1. Natural and oxidized poly(L-methionine), **M**. (a) Structures and schematic drawings of

M, $\mathbf{M^{O}}$, and $\mathbf{M^{O2}}$ homopolymers. Circular dichroism spectra of (b) $\mathbf{M_{80}}$ prepared as a thin film cast from a 0.25 mg/mL solution in THF, 20 ºC, with ellipticity is reported in degrees·cm^2; (c) $\mathbf{M^{O}_{80}}$ at 0.25 mg/mL in water, 20 ºC; and (d) $\mathbf{M^{O2}_{80}}$ at 0.25 mg/mL in water, 20 ºC. [O] = oxidation step. Reprinted with permission from *Biomacromolecules* **2013**, *14*, 3610-3614. Copyright 2013 American Chemical Society.

The ability of **M** segments to undergo reversible changes in both chain conformation and water solubility upon oxidation to $\mathbf{M^{O}}$ is attractive for development of stimuli responsive macromolecular assemblies. It is also worth noting that $\mathbf{M^{O}}$ has been reported to be non-toxic when given intravenously up to 2 g/kg in rats, a good preliminary indication of biocompatibility.[36] In an effort to create enzyme responsive polypeptide assemblies, Deming and coworkers designed amphiphilic copolypeptides containing segments of water soluble $\mathbf{M^{O}}$, which were prepared via synthesis of a fully hydrophobic precursor diblock copolypeptide, poly(L-methionine)$_{65}$-*block*-poly(L-leucine$_{0.5}$-*stat*-L-phenylalanine$_{0.5}$)$_{20}$, $\mathbf{M_{65}(L_{0.5}/F_{0.5})_{20}}$, followed by its direct oxidation in water to give the amphiphilic $\mathbf{M^{O}}$ derivative, $\mathbf{M^{O}_{65}(L_{0.5}/F_{0.5})_{20}}$ (Figure 2).[33] Assembly of $\mathbf{M^{O}_{65}(L_{0.5}/F_{0.5})_{20}}$ in water gave vesicles with average diameters of a few microns that could then be extruded to nanoscale diameters. The $\mathbf{M^{O}}$ segments in the vesicles were found to be substrates for MSR enzymes *in vitro*, which regenerated hydrophobic **M** segments and resulted in changes in supramolecular morphology that caused vesicle disruption and release of cargos (Figure 2).[33] Since MSR enzymes are found only intracellularly in nature,[16-18] these vesicles may have utility for selective intracellular delivery of molecules.

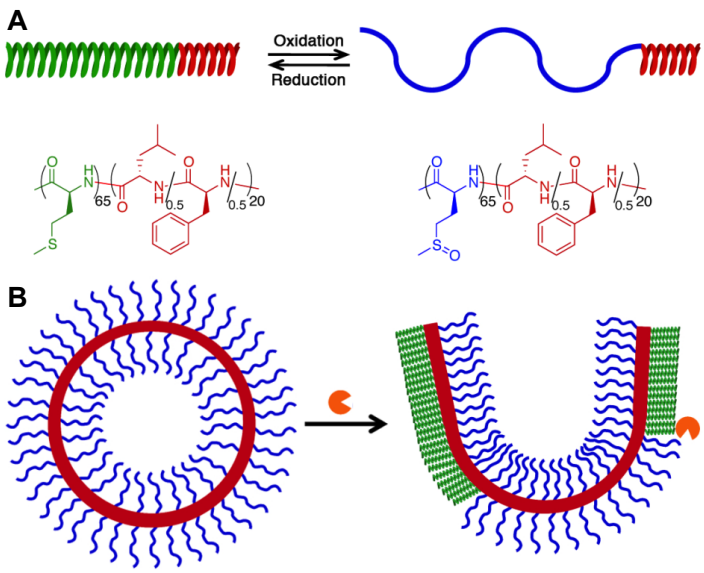
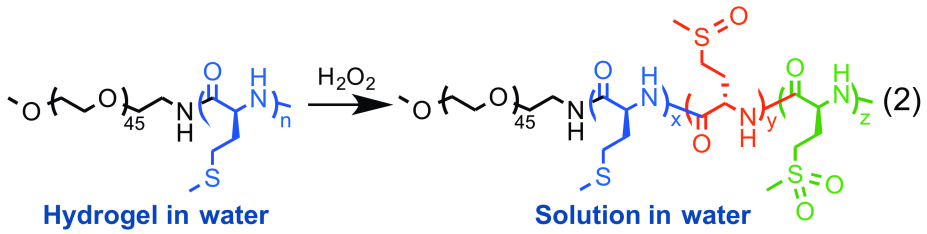


Figure 2. (a) Schematic showing structure, redox properties, and chain conformations of $\mathbf{M^{O}_{65}(L_{0.5}/F_{0.5})_{20}}$ copolypeptides. (b) Schematic showing how enzymatic reduction of vesicle surface $\mathbf{M^{O}}$ segments to **M** segments can result in vesicle disruption. Adapted with permission from *Biomacromolecules* **2013**, *14*, 3610-3614. Copyright 2013 American Chemical Society.

To pursue intracellular vesicle delivery, Deming and coworkers next introduced short poly(L-homoarginine), $\mathbf{R^{H}}$, segments in the vesicles through the synthesis of the triblock copolypeptides, e.g. $\mathbf{R^{H}_{10}M^{O}_{55}(L_{0.5}/F_{0.5})_{20}}$,[37] since $\mathbf{R^{H}}$ segments have been shown to promote cellular uptake.[38] These triblock copolypeptides were physically blended with $\mathbf{M^{O}_{65}(L_{0.5}/F_{0.5})_{20}}$ at different ratios to give vesicles with tunable $\mathbf{R^{H}}$ content on the vesicle surfaces, and these mixed vesicles were assayed for cytotoxicity and their uptake into HeLa cells. The vesicles composed of 1:1 mixtures of diblock and triblock copolymers were found to be optimal nanovesicle formulations that possessed an advantageous combination of low cytoxicity and good cell uptake.[37] The switchable hydrophobicity obtained by oxidation of **M** segments was also used by Chen and coworkers to develop hydrogels capable of cell encapsulation, which could then be

degraded by oxidation.[39] They prepared block copolymers of poly(ethylene glycol), PEG, and **M** (Eq. 2), which formed hydrogels in water at elevated temperature due to association of hydrophobic **M** segments. Upon incubation with hydrogen peroxide, either supplied chemically or produced by macrophages, Met residues were oxidized to MetO and MetO2, and the hydrogels were observed to degrade and release entrapped cargos.



(2)

In the examples above, interconversion of **M** and $\mathbf{M^O}$ resulted in simultaneous changes in both water solubility and chain conformation. In a study of the conformational properties of glycosylated polypeptides, Deming and coworkers observed that monosaccharide functionalized L-homocysteine (Hcy) polypeptides underwent changes in chain conformation upon stepwise oxidation that followed the same pattern as those observed for **M** (Figure 3).[40] This result revealed that the Hcy backbone was a key factor in stabilization of these chain conformations, and that replacement of the terminal methyl groups in **M** with other alkyl groups, e.g. monosaccharides, had little influence on conformation. Using this insight, Deming and coworkers realized that changes in water solubility and chain conformation due to interconversion of thioether and sulfoxide groups could be entirely decoupled in poly(alkyl-L-homocysteine), $\mathbf{R\text{-}C^H}$, materials by utilizing hydrophilic **R** functionalities. Using both monosaccharide and oligoethylene glycol functionalized $\mathbf{R\text{-}C^H}$ ($\mathbf{\alpha\text{-}gal\text{-}C^H}$ and $\mathbf{EG_4\text{-}C^H}$, respectively), highly water soluble homopolypeptides were obtained that could be reversibly switched between soluble α-helical and disordered conformations via reversible oxidation of the thioether linkages under mild conditions (Figure 3).[41] Such simple polypeptides, which can

readily switch between ordered and disordered conformations via chemical triggers, are promising components for development of stimuli responsive biomimetic assemblies as well as molecular devices.

Thioether containing polypeptides are also readily prepared as poly(alkyl-L-cysteines), **R-C**. While a large variety of **R-C** homopolypeptides have been prepared, many of these possess ionic side-chains, and are disordered in water, or hydrophobic side-chains, and form insoluble β-sheets in water.[42] Recently, a number of oligoethylene glycol functionalized **R-C** polypeptides have been prepared, which either form poorly soluble β-sheets in water, or form β-sheets at elevated temperature in water with longer ethylene glycol repeats (i.e. > 3-4).[43-45] An example to the contrary is the L-menthyloxycarbonyl functionalized **R-C** polypeptide reported by Hayakawa and coworkers, which adopts stable α-helical conformations in organic solvents.[46] Using the hypothesis that the bulky menthyl groups, at some distance from the polypeptide backbone, adopt a packing arrangement that favors the α-helical conformation, Deming and coworkers designed structurally similar **R-C** polymers, where hydrophilic monosaccharides replaced the hydrophobic menthyl groups (Figure 3).[40] These glycosylated **R-C** homopolypeptides were also found to adopt stable α-helical conformations in water, which is quite atypical for Cys based polypeptides.[42]

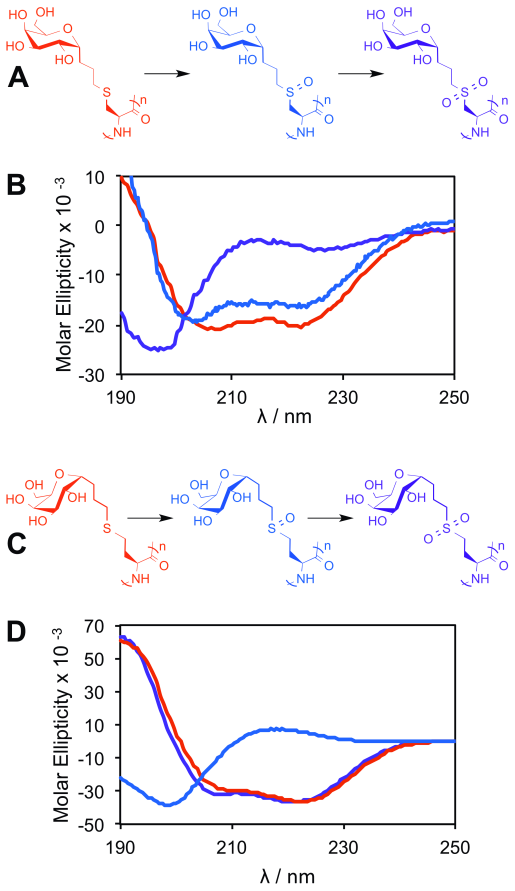


Figure 3. Structures and corresponding circular dichroism spectra of parent and oxidized glycopolypeptides. (a) Oxidation of **α-gal-C** to **α-gal-C^{O}** and **α-gal-C^{O2}**. (b) Circular dichroism spectra of **α-gal-C** (red line), **α-gal-C^{O}** (blue line), and **α-gal-C^{O2}** (purple line). (c) Oxidation of **α-gal-C^{H}** to **α-gal-C^{HO}** and **α-gal-C^{HO2}**. (d) Circular dichroism spectra of **α-gal-C^{H}** (red line), **α-gal-C^{HO}** (blue line), and **α-gal-C^{HO2}** (purple line). All samples analyzed at concentrations of 0.5 mg/mL in PBS buffer at 20 ºC. Adapted with permission from *J. Amer. Chem. Soc.* **2012**, *134*, 4112-4115. Copyright 2012 American Chemical Society.

Oxidation of thioether containing **R-C** polypeptides has also been studied, and the results provide some interesting contrasts compared to homologous **R-C^{H}** materials. Oxidation of the galactosylated **R-C**, i.e. **α-gal-C**, polypeptides described above to sulfoxide and sulfone

containing derivatives resulted in a reversal of conformational changes compared to the **R-C^{H}** polypeptides (Figure 3).[40] The sulfoxide derivative, **α-gal-C^{O}**, was found to predominantly retain the α-helical conformation of the parent thioether containing polypeptide, and the fully oxidized sulfone derivative, **α-gal-C^{O2}**, switched to a disordered conformation in water. Here, it was observed that the subtle difference in placement of the sulfur atom by one bond length from the polypeptide backbone is sufficient to dramatically reverse the conformational trends upon oxidation for **α-gal-C** and **α-gal-C^{H}**. Although the molecular interactions that drive these changes have not yet been determined, the different conformations of these polypeptides must be close enough in energy such that slight variations in side-chain sterics, sulfur oxidation state, and sulfur placement are enough to select between α-helical and disordered states.

Research groups have begun to utilize oxidized **R-C** polypeptides in supramolecular assemblies and biomaterials. Deming and coworkers reported incorporation of **α-gal-C^{O2}** as non-ionic, hydrophilic segments in vesicle forming block copolypeptides, where the disordered chain conformations were found to aid vesicle assembly.[47] Similarly, Li and coworkers has reported the preparation of diethyleneglycol methacrylate-cysteine functionalized polymers (**EG$_2$MA-C**), which assemble into micelles in water due to β-sheet formation of the polypeptide segments (Figure 4).[48] Upon oxidation of thioether groups to sulfones (**EG$_2$MA-C^{O2}**), the polypeptide segments become disordered, and the micelles undergo triggered disassembly. A related study by Dong and coworkers utilized *o*-nitrobenzyl functionalized **R-C** segments as a means to obtain multistimuli responsive polymer assemblies.[49] The parent thioether containing PEG-(*o*-nitrobenzyl functionalized **R-C**) block copolymers were found to form vesicles in water, and upon oxidation to sulfone derivatives, yielded vesicles whose diameters were substantially reduced 3-fold. Subsequent UV photolysis of the *o*-nitrobenzyl groups resulted in a further

change to micellar assemblies. As can be seen from the studies described above, oxidation of thioether groups in peptide, polypeptides, and proteins has tremendous potential utility as a chemically activated switch to readily alter properties.

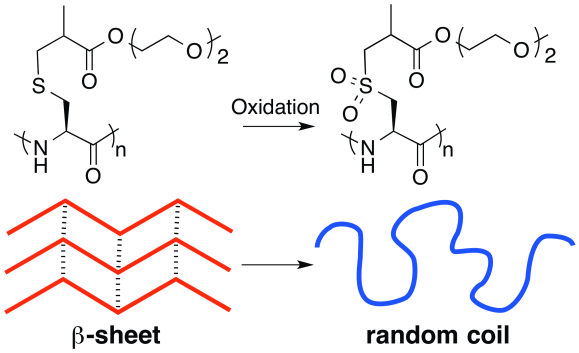


Figure 4. Schematic showing oxidation of β-sheet forming **EG$_2$MA-C** to random coil **EG$_2$MA-C^{O2}**. Upon oxidation, the poorly water soluble β-sheet forming polypeptide chains were converted to random coil chains with good water solubility. Adapted with permission from *Biomacromolecules* **2014**, *15*, 1055–1061. Copyright 2014 American Chemical Society.

Alkylation of thioether groups

In biological systems, Met amino acids are often present as alkylated sulfonium derivatives, which play critical roles as cosubstrates in enzyme mediated methyl transfer as well as other biological reactions.[14] These sulfonium salts, primarily S-adenosyl-L-methionine, abundant in animals,[14] and S-methyl-L-methionine, abundant in plants,[50] are stable molecules that are also marketed as nutritional supplements in many countries (Figure 5). The first chemical synthesis of Met sulfonium salts, via reaction of Met with alkyl halides, was reported in the 1940s,[51,52] and was subsequently followed by alkylation reactions of Met residues in peptides, proteins, and polypeptides.[53-55] Early studies on reactions of proteinaceous functional groups with simple alkyl halides revealed that many residues (e.g. Cys, L-histidine, L-lysine) are

highly reactive at neutral pH and above, but their reactivity diminishes greatly with decreasing pH and they are essentially unreactive at pH < 3.[56,57] This trend is due to the protonation of the nucleophilic functional groups in these amino acids at low pH, which greatly decreases their reactivity. Since the thioether in Met is highly resistant to protonation, it becomes the only available reactive nucleophilic amino acid at pH < 3, allowing chemoselective Met alkylation under these conditions.[56,57] In pioneering bioconjugation studies, this methodology was used to add labels and tags to, or to inactivate, proteins via chemoselective alkylation at Met residues.[14]

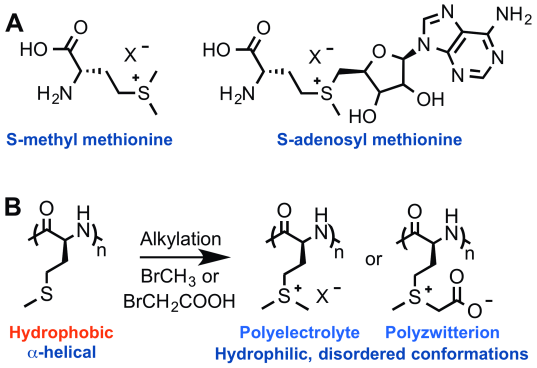


Figure 5. Methionine based sulfonium salts. (a) Structures of biological molecules S-methyl-L-methionine and S-adenosyl-L-methionine. (b) Initial studies on alkylation of hydrophobic, α-helical **M** to give disordered and hydrophilic $\mathbf{M}^{\mathbf{R}}$ alkyl sulfonium derivatives.

Met alkylation was first applied to **M** polymers by Katchalski, Berger, and coworkers, who reported quantitative conversion of thioether groups in **M** to methyl and carboxymethyl sulfonium derivatives ($\mathbf{M}^{\mathbf{R}}$ polymers) by reaction with the corresponding alkyl bromides in neat formic acid (Figure 5).[27,28] These polysulfoniums were found to be stable and were studied for their conformational and polyelectrolyte behavior. Similar to oxidation reactions, alkylation of thioether groups on **M** results in changes in both chain conformation and solubility, where the sulfonium derivatives possess disordered conformations and good water solubility.[58] Despite the

ease and potential utility of **M** alkylation, which gives quantitative alkylation with no side-products, there have been few further applications of these reactions until recently. This lack of development over a 50 year period may be due to traditional difficulties in synthesis of **M** polymers,[59,60] as the monomer, L-methionine N-carboxyanhydride (Met NCA), is difficult to purify by traditional methods, and the living homopolymerization of Met NCA had not been demonstrated until recently. Realizing that the alkylation of Met thioether groups had potential to be much broader in scope than had been reported, Deming and coworkers worked to develop the synthesis of well-defined **M** polymers, and subsequently studied and expanded Met alkylation chemistry as a bioconjugation tool to introduce diverse and reactive functionality into peptides, proteins and polypeptides.

Deming and coworkers recently reported a method for straightforward preparation of Met NCA in high yield and high purity,[61] which enabled the further development of **M** polymers. They subsequently showed that **M** segments can be prepared with controlled lengths, and are readily incorporated into block copolymers in NCA polymerizations using cobalt initiators.[61] Using well defined **M** polymers, Deming and coworkers showed that Met thioether groups can be functionalized quantitatively using a wide variety of activated alkyl halides or alkyl triflates (Figure 6).[10] Alkylations with unactivated alkyl halides required addition of silver tetrafluoroborate to increase alkyl halide reactivity. Combined, these methodologies allowed facile introduction of a broad range of functional groups into readily prepared, inexpensive polypeptides, including reactive groups, such as alkyl halides and ketones, that are difficult to introduce or incorporate via other methods.

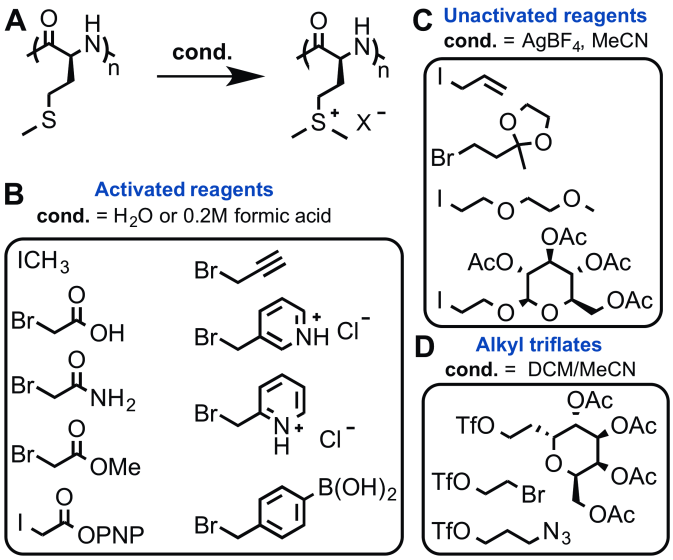
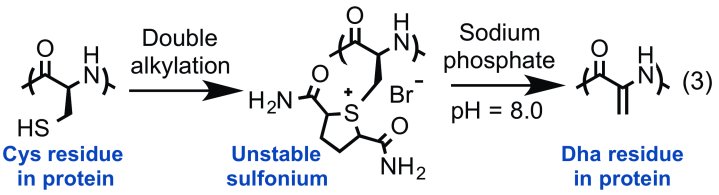


Figure 6. Preparation of functional polypeptides via alkylation of **M** using different reagents. (a) Reaction schematic. (b) Activated alkyl halide reagents that can be used directly. (c) Alkyl halide reagents that require activation with $AgBF_4$. (d) Alkyl triflate reagents.

Follow up studies by Deming and coworkers showed that sequence specific peptides could be chemoselectively alkylated at Met residues in high yield to give functional derivatives, even in the presence of other highly nucleophilic amino acid residues.[62] This methodology was also recently applied to an elastin-like recombinant protein, which was engineered to contain Met residues in all pentapeptide repeats of the sequence. The multiple Met residues were quantitatively alkylated to give uniform sulfonium derivatives whose properties could be tuned by variation of the alkylating groups.[63] Contrary to the broad applicability of Met alkylation to give functionalized products, alkylations of thioether groups created from Cys residues are known to generally be unstable, eliminating thioether groups and yielding dehydroalanine (Dha) residues under basic conditions (Eq. 3).[64]



While simple alkyl halides can be used to generate functionalized Met sulfonium residues in a straightforward process, some of the resulting products can also be unstable in the presence of nucleophiles, especially those with unsaturation in the alkylating group (*vide infra*). Deming and coworkers showed that sulfonium products with increased stability are obtainable, but this required use of anhydrous conditions and highly reactive or expensive reagents (i.e. triflates),[10] which may limit the applicability of this methodology. In searching for ways to improve synthesis of stable $\mathbf{M}^{\mathbf{R}}$ polymers, Deming and coworkers were inspired by early studies on reactions of ethylene oxide (EO) with protein functional groups.[65,66] Similar to reactions of alkyl halides with Met, the reaction of EO with proteins was observed to be selective for Met residues at pH < 3.[67] Since a large variety of functional epoxides are either commercially available or readily prepared, the addition of epoxides to Met residues appeared promising as a potentially chemoselective reaction to prepare stable Met sulfonium products in protic media.

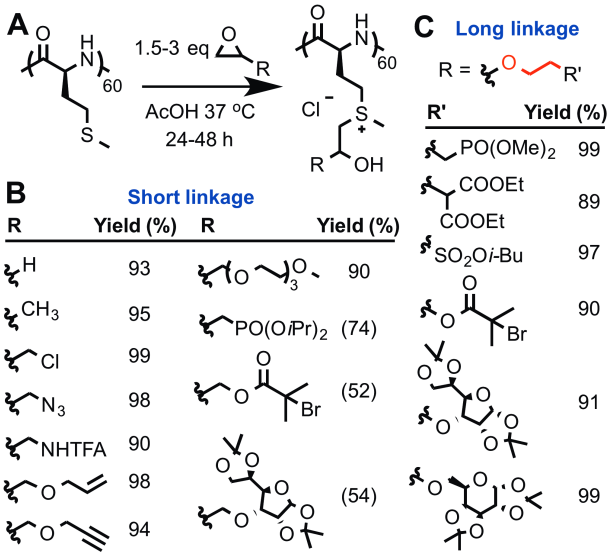


Figure 7. Alkylation of $\mathbf{M_{60}}$ with functional epoxides in acetic acid. (a) Reaction schematic. Counterion exchange during purification gave samples as chloride salts. (b) Reactions using epoxides with a short linkage between epoxide and functional groups. Yield is total isolated yield of completely functionalized polypeptide, except for values in parentheses, which are percent modification for incomplete functionalizations. (c) Reactions using epoxides with a long linkage, an oxoethylene spacer (red), between epoxide and functional groups. Yield is total isolated yield of completely functionalized polypeptide.

Deming and coworkers subsequently studied alkylation reactions using **M** polymers and a variety of commercially available epoxides under different conditions in protic media, and found that high degrees of functionalization and short reaction times were obtained in glacial AcOH at 37 ºC (Figure 7).[11] In these reactions, the sulfur of Met residues was found to add primarily to the less hindered side of the epoxide to give β-alkyl-β-hydroxyethyl Met sulfoniums (Figure 7). This methodology allowed introduction of many desirable functional groups, including amine, alkyl chloride, alkene, alkyne, azido, and oligoethyleneglycol. An advantage in using this methodology can be seen by the introduction of azido groups via epoxides in wet media, which previously was only obtained using azido triflates in anhydrous solvent.[10] However, the use of more sterically demanding epoxides was found to limit conversion of Met thioethers to sulfoniums so that fully functionalized $\mathbf{M^R}$ polymers could not be obtained (Figure 7). To relieve crowding of neighboring groups on the polymer backbone, functional epoxides containing oxoethylene spacers were prepared to increase the distance between functional groups and epoxides, which then allowed for quantitative functional group incorporation at all Met residues (Figure 7).[11] In general, these sulfonium products exhibited sufficient stability to allow full removal of protecting groups after alkylation, were stable toward secondary bio-orthogonal

functionalizations, such as azide-alkyne cycloadditions, and were stable against dealkylation by nucleophilic 2-mercaptopyridine. After optimization of this methodology using the **M** polymer platform, Deming and coworkers showed it could also be used for chemoselective tagging and functionalization of the **PHCKRM** peptide, which contains many nucleophilic functional groups (Figure 8).

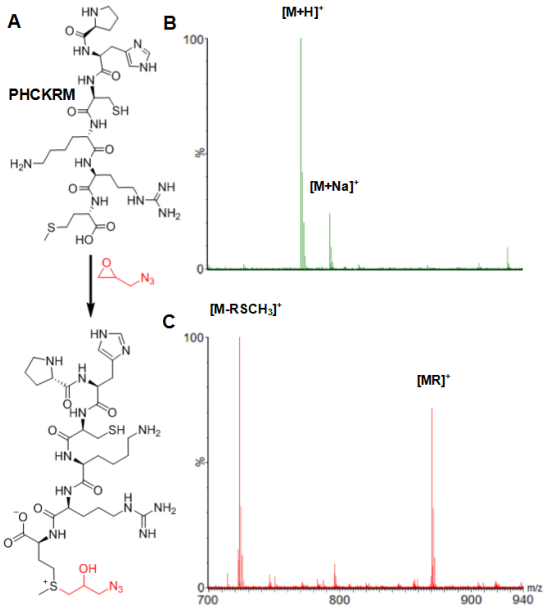


Figure 8. Chemoselective alkylation of the **PHCKRM** peptide. (a) Reaction scheme for alkylation of **PHCKRM** with glycidyl azide. (b) ESI-MS spectrum of **PHCKRM** with the $[M+H]^+$ and $[M+Na]^+$ peaks labeled. (c) ESI-MS of product after alkylation showing $[MR]^+$, as well as the characteristic $[M\text{-}RSCH_3]^+$ fragment. Reprinted with permission from *Biomacromolecules* **2015**, *16*, 1802-1806. Copyright 2015 American Chemical Society.

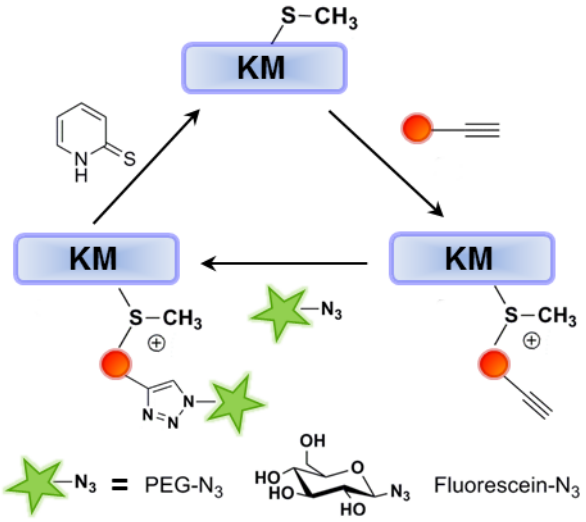


Figure 9. Schematic showing tag, modify, and release of different functional molecules on a statistical copolymer of methionine and lysine (**KM**). Red circle = benzylic halide alkylating reagent. Reprinted with permission from *Chem. Commun.* **2013**, *49*, 5144 - 5146. Copyright 2013 Royal Society of Chemistry.

Follow up studies by Deming and coworkers described the reversible modification of thioether groups in polypeptides via the chemoselective dealkylation of select methionine sulfonium residues (Figure 9).[62] A series of studies were used to identify alkyl substituents on Met sulfoniums that were more prone to nucleophilic attack. While simple alkyl (e.g. methyl) and anionic (e.g. carboxymethyl) groups were found to be reasonable stable, groups that contained unsaturation (e.g. carbonyl or phenyl) were more electrophilic and could be selectively cleaved from Met sulfonium ions in high yield by nucleophiles. Using these insights, functional benzylic halides were designed for reaction with Met residues to give stable sulfonium products, allow secondary modifications, and then allow selective "tag" removal by addition of a nucleophile under mild conditions.[62] Such 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 (Figure 9). These Met alkylation and dealkylation reactions were found to be compatible with deprotection of other functional groups, and use a natural amino acid that is readily incorporated into peptide, protein or polymer sequences, enhancing the utility of this methodology. Benzylic halide reagents were also used to alkylate thioether groups in **EG$_4$-C^H** polymers, resulting in reversible switching of their conformations between α-helical and disordered states (Figure 10),[41] similar to their reversible oxidation to sulfoxide derivatives as described above.

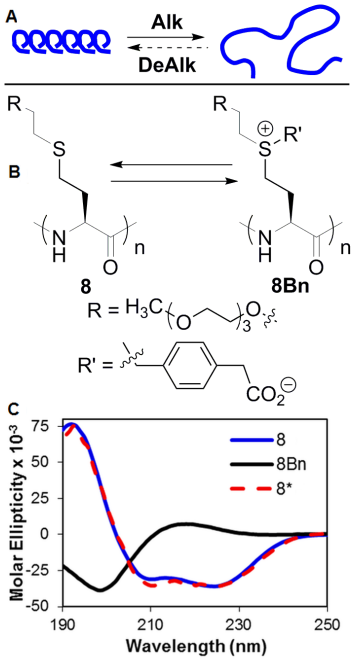


Figure 10. Reversible conformational switching of **EG$_4$-C^H** (**8**) via alkylation and dealkylation of thioether linkages in aqueous solution. (a) Schematic showing conformational changes. (b) Alkylation of **8** using 4-bromomethyl phenyl acetic acid to give **8Bn**, and dealkylation of **8Bn** using 2-mercaptopyridine to go back to **8** (designated **8***). (c) Circular dichroism spectra

showing changes in chain conformation upon reversible alkylation of **8**. Polymer concentration is 0.1 mg/mL in DI water, 20 °C. Adapted with permission from *J. Amer. Chem. Soc.* **2014,** *136*, 5547–5550. Copyright 2014 American Chemical Society.

In terms of applications of Met sulfonium groups, it is important to recognize that alkylation of thioether groups introduces cationic charge in addition to alkyl functionality. Cationic peptides are well-known to possess cell-binding ability, and can function as either cell penetrating peptides (CPPs), or antimicrobial peptides, where the difference between these is in the way they perturb cell membranes and typically increased cell toxicity of the latter.[68,69] Deming and coworkers studied the cytotoxicity, cell uptake, and membrane interactions of a series of cationic $\mathbf{M^R}$ sulfonium polymers, where the degree of alkylation and the nature of the alkylating groups were varied.[70] The alkylating functional groups included a neutral methyl group, hydrophobic allyl and benzyl groups, and hydrophilic monosaccharides. While the more hydrophobic $\mathbf{M^R}$ polymers were found to be highly cytotoxic, akin to antimicrobial peptides, the hydrophilic monosaccharide $\mathbf{M^R}$ polymers (Figure 6d) were well tolerated by cells in vitro. Remarkably, the monosaccharide $\mathbf{M^R}$ polymers were found to be taken up by many cell lines, showing excellent CPP properties, and were found to restructure model lipid membranes in the same manner as the "gold standard" CPP, oligoarginine.[70] These findings were significant since sulfonium cations, unlike guanidinium groups, cannot use H-bonding to induce membrane curvature, and thus must achieve similar membrane modification via a completely different process.[68,69] These hydrophilic Met sulfonium polymers show that other cationic structures, beyond guanidiniums, can be used to obtain high CPP activity and low cytotoxicity.

As shown above, cationic Met sulfonium residues can provide useful properties. However, for many potential bioconjugate and biomaterials applications, introduction of cationic

charge simultaneously with functionality is not always desirable. To address this challenge, and building on the Met sulfonium dealkylation results shown above, Deming and coworkers pursued the development of methodology for selective removal of methyl, as opposed to alkyl, groups (i.e. demethylation) from Met sulfonium residues. Such methodology would allow the transformation of Met residues into functionalized, stable Hcy residues that lack the sulfonium charge and can adopt ordered chain conformations (Figure 11).[71] The strategy used relied on potent nucleophiles in combination with more sterically demanding, non-labile R groups to favor the demethylation pathway over dealkylation. Using $\mathbf{M}^{\mathbf{R}}$ polymers prepared by reaction of **M** with functional epoxides, which had been shown to be stable against dealkylation under a variety of conditions,[11] successful selective demethylation was achieved using an optimized combination of resonance stabilized anionic nucleophiles (thioacetate or **APDC**) with less polar, EtOH rich solvent mixtures that favor ion-pairing (Figure 11).[71] Using this methodology, a variety of epoxide-functionalized $\mathbf{M}^{\mathbf{R}}$ polymers were prepared, and subsequently demethylated to give new, functional $\mathbf{R\text{-}C}^{\mathbf{H}}$ polymers.

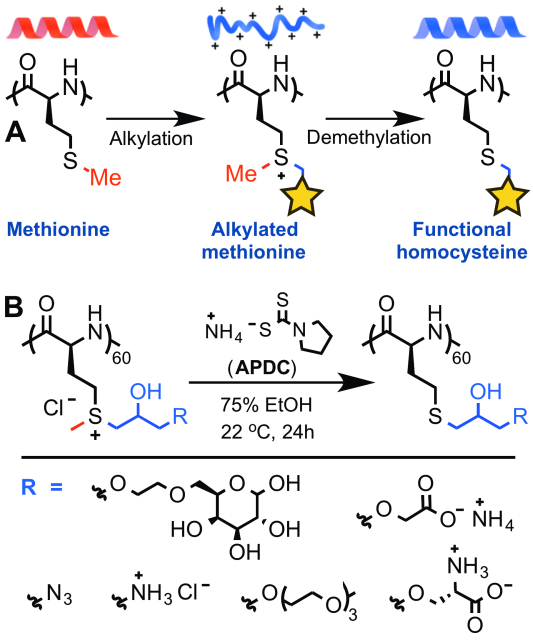


Figure 11. Demethylation of $\mathbf{M^{R}_{60}}$ polysulfoniums to yield functional $\mathbf{R\text{-}C^{H}_{60}}$ derivatives. (a) Schematic showing conversion of methionine to functional homocysteine residues via chemoselective alkylation using a functional reagent followed by selective demethylation. Cartoons at top show conformational and solubility changes of $\mathbf{M_{60}}$ (hydrophobic α-helix) during transformation to $\mathbf{R\text{-}C^{H}_{60}}$ derivatives (hydrophilic α-helix). (b) Reaction showing complete conversion of different $\mathbf{M^{R}_{60}}$ to functional $\mathbf{R\text{-}C^{H}_{60}}$. **APDC** = ammonium pyrrolidinedithiocarbamate.

The alkylation/demethylation strategy described above was also used to convert a single batch of **M** polymer into a series of α-helical thermoresponsive polypeptides ($\mathbf{R\text{-}C^{H}}$) containing a variety of side-chain oligoethylene glycol groups (Figure 12).[72] By addition of different epoxides to **M**, the number of oligoethylene glycol repeats and the nature of terminal groups were systematically varied. The lower critical solution temperature (LCST) properties of the non-ionic $\mathbf{R\text{-}C^{H}}$ polymers in water were then evaluated and it was found that, in addition to the number of ethylene glycol repeats in the side-chains, terminal and linker groups also have substantial and predictable effects on LCSTs (Figure 12). The thioether linkages regenerated in the $\mathbf{R\text{-}C^{H}}$ polypeptides were also shown to provide additional functionality for switching of both polypeptide conformation and thermoresponsive properties via reversible oxidation to sulfoxide groups, where oxidation gave water soluble disordered chains with no LCST.[72] The mild, two-step alkylation-demethylation process described above is chemoselective, high-yielding, functional group tolerant, and a promising means for creation of new functional biopolymers, site-specific peptide tagging, and synthesis of biomimetic and structural analogs of peptides.

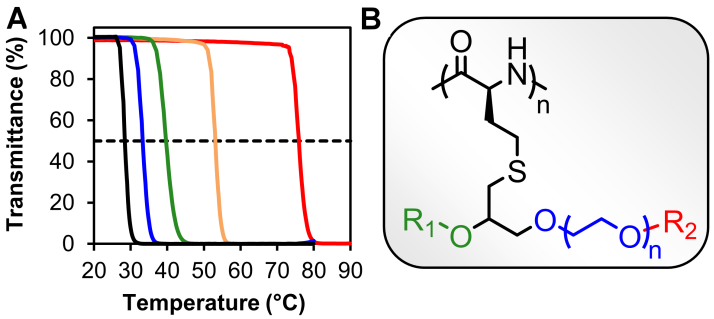


Figure 12. Thermoresponsive **R-C$^{H}_{60}$** derivatives prepared from **M$_{60}$**. (a) A collection of heating curves showing polymer phase separation at different elevated temperatures for solutions of a variety of oligoethylene glycol **R-C$^{H}_{60}$** derivatives in DI water. Measurements performed with polypeptide (3.0 mg/mL) in H_2O with heating rates of 1 °C/min. (b) Schematic structure showing different parameters (R_1, R_2, n) that can be readily varied during synthesis of oligoethylene glycol **R-C$^{H}_{60}$** derivatives. Adapted with permission from *J. Phys. Chem. B*, **2016**, *120*, 6096-6101. Copyright 2016 American Chemical Society.

Conclusions

The thioether functionality found naturally in Met residues, but also available in alkylated Cys and Hcy residues, has begun to emerge as a site useful for chemoselective modification and conjugation of functional molecules. Recently, the strategies and methods for thioether functionalization in Met, Hcy and Cys based residues have also been applied to other polymeric materials. Li and coworkers have reported the synthesis of thioether containing poly(L-glutamate) derivatives via addition of thiols to alkyne groups.[73] Subsequent partial oxidation of the resulting thioether groups was found to alter thermoresponsive properties of solutions of these polymers in water. Non-peptidic acrylate and methacrylate polymers bearing Met inspired

thioether and S-methyl-sulfonium side-chains were also recently reported by Long and coworkers and Matyjaszewski and coworkers,[74,75] where the cationic sulfonium derivatives were evaluated for both gene and siRNA delivery. Met inspired side-chains were incorporated into epoxide monomers by Frey and coworkers,[76,77] and used to prepare well-defined block and hyperbranched copolymers. These copolymers could be oxidized or alkylated, in a manner similar to methods used for **M**, to yield functionalized materials as well as stimuli responsive assemblies. Hedrick and coworkers also prepared Met inspired thioether containing cyclic carbonate monomers, which were used to prepare polycarbonates that could be post-polymerization alkylated with epoxides similar to reactions of epoxides on **M**.[78]

As shown in the many examples above, reactions for thioether functionalization proceed under mild and protic conditions, are high yielding, and are compatible with polymers, peptides, polypeptides, and in some cases, proteins as well. In many examples in this review, **M** polymers were used as a test platform to develop and optimize thioether modification reactions. Use of polymers for development of bioconjugation reactions on amino acid residues, instead of model peptides or proteins, can be advantageous since they are easily prepared and inexpensive, as copolymers with other functional residues can be used to assess chemoselectivity, allow for facile purification of products via precipitation and/or dialysis, and their simple sequences also allow for facile product characterization by routine techniques (such as NMR). Overall, functional modification of thioether containing amino acids has many advantages, and is complimentary methodology to the widely utilized methods for modification at Cys residues.

Corresponding Author *demingt@seas.ucla.edu Address: Department of Bioengineering, 5121 Engineering 5, HS-SEAS, University of California, Los Angeles, CA 90095 Fax: (+1) 310-794-5956

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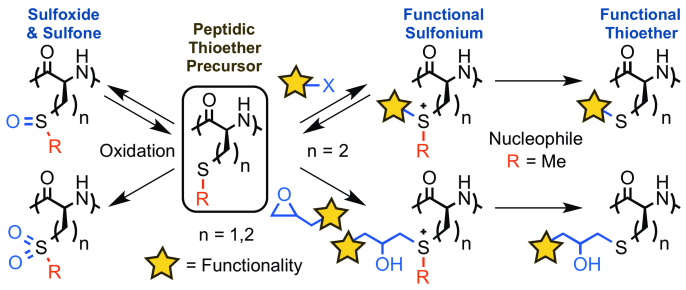
TOC graphic:


Sulfoxide & Sulfone
Peptidic Thioether Precursor
Functional Sulfonium
Functional Thioether
Oxidation
n = 2
Nucleophile
R = Me
n = 1,2
= Functionality