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Tuning Thermoresponsive Properties Of Cationic Elastin-Like Polypeptides By Varying Counterions And Side-Chains

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ABSTRACT. We report the synthesis of methionine-containing recombinant elastin-like polypeptides (ELPs) of different lengths that contain periodically spaced methionine residues. These ELPs were chemoselectively alkylated at all methionine residues to give polycationic derivatives. Some of these samples were found to possess solubility transitions in water, where the temperature of these transitions varied with ELP concentration, nature of the methionine alkylating group, and nature of the sulfonium counterions. These studies show that introduction and controlled spacing of methionine sulfonium residues into ELPs can be used both as a means to tune their solubility transition temperatures in water using a variety of different parameters, and to introduce new side-chain functionality.

Introduction

Thermoresponsive polymers presenting lower or upper critical solution temperature (*LCST* or *UCST*, respectively) phase transition behavior in water are of great interest for biomedical applications.[1,2,3] In particular, recombinant elastin-like polypeptides (ELPs) have been shown to be excellent biomaterials in the fields of drug delivery and tissue engineering.[4,5,6] Derived from the hydrophobic domain of tropoelastin, ELPs are composed of repeating sequences of [-Val-Pro-Gly-*Xaa*-Gly-] pentapeptides, (VPG*X*G), where the guest residue *Xaa* (*X*) can be any amino acid except proline.[7] ELPs are characterized by a reversible *LCST* phase transition: below a critical transition temperature (*T*t), ELP chains are soluble in aqueous solution, while above the *T*t they desolvate and aggregate.[8,9] Precise tuning of the *T*t can be achieved at the macromolecular level mainly through adjustment of an ELP's primary sequence, including

amino acid composition (*i.e.* identity of *Xaa*) and chain length (*i.e.* molecular weight), which are two parameters exquisitely controlled by the initial gene design.[10,11,12]

Although recombinant DNA and protein engineering techniques are powerful methods to access precision protein-like polymers, tedious molecular cloning steps can sometimes hamper the development of large libraries of ELPs with different sizes, diverse functionalities and tunable properties. Moreover, some functional groups can only be introduced by the use of unnatural amino acids, which in general significantly lower the production yields.[13] The use of chemoselective reactions to selectively modify specific residues in ELPs post-synthesis has been shown to be a promising means to introduce new functionalities and impart new properties to ELPs, especially at their chain ends,[14,15,16,17] and more scarcely at ELP side-chain residues.[18,19]

In a previous study, we reported the chemoselective alkylation of all methionine (Met) residues in the ELP sequence $(VPGMG)_{20}$, which contains Met residues in every repeat, and used this modification to tune the *LCST* of the resulting polysulfonium ELP derivatives.[20] However, a limitation of this system was the need to use hydrophobic alkylating reagents, *i.e.* benzyl bromide, to retain the *LCST* after sulfonium formation. Alkylation with less hydrophobic groups, *i.e.* methyl, resulted in a complete loss of thermoresponsive properties in the polysulfonium ELP derivative. The goal of the present work was to improve the versatility of this post-synthesis ELP modification strategy to accommodate a wider range of side-chain functionality, while also being able to retain and tune polysulfonium ELP derivative thermoresponsive properties.[17]

To accomplish this goal, we have redesigned ELP sequences to contain fewer Met residues, since the high charge density obtained when sulfonium ions were generated in every pentapeptide repeat in $(VPGMG)_{20}$ resulted in very hydrophilic polymers. Decreasing the density of functionalization sites was envisioned to help retain thermoresponsive properties, while also

allowing reasonable levels of functional modification. With this system, we also studied how the *T*t was affected by ELP concentration, different alkylating functional groups, different sulfonium counterions, and by introduction of functionality in the absence of sulfonium charge. Overall, our results show that the thermoresponsive properties of Met containing ELP sequences can be finely adjusted by a number of post-synthesis modifications.

Results and discussion

Development and characterization of ELP-M-20 and ELP-M-40.

For the present study, two recombinant methionine-containing ELPs with the primary structures MW[VPGVGVPGMG(VPGVG)$_2$]$_{n/4}$, where n = 20 or 40, hereafter designated as **ELP-M-n**, were designed and produced using recombinant DNA and protein engineering techniques (Figure 1A). One Met residue was incorporated per every four ELP pentapeptide repeats for subsequent post-synthesis modifications, and non-reactive valine occupied the guest residue position in all the other repeats, giving a Val:Met ratio at the guest residue position of 3:1. The periodic spacing of Met residues along the ELP backbone, as compared to our previous fully Met substituted ELP,[20] was designed to moderate the perturbation of ELP properties upon Met sulfonium generation, as well as to allow introduction of more sterically demanding side-chain modifications. Met and tryptophan (Trp) residues were also introduced at the *N*-terminus of the ELPs (named as “Leader” in Figure 1A) for proper initiation of translation in *Escherichia coli* (*E. coli*) and UV-Vis detection purposes, respectively.

The **ELP-M-40**-encoding gene was obtained by recursive directional ligation[21] of the gene for **ELP-M-20**, which was obtained from a commercial source (See SI). After cloning, **ELP-M-20** and **ELP-M-40** were expressed in *E. coli* for 21 h after induction by isopropyl-β-D-thiogalactoside (IPTG) (Figure S1A). The ELPs were extracted from cell lysates, purified by

Inverse Transition Cycling (ITC),[22] dialyzed extensively against ultrapure water, and lyophilized to provide **ELP-M-20** and **ELP-M-40** in 65 and 150 mg/L culture yields, respectively (*ca*. 8 mol/L). The samples were found to be of satisfactory purity as assessed by SDS-PAGE (Figure S1B). The molecular weights of **ELP-M-20** and **ELP-M-40** (8,685.4 and 17,035.2 Da, respectively) were determined using MALDI mass spectrometry and found to be in excellent agreement with theoretical values (8,685.4 and 17,035.4 Da, respectively) (Figure S2). Both ELPs were characterized by 1D and 2D NMR spectroscopy (Figures S3 and S4).

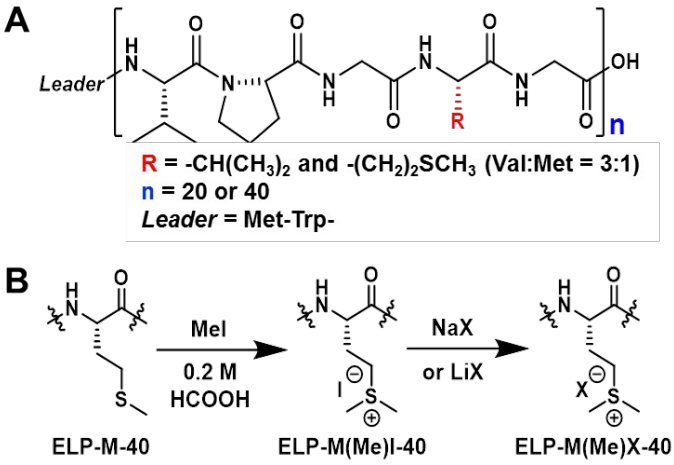


Figure 1. (A) General chemical structures of **ELP-M-20** (n=20) and **ELP-M-40** (n=40). (B) Synthetic scheme for methylation of **ELP-M-40** and counterion exchange. Iodide was replaced by different anions using sodium or lithium salts.

Counterion exchange.

With these new parent ELP samples in hand, we first sought to prepare methyl sulfonium derivatives of **ELP-M-20** and **ELP-M-40** as analogs of our previously reported methylated $(VPGMG)_{20}$ sample, to study the effects of Met sulfonium "dilution" along the ELP backbone on

thermoresponsive properties. The parent **ELP-M-20**, **ELP-M-40**, and $(VPGMG)_{20}$ samples all are thermoresponsive and possess *T*t values in water in the 25-45 °C temperature range. (Figure S5) It is important to note that although **ELP-M-20** and $(VPGMG)_{20}$ have identical numbers of ELP repeats and similar hydrophobicity within ELP repeat units, they display significantly different *T*t values due to the presence of additional *N*-terminal leader sequence residues, MGTELAAASEFTH, used in the recombinant production of $(VPGMG)_{20}$,[20] which contain both charged and apolar residues that likely affect the physico-chemical properties of this short ELP. Hence, only limited comparisons between **ELP-M-20** and $(VPGMG)_{20}$ samples and derivatives can be made. Within the new samples containing identical and short leader sequences, more direct comparisons can be made. In particular, the lower *T*t of **ELP-M-40** compared to **ELP-M-20** can be readily explained by its doubled molecular weight.

We previously reported that quantitative methylation of Met residues in $(VPGMG)_{20}$ led to a complete loss of the *T*t due to introduction of multiple hydrophilic sulfonium groups.[20] With **ELP-M-20**, **ELP-M-40**, we also observed complete loss of *T*t upon full methylation of Met groups, even though the sulfonium charge density on the chains was decreased. Because of their lower charge density, we hypothesized that thermoresponsive behavior of these methylated **ELP-M-20** or **ELP-M-40** ELPs might be obtained, and potentially tuned, by introduction of more hydrophobic counterions to these polymers. Counteranions are indeed known to greatly affect the solubility of polycations in water,[23,24] and can also alter the micellization properties of gemini surfactants.[25,26,27]

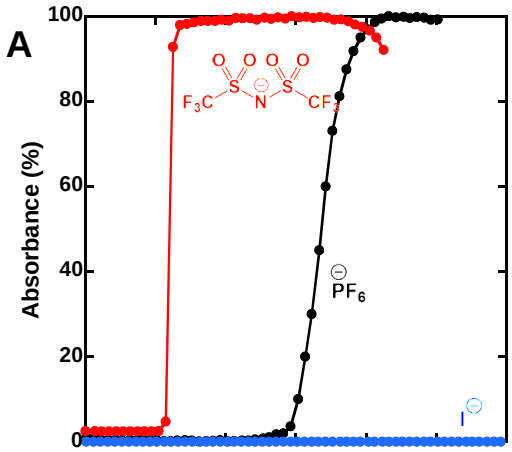
To study the effects of different counterions on thermoresponsive behavior, we focused on the fully methylated **ELP-M-40** sample, as the parent ELP possesses a lower *T*t than **ELP-M-20** due to its larger molecular weight (Figure S5). A sample of **ELP-M-40** was fully methylated using

established procedures[20] to give **ELP-M(Me)I-40**, where iodide served as the baseline counterion for comparisons (Figure 1B). The iodide counterions were then exchanged for six different counteranions of varying hydrophobicity using the corresponding sodium or lithium salts (NaX or LiX, Figure 1B). The series of anions studied included $(CF_3SO_2)_2N^-$ (NTf_2^-), PF_6^-, CF_3COO^- (Tfa^-), CH_3COO^- (Ac^-) as well as two chiral anions with opposite configurations: (R)-(+)-Mosher's carboxylate (RMC^-) and (S)-(-)-Mosher's carboxylate (SMC^-). Anion exchange was performed by dropwise addition of a solution of **ELP-M(Me)I-40** into an aqueous solution of the appropriate salt using 5 equiv. NaX or LiX per sulfonium ion. After overnight stirring followed by desalting, **ELP-M(Me)X-40** derivatives were lyophilized and analyzed using NMR spectroscopy to assess the degree of counterion exchange.

For fluorinated anions, ^{19}F NMR was used to qualitatively confirm the presence of these counterions in the products (Figures S6-S10). In addition, quantitative evidence for counterion exchange was obtained by using ^{1}H and ^{13}C NMR spectroscopy in D_2O and CD_3OD, respectively (Figures S10-S15). Deuterated methanol was preferred to D_2O for ^{13}C NMR spectra acquisition since it facilitated ion-pairing that led to shifted resonances for different counterions. For example, complete exchange from I^- to PF_6^- was confirmed by shifting resonances of the $-S^+(\underline{C}H_3)_2$ carbons in the ^{13}C NMR spectra of **ELP-M(Me)I-40** (25.56 ppm and 25.38 ppm) and **ELP-M(Me)PF_6-40** (25.48 ppm and 25.26 ppm) (Figure S13). The complete disappearance of one set of resonances, and appearance of only the new set confirmed quantitative counteranion exchange. For the **ELP-M(Me)RMC-40** and **ELP-M(Me)SMC-40** derivatives, complete anion exchange was confirmed using ^{1}H NMR by appearance of resonances for methoxy protons (3.5 ppm, 33 H) and phenyl protons (7.48, 33 H and 7.58 ppm, 22 H) from RMC^- and SMC^- anions

(Figure S15, S16). Since **ELP-M-40** contains 11 Met residues, the observed integrations matched expected values for quantitative exchange.

The effects of sulfonium counterions on thermoresponsive behavior were evaluated by cloud point measurements using light absorption at 600 nm, with **ELP-M(Me)X-40** samples solubilized at 2 mM in ultrapure water at 20 °C (Figure 2). The *T*t values were determined as the temperature corresponding to the onset of turbidity. The most significant effects were observed with the highly hydrophobic counterions NTf_2^- and PF_6^-, where strong thermal transitions were observed for both samples, showing that counterion exchange is able to restore robust thermoresponsive properties in these ELPs (Figure 2A). While **ELP-M(Me)I-40** possesses no measurable *T*t at 2 mM (*i.e.* *T*t > 80 °C), relatively low *T*t values were observed for **ELP-M(Me)NTf_2-40** and **ELP-M(Me)PF_6-40** (31 °C and 49 °C, respectively), following a trend of lower *T*t as hydrophobicity of the anions was increased.



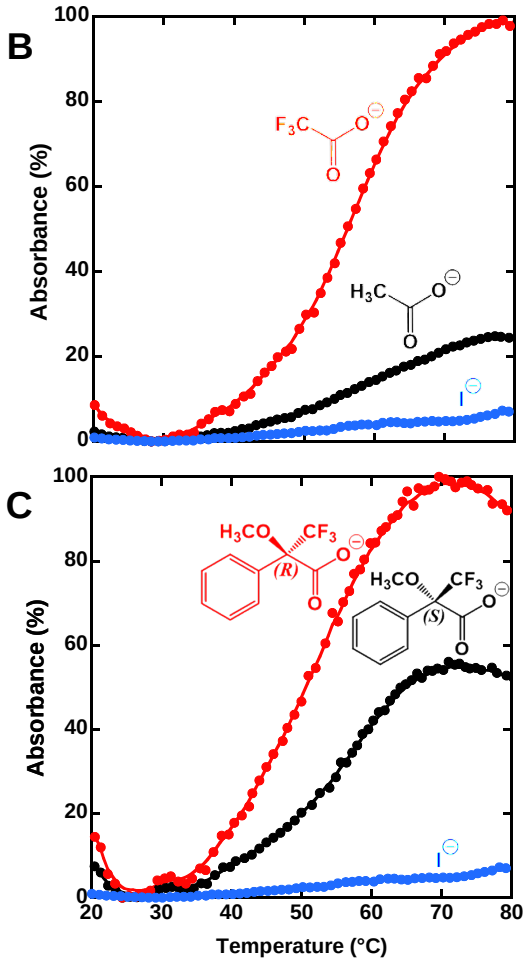


Figure 2. Normalized relative absorbance of 2.0 mM aqueous solutions of **ELP-M(Me)X-40** derivatives as functions of temperature. (A) **ELP-M(Me)NTf$_2$-40**, **ELP-M(Me)PF$_6$-40**, and **ELP-M(Me)I-40**. (B) **ELP-M(Me)Tfa-40**, **ELP-M(Me)Ac-40**, and **ELP-M(Me)I-40**. (C) **ELP-M(Me)RMC-40**, **ELP-M(Me)SMC-40**, and **ELP-M(Me)I-40**.

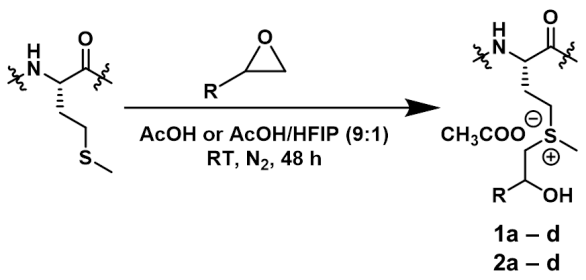
Substitution of **ELP-M(Me)I-40** with Tfa$^-$ and Ac$^-$ counterions resulted in samples with weak thermoresponsive properties, where *T*t of **ELP-M(Me)Tfa-40** and **ELP-M(Me)Ac-40** were identified around 34 °C and 39 °C, respectively (Figure 2B). Substitution of **ELP-M(Me)I-40** with the optically active anions RMC$^-$ and SMC$^-$, which present hydrophobic phenyl and trifluoromethyl groups, resulted in stronger thermoresponsive transitions compared to Tfa$^-$ and

Ac^-, and gave *T*t values around 35 °C (Figure 2C). However, only a slight difference in thermoresponsive behavior was observed for the opposite configurations of these counterions. Since no significant changes in ELP conformations (*i.e.*, relative content of random coils or type II β-turns secondary structures) was observed between **ELP-M-40**, **ELP-M(Me)I-40**, **ELP-M(Me)RMC-40**, and **ELP-M(Me)SMC-40** by circular dichroism (Figure S18), it may be that sulfonium/counterion pairs are too separated along the ELP backbone from each other to significantly affect the chain conformations in solution.

Introduction of functionality by thioether alkylation.

A separate objective of this work was to further extend the variety of functional groups that can be introduced at the Met side chains in ELPs *via* alkylation reactions.[20] A variety of epoxide alkylating agents were used for this purpose, which were preferred over the alkyl halides and triflates used previously since many functional epoxides are commercially available or easily synthesized, and epoxides are generally more easily handled than alkyl triflates.[28] **ELP-M-20** and **ELP-M-40** were reacted with a variety of functional epoxides in acidic conditions following procedures previously established by Deming *et al.* for poly(L-methionine).[28,29] Since Met residues in ELPs are less reactive than those in poly(L-methionine),[20] epoxides were used in excess (10 equiv. per Met residue), and added in two portions to limit epoxide hydrolysis. In order to minimize Met oxidation under these conditions, the reactions were run under inert N_2 atmosphere. Glacial acetic acid was used as solvent in the reactions with **ELP-M-20**. However, 10 v/v% HFIP in acetic acid was found to be necessary for complete alkylation of **ELP-M-40**, where the HFIP likely helped increase accessibility of Met groups (Figure 3).

A



B

R	Functionalization %	
	ELP-M-20	ELP-M-40
H_3C	1a, > 99	2a, > 99
O O 3	1b, 98	2b, 98
O	1c, > 99	2c, 97
O	1d, 96	2d, 96

Figure 3. (A) General reaction scheme of **ELP-M-n** with different epoxides. (B) Table summarizing **ELP-M-n** derivatives and their percent functionalization.

The extent of ELP alkylation from epoxide reactions was assessed by ^{1}H NMR spectroscopy. All spectra were calibrated using the unchanging resonances centered at 4.45 ppm, which correspond to the αC*H* protons of proline and the initial valine in each (***VP***GXG) repeat, integrating as 40 total protons in **ELP-M-20** and 80 total protons in **ELP-M-40**. Extent of ELP alkylation was calculated by integrating the resonance at *ca*. 3 ppm that corresponds to the Met sulfonium methyl group in the product. This resonance is highlighted in blue for the derivative **1c** (Figure 4A), and should integrate as 18 protons (6 Met sulfonium groups) upon complete alkylation of **ELP-M-20** (for **ELP-M**-40, this resonance will integrate as 33 protons for 11 Met sulfonium groups). Other resonances were also used to confirm degree of alkylation, such as the singlet at 4.3 ppm corresponding to the methylene group highlighted in red for derivative **1c** (Figure 4A), which integrates as 12 protons for **ELP-M-20** derivatives.

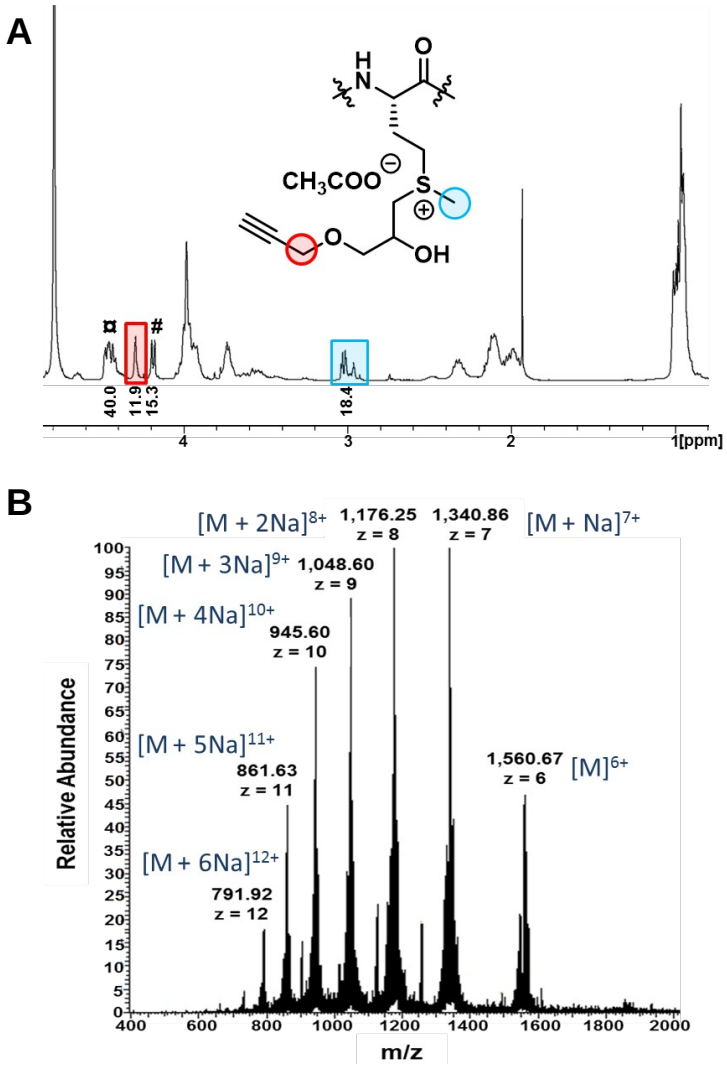


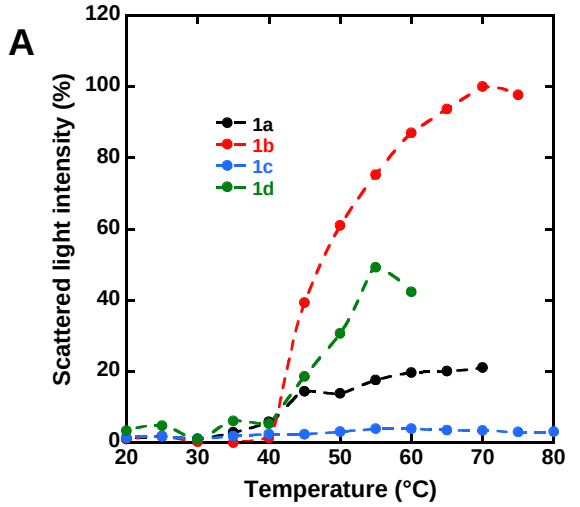
Figure 4. (A) Partial ^{1}H NMR spectrum of compound **1c** in D_2O. Resonance # corresponds to ***Val*** αC*H* in VPG***V***G repeat units, and resonance ¤ corresponds to ***Val*** αC*H* and ***Pro*** αC*H* of ***VP***GXG repeats. Resonance ¤ was used to calibrate the integrals. Red and blue areas = protons used to calculate the degree of functionalization. (B) ESI mass spectrum (positive ion mode) of compound **1c**. Theoretical mass of **1c**, M: 9,363.8 Da ($[M]^{6+}_{theo}$ = 1,560.63).

Four different functionalities were conjugated to the Met side chains of **ELP-M-20** and **ELP-M-40**, ranging from simple methyl groups, to oligo(ethyleneglycol) chains, as well as alkyne and

alkene groups that allow for further functionalization reactions. The structures of the different derivatives as well as percent functionalization are shown in Figure 3B. Excellent degrees of functionalization (> 95 %) were obtained for all compounds synthesized, with isolated yields ranging from 83 to 94%. Quantitative alkylation of all Met residues was achieved for compounds **1a**, **2a**, and **1c**. The slightly decreased degrees of alkylation obtained for compounds **1b**, **2b, 2c**, **1d** and **2d** were due to a small amount of Met oxidation into Met sulfoxides during the reaction, as evidenced by appearance of resonances at 2.7 ppm in their ^{1}H NMR spectra, which correspond to –S(O)CH_3 protons. Full ^{1}H NMR spectra of all derivatives are provided in SI (Figures 4A, S19-27). All compounds were also analyzed using ESI mass spectrometry, in positive ion mode, to confirm their molecular weights (Figure 4B, S28-35). The ESI mass spectra showed the characteristic charge state distribution corresponding to the desired polysulfonium product. For instance, compound **1c** was identified by the peak at m/z 1,560.67 Da corresponding to the m/z value of **1c** with a +6 charge (Figure 4B).

Since light scattering is more sensitive than light absorption for detection of aggregation phenomena when small particles are formed, solutions of all **ELP-M-20** and **ELP-M-40** derivatives were initially studied using dynamic light scattering (DLS). The scattered light intensity at 90 ° for 1 mM aqueous solutions of **1a-d** and **2a-d** was monitored over a temperature range of 20 °C to 80 °C. For each sample, the scattered light intensity in arbitrary units (a.u.) was plotted versus temperature (Figure 5), where an increase of scattered light identifying the onset of assembly. Most samples showed a transition at increased temperatures, with a wide range in degree of scattering response. Also, only irregular aggregates with a broad range of sizes were observed, consistent with a coacervation process. All the thermal transitions were found to be reversible. When the onset aggregation temperatures of alkylated ELP derivatives were

compared to that of the parent ELP, most derivatives were found to have higher *T*t values, which was likely due to the increased hydrophilicity of the sulfonium groups.



C

Sample	ELP-M-20	1a	1b	1c	1d
Tt (°C)	31	35	40	none	40
Sample	ELP-M-40	2a	2b	2c	2d
Tt (°C)	25	45	44	68	30

Figure 5. Scattered light intensity of (A) **ELP-M-20** derivatives **1a-d,** and (B) **ELP-M-40** derivatives **2a-d** as functions of temperature at 1 mM in ultrapure water. (C) Table summarizing the onset temperature of aggregation (*T*t) of the different derivatives at 1 mM in water.

Notably, the 2-hydroxypropyl derivatives **1a** and **2a** were found to have *T*t values at relatively low temperatures, especially when compared to the methyl sulfonium derivative **ELP-M(Me)Ac-40** reported above. The epoxide-derived polysulfonium ELPs, although they show limited aggregation, do appear to be able to retain thermoresponsive properties after Met alkylation better than simple alkyl derivatives.

The derivatives **1c** and **2c**, which contain pendant alkyne groups, showed the least aggregation, with only **2c** showing some increased scattering above *ca*. 68 °C. This result was surprising since the related alkene derivatives **1d** and **2d** showed substantially lower aggregation onset temperatures (in the range 30 to 40 °C). This behavior may be due to the increased polarizability of the alkyne groups. We also observed that the *T*t values for **ELP-M-40** derivatives **2a** and **2b** were higher than those for the analogous **ELP-M-20** derivatives **1a** and **1b**, which was surprising since higher molecular weight ELPs generally possess lower *T*t values.

Samples **1b** and **2b** were found to give the greatest phase separation above *T*t, and so the thermal transition of **2b** could be quantified in more detail by absorbance measurements at 600 nm. The *T*t values for **2b** were measured in the concentration range of 250-1,500 μM (Figure 6A), and compared to *T*t values for the unmodified **ELP-M-40** (Figure 6B). Due to the charged sulfonium groups and the hydrophilic ethylene oxide chains, the *T*t values of **2b** (42 °C to 51 °C) were greater than those of **ELP-M-40** (25 °C to 32 °C). In addition, while a *T*t for **ELP-M-40** could be measured at a concentration as low as 10 μM, 250 μM was the lowest concentration at which a *T*t for **2b** could be detected. For both samples, plots of *T*t versus concentration were fit using the empirical equation established by Chilkoti and coworkers[10] (eq. 1), and allowed accurate estimation of *T*t values at arbitrary concentrations (Figure 6B). Similar to our previous observations with sulfoxide and sulfone derivatives of **ELP-M-40**,[30] the slope of the fit for **2b**

was steeper than the one for **ELP-M-40**. This trend is similar to that found for the increased hydrophilicity of ELPs as chain length is decreased as described by Chilkoti *et al.*[10]

$$T_t = T_{t,c} + \frac{k}{L} \ln\left(\frac{C_C}{C}\right) \qquad (1)$$

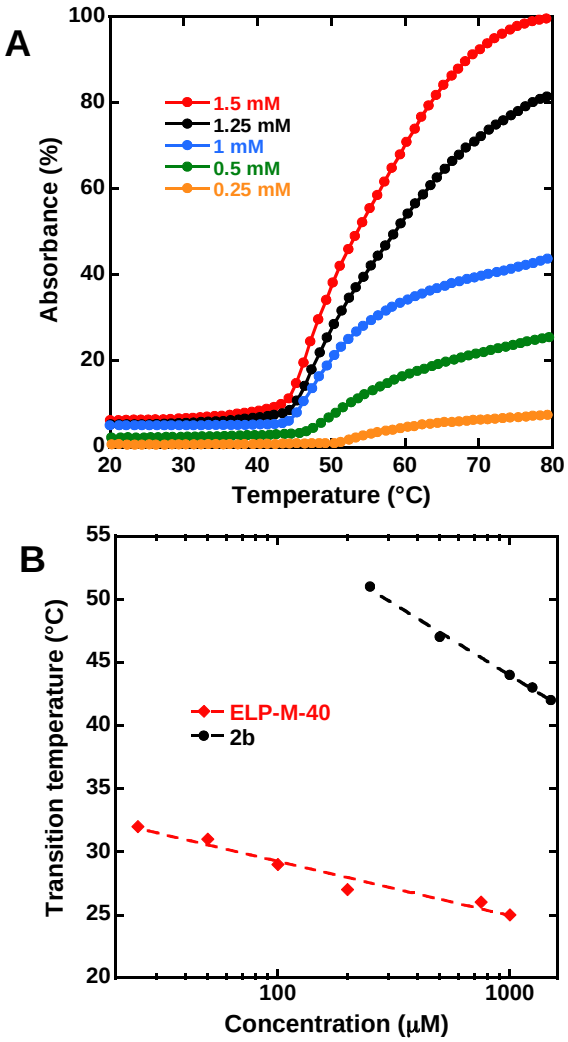
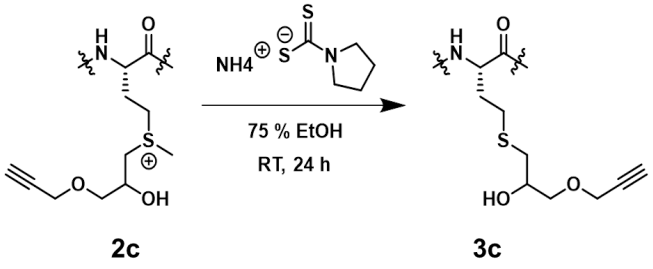


Figure 6. (A) Plots of absorbance at 600 nm for sample **2b** at different concentrations in water. (B) *T*t values of **ELP-M-40** and sample **2b** as functions of concentration. The dotted lines correspond to the fits of the data using **equation 1**.

In effort to distinguish the roles of the sulfonium charges from introduced functionality on the thermoresponsive properties in ELPs modified by alkylation with epoxides, we attempted to demethylate the sulfonium groups in **2c** using ammonium pyrrolidinedithiocarbamate (APDC) in 75% ethanol (eq. 2).[31] This transformation to create S-alkyl-L-homocysteine residues from Met sulfoniums was optimized and demonstrated on structurally related poly(L-methionine) sulfoniums by Deming *et al.*[31] Using these conditions, the **ELP-M-40** derivative **2c** was found to be efficiently demethylated to give exclusively the S-alkyl-L-homocysteine containing product **3c** as confirmed by ^{1}H NMR analysis (Figure S36) and ESI mass spectrometry (Figure S37). The thermoresponsive properties of **3c** were measured using DLS at 1 mM in ultrapure water and it was found to have a strong *LCST* transition at 30 °C (Figure S38). This *T*t was much lower than that found for **2c** (68 °C), and only slightly higher than the parent **ELP-M-40** (25 °C). These data show that the multiple charged sulfonium groups, not surprisingly, substantially affect ELP *T*t values, and that functional substitutions without charge, such as the introduction of alkyne groups shown here, can be made on ELPs with only limited perturbation of thermoresponsive properties.



(2)

Conclusion

New ELP sequences with precisely positioned Met residues were synthesized and obtained in high purity. The Met residues were used as post-synthesis functionalization sites, *via* chemoselective alkylation reactions, to modify thermoresponsive solution properties of the ELPs. With this system, we found that the resulting polysulfonium ELPs could retain *LCST* behavior through suitable choice of sulfonium counterions or alkylating functional groups. Subsequent demethylation of Met sulfonium groups was also found to yield S-alkyl-L-homocysteine containing ELPs with robust thermoresponsive properties. Overall, our results show that the thermoresponsive properties of Met containing ELP sequences can be finely adjusted by a number of post-synthesis modifications, allowing the preparation of a wide variety of ELP derivatives, each with distinct properties, from single precursor sequences.

Experimental procedures

Materials.

LB medium was purchased from Sigma-Aldrich (FR). Bacto-tryptone, and yeast extract were purchased from Biokar Diagnostics (FR). Ampicillin was obtained from Eurobio (FR). Glycerol and isopropyl β-D-thiogalactopyranoside (IPTG) were purchased from Euromedex (FR). Complete mini EDTA-free protease inhibitors were purchased from Roche Diagnostics (D). Bis(trifluoromethane)sulfonamide lithium salt was purchased from TCI Europe (BEL). Sodium acetate and sodium trifluoroacetate were purchased from Sigma-Aldrich (FR). Sodium hexafluorophosphate, (R)-(+)-α-methoxy-α-(trifluoromethyl)phenylacetic acid (R-Mosher's acid)

and (S)-(-)-α-methoxy-α-(trifluoromethyl)phenylacetic acid (S-Mosher's acid) were obtained from Arcos Organics (FR). All epoxides were obtained from Sigma-Aldrich (USA or Saint-Quentin-Fallavier, FR) except 2-(2,5,8,11-tetraoxadodecyl)oxirane (EG_3-epoxide) which was synthesized. Glacial acetic acid was obtained from Fisher Scientific (USA) or Sigma-Aldrich (FR). HFIP was obtained from Sigma-Aldrich (USA) or TCI Europe (BEL). Deionized water (18 MΩ-cm) was obtained by passing in-house deionized water through a Millipore Milli-Q Biocel A10 purification unit.

Construction of the expression vector.

A synthetic gene corresponding to the MW[VPGVGVPGMG$(VPGVG)_2]_5$ sequence (**ELP-M-20**) was designed and purchased from Eurofins (Ebersberg, GE). The gene sequence was selected according to *E. coli* codon usage while minimizing sequence repetition. The DNA fragment was extracted from the pEX-A plasmid by a double digestion with *Eco*RI and *Hind*III, and was ligated with the Quick ligation™ kit into similarly digested and dephosphorylated pUC19. After transformation into NEB 5α-F'Iq *E. coli* competent cells, a positive clone was selected by colony PCR with OneTaq® hot start DNA polymerase and verified by DNA sequencing. The sequence coding for MW[VPGVGVPGMG$(VPGVG)_2]_{10}$ (**ELP-M-40**) was obtained by using a variation of the recursive directional ligation method,[21] as described previously.[32] Cloning in the expression vector was performed as follows: The ELP sequence was extracted from pUC19 by a double digestion with *Nde*I and *Bam*HI, and ligated with the Quick ligation™ kit into similarly digested and dephosphorylated pET-44a(þ) plasmid. The different ligation products were then used to transform *E. coli* BLR(DE3)-competent cells for production. The sequence of the

resulting plasmid was confirmed by DNA sequencing. The sequences of the **ELP-M-40** gene and of the corresponding protein are shown below.

```
atgtgggttccaggcgttggagtgccaggcatgggcgtaccaggtgtgggagttccaggt
 M  W  V  P  G  V  G  V  P  G  M  G  V  P  G  V  G  V  P  G
gttggggtaccgggcgtcggagttcctgggatgggagttccgggagttggtgtgccgggt
 V  G  V  P  G  V  G  V  P  G  M  G  V  P  G  V  G  V  P  G
gtcggtgtgcctggggtgggtgttccaggtatgggggttccgggtgtcggcgttcccggc
 V  G  V  P  G  V  G  V  P  G  M  G  V  P  G  V  G  V  P  G
gttggtgttccaggcgtaggtgtgccgggaatgggggttccgggagttggtgtacctggc
 V  G  V  P  G  V  G  V  P  G  M  G  V  P  G  V  G  V  P  G
gtgggagtacctggagtcggcgtgcctggtatgggcgtgcctggcgtcggcgtacctggc
 V  G  V  P  G  V  G  V  P  G  M  G  V  P  G  V  G  V  P  G
gtaggtgttccaggcgttggagtgccaggcatgggcgtaccaggtgtgggagttccaggt
 V  G  V  P  G  V  G  V  P  G  M  G  V  P  G  V  G  V  P  G
gttggggtaccgggcgtcggagttcctgggatgggagttccgggagttggtgtgccgggt
 V  G  V  P  G  V  G  V  P  G  M  G  V  P  G  V  G  V  P  G
gtcggtgtgcctggggtgggtgttccaggtatgggggttccgggtgtcggcgttcccggc
 V  G  V  P  G  V  G  V  P  G  M  G  V  P  G  V  G  V  P  G
gttggtgttccaggcgtaggtgtgccgggaatgggggttccgggagttggtgtacctggc
 V  G  V  P  G  V  G  V  P  G  M  G  V  P  G  V  G  V  P  G
gtgggagtacctggagtcggcgtgcctggtatgggcgtgcctggcgtcggcgtacctggc
 V  G  V  P  G  V  G  V  P  G  M  G  V  P  G  V  G  V  P  G
gtaggttaa
 V  G  -
```

Bioproduction of recombinant ELP-M-20 and ELP-M-40 (same procedure used for both ELPs).

A single bacterial colony was selected and cultured overnight at 37 °C on a rotary shaker at 200 rpm in 50 mL rich LB medium (1% bacto-tryptone, 0.5% NaCl, 1% yeast extract) containing 100 $\mu g \cdot mL^{-1}$ ampicillin. The seed culture was inoculated into 0.95 L rich LB medium supplemented with glycerol (1 $g.L^{-1}$) and ampicillin (100 $\mu g.mL^{-1}$), and bacteria were cultivated at 37 °C in 5 L flasks. When the optical density at 600 nm (OD_{600}) reached the value of 0.8, IPTG was added to a final concentration of 0.5 mM and the temperature of cultivation was decreased to 25 °C. Samples were then collected every hour for measurement of OD_{600}.

Isolation and purification of recombinant ELP-M-20 and ELP-M-40 (same procedure used for both ELPs).

After 21 h IPTG-induction, the culture was harvested by centrifugation at 7,500 g and 4 °C for 15 min. The cell pellet was resuspended with 10 $mL \cdot g^{-1}$ (wet weight) phosphate buffer (PBS; NaCl 137 mM, KCl 2.7 mM, Na_2HPO_4 8 mM, KH_2PO_4 2 mM, pH 7.4) supplemented with one tablet/10 mL of Complete mini EDTA-free protease inhibitors. The mixture was incubated overnight at -80 °C and defrosted by incubation at 4 °C. Cell lysis was completed by sonication at 15 °C with sequential 4 sec-pulses at 125 W separated by 9 sec-resting time periods for a total duration of 15 min. Insoluble debris were removed by centrifugation at 16,000 g and 4 °C for 30 min. The cleared lysate was thereafter subjected to three successive rounds of *Inverse Transition Cycling* (ITC).[22] The ELP was precipitated with NaCl and retrieved by centrifugation at 16,000 g and 25 °C for 30 min ("warm spin"). After removal of soluble proteins in the supernatant, the ELP-containing pellet was resuspended in cold PBS. Insoluble, heat denatured proteins from *E. coli* were eliminated in the pellet after centrifugation at 16,000 g and 4 °C for 15 min ("cold spin"), while the ELP-containing supernatant was subjected to an additional ITC round. The soluble ELP was then extensively dialyzed against ultrapure water at 4 °C using 1 kDa MWCO-dialysis tubing (Spectra Por7) and lyophilized. The purity and average MW of the ELP were assessed by SDS-PAGE using 15% TRIS-glycine gels stained with colloidal blue G250.

Synthesis of ELP-M(Me)-40 and counterion exchange.

Synthesis of ELP-M(Me)I-40

MW-$[(VPGVG)(VPGMG)(VPGVG)_2]_{10}$ (**ELP-M-40**) was dissolved in 0.2 M aqueous formic acid (20 mg/mL). Iodomethane (15 equiv. per Met residue) was added as a solution in THF (50

mg/mL). The reaction was sealed, covered with foil, and stirred for 4 days at room temperature. Diethyl ether (equal to about half the reaction volume) was added to the reaction to extract the excess alkyl halide. The biphasic reaction was vortexed briefly and then allowed to sit until separation of the phases. The ether layer was pipetted off and discarded. The reaction mixture was transferred to a 3,000 MWCO ultra-centrifugal filter tube and purified with 40 mL DI water. The contents of the cartridge were then lyophilized to provide the modified ELP as a white solid.

Counterion exchange (general procedure)

The **ELP-M(Me)I-40** previously synthesized was suspended in water (ca. 50 mg in 1 mL) and added dropwise to a solution of the desired salt (NaX or LiX, 5 equiv. per sulfonium, ca. 40 mg in 400 µL). The mixture was shaken overnight at room temperature, or for the most hydrophobic counterions at 10 °C. The solution was transferred to a 3,000 MWCO ultra-centrifugal filter tube and purified with 20 mL DI water to remove the excess salt. The contents of the cartridge were then added dropwise to a new solution of the desired salt (5 equiv. per sulfonium, *ca*. 40 mg in 400 µL) and stirred overnight. The solution was then purified with 40 mL DI water using a 3,000 MWCO ultra-centrifugal filter tube. The contents of the cartridge were then lyophilized to provide the modified ELP as a white solid.

Modification of ELP-M-20 and ELP-M-40 using epoxides (general procedures).

Procedure A

MW-[(VPGVG)(VPGMG)$(VPGVG)_2]_5$ (**ELP-M-20**) was dissolved in glacial acetic acid (*ca*. 2.5 mL, 20 mg/mL) and the solution was degassed by bubbling N_2 into the solution for 1 hr and then stirring under N_2. The epoxide was added (10 equiv. per methionine residue) and the mixture was

stirred for 48 hr under N_2 at room temperature. The reaction mixture was transferred to a 3,000 MWCO ultra-centrifugal filter tube and washed with 40 mL DI water. The contents of the cartridge were then lyophilized to provide the modified ELP as a white solid.

Procedure B

MW-$[(VPGVG)(VPGMG)(VPGVG)_2]_{10}$ (**ELP-M-40**) was dissolved in an AcOH/HFIP mixture (9/1, v/v) (*ca*. 2.5 mL, 20 mg/mL) and the solution was degassed by bubbling N_2 into the solution for 1 hr, followed by stirring under N_2. The epoxide was added (10 equiv. per methionine residue) and the mixture was stirred for 48 hr under N_2 at room temperature. The reaction mixture was transferred to a 3,000 MWCO ultra-centrifugal filter tube and washed with 40 mL DI water. The contents of the cartridge were then lyophilized to provide the modified ELP as a white solid.

Synthesis of 2-(2,5,8,11-tetraoxadodecyl)oxirane (EG_3-epoxide)

Triethylene glycol monomethyl ether (1 equiv.) and water (1 mL) were mixed together and stirred on ice before addition of NaOH (2.95 equiv.) and 0.4 M tetrabutylammonium hydroxide (aq) (0.05 equiv.). Once the mixture had cooled to 0 °C, epichlorohydrin (2.95 equiv.) was added portion-wise over 3 min. The mixture was stirred at room temperature for 16 hours. H_2O (15 mL) was then added and the mixture was extracted with EtOAc (4x 30 mL). The combined extracts were washed with brine (30 mL) and dried over Na_2SO_4. The extracts were concentrated under reduced pressure. The residue was distilled under vacuum, providing EG_3-epoxide as a colorless liquid boiling at 110-117 °C (0.1 mmHg). ^1H NMR (300 MHz, $CDCl_3$, 25 °C): δ 3.80-3.44 (br m,

14 H, C*H*$_2$O(C*H*$_2$C*H*$_2$O)$_3$), 3.37 (s, 3 H, OCH$_3$), 3.15 (m, 1 H, CHb), 2.79 (dd, J = 6.6, 5.6 Hz, 1 H, CHa), 2.61 (dd, J = 6.7, 3.6 Hz, 1 H, CHa').

Details of compounds 1a-d and 2a-d.

ELP-M(2-hydroxypropyl)-20, 1a

1a was prepared from **ELP-M-20** and **propylene oxide** using **Procedure A**.

^{1}H NMR (400 MHz, D_2O, 25 °C): (main peaks): δ 4.5-4.4 (m, 40 H, αC*H* *V*PGXG and αC*H* V*P*GXG), 4.2-4.15 (d, 15 H, αC*H* VPG*V*G), 3.04-2.9 (m, 18 H, SC*H*$_3$), 1.4-1.35 (d, 18 H, CHC*H*$_3$ Met), 1.00-0.75 (br m, 210 H, C*H*$_3$ Val)

MS-ESI: Theoretical MW = 9,039.7 Da, Experimental $[M_6 + Na - H]^{6+}$= 1,510.2 Da

ELP-M(EG$_3$)-20, 1b

1b was prepared from **ELP-M-20** and **EG$_3$-epoxide** using **Procedure A**.

^{1}H NMR (400 MHz, D_2O, 25 °C): (main peaks): δ 4.5-4.4 (m, 40 H, αC*H* *V*PGXG and αC*H* V*P*GXG), 4.2-4.15 (d, 15 H, αC*H* VPG*V*G), 3.40 (s, 18 H, OC*H*$_3$ Met), 3.06-2.9 (m, 18 H, SC*H*$_3$), 1.00-0.75 (br m, 210 H, C*H*$_3$ Val)

MS-ESI: Theoretical MW = 10,012.2 Da, Experimental $[M_6 + Na - H]^{6+}$= 1,672.6 Da

ELP-M(alkyne)-20, 1c

1c was prepared from **ELP-M-20** and **glycidyl propargyl ether** using **Procedure A**.

^{1}H NMR (400 MHz, D_2O, 25 °C): (main peaks): δ 4.5-4.4 (m, 40 H, αC*H* *V*PGXG and αC*H* V*P*GXG), 4.3 (s, 12 H, OC*H*$_2$CCH Met) 4.2-4.15 (d, 15 H, αC*H* VPG*V*G), 3.06-2.9 (m, 18 H, SC*H*$_3$), 1.00-0.75 (br m, 210 H, C*H*$_3$ Val)

MS-ESI: Theoretical MW = 9,363.8 Da, Experimental $[M_6]^{6+}$= 1,560.7 Da

ELP-M(alkene)-20, 1d

1d was prepared from **ELP-M-20** and **allyl glycidyl ether** using **Procedure A**.

^{1}H NMR (400 MHz, D_2O, 25 °C): (main peaks): δ 6-5.9 (m, 6 H, OCH_2CHCH_2 Met), 5.4-5.2 (m, 12 H, SCH_2CHCH_2), 4.5-4.4 (m, 40 H, αC*H V*PGXG and αC*H* V*P*GXG), 4.2-4.15 (d, 15 H, αC*H* VPG*V*G), 4.15-4.05 (d, 12 H, OCH_2CHCH_2 Met), 3.06-2.9 (m, 18 H, SCH_3), 1.00-0.75 (br m, 210 H, CH_3 Val)

MS-ESI: Theoretical MW = 9,375.9 Da, Experimental $[M_6+H]^{7+}$= 1,339.5 Da

ELP-M(2-hydroxypropyl)-40, 2a

2a was prepared from **ELP-M-40** and **propylene oxide** using **Procedure B**.

^{1}H NMR (400 MHz, D_2O, 25 °C): (main peaks): δ 4.5-4.4 (m, 80 H, αC*H V*PGXG and αC*H* V*P*GXG), 4.2-4.15 (d, 30 H, αC*H* VPG*V*G), 3.04-2.9 (m, 33 H, SCH_3), 1.4-1.35 (d, 33 H, $CHCH_3$ Met), 1.00-0.75 (br m, 420 H, CH_3 Val)

MS-ESI: Theoretical MW = 17,684.9 Da, Experimental $[M_{11}]^{11+}$ = 1,607.4 Da

ELP-M(EG_3)-40, 2b

2b was prepared from **ELP-M-40** and **EG_3-epoxide** using **Procedure B**.

^{1}H NMR (400 MHz, D_2O, 25 °C): (main peaks): δ 4.5-4.4 (m, 80 H, αC*H V*PGXG and αC*H* V*P*GXG), 4.2-4.15 (d, 30 H, αC*H* VPG*V*G), 3.40 (s, 33 H, OCH_3 Met), 3.06-2.9 (m, 33 H, SCH_3), 1.00-0.75 (br m, 420 H, CH_3 Val)

MS-ESI: Theoretical MW = 19,467.9 Da, Experimental $[M_{10} + H]^{11+}$ = 1,748.9 Da

ELP-M(alkyne)-40, 2c

2c was prepared from **ELP-M-40** and **glycidyl propargyl ether** using **Procedure B**.

^{1}H NMR (400 MHz, D_2O, 25 °C): (main peaks): δ 4.5-4.4 (m, 80 H, αC*H V*PGXG and αC*H* V*P*GXG), 4.3 (s, 22 H, OCH_2CCH Met) 4.2-4.15 (d, 30 H, αC*H* VPG*V*G), 3.06-2.9 (m, 33 H, SCH_3), 1.00-0.75 (br m, 420 H, CH_3 Val)

MS-ESI: Theoretical MW = 18,279.1 Da, Experimental $[M_{11}]^{11+}$ = 1,661.8 Da

ELP-M(alkene)-40, 2d

2d was prepared from **ELP-M-40** and **allyl glycidyl ether** using **Procedure B**.

^{1}H NMR (400 MHz, D_2O, 25 °C): (main peaks): δ 6-5.9 (m, 11 H, OCH_2CHCH_2 Met), 5.4-5.25 (m, 22 H, SCH_2CHCH_2), 4.5-4.4 (m, 80 H, αC*H V*PGXG and αC*H* V*P*GXG), 4.2-4.15 (d, 30 H, αC*H* VPG*V*G), 3.05-2.9 (m, 33 H, SCH_3), 1.05-0.9 (br m, 420 H, CH_3 Val)

MS-ESI: Theoretical MW = 18,301.3 Da, Experimental $[M_{10} + 6H]^{16+}$= 1,137.3 Da

*Demethylation of compound **2c**.*[31]

2c was dissolved in 75% EtOH(aq) at a 10 mM concentration and then treated with APDC (5.0 eq per Met residue). The solution was briefly flushed with a stream of N_2 and rapidly capped. The reaction mixture was vortexed until homogenous, and then allowed to stand for 24h at room temperature. The reaction mixture was transferred to a 1 kDa MWCO dialysis bag and dialyzed against 50% MeOH(aq) during 24h with 3 solvent changes followed by 8h dialysis against DI water with 3 changes. The dialysis bag contents were then lyophilized, to provide compound **3c**.

^{1}H NMR (400 MHz, D_2O, 25 °C): (main peaks): δ 4.5-4.4 (m, 80 H, αC*H V*PGXG and αC*H* V*P*GXG), 4.25 (s, 22 H, OCH_2CCH Met) 4.2-4.15 (d, 30 H, αC*H* VPG*V*G), 2.8-2.55 (m, 44 H, CH_2SCH_2), 1.00-0.75 (br m, 420 H, CH_3 Val)

MS-ESI: Theoretical MW = 18,113.8 Da, Experimental $[M_{11} + 2H]^{13+}$= 1,394.7 Da $[M_{11} + 3Na]^{14+}$= 1,298.2 Da

*Mass spectrometry analysis of **ELP-M-20** and **ELP-M-40**.*

Mass spectrometry analyses were performed on a MALDI-ToF-ToF (Ultraflex III, Bruker Daltonics, Bremen, Germany) equipped with a SmartBeam laser (Nd:YAG, 355nm). Solutions of ELPs were prepared as follows: lyophilized ELPs were resuspended in water/acetonitrile (1/1, v/v) to obtain a final concentration lower than 100 µM. Samples were then mixed with the matrix solution of sinapinic acid prepared at the concentration of 10 mg/mL in water/acetonitrile (1/1, v/v). All MALDI-MS measurements were acquired in the linear positive mode and a mixture of standard proteins was used for external calibration in the suitable mass range (10-20 kDa).

*Mass spectrometry analysis of compounds **1a-d** and **2a-d**.*

Mass spectrometry analysis was performed on a ESI-Q-TOF (Q-TOF Premier, Waters, Manchester, UK). All solvents used were HPLC grade. Lyophilized compounds were resuspended in DMSO and then diluted in H_2O/MeOH (1:1 v/v). The solution was diluted in methanol/0.1% aqueous formic acid (1:1 v/v) to a final concentration of around 10 pmol/µL and infused into the electrospray ionization source at a flow rate of 10 µL/min. The mass spectrometer was operated in the positive mode with external calibration performed with a solution of the standard protein apomyoglobin at a concentration of 1 pmol/µL in acetonitrile/0.1% aqueous formic acid (1:1 v/v).

*NMR spectroscopy analysis of **ELP-M-20**, **ELP-M-40**, compounds **1a-d** and compounds **2a-d**.*

NMR spectra were acquired in D_2O at 283 K (**ELP-M-40**) or 298 K (**ELP-M-20** and all derivatives) either on a Bruker AV400 NMR spectrometer (UCLA) operating at 400 mHz or a Bruker AV800 NMR spectrometer (NMR platform of Institut Européen de Chimie et Biologie) operating at 800 MHz. The solvent signal was used as the reference signal (δ = 4.70 ppm). Data

processing was performed using Topspin software. Chemical shifts of amino acids are well known in the literature.[33,34] We have identified the CHα and of the proline (*V*PGXG) at 4.5-4.4 ppm (60.5-57 ppm for ^{13}C) and used it as reference for the calibration of integrations. Full assignment of **ELP-M-40** (Figure S1) was done with the help of the COSY and HSQC spectra.

*Transition temperature measurements of **ELP-M(Me)X-40** (X = I, Ac, Tfa, RMC, SMC, PF_6, NTf_2).*

Transition temperatures (*T*t) were determined by measuring the turbidity at 600 nm between 20 and 80 °C at a 1 °C/min scan rate at concentrations of 2 mM in DI water. Data were collected on a Cary 100 UV−Vis spectrophotometer equipped with a multi-cell thermoelectric temperature controller from Agilent Technologies (Les Ulis, FR). The *T*t is defined as the temperature corresponding to the point where the absorbance starts to increase in the absorbance versus temperature plot.

*Transition temperature measurements of compounds **1a-d** and **2a-d**.*

Dynamic light scattering (DLS) measurements were performed on a NanoZS instrument (Malvern, U.K.) at a 90 ° angle at a constant position in the cuvette (constant scattering volume). Solutions of compounds **1a-d** and **2a-c** were prepared at 1 mM concentration in ultrapure water. Three independent measurements of fifteen 10 s-runs were recorded and averaged. Temperature was raised from 20 to 80 °C and measurements were performed every 5 degrees after a 2 min-temperature equilibration time. The derived count rate (DCR) was defined as the mean scattered intensity normalized by the attenuation factor. The DCR was plotted against temperature and the

*T*t is defined as the temperature corresponding to the point where the DCR starts increasing on this plot.

ASSOCIATED CONTENT

Supporting Information. Additional figures and spectral data. This material is available free of charge via the Internet at http://pubs.acs.org

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The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

We declare no competing financial interests.

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