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Versatile synthesis of stable, functional polypeptides via reaction with epoxides

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Abstract

Methodology was developed for efficient alkylation of methionine residues using epoxides as a general strategy to introduce a wide range of functional groups onto polypeptides. Use of a spacer between epoxide and functional groups further allowed addition of sterically demanding functionalities. Contrary to other methods to alkylate methionine residues, epoxide alkylations allow the reactions to be conducted in wet protic media and give sulfonium products that are stable against dealkylation. These functionalizations are notable since they are chemoselective, utilize stable and readily available epoxides, and allow facile incorporation of an unprecedented range of functional groups onto simple polypeptides using stable linkages.

KEYWORDS: polypeptide, polymer functionalization, sulfonium

Introduction

The development of robust methods for facile synthesis of well-defined functional polymers is an ongoing challenge. To circumvent the common incompatibility of reactive side-chain functional groups with polymerization chemistries, there has been considerable effort to develop selective and efficient methods for post-polymerization modification.¹⁻³ This is especially true for synthesis of functional polypeptides, which are desirable as mimics of post-translationally modified proteins and for uses in biological and medical applications.⁴ Consequently, a variety of precursor polypeptides, and their reactions with functional molecules, have been reported in recent years.⁵⁻¹⁴ Our lab has reported the reaction of methionine, Met, residues with alkylating agents as an efficient means to prepare functional polypeptides, which utilizes an inexpensive amino acid precursor and is broad in scope.¹⁵ In this system, some functional groups can be installed using commercial reagents in water, while others require use of stoichiometric silver salts or preparation of reactive alkyl triflates and anhydrous conditions. We now report the development of Met alkylation using epoxides as an efficient, general method to introduce a wide range of functional groups onto polypeptides. These functionalizations are notable since they can be conducted in wet protic media and are chemoselective, they utilize stable, easily accessible epoxides, and they allow facile incorporation of an unprecedented range of functional groups onto polypeptides using stable linkages.

In our previous work, activated alkyl halides were found to react readily with poly(L-methionine), **M**, in protic media to give high yields of fully alkylated polysulfonium products.¹⁵ **M** polymers are α -helical and hydrophobic, while the polysulfoniums are typically water soluble, and we have previously shown that they are in disordered conformations in water.¹⁵ Many of the activating groups in these previous examples (i.e. carbonyl, alkyne, aryl), also made the product sulfonium ions unstable toward nucleophiles, resulting in dealkylation.¹⁶ While such reversibility is desirable for temporary modifications, the ability to prepare permanently functionalized materials under mild conditions is also important for many uses. We had found that stable sulfonium products could be obtained, but required use of anhydrous conditions and use of highly reactive or expensive reagents,¹⁵ which may limit their applicability. In search for an improved methodology, we were inspired by early studies on reactions of ethylene oxide

(EO) with protein functional groups.¹⁷⁻¹⁸ It was found that EO reacts with many functional amino acids, including Met residues, which gave stable β -hydroxyethyl sulfonium products (Figure 1).¹⁹ Similar to reactions of alkyl halides with Met,¹⁵ the reaction of EO with proteins was observed to be selective for Met residues at pH < 3, where all other nucleophilic functional groups are protonated and unreactive.¹⁷⁻¹⁸ Subsequent studies, utilizing N-protected Met amino acid, showed that substituted epoxides, such as propylene oxide and *tert*-butyl glycidyl ether, also react with Met to give sulfonium ions that were stable to acid and mild base.²⁰⁻²² In these cases, the sulfur of Met adds primarily to the least hindered side of the epoxide to give the β -alkyl- β -hydroxyethyl sulfonium (Figure 1). These data showed that addition of epoxides to Met residues is promising as a potentially chemoselective reaction to prepare stable sulfonium products, which can be accomplished in protic media. Since a large variety of epoxides are either commercially available or readily prepared, we sought to further develop this reaction as a general means to synthesize a broad range of functional and stable Met derivatives under mild conditions.

Experimental Section

Materials and Methods

Unless otherwise stated, all polymer functionalization reactions were performed in glass vials, under ambient atmosphere. Reactions at elevated temperature were controlled using a Corning PC 420D thermostated hotplate equipped with a thermocouple probe. Room temperature reactions were performed at ca. 20 °C ambient temperature. Glacial acetic acid, AcOH was used as received from Fisher Scientific. The **PHCKRM** peptide was obtained from NeoBioLab and was reported 96.7% pure. Poly(*S*-methylnmethionine sulfonium chloride), **M^{Me}**, and poly(*S*-benzylmethionine sulfonium chloride), **M^{Bn}**, were prepared as previously described.¹⁵ Epoxides were either purchased and used as received, or synthesized (see SI). Dialysis was performed using deionized water (18.2 M Ω -cm) prepared by passing in-house deionized water through a Millipore Milli-Q Biocel A10 unit. Regenerated cellulose dialysis tubing obtained from Spectrum Labs. ESI-MS was performed using a Waters LCT Premier spectrometer.

General Synthetic Procedures

Poly(L-methionine)₆₀, M₆₀

Prepared by previously reported method.¹⁵ L-Methionine N-carboxyanhydride was polymerized with Co(PMe₃)₄ using a 20:1, monomer to initiator ratio. The DP was determined by endcapping a small aliquot from the polymerization mixture with 2 kDa PEG-isocyanate (CH₃(OCH₂CH₂)₄₅N=C=O) followed by ¹H NMR analysis.¹⁵ Found Composition, DP = 59.

M₆₀ alkylation procedure A (Procedure A)

M₆₀ was suspended in glacial AcOH (16 mg/mL). The epoxide (3 eq per Met residue) was added in one portion. The mixture was stirred vigorously at 37 °C. After 24 h, the limpid solution was transferred to a 2 kDa MWCO dialysis bag and dialyzed against 3 mM HCl_(aq) (24 h, 3 H₂O changes). The retentate was lyophilized, to provide the functionalized polypeptide.

M₆₀ alkylation procedure B (Procedure B)

M₆₀ was suspended in glacial AcOH (27 mg/mL). The epoxide (1.5 eq per Met residue) was added. The mixture was stirred vigorously at 37 °C. After the peptide dissolved (*ca.* 2-6 h), a second portion of epoxide (1.5 eq per Met residue) was added. After 24 h, the limpid solution was transferred to a 2 kDa MWCO dialysis bag and dialyzed against 3 mM HCl_(aq) (24 h, 3 H₂O changes). The retentate was lyophilized, to provide the functionalized polypeptide.

M₆₀ glycosylation (Procedure C)

The procedure was analogous to *Procedure B*, however before transfer to the dialysis bag, 1 mL of 2 M HCl_(aq) was added. The solution was allowed to stand at RT for 16 h. After dialysis and lyophilization, the deprotected, fully glycosylated peptide was recovered.

Alternative M₆₀ alkylation using 1.5 eq of epoxide

M₆₀ (6.0 mg) was suspended in glacial AcOH (0.20 mL). Glycidyl Azide (**4a**) (3.4 mg, 0.034 mmol, 0.75 eq per Met residue) was added. The suspension was stirred vigorously at RT and became homogenous over 24 h. Another addition of **4a** (3.4 mg, 0.034 mmol, 0.75

eq per Met residue) was performed and stirring was continued for an additional 24 h. The reaction mixture was transferred to a 2 kDa MWCO dialysis bag and dialyzed against 3 mM HCl_(aq) (24 h, 3 H₂O changes). The retentate was lyophilized, to provide **4** (12 mg, 94% yield, >99% functionalized (¹H NMR)).

Poly(*S*-alkyl-L-methionine) stability studies

Polymer stock solutions (25 mmol Met residue per mL) were prepared in H₂O. Buffers were prepared by titrating 0.1 M solutions of the parent acid with 1 N NaOH. PBS 10x was prepared by dissolving a PBS tablet and adjusted to pH 7.4. The polymer stock (0.9 mL) was diluted with the buffer stock (0.1 mL) and if necessary, nucleophile (0.1 mmol) was added. The mixture was incubated on a 37 °C H₂O bath for 24 h. The solution was transferred to a 2 kDa MWCO dialysis bag and dialyzed against 3 mM HCl_(aq) (24 h, 3 H₂O changes). The retentate was lyophilized. The products were analyzed by ¹H NMR, and the ratio of *S*-alkyl-Met/Met was determined. In all studies of **4**, **8** and **M^{Me}** mass recoveries were greater than 90%.

Results and Discussion

Figure 1. Alkylation of **M₆₀** with epoxides in acetic acid, 37 °C for 24-48h followed by counterion exchange. Yield is total isolated yield of completely functionalized polypeptide,

except for **9,10**, and **11**. a = Percent modification for incomplete functionalizations are in parentheses.

To test epoxide reactivity with polypeptides, we reacted a 60-mer Met polymer, **M**₆₀, with EO, propylene oxide or glycidyl azide under different conditions in protic media. We found that highest degrees of functionalization and shortest reaction times were obtained using a small excess of epoxide (1.5 to 3 equivalents) in glacial AcOH at 37 °C (Figure 1). The reaction is significantly faster under acidic conditions compared to neutral pH although some epoxide is consumed by the acidic solvent, hence the use of 3 equivalents of epoxide per Met residue. If the epoxide is added in stages over time, quantitative functionalization can also be obtained using 1.5 equivalents of epoxide (see SI). Use of other solvents, such as water, methanol, formic acid, or DMF gave slower or incomplete functionalizations (see SI). High degrees of functionalization were also obtained with epoxides containing other desirable functional groups, such as protected amine, alkyl chloride, alkene, alkyne, and oligoethyleneglycol (Figure 1). Many of these are reactive groups that can be utilized for secondary functionalization using a diverse range of chemistries.¹⁻³ Reaction of a PEG end-capped Met polymer with glycidyl azide, and analysis of the product by ¹H NMR, showed that no polypeptide chain cleavage occurred during the alkylation process (see SI). The resulting sulfoniums were all highly water soluble and exclusively contained the alkyl substituents at the β -position except in the case of **2**, which showed a trace of the α -alkylation product due to low steric demand of the substrate (see SI). The potential advantages of this methodology can be seen by the introduction of azido groups via an epoxide in wet media, which previously required use of an azido triflate in anhydrous solvent.¹⁵

Figure 2. Alkylation of **M**₆₀ with epoxides containing an oxoethylene spacer (red) in acetic acid, 37 °C for 36h followed by ion exchange. Yield is total isolated yield of completely functionalized polypeptide. a = Sulfonate partially deprotected under reaction conditions. b = Yield is of fully deprotected glycopolypeptide.

When we attempted to functionalize **M**₆₀ with more sterically demanding epoxides, including those containing monosaccharides, ATRP initiating groups, and phosphonates, we found that complete conversion of all Met residues to sulfoniums could not be readily obtained (Figure 1). Such functional groups are difficult to introduce onto polypeptides by other methods, and are useful for a variety of applications including binding to biomolecules,²³ synthesis of hybrid copolymers,²⁴ or mimicking biomineralization processes.²⁵ We reasoned that the inability to completely functionalize **M** polymers with bulky epoxides was simply due to crowding of neighboring groups on the polymer backbone preventing further functionalization. To circumvent this issue, we prepared functional epoxides containing oxoethylene spacers that increased the distance between functional groups and the epoxides (Figure 2). With these longer tethers, we found that quantitative alkylations of **M**₆₀ polymers with a wide array of large functional groups was achieved, allowing facile preparation of polypeptides containing a variety of unprecedented or difficult to introduce functional groups such as sulfonate, phosphonate, and malonate

(Figure 2). These polybetaines should possess interesting properties potentially useful for binding metal ions or creating non-fouling surfaces.²⁶

Some of the functional epoxides above required use of protecting groups during synthesis. In general, the sulfonium products exhibited sufficient stability to allow full removal of these protecting groups after alkylation without loss of the functional groups (Scheme 1). Note that the saccharides in the deprotected glycosylated polysulfoniums (Scheme 1) are able to equilibrate to a mixture of anomers in water. The sulfonium products were also stable toward secondary bio-orthogonal functionalizations, such as azide-alkyne cycloadditions (eq. 1). To study the stability of the sulfonium **M** polymers in more detail, we subjected a select group of samples to different aqueous conditions (Figure 3). Methylated (**M**^{Me}) and benzylated (**M**^{Bn}) samples were chosen as points of reference since these have been shown respectively to be unreactive and highly reactive towards dealkylation by nucleophiles.¹⁶ Polypeptides **4** (**M**^{N3}) and **8** (**M**^{EG}), prepared by alkylation with functional epoxides, were found to display good stability in aqueous buffers ranging in pH from 5 to 9. These polymers were also quite stable against dealkylation by the potent nucleophile 2-mercaptopyridine. These results show that the polysulfonium structures obtained from epoxide alkylation are significantly more stable than those prepared from activated alkyl halides (e.g. benzyl bromide).

Scheme 1. Removal of protecting groups from representative functionalized **M**₆₀ polymers.

To test the chemoselectivity for epoxide alkylation of Met over other nucleophilic functional groups, we prepared a statistical copolymer of Met and L-lysine and studied its alkylation. We chose lysine as a competing nucleophile since it is the most abundant nucleophile found in proteins, it is more widely used in synthetic polypeptides compared to histidine or cysteine, and it is known to compete with thiol and imidazole groups in protein alkylations.^{27,28} Similar to results obtained in other Met alkylations,¹⁵ we found that the Met residues in the copolymer could be alkylated chemoselectively with glycidyl azide in acidic media in the presence of a fourfold excess of amine groups (eq. 2).

Figure 3. Dealkylation studies on sulfonium **M**₆₀ polymers.

For a more demanding test of chemoselectivity, we attempted to alkylate only the Met residues in the antioxidant peptide **PHCKRM**, which also contains highly nucleophilic histidine, cysteine and lysine residues (Figure 4).¹⁶ Treatment of **PHCKRM** with glycidyl azide in glacial AcOH gave a dominant product (**18**), where the Met residue was alkylated. The identity of **18** was determined using ESI-MS (Figure 4, see SI), where the parent ion (MR^+ , R = 3-azido-2-hydroxypropyl group) showed primarily addition of a single 100 Da 3-azido-2-hydroxypropyl group to the peptide. The additional presence of a single fragment corresponding to the loss of the thioether $RSCH_3$, which is commonly observed in MS analysis of Met sulfonium ions,^{29,30} confirmed that alkylation was primarily occurring at the Met residue. These results also demonstrate that, in addition to polypeptides, peptides can be chemoselectively modified at Met residues via epoxide alkylation at low pH.

Figure 4. Chemoselective alkylation of **PHCKRM**. (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) Product after alkylation showing **18**, [MR]⁺, as well as the characteristic [M-RSCH₃]⁺ fragment.

Conclusions

The alkylation of Met residues in polypeptides using functional epoxides was developed to give high yields of fully functionalized Met sulfonium containing materials, which were found to possess high water solubility and good stability against dealkylation. The epoxide reagents were optimized to provide chemoselective functionalization of Met, even when multiple sterically demanding functional groups were added to polypeptides. This methodology provides a simple solution for preparation of a diverse array of functional polypeptides in wet conditions using readily available or easily prepared reagents. Since **M** polymers are readily prepared from an inexpensive amino acid without need of protecting groups,¹⁵ we expect that this economical approach to functional polypeptides will allow their use in an expanded array of applications.

Acknowledgements

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Supporting Information Available.

Experimental procedures and spectral data for all new compounds, procedures for alkylation reactions, and methods for stability studies. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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