Expanding the Toolbox of Chemoselective Modifications of Protein-Like Polymers at Methionine Residues

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Abstract:
Selective modifications at methionyl residues in proteins have attracted particular attention in recent years. Previously described methods to chemoselectively modify the methionine side chain in elastin-like polypeptides (ELPs) involved nucleophilic addition using alkyl halides or epoxides yielding a sulfonium group with a positive charge strongly affecting ELPs' physico-chemical properties, in particular their thermal responsiveness. We herein explored the recently reported ReACT method (Redox-Activated Chemical Tagging) based on the use of oxaziridine derivatives yielding an uncharged sulfimide as an alternative route for chemoselective modifications of methionine-containing ELPs in aqueous medium. The different synthetic strategies are herein compared in order to provide a furnished toolbox for further biorthogonal post-modifications of any protein polymers.

Main text:
Protein-based polymers are attractive supplies for the design of high performance biologically-inspired materials for a variety of applications such as biomaterials, tissue engineering, therapeutic drug delivery and targeting, and nanotechnology.[1–4] Inspired from natural proteins of various living systems and designed by genetic engineering, protein polymers presenting a high degree of control over the macromolecular architecture and a high sequence complexity are attractive building units to access functional precision polymer materials. [4–7] Among the most common structural proteins in mammals, elastin is a well-known fibrous protein,[8–10] whose sequence and properties have inspired the design of elastin-like polypeptides (ELPs).[11–15] ELPs are artificial biopolymers composed of repeating peptide sequences from the hydrophobic domain of tropoelastin presenting similar thermal properties to elastin.[11,16] Constituted of repetitive -[Val-Pro-Gly-Xaa-Gly]- pentapeptide units, where Xaa corresponds to any amino acid except proline,[11] ELPs present a reversible temperature-triggered soluble-to-insoluble phase transition behavior in aqueous medium. The thermal responsiveness of a specific ELP can be quantified by its transition temperature (*T*t) defined as the characteristic temperature at which soluble ELP chains self-associate and aggregate into an insoluble ELP-rich phase. Interestingly, this unique feature is highly tunable playing with molecular parameters (*e.g.*, guest residue composition, molar mass) as well as with environmental parameters (*e.g.*, salts and cosolutes, ionic strength, pH). Bioproduction of ELPs using recombinant DNA and protein-engineering techniques allows utter control over their macromolecular characteristics,[2] including amino acid composition, sequence and chain length, and therefore over their thermal properties. As such, ELPs constitute attractive biocompatible, biodegradable and inert thermo-responsive protein polymers for a wide range of biomedical applications.[17,18] An alternative to the design of recombinant ELPs with different primary structures by genetic engineering to access ELPs with different *T*ts relies on the chemical modification of a unique ELP backbone applying orthogonal chemoselective bioconjugation reactions as an easy means to access a variety of ELP derivatives with different thermal responsiveness.[19–21]

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Post-translational modifications of proteins, involving chemical changes after proteins' translation play a fundamental role in the regulation of proteins' structures and functions.[22] This has strongly motivated the development of different bioconjugation strategies to mimic nature's ability to site-specifically modify proteins in order to modify or enhance their properties.[23,24] To this end, one alternative relies on the use of functional non-natural amino acids. However, despite significant technological advancements, incorporation of non canonical residues into recombinant proteins remains non trivial and sometimes incomplete.[25–28] A second method relies on synthetic strategies to selectively modify natural amino acid residues presenting functional side chains or employing proteins' terminal ends. Biorthogonal reactions have been and are still extensively developed, and various chemical methods are currently available to selectively modify cysteine, lysine, tyrosine or tryptophan residues by carefully balancing reactivity and selectivity.[29–32]

Because of its relatively low nucleophilicity in physiological conditions, post-modifications at methionyl residue remain less explored in proteins and peptides as compared to lysine and cysteine, the most commonly modified amino acids. However, the unique reactivity of the thioether group under acidic conditions allows specific biorthogonal alkylation of methionine's side chain at low pH without the need for any protecting group for other residues, as pioneered by Toennies using alkyl halides.[33,34] Similar strategies were applied on synthetic poly(methionine) polymers, copolymers and block copolymers,[35–37] before the development by Deming and co-workers of a more versatile method based on the use of epoxide derivatives that significantly enlarged the scope of methionine modifications.[38] Recently, Lin and co-workers reported a potent strategy to chemoselectively functionalize proteins at methionyl residues using oxaziridine derivatives.[39] Termed redox-activated chemical tagging (ReACT), this strategy relies on the electrophilic amination of the thioether group of methionine with an appropriately-designed oxaziridine derivative leading to the formation of a sulfimide conjugate. Indeed, with an extremely reactive strained -C-N-O- three-membered ring, oxaziridines have been widely explored for their ability to function as aminating or oxygenating reagents, as firstly described by Emmons.[40] The nature of the nitrogen substituent as well as of the nucleophile involved have been shown to play a crucial role in the reactivity of oxaziridine derivatives towards different nucleophiles. Oxaziridines containing small *N*-substituent are mostly involved in *N*-transfer reactions, whereas those bearing electron withdrawing groups behave more reactive toward oxygen transfer.[41–43]

A few years ago, our group started to explore chemoselective modifications of ELPs at methionyl residues as a convenient means to modulate their thermo-responsive properties and introduce various functionalities.[19–21] In particular, applying nucleophilic addition reactions using alkyl halides and epoxides, methionine-containing ELPs were functionalized with different pendant groups yielding polycationic derivatives. Herein, we aimed at expanding the scope of chemoselective modifications of ELPs at methionine exploring the ReACT strategy giving access to uncharged sulfimide ELP derivatives. We were particularly interested in performing a comparative study between three main synthetic methods to achieve quantitative chemoselective modifications of ELPs and evidence their advantages and drawbacks, especially for subsequent functionalization with bioactive motifs.

We specifically focused the present study on a forty repeat unit-ELP containing periodically spaced methionine residues produced recombinantly in *Escherichia coli* bacteria and purified by *inverse transition cycling*[44] as described previously.[30] Namely ELP[M_1V_3-40], following established nomenclature,[45] was chosen for its intermediate methionine content (one Met every four pentapeptide units, corresponding to a total of 10 methionine in addition to the *N*-terminal Met residue) and *T*t in a suitable temperature range for further turbidimetry studies (*i.e.*, 32°C at 250 µM in water). ELP[M_1V_3-40] was chemoselectively modified on methionine residues using either the alkyl halide method (Figure 1, route A), epoxide chemistry (Figure 1, route B), or the ReACT method[39] (Figure 1, route C). A series of ELP derivatives

was synthesized following each of the three synthetic routes so as to introduce various pendant groups (R groups in Figure 1) onto methionyl residues' side chain. (Table 1) Short alkyl chains, such as methyl or ethyl, were introduced in order to compare, in similar conditions, the influence of each type of modification onto the thermo-responsive properties of the resulting ELP derivatives as compared to the pristine ELP. ELP derivatives containing functional reactive groups such as alkyne, alkene, or azido groups were also designed with the aim of further investigating post-modification reactions with biomolecules (*i.e.*, monosaccharides).

The modifications through the alkyl halide route (A) were performed in aqueous formic acid under argon atmosphere and required large excess of alkyl halides together with long reaction time (5 days) as reported previously with ELP[M-20] and ELP[M_1V_3-*n*] (*n*=20,40). [19,21] Using these reaction conditions, ELP[M_1V_3-40] was reacted with iodomethane and compound 1A was obtained after purification by dialysis using ultra-centrifugal filter tube against Milli-Q water. In order to incorporate a functional group onto ELP[M_1V_3-40] and inspired by the work of Kramer and co-workers[37], propargyl bromide was used in similar conditions but the reaction did not go to completion. Despite several attempts for optimization playing with different reaction conditions (*e.g.*, propargyl bromide equivalents, reaction time, nature of organic co-solvent and aqueous conditions), we did not succeed in exceeding a 40 % functionalization degree (compound 2A).

New batches of compounds 1B, 2B and 3B were synthesized using epoxide chemistry (route B) following previously established procedures[21,46] in order to perform complementary analyses for further comparative studies. A mixture of AcOH/HFIP (9/1, v/v) was necessary to achieve complete functionalization within 2 days of reaction. It is worth mentioning that, under these conditions, methionine residues in ELPs are highly sensitive to oxidation, and therefore careful attention was paid to maintain all reagents, solvents and reaction mixture under argon atmosphere in order to avoid undesired sulfoxide formation.

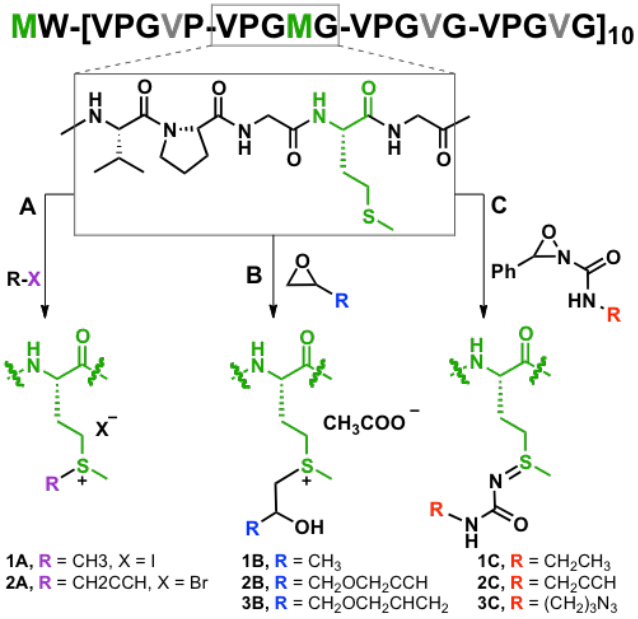


Figure 1. Primary structure of ELP[M_1V_3-40] and chemoselective modifications at methionine using alkyl halide (A), epoxide (B) or oxaziridine (C) derivatives. Solvent conditions: A) THF/0.2 M aqu. formic acid; B) AcOH/HFIP (9:1); and C) H_2O/DMF (99:1).

The ReACT strategy was also explored to introduce different functionalities onto ELP[M_1V_3-40] through a sulfimide bond using suitable oxaziridine derivatives for oxidative sulfur imidation reaction to occur. The reaction has been described to occur under mild conditions (aqueous conditions at neutral pH) compatible with sensitive biomolecules, in short reaction times and high selectivity to methionine over other amino acids as demonstrated by experimental[39] and theoretical[47] investigations. As mentioned previously, a wise selection of *N*-substituents is also essential to favor the amination process (nitrogen-transfer product, NTP) over the competitive oxidation (oxygen-transfer product, OTP) at the thioether.[47,48] Three oxaziridine derivatives substituted with a weak electron-withdrawing urea linkage allowing high selectivity for the NTP were therefore prepared following the procedure described by Lin, Yang and co-workers.[39] Imidation reactions with ELP[M_1V_3-40] were performed under argon atmosphere in aqueous conditions containing 1 % DMF and completed within 30-60 minutes after addition of the oxaziridine derivative. Excess oxaziridine and benzaldehyde side product were removed by liquid-liquid extraction and the aqueous phase was purified by dialysis to provide compounds 1C, 2C and 3C. All compounds were synthesized and obtained with isolated yields ranging from 75 % to 90 %. (Table 1) Functionalization degrees, corresponding to the percentage of functionalized methionine, were determined by ^{1}H NMR spectroscopy by integration of the resonance peak of the Met methyl protons (-S*CH*$_3$) relative to the resonance peak of the asymmetric protons of valine at the guest residue position (signal i) integrating for 15 H.

Table. 1. Characteristics of ELP[M_1V_3-40] derivatives.

	Compound	*M* (g.mol^{-1})	Funct.[a]	Yield[b]	Tt (125 µM)[c]
A) Alkyl halide	1A[d]	18,597	100 %	75 %	> 70 °C
	2A	18,344	ca. 40 %	81 %	n.d.
B) Epoxide	1B[d]	18,335	100 %	88 %	> 70 °C
	2B[d]	18,929	> 95 %	90 %	> 70 °C
	3B[d]	18,952	> 95 %	89 %	n.d.
C) Oxaziridine	1C	17,982	100 %	88 %	57 °C
	2C	18,092	100 %	92%	54 °C
	3C	18,588	100 %	85 %	48 °C

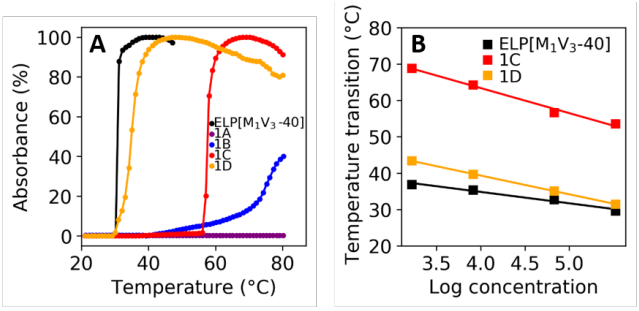
[a] Functionalization degrees determined by ^{1}H NMR, [b] Yield of reaction, [c] Transition temperature in Tris buffer determined by DLS, [d] Synthesized following reported procedures.[21]

^{1}H and 2D NMR (HSQC or COESY) spectra of all derivatives are provided in Figures S1-S17. Small alkyl chains were readily introduced quantitatively onto ELP[M_1V_3-40] whatever the chemical route used, derivatives 1A, 1B, and 1C being obtained with complete functionalization degrees (100 %) in excellent purity as evidenced by size-exclusion chromatography (Figure S18). Regarding the introduction of functional groups, the ReACT method showed excellent results with total incorporation of either alkyne or azide handles in less than one hour yielding, respectively, compounds 2C and 3C. With slightly lower functionalization degree (> 95 %), attributed to minor methionine oxidation into sulfoxide (0.5 Met over 11 total), compounds 2B and 3B were also successfully synthesized using the epoxide chemistry strategy. In contrast, introduction of an alkyne group using the alkyl halide route proved more challenging and a maximum functionalization degree of 40 % was obtained for compound 2A. Concurrent oxidation of the thioether was not found responsible for the incompleteness of the reaction and further addition of propargyl bromide did not lead to any success.

Overall, with a longer reaction time (5 days) and a lower functionalization degree for compound 2A, the alkyl halide route (A) did not prove as efficient as the epoxide (B) or oxaziridine (C) routes. As pre-

viously reported,[21,46] we confirmed that epoxide chemistry is also more versatile than the use of alkyl halides for the thioalkylation of ELP[M_1V_3-40]. Specific care is however needed to avoid oxidation of the sensitive thioether group. The ReACT approach[39] based on the use of oxazidine derivatives was found very efficient with short reaction times (less than 1 hour) and quantitative functionalization. One minor drawback of this strategy is however the tedious synthesis and purification of oxaziridine reagents obtained in poor yields (30 % - 40 %) as already mentioned by Lin *et al*.[39]

In order to evidence the impact of each type of modification onto the thermo-responsive properties of ELPs, turbidity experiments were performed on derivatives with similar alkyl R groups, namely compounds 1A, 1B and 1C. Transition temperature measurements were performed at different ELP concentrations in Tris buffer using light absorption at 600 nm (Figure 2, Figures S19-S22). The *T*t values were determined as the temperature corresponding to the onset of turbidity (Figure 2 and Table S1). While the *T*t of the pristine ELP[M_1V_3-40] ranged between 30°C and 37°C in the 25–250 µM concentration range, in good agreement with previous results obtained in pure water (*T*t values ranging from 25°C to 32°C in 250–1500 µM solutions)[21], thioalkylated derivatives 1A and 1B presented none or very weak thermo-responsive properties in the temperature and concentration ranges studied. As already reported by our group, this can be directly attributed to the increased hydrophilicity and solubility afforded by the sulfonium groups present along the polypeptide chain.[21] Compound 1C obtained from the modification of ELP[M_1V_3-40] through the ReACT strategy presented high *T*ts (54-69 °C) in the 25–250 µM concentration range despite a higher molecular weight than ELP[M_1V_3-40] and the uncharged linkage between the ethyl R group and the methionine side chain. This can be explained by the very polar sulfimide bond having a similar character as the sulfoxide bond, its oxygen analog.[20] While the overall hydrophilicity of 1C is enhanced by the polar sulfimide moieties, it is however likely counterbalanced by the keto-enol equilibrium of the adjacent urea moiety, allowing possible H-bonds formation leading to intra- and intermolecular interactions and a decrease of water solubility. As a consequence, compound 1C exhibited *T*t values in a similar range than the sulfone derivative from our previous study and significantly lower than the *T*ts of the sulfoxide derivative.



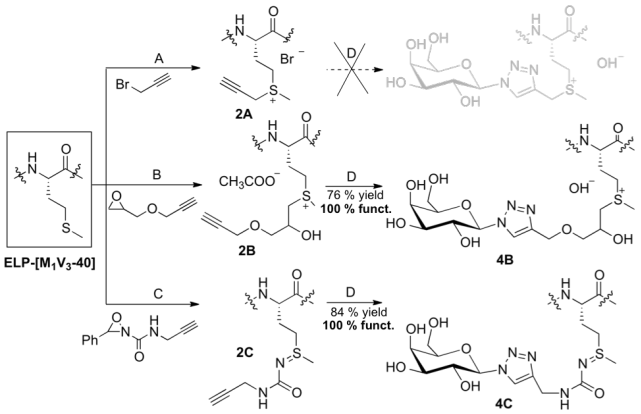
ELP-[M_1V_3-40]	1A	1B	1C	1D
33°C	> 80°C	> 70°C	57°C	35°

Figure 2. (A) Relative absorbance at 600 nm of ELP[M_1V_3-40], 1A, 1B, 1C and 1D at 125 µM in Tris buffer as a function of temperature. (B) Tt values of ELP[M_1V_3-40], 1C and 1D as functions of concentration in Tris buffer. Lines correspond to the fits of the data using Chilkoti and co-workers empirical equation.[49]

(C) Chemical structures of the methionine residues' side chain for each derivative and their corresponding onset transition temperature (*T*t) at 125 µM in Tris buffer.

In order to have access to a synthetic strategy leading to chemoselectively modified methionine side chains while retaining the thermo-responsive properties of the ELP, we have performed the demethylation of compound 1B.[21,38] (Scheme S1 and Figure S18) The resulting uncharged compound 1D was indeed found to recover its thermal responsiveness with a range of *T*ts closer to those of ELP[M_1V_3-40]. The presence of the hydroxyl group in the *S*-alkyl-L-homocysteine-containing derivative is however likely responsible for the slightly higher *T*ts of 1D (31-43°C) as compared to those of ELP[M_1V_3-40] (30-37°C). Tt versus concentration curves were measured for uncharged derivatives 1C and 1D for comparison with ELP[M_1V_3-40] and fitted plots were obtained using the empirical equation established by Chilkoti and co-workers.[49] (Figures 2B) For both 1C and1D, the slope of the fits were found steeper than the one observed with the pristine ELP[M_1V_3-40] confirming their greater hydrophilic character. Importantly, additional experiments confirmed the retained reversibility of the thermal transition for both 1C and 1D derivatives (Figure S19-S22). Overall, with different synthetic routes in hands, chemoselectively-modified ELPs with either none, weak or high thermal responsive behavior in aqueous solutions can be obtained.

Scheme. 1. Synthetic strategies to multivalent galactose-ELP bioconjugates.



Thereafter, we were particularly interested in exploring subsequent orthogonal post-modifications to allow the grafting of relevant bioactive motifs onto ELPs. With potentially three strategies in hand to chemoselectively modify ELPs at methionine residues, we wished to investigate the functionalization of ELP[M_1V_3-40] with galactose as illustrated on Scheme 1. As unsatisfying functionalization was obtained for compound 2A, this strategy was not further continued. In contrast, 2B and 2C were quantitatively functionalized with azido-galactose through Huisgen's copper-catalyzed azide-alkyne cycloaddition (CuAAC).[50] Compound 4B was obtained in aqueous conditions using copper sulfate as catalyst and *N*,*N*,*N*′,*N*′′,*N*′′-Pentamethyldiethylenetriamine (PMDETA) as ligand (0.26 equiv. $CuSO_4$ per alkyne, 1.3 equiv. Na ascorbate per alkyne and 0.26 equiv. PMDETA per alkyne in Milli-Q water), as confirmed by ^{1}H and HSQC NMR spectroscopy (Figures S23-S24), in particular thanks to the resonance peak of the triazole proton at 8.34 ppm integrating as 11 H corresponding to a full bioconjugation. We also noticed that the original resonance of the methylene in α position of the alkyne function shifted from 4.29 ppm to 4.79 ppm, this being the result of the deshielding induced by the increased electron density of the triazole as compared to the alkyne bond (Figure S25). These reaction conditions being unsuccessful for the complete functionalization of compound 2C, slight optimization of the procedure was needed, especially playing with catalytic conditions. Total functionalization of compound 2C with azido-galactose

was achieved using DMSO as solvent, a catalytic amount of copper sulfate and sodium ascorbate system (0.05 equiv. and 0.2 equiv. per methionine, respectively) and 0.05 equiv. per methionine of tris((1-benzyl-4-triazolyl)methyl)amine (TBTA) as a ligand for CuI. The reaction was stirred for 3 days under argon atmosphere and purified by dialysis using an ultra-centrifugal filter tube against Milli-Q water. Similarly to compound 2B, the completeness of the reaction was assessed by analysis of the ^{1}H and HSQC NMR spectra (Figures S26-S27) thanks to the triazole proton signal integrating as 11 H at 8.06 ppm and the deshielding of the methylene in α position of the alkyne group from 3.79 ppm to 4.35 ppm. The purity of compounds 4B and 4C was also assessed by size-exclusion chromatography (Figures S28 and S29).

Overall, the ReACT strategy was confirmed to be a very efficient synthetic method for the quantitative functionalization of ELP[M_1V_3-40] with alkyne handles for subsequent post-modification with azido-galactose units by CuAAC click chemistry. Advantageously, the latter yields uncharged ELP bioconjugates in contrast with the epoxide route where an additional demethylation reaction is required to remove the positive charge.

To conclude, we have herein explored a new route for chemoselectively modifying methionine-containing ELPs using oxaziridine-based reagents, providing an additional means for easily tuning the thermo-responsive properties of recombinant ELPs. This enlarges the variety of accessible ELP derivatives through sulfonium, thioether or sulfimide linkages. With commercially available reagents, reasonable reaction times and high efficiency, the epoxide-based route is highly attractive. The possibility to recover a thioether bond, and therefore thermo-responsive properties, by sulfonium demethylation adds on to the list of advantages of this synthetic route. The ReACT strategy also proved an excellent alternative route with high efficiency, short reaction times and the possibility to maintain ELP thermal responsiveness. Altogether, this set of reactions opens up a whole range of possibilities to functionalize ELPs with a large variety of molecules such as bioactive moities, active drugs, or contrast agents, for the design of new ELP bioconjugates. In a more general way, with very high chemoselectivity towards unprotected elastin-like polymers, the different strategies studied in this work can easily be extended to other methionine-containing polymers or protein like-polymers,[36,39] expanding the toolbox of selective modifications at methionyl residues.

ASSOCIATED CONTENT

Supporting Information. Experimental procedures, SEC chromatograms, onset temperatures of aggregation, Tt as function of temperature for ELP derivatives, Tt as function of concentration, DLS temperature sweeps and NMR spectra ^{1}H and HSQC are available in the supporting information.

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Author Contributions

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