Communication

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Preparation of Multifunctional and Multireactive Polypeptides via Methionine Alkylation

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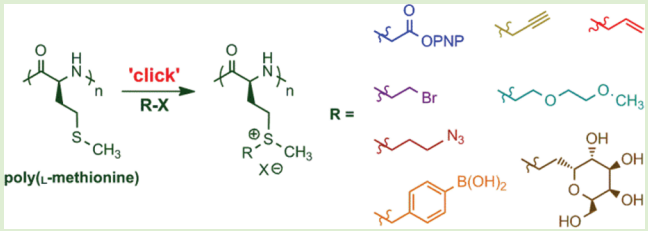
Supporting Information

ABSTRACT: We report the development of a new "click"-type reaction for polypeptide modification based on the chemoselective alkylation of thioether groups in methionine residues. The controlled synthesis of methionine polymers and their alkylation by a broad range of functional reagents to yield stable sulfonium derivatives are described. These "methionine click" functionalizations are compatible with deprotection of other functional groups, use an inexpensive, natural amino acid that is readily polymerized and requires no protecting groups, and allow the introduction of a diverse range of functionality and reactive groups onto polypeptides.



The postpolymerization modification of polymers to introduce a diverse range of functional groups has received much recent attention.[1,2] This approach, where different molecules are conjugated to reactive side-chain groups in a polymer, is advantageous because it avoids the need to prepare many different functional monomers and individually optimize their polymerization conditions. Because of the need for highly efficient side-chain conjugations and compatibility of reactive groups with polymerization, "click"-type reactions are typically employed.[1−3] We are interested in the preparation of side-chain-functionalized polypeptides because these can mimic the diversity of post-translationally modified proteins found in biology and may be useful for medical applications.[4] Reactive precursor polypeptides have been prepared that contain side-chain alkyne,[5−8] alkene,[9−13] azide,[14] or thiol[15] groups for modification using thiol/ene, thiol/yne, or azide/alkyne click chemistry. However, these methods require the use of expensive unnatural amino acids or prior introduction of unnatural functional groups or protecting groups onto polypeptide side chains, adding substantial cost, and often use linkages (i.e., esters)[5−14] that are unstable to deprotection conditions necessary to create copolypeptides containing other functional residues (e.g., lysine, glutamic acid). Here we report the development of a new "click"-type reaction for polypeptides that utilizes the unique reactivity of the thioether group found in the natural amino acid methionine. Because it is not protonated at low pH methionine is the most reactive nucleophile present in peptides and proteins under acidic conditions.[16−19] We have found that methionine can undergo chemoselective, broad scope, highly efficient alkylation reactions in homo- and copolypeptides yielding stable sulfonium derivatives. These "methionine click" functionalizations are compatible with deprotection of other functional groups, use an inexpensive, natural amino acid that is readily polymerized and requires no side-chain modification or protecting groups, and allow the introduction of a diverse range of functionality and reactive groups onto polypeptides.

Because of its role in biology, the alkylation of thioether groups in methionine has been studied for some time, with pioneering studies being done by Toennies in the 1940s.[20,21] The majority of work in this area has employed simple alkylating agents, such as iodomethane to give the naturally occurring, dietary supplement *S*-methyl-methionine,[22,23] as well as iodoacetic acid to form soluble derivatives and probe the active sites of proteins.[24−26] There are some reports on the use of other activated alkyl halides to alkylate methionine, including those containing alkene and alkyne groups, yet no reactions were performed on the resulting sulfonium derivatives.[27−31] Katchalski was the first to alkylate poly(L-methionine), poly(Met), preparing both methyl and carboxymethyl sulfonium derivatives from the corresponding alkyl bromides and poly(Met) in neat formic acid.[32] These polysulfoniums were found to be stable and water-soluble and were studied for their conformational and polyelectrolyte behavior. Despite the simplicity of this reaction, which gives quantitative alkylation with no side products, there have been few further studies on these materials.[33,34] More significantly, there have been no reports on the use of any other functional reagents for alkylation of poly(Met). This lack of activity may be due to traditional difficulties in synthesis of poly(Met),[35,36] as the monomer, L-methionine *N*-carboxyanhydride (Met NCA), is difficult to purify by standard methods, and the living homopolymerization of Met NCA has not been demonstrated. Realizing that the alkylation of methionine residues has potential to be much broader in scope, we sought to develop

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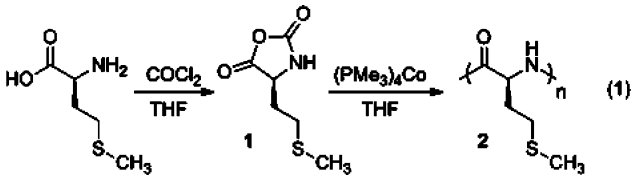
the synthesis of well-defined poly(Met) and subsequently expand its alkylation chemistry as a means to create polypeptides containing diverse and reactive functionality.

Our lab recently reported a method for straightforward preparation of Met NCA in high yield and high purity,[37] which enables the further development of methionine polymers. We also showed that statistical copolymers of equimolar Met NCA with N_ε-CBZ-L-lysine *N*-carboxyanhydride (Z-Lys NCA) could be prepared with controlled chain lengths and narrow chain length distributions using $(PMe_3)_4Co$ initiator in THF. We now report that homopolymerizations of Met NCA (eq 1) also



go to completion within a few hours at room temperature, yet molecular weight analysis of these chains was prohibited by aggregation of poly(Met).[38] To determine chain lengths, we polymerized Met NCA at different monomer-to-$(PMe_3)_4Co$ initiator ratios, and after complete monomer consumption, active chains were end-capped with isocyanate terminated PEG (M_n = 2000 Da).[39] Compositional analysis of purified, end-capped polymers by ^1H NMR gave average poly(Met) chain lengths that increased linearly with stoichiometry (Figure 1).

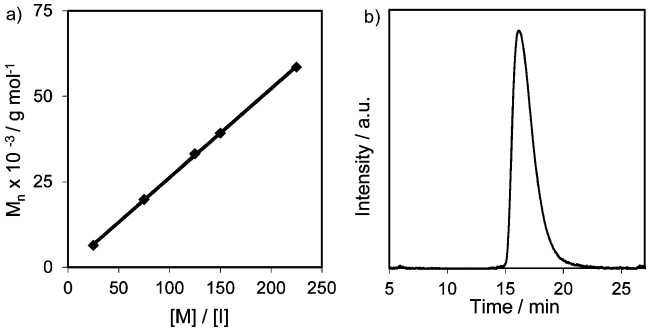
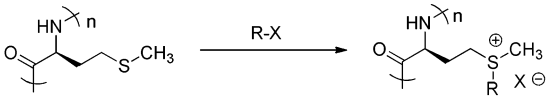


Figure 1. (a) Molecular weight (M_n, ◆) as a function of monomer to initiator ratio ([M]/[I]) for poly(Met) prepared by polymerization of Met NCA using $(PMe_3)_4Co$ in THF at 20 °C. (b) GPC chromatogram (normalized LS intensity versus elution time in arbitrary units (a.u.)) of glycopolypeptide **15**, M_w/M_n = 1.14.

Although chain length distributions of these poly(Met) samples could not be obtained, GPC analysis of an alkylated poly(Met) (vide infra) was found to possess a narrow polydispersity index (M_w/M_n) of 1.14, indicating that the parent poly(Met)s are also well-defined (Figure 1). Poly(Met) was prepared in high yield with precisely controlled chain lengths up to over 400 residues long and could also be prepared as statistical and diblock copolymers with other amino acids. (See the Supporting Information (SI).) Overall, these data show that Met NCA, similar to other NCAs,[40,41] is able to undergo living polymerization when initiated with $(PMe_3)_4Co$.

Although α-helical poly(Met) has low solubility in many solvents, DCM and TFA being notable exceptions, it is soluble enough in a variety of different media to allow facile alkylation. This property provides a significant advantage over poly(L-cysteine), which forms insoluble β-sheets that prohibit preparation of long chains as well as make high yield modification (i.e., alkylation) of the free thiol groups challenging.[15] For initial studies, poly(Met) was reacted with a variety of alkylating reagents in DMF, deionized water, or 0.2 M aqueous formic acid. We observed, similar to previously reported reactions on methionine amino acid,[27−31] that only activated alkyl bromides and iodides were able to react efficiently with poly(Met) under mild conditions (Figure 2).



R-X	Product	Yield
ICH_3	**3**	99%[a]
Br, OH	**4**	97%[a]
Br, NH_2	**5**	99%[a]
Br, OMe	**6**	96%[b]
I, OPNP	**7**	98%[c]
Br	**8**	99%[a]
Br, NHCl	**9** (*ortho*)	97%[a]
	10 (*meta*)	96%[a]
Br, $B(OH)_2$	**11**	99%[a]

Figure 2. Alkylation of poly(Met). Reagents and conditions: R-X in DMF, H_2O, or 0.2 M aqueous formic acid, 20 °C. Yield is total isolated yield of completely functionalized polypeptide. (a) Product was dialyzed against 0.1 M aqueous NaCl to give X = Cl. (b) X = Br. (c) X = I.

The significant exception in this comparison was allyl bromide/iodide, which has been reported to alkylate methionine amino acid,[42] yet was found by us to react only sluggishly with poly(Met) (vide infra). In addition to the previously described products **3** and **4**,[32] these direct alkylation reactions were used to prepare a number of new sulfonium derivatives of poly(Met). Various haloacetyl derivatives reacted readily and quantitatively to generate polysulfoniums bearing amide (**5**), ester (**6**), and active ester (**7**) functionalities, where the latter may be useful for further derivatization with nucleophiles such as primary amines. Propargylic and benzylic/pseudobenzylic halides also reacted efficiently with poly(Met) and allow the introduction of a variety of useful functional groups into polypeptides. This method for introduction of alkyne functionality (**8**) is straightforward and is more economical than other routes to install this click reactive group onto polypeptides.[5−8] The facile formation of pyridine-containing sulfoniums (**9**, **10**) allows incorporation of this basic functionality that is otherwise difficult to introduce in polypeptides. Phenyl-boronic-acid-containing polypeptides (**11**) have also been of interest for their sugar-binding abilities[43,44] and now can be readily prepared with high degrees of incorporation in a single step.

In contrast with the results above, unactivated alkyl halides, especially those without adjacent multiple bonds, reacted either sluggishly or not at all with poly(Met) under similar conditions. This result is not surprising because in all previous work on methionine alkylation using amino acids, peptides, or proteins there are no reports of sulfonium formation using unactivated

reagents.[16−19,27−31,42] To expand further the scope of poly(Met) alkylation, we explored different means to increase the reactivity of unactivated alkyl halides. Silver tetrafluoroborate is known to promote thioether alkylation in small molecules,[45,46] and we found that the addition of this reagent to alkylations in acetonitrile promoted the complete reaction of poly(Met) with unactivated alkyl halides (e.g., haloethyl compounds) (Figure 3). This strategy allowed the introduction of an even wider

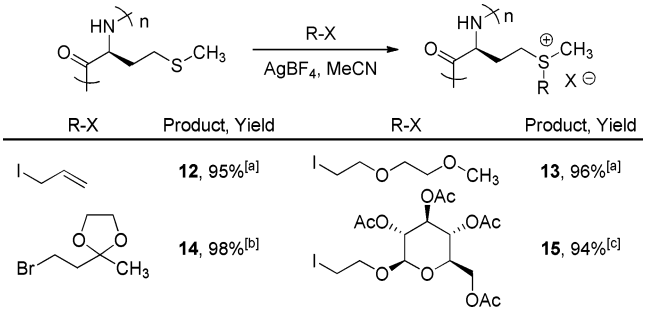


Figure 3. Alkylation of poly(Met) using $AgBF_4$. Reagents and conditions: R-X, $AgBF_4$, MeCN, 50 °C. Yield is total isolated yield of completely functionalized polypeptide. (a) Product was dialyzed against 0.1 M aqueous NaCl to give X = Cl. (b) Product was dialyzed against aqueous HCl at pH 2 with 0.1 M NaCl to give the polyketone, **14a**, and X = Cl. (c) X = BF_4.

variety of reactive and functional groups onto poly(Met), giving additional new reactive and functional polypeptides. In addition, use of silver salts now facilitated the facile incorporation of alkene functionality (**12**) onto polypeptides, which is useful for a variety of further modifications including thiol/ene click reactions.[9,10] Ethylene glycol derivatives (**13**) were added to impart the water solubilizing and passivating properties of PEG.[47,48] Reactive ketone groups, useful for conjugation in aqueous environments,[49] were introduced in a single step using the 1,3-dioxolane derivative (**14**) that deprotects to give the water-soluble polyketone during acidic workup. Glycopolypeptides, of interest as mimics of natural glycoproteins,[50,51] often require many synthetic steps, especially for preparation of longer chains with high sugar content. Using iodoethyl glycosides, high-molecular-weight and well-defined, fully glycosylated polypeptides (**15**) were readily prepared in excellent yield by poly(Met) alkylation.

Because the use of silver salts may not be desirable in some applications, we also explored functionalization of poly(Met) with alternative reactive reagents. Alkyl triflates are known to be powerful alkylating agents,[52] and, although these have not been used to alkylate methionine in amino acids, peptides, or proteins, we found that these react efficiently and cleanly with poly(Met). Functional alkyl triflates were prepared in a straightforward manner from a variety of hydroxyalkyl compounds. These reagents reacted efficiently with poly(Met) in organic solvents under mild conditions to give the fully alkylated polymers (Figure 4). Because of the significant difference in reactivity between alkyl triflate and bromide, this method also allowed incorporation of alkyl bromide functionality onto poly(Met) (**16**). This electrophilic functionality can be readily modified by subsequent reaction with different nucleophiles, such as amines or thiols. We reacted polysulfonium **16** with aminomethane sulfonic acid, which gave quantitative incorporation of sulfonate functionality (**16a**) that may be useful in mimicking sulfonated biopolymers (eq 2).

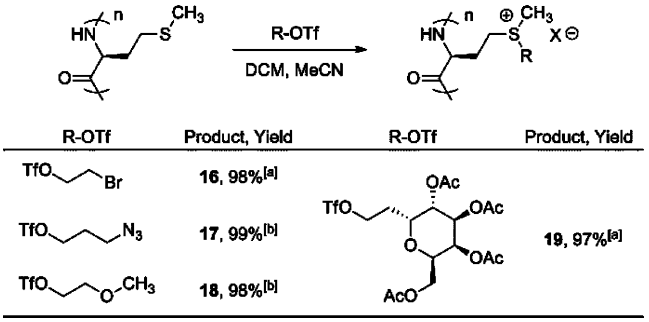
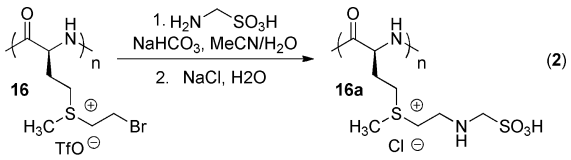


Figure 4. Alkylation of poly(Met) using alkyl triflates. Reagents and conditions: R-OTf, DCM/MeCN, 20 °C. Yield is total isolated yield of completely functionalized polypeptide. (a) X = OTf. (b) Product was dialyzed against 0.1 M aqueous NaCl to give X = Cl.



Alkyl triflate modification of poly(Met) was also found to be a facile method to introduce the azide functional group (**17**), which gave polypeptides bearing the click reactive counterpart to the alkyne groups introduced above. Other functional groups that required silver salts for introduction onto poly(Met) could also be introduced by the use of the corresponding alkyl triflates. Therefore, ether (**18**) and glycoside (**19**) functionalities were added to poly(Met) via the corresponding alkyl triflates. Removal of the acetyl protecting groups from the sugars in polypeptide **19** gave a water-soluble glycopolypeptide with no signs of any degradation. (See the SI.) In general, all of the above poly(Met) alkylations were found to cause no polypeptide chain cleavage and gave polysulfoniums that were stable in a variety of media, at different pH (2 to 10), at elevated temperature (80 °C), and after storage for more than 3 months. (See the SI.)

The results above confirm that alkylation of poly(Met) is a highly efficient process for preparation of functionalized polypeptides. Many of the polysulfoniums reported here are the first examples of methionine derivatives bearing these reactive or functional groups, and, compared with other methods, these reactions are also very cost-effective and straightforward ways to introduce these groups onto polypeptides. We have shown that alkylation of poly(Met) is broad in scope, is high yielding, occurs readily, can use equimolar reagents, has a single reaction trajectory, gives stable products, and purification of products is facile: characteristics that define "click"-type reactions.[3] For methionine alkylation to be considered a "click" reaction, it must also be chemoselective.[3] In peptides, polypeptides, and proteins, there can be many nucleophilic functional groups that react with alkylating reagents.[42] Of these, the cysteine thiol group is most widely utilized in protein and peptide alkylation reactions,[53] as it typically gives single products, in contrast with lysine amines and histidine imidazoles, which give multiple alkylation products. It is noteworthy that in water the thiol, amine, and imidazole functional groups are highly reactive with alkylating agents at near neutral pH and above, but their reactivity diminishes greatly with decreasing pH.[42,54] This trend is due to protonation of all these nucleophilic amino acids at low pH,

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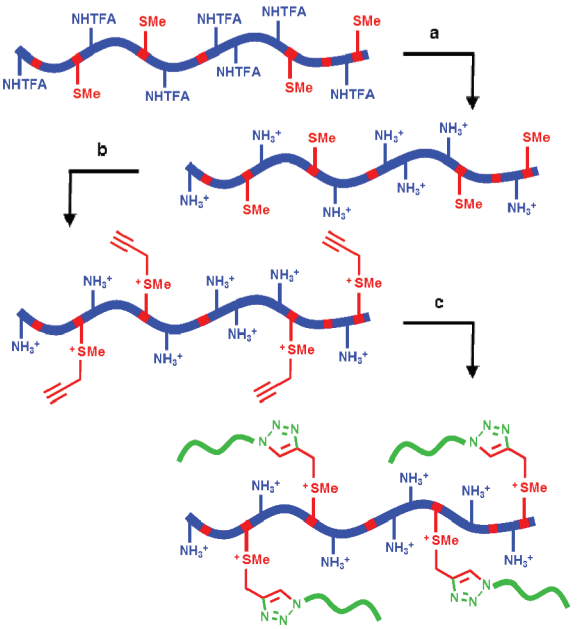
which greatly decreases their reactivity. Because methionine is more resistant to protonation, it is consequently the only highly reactive nucleophilic amino acid at pH <3.[16−19,27−31] To highlight this chemoselectivity and show its utility for selective alkylation of methionine in polypeptides, we prepared a statistical copolymer of methionine and lysine and studied its alkylation (Scheme 1). We chose lysine as a competing nucleophile because it is the most abundant nucleophile found in proteins, it is more widely used in synthetic polypeptides compared with histidine or cysteine, and it is known to compete with thiol and imidazole groups in protein alkylations.[42,53] As is also true for cysteine and histidine polypeptides, the synthesis of poly(L-lysine) requires the use of protecting groups, and methionine alkylation needs to be compatible with and orthogonal to deprotection chemistry to be useful as a "click" reaction. Here we found that the methionine residues of our copolymer could be alkylated with propargyl bromide either before or after lysine deprotection to give the same final product. These results show that methionine sulfonium groups are stable to deprotection reactions (see the SI) and alternatively that methionine residues can be alkylated chemoselectively at pH 2.4 in the presence of a four-fold excess of free amine groups (Scheme 1). Confirming this selectivity, a control reaction of propargyl bromide with pure poly(L-lysine) under identical conditions gave no alkylation products. (See the Supporting Information.) All of the alkyne groups in the copolymer prepared above were then quantitatively conjugated with azide-terminated PEG chains (Scheme 1), showing that alkyne reactivity is not compromised and that the sulfonium groups are stable and useful for further polypeptide modifications.

Scheme 1. Schematic Showing Chemoselective Alkylation of Methionine Residues in the Presence of Excess Amine Groups, Followed by Clicking of PEG-N_3 to the Alkylated Methionine Residues[a]



[a]Initial copolymer is poly[$(N_\varepsilon$-TFA-L-lysine$)_{0.8}$-*stat*-$(Met)_{0.2}]_{206}$, where 0.8 and 0.2 are mole fractions of lysine and methionine residues, respectively. Reagents and conditions: (a) K_2CO_3, MeOH, H_2O (99%); (b) propargyl bromide, 0.2 M formic acid (94%); (c) α-methoxy-ω-azidoethyl-poly(ethylene glycol) (M_n = 1000 Da), $CuSO_4$, ascorbic acid, PMDETA, H_2O (95%).

In summary, the alkylation of methionine residues in polypeptides has all the features of a "click" reaction,[2] and consequently is an attractive means for preparation of functionalized and side-chain reactive polypeptides. Aside from the examples given here, these methods should also be applicable to a variety of other alkylating reagents and thioether-containing residues, such as S-alkyl cysteines. In comparison with other methods for installation of functional and click reactive groups onto polypeptides, the starting material methionine is substantially less expensive than the side-chain-modified or unnatural amino acids typically employed, and poly(Met) is readily prepared with controlled and high molecular weights, which makes these "methionine click" reactions attractive for large-scale use. Facile incorporation of other click-reactive functional groups (e.g., alkyne, azide, or alkene) by methionine alkylation also allows for further chemoselective modification of polypeptides. Such "secondary click" strategies, as shown in Scheme 1, allow methionine alkylation to also utilize the broad diversity of reagents already developed and available for existing click conjugation methods.[1,2]

■ ASSOCIATED CONTENT

Supporting Information

Experimental procedures and spectral data for all new compounds, polymerization data, and M_n versus [M]/[I] plots. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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