Quantitative side-chain modifications of methionine-containing elastin-like polypeptides

Jessica R. Kramer,[‖,†] Rosine Petitdemange,[‖,§,¤] Laure Bataille,[§,¤] Katell Bathany,[#] Anne-Laure Wirotius,[§] Bertrand Garbay,[‡] Timothy J. Deming,[†] Elisabeth Garanger[§,¤] and Sébastien Lecommandoux*[,§]

[†] Department of Chemistry and Biochemistry, and Department of Bioengineering, University of California, Los Angeles, California 90095, United States

[§] Université de Bordeaux/Bordeaux INP, ENSCBP, 16 avenue Pey-Berland, Pessac 33607, France, and CNRS, Laboratoire de Chimie des Polymères Organiques (UMR5629), Pessac, France

[‡] Université de Bordeaux, EA4135, Bordeaux 33000, France

[#] CBMN, UMR5248, CNRS, Université Bordeaux, Bordeaux 33000, France

[¤] Institut Européen de Chimie et Biologie (IECB), Pessac 33607, France

[‖] Authors contributed equally to the work.

Supporting Information Placeholder

ABSTRACT: Tuning the *LCST* of temperature-responsive recombinant elastin-like polypeptides has usually been achieved by designing different protein sequences, in terms of amino acid composition and length, implying tedious molecular cloning steps. In the present work, we have explored the chemoselective alkylation of methionine as a means to modify elastin repeat side chains and modulate the *LCST* of the polypeptides.

Introduction

While commonly exploited in biology basic research, as well as in biotech and pharmaceutical industries to produce recombinant proteins and therapeutics, protein engineering techniques are rapidly advancing the field of polymer science, paving the way to precision protein-like polymers with exquisite control over primary structure, namely monomer sequence, and molecular weight.[1–3] In addition to silk-based proteins that have been developed mainly for tissue engineering and drug-delivery,[4,5] recombinant elastin-like polypeptides (ELPs) are emerging as a unique class of precision polymers with stimuli-responsive self-assembly properties for specific biomedical and biotechnological applications.[6] ELPs are repeating sequences of [-Val-Pro-Gly-*Xaa*-Gly-] pentapeptide, the guest residue *Xaa* being any amino acid except proline, originally inspired from the hydrophobic domain of tropoelastin.[7] ELPs exhibit a lower critical solution temperature (*LCST*), also referred as an inverse temperature transition (*T*t), similar to synthetic polymers such as poly(*N*-isopropylacrylamide) (pNIPAM). ELP chains are fully soluble in water below the *LCST*, while switching to an insoluble state above the *LCST*. Fully reversible, the aggregation is influenced by different parameters such as the nature of the *Xaa* guest residues within the ELP repeats, the overall molecular weight and molar concentration of the ELP, and the ionic strength of the solution.[8,9] This solubility switch has proven to be a major advantage for the purification of recombinant ELPs from bacterial lysates,[10] as well as for the controlled self-assembly of individual ELP blocks.[11,12] This property has also been used to develop nanocarriers for drug-delivery systems, the resulting micellar systems being able to self-assemble spontaneously with temperature and to load different drugs.[11,13-15]

Post-polymerization modifications of ELPs reported so far have mostly involved their chain ends. Different ELP sequences have been conjugated to small organic molecules,[16] oligonucleotides,[17] drugs,[18,19] or PEG.[20] Modifications at the amino acid side chains within the ELP domain have been more scarcely reported.[21,22] Such modifications require the use of highly efficient reactions in order to successfully modify all the repeating functional groups in these sequences in high yield. These modifications also require the use of bioorthogonal ligation strategies to chemoselectively modify residue-specific side chains without affecting the amino acid backbone, *C*- and *N*-terminal ends and side chain groups of other residues. Introducing specific reactive groups in synthetic polypeptides can be relatively easily achieved, allowing their subsequent site-specific chemical modifications.[23-28] However for recombinant polypeptides, as with natural or recombinant proteins, this requires the incorporation of unnatural or non-canonical amino acids.[29] This strategy has been successfully explored to functionalize proteins by Pictet-

Spengler or Staudinger ligations.[30,31] However, replacement of natural residues by amino acid surrogates is non-trivial and modest yields of production are often obtained, especially if multiple substitutions are desired.

In this context, the recent development of thioether alkylation reactions for the modification of methionine and thioether containing residues in synthetic polypeptides by Deming *et al.* is of significant interest. Because the thioether of methionine is the most nucleophilic group at low pH over primary amines of the *N*-terminal end and of lysines' side chains, imidazole groups of histidines as well as thiols of cysteines, various alkylating agents such as triflates or alkyl halides were found to be chemoselectively attacked by thioether groups of polypeptides allowing functionalization at each methionine residue with a wide range of compounds.[32-33]

The goal of the present work was thus to apply these alkylation reactions to chemoselectively modify methionine side chains of recombinant ELPs. We have thus designed and produced recombinantly in *Escherichia coli* a 20-repeat ELP featuring methionine at the guest residue position of every ELP repeat, namely [-Val-Pro-Gly-*Met*-Gly-]$_{20}$ designated as ELP20. Methionine thioether groups were subsequently alkylated chemoselectively to introduce small methyl or bulkier benzyl groups to investigate the effect of alkylation on the *LCST*.

Results and discussion

Design, production, purification and characterization of ELP20

Recombinant ELP20 was produced in *E. coli* using protein-engineering techniques. To this aim, a synthetic gene was first designed to code the protein sequence *Leader*-(VPGMG)$_{20}$ corresponding to ELP20. Methionine was used as guest residue in the twenty pentapeptide repeats for subsequent "post-polymerization modification" by chemoselective alkylation. A 13 amino acid-long sequence termed *Leader* (detailed sequence provided in the supporting information) was introduced at the *N*-terminal end of the ELP domain to provide an initial methionine for proper initiation of translation in *E. coli*, a tryptophan for detection purposes and additional residues for optimal production of ELP20 in the bacterial host. After cloning of the ELP20-encoding gene, the protein polymer was expressed in the T7express *E. coli* strain cultivated in three independent and process-controlled 1 L fermentors. The production yield was found to be optimal 5 h after induction by isopropyl-□-D-thiogalactoside (IPTG), (Figure 1A) as degradation of ELP20 progressively occurred at longer production times. ELP20 was extracted from cell lysates and purified by *Inverse Transition Cycling* (*ITC*) avoiding the use of time-consuming and expensive chromatography purification techniques.[10] The purity of the recovered ELP20 fraction was assessed by SDS-PAGE. (Figure 1B)

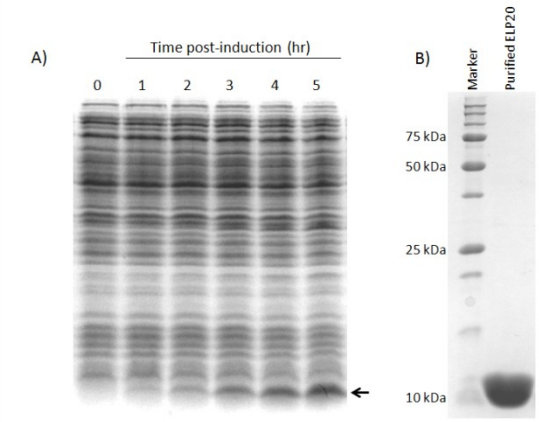


Figure 1. Expression and purification of recombinant ELP20 as monitored by 15% SDS-PAGE. A) Expression of ELP20 during fermentation. The band corresponding to ELP20 is indicated with an arrow. Lane 0, non-induced culture; lanes 1-5, total proteins after IPTG induction. B) Analysis of ELP20 after purification by *ITC*. Gels were stained with colloidal blue G250.

ELP20 was extensively dialyzed against water to remove excess salts used in the purification process, then lyophilized and subjected to mass spectrometry analysis. ELP20 was identified by the peaks of its multi-charged species. (Figure 2) Deconvolution of the mass spectrum was performed by processing the raw data using a maximum entropy-based approach providing ELP20's experimental mass. (Figure 2, inset) The latter was found as 10,382 Da, corresponding to the theoretical mass of ELP20 without its *N*-terminal methionine, suggesting that it was removed after translation. ELP20 was also characterized by 1D and 2D NMR spectrometry (Figure S1) and its *LCST* was measured by absorbance spectroscopy and found to be 29°C at 100 □M in phosphate buffer.

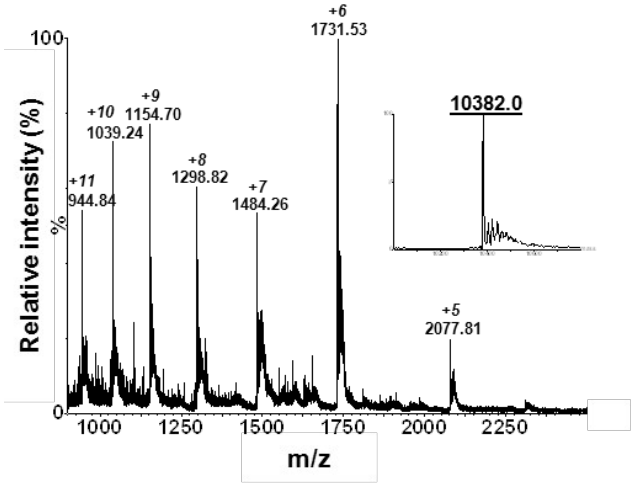
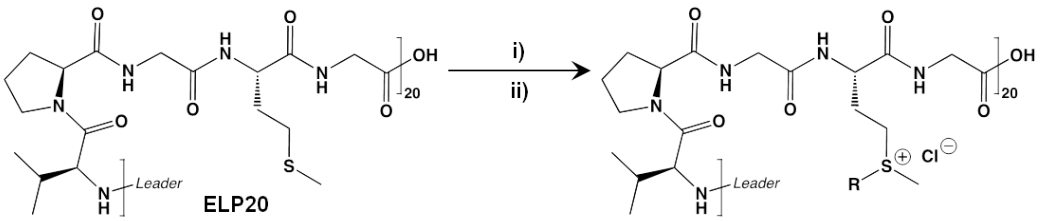


Figure 2. ESI mass spectrum showing the charge state distribution of ELP20. Charge states of the highest intensities are labeled with their charges. Inset: MaxEnt-deconvoluted ESI mass spectrum of ELP20.

Chemoselective methionine alkylation of ELP20

ELP20 samples were reacted with methyl or benzyl alkylating reagents with the aim of both evaluating the efficacy of this bioconjugation reaction on these protein substrates, and for measuring the resulting effect on the chain properties (*LCST* and conformation). Methionine residues of ELP20 were first methylated using methyl iodide to measure the effect of the conversion from a thioether group to a positively-charged sulfonium on the *LCST*. Quantitative methylation was obtained with 15 equivalents of MeI per methionine, and the reactions were performed under acidic conditions (0.2 M formic acid in water) to favor reaction at methionine.[32-33] Excess methyl iodide was easily removed after reaction by liquid-liquid extraction with diethyl ether. While alkylations of methionine residues in synthetic polypeptides proceed rapidly with near stoichiometric amounts of alkylating reagent,[32] we found that ELP20 was slower to react, and excess alkylating agents were used to ensure complete modification of all methionine residues. The diminished reactivity of methionine residues in ELP20 may be due to steric hindrance by neighboring residues related to the chain conformations of ELPs.

Scheme 1. Chemoselective methionine alkylation reaction of ELP20.



i) 0.2 M HCOOH, R-X (CH_3I or BnBr) in THF, rt, 5 days
ii) Dialysis (0.1 M NaCl and H_2O)

ELP20(Me): R = CH_3
ELP20(Bn): R = CH_2-C_6H_5

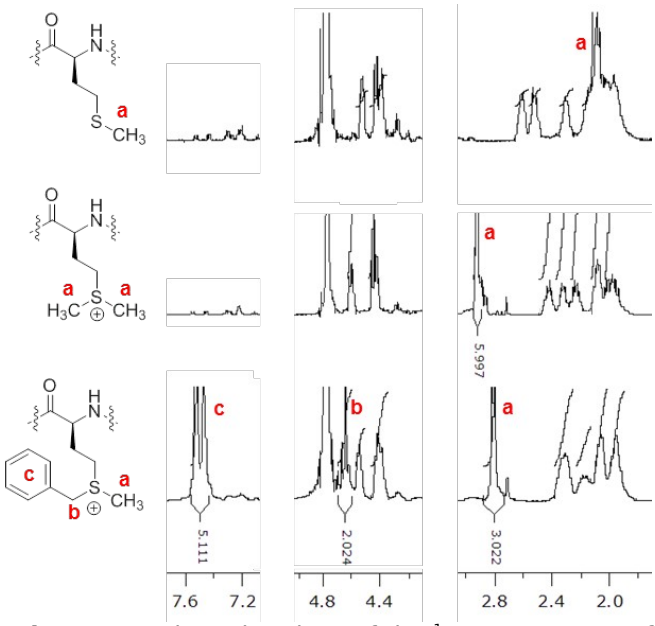


Figure 3. Selected regions of the ^{1}H NMR spectra of ELP20 (upper spectrum), ELP20(Me) (middle spectrum) and ELP20(Bn) (lower spectrum).

The degree of methylation in ELP20(Me) was assessed by ^{1}H NMR analysis on the lyophilized product. The original resonance at δ = 2.09 ppm in ELP20, corresponding to the 3 protons of the methionine methyl group (protons **a**), was found to shift to δ = 2.94 ppm and integrated for 6 protons in ELP20(Me). (Figures 3 and S2, lower and middle spectra). These integrations were calibrated by assigning the resonances at δ = 0.9 ppm to the 6 methyl protons of the valine side chain. (Figure S2).

Similar to above, a bulkier benzyl group was introduced at each methionine side chain of ELP20 using 15 equivalents of benzyl bromide. ELP20(Bn) was separated from the excess BnBr after extraction with Et_2O, followed by dialysis and lyophilization. The degree of benzylation was again assessed using ^{1}H NMR analysis. Calibrating our integrations to valine methyl groups as above, we observed the appearance of resonances at δ = 7.47 and 7.54 ppm that integrated for 5 protons and were assigned to the phenyl group of the sulfonium (protons **c**). The resonance at δ = 4.66 ppm that integrated for two protons was assigned to the methylene of the benzyl group. The resonance of the methionine methyl group **a** was shifted from δ = 2.09 ppm in the ELP20 spectrum to δ = 2.81 ppm in ELP20(Bn). (Figures 3 and S2, lower and upper spectra).

Effect of alkylation on LCST

To investigate the effect of methylation and benzylation of the ELP backbone on the *LCST*, turbidity assays were carried out in phosphate buffer at different concentrations. (Figure 4) ELP20's *LCST* ranged between 27°C and 33°C depending on the concentration (50-200 μM). After methylation, the resulting ELP20(Me) did not exhibit any *LCST* in the range of temperature studied. This is most likely due to the presence of positive charges of the sulfoniums increasing the hydrophilicity of the whole polypeptide. Benzylation resulted in polypeptides that retained *LCST* behavior, but *LCST*s were shifted to higher values indicating that the benzyl group is hydrophobic enough to partly counterbalance the hydrophilic effect of the positive charge. In order to determine whether alkylation of ELP20 also translates into changes in secondary structure, circular dichroism (CD) spectra of ELP20, ELP20(Me) and ELP20(Bn) were measured in PBS at 20 °C, below all *LCST* val-

ues. (Figure S3) As usually observed with these polypeptides, ELP20 exhibited both random coil and type II β-turn secondary structure characterized by two minima at *ca*. 197 nm and 225 nm, respectively. Alkylation (methylation and benzylation) resulted in an overall increase of random coil structures and decrease of type II β-turns, confirming a decrease of order with increasing hydrophilicity of the ELP.

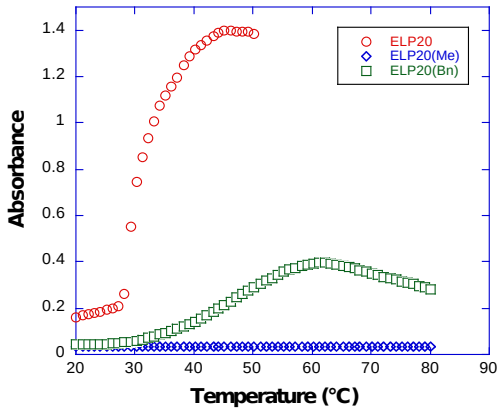


Figure 4. Turbidity assay of ELP20, ELP20(Me) and ELP20(Bn), 100 μM in PBS.

We have reported a synthetic strategy to introduce side chain modifications in elastin-like polypeptides and explored the effect of such modifications on the *LCST* behavior. An ELP with repeating methionine residues was designed and efficiently produced recombinantly in *E. coli* allowing selective and quantitative side-chain modifications at multiple sites to be performed by chemoselective methionine alkylation. ELPs conformation and *LCST* were modified depending on the alkylation agent, allowing for selective modification of protein properties. This work opens new avenues to study the effect of side chain modifications on physical and biological properties of elastin-like polypeptides.

ASSOCIATED CONTENT

Experimental procedures and spectral data. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

* Mailing address: Université de Bordeaux/IPB, ENSCBP, 16 avenue Pey-Berland, 33607 Pessac Cedex, France. E-mail: lecommandoux@enscbp.fr

Author Contributions

‖ Authors contributed equally.

Notes

We declare no competing financial interests.

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