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THÈSE PRÉSENTÉE
POUR OBTENIR LE GRADE DE
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SPÉCIALITÉ : POLYMERES

Par **Rosine PETITDEMANGE**

**Chemoselective modifications of recombinant
elastin-like polypeptides:
Tuning thermosensitivity and bioactivity**

Sous la direction de : Sébastien Lecommandoux

Soutenue le 12 décembre 2016

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TITRE ET RESUME EN FRANÇAIS

MODIFICATIONS CHIMIOSELECTIVES DE POLYPEPTIDES RECOMBINANT A BASE DE MOTIFS ELASTINE : MODULATION DE LA THERMOSENSIBILITE ET DE L'ACTIVITE BIOLOGIQUE

Au cours des dernières décennies, l'usage de polymères pour les applications biomédicales a largement impacté le développement de la médecine moderne. Les polymères biodégradables, tels que les polyesters par exemple, ont suscité un intérêt particulier du fait de leur capacité à se décomposer et à être métabolisé ou excrété une fois leur fonction accomplie. D'autres polymères, non biodégradables mais bio-éliminables tel que le poly(ethylene glycol), ont quant à eux également été l'objet de nombreuses études. Dans le domaine des applications biomédicales ces polymères, qu'ils soient d'origine naturelle ou synthétique, sont avant tout intéressants en raison de leur biocompatibilité. Les études concernant ces polymères tournent principalement autour de trois types d'applications : les biomatériaux, l'ingénierie tissulaire et les systèmes de vectorisation de principes actifs. Actuellement les scientifiques cherchent en outre à conférer une fonction bioactive à ces polymères en les conjuguant à des groupements biofonctionnels tels que des peptides, des acides nucléiques, des anticorps, des saccharides, *etc*, et ce afin d'améliorer leur capacité d'adhésion cellulaire ou de ciblage.

Dans ce contexte, notre but est d'introduire à l'échelle macromoléculaire des groupements fonctionnels spécifiques qui permettront par la suite des modifications chimiques post-polymérisation et notamment l'introduction de fonctions bioactives. Pour cela il est nécessaire de contrôler la séquence de monomères afin d'obtenir une structure macromoléculaire précise qui pourra ensuite être modifiée via des réactions chimiques bioorthogonales.

Par conséquent, dans le cadre de ce projet de thèse nous nous sommes intéressés plus particulièrement aux polypeptides recombinants à base de motifs élastine, appelés « elastin-like polypeptides » (ELPs). Ces polypeptides dérivés de l'élastine, une protéine omniprésente dans le corps humain au niveau des tissus conjonctifs (peau, artères, ligaments, etc), sont produits de manière recombinante. Cette méthode de production basée sur le design d'un gène codant directement pour la protéine désirée permet d'accéder à un composé parfaitement monodisperse avec une structure primaire contrôlée et ce de manière reproductible d'une production à l'autre. Ces techniques de production par ingénierie des protéines permettent également d'obtenir une large variété de

polypeptides de fortes masses molaires non accessibles par d'autres méthodes telles que la synthèse sur support solide. Les ELPs sont constitués de séquence de répétition du pentapeptide [Val-Pro-Gly-Xaa-Gly], le résidu hôte Xaa pouvant être n'importe quel acide aminé excepté la proline. Les ELPs ont une propriété particulière appelée température de transition (T_t), c'est-à-dire que pour une concentration donnée, en dessous de cette température l'ELP est soluble en solution aqueuse alors qu'au-dessus il s'agrège. Cette température de transition peut-être un moyen de contrôler le phénomène d'auto-assemblage et elle permet également d'isoler et de purifier le polypeptide après la phase de production. La T_t peut être modulée étant donné qu'elle dépend de différents paramètres. Des paramètres environnementaux tels que la concentration de la solution ou la présence de co-solutés, ou bien des paramètres macromoléculaires tels que la masse molaire ou la nature du résidu hôte. En effet, plus la masse molaire de l'ELP augmente plus la T_t diminue, et plus le résidu hôte est hydrophobe plus la T_t sera basse. L'équipe de Chilkoti a effectivement montré que la T_t peut être modulée de manière précise en faisant varier ces deux paramètres macromoléculaires permettant ainsi de balayer une large gamme de températures. Cependant faire varier la T_t de cette manière reste relativement complexe car cela implique de passer par des étapes fastidieuses de biologie moléculaire afin de concevoir un nouveau gène pour chaque nouvelle structure d'ELP souhaité.

Par conséquent, il est nécessaire de faire appel à d'autres méthodes pour moduler la T_t de manière plus modulable, par exemple en post-modifiant précisément ces ELPs *via* des réactions chimiques bioorthogonales. Ces modifications permettraient d'introduire différents groupements sur la chaîne d'ELP ce qui pourrait engendrer un changement de T_t . L'introduction orthogonale de groupements fonctionnels pourrait également être envisagée afin d'accéder à des structures potentiellement bioactives. Jusqu'à présent les ELPs ont majoritairement été modifiés chimiquement au niveau de leurs bouts de chaîne. Chilkoti a par exemple conjugué un oligonucléotide au N-terminus d'un ELP, alors que le groupe de van Hest a couplé un PEG et un ELP *via* leur extrémité de chaîne. Dans notre cas, nous cherchons à modifier les ELPs en ciblant précisément des acides aminés fonctionnalisables qui auront été préalablement introduits en tant que résidus hôtes tout en évitant d'affecter les bouts de chaîne. Pour cela, il nous faut choisir comme résidu hôte un acide aminé qui soit fonctionnalisable de manière sélective vis-à-vis des fonctions *N* et *C* terminales, ce qui n'est par exemple pas le cas de la lysine et de l'acide glutamique ou aspartique. De plus, ces deux acides aminés sont hydrophiles, ce qui engendrerait des ELPs avec des T_t s relativement élevées rendant donc l'étape de purification plus compliquée. La cystéine quant à elle est un acide aminé difficile à contrôler car il a tendance à former

des ponts disulfures. Finalement nous avons choisi d'introduire la méthionine en tant que résidu hôte. En effet, la méthionine est un acide aminé plutôt hydrophobe et qui est le nucléophile le réactif en condition acide. Le groupe de Deming à UCLA avec lequel nous collaborons a d'ailleurs récemment remis au goût du jour la modification chimiosélective de résidus méthionine par thioalkylation en utilisant différents types de réactifs : des halogénures d'alkyle, des triflates, des époxydes, *etc.* Nous nous sommes donc inspirés de ces réactions afin de les adapter à la modification d'ELPs contenant des résidus méthionine produits par voie recombinante.

La production de ces ELPs par ingénierie des protéines a donc tout d'abord nécessité le design d'un gène codant pour le polypeptide à produire, et ce pour chacun des ELPs souhaités. Ce gène est ensuite introduit dans un ADN plasmidique afin d'être cloné. Après clonage, l'ADN plasmidique est introduit dans une souche de bactérie *E.coli*. Ces bactéries seront donc capables de produire le polypeptide d'intérêt encodé dans l'ADN plasmidique en plus des protéines encodées dans l'ADN génomique. Après ces étapes de biologie moléculaire les bactéries modifiées sont ensuite cultivées. Dans un premier temps on cherche à faire croître leur nombre avant d'induire la production des protéines. Le bon déroulement de la production peut être suivi par gel d'électrophorèse, on note ainsi que la bande correspondant à l'ELP produit s'intensifie bien au fil du temps après l'induction (Figure 1).

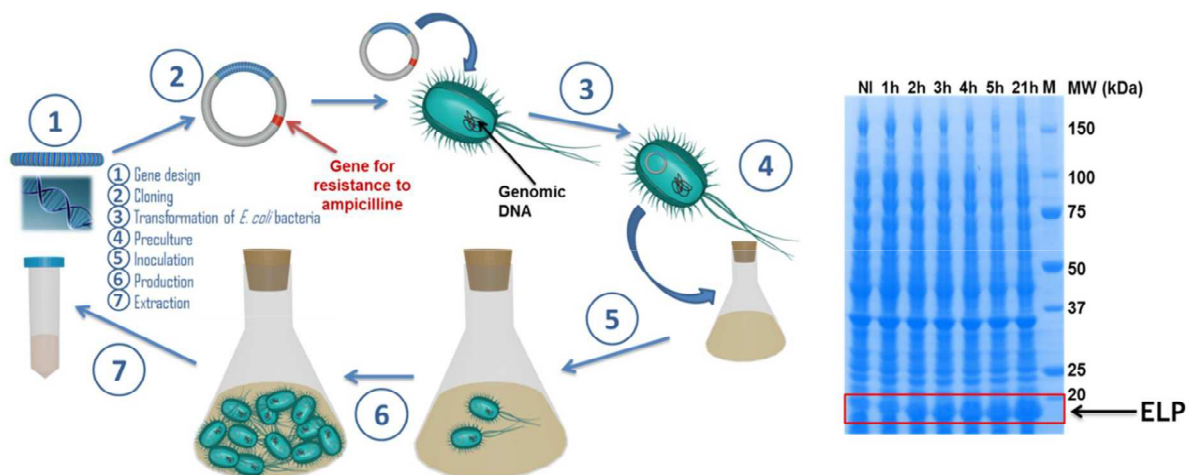


Figure 1. Production recombinante d'ELP et caractérisation par gel d'électrophorèse

Suite à la production, les bactéries sont récoltées et séparées du milieu de culture par centrifugation avant de subir une lyse cellulaire par sonication afin de libérer les protéines produites. Les protéines

sont ensuite séparées des débris cellulaires par centrifugation à froid pour s'assurer qu'elles restent bien solubles dans le surnageant. Ce surnageant contenant à la fois l'ELP d'intérêt mais également toutes les autres protéines produites doit ensuite être soumis à une étape de purification afin de séparer les ELPs des autres protéines. Pour être cela on fait appel à une méthode développée par le groupe de Chilkoti, appelée *Inverse Transition Cycling (ITC)*. Dans un premier temps, du sel est ajouté dans la solution obtenue après la lyse cellulaire, ce qui a pour effet de faire précipiter l'ELP. En centrifugeant à 25°C on peut donc récupérer l'ELP dans le culot alors que les protéines restées solubles sont éliminées dans le surnageant. L'ELP est ensuite resolubilisé dans du PBS froid avant d'être à nouveau centrifugé, à froid cette fois-ci, afin d'éliminer les protéines insolubles dans le culot tandis que l'ELP reste soluble dans le surnageant. Ce cycle est répété plusieurs fois jusqu'à avoir bien purifié l'ELP, en général trois fois, ce qui peut être vérifié à nouveau par gel d'électrophorèse (Figure 2).

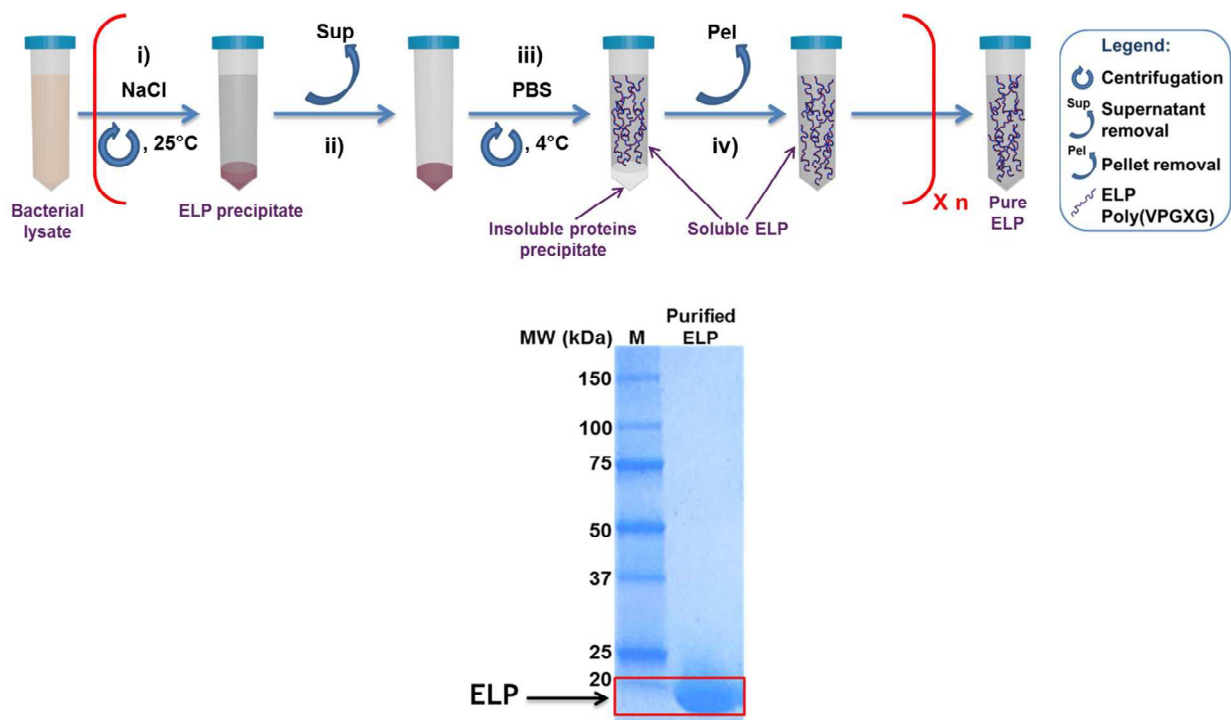


Figure 2. Purification des ELPs par Inverse Transition Cycling (ITC) et caractérisation par gel d'électrophorèse.

En utilisant la production par voie recombinante ainsi que la purification par ITC nous avons pu obtenir trois ELPs différents : le $(VPGMG)_{20}$ avec un résidu méthionine à chaque répétition, l'ELP-M-20 avec une alternance de résidus valine et méthionine afin d'espacer ces derniers, et l'ELP-M-40 possédant la même structure que l'ELP-M-20 mais avec une masse molaire deux fois plus élevée.

Etant donné que notre objectif est de modifier précisément ces ELPs il était important de parfaitement les caractériser pour pouvoir ensuite identifier et quantifier ces modifications chimiques. Les trois ELPs ont donc été caractérisés par spectrométrie de masse et RMN. L'analyse par spectrométrie de masse a notamment permis de vérifier la parfaite monodispersité de nos trois polypeptides. La RMN ^1H des trois ELPs a été réalisées et chaque spectre a été complètement attribué grâce à la RMN 2D afin de pouvoir identifier les pics les plus importants qui nous permettront ensuite d'évaluer la bonne fonctionnalisation de nos composés (Figure 3).

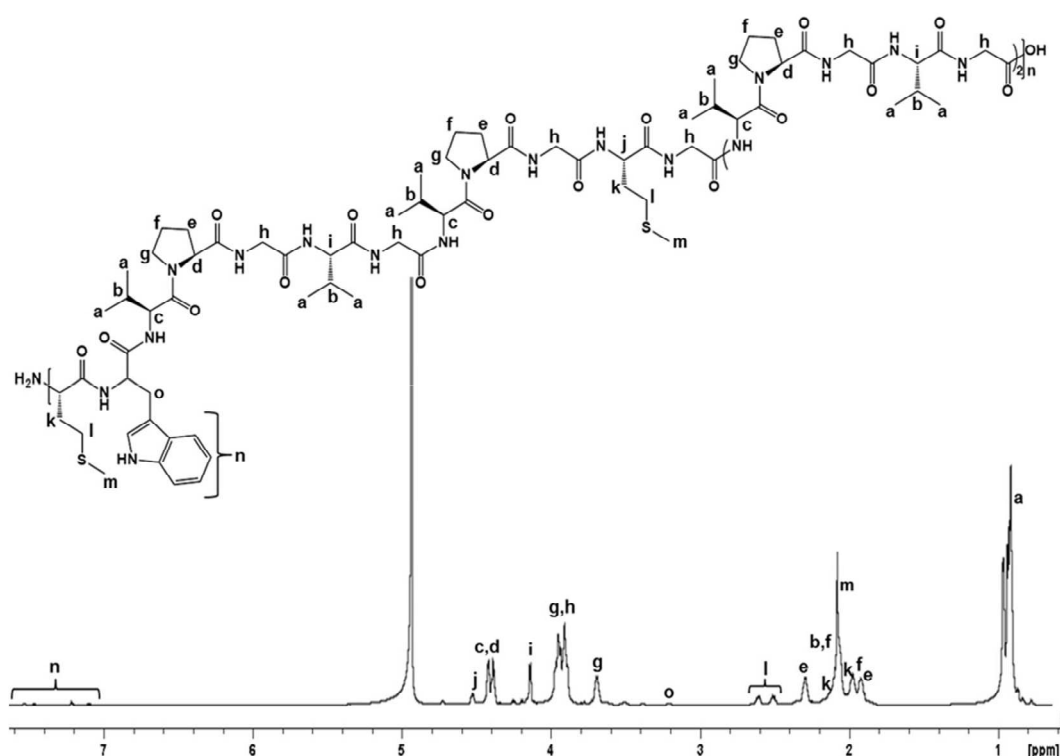


Figure 3. Spectre RMN ^1H de l'ELP-M-40

Une fois les trois ELPs obtenus et parfaitement caractérisés, nous avons pu procéder aux premières modifications chimiques. Pour cela nous avons adapté la méthode développée par le groupe de Deming utilisant des halogénures d'alkyl en milieu acide afin de thioalkyler l'ELP (VPGMG) $_{20}$. Grâce à cette méthode nous avons pu introduire un groupement méthyle et un groupement benzyle sur les résidus méthionine de cet ELP. La fonctionnalisation du (VPGMG) $_{20}$ a été vérifiée par RMN en observant le déplacement du pic correspondant au méthyle de la méthionine ainsi que l'apparition des pics aromatiques en ce qui concerne le groupement benzyle. Le taux de fonctionnalisation des deux composés obtenus, (VPGM(Me)G) $_{20}$ et (VPGM(Bn)G) $_{20}$ a également été établi par RMN en intégrant les pics d'intérêt et a ainsi permis de montrer qu'ils étaient totalement fonctionnalisés.

Afin d'évaluer l'impact de ces modifications chimiques sur les propriétés thermosensibles du $(\text{VPGMG})_{20}$, le point de trouble des trois composés a été mesuré (Figure 4). Nous avons pu observer la perte de la T_t dans le cas du $(\text{VPGM}(\text{Me})\text{G})_{20}$ du fait de l'introduction des charges positives suite à la réaction de thioalkylation, rendant ainsi l'ELP plus soluble. A l'inverse, le $(\text{VPGM}(\text{Bn})\text{G})_{20}$ conserve une T_t , bien que plus élevée, du fait du caractère plus hydrophobe du groupement benzyl par rapport au groupement méthyl permettant de contrebalancer l'effet des charges positives.

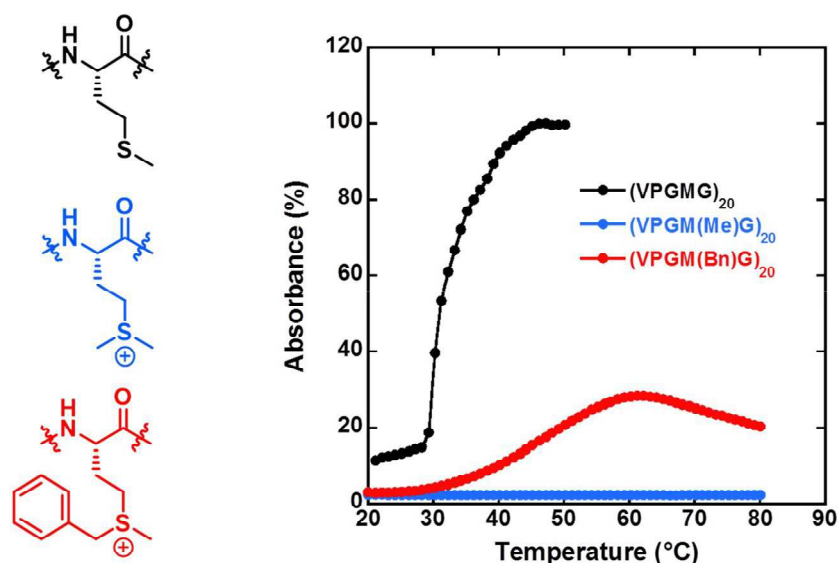


Figure 4. Mesure du point de trouble de $(\text{VPGMG})_{20}$, $(\text{VPGM}(\text{Me})\text{G})_{20}$ et $(\text{VPGM}(\text{Bn})\text{G})_{20}$

Les études effectuées sur ce premier système nous ont donc donné d'intéressants résultats préliminaires sur la modification des résidus méthionine ainsi que leur impact sur la T_t . En revanche cet ELP s'est révélé relativement difficile à produire ne nous permettant pas d'accéder à des rendements très élevés. De plus, la présence de méthionines à chaque répétition induit une trop forte densité de charges positives suite aux réactions de thioalkylation. C'est pourquoi nous avons basé les études suivantes sur les deux autres systèmes ELP-M-20 et ELP-M-40.

Dans un premier temps, l'ELP-M-40 a été soumis à des conditions oxydantes afin de modifier les groupements thioether de la méthionine en sulfoxyde ou sulfone (Figure 5).

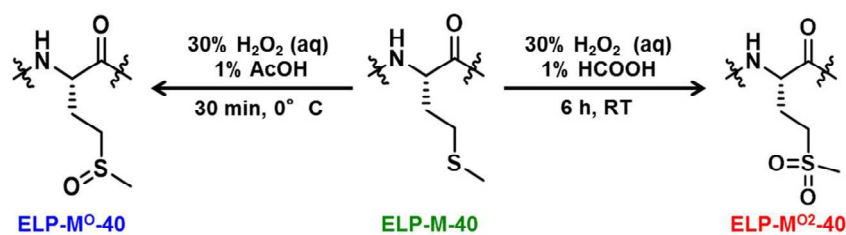


Figure 5. Réaction d'oxydation de l'ELP-M-40

La fonctionnalisation des composés obtenus a été vérifiée et quantifiée de nouveau par RMN ainsi que par spectrométrie de masse MALDI.

L'étude de l'impact de ces modifications sur le caractère thermosensible de l'ELP-M-40 a montré que l'oxydation augmente la solubilité de l'ELP, en particulier dans le cas de l'ELP-M^O-40 pour lequel la T_t a été augmenté de 30°C par rapport à l'ELP-M-40, et dans une moindre mesure pour l'ELP-M^{O2}-40 (Figure 6A).

Nous avons également cherché à vérifier si les composés modifiés suivent l'équation établie par Chilkoti :

$$T_t = T_{t,c} + k/L \ln(C_c/C)$$

liant la T_t et la concentration C de la solution. En effet, cette équation a été établie expérimentalement à partir d'une série d'ELP non modifié, il n'est donc pas évident qu'elle soit également vérifiée par des ELPs modifiés. Nous avons finalement constaté que l'ELP-M^O-40 et l'ELP-M^{O2}-40 suivent bien la loi expérimentale de même que l'ELP-M-40 (Figure 6B). La T_t d'un de ces composés pourra donc être estimée à une concentration spécifique grâce à ce diagramme ou de cette relation.

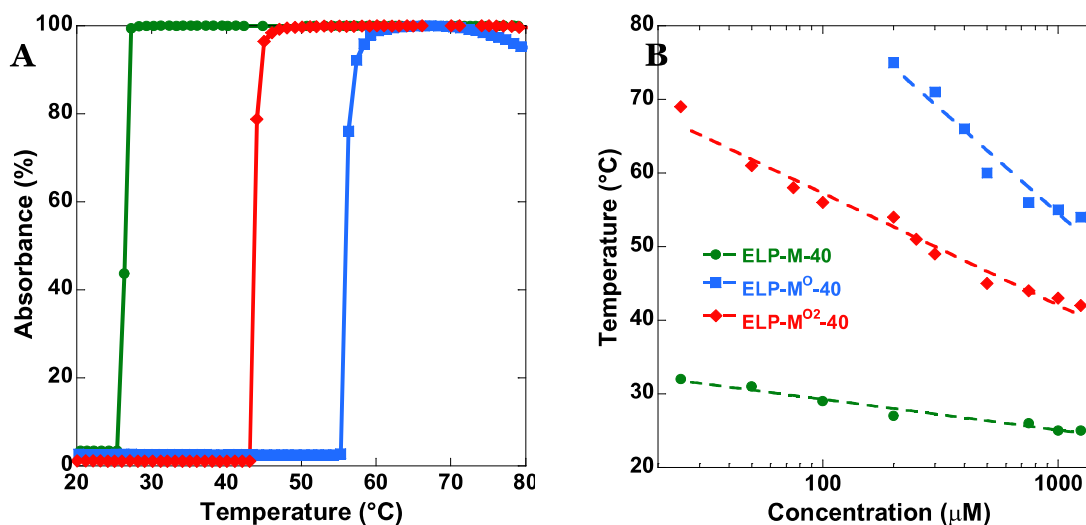


Figure 6. Etude des propriétés thermosensibles de ELP-M-40, ELP-M^O-40 et ELP-M^{O2}-40

Dans un second temps l'impact de deux anions faisant partie de la série de Hofmeister, NO_3^- et I^- , sur la solubilité de l'ELP-M-40 et de ses deux dérivés oxydés a été étudié, montrant une variation différente de la T_t selon l'anion mais aussi selon le composé.

Tout comme l'alkylation, l'oxydation des résidus méthionine permet donc de moduler les propriétés thermosensibles de nos ELPs.

Nous avons ensuite envisagé la possibilité de moduler la T_t des ELPs non pas en introduisant différents groupement sur les résidus méthionine, mais en jouant sur un échange de contre-ion. En effet, suite aux réactions de thioalkylation précédemment réalisées, une charge positive est introduite suite à la formation du groupement sulfonium. Ceci induit la présence de contre-ions que l'on peut échanger pour d'autres contre-ions plus ou moins hydrophobes : CH_3COO^- , CF_3COO^- , PF_6^- , NTf_2^- , etc. Nous avons ainsi observé qu'en faisant varier le contre-ion du sulfonium on pouvait également moduler les propriétés thermosensibles des ELPs après l'étape de thioalkylation.

Afin de varier le type de groupements introduits sur les résidus méthionine par thioalkylation nous avons ensuite utilisé des époxydes comme réactifs plutôt que des halogénures d'alkyle. En effet, les époxydes sont plus stables, ils peuvent se trouver commercialement pour certains ou être synthétisé assez simplement pour d'autres. Une méthode de thioalkylation a récemment été développé dans le groupe Deming pour la modification de polyméthionines que nous avons adapté aux ELPs : ELP-M-20 et ELP-M-40. Pour l'ELP-M-20 la réaction est effectuée dans l'acide acétique à température ambiante, sous atmosphère inerte pendant deux jours. Grâce à cette méthode nous avons introduit quatre groupements différents sur l'ELP-M-20 avec d'excellents taux de fonctionnalisation (Figure 7). En ce qui concerne l'ELP-M-40, il a été plus compliqué d'atteindre 100% de fonctionnalisation. En effet, même en augmentant la quantité de réactif utilisée ou en rallongeant le temps de réaction le taux maximal atteint était de 70%. Finalement, il a fallu changer le solvant réactionnel car l'acide acétique ne permettait pas de suffisamment solubiliser l'ELP. Le problème a été résolu en utilisant un mélange acide acétique/HFIP ce qui rend chaque résidu méthionine plus accessible pour le réactif, permettant ainsi de totalement fonctionnalisé l'ELP-M-40.


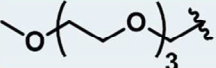
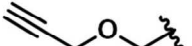

R	Functionalization %	
	ELP-M-20	ELP-M-40
	1a, > 99	2a, > 99
	1b, 98	2b, 98
	1c, > 99	2c, 97
	1d, 96	2d, 96

Figure 7. Fonctionnalisation de ELP-M-20 et ELP-M-40 avec des époxydes

En plus de l'analyse par RMN qui a permis d'obtenir les taux de fonctionnalisation, le succès de ces réactions a également été confirmé par spectrométrie de masse ESI, tous les composés ont donc tous été parfaitement caractérisés (Figure 8).

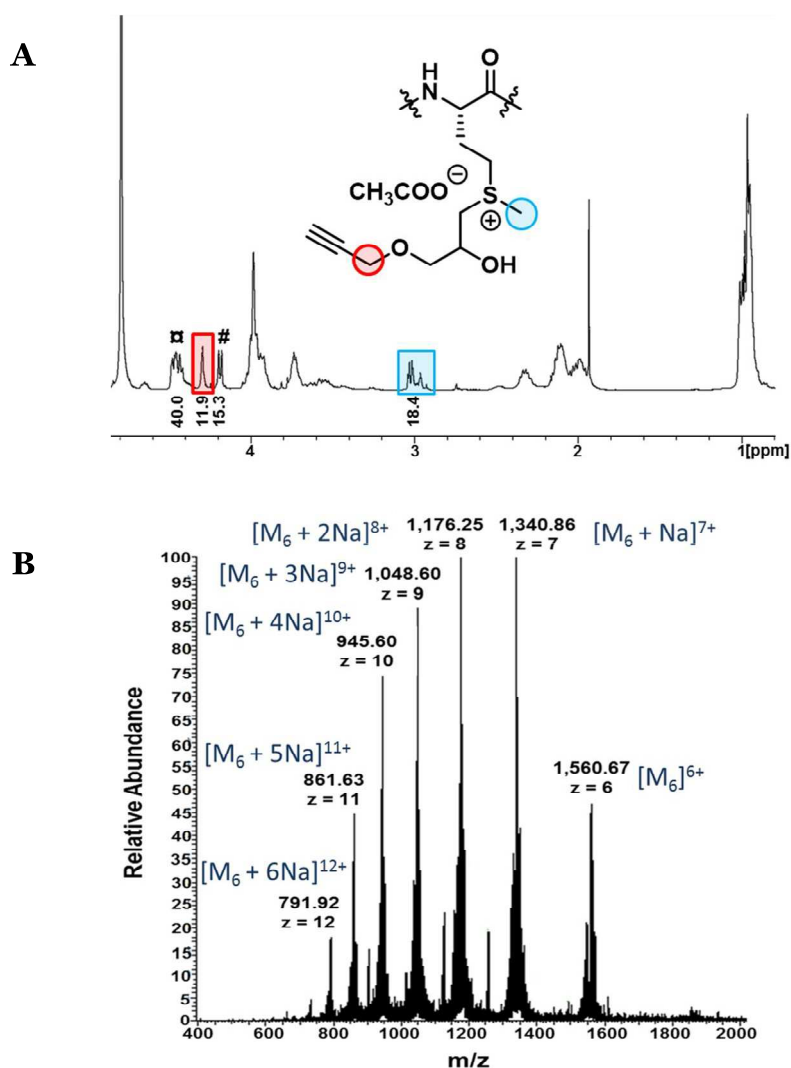


Figure 8. Caractérisation de l'ELP-M(alkyne)-20 par RMN ^1H (A) et spectrométrie de masse ESI (B)

De plus, les propriétés thermosensibles de chaque composé ont été évaluées par diffusion de lumière dynamique (DLS), montrant qu'en faisant varier le groupement pendant on peut accéder à une gamme de T_t de 35°C à des températures plus élevées voire non observables.

Grâce à l'utilisation des époxydes un groupement alcyne a été introduit sur chaque méthionine des deux ELPs. Cet alcyne peut être utilisé dans second temps pour encore modifier nos ELPs de manière versatile par cycloaddition de Huisgens, permettant ainsi d'accéder à toute une gamme de groupements fonctionnels. Nous avons utilisé cette méthode pour introduire des monosaccharides sur les ELP-alcyne. Les monosaccharides choisis sont le glucose (Glc), le galactose (Gal) et le mannose (Man), ce sont des groupements simples, qui peuvent servir de modèle, et ils sont également capables de se lier à des lectines de manière sélective : le glucose et le mannose se lient à la ConA tandis que le galactose se lie à la RCA120. Grâce à la réaction de chimie click les trois monosaccharides ont été introduits sur chaque ELP avec à nouveau de très bons taux de fonctionnalisation, ce qui a été vérifié par RMN.

Nous avons ensuite vérifié la capacité de ces ELPs glycoconjugués à se lier sélectivement à des lectines. Pour ces études préliminaires la lectine choisie a été la RCA120. Nous avons donc évalué la capacité de l'ELP-M(Gal)-40 à se conjuguer à cette lectine tout en vérifiant la sélectivité de la conjugaison en testant aussi l'ELP-M(Glc)-40 qui ne devrait pas se lier à la RCA120. Finalement les résultats obtenues par diffusion de la lumière multi-angles nous suggèrent que l'ELP-M(Gal)-40 (2c) se conjuguent bien à la RCA120, tandis que l'ELP-M(Glc)-40 ne semblent pas se conjuguer (Figure 9).

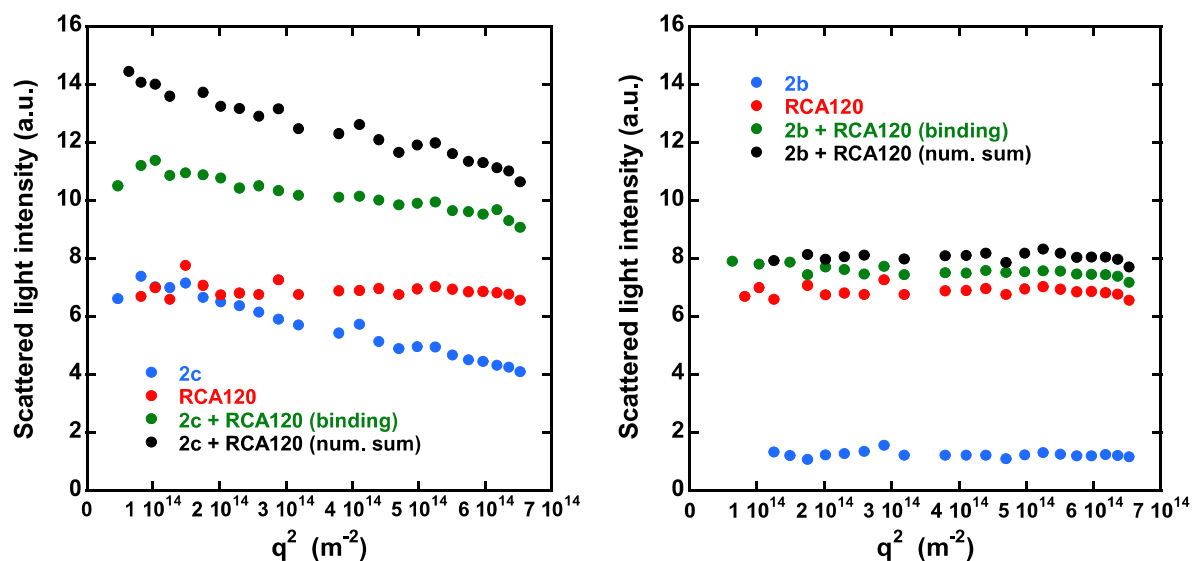


Figure 9. Etude de conjugaison entre l'ELP-M(Gal)-40 (2c) et ELP-M(Glc)-40 avec la RCA120 par diffusion de lumière multi-angles

Dans un second temps nous avons étudié la possibilité de tirer parti des propriétés thermosensibles des ELPs ainsi que de la capacité de l'ELP-M(Gal)-40 à se lier à la RCA120 de manière sélective pour faire de la séparation sélective de protéines. Cette méthode a été testée de manière visuelle et également par spectrométrie de fluorescence grâce à une lectine conjuguée fluorescéine. Les résultats obtenus suggèrent qu'il est en effet possible de récupérer la RCA120 en solution de manière sélective par une méthode de chauffage et centrifugation.

Finalement ces travaux de thèse nous ont permis d'accéder à une large gamme d'ELPs modifiés avec différents groupements fonctionnels. Grâce à ces modifications, nous avons pu moduler les propriétés thermosensibles des trois ELPs que nous avons produits. Nous avons également montré que la Tt peut également varier par échange de contre-ions, ce qui étend encore le champ des possibilités. Enfin, on peut également obtenir des ELPs bioactifs en introduisant des groupements biofonctionnels tels que des monosaccharides. Cette méthode peut bien sûr être étendue à d'autres groupements biofonctionnels tels que des peptides ou autres ligands.

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Rosine

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GENERAL INTRODUCTION

The use of polymers as biomaterials for biomedical applications raised great interest during the past decades and had a great impact on the development of modern medicine. Biodegradable polymers are particularly interesting as they have the ability to break-down and to be metabolized or excreted after having accomplished their function. Therefore a broad range of natural and synthetic biodegradable polymers have already been studied and new materials are constantly developed to make progresses in the fields of tissue engineering and drug delivery.

To design such materials, it is particularly interesting to get inspiration from Nature. Indeed through billions of years of evolution, Nature has created and assembled macromolecules with remarkable structures and functionalities. Therefore scientist often rely on biomimicry for preparing biomaterials as they are simplified analogues of biomolecules capable of mimicking their properties.

In particular polypeptides are very interesting biopolymers in the scope of biomedical applications. Polypeptides can be produced by different techniques which all have their advantages and drawbacks. For instance, solid-phase peptide synthesis allows the production of sequence specific peptides but the chain length of the synthesized peptides is limited. This drawback can be overcome by producing polypeptides *via* ring opening polymerization of amino acid N-carboxyanhydrides. Indeed well-defined, high molecular weight polypeptides have been obtained by this method although sequence specificity is still a limitation. On the contrary, polypeptides obtained by protein engineering techniques have the advantage to combine defined sequence and high molecular weights. Recombinant synthesis has however the disadvantage to require long and complex molecular biology steps to obtain the genetically encoded polymers. However, recent developments in this field have allowed great progress in the production and purification of those polypeptides.

In this context our lab is interested in the use of recombinantly produced proteins, especially elastin-like polypeptides (ELPs). Along with exquisite features such as precise sequence, monodispersity and biocompatibility, ELPs are very appealing biomimetic polypeptides because of their stimuli-responsive properties. Indeed stimuli-responsive materials are of great interest in medicine as they can respond to specific internal or external triggers such as pH or temperature variations that occur in the body, which is useful for example in drug delivery applications. Depending on their sequence, ELPs can present pH-responsive properties, but they are mostly used for their thermoresponsive properties.

These thermoresponsive properties can be fine tuned by different manners for example by changing the ELP sequence or its molecular weight both depending on the gene design.

As mentioned before, changing the gene sequence is very tedious as it relies on molecular biology. Therefore, alternative strategies to tune the thermoresponsive properties as well as the functionality of ELPs represent an interesting challenge. The aim of this thesis was then to develop chemoselective modifications as versatile tools for tuning properties of ELPs as well as introducing functional groups on their backbone.

In the context of biomedical applications it is important to synthesize functional polypeptides as mimics of post-translationally modified proteins. Indeed, modified proteins such as glycosylated proteins play important roles in living systems as they are involved in many biological processes, for instance cell communication, interaction and motility. Therefore such biomimetic structures could be interesting to create biomaterials that could for example interact with specific targets or help understanding structure-function relationship of their natural counterparts.

In order to mimic these glycoproteins, precise materials with controlled structures need to be synthesized. In order to modify the ELP backbone at specific sites two requirements are necessary: chemical modifications have to be selective towards one specific amino acid and this chosen amino acid has to be genetically introduced at specific and desired positions depending on the targeted final polypeptide. Several reactions have been developed for the selective modification of natural amino acids such as cysteine or lysine residues. In this project, we decided to introduce methionine residues in our ELPs sequences and to use these residues as targets for the chemoselective modification of the ELP backbone. Indeed, recent developments in methionine thioalkylation for the selective modification of synthetic polypeptides have attracted our attention. We are thus aiming at developing a post-modification strategy of ELPs containing methionine residues, in order to tune their properties (mainly temperature responsive character) and to introduce specific functional groups such as sugars. The versatility of this approach, combining protein engineering and chemoselective post-modification, represent a unique synthetic toolbox for biomaterials design.

The first part of this manuscript will be dedicated to a state-of-the-art review of the field. We will then focus on the chemoselective modification of one of our elastin-like polypeptides by alkylation of the methionine residues side-chain to tune its properties. In the third part we will describe another method for tuning the thermoresponsive properties of our ELPs: the selective oxidation of the

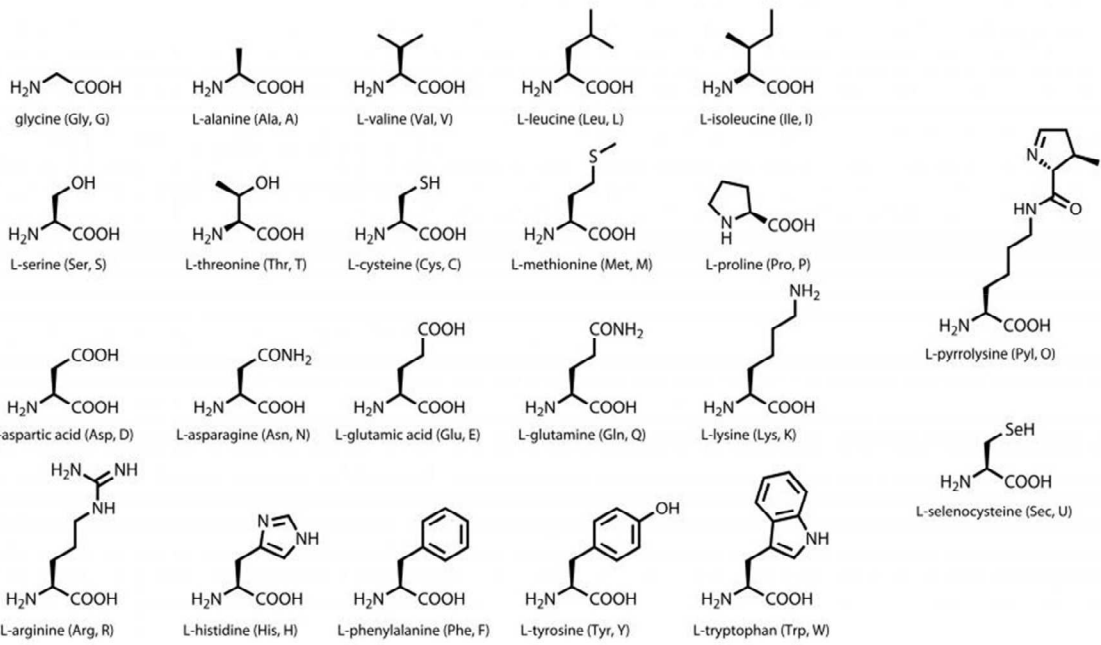
methionine residues. We will then study the versatile introduction of different functional groups on the ELP backbone as well as the exchange of the counteranions of the formed polycationic derivatives. The influence of these two variations on the ELP properties will be studied. Finally the fifth part of the thesis will focus on the glycosylation of two ELPs and describe preliminary studies on the bioactivity of these bioconjugates.

LIST OF ABBREVIATIONS

BFP	Blue Fluorescent Protein
BSA	Bovine Serum Albumin
COSY	Correlation spectroscopy
CP	Capsid Protein
CPP	Cell-Penetrating Peptide
DLS	Dynamic Light Scattering
DMSO	Dimethyl Sulfoxide
DOX	Doxorubicin
EBP	Elastin-Binding Protein
ELP	Elastin-like Polypeptide
EPL	Expressed Protein Ligation
EPR	Enhanced Permeation and Retention
ESI	Electrospray Ionization
HSQC	Heteronuclear Single Quantum Coherence
ITC	Inverse Transition Cycling
LCST	Lower Critical Solution Temperature
MALDI	Matrix Assisted Laser Desorption Ionization
NCA	N-carboxyanhydride
NCL	Native Chemical Ligation
NHS	N-hydroxysuccinimide
NMR	Nuclear Magnetic Resonance
OERCA	Overlap Extension Rolling Circle Amplification
PBLG	Poly(γ -benzyl glutamate)
PEG	Poly(ethylene glycol)
POEGMA	poly(oligo(ethylene glycol) methyl methacrylate)
RDL	Recursive Directional Ligation
ROP	Ring Opening Polymerization
SBL	Subtilisin <i>Bacillus Lentus</i>

SPAAC Strain Promoted Azide Alkyne Cycloaddition
SPPS Solid-Phase Peptide Synthesis

NATURAL AMINO ACIDS



CHAPTER 1

BIBLIOGRAPHIC REVIEW

I. ELASTIN-LIKE POLYPEPTIDES: FROM NATURAL ELASTIN TO BIOMEDICAL APPLICATIONS

I.1. ELASTIN-LIKE POLYPEPTIDES: DEFINITION AND PROPERTIES

I.1.1. Tropoelastin/Elastin

Elastin is a natural protein found in the extracellular matrix of vertebrates' connective tissues.^{1,2,3} It is found in various tissues which need to deform repetitively and reversibly, such as elastic ligaments, arteries, lungs, tendons and skin,^{1,2,4,5,6} providing them elasticity and resilience.^{3,7} Elastin is an extremely insoluble protein due to its high content in hydrophobic amino acids (75 % of its entire primary sequence is composed of Gly, Val, Ala, Pro), as well as to the high level of interchain crosslinks between Lys residues.^{1,2,8}

Tropoelastin (≈ 70 kDa), the soluble precursor of human elastin, is synthesized by several types of cells: smooth muscle cells, endothelial cells, fibroblasts and chondrocytes.^{1,2} Once synthesized, it is immediately bound to the elastin-binding protein (EBP) preventing its premature aggregation (Figure 1).⁹

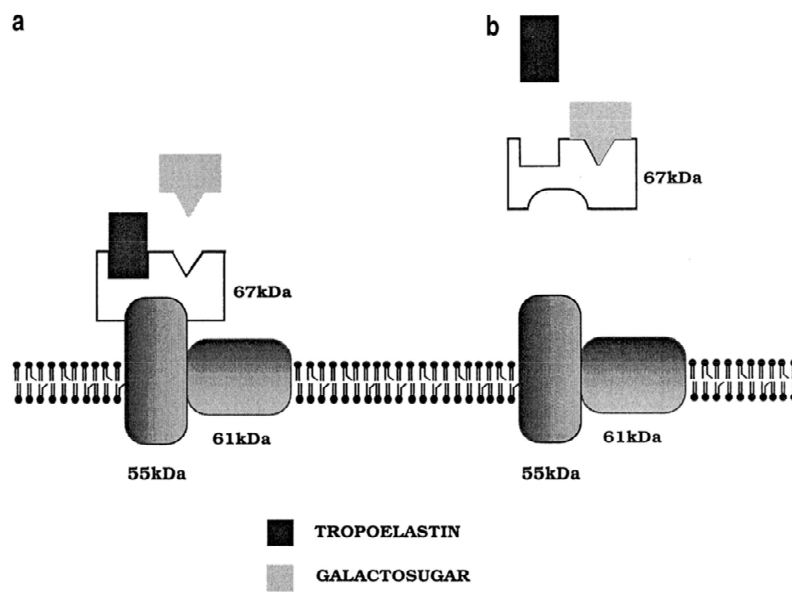


Figure 1. Model of the elastin-binding protein (EBP) complex, reproduced from Vrhovski and Weiss²

Formation of this EBP-tropoelastin complex allows its secretion from the cell. After excretion in the extracellular space, the EBP lectin domain interacts preferentially with galactosugars (galactose, galactosamine) of microfibrils of the extracellular matrix, thereby decreasing its affinity for

tropoelastin and leading to the local release of latter. These microfibrils act as a scaffold for the alignment of tropoelastin macromolecules (Figure 2).^{3,7,10}

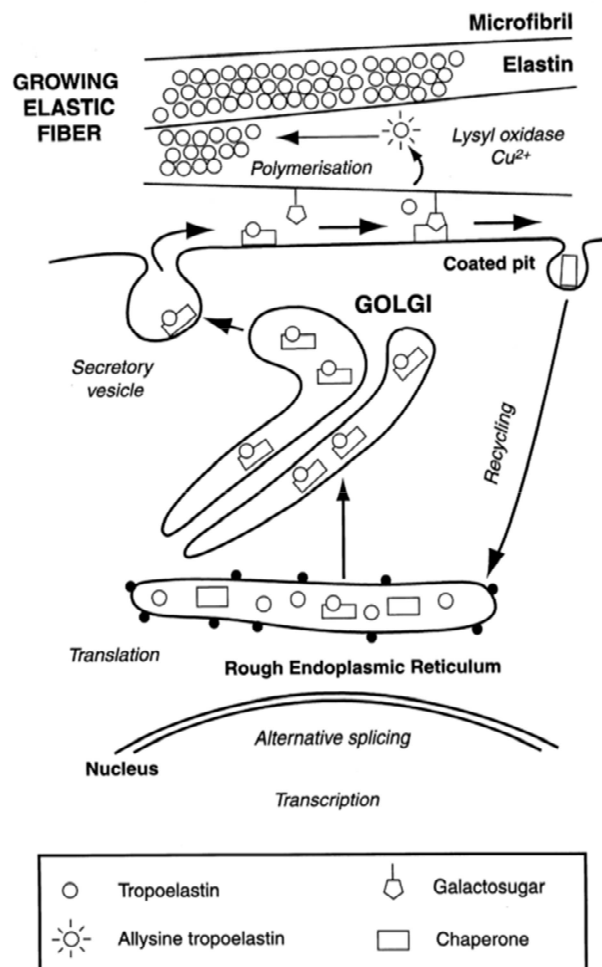


Figure 2. Schematic description of elastin deposition, reproduced from Debelle and Tamburro³

The composition of tropoelastin also plays a key role in its correct deposition onto the microfibrillar structure. Indeed, it consists in two domains: the hydrophobic one mainly composed of repeats such as Gly-Val-Gly-Val-Pro (GVGVV), Gly-Val-Pro-Gly-Val (GVPGV) or Gly-Val-Gly-Val-Ala-Pro (GVGVAP), and the hydrophilic one mainly composed of Lys and Ala residues.⁵ Tropoelastin molecules have the ability to self-assemble under physiological conditions, a process named coacervation,² which enables the alignment of the hydrophobic domains and the juxtaposition of lysine residues necessary for crosslinking (Figure 3).^{11,12}

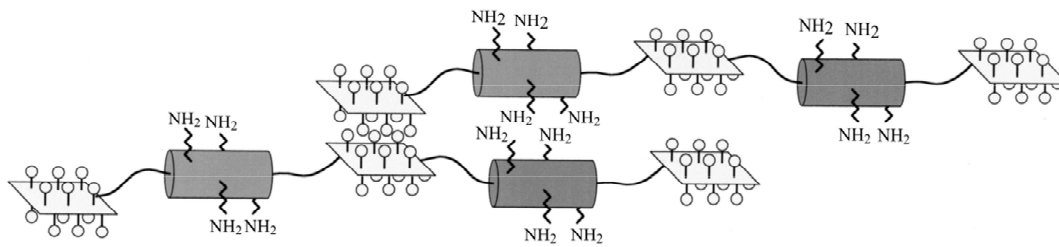


Figure 3. Alignment of tropoelastin molecules during coacervation. Hydrophobic domains are represented by square planar structures with large hydrophobic side chains protruding above and below the plane. Cross-linking domains are represented by cylinders, with pairs of lysine residues protruding on the same side of the structure, reproduced from Keeley *et al.*¹³

Most Lys residues are then deaminated and oxidized to allysine by lysyl oxidase. Four allysine residues from two separate tropoelastin molecules then react with each other (or with unmodified Lys) to form tetrafunctional crosslinks such as desmosine and isodesmosine.^{1,2,14} This mechanism results in the formation of elastic fibers with remarkable properties (Figure 4).

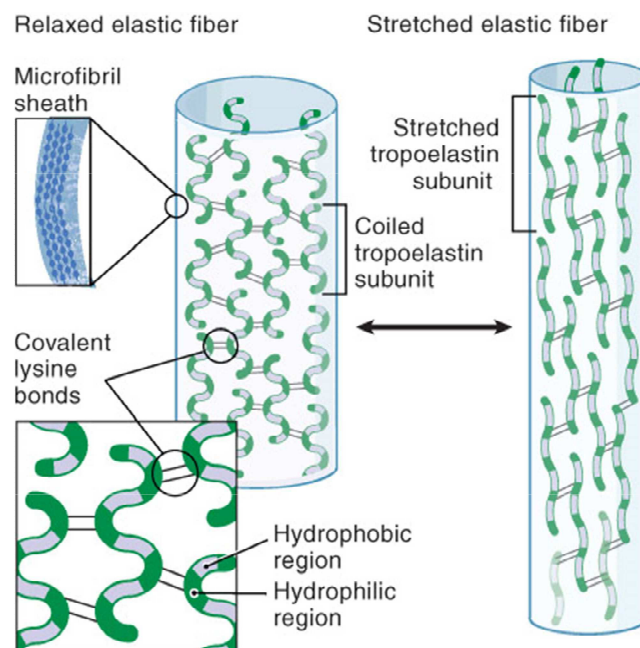


Figure 4. Elastin structure, reproduced from Plopper¹⁵

In 1974, Hovee and Flory demonstrated that the basis of elastin elasticity is entropic.¹⁶ Stretching decreases the entropy and the spontaneous return to maximum entropy drives the recoil of the system.¹⁷ Several studies have then been carried out to determine the structure–function relationships of elastin, leading to several models.^{3,18,19,20} The random chain model¹⁶ compares elastin with natural

rubber, consisting of random, flexible chains, kinetically free and in permanent motion due to thermal agitation. However several features of elastin are not compatible with this model such as the ability of tropoelastin to coacervate or the fibrous nature of elastin. Therefore several other models have been developed. First of all, the liquid drop model which considers that elastin swollen in water might be seen as a two-phase system consisting in globular domains connected by cross-links.²¹ Another model, the oiled coil model, proposed a structure based on fibrillar units of alternating α -helical cross-linking domains and a broad coil composed of hydrophobic repeat units. However both of these models have been rejected for similar reasons, for instance the high density of these structures is not compatible with the mobility required for elastin's stretching.¹⁶ Finally the fibrillary model, first introduced by Urry²², is based on the study of the repeating hydrophobic segments of tropoelastin such as APGVGV, VPGVG and VPGG and is supported by a significant amount of experimental data. The structure of elastin proposed in this model consists of alternating β -spirals formed by the hydrophobic domains and α -helical domains, with the chains held together by cross-links and hydrophobic interactions.

Elastin is an extremely stable protein, with a very slow turnover, that can last for almost an entire lifespan.²³ One of the reasons explaining such stability is the resistance of elastin to proteolytic degradation. However a group of proteases known as elastases are still able to degrade elastin. Elastolysis plays a role in many physiological processes such as growth, pregnancy, or wound healing.²⁴ Nonetheless, inappropriate and uncontrolled elastin degradation can lead to disorders such as emphysema in the lungs, atherosclerosis in arteries, cutis laxa in skin, and is also a feature of normal ageing.^{2,25}

I.1.2. General features of elastin-like polypeptides

Elastin-like polypeptides (ELPs) are artificial polypeptides derived from recurring sequences found in the hydrophobic domain of tropoelastin. Most ELPs consist of repeats of $(VPGXG)_n$ pentapeptides where X, the guest residue, can be any amino acid except proline,²⁶ and n represents the number of repeats in the ELP. Urry and coworkers have carried out the first pioneering works in this field being the first to chemically synthesize the repetitive peptides, their oligomers and polymers.²⁷ They were thereby able to study and understand the conformation and interactions inside the hydrophobic domain of tropoelastin. In particular, they observed that the polypentapeptides $(VPGVG)_n$ ($10 \leq n \leq 15$) exhibit an inverse temperature phase transition in aqueous medium, in a similar way as tropoelastin coacervation, occurring at a specific temperature, called transition temperature (T_t)

(Figure 5).^{27,28} This T_t is comparable with the *lower critical solution temperature (LCST)* observed with certain synthetic polymers such as polyNIPAM.²⁹ They used the VPGVG repeat sequence as a simple model to explain the mechanism and the structural changes leading to coacervation of tropoelastin upon heating above its phase transition temperature.

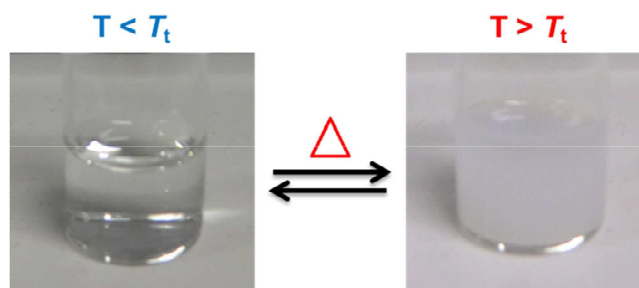


Figure 5. ELP aggregation upon heating above T_t

According to Urry *et al.*, below the T_t ELP chains adopt a random coil conformation, and a β -spiral conformation resulting from consecutive type II β -turn above the T_t . This conformational change comes with a simultaneous release of hydrating water molecules.^{30,31,32} The β -turns result from the presence of Pro-Gly dipeptides while the adjacent amino acids can be substituted by any amino acid except Pro (Figure 6).^{30,33,34}

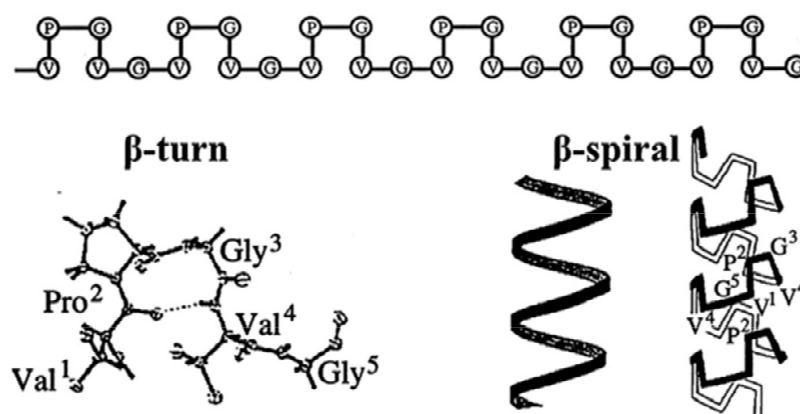


Figure 6. Poly(VPGVG) structure above T_t . The VPGVG repeats form β -turns, stabilized by intramolecular hydrogen bonds between the backbones of the first and fourth residues of the pentapeptide. These β -turns arrange into helical β -spirals, reproduced from Smits *et al.*³⁵

However, the mechanism explaining the phase transition behavior of ELPs is still not clearly established and subject of controversy. Indeed, different studies also suggest that other mechanisms are responsible for this behavior as they have shown that β -turns can also be found in ELPs below T_t .³⁶ A study from Li and coworkers concluded that several parameters such as thermal disruption of the

water network, loss of hydrating water molecules, and increasing hydrophobicity of the ELP underlie the *LCST* behavior. According to their molecular modeling simulations, β -turns are present at all temperatures but there is an increase in ordering at higher temperatures because the polypeptide tends to adopt β -strand conformations.³⁷

This phase transition behavior has been shown to be a very interesting feature for ELPs applications, especially because their T_t can be tuned in many different ways. First of all, the guest residue of the VPGXG pentapeptide repeats can be substituted by any natural or non-natural amino acid except proline leading to ELP with different T_t s depending on the nature of the amino acid (hydrophilic, hydrophobic, polar, non-polar, *etc*) introduced.³⁰ Along with the guest residue modification, the chain length of the ELP also influences the phase transition: at equivalent composition, longer ELP chains have lower T_t s. These two alternatives for tuning the T_t of ELPs, both related to macromolecular parameters (amino acid composition and chain length), are very often used because their influence can be predicted, allowing the ELP to be initially designed accordingly. Indeed different models have been developed describing the influence of the hydrophobicity,^{30,38,39} or the charge state⁴⁰ of the guest residue or of the ELP chain length⁴¹ on the T_t . Chilkoti and coworkers have established an empirical model that correlates the T_t value to the ELP composition, chain length and concentration in a single equation:⁴²

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

This equation also involves different parameters: the critical temperature $T_{t,c}$ ($^{\circ}\text{C}$), the critical concentration C_c (μM), k a constant ($^{\circ}\text{C}$) and L the number of pentapeptide units. This equation allows predicting the T_t of a defined ELP sequence at a specific concentration. Indeed the concentration of ELP in solution also plays an important role on the T_t : the higher the concentration, the higher the T_t . Beside the ELP design or its concentration, external parameters can also be used to tune the T_t . The cosolutes present in the ELP solution and their concentration are also important factors.^{43,44} The influence of several anions from the Hofmeister series (CO_3^{2-} , SO_4^{2-} , $\text{S}_2\text{O}_3^{2-}$, H_2PO_4^- , F^- , Cl^- , Br^- , NO_3^- , I^- , ClO_4^- , SCN^-) at different concentrations has, for instance, been studied and showed that in most cases increasing salt concentrations decreases the T_t although this decrease was found to be linear for kosmotropes (strongly hydrated ions) and non-linear for chaotropes (more weakly hydrated ions).^{45,46} Since difficult to predict *in vivo*, these two alternatives (*i.e.*, addition of cosolutes and concentration) for tuning the aggregation state of an ELP have been much less exploited.

In the context of biomedical applications, a crucial parameter to take into account is the biocompatibility and biodegradability of ELPs. Biocompatibility of ELPs was shown by Urry *et al.* by performing several tests such as, mutagenicity test, cytotoxicity test, or intracutaneous toxicity, on their model system poly(GVGVP), proving that ELPs are non-toxic, non-mutagenic, and non-immunogenic.⁴⁷ The biodegradability of ELPs has been studied *in vitro* as well as *in vivo* on mice and has demonstrated their sufficient stability to accumulate in a solid tumor, but also their ability to degradation at a sufficient rate to be effectively cleared from the body over a few weeks.⁴⁸

I.1.3 Production and purification of elastin-like polypeptides

Due to the difficult sequence control of polymerization reactions and the tedious and long syntheses *via* solid phase peptide synthesis (SPPS), ELPs are most of the time produced using recombinant DNA and protein engineering techniques. The construction of the genes can be performed using different methods for assembling repeating sequences with precise control over gene length. One of these techniques is the *concatemerization* which allows the creation of a library of repetitive ELP genes with varying lengths. It is a rapid method to assemble genes of different lengths but the achievement of the desired gene length and thus the desired ELP molecular weight is not ensured. Also the *concatemerization* method is limited to low degree of polymerization (≤ 30 pentapeptides). It can however be used in combination with other techniques such as the *recursive directional ligation* (RDL). This method involves the stepwise oligomerization of short ELP genes derived from *concatemerization* using type II restriction enzymes.⁴⁹ RDL is a practical method to rapidly assemble a specific number of repeats in a defined orientation. It allows the design of ELPs with complex architectures, such as multi-block copolymers. Other peptides or protein sequences can be inserted at the *N*- and *C*-terminal ends or within the ELP gene, which can be useful for various applications. Nonetheless, this method is quite tedious as it requires multiple cloning steps to create an ELP gene with a high degree of polymerization. In the case of ELP homopolymers another technique can be considered for the rapid synthesis of ELP genes with a high degree of polymerization: the *overlap extension rolling circle amplification* (OERCA).⁵⁰ This method is powerful to synthesize genes longer than the one obtained by *concatemerization*. However only homopolymer ELPs are obtained *via* this technique.

ELP genes are most often expressed in *Escherichia coli* (*E. coli*), but in some cases ELPs have also been expressed in yeast,^{51,52,53} fungi⁵⁴ and plants.^{55,56,57} However these methods need further

development as the yield obtained were in general lower than those obtained with *E. coli* culture. For instance, yields obtained *via* yeast expression were *Tt* dependent: ELPs with relatively low *Tt* led to lower yield while ELPs with high *Tt*s were quite efficiently produced. Moreover methods such as plant expression have shown post-translational modifications of proteins, such as glycosylation, which is not always desirable.

After culture of the selected bacterial clone, as the ELPs are usually not secreted in the medium, the bacteria are subjected to cell lysis and the cell lysate is centrifuged at cold temperature in order to retrieve the soluble ELP in the supernatant and to discard the cell residues in the pellet (Figure 7).

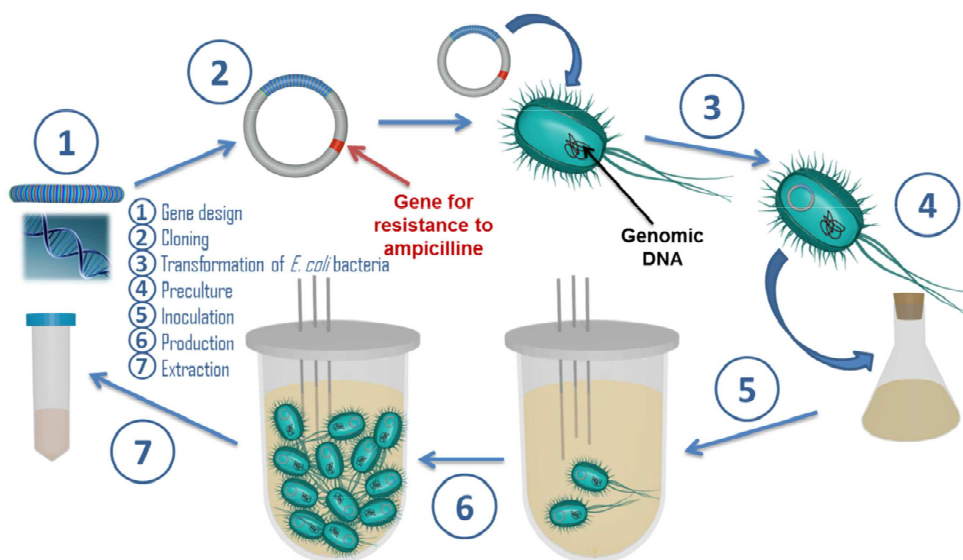


Figure 7. Production of ELPs in *E. coli*

ELPs are then purified from other bacteria proteins by exploiting the ELP's thermoresponsive properties. Indeed the use of heat or salts to trigger ELP's aggregation and separate it from other proteins is very advantageous compared to purification methods based on chromatography which are more tedious and expensive. This method is called *inverse transition cycling* (ITC) (Figure 8).⁴³ ITC consists of four consecutive steps: i) the ELP is aggregated by raising the solution temperature above the *Tt* and/or by adding salts such as sodium chloride; ii) then the solution is centrifuged above the *Tt* therefore the ELP is spinned down in the pellet and soluble contaminants are discarded in the supernatant; iii) the ELP pellet is then resuspended in a buffer below the *Tt*; iv) finally centrifugation below the *Tt* allows elimination of the insoluble proteins in the pellet while the soluble ELP is

recovered in the supernatant. Repeated ITCs followed by extensive dialysis have been found an easy and efficient purification method for the extraction of ELPs with sufficient purity.

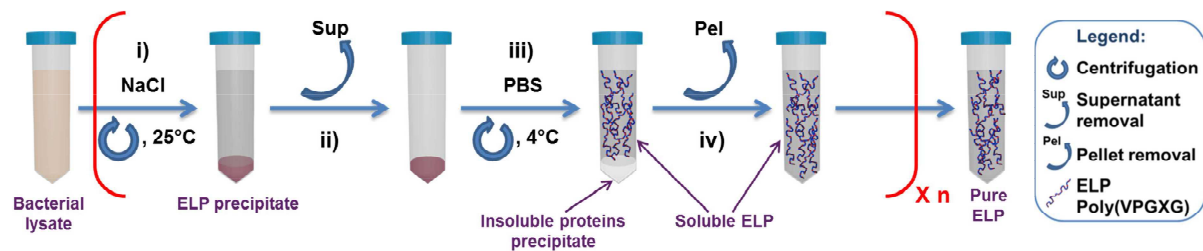


Figure 8. ELP purification by inverse transition cycling (ITC)

I.2. APPLICATIONS OF ELASTIN-LIKE POLYPEPTIDES

I.2.1. Protein purification

ELPs have been used as tags for non-chromatographic purification of recombinant proteins. The purification of ELP fusion proteins has in particular been studied by Chilkoti and coworkers.^{43,58} They have used this method to purify proteins such as thioredoxin, chloramphenicol acetyltransferase, calmodulin, green fluorescent protein, and blue fluorescent protein.^{59,60,61,62} These proteins were selected to investigate the performance of ELPs as protein purification tags because they constitute a broad range of target proteins with respect to their molecular weight, surface hydrophobicity, and surface charge. Also the fluorescent aspect of some of these proteins has been useful for following their progression during the process. The fusion of the ELP to a recombinant protein, at the gene level, induces transmission of the ELP thermoresponsive properties to the fused protein. The purification technique thus relies on the thermoresponsive properties of the ELP segment as the fusion protein will be subjected to the previously described *inverse transition cycling (ITC)* method.⁴³ Once purified the fusion protein can be released from ELP by enzymatic cleavage of a proteolytic linker introduced at the gene level and the protein can finally be separated from the ELP by additional rounds of *ITC* (Figure 9).

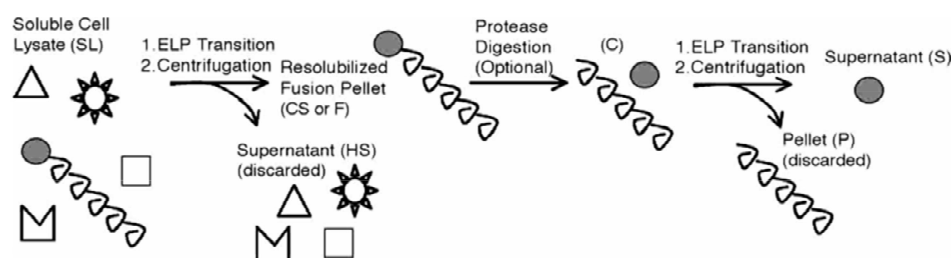


Figure 9. Purification of ELP fusion protein by ITC, reproduced from Chilkoti⁵⁹

This method provides an easy and efficient way of purifying proteins that has many advantages over the usual chromatographic techniques: its low cost, its simplicity (only requires centrifuge or filters), the possibility to reach high yield, etc. Purification of scarce amount of ELP fusion proteins has even been performed by a coaggregation process. The addition of free ELP to the cell lysate increases the overall concentration of ELP thereby inducing aggregation at low level of ELP fusion protein and enabling *ITC* purification.^{63,64}

Another method called ELP-mediated affinity capture (EMAC) combines temperature triggered aggregation and affinity capture. The ELP is chemically or genetically linked to a capture reagent, which can be a DNA-binding protein, an antibody-binding domain, a metal-binding ligand, *etc.* This method avoids the ELP cleavage step after purification thus allowing potential recycling of the ELP. This method has been used to purify proteins,^{65,66,67,68,69} but also plasmids,^{70,71} and to remove pollutants from a solution.^{72,73,74}

I.2.2. Drug delivery

The self-assembly of ELPs and their application in drug delivery has been the subject of several reviews.^{35,58,75, 76} ELPs are indeed very attractive biocompatible polymers for the design of macromolecular carriers because of the precise control over their size, sequence, their stimuli responsive properties and their ability to self-assemble without using organic solvents. Also as ELPs monomers are amino acids, their degradation by specific enzymes can be programmed *via* proper design of the ELP.

I.2.2.1. Self-assembly

Different examples of ELP-based nanoparticles have been proposed in the literature, some of them being solely composed of ELP blocks while others being hybrid systems.

i) ELP block copolymers

- ELP diblock copolymers

Self-assembly of ELP diblock copolymers is based on the difference of T_t between the two blocks. The blocks are usually designed to possess significantly different T_t s, by introducing residues of different hydrophobicity or by playing with the sizes of the different blocks. The self-assembly process triggered by temperature can then be divided in three steps: i) the ELP copolymers are first present as soluble

unimers below the T_t of both blocks, ii) then when the T_t of the more hydrophobic block is reached the spherical structures are formed, iii) finally while increasing the temperature water is expelled from the core of the nanoparticle inducing its densification until the T_t of the more hydrophilic block is reached leading to the formation of polydisperse aggregates (Figure 10).⁷⁷

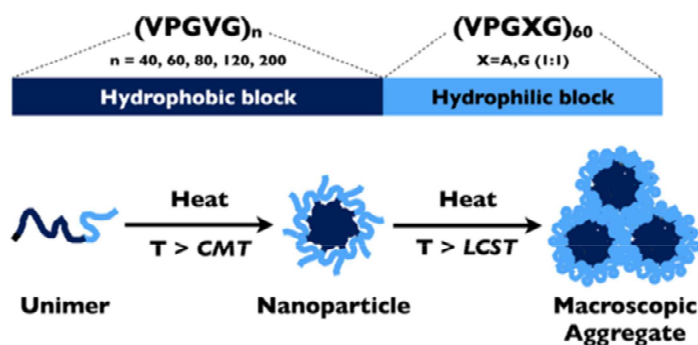


Figure 10. Schematic representation of ELP diblocks self-assembly, reproduced from Garanger *et al.*

The first example of thermoresponsive ELP diblock copolymers forming worm-like and spherical micelles nanoparticles was developed by Conticello *et al.*^{78,79} In these studies ELPs were composed of the following sequences: $[VPGEG-(IPGAG)_4]_{14}$ as the hydrophilic block and $[VPGFG-(IPGVG)_4]_{14}$ as the hydrophobic block. This study showed that changing the temperature or the pH enabled adjusting the dynamics of nanoparticle formation thereby allowing a high degree of control over the morphology of the particles, which is of great importance in drug delivery applications. Indeed the different hydrophobicities of the guest residues present in the two blocks allow formation of micelles at room temperature while the block ELPs were present as unimers at cold temperature. The presence of glutamic acid residues allows shifting the temperature of micellization depending on the pH.

ELP-based nanoparticles have then been extensively studied by Chilkoti and coworkers.^{49,80} They created a series of diblock copolymer based on a high T_t domain $[VPGVG-(VPGAG)_8-(VPGGG)_7]_n$ and a lower T_t domain $[VPGVG]_m$ of different chain lengths ($n = 64, 96, 128$; $m = 60, 90, 120$) and studied the influence of the more hydrophobic block size and of the hydrophobic/hydrophilic ratio on the resulting self-assembly. Indeed only ELP block copolymers with a hydrophilic-to-hydrophobic ratio between 1:2 and 2:1 were able to assemble into nanoparticles. The size of the micelle was controlled by both the total ELP length and hydrophilic-to-hydrophobic block ratio.⁸⁰ It has also been shown that a

minimum hydrophobic block size of around 48 ELP repeat units was required to form stable nanoparticle.⁸¹

- ELP triblock copolymers

Nanoparticles have also been obtained using ELP triblocks instead of ELP diblocks and different morphologies have been observed. ELP triblocks consisting of a hydrophilic block surrounded by two identical hydrophobic blocks spontaneously formed nanoparticles below the T_t of the hydrophobic blocks. Raising the temperature above this T_t lead to more compact and smaller micelles This structural change was induced by an helix-to-sheet switch.⁸² On the other hand, using ELP triblocks consisting of hydrophobic block placed in between two identical hydrophilic blocks formation of vesicle-like structures was suggested by TEM and AFM measurements.⁸³

ii) Hybrid copolymers

ELPs have also been conjugated to a large variety of other molecules to form hybrid systems.

They have for instance been combined to other recombinant protein such as silk-like polypeptides. These silk-elastin-like polypeptides have been studied either only utilizing the precipitation behavior of the silk domain^{84,85,86} or taking advantage of the thermoresponsive behavior of both domains.^{87,88}

Hybrid systems made of ELP and synthetic polymers have also been studied. For example copolymers of ELP-polyaspartic acid were synthesized and formed micelles able to retain an hydrophobic dye.⁸⁹ ELP-PEG conjugates of different sizes were also synthesized and self-assembled into well-defined micelles upon inducing ELP phase transition.⁹⁰ Poly(OEGMA) brushes on an ELP triblock copolymers have also been obtained. Interestingly the self-assembly process was directed by the $LCST$ of POEGMA not by the T_t of the ELP.⁹¹

ELPs combined with DNA were able to assemble combining the molecular recognition of complementary oligonucleotides with the $LCST$ behavior of the ELP allowing control of particle size, complexity and dynamics.⁹² This kind of hybrid materials were also studied for gene delivery applications.^{93,94}

Hybrid systems such as ELP-protein conjugates have also been proposed in the literature. ELPs were for instance successfully fused to 4 different proteins, blue fluorescent protein (BFP), chloramphenicol acetyltransferase (CAT), thioredoxin (Trx), and interleukin-1 receptor agonist (IL1Ra), either to the N -terminal or the C -terminal end of the ELP. This study proved that it is better to link them to the N -terminus to preserve a good expression and activity of the proteins.⁹⁵ Capsid protein (CP) conjugated

to a short ELP were also studied and formed virus-like particles of different sizes upon two different triggers: pH and temperature.⁹⁶

ELPs have also been fused to small molecules such as peptides or drugs. ELPs conjugated to an alpha helical amphipatic peptide (L4F peptide) were for example capable of forming nanoparticles.⁹⁷ Besides Chilkoti and coworkers have shown that small molecules with sufficient hydrophobicity ($\log D > 1.5$) introduced enough amphiphilicity for inducing self-assembly.⁹⁸ Moreover genetic engineering of ELPs allows introduction of residues for conjugation of therapeutics. For instance incorporation of either a lysine residue (Figure 11) or a cysteine residue in a hydrophilic ELP have been used to conjugate the appropriately functionalized drug Doxorubicin (Dox).^{99,100} An hydrazone linker between the ELP and the small hydrophobic molecule induced disassembly and release of the small molecule at low pH while having a minimal influence on the ELP *T_t*. An example of these ELP-drug conjugates was synthesized, not only with one drug molecule per ELP but with multiple copies of Dox and led to complete tumor regression due to good pharmacokinetics and accumulation of the nanoparticles.¹⁰¹

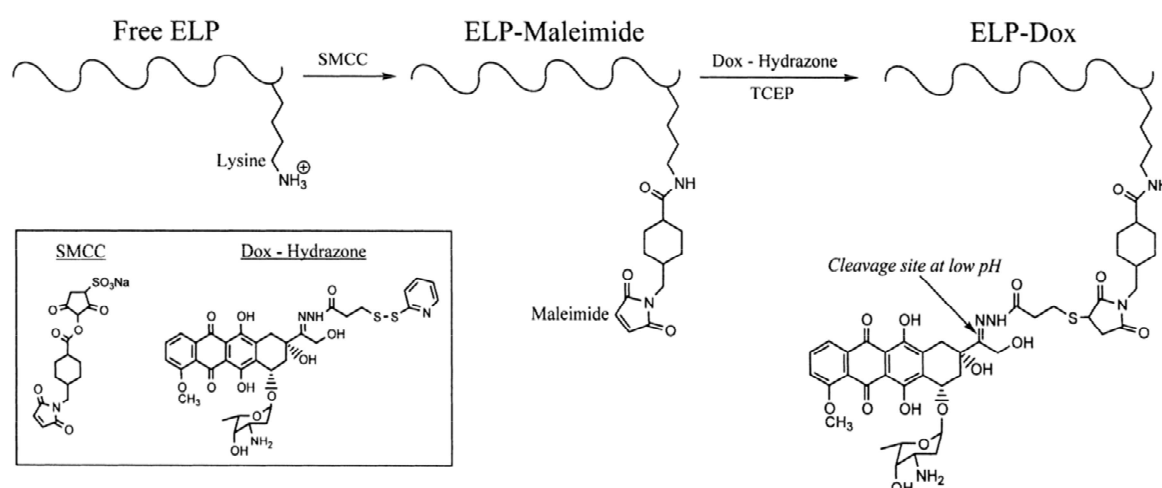


Figure 11. ELP-doxorubicin conjugation scheme, reproduced from Chilkoti⁹⁹

iii) Stability and degradation of the nanoparticles

For *in vivo* applications the disassembly of nanoparticles is an interesting parameter once the drug carrier has reached its target, however the premature disassembly is not desirable, it is therefore necessary to stabilize them. This has been done for instance by crosslinking cysteine-containing ELP nanoparticles with disulfide bonds.¹⁰² Incorporation of lysine residues at the ELP *N*-terminus can also help stabilizing particles. Van Hest *et al.* for instance used genipin to crosslink the lysine amine groups or turned the amines into azides then using strain promoted azide-alkyne cycloaddition (SPAAC).¹⁰³

Once ELP particles have reached their target and released their drug, it is then important to think of the future of these particles after degradation. As mentioned before, the biocompatibility of ELPs has been studied by Urry and coworkers through their model system. More recently the biodegradation of ELP-based nanoparticles subjected to elastases and collagenases has been investigated.¹⁰⁴ This study showed that self-assembled ELP nanoparticles are mildly protected against collagenases but not elastases. Moreover, in this study, the biodegradation of ELP nanoparticles occurred within low pH-compartment of cultured liver cells thereby supporting the use of ELP nanoparticles for the development of biodegradable drug carriers as hepatic lysosomes would likely be the final destination of these nanoparticles.

1.2.2.2. Targeted drug delivery

Studies on the self-assembly of ELP based polymers especially look for control over parameters such a morphology, size and polydispersity of nanoparticles which is of major importance for application as drug carrier to achieve good tissue penetration, encapsulation capacity and renal clearance for example. ELP nanoparticles are in fact very interesting candidates for the encapsulation and delivery of hydrophobic drugs as indicated by the large number of studies published in this field.^{98,102,105,106}

One of the purposes of drug delivery systems is to reach a specific site *in vivo*. This can be achieved by different manners.

i) Thermal targeting

To this end, hyperthermia treatment can be employed. An ELP whose T_t lies between physiological temperature (37°C) and the temperature that is used in “mild” hyperthermia (42°C) was reported (ELP1).¹⁰⁷ Another ELP with a T_t of 55°C served as a control insensitive to the thermal trigger (ELP2). The accumulation of these two fluorescently tagged ELPs in solid tumors was monitored. *In vitro* as well as *in vivo*, local heating of the tumor to 42°C resulted in aggregation of the first ELP and in a twofold increase in accumulation compared to tumors that were not heated or compared to the temperature insensitive ELP control.^{108,109} Upon cooling of the tumor, the ELP is resolubilized creating a gradient that pumps the ELP from the vasculature into the extravascular tumor space. The accumulation can even be improved by applying three thermal cycling to the tumors which nearly doubled the concentration of the thermoresponsive ELP (Figure 12).¹¹⁰

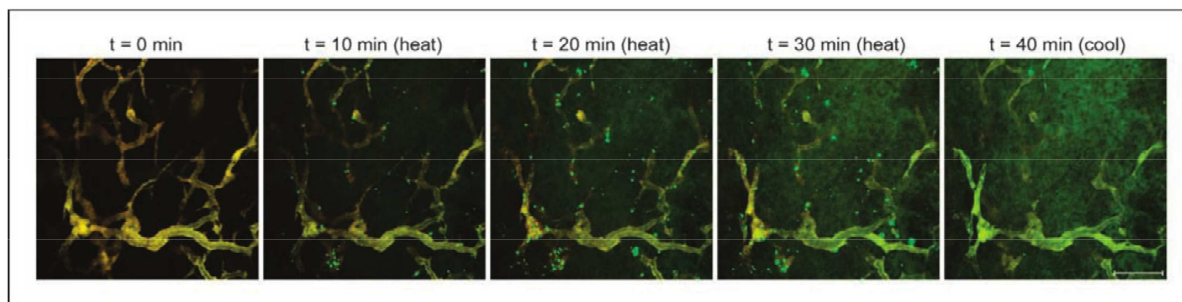


Figure 12. Images of thermally sensitive ELP1 (green) and thermally insensitive ELP2 (red) in a solid tumor before, during, and after a hyperthermia treatment. (...) reproduced from Chilkoti¹¹⁰

The combination of temperature responsive polymer carriers with hyperthermia showed several advantages. First, the ELP carrier improves drug's pharmacokinetics and lead to passive targeting by the enhanced permeability and retention (EPR) effect. Second, local hyperthermia of a solid tumor increases the permeability of tumor vessels, which helps the diffusion of the drug carrier into the extra-vascular space.^{111,112} Third, aggregation of ELP within the tumor under local hyperthermia, followed by its dissolution upon cessation of hyperthermia, increases the local concentration of the ELP in the tumor. The demonstration that combining the thermoresponsive properties of ELP with mild hyperthermia may improve delivery of anti-cancer drugs to tumors was pursued using radiolabeled ELPs.⁴⁸ This method facilitated the determination of the ELP's pharmacokinetics, tumor localization and tumor special distribution. It confirmed the accumulation results previously mentioned and also showed that the thermally sensitive ELP had a more homogeneous tumor distribution with a greater concentration in the tumor center compared to the control treatments (temperature insensitive ELP or temperature insensitive ELP without hyperthermia).

ii) pH targeting

Another particularly interesting approach in the scope of tumor targeting is the pH-sensitivity. To this end the introduction of histidine as guest residues in the ELP sequence is one option. The pH-sensitive ELP [VPGVG-(VPGGG)₇-(VPGAG)₈]₅-[VPGVG(VPGHG)₄]₂₀ for example turned out to form aggregates at low pH (≤ 6.4) because of the protonated histidines that increased the T_t of the more hydrophobic block approaching the T_t of the hydrophilic block leading the system to behave like one single ELP block.¹¹³ This ability to disassemble at low pH is interesting to target the tumor environment or to release a drug upon internalization in endosomes.

iii) Targeting using specific interactions

Precise delivery can also be achieved by taking advantage of specific interactions, for example between a cell penetrating peptide (CPP) and the components of cell membrane. The lab of Chilkoti for example designed an ELP diblock with a CPP domain at the hydrophilic end. Upon thermal trigger these block copolymers formed micelles with a multivalent display of the CPP, enabling the internalization of these particles (Figure 13).^{114,115} This multivalency led to an amplified uptake into targeted cells. The cellular uptake of ELPs conjugated to CPP such as Tat or penetratin has also been evaluated, leading to conjugation to the kinase inhibitor p21, stopping the cell proliferation.¹¹⁶ Cellular CPP delivery was also investigated combined with hyperthermia.¹¹⁷

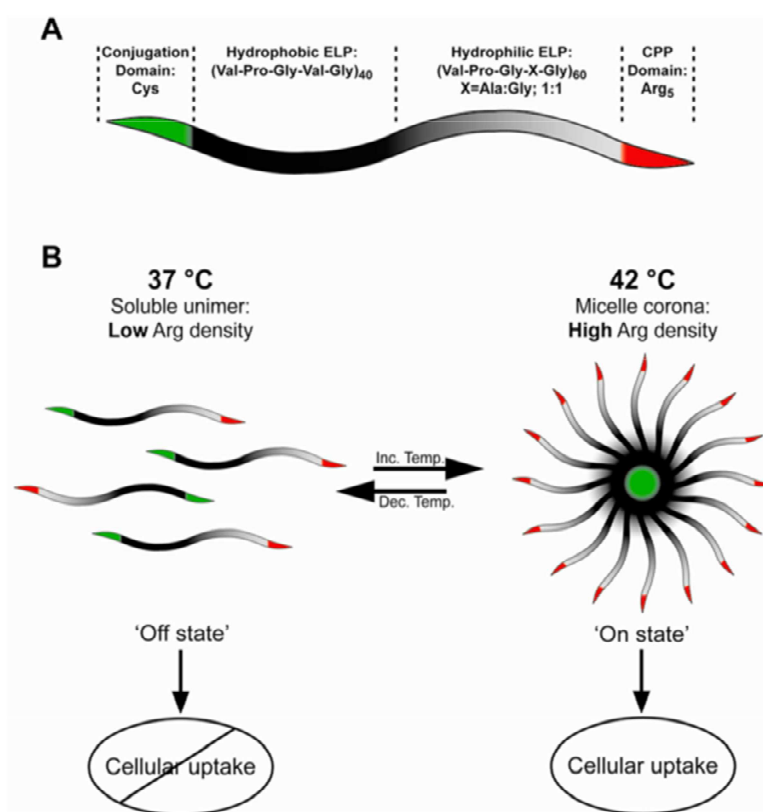


Figure 13. (A) Controlled cellular uptake by modulation of local Arg density is achieved by temperature-triggered micelle assembly of a genetically encoded Arg5-ELPBC. (B) ‘Off state’: At 37 °C, below the CMT, Arg5-ELPBCs exist as soluble unimers, whose limited number of Arg residues is below the threshold necessary for efficient cellular uptake. **‘On state’:** Above the CMT at 42 °C (a temperature that can be achieved with mild clinical hyperthermia) Arg5-ELPBCs assemble into spherical micelles, reproduced from Chilkoti¹¹⁴

Combination of specific targeting with hyperthermia is an interesting method in order to develop dynamic affinity modulation. Indeed even if high affinity carriers show increased accumulation at the targeted site¹¹⁸ these methods can also lead to reduced specificity,¹¹⁹ due to off-site targeting if healthy

tissue express the same receptor even though at lower concentration, thereby resulting in higher toxicity.¹²⁰ Therefore combination of ligand receptor targeting and hyperthermia was explored using GRGDS peptide, incorporated to the ELP at the gene level, as the ligand and $\alpha_v\beta_3$ integrin as the receptor.⁸⁰ Nanoparticles formed upon thermal trigger were massively internalized in the cells overexpressing the receptor while monovalent RGD-ELP was not significantly uptaken by these cells.¹²¹

Tunable binding has been shown using mixed micelles made of two different ELP block copolymers, [(VPGAG)₄-(VPGVG)]₁₆-[(VPGIG)₄-(VPGVG)]₁₂ and [(VPGPG)₄-(VPGVG)]₁₆-[(VPGIG)₄-(VPGVG)]₁₂, the former one bearing a fibrinogen-binding peptide on its N-terminus. The micelles formed upon increasing the temperature above the first T_t were shown to bind fibrinogen due to multivalent presence of binding peptides on the micelles. After increasing the temperature above the T_t of the block bearing the peptide the block collapses onto the particles resulting in release of fibrinogen. These double temperature responsive micelles allow switchable fibrinogen binding.¹²²

iv) Local drug delivery

Apart from targeted drug delivery using nanoparticles, ELPs can also be employed for local delivery. Several strategies can be considered for local delivery: coacervation of a soluble ELP triggered by body temperature, formation of an ELP drug depot by the *in situ* chemical crosslinking of ELPs, or crosslinked ELP depots containing a drug can be made *ex vivo* and implanted *in vivo* for the sustained drug release.

In the first strategy, the designed ELP has a T_t between room temperature and physiological temperature so that highly viscous ELP coacervates can be formed upon local injection. ELP fusion proteins have been delivered *via* this technique and exhibited a half-life 25-fold longer than the temperature insensitive, soluble ELP control. The ELP coacervate is then cleared over time by disaggregation and degradation into biocompatible units.¹²³ Another example is the ELP-IL-1Ra fusion protein that was able to coacervate at a temperature between room and body temperature.¹²⁴ As for the tumor necrosis factor receptor type II (sTNFR_{II}) conjugated to an ELP it exhibited a sevenfold increased half-life compared to a soluble ELP control.¹²⁵

Chemically crosslinked depots were also made for local delivery of encapsulated antibiotics.¹²⁶ Antibiotics were mixed with the ELP before its crosslinking leading to entrapment of the therapeutics within the ELP hydrogel.

I.2.3. Tissue engineering

ELPs are inspired by elastin, an extracellular matrix protein, so their properties are very well-suited for tissue engineering applications. In fact they have already been applied several times in this field to make for example: vascular grafts, cell sheets, cartilage repair, ocular surface scaffolds, or substrate for stem cell differentiation.^{127,128,129,130,131}

There are several strategies to fabricate ELP scaffolds for tissue engineering: coacervation of a soluble ELP,^{131,132} physical crosslinking,^{129,133} chemical crosslinking (including photocrosslinking),¹³⁴ or enzymatic crosslinking.¹³⁵

The thermally triggered formation of ELP coacervates is the easiest method. Indeed cellular loading can simply be carried out *ex vivo* by increasing the temperature of an ELP-cells suspension above the T_t of the ELP.¹³¹ These ELP coacervates have been used for cartilage tissue engineering¹³² and also to create cell sheets.¹²⁷ However this technique is limited to some applications because contrary to hydrogels, ELP coacervates have limited structural stability and stiffness.

Besides, ELP triblocks consisting in a central hydrophilic domain surrounded by two hydrophobic blocks have demonstrated successful physical crosslinking.¹³⁶ These hydrogels are very versatile as their mechanical properties can be tuned either by the solvent in which ELP gels are formed or by the hydrophobic content of the ELP triblock, larger hydrophobic domains giving higher elastic modulus.¹³⁷ However, physically crosslinked ELP hydrogels are not suitable for some tissue engineering applications because of insufficient strength.

This problem can be solved by the formation of chemical crosslinks between the ELP chains. ELP crosslinking can be achieved by introducing lysine residues in their sequence and binding these residues *via* an electrophilic crosslinker such as bis(sulfosuccinimidyl),^{138,139} or trifunctional crosslinker such as tris-succinimidyl aminotriacetate.¹⁴⁰ Modulation of the mechanical properties is possible by changing polypeptide concentration, lysine frequency, or the molecular weight of the ELP. Maximum stiffness was observed with high concentration, high molecular weight and high lysine content. The choice of crosslinker is also of major importance, it should be non-toxic and the use of organic solvents for the chemical crosslinking reactions should be avoided. Therefore crosslinkers that can be used in aqueous conditions such as hydroxymethylphosphines (HMP) are usually chosen.¹⁴¹ The mechanical properties of these hydrogels are controlled by the number of lysine and the pH of the crosslinking reaction. Therefore a library of ELPs was designed with varied molecular weights and

lysine spacing, these ELPs were crosslinked at different concentrations in order to investigate the effect of these variables on the structural properties and morphology of the crosslinked ELP hydrogels. In a study β -[tris(hydroxymethyl)phosphino]propionic acid (THPP) was used as a crosslinker. The lysine amount, the molecular weight of the ELP, the pH of the crosslinking reaction, and the concentration of the crosslinker were all optimized to investigate their effect on the properties of the hydrogel and to allow mixing of the cells with the ELP before crosslinking and still preserving the cells viability. One of these hydrogels was used for cartilage repair¹⁴² and showed good results after 3 months but these results were not confirmed after 6 months. Use of the same crosslinker (THPP) but with ELP triblocks composed of a central hydrophilic block and two terminal hydrophobic blocks containing lysine residues formed larger pore diameters compared to hydrogels made of single segment ELPs. Varying length of the hydrophilic block allowed modulation of the structure, swelling and mechanical properties. This architecture turned out to be more favorable to fibroblast viability and proliferation than hydrogels formed with single block ELPs (Figure 14).¹⁴³

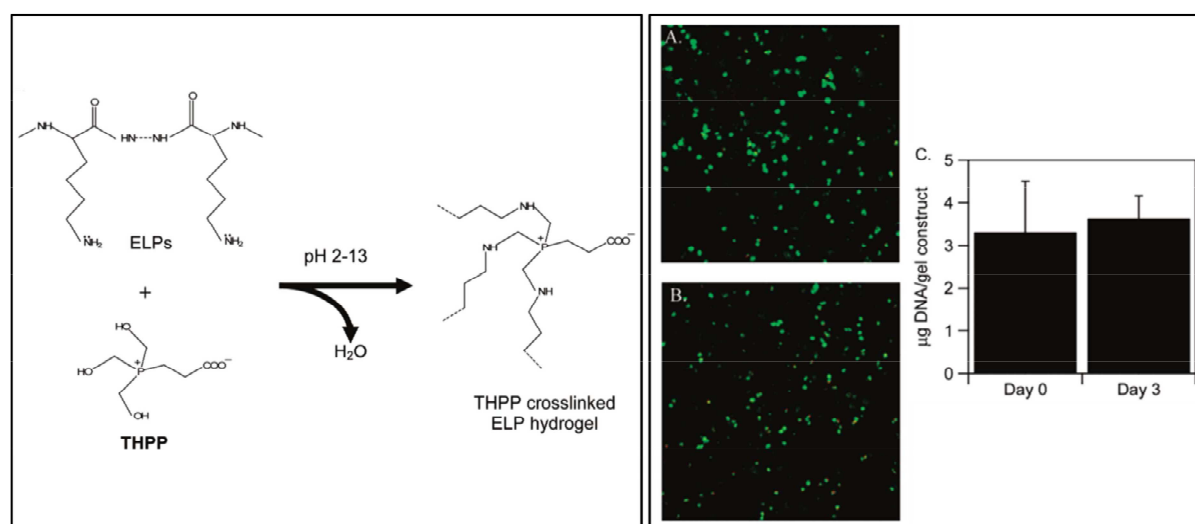


Figure 14. Left panel: cross-linking mechanism between Lys residues of ELPs and THPP. Right panel: Fluorescence microscopy of fibroblasts encapsulated in rapidly cross-linked ELP hydrogels and their DNA content per hydrogel. Cell survival of encapsulated fibroblasts was evaluated at (A) day 0 and (B) day 3 via a fluorescent cell viability/cytotoxicity assay. The DNA content of fibroblasts within each hydrogel was quantitatively measured by Picogreen DNA assay. adapted from Chilkoti¹⁴¹

To perform enzymatic crosslinking on ELPs, either glutamine or lysine were introduced at the guest residue position and tissue transglutaminase (tTG) was used as the enzyme. This allowed crosslinking between glutamine residue and the amine of the lysine.¹³⁵ The stiffness of the obtained hydrogel was compared with a coacervate from the two ELPs and gave better results. The same enzyme has also

been tested to crosslink ELP and collagen through their lysine and glutamine residues.¹⁴⁴ The mechanical properties were improved compared to a material composed of collagen alone.

In order to improve the interactions between the ELP scaffold and the cells, peptides can be introduced within the ELP chain. For instance, mimicking ligands found in the extracellular matrix ECM allowed targeting the integrin class of cell surface receptors.^{129,133,145} REDV peptide from CS5 domain of fibronectin could for instance support endothelial cells adhesion and spreading.¹⁴⁶ Increase of bovine aortic endothelial cells adhesion to ELP hydrogels containing RGDS peptide was observed^{147,148} and the mechanical properties of these gels could be improved by crosslinking.^{149,129,150} Moreover, cellular adhesion can be tuned depending on the density of binding domains.¹⁴⁹ ELP hydrogels displaying CS5 fibronectin domains and reactive lysine residues were also designed.¹⁵¹ The one formed with high cross-link density and thus low molecular weight ELP demonstrated mechanical properties close to those of elastin. ELP films were also made with comparable design but with lysine residues close to the ELP terminal end.^{152,153,154} The strength of human umbilical vein endothelial cells (HUVEC) attachment and spreading was higher on ELP gels crosslinked via chain termini than on the ELP gels crosslinked *via* lysine residues spread along the ELP chains (Figure 15).

It should be noted that non-specific cell binding can sometimes occur. To avoid this problem, conjugation of PEG to ELP chains allowed decreasing of nonspecific binding. Indeed significant reduction in cellular attachment was observed, without affecting the specific binding associated with the RGD binding ligand.¹⁴⁹

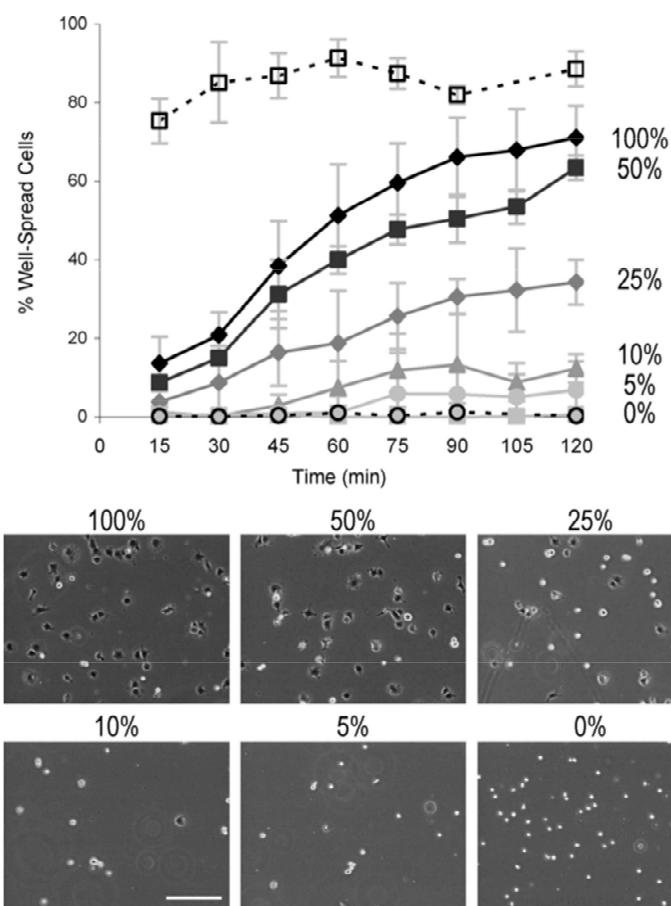


Figure 15. HUVEC spreading on cross-linked aECM films. Cell spreading kinetics can be modulated by varying the density of adhesion ligands. Fibronectin (\square) and BSA (\circ) controls are represented by dotted lines. The percentages to the right of the traces indicate the percentage of aECM-RGD-PEG in each film. Phase contrast micrographs show that substrates with higher aECM-RGD-PEG content have a larger number of dark, well-spread cells and fewer bright, rounded cells after 90 min of incubation. Reproduced from Tirrell¹⁴⁹

After a while these ELP scaffolds should however be eliminated from the body. The incorporation of protease cleavage sites, such as the VGVAPG sequence (found in natural elastin) within the ELP sequence is one method that has been considered for sustained biodegradation of the scaffolds.^{130,133}

II. BIOCONJUGATION REACTIONS ON POLYPEPTIDES/PROTEINS

In nature the post-translational modification of proteins is very common and allows increasing the variety of their structures and functions.^{155,156} Scientists have tried to mimic nature's ability to introduce so diverse and so precise modifications, however they are limited by the lack of chemical methods available. Indeed chemoselective modification of a selective amino acid is very tedious when surrounded by a plethora of reactive functions: amines, alcohols, carboxylic acids, thiols. The search for chemical strategies to selectively modify proteins has thus been widely studied and reviewed in several publications.^{157,158,159,160}

Proteins have been modified either by chemical reactions on natural amino acids or by the introduction of unnatural amino acids and subsequent reactions. We will mostly focus here on the modification of natural amino acids and refer to the published reviews concerning unnatural amino acids.¹⁶¹

II.1. BIOCONJUGATION OF POLYPEPTIDES/PROTEINS CONTAINING NATURAL AMINO ACIDS

II.1.1. Bioorthogonal reactions on natural amino acids (except methionine) and N-terminus

The most modified residues in proteins are cysteine, lysine (Figure 16), tyrosine, tryptophan as well as the *N*-terminal end.

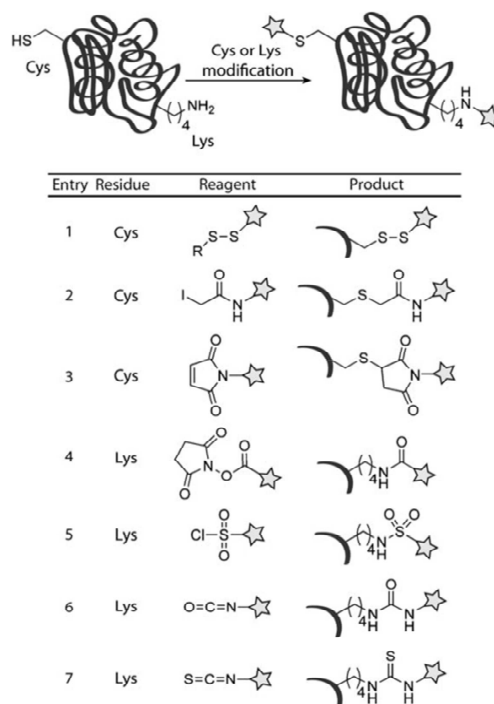


Figure 16. Classical bioconjugation reactions for the modification of Cys and Lys residues, reproduced from Bertozzi¹⁵⁷

II.1.1.1. Cysteine

As seen in nature the thiol group of cysteine is a very robust nucleophile and a unique reactive moiety in proteins. Selective reactions at cysteine over other nucleophilic amino acids such as lysine and histidine can be achieved under controlled pH. Also the low abundance of cysteine in proteins (< 2%) allows modification at a single site.¹⁶² The selective reaction of electrophiles such as α -halocarbonyls or maleimides has for example been reported.¹⁶³ For instance bromomaleimides derivatives have been used for reversible cysteine modification in Grb2 protein, allowing modulation of activity and *in vivo* monitoring (Figure 17).¹⁶⁴ Moreover glycosylated bovin hemoglobin could be synthesized by coupling maleimide-lactoside conjugates to cysteine residues.¹⁶⁵ A vaccine candidate has also been synthesized through the coupling of maleimide terminated KLH protein and a cysteine bearing antigen.¹⁶⁶ More recently, Ball and coworkers reported a cysteine modification reaction with diazo reagents through rhodium(II) catalysis leading to good selectivity under mild conditions.¹⁶⁷

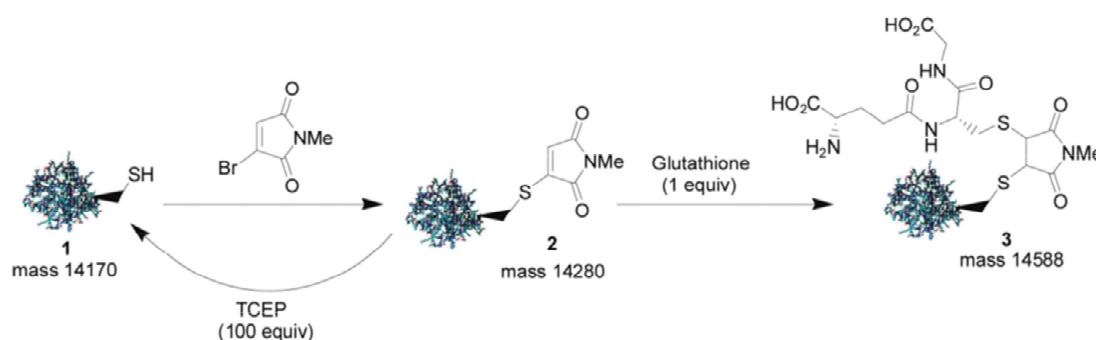


Figure 17. Modification of the Grb2 SH2 Domain (L111C) with N-Methylbromomaleimide, reproduced from Baker¹⁶⁴

Sulfur also has the ability to engage in disulfides bonds which are essential in maintaining protein tertiary and quaternary structure through interchain bridges. Disulfides bonds between thiol and cysteine can form at ambient temperature and can thus be used for synthetic modification of proteins. For instance chemical modification of SBL (subtilisin *Bacillus lentus*) enzyme with methanethiosulfonate reagents lead to a novel enzyme with better catalytic activity as compared to the wild type enzyme.¹⁶⁸ Moreover glycosylation of proteins such as BSA or SBL has been achieved thanks to glycomethanethiosulfonate or glycophenylthiosulfonate reagents *via* disulfide bridges formation.^{169,170,171} Disulfides coupling can also occur *via* the use of selenenyl–sulfides either placed on

the protein then coupled to a thiol functionalized sugar (electrophilic strategy) or placed on the sugar reagent to be directly coupled to a cysteine residue (nucleophilic strategy).¹⁷²

When positioned at the *N*-terminal end, cysteines can also react through native chemical ligation (NCL) leading to the formation of an amide bond. Kent et al. first reported the ligation of thioesters to *N*-terminal cysteine in 1994.¹⁷³ NCL is a powerful technique for modification, synthesis and semisynthesis of proteins under physiological conditions and has allowed the synthesis of proteins larger than the one obtained through traditional solid-phase peptide synthesis.¹⁷⁴ It has also allowed to isotopically label segments of proteins which facilitated NMR structural studies.^{175,176}

II.1.1.2. Lysine

Unlike cysteine, lysine is a highly abundant amino acid in proteins. It however remains a commonly used residue for protein modifications as its highly nucleophilic amine can be subjected to many reactions.¹⁵⁷ Conjugation to lysine residues has for example been used to coat virus particles with proteins antigens, where multiple conjugations were needed.¹⁷⁷ In the case where selective reaction on lysine's amine over cysteine's thiol was necessary, harder electrophiles have been used such as activated esters,¹⁷⁸ for mass spectrometry studies, isothiocyanates,¹⁷⁹ to investigate the reactivity of this reagent under physiological conditions, or unsaturated aldehydes esters¹⁸⁰ for PET (Positron Emission Tomography) imaging purpose (Figure 18).

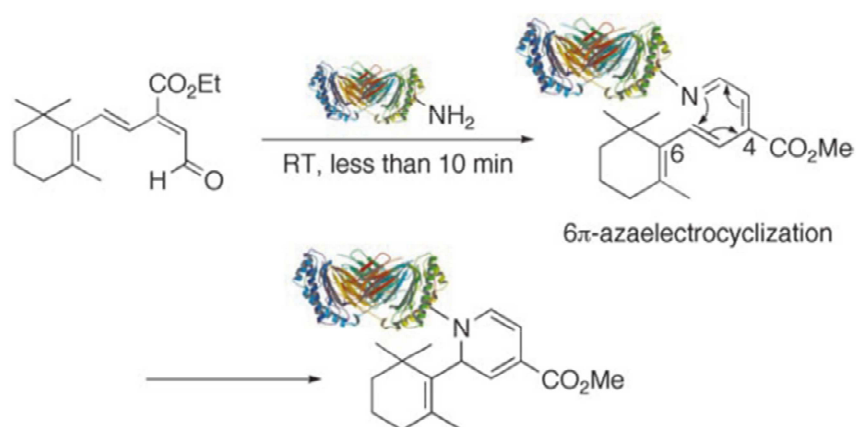


Figure 18. Rapid reaction with lysine through 6π-aza-electrocyclization, reproduced from Fukase¹⁸⁰

Aldehydes in the presence of sodium cyanoborohydride have also been used to perform reductive alkylation of lysine residues.¹⁸¹ Sodium cyanoborohydride was preferred to sodium borohydride because it allows more efficient labeling and avoid the side reactions caused by the latter. The use of

harsh reagent such as cyanoborohydride has also been avoided for this type of reaction by using iridium based catalyzer.¹⁸²

II.1.1.3. Tyrosine

Unlike cysteine and lysine residues, tyrosine has raised interest for protein modification purpose only recently. As tyrosine residues are relatively rare in proteins they are a good choice for modification at a single position. Reactions on tyrosine residues often involved metal mediated modifications. This strategy has first been explored by Kodadek *et al.* who explored the coupling of two proteins by nickel(II) catalyzed oxidative cross-linking.^{183,184} This method has been used by Finn *et al.* to couple alkyne dies to viral capsid proteins.¹⁸⁵

As shown by Francis and coworkers the use of Mannich type reaction involving aldehydes and anilines has allowed highly selective coupling of a chromophore to the tyrosine containing proteins: chymotrypsinogen A or lysozyme.^{186,187,188} Chymotrypsinogen A was also fluorescently labeled through palladium-catalyzed reactions on its tyrosine residues.¹⁸⁹

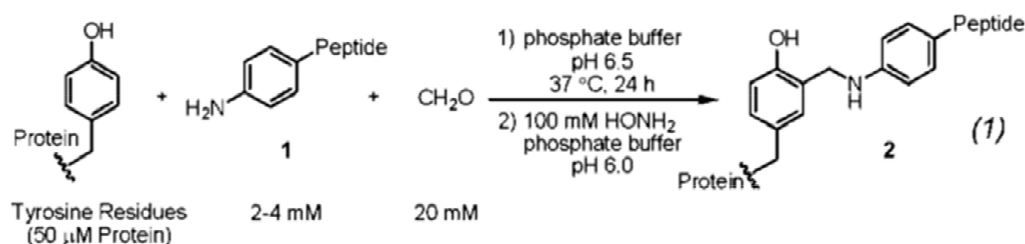


Figure 19. Mannich-type coupling reaction for the conjugation of synthetic aniline-containing peptides (1) to native tyrosine residues on proteins, reproduced from Francis¹⁸⁸

II.1.1.4. Tryptophan

As well as for tyrosine much recent studies have focused on the modification of tryptophan residues. Tryptophan is the rarest amino acid, so it allows site-specific modification of proteins. It has been proved that tryptophan-containing proteins such as myoglobin or subtilisin can be modified in the presence of metallocarbenes in aqueous solution.¹⁹⁰ While this reaction initially required harsh conditions it has been more recently shown that it could be run at pH 6 instead of pH 3 by adding *t*BuNHOH in order to promote the formation of rhodium carbenoids under mild aqueous conditions.¹⁹¹

Moreover Pictet-Spengler reaction has been used to couple the *N*-terminal tryptophan of a peptide to an aldehyde terminated peptide (Figure 20).¹⁹²

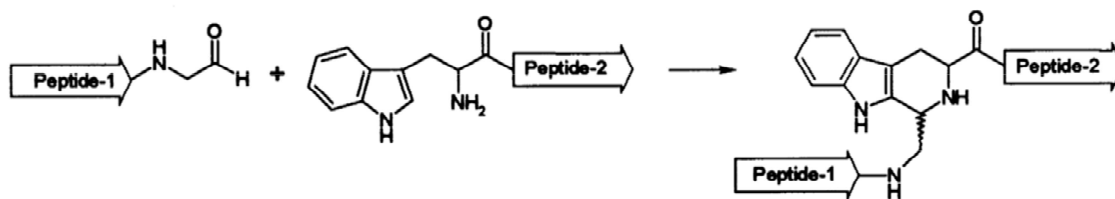


Figure 20. Ligation of unprotected peptide segments using the Pictet–Spengler reaction, reproduced from Tam¹⁹²

II.1.1.5. *N*-terminus

Because of its pH-dependent reactivity, the *N*-terminal end of proteins is an interesting target for site-specific modifications. The amino group at the *N*-terminus has a lower pKa compared to the lysine's amino group which allows selective acylation or alkylation, albeit difficult when surrounded by many lysines.¹⁹³ Transamination reaction on the other hand were proved to be particularly effective since pioneered by Bonetti *et al.*¹⁹⁴ However, in that first study very high and denaturing temperature was used. This problem was solved later on by Dixon by using a cupric salt and pyridine as catalysts.¹⁹⁵ Further improvements of transamination reaction were done by Francis and coworkers who managed to perform myoglobin transamination under physiological conditions (Figure 21).¹⁹⁶

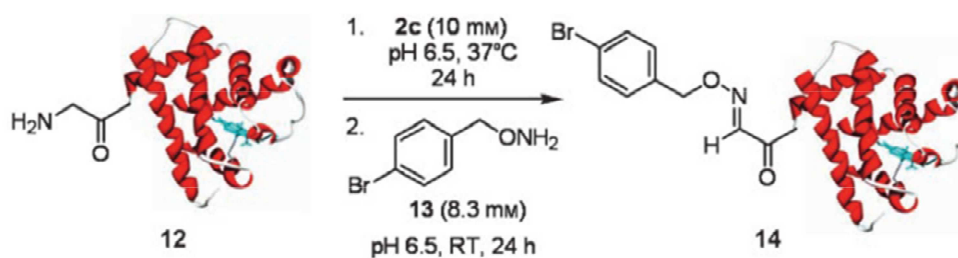


Figure 21. Myoglobin site-selective modification via transamination reaction under physiological conditions, reproduced from Francis¹⁹⁶

II.1.2. The case of methionine

Among the natural amino acids methionine is a particularly interesting residue because of the unique reactivity of its thioether group. Indeed methionine has the interesting feature to be the most reactive nucleophile present in peptides and proteins under acidic conditions because it is not protonated at low pH.^{197,198,199} Therefore methionine side-chain can be subjected to very efficient alkylation reactions leading to stable sulfonium derivatives without requiring any protecting groups. The orthogonal post-

modification of methionine residues has been pioneered decades ago by Toennies.^{200,201} Several alkylating agents have then been used to modify methionine, for example alkyl halides such as iodomethane²⁰² or iodoacetic acid.^{203,204,205}

Alkylation of synthetic polymethionine was first performed by Katchalski using alkyl bromides to form stable and water-soluble methyl and carboxymethyl sulfoniums.²⁰⁶ The configuration of these polymers was evaluated in different solvents and their polyelectrolyte behavior in aqueous solution was studied. More recently the tensile properties of sulfonium-containing polymer fibers were tested.²⁰⁷ Deming and coworkers really developed the alkylation of polymethionine by introducing a large variety of functional group using different alkylating agents. Polymethionine and methionine containing polypeptides were modified by a series of alkyl halides and alkyl triflates and allowed the synthesis of a broad library of functional materials with controlled and high molecular weights.^{208,209} The reversibility of the sulfonium formation was also shown by performing stability and dealkylation tests on these polysulfoniums.²⁰⁹ In order to introduce new functional groups in their methionine containing polypeptides using more stable and more easily accessible reagents, Deming *et al.* investigated the use of epoxides as alkylating agents (Figure 22). This method proved to be very versatile as it allowed them to introduce a large number of functional groups even when bulkier reagents were used. The obtained polypeptides exhibited high water solubility and good stability against dealkylating conditions.²¹⁰

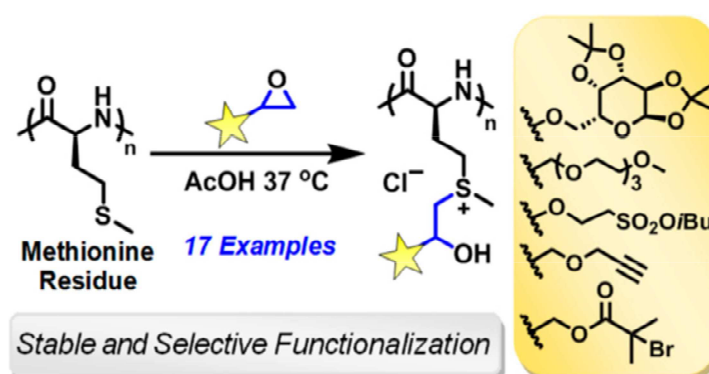


Figure 22. Versatile functionalization of methionine residues using epoxides, reproduced from Deming²¹⁰

II.2. MODIFICATION OF ELASTIN-LIKE POLYPEPTIDES

II.2.1. Modification of ELPs chain-ends

Chemical modification of ELP chain ends has been explored in several studies and allowed conjugation of ELPs to several types of molecules.

Chilkoti and coworkers for instance studied the conjugation of ELPs to different drug molecules. They first carried out their experiments using small organic molecules as model compounds.⁹⁸ This allowed them to observe the self-assembly behavior of their ELP conjugates depending on the hydrophobicity of the small molecules. A cysteine rich segment introduced at the C-terminus of the ELP allowed introduction of the different maleimide derivatives *via* Michael addition reaction. They then pursued their study by introducing three therapeutic molecules: gemcitabine, oxycodone and paclitaxel, to the ELP chain end. Chilkoti *et al.* also investigated the chemical conjugation of doxorubicin to ELPs by two different methods. The first strategy involved an ELP with a lysine residue incorporated in the leader sequence. The lysine residue was modified with succinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (SMCC) to provide an ELP-maleimide conjugate which was then conjugated to the doxorubicin-thiol derivative.⁹⁹ The other strategy involved an ELP with a C-terminal cysteine residue that could be conjugated to a maleimide-activated doxorubicin.¹⁰⁰

ELPs have also been conjugated to oligonucleotide sequences for gene delivery applications.⁹² In this study an NHS-activated oligonucleotide (ODN) was coupled to the *N*-terminal-end of an ELP composed of 40 VPGVG repeats. The molecular recognition of a mixture of ODN-ELPs in which each ODN was designed to be complementary to two other ODNs led to the formation of monodisperse starlike architecture composed of a DNA core and a pendant polymer. In another study, an ELP was used as a macroinitiator for the ring opening polymerization of β -benzyl-L-aspartate *N* carboxyanhydride (NCA) to yield an ELP-p(BLA) conjugate. The p(BLA) segment was further modified using diethylenetriamine to form a cationic diblock copolymer that was able to form polyplexes in the presence of pGL4 plasmid. These polyplexes showed appreciable gene transfection efficiency with low cytotoxicity.

This last example showed the conjugation of an ELP to a synthetic polypeptide, but ELPs have also been conjugated to synthetic polymers such as polyethylene glycol (PEG).⁹⁰ Van Hest and coworkers developed two different strategies allowing the conjugation of ELP segments (ELP40, ELP90 or ELP130) to either one or two PEG segments (Figure 23). The key step leading to one strategy or the other was the metal-free and pH controlled diazotransfer. Carried out at pH 8.5 this step only led to the conversion of the *N*-terminal amine into an azide moiety while when carried out at pH 11 both the *N*-terminal amine and the amine of the lysine residue incorporated in the ELP leader sequence were converted to azide moieties. These azides were then clicked to bicyclo[6.1.0]nonyne(BCN)-PEG derivatives by strain-promoted alkyne-azide cycloaddition.

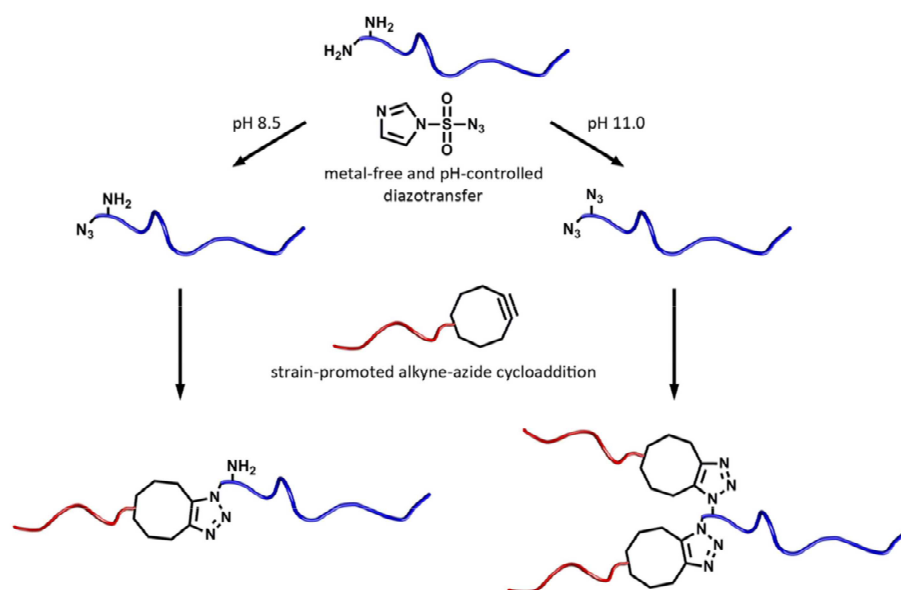


Figure 23. General scheme of the ELP-PEG block copolymers synthesis, reproduced from van Hest⁹⁰

II.2.2. Modification of ELPs backbone

Modification of ELPs backbone have been more scarcely reported and mostly applied to lysine-containing ELPs.

For example ABA triblock ELPs composed of two lysine rich blocks (A) and one neutral, hydrophilic, lysine-free block (B) were designed and ATRP initiator were introduced on the lysine residues. Then *in situ* ATRP was carried out to grow a poly(OEGMA) brush from the ELP backbone.⁹¹

ELP backbone modifications were also used to chemically crosslink gels for tissue engineering applications. In every cases the ELP chains were crosslinked through their lysine residues using different crosslinking agents: bis(N-hydroxysuccinimidyl),¹³⁹ tris-succinimidyl aminotriacetate¹⁴⁰ or β -[tris(hydroxymethyl)phosphino]propionic acid (THPP).¹⁴¹ Lysine-containing ELPs were also used to form ELP-PEG gels in aqueous solution *via* Mannich-type condensation using tris(hydroxymethyl)phosphine (THP) as a crosslinking agent (Figure 24).²¹¹ These hybrid ELP-PEG hydrogel systems combined both the tunability of ELP hydrogels and the optical advantages of PEG hydrogels improving the transparency of the gel for better imaging of fibroblasts encapsulation.

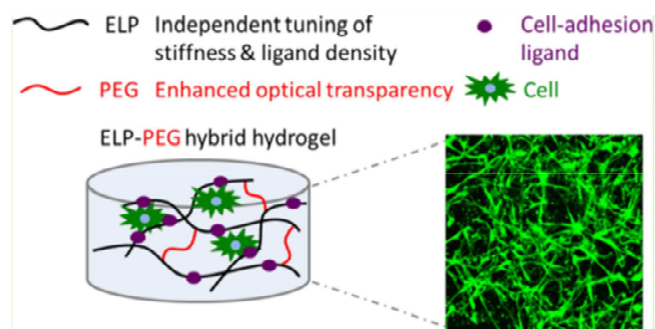


Figure 24. Sketch representing ELP-PEG hybrid hydrogel and cell encapsulation imaging, reproduced from van Hest²¹¹

Proteins can also be modified by residue specific incorporation of non-canonical amino acids as reviewed by Tirrell *et al.*¹⁶¹ This method has been used by van Hest and coworkers to synthesize clickable ELPs.²¹² Non-natural amino acids were introduced in the ELP sequence: either azidohomoalanine (AHA-ELP) or homopropargylglycine (HPG-ELP). Successful ELP functionalization was demonstrated with small fluorescent probes as well as with larger biomolecule such as Cal B enzyme.

III. GLYCOPROTEINS AND GLYCOPOLYPEPTIDES

III.1. GLYCOPROTEINS

III.1.1. Natural glycoproteins

Glycoproteins are an important class of biomacromolecules, ubiquitous in nature and involved in many biological processes.^{213,214} Indeed glycoproteins cell receptors are involved in cell membrane interactions with bacteria and viruses as well as communication between cells (Figure 25). For instance it has been reported that glycoproteins have a critical role in neuronal development, hormone activities, immune surveillance, and inflammatory responses.^{215–218} Along with their central role in communication processes, glycans also have an important role in proteins structure as they stabilize their tertiary structure.²¹⁹ In fact, without proper glycosylation many proteins will not fold correctly.²²⁰ It has also been observed that glycosylation result in enhanced proteolytic stability,^{221–224} *In vivo* glycosylation is one of the most complex post-transcriptional mechanism²²⁵ which relies on a large range of enzymes producing a diversity of oligosaccharides called glycoforms. These glycans are linked to the protein backbone in a controlled manner leading to the formation of glycoproteins with complex structures.²²⁶ However these structures and the role of glycoproteins are not completely understood yet. Because of the difficulty to obtain pure glycoforms, the preparation of synthetic glycoproteins is an interesting alternative to study these biomolecules in order to better understand their functions as highlighted by the number of studies carried out in this field for the past few years.^{213,214,227–230}

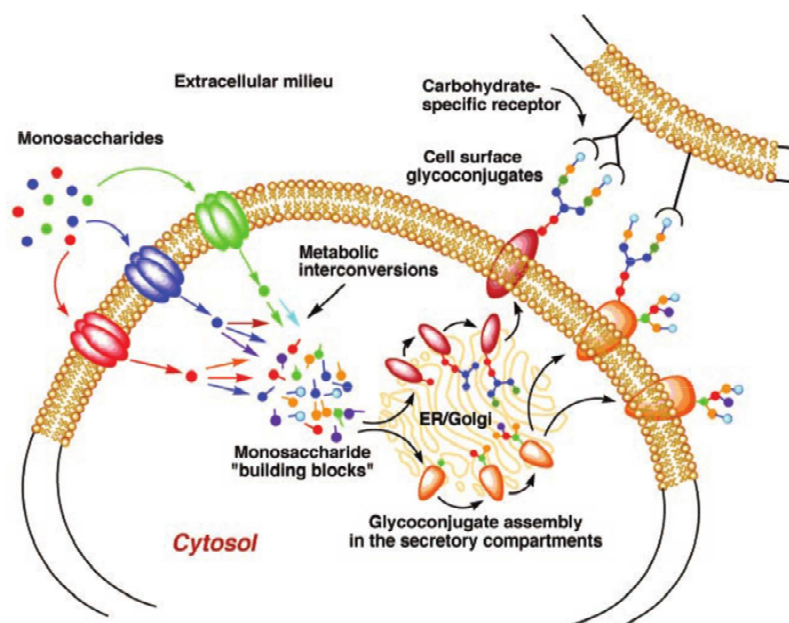


Figure 25. Glycoconjugate biosynthesis and cell surface recognition, reproduced from Kiessling²¹⁸

III.1.2. Synthetic glycoproteins

Synthetic techniques have been recently developed to produce glycoproteins as an alternative to biological production.^{218,231} The methodology consists in combining both peptide and carbohydrate chemistry, which is really challenging, especially to form carbohydrate-amino acid bonds. In nature these bonds are generally N-glycosidic bonds or O-glycosidic bonds. In synthetic glycoproteins, these natural linkages can be used as well as unnatural ones such as thioethers, oximes, triazoles, allyls, etc.²¹³

Therefore several methods are available for the synthesis of glycoproteins: solid-phase peptide synthesis, native chemical ligation (NCL), expressed protein ligation (EPL), enzymatic synthesis.

III.1.2.1. Solid-phase peptide synthesis

The synthesis of glycoproteins *via* solid-phase peptide synthesis (SPPS) can be achieved through two different strategies: a linear strategy (Figure 26) and a convergent strategy. In the linear strategy, glycosylated amino acid derivatives are assembled with each other and with other amino acids step by step. Complex glycoproteins have been obtained through this strategy but it required the use of protecting groups.²³² In the convergent strategy, the peptidic backbone is first obtained, and then followed by the chemical coupling of carbohydrates.²³³ The SPPS methods has allowed the preparation of natural glycopeptides such as HIV-1 V3 domain or several antifreeze glycoprotein analogues.^{234,235} However the size of the peptides obtained by this method is limited to a maximum of around 50 residues and the yields obtained decrease with the length of the peptide. Therefore synthesis of glycoproteins is limited through this method but can be overcome by using chemical ligation techniques.

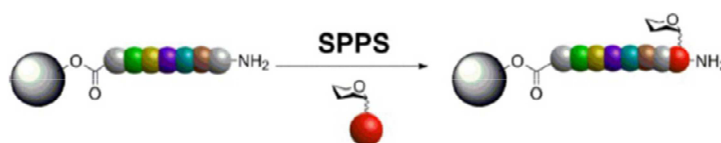


Figure 26. Representative scheme of glycoprotein synthesis via SPPS using a linear strategy, reproduced from Lecommandoux²¹⁴

III.1.2.2. Native Chemical ligation (NCL) and Expressed Protein Ligation (EPL)

In order to pursue the convergent strategy mentioned above different chemical ligation methods have been developed. Therefore individual peptides can be assembled stepwise by these methods to achieve

the synthesis of larger glycoproteins.²³³ One of these methods, the native chemical ligation (NCL), involves the chemoselective reaction between two peptides, one with an N-terminal cysteine the other one with a C-terminal thioester (Figure 27). Over the last decades this method has been significantly improved and allowed the synthesis of several glycoproteins.^{236,237,238,239}

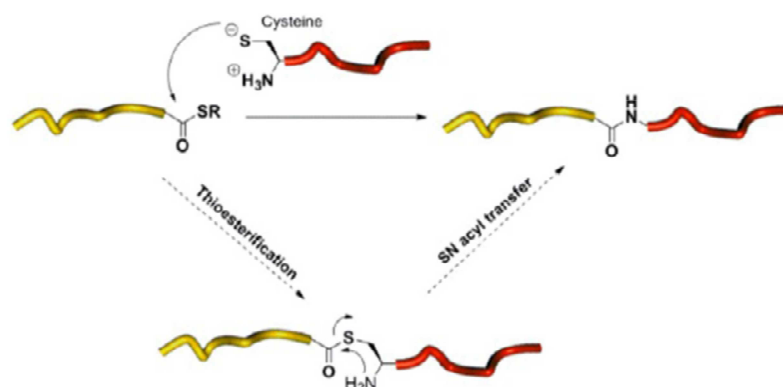


Figure 27. Representative scheme of glycoprotein synthesis via NCL, reproduced from Lecommandoux²¹⁴

In order to reach even larger biomacromolecules than the one obtained by classical NCL, a slightly different strategy called expressed protein ligation (EPL) has been considered. This strategy involves two peptides/proteins that will go through the same two-step mechanism as in NCL, namely transesterification and S-N acyl transfer, but one of the peptides/proteins is produced by recombinant methods.²⁴⁰ Several glycoproteins have been synthesized by EPL, for example GlyCAM-1 glycoprotein or RNase C39 enzyme.^{241,242} Although NCL and EPL are both interesting methods for the synthesis of glycoproteins, they require the presence of an N-terminal cysteine, a rare amino acid in human proteins, on one of the peptides. Therefore the cysteine residue sometimes need to be artificially introduced or substituted by a removable thiol-based molecule.²⁴³ This problem can also be overcome by sugar-assisted ligation,²⁴⁴ or desulfurization.²⁴⁵

III.1.2.3. Enzymatic synthesis

The introduction of complex oligosaccharides still remains a challenge, at least when attached to the peptidic backbone through a native linkage. Therefore enzymatic synthesis can be an alternative. In such strategy a single carbohydrate is introduced on the peptidic backbone during SPPS. This sugar then serves as a handle for the enzymatic reaction using glycosyltransferases.²⁴⁶ This methods has for instance been used for the preparation of ribonuclease glycoforms.²⁴⁷

Despite the recent progresses that have allowed the preparation of several glycoproteins and the reproduction of some biological modifications, the synthesis of glycoproteins still remains a challenge because of their complex structure. Therefore biomimetic approaches based on the development of synthetic glycoprotein analogues represent an interesting alternative as they are based on more simple biomacromolecules that are able to mimic the properties of natural glycoproteins.

III.2. GLYCOPOLYPEPTIDES

The biomimetic analogues of glycoproteins, called glycopolypeptides, are made of a polypeptide backbone with pendant carbohydrates. The aim of these analogues is to mimic the structure of glycoproteins in order to better understand their properties and their structure-function relationship.

III.2.1. Synthesis of glycopolypeptides

Two different pathways can be followed for the synthesis of glycopolypeptides (Figure 28). The first pathway involves the polymerization of previously synthesized glyco-NCA, while the second pathway is based on the post-polymerization introduction of carbohydrates on previously synthesized polypeptides. The polypeptides used in the second strategy are mostly prepared by ring opening polymerization of NCAs but can also be prepared by other methods such as protein engineering.

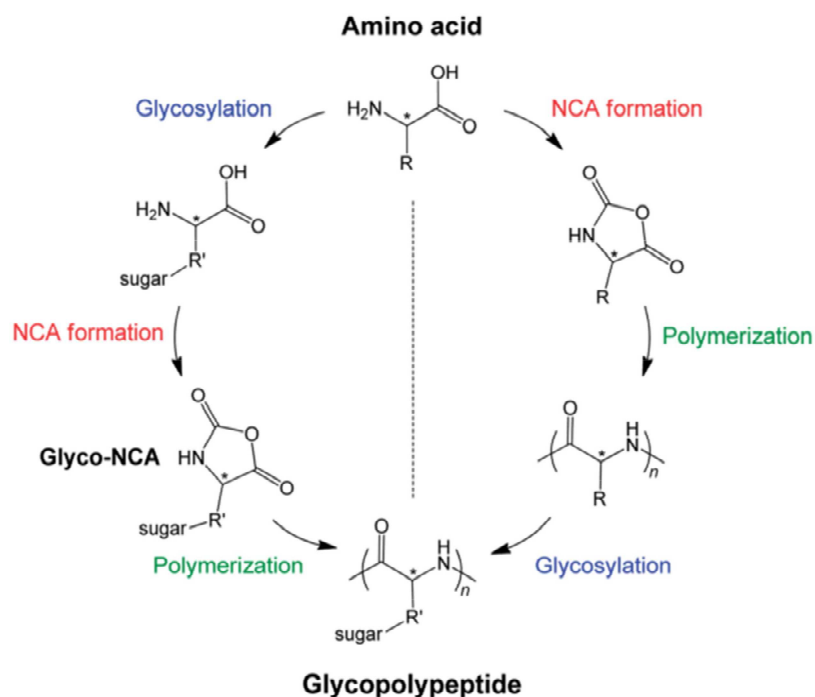


Figure 28. Two strategies toward glycopolypeptides synthesis: polymerization of glyco-NCAs (left) or post-glycosylation of polypeptides synthesized by NCA ROP, reproduced from Schlaad²⁴⁸

III.2.1.1. Synthesis of glycopolypeptides using glyco-NCAs

The first glyco-NCA was obtained by Rde et al. in 1966 by coupling tetra-O-acetylated glucose to serine and the subsequent reaction of the amino acid derivative with phosgene.²⁴⁹ This glyco-NCA was then used by Okada and coworkers to form the first synthetic glycopolypeptide by ring opening polymerization (ROP).²⁵⁰ Using primary amine initiators, they managed to obtain well-defined polymers with a M_n of about 10 kg.mol⁻¹ and low dispersity ($M_w/M_n \approx 1.1$). They also produced block copolypeptides by living polymerization after addition of a second monomer: alanine-NCA.

In order to achieve glycopolypeptides with higher molar masses, highly pure glyco-NCAs are required. This difficult challenge was recently tackled by Deming and coworkers who managed to get highly pure glycosylated L-lysine-NCAs. They were thus able to synthesize glycopolypeptides with high molecular weight (160 kg.mol⁻¹) with low dispersity (1.1-1.2).^{251,252} Their method was based on the coupling of peracetylated sugars to L-lysine amino acid which was then converted to NCA by treatment with dichloro(methoxy)methane (Figure 29). The resulting glyco-NCAs were purified by chromatography under anhydrous conditions. Homo and block copolypeptides were then obtained by polymerization using (PMe₃)₄Co as a catalyst.

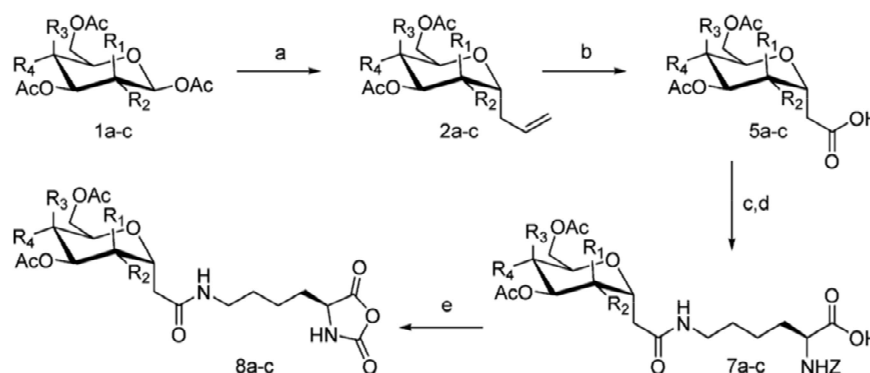


Figure 29. Synthesis of glycosylated lysine NCA, reproduced from Deming²⁵²

Glycosylated L-lysine NCAs have also been obtained by another method developed by Sen Gupta *et al.*^{253,254} They coupled the 1,2 orthoester of glucose or mannose with a protected lysine using a gold catalyst. The protecting group was then removed by hydrogenation and the amino acid converted into NCA using triphosgene. The resulting NCAs were purified by recrystallization and polymerized using 1-hexylamine as an initiator. The resulting homo and block copolymers exhibited higher molar masses than expected and low dispersity. A third method to obtain glycosylated lysine-NCAs was followed

by Wenz and coworkers.²⁵⁵ They synthesized an isocyanate tetra-O-acetyl-glucopyranose derivative and subsequently coupled the isocyanate moiety to the ϵ -amino group of the Cbz-lysine. The NCA was obtained by treatment with dichloromethyl(methoxy)methane and copolymerized with PEGylated lysine and ϵ -TFA-lysine-NCA using trimethylamine or (bipy)Ni(COD) as a catalyst.

Other glyco-NCAs such as glyco-L-cysteine have also been obtained by coupling the cysteine thiol to 1-allyl-2,3,4,6-tetra- O-acetyl- α -D-glucose or -galactose through photochemical addition and subsequent NCA formation using dichloro(methoxy)methane.²⁵⁶ After purification by chromatography or aqueous workup the glyco-NCAs were polymerized using $(\text{PMe}_3)_4\text{Co}$ as a catalyst resulting in glycopolypeptides with molar masses up to $90 \text{ kg}\cdot\text{mol}^{-1}$ and low dispersity (<1.1).

Threonine, serine and cysteine based glyco-NCAs have also been obtained by the coupling of sugars acetobromo derivatives to the chosen amino acid in the presence of iodine and subsequent conversion into NCAs using triphosgene and α -pinene. However these glyco-NCAs have not yet been used in polymerization reactions.²⁵⁷

Several glyco-NCAs have been obtained and successfully polymerized. However this strategy requires the use of tedious multi-step synthesis and efficient purification processes sometimes leading to low yields. Therefore post-polymerization methods have also been developed.

III.2.1.2. Synthesis of glycopolypeptides using post-polymerization glycosylation

i) Glycosylation of the end extremity of polypeptides

The initiation step of NCA polymerization has also allowed the introduction of reactive moieties such as alkynes or azides at the extremity of the formed polypeptide enabling its subsequent conjugation.²⁵⁸ This method was for instance used conjugate oligosaccharides with an azido functionalized poly(γ -benzyl-L-glutamate) using copper catalyzed azide-alkyne cycloadditions.^{259,260} Lecommandoux et al. indeed coupled an alkyne functionalized dextran to a PBLG block leading to the formation of copolymers that were able to self-assemble in water into small polymersomes with low polydispersity.²⁵⁹ Similar glycopolypeptides were also prepared by the same group using PBLG as the polypeptide block and hyaluronan instead of dextran as the oligosaccharide block that were able to form stable vesicular structures.²⁶⁰ These biocompatible and biodegradable polymersomes allowed loading and controlled release of doxorubicin. Hyaluronan was also chosen for its specific ligand properties to CD44 glycoprotein receptors overexpressed in several cancers thus providing biofunctionality to the formed nanoparticles.

ii) Glycosylation of polypeptides obtained by ROP of NCAs

Natural amino-acid based NCAs have been used to synthesize polypeptide backbones that could be further modified by introducing saccharide units on along this backbone. For instance, ϵ -benzyloxycarbonyl-L-lysine NCA have been polymerized by ROP and, after removing the side-chain protecting groups, the lysine amines were reacted with isothiocyanate or glycosyl lactones.^{261,262} However steric hindrance lead to limited degree of functionalization. Higher functionalization degrees were obtained by addition of glycosyl amine onto poly(glutamic acid) using either HOBt or DMT-MM as peptidic coupling agents.^{263,264,265}

Quantitative saccharides introductions of up to 97% on polypeptide backbones obtained by ROP of natural amino acid NCAs were recently obtained by Deming and coworkers. They alkylated poly(L-methionine) using iodoethyl glycosides, alkyl triflate glycosides or epoxide glycosides,^{208,210} on the thioether group of the methionine.

Instead of natural amino acid-NCAs, unnatural amino-acid-NCAs have also been used for post-polymerization glycosylation. For example, propargylglycine NCA, γ -propargyl-L-glutamate NCA, γ -3-chloropropyl-L-glutamate NCA or azido containing NCA were polymerized and subsequently modified by copper-catalyzed azide-alkyne cycloaddition using CuBr, CuSO₄ or Cu(PPh₃)Br.^{266,267,268,269} All the resulting polypeptides were quantitatively functionalized with carbohydrates. Schlaad and coworkers also developed thiol-ene clickable polypeptides using DL-allylglycine NCA monomers polymerized by ROP. 2,3,4,6-tetra-O-acetyl-1-thio- β -D-glucopyranose was then used for the thiol-ene addition step.²⁷⁰ A similar approach involving thiol-yne additions was recently developed with copolypeptides based on γ -benzyl-L-glutamate NCA and DL-propargylglycine NCA.²⁷¹

ROP of NCAs is the most efficient synthetic strategy to obtain well-defined, high molecular weight polypeptides. However sequence specificity at the single residue level cannot be achieved yet.

iii) Glycosylation of polypeptides obtained by protein engineering

Protein engineering techniques allow the production of protein-based polymers with precise control of amino acid sequence. Kiick *et al.* have demonstrated the precise and selective glycosylation of glutamic acid containing recombinant proteins using peptidic coupling agents.²⁷² These type of glycopolypeptides were used to evaluate the influence of the sugar branching on the targeting of toxins or lectins.^{273,274}

III.2.2. Applications of glycopolypeptides in biomedicine

III.2.2.1. Self-assembly

In the scope of biomedical applications, glycopolypeptides self-assembly represent a very interesting approach. Indeed drug loaded particles based on glycopolypeptides can participate to specific carbohydrate protein interactions/recognition leading to targeted drug delivery and the efficiency of those interactions is improved by the multivalent effect of the carbohydrate decorated particles.

For instance, Lecommandoux and coworkers have studied several systems based on glycopolypeptides and studied their self-assembly behavior. One of these systems is based on amphiphilic PBLG-*b*-poly(galactosylated propargylglycine) (PBLG-*b*-PGG) copolypeptides obtained by ring-opening polymerization of benzyl-L-glutamate (BLG) and propargylglycine (PG) NCAs and the glycosylation of the poly(PG) block by Huisgens cycloaddition “click” reaction using an azido-galactose derivative. A library of different PBLG₂₀-*b*-PGG_n (n = 5, 9, 18, 25, 32) copolypeptides, with low polydispersity index (≈ 1.1), was obtained. These glycopolypeptides were self-assembled by nanoprecipitation with different order of addition: water to DMSO or DMSO to water. DLS measurements and TEM imaging proved the formation of different morphologies from worm-like to spherical structures depending on the composition of the copolypeptides, the order and the speed of addition. One of the glycopolypeptides, PBLG₂₀-*b*-PGG₂₅, allowed the formation of stable vesicles.²⁷⁵

The self-assembly behavior of “tree-like” amphiphilic glycopolypeptides was also studied by Lecommandoux, Heise and coworkers (Figure 30). These “tree-like” glycopolypeptides were based on the same type of polypeptide backbone described before, PBLG-*b*-PGG, but with a larger PBLG block (PBLG₄₀) and a short PGG block of 5 repeats in average. Two different oligosaccharides were clicked on the PGG block: dextran and hyaluronan. The structures formed were comparable with tree-like structures with the rod-like PBLG being the trunk and the polysaccharide chains being the branches. The dextran based glycopolypeptides were able to form homogeneous micelles of around 50 nm while the hyaluronan based glycopolypeptides formed smaller micelles of around 35 nm. This difference in size is probably due to the difference in molecular weight between the two polysaccharides.

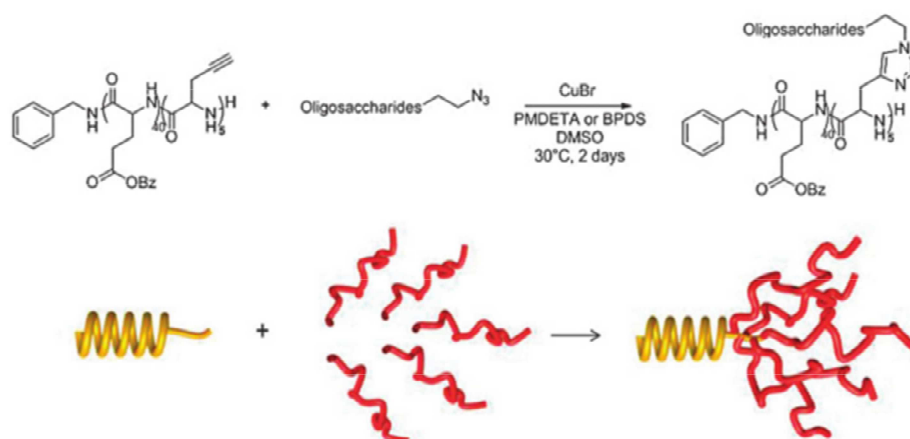


Figure 30. Coupling reaction between polysaccharides and PBLG-b-PGG copolypeptide, reproduced from Lecommandoux²⁷⁶

Deming *et al.* studied the influence of glycopolypeptides conformation on the morphology of the formed nanoparticles (Figure 31). The two copolypeptides studied, $(\alpha\text{-gal-C}^{O2})_{65}\text{L}_{20}$ and $(\alpha\text{-gal-K})_{65}\text{L}_{20}$, were both based on the same hydrophobic α -helical block, poly(L-leucine), and a hydrophilic block of either the α -helical poly(α -D-galactopyranosyl-L-lysine) or the random coil poly(α -D-galactopyranosyl-L-cysteine sulfone). The fully α -helical copolypeptide assembled into micron sized aggregates and plate-like objects because of the rigidity of the hydrophilic segments. On the other hand, the more flexible random coil hydrophilic block (poly(α -D-galactopyranosyl-L-cysteine sulfone)) allowed the formation of vesicles with diameter ranging from 100 nm to a few microns.²⁷⁷

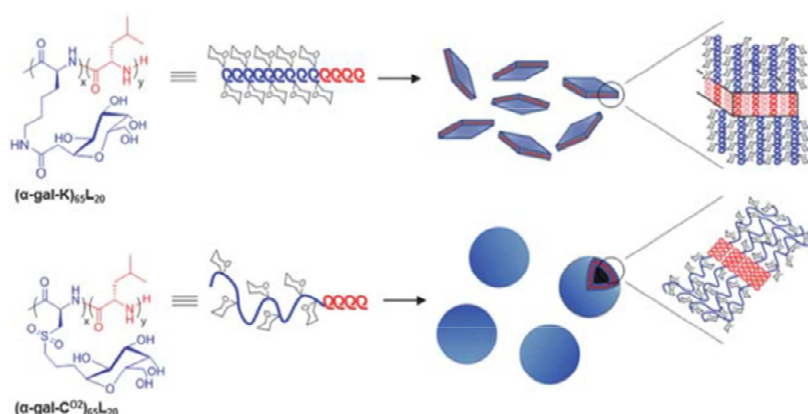


Figure 31. Chemical structures of glycosylated copolypeptides and observed self-assemblies, reproduced from Deming²⁷⁷

Another study from Sen Gupta and coworkers described the self-assembly behavior of glycopolypeptide-dendron block copolymers composed of a glycosylated poly(L-lysine) and an

aromatic dendron linked together with a short PEG segment. Depending on the glycopolypeptide and the dendron lengths, different objects were obtained: organogels in DMSO or nanorods and micelles in aqueous solution. The formation of compartmentalized rod-like structures could be explained by the structural rigidity of the system and the π - π interactions of the dendrons. Moreover, strong correlation between the conformation of the polypeptide and the self-assembly behavior could be observed as the racemic version did not form rod-like structures contrary to the α -helical L-glycopolypeptide.²⁷⁸

The bioactivity and the possibility of using these different glycopolypeptide assemblies for biomedical applications have then been studied for instance by lectin-binding assays.

III.2.2.2. Lectin binding

As mentioned before, glycoproteins are involved in many biological communication processes. These events are based on specific carbohydrate-protein interactions occurring at the surface of the cells.²⁷⁹

It has been shown that lectin binding to carbohydrates can be improved by combining several carbohydrates in the same glycopolypeptide as it lead to multiple binding.^{280,281} Several studies have focused on the binding mechanisms and the influence of the glycopolypeptide structure on the multivalent interactions. In these studies, to prove the bioactivity of the prepared glycopolypeptides mostly two lectins were used: Concanavalin A (ConA) for binding of glucose or mannose, and Ricinus Communis Agglutinin (RCA120) for binding of galactose. The recognition and specific interaction were observed by turbidity assays,^{254,267,271,275,277} surface plasmon resonance (SPR)²⁶³ or isothermal titration calorimetry (ITC).²⁵⁴

It has been shown that the degree of glycosylation affects the kinetics of the lectin binding²⁸² while the secondary structure of the glycopolypeptide does not seem to significantly influence the binding affinity.²⁶⁷

Wenz and coworkers also showed that galactose conjugated fluorescent poly(L-lysine) could specifically recognized T lymphocytes in the body, a process that could be useful for selective staining of cells or targeted drug delivery (Figure 32).²⁵⁵

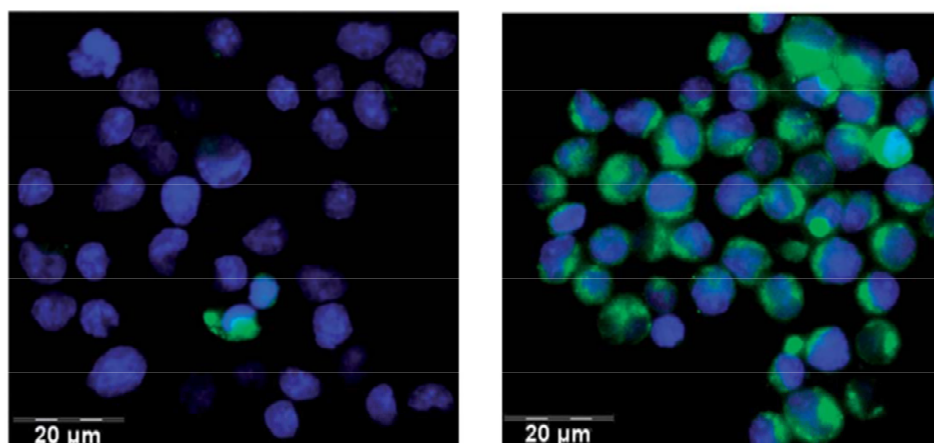


Figure 32. Imaging of fluorescein (green) in the T lymphocytes by fluorescence microscopy after 6 h incubation at 4°C (left image) and 37°C (right image) with the galactose conjugated fluorescent poly(L-lysine). The cell nuclei were stained blue by DAPI, reproduced from Wenz²⁵⁵

III.2.2.3. Drug delivery

Feng and coworkers reported the use of particles of glycosylated poly(L-lysine)-*block*-poly(tetrahydrofuran)-*block*-poly(L-lysine) for loading and *in vitro* release of doxorubicin.²⁶² These particles were spherical with a diameter of 100-150 nm. The release study of doxorubicin showed a rapid release during the first 2 hours followed by stable release for 10-15 hours and a faster release in acidic environment. They also proved that the biocompatibility of the poly(L-lysine) was improved by the presence of the sugars. Other studies involving the use of glycopolyptide dendrimers have been reported.^{283,284}

The use of polypeptide-*b*-oligosaccharide copolymers such as poly(γ -benzyl L-glutamate)-*b*-hyaluronan for drug delivery applications has also been reported.^{260,285,286,287} The encapsulation of two drugs, doxorubicin (Dox) and docetaxel (Doc), into PBLG-*b*-HYA polymersomes allowed the controlled release of these drugs.^{260,285} *In vitro* studies showed that docetaxel encapsulated in polymersomes (PolyDoc) was equipotent or more potent than the free docetaxel. Radiolabelled samples proved that circulation time was significantly increased for PolyDoc compared to the docetaxel solution (DS). Further *in vivo* studies on Ehrlich ascites tumor (EAT) bearing mice showed that PolyDoc tumor uptake was greater than with the DS formulation due to both passive accumulation (Enhanced Permeation and Retention (EPR) effect) and active targeting (CD44 mediated endocytosis).²⁸⁵ Similar good results were obtained *in vivo* on EAT bearing mice using polymersomes loaded with doxorubicin (PolyDox).²⁸⁶ Further *in vitro* and *in vivo* studies on lung tumor cells involving PBLG-*b*-HYA copolymers demonstrated that the nanoparticles size, the CD44

receptors level and the administration route play a critical role in cellular uptake. Indeed smaller nanoparticles of 30 nm were internalized more efficiently compared to 300 nm nanoparticles. Direct administration into the airways did not improved tumor uptake while intravenous injection showed a preferential targeting of CD44 positive tumors at least for the 30 nm nanoparticles.²⁸⁷

CONCLUSION

To conclude, regarding the different informations given by the literature, elastin-like polypeptides represent versatile materials with very interesting features in the context of biomedical applications. Their thermoresponsive behavior for instance is particularly interesting as it facilitates isolation and purification of elastin-like polypeptides and it is a mean to control the self-assembly process. Until now most modifications done on ELPs to functionalize them and tune their properties have involved their chain ends while ELPs backbone modifications have been more scarcely reported. Therefore it would be interesting to focus on the chemoselective modification of ELPs backbone.

Among all amino acids, methionine appears to be a particularly interesting target for chemoselective reactions. In particular the methods recently developed by Deming et al. seem to be efficient and versatile techniques to functionalize polypeptides. Therefore introducing methionine as guest residue in ELPs could be a way to functionalize their backbone.

Functionalizing ELPs using chemoselective reactions could also allow access to precise glycopolypeptides which very attractive materials in the context of biomedical applications as proved by the recent studies in the literature.

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CHAPTER 2

QUANTITATIVE SIDE-CHAIN MODIFICATIONS OF METHIONINE- CONTAINING ELASTIN-LIKE POLYPEPTIDES AS A VERSATILE TOOL TO TUNE THEIR PROPERTIES

Abstract: Tuning the T_t 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 an easy means to modify elastin repeat side chains and easily modulate the T_t of the polypeptides. Such a versatile synthetic method shall practically be exploited to modulate any properties of recombinant polymers.

The work presented in this chapter was the subject of a publication.¹

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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 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 applications,^{4,5} recombinant elastin-like polypeptides (ELPs) are emerging as a unique class of precision protein-like polymers with stimuli-responsive self-assembly properties for specific biomedical and biotechnological applications.⁶ ELPs are repeating sequences of [-Val-Pro-Gly-*Xaa*-Gly-] pentapeptides, the guest residue *Xaa* being any amino acid except proline, originally inspired from the hydrophobic domain of tropoelastin.⁷ In water, ELPs exhibit a lower critical solution temperature (*LCST*), also referred as an inverse temperature transition (*T_i*), similar to synthetic polymers such as poly(*N*-isopropylacrylamide) (pNIPAM). ELP chains are fully soluble in water below the *LCST*, but switch to a phase-separated 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 type and concentration of cosolutes.⁸ Fine tuning of the *LCST* can thus be achieved either by controlling macromolecular parameters implying the *de novo* design of ELPs, or by playing with environmental parameters such as pH, concentration or additional cosolutes.^{9,10} This solubility switch is a defining property of ELPs and has proven to be a major advantage for the purification of recombinant ELPs from bacterial lysates,¹¹ as well as for the controlled self-assembly of individual ELP blocks.^{12,13} This property has also been used to develop nanocarriers for drug-delivery, where the resulting micellar systems are able to self-assemble spontaneously with temperature and to encapsulate different drugs.^{12,14-17}

Post-polymerization modifications of ELPs reported so far have mostly involved their chain ends. Different ELP sequences have been conjugated to small organic molecules,¹⁸ oligonucleotides,¹⁹ drugs,^{20,21} or PEG.²² Modifications at the amino acid side chains within the ELP domain have been more scarcely reported.^{23,24} 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.²⁵⁻³⁰ However, for recombinant polypeptides, as with natural or recombinant proteins, this requires the incorporation of unnatural or non-canonical amino acids.³¹ This strategy has been successfully explored to functionalize proteins by Pictet-Spengler or Staudinger ligations.^{32,33} 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 in proteins at low pH, over N-terminal and lysine primary amines, histidine imidazoles as well as cysteine thiols, various alkylating agents such as triflates or alkyl halides were found to be chemoselectively attacked by thioether groups of polypeptides allowing efficient functionalization of every methionine residue with a wide range of compounds.³⁴⁻³⁶

The goal of the present work was to apply these alkylation reactions to chemoselectively modify methionine side chains of a recombinant ELP and thereby easily tune its T_t . 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 **(VPGMG)₂₀**. Methionine thioether groups were subsequently alkylated chemoselectively to introduce small methyl or bulkier benzyl groups to investigate the effect of alkylation on the T_t .

I. DESIGN, PRODUCTION, PURIFICATION AND CHARACTERIZATION OF (VPGMG)₂₀

I.1. DESIGN OF (VPGMG)₂₀

Recombinant **(VPGMG)₂₀** 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 **(VPGMG)₂₀**. Methionine was used as guest residue in the twenty pentapeptide repeats for subsequent “post-polymerization modification” by chemoselective thioalkylation. A 13 amino acid-long sequence termed *Leader* (detailed sequence provided in the experimental section) 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 **(VPGMG)₂₀** in the bacterial host.

I.2. PRODUCTION, PURIFICATION AND CHARACTERIZATION OF ELP-M-N

After cloning of the **(VPGMG)₂₀**-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 hrs after induction by isopropyl- β -D-thiogalactoside (IPTG), (Figure 1A) as degradation of **(VPGMG)₂₀** progressively occurred at longer production times. **(VPGMG)₂₀** was extracted from cell lysates and purified by *Inverse Transition Cycling (ITC)* avoiding the use of time-consuming and expensive chromatography purification techniques.¹¹ The purity of the recovered **(VPGMG)₂₀** fraction was assessed by SDS-PAGE (Figure 1B).

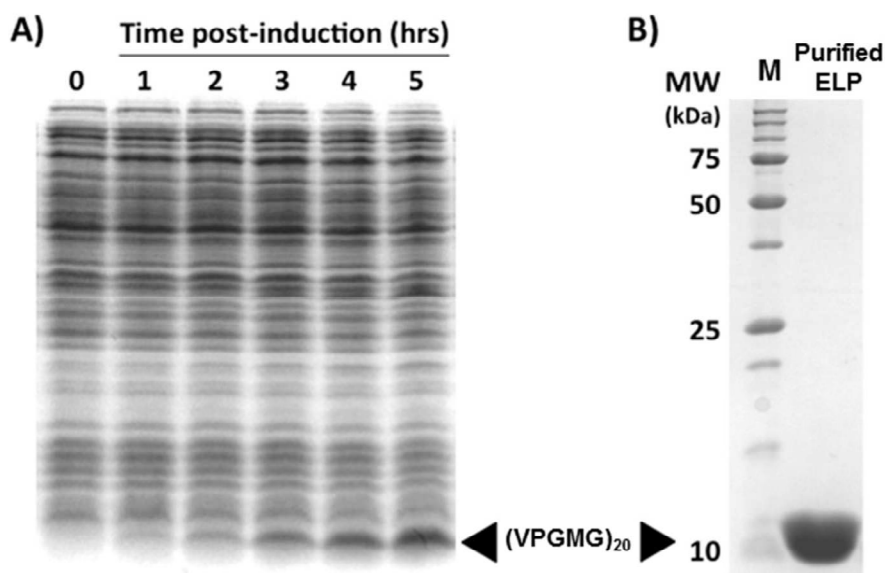


Figure 1. Expression and purification of recombinant **(VPGMG)₂₀ as monitored by 15% SDS-PAGE.**

A) Expression of **(VPGMG)₂₀ during fermentation. The band corresponding to **(VPGMG)₂₀** is indicated with an arrow. Lane 0, non-induced culture; lanes 1-5, total proteins after IPTG induction. B) Analysis of **(VPGMG)₂₀** after purification by *ITC*. Gels were stained with colloidal blue G250. Protein markers (M) were used as controls.**

(VPGMG)₂₀ was extensively dialyzed against water to remove excess salts used in the purification process, then lyophilized and subjected to mass spectrometry analysis. **(VPGMG)₂₀** 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 **(VPGMG)₂₀**'s

experimental mass (Figure 2, inset). The latter was found as 10,382 Da, corresponding to the theoretical mass of (VPGMG)₂₀ without its *N*-terminal methionine, suggesting that it was removed after translation. (VPGMG)₂₀ thus contained a total of 21 Met residues including the one present in the *Leader* sequence.

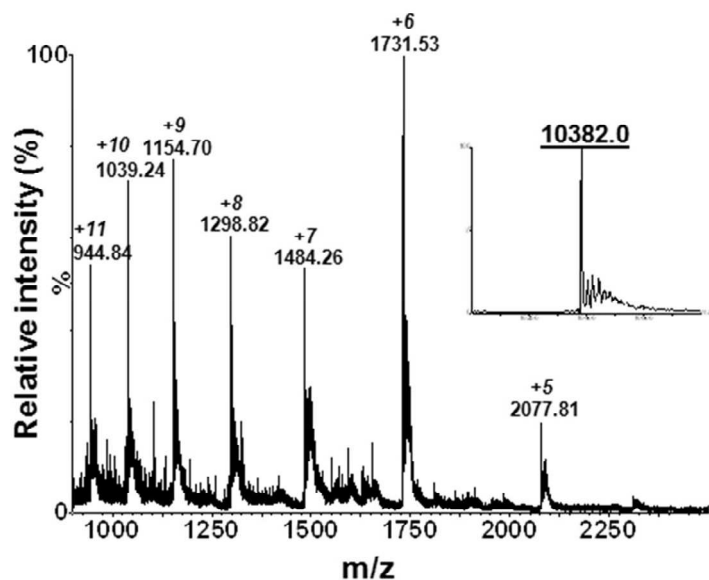


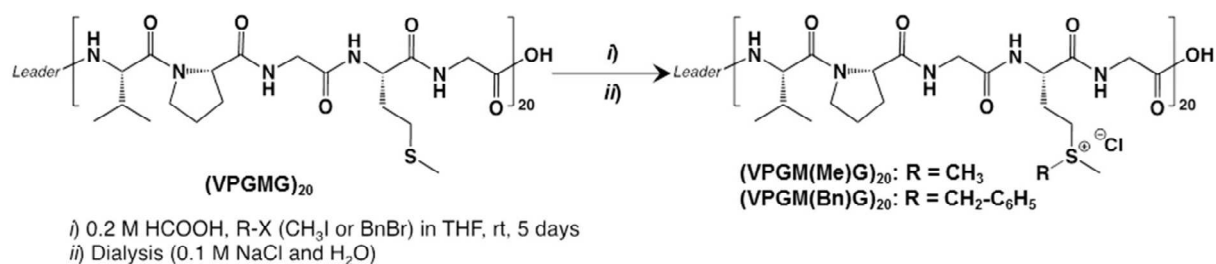
Figure 2. ESI mass spectrum showing the charge state distribution of (VPGMG)₂₀. Charge states of the highest intensities are labeled with their charges. Inset: MaxEnt-deconvoluted ESI mass spectrum of (VPGMG)₂₀.

(VPGMG)₂₀ 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 μM in phosphate buffer (Figure 4).

II. CHEMOSELECTIVE METHIONINE ALKYLATION OF (VPGMG)₂₀

II.1. CHEMICAL STRATEGY

(VPGMG)₂₀ 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 (*T_t* and conformation).



Scheme 1. Chemoselective methionine alkylation reaction of (VPGMG)₂₀.

Methionine residues of (VPGMG)₂₀ were first methylated using methyl iodide to measure the effect of the conversion from a thioether group to a positively-charged sulfonium on the Tt. 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.³⁴⁻³⁶ 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,³⁴ we found that (VPGMG)₂₀ 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 (VPGMG)₂₀ may be due to steric hindrance by neighboring residues related to the chain conformations of ELPs.

II.2. FUNCTIONALIZATION ASSESSMENT

The degree of methylation in (VPGM(Me)G)₂₀ was assessed by ¹H NMR analysis on the lyophilized product (Figure 3). The original resonance at $\delta = 2.09$ ppm in (VPGMG)₂₀, corresponding to the 3 protons of the methionine methyl group (protons **a**), was found to shift to $\delta = 2.94$ ppm and integrated as 6 protons in (VPGM(Me)G)₂₀ (Figures 3 and S2, upper and middle spectra). These integrations were calibrated by assigning the resonances at $\delta = 0.9$ 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 (VPGMG)₂₀ by reaction with 15 equivalents of benzyl bromide. (VPGM(Bn)G)₂₀ was separated from the excess BnBr after extraction with Et₂O, followed by dialysis and lyophilization. The degree of benzylation was again assessed using ¹H NMR analysis. Calibrating our integrations to valine methyl groups as above, we observed the appearance of resonances at $\delta = 7.47$ and 7.54 ppm that integrated for 5 protons and were assigned to the phenyl group protons of the sulfonium (Figure 3,

protons **c**). The resonance at $\delta = 4.66$ ppm that integrated as two protons was assigned to the methylene protons of the benzyl group. The resonance of the methionine methyl group **a** was shifted from $\delta = 2.09$ ppm in the **(VPGMG)₂₀** spectrum to $\delta = 2.81$ ppm in **(VPGM(Bn)G)₂₀** (Figures 3 and S2, upper and lower spectra).

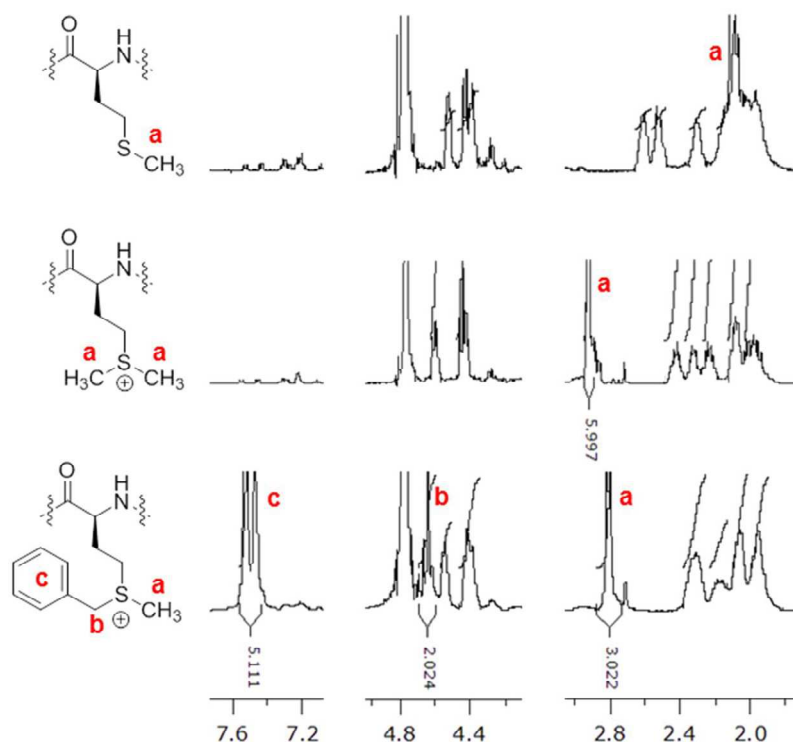


Figure 3. Selected regions of the ^1H NMR spectra of **(VPGMG)₂₀** (upper spectrum), **(VPGM(Me)G)₂₀** (middle spectrum) and **(VPGM(Bn)G)₂₀** (lower spectrum).

III. EFFECT OF ALKYLATION ON THE THERMORESPONSIVE BEHAVIOR

To investigate the effect of methylation and benzylation of the ELP backbone on the T_t , turbidity assays were carried out in phosphate buffer at different concentrations (Figure 4). The T_t values of **(VPGMG)₂₀** ranged between 27°C and 33°C depending on the concentration (50-200 μM). After methylation, the resulting **(VPGM(Me)G)₂₀** did not exhibit any T_t values 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 T_t behavior, but T_t s were shifted to higher values indicating that the benzyl groups are hydrophobic enough to partly counterbalance the hydrophilic effect of the positive charges. As reflected by the lower

turbidity of the solution above the T_t , aggregates of $(\text{VPGM}(\text{Bn})\text{G})_{20}$ are likely less dense than those formed by $(\text{VPGMG})_{20}$ due to electrostatic repulsions between the sulfonium groups.

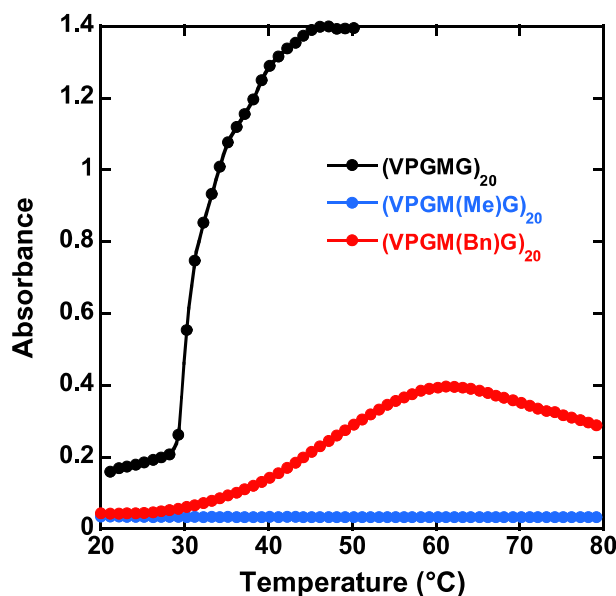


Figure 4. Turbidity assay of $(\text{VPGMG})_{20}$, $(\text{VPGM}(\text{Me})\text{G})_{20}$ and $(\text{VPGM}(\text{Bn})\text{G})_{20}$, 100 μM in PBS.

To determine whether alkylation of $(\text{VPGMG})_{20}$ also translates into changes in secondary structure, circular dichroism (CD) spectra of $(\text{VPGMG})_{20}$, $(\text{VPGM}(\text{Me})\text{G})_{20}$ and $(\text{VPGM}(\text{Bn})\text{G})_{20}$ were measured in PBS at 20°C, below all T_t values (Figure S3). As usually observed with these polypeptides, $(\text{VPGMG})_{20}$ 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 conformational order with increasing hydrophilicity of the ELP.

CONCLUSION

We herein reported a synthetic strategy to introduce side chain modifications in elastin-like polypeptides and explored the effect of such modifications on their thermoresponsive behavior. An ELP with repeating methionine residues was designed and efficiently produced recombinantly in *E. coli* allowing quantitative side-chain modifications at multiple sites to be performed by chemoselective sulfonium formation. ELP conformations and Tt values 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.

EXPERIMENTAL SECTION

Materials.

LB medium, bacto-tryptone, and yeast extract were purchased from BD Biosciences (Le Pont-de-Claix, FR). Ampicillin was obtained from Eurobio (Courtaboeuf, FR). Glucose and polyethyleneimine (PEI) were purchased from Sigma-Aldrich (Lyon, FR). Isopropyl β -D-thiogalactopyranoside (IPTG) was obtained from QBiogene (Illkirch, FR). Complete mini EDTA-free protease inhibitors were purchased from Roche Diagnostics (Mannheim, DEU). Deionized water (18 M Ω -cm) was obtained by passing in-house deionized water through a Millipore Milli-Q Biocel A10 purification unit. THF was purchased from Thermo Fisher Scientific (Waltham, MA USA). Methyl iodide, benzyl bromide, and formic acid were purchased from Sigma Aldrich (St. Louis, MO, USA).

Design of (VPGMG)₂₀-encoding gene.

Oligonucleotide and protein sequences of (VPGMG)₂₀ synthetic gene and polypeptide are provided below. The leader sequence is indicated in italics.

```
catatgggtaccgagctcgcggccgcaagcgaattcacacatatgtgggtaccgggaatg
    M G T E L A A A S E F T H M W V P G M
ggtgtgcccggcatgggcgtacctggtatgggagttcccgggatgggcggtccagggatg
    G V P G M G V P G M G V P G M G V P G M
ggagtcccagggatgggtgtaccgggcatgggagtgccgggaatgggcgtaccggggatg
    G V P G M G V P G M G V P G M G V P G M
ggtgtgcttgggtatgggtgtccccgggatgggagttcccggcatgggcggtccagggcatg
    G V P G M G V P G M G V P G M G V P G M
ggtgtgccgggtatgggagttaccgggtatgggtgtaccaggaatgggcgtgcttggcatg
    G V P G M G V P G M G V P G M G V P G M
ggagtaccagggcatgggcgtccccgggatgggtgttcccgggatggggtaaaggatccaa
    G V P G M G V P G M G V P G M G -
agctt
```

Bioproduction of recombinant (VPGMG)₂₀.

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 µg·mL⁻¹ ampicillin. The seed culture was inoculated into 0.95 L rich LB medium supplemented with glucose (1 g·L⁻¹) and ampicillin (100 µg·mL⁻¹), and bacteria were cultivated at 37°C in a multi-bioreactor system with three independent 1 L-vessels process control (BIOSTAT® Qplus, Sartorius Stedim Biotech, Germany). The pH was maintained at 7 with 1 M phosphoric acid and 1 M sodium hydroxide. The dissolved oxygen tension pO₂ was kept above 15% by adjusting agitation rate (150-600 rpm) and air flow (1-1.5 L·min⁻¹). When the optical density at 600 nm (OD₆₀₀) reached the value of 1.2, 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 OD₆₀₀.

Isolation and purification of recombinant (VPGMG)₂₀.

After 5 hrs 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·g⁻¹ (wet weight) phosphate buffer (PBS; NaCl 137 mM, KCl 2.7 mM, Na₂HPO₄ 8 mM, KH₂PO₄ 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 slowly 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. PEI was then added at a final concentration of 0.44% (v/v) to precipitate bacterial DNA. Insoluble debris was 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). Briefly, (VPGMG)₂₀ polypeptide was precipitated with NaCl at 25°C and retrieved by centrifugation at 16,000 g and 25°C for 30 min (“warm spin”). After removal of soluble proteins in the supernatant, (VPGMG)₂₀-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 (VPGMG)₂₀-containing supernatant was subjected to an additional ITC. Final concentrations of NaCl used before each “warm spin” were 1.5, 0.7 and 0.5 M for the first, second and third ITC rounds, respectively. Soluble (VPGMG)₂₀ 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 (VPGMG)₂₀ were assessed by SDS-PAGE using 15% TRIS-glycine gels stained with colloidal blue G250.

Mass spectrometry analysis of (VPGMG)₂₀.

Mass spectrometry analysis was performed on a ESI-Q-TOF (Q-TOF Premier, Waters, Manchester, UK). All solvents used were HPLC grade. Lyophilized ELP20 was resuspended in DMSO and then diluted in H₂O/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⁻¹ and infused into the electrospray ionization source at a flow rate of 10 μL·min⁻¹. 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⁻¹ in acetonitrile/0.1% aqueous formic acid (1:1 v/v). The (VPGMG)₂₀ spectrum was deconvoluted by the Waters software MaxEnt using a maximum entropy-based approach. Theoretical average molecular mass of (VPGMG)₂₀ is 10,381.64 Da. Experimental [M+H]⁺=10,382.0.

NMR spectrometry analysis of (VPGMG)₂₀.

Phase-sensitive HSQC experiment was used to acquire the 2D spectra giving DEPT-type information in addition to the ¹H–¹³C connectivity. This experiment was performed at 298 K on a Bruker Avance III HD NMR spectrometer operating at 400.3 MHz (100.7 MHz for the carbon). The ¹JCH used was 145 Hz. 128 scans were used with a recycle delay of 2 sec. COSY experiment was performed at 298 K on the same spectrometer. 256 scans were recorded with a recycle delay of 2 sec. The DMSO signal was used as the reference signal (δ = 2.50 ppm). Data processing was performed using Topspin software. Chemical shifts of amino acids are well known in the literature. With Phase-sensitive HSQC, CH_n spin vectors develop differently after a 90° pulse depending on how many hydrogens are bonded to the carbon atom. CH and CH₃ vectors have opposite phase compared with CH₂. We have identified the CH₃ of valine at 0.85-0.90 ppm (18.9-19.5 ppm for ¹³C) and used it as reference for the calibration of integrations. After examination of COSY spectra, we determined the resonances for each amino acid. (Figure S1-A) Assignments of (VPGMG)₂₀ were done with the help of COSY and HSQC. (Figure S1)

Alkylation of (VPGMG)₂₀ (general procedure).

(VPGMG)₂₀ was dissolved in 0.2 M aqueous formic acid (20 mg·mL⁻¹). Alkyl halide (15 equiv. per Met residue) was added as a solution in THF (50 mg·mL⁻¹). The reaction was sealed, covered with foil, and

stirred for 5 days at room temperature. Diethyl ether (equal to about half the reaction volume) was then added to the reaction to extract the excess alkyl halide. The biphasic reaction was vortexed briefly and allowed to sit to allow separation of the organic and aqueous phases. The ether layer was pipetted off and discarded. The reaction mixture was then transferred to 2,000 MWCO dialysis tubing and dialyzed against 0.1 M NaCl for 24 hrs to exchange all counterions to chloride, followed by dialysis against ultrapure water for 48 hrs with frequent water changes. The product was lyophilized to dryness to give a white powder in ~ 85% yield.

Synthesis and characterization of ELP20(Me) and ELP20(Bn).

(VPGM(Me)G)₂₀ and (VPGM(Bn)G)₂₀ were prepared from 10.3 mg (VPGMG)₂₀ and either methyl iodide or benzyl bromide, respectively, using the general procedure described above. (VPGM(Me)G)₂₀ and (VPGM(Bn)G)₂₀ were characterized by ¹H NMR. ¹H NMR spectra were recorded on Bruker spectrometers at 500 MHz at ambient temperature, and are calibrated relative to the D₂O solvent signal at 4.78 ppm. Integrations were calibrated to the 6 valine methyl protons at 0.9 ppm. (Figure S2)

Transition temperature measurements.

Transition temperatures (LCSTs) were determined by measuring the turbidity at 350 nm between 20 and 80°C at a 1°C·min⁻¹ scan rate for (VPGMG)₂₀, (VPGM(Me)G)₂₀ and (VPGM(Bn)G)₂₀ in PBS at three concentrations (50, 100 and 200 μM). Data were collected on a Cary 100 Bio UV–visible spectrophotometer equipped with a multi-cell thermoelectric temperature controller from Varian (Palo Alto, CA). The T_t is defined as the temperature corresponding to the maximum of the first derivative of the turbidity versus temperature curve.

Circular dichroism (CD).

CD spectra were recorded on a Jasco J-815 spectropolarimeter equipped with a PFD-425S Peltier temperature control unit in 1 mm quartz cells. Spectra of (VPGMG)₂₀, (VPGM(Me)G)₂₀ and (VPGM(Bn)G)₂₀ in PBS (25, 10 and 10 μM, respectively) were recorded at 20°C and 10 nm·min⁻¹ scanning speed from 260 to 180 nm with 0.1 nm resolution, 1 sec DIT and high sensitivity. CD spectra represented the average of three measurements. CD data are reported as mean residue ellipticity [θ] (deg·cm²·dmol⁻¹). (Figure S3)

SUPPLEMENTARY FIGURES

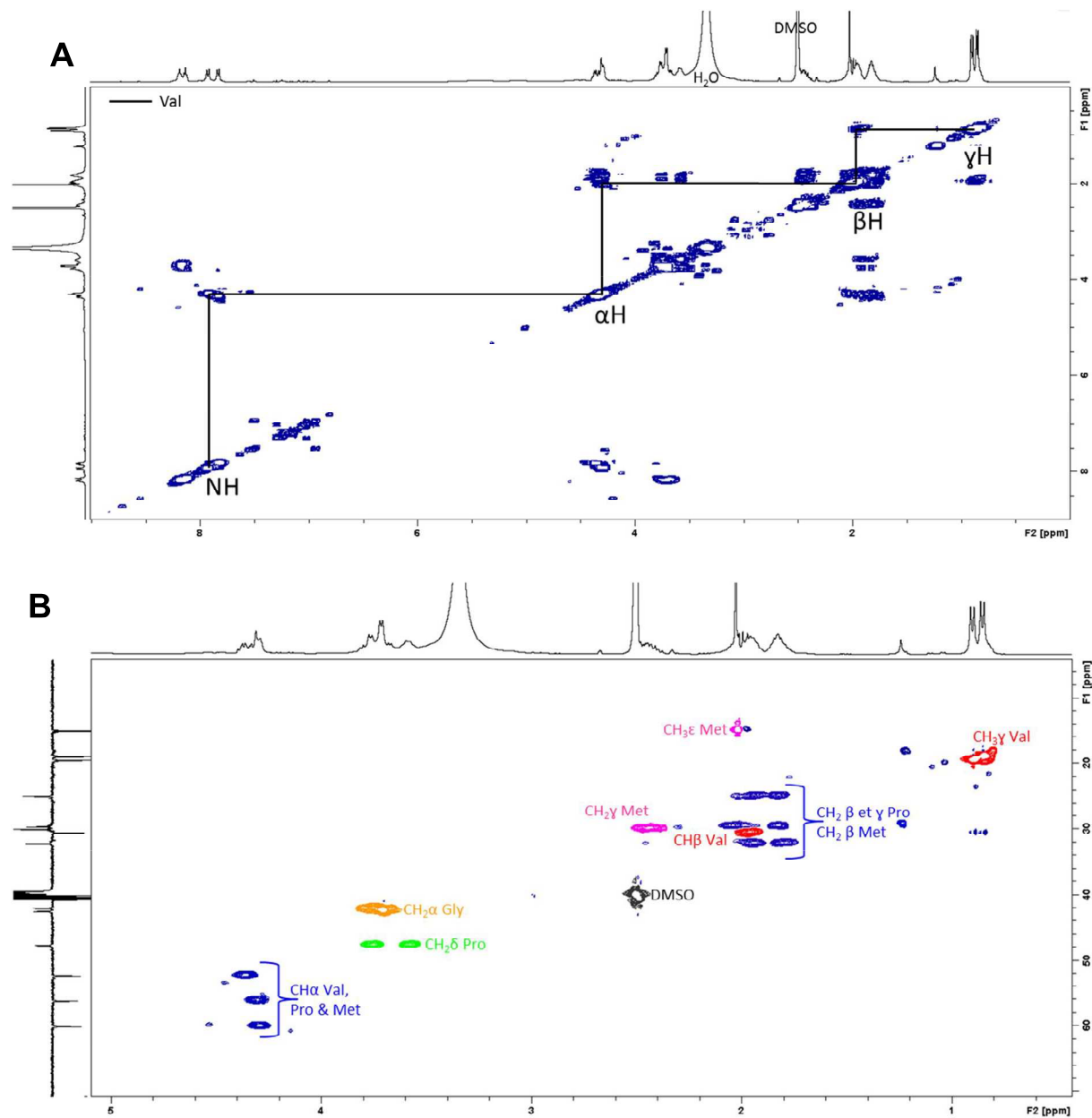


Figure S1. 2D NMR spectra of (VPGMG)₂₀ in d₆-DMSO. (A) COSY, (B) HSQC.

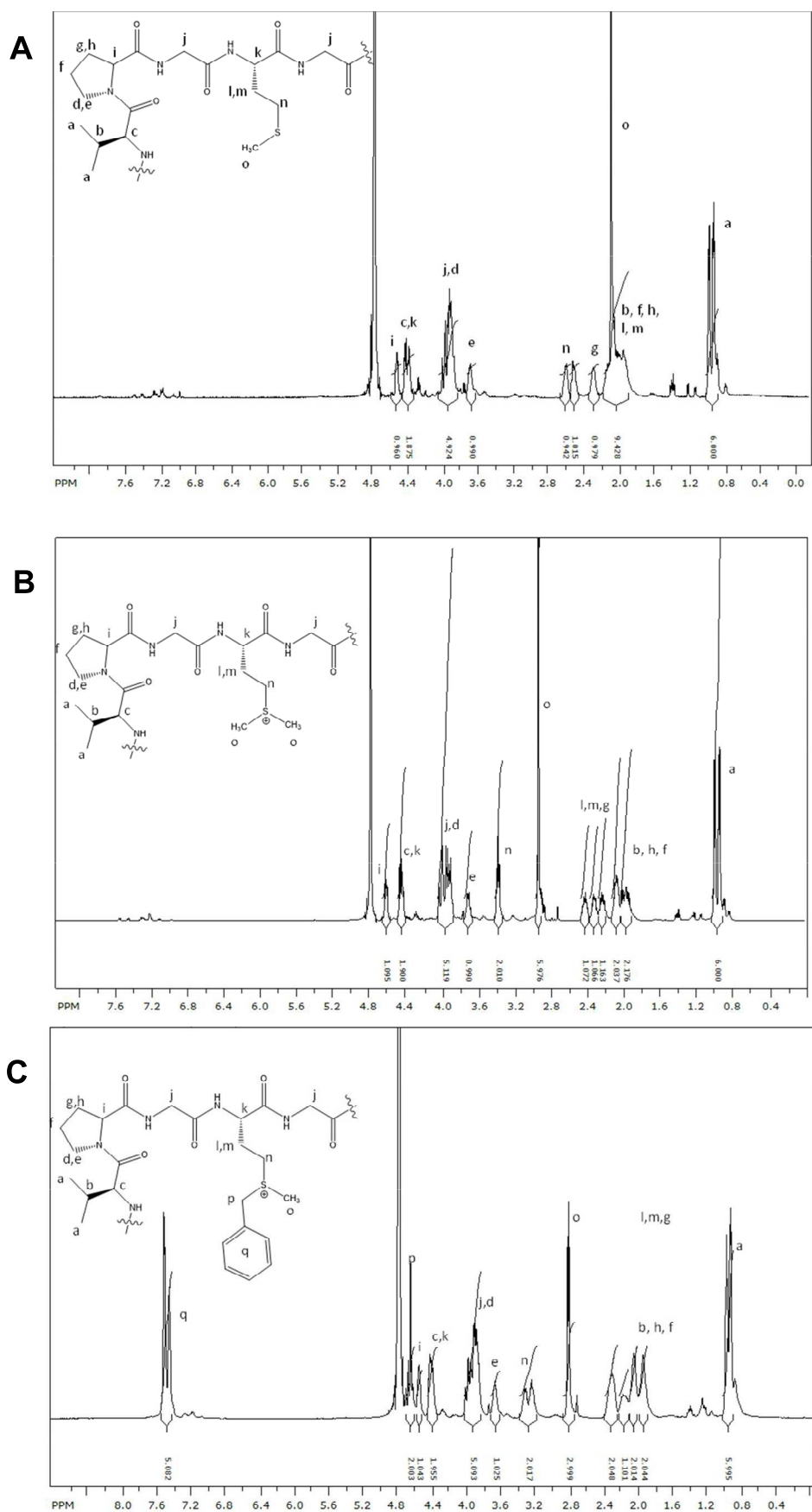


Figure S2. Full ^1H NMR spectra of (A) $(\text{VPGMG})_{20}$, (B) $(\text{VPGM}(\text{Me})\text{G})_{20}$ and (C) $(\text{VPGM}(\text{Bn})\text{G})_{20}$ in D_2O .

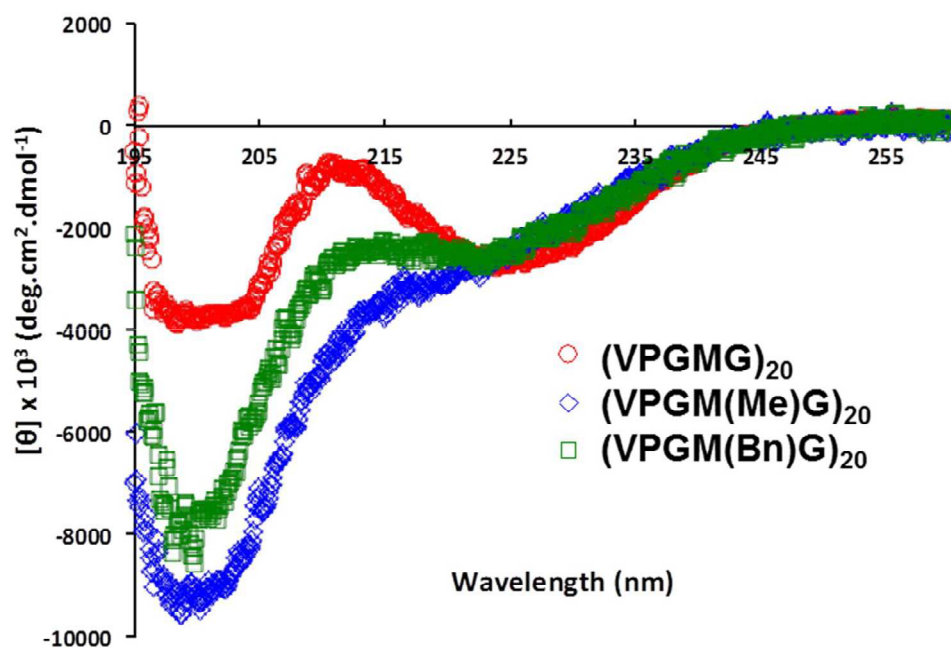


Figure S3. Circular dichroism spectra of (VPGMG)₂₀, (VPGM(Me)G)₂₀ and (VPGM(Bn)G)₂₀ at 20°C.

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CHAPTER 3

SELECTIVE TUNING OF ELASTIN-LIKE POLYPEPTIDE PROPERTIES *VIA* METHIONINE OXIDATION

Abstract: We have designed and prepared a recombinant elastin-like polypeptide (ELP) containing precisely positioned methionine residues, and performed the selective and complete oxidation of its methionine thioether groups to both sulfoxide and sulfone derivatives. Since these oxidation reactions substantially increase methionine residue polarity, they were found to be a useful means to precisely adjust the temperature responsive behavior of ELPs in aqueous solutions. In particular, lower critical solution temperatures were found to be elevated in oxidized sample solutions, but were not eliminated. These transition temperatures were found to be further tunable by the use of solvents containing different Hofmeister salts. Overall, the ability to selectively and fully oxidize methionine residues in ELPs proved to be a convenient post-modification strategy for tuning their transition temperatures in aqueous media.

INTRODUCTION

Along with their fundamental role as initiating molecules in protein synthesis, proteinaceous methionine residues also play an important role in biology as common substrates for redox reactions.¹ The thioether groups of methionine are readily oxidized into sulfoxides by a variety of reactive oxidative species (ROS), and can be reduced back to native methionine by endogenous methionine sulfoxide reductase (MSR) enzymes.^{1,2,3} Deming and coworkers have taken advantage of these redox properties by developing enzyme-responsive vesicles from amphiphilic block copolypeptides containing poly(L-methionine sulfoxide) hydrophilic blocks.⁴ In the case of recombinantly produced polypeptides, the use of redox chemistry on methionine residues has been reported in one instance for silks^{5,6}), but not for elastin-like polypeptides (ELPs). Here, we report the design and preparation of ELPs containing precisely positioned methionine residues, and their controlled oxidation to sulfoxide and sulfone derivatives. We found these oxidation reactions, since they substantially alter methionine residue polarity, are a convenient means to precisely adjust the temperature responsive behavior of ELPs in aqueous solutions.

Oxidation of methionine in naturally occurring proteins has been widely studied by the labs of Stadtman and Weissbach,^{1,2,7,8} to better understand its mechanism and exact role in the biological activity of proteins. Methionine residues were found to act as ROS scavengers thereby preventing irreversible oxidation of other amino acids such as those in active sites of proteins.^{1,7} MSR enzymes can also reduce sulfoxide moieties to regenerate native methionine residues thereby allowing the catalytic consumption of ROS.^{8,9} In applications of methionine biochemistry to synthetic materials, the influence of reversible thioether group oxidations on the conformations and thermoresponsive properties of poly(L-methionine) and poly(S-alkyl-L-homocysteine) derivatives has been reported.^{10,11} In these studies, reversible oxidation of thioether groups has been used as a conformational switch between α -helical and disordered conformations that depend on the oxidation state. However, in these examples all of the oxidized sulfoxide containing polypeptides were found to be fully water-soluble and did not exhibit any thermoresponsive properties in aqueous solution.

Recombinant ELPs are a special class of precision protein-like polymers with stimuli-responsive self-assembly properties, mainly developed for their potential use in biomedical applications.^{12,13} ELPs are composed of repeating sequences of [-Val-Pro-Gly-*Xaa*-Gly-] pentapeptides, derived from the hydrophobic domain of tropoelastin, in which the guest residue *Xaa* can be any amino acid except

proline.¹⁴ ELPs with precisely controlled sequences and molecular weights can be produced by protein engineering techniques. In addition, ELPs with sufficient molecular weight and hydrophobicity have been efficiently produced at large scale using *Escherichia coli* as expression system.^{15,16} The reversible aqueous solubility transition of ELPs, at a lower critical solution temperature (LCST), make them particularly attractive candidates in biomaterials design. Soluble in aqueous media at low temperatures, ELPs phase separate as aggregates at an experimentally determined temperature called the inverse transition temperature (T_t).^{17,18} The T_t can be tuned by modifying macromolecular parameters such as ELP molecular weight, the nature of the guest residue in the polypeptide repeats, and also by adjusting polypeptide concentration and composition of the aqueous medium.^{19,20,21} In previous studies, we have shown that properties of methionine containing ELPs can also be tuned by chemoselective alkylation of methionine thioether groups.²² This study was focused on examining the influence of oxidation on the temperature responsive properties of methionine-containing ELPs in aqueous solution. Oxidation of precisely positioned methionine residues in ELP chains was envisioned as a straightforward and convenient means to tune the hydrophilic character of these polypeptides. A new ELP was designed to contain periodically spaced methionine residues and produced recombinantly. This ELP was then selectively oxidized at low pH to form either sulfoxide- or sulfone-containing ELP derivatives whose properties in aqueous solution were then characterized and analyzed.

I. DESIGN, PRODUCTION, PURIFICATION AND CHARACTERIZATION OF ELP-M-40

I.1. DESIGN OF ELP-M-40

ELP-M-40 (Figure 1A), containing a total of 40 ELP repeat units (n), was recombinantly produced in *Escherichia coli* (*E. coli*) using protein engineering techniques. To create an ELP sequence with precise periodic placement of methionine residues, we constructed a gene that encodes the following amino acid sequence: MW[VPGVGVPGMG(VPGVG)₂]₁₀ (see SI). Methionine and valine were encoded at a 1:3 ratio, respectively, as guest residues in the VPGXG repeat units. Methionine residues were encoded as substrates for subsequent oxidation reactions, while valine residues were used as non-reactive hydrophobic residues to dilute the methionine content.²² Additional methionine and tryptophan residues at the *N*-terminus of the ELP sequence were introduced for proper initiation of translation in *E. coli* and UV-Vis detection purposes, respectively.

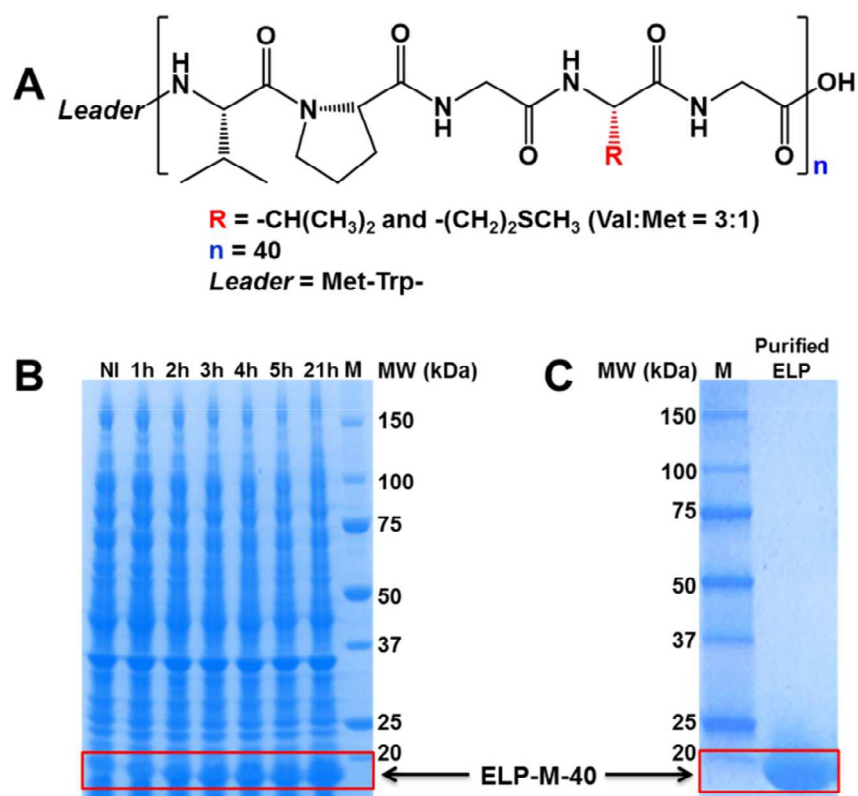


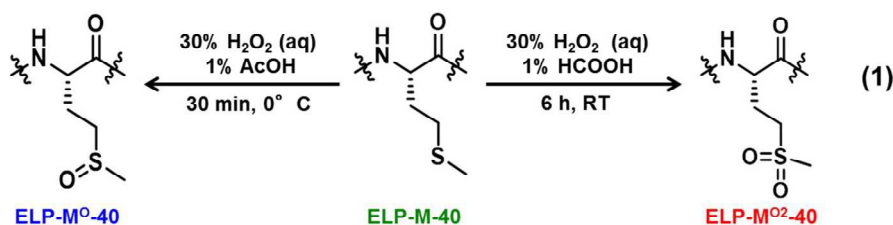
Figure 1. (A) Chemical structure of ELP-M-40. (B) Expression of recombinant ELP-M-40 during bacterial fermentation as analyzed by SDS-PAGE. Lane NI = non-induced culture; lanes 1-21 h = time after IPTG induction in hours. M = molecular weight marker. (C) Purified ELP-M-40 as analyzed by SDS-PAGE.

I.2. PRODUCTION, PURIFICATION AND CHARACTERIZATION OF ELP-M-40

A clone expressing the **ELP-M-40** polypeptide was cultured in 5 L flasks. After induction of recombinant protein synthesis using isopropyl- β -D-thiogalactoside (IPTG), the culture was allowed to continue for 21 h (Figure 1B). Soluble proteins were then extracted from the cell lysates, and **ELP-M-40** was purified by inverse transition cycling (ITC),²³ extensively dialyzed against ultrapure water and lyophilized. The purity of **ELP-M-40** was assessed by SDS-PAGE (Figure 1C). The yield of the purified ELP was 150 mg/L culture. The MALDI mass spectrum of **ELP-M-40** (Figure 2A) provided an experimental mass of 17,035.2 Da in excellent concordance with the expected mass (17,035.4 Da). **ELP-M-40** was also characterized by 1D and 2D NMR spectrometry (Figures S1 and S2).

II. OXIDATION OF ELP-M-40

II.1. CHEMICAL REACTIONS



Samples of **ELP-M-40** were then subjected to different chemical oxidation conditions in order to obtain the sulfoxide-containing derivative (**ELP-M⁰-40**) and the sulfone-containing derivative (**ELP-M^{O2}-40**) (eq. 1).^{4,24} These derivatives were used to subsequently evaluate the effect of oxidation on the temperature-induced aggregation of the ELPs in aqueous solutions. **ELP-M⁰-40** was obtained using mild oxidation conditions (hydrogen peroxide, acetic acid, 30 min, 0 °C), while stronger oxidizing conditions were necessary to obtain **ELP-M^{O2}-40** (hydrogen peroxide, formic acid, 6 h, 20°C). Initial attempts to obtain **ELP-M^{O2}-40** using milder conditions (hydrogen peroxide, acetic acid, 3 h, 20°C) resulted in only 14 % conversion to sulfone groups, with the remaining 86 % thioether groups converted into sulfoxides. Acetic acid was therefore replaced for formic acid and the reaction time was increased to 6 h to fully oxidize all 11 thioether groups in **ELP-M^{O2}-40** into sulfones.

II.2. OXIDATION ASSESSMENT BY NMR AND MASS SPECTROMETRY

After isolation, **ELP-M⁰-40** and **ELP-M^{O2}-40** were both subjected to MALDI mass spectrometry and ¹H NMR spectrometry analyses to confirm their molar masses and chemical structures, respectively. The MALDI mass spectra (Figure 2B and 2C) provided experimental masses of 17,215.8 Da for **ELP-M⁰-40** and 17,382.1 Da for **ELP-M^{O2}-40** in concordance with the expected theoretical values. MS analysis also confirmed that monodispersity of **ELP-M-40** derivatives was retained after the oxidation reactions.

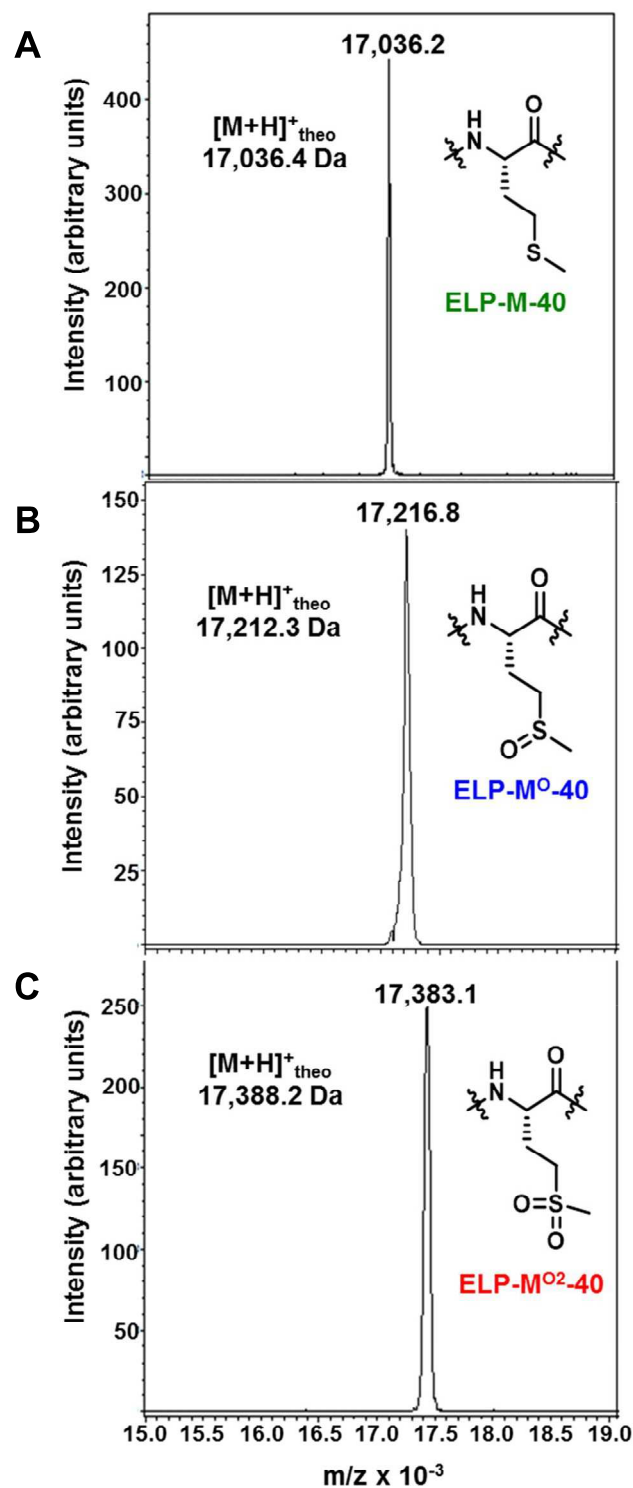


Figure 2. MALDI mass spectra of (A) ELP-M-40, (B) ELP-M^O-40, (C) ELP-M^{O2}-40. [M+H]⁺_{theo} = theoretical mass for each sample.

The complete conversion of all 11 thioether groups of **ELP-M-40** into sulfoxide and sulfone groups was also confirmed by ¹H NMR (Figure 3). The resonance for the protons of the methionine methyl group at $\delta = 2.12$ ppm was shifted to 2.75 ppm in **ELP-M^O-40** (Figure 3B) and to 3.1 ppm in **ELP-**

M⁰²-40 (Figure 3C), while the resonance of the methylene protons bonded to sulfur in methionine was shifted from 2.6 ppm in **ELP-M-40** to 3 ppm in **ELP-M⁰-40** and to 3.4 ppm in **ELP-M⁰²-40**. These shifts can be explained by the deshielding effect of the oxygen atom(s) present in **ELP-M⁰-40** and **ELP-M⁰²-40** compared to **ELP-M-40**, and are also consistent with NMR data on oxidized derivatives of poly(L-methionine).⁴ Integrations confirming full conversions of **ELP-M-40** to sulfoxide and sulfone derivatives were accomplished by comparing the methyl and methylene resonances of all samples described above to the unchanging resonances centered at 4.45 ppm, which correspond to the α CH protons of proline and the initial valine in each repeat (**VPGXG**) (see SI, Figure S3).

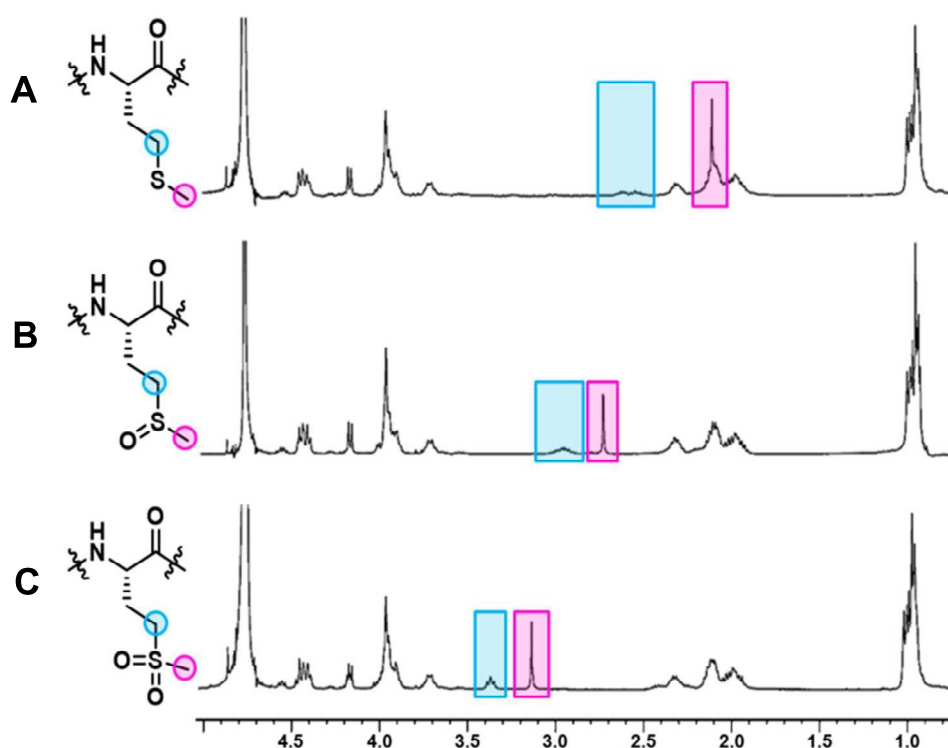


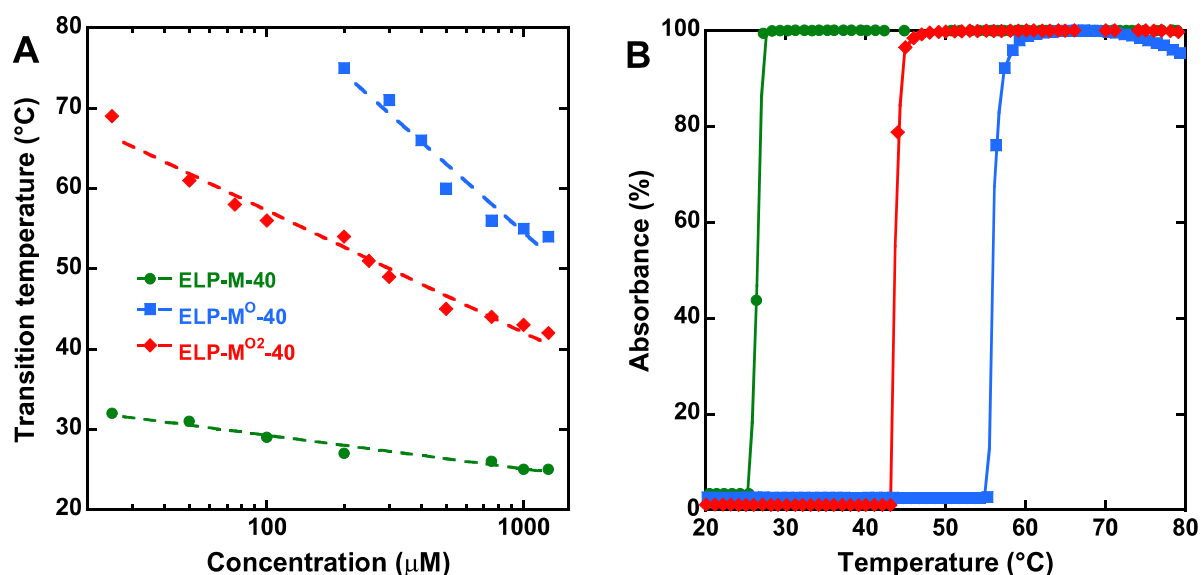
Figure 3. ^1H NMR spectra of (A) **ELP-M-40**, (B) **ELP-M⁰-40**, (C) **ELP-M⁰²-40**. Blue boxes highlight resonances for CH_2 groups bonded to sulfur, and red boxes highlight resonances for CH_3 groups bonded to sulfur.

III. EFFECT OF OXIDATION ON THE THERMORESPONSIVE BEHAVIOR

III.1. **ELP-M-40**, **ELP-M⁰-40**, **ELP-M⁰²-40** TEMPERATURE RESPONSIVENESS

The effect of methionine oxidation on temperature responsiveness of the ELP solutions was evaluated by carrying out cloud point measurements in DI water at different concentrations to determine T_t values (Figures 4A and 4B). Depending on sample concentration (25 to 1,250 μM), the T_t of **ELP-M-**

40 ranged from 25 to 32 °C. After oxidation to give **ELP-M⁰-40**, *T_t* were shifted to higher values ranging from 54 to 75 °C depending on sample concentration (200 to 1,250 μM), and no measurable *T_t* could be determined below 200 μM concentration (*i.e.*, *T_t* > 80°C). The lack of *T_t* for low concentrations of **ELP-M⁰-40** was likely due to increased water-solubility afforded by the sulfoxide groups as compared to thioether groups.^{4,25,26} With *T_t* values greater than those of **ELP-M-40**, but lower than those of **ELP-M⁰-40** at identical concentrations, the sulfone derivative **ELP-M^{O2}-40** (*T_t* = 42 to 69°C, concentration = 25 to 1250 μM) displayed intermediate temperature dependent solubility compared to the other ELPs. Although sulfone groups have greater dipole moments compared to sulfoxides, the large dipoles of the sulfone groups can lead to sulfone-sulfone and sulfone-protein interactions, that can lead to decreased water solubility.^{4,27}



C

	Transition temperature (°C)		
	DI water	0.1 M NaNO ₃	0.1 M NaI
ELP-M-40	25	26 (+1) ^a	26 (+1) ^a
ELP-M⁰-40	55	54 (-1) ^a	59 (+4) ^a
ELP-M^{O2}-40	43	38 (-5) ^a	44 (+1) ^a

Figure 4. (A) *T_t* values of **ELP-M-40**, **ELP-M⁰-40** and **ELP-M^{O2}-40** plotted as functions of sample concentration in DI water; data fitted using equation 2. (B) Absorbance of solutions of **ELP-M-40**, **ELP-M⁰-40** and **ELP-M^{O2}-40** (1.0 mM in DI water) as functions of temperature. (C) Table showing *T_t* values of ELP samples at 1.0 mM in DI water or 0.1 M Hofmeister salt solutions. ^a = values in parentheses are the differences compared to the corresponding *T_t* in DI water.

The T_t values of the 3 ELPs were plotted as functions of concentration (Figure 4A) and the data were fitted using the empirical equation established by Chilkoti *et al.* (eq 2).¹⁹

$$T_t = T_{t,c} + k/L \ln(C_c/C) \quad (1)$$

This equation provided a satisfactory fits of all three data sets enabling accurate estimations of the T_t of **ELP-M-40** as well as the oxidized **ELP-M⁰-40** and **ELP-M⁰²-40** at specific concentrations. We also noticed as hydrophilicity of the ELP increased, the slope of the fit became steeper. This result is comparable with observations by Chilkoti *et al.* regarding the increase of the slope with decreasing ELP chain length, which also correlates to improved hydrophilicity.

III.2. EFFECT OF HOFMEISTER SALTS ON THE THERMORESPONSIVE BEHAVIOR

The impact of two different salts of the Hofmeister series on the thermoresponsive properties of the ELPs in aqueous solution was also studied (Figure 4C).²⁸ Knowing that anions tend to have more influence on thermoresponsive properties of polymer solutions than cations,²⁹ we studied the effects of two anions, namely NO_3^- and I^- (Na^+ counterions), on ELP solutions. The trends we noticed were similar to those observed with other biological and synthetic polymers.^{10,11,29,30,31,32} In the presence of either 0.1 M NO_3^- or I^- salt, **ELP-M-40** was found to have only a minimal change in its T_t . In contrast, the T_t values of oxidized derivatives showed larger changes in the presence of salts. In particular, the T_t of **ELP-M⁰-40** increased in the presence of I^- , while the T_t of **ELP-M⁰²-40** decreased in the presence of NO_3^- . Although these two anions are both commonly considered to be chaotropic, they probably do not affect the T_t values of oxidized **ELP-M-40** derivatives in a similar manner. This can be explained by the interactions involved in the phase transition process where poorly hydrated anions, such as NO_3^- or I^- , can influence the phase transition by modifying the interfacial tension at the polymer/water boundary and by binding to hydrophobic surfaces of the biomacromolecules.³⁰

CONCLUSION

The design and preparation of a new methionine-containing recombinant elastin-like polypeptide **ELP-M-40**, along with its oxidized sulfoxide **ELP-M⁰-40** and sulfone **ELP-M⁰²-40** derivatives, were reported. Complete and selective oxidization of all methionine thioether groups in **ELP-M-40** gave a total of three different ELP variants with significantly different T_t values in water. These T_t values were found to be further tunable by use of solvents containing different Hofmeister salts. Unlike homopolymers containing high densities of thioether groups where oxidation eliminates *LCST* behavior,^{4,10,11} the precise spacing of methionine residues in the ELPs reported here allowed for the controlled tuning and retention of *LCSTs* in oxidized samples. Ultimately, the ability to selectively and fully oxidize methionine residues in ELPs proved to be a convenient post-modification strategy for tuning their transition temperatures in aqueous media, without requiring any additional molecular cloning steps.

EXPERIMENTAL SECTION

Materials.

LB medium was purchased from Sigma Aldrich (Saint-Quentin-Fallavier, FR). Bacto-tryptone, and yeast extract were purchased from Biokar Diagnostics (Allone, FR). Ampicillin was obtained from Eurobio (Courtaboeuf, FR). Glycerol and isopropyl β -D-thiogalactopyranoside (IPTG) were purchased from Euromedex (Souffelweyersheim, FR). Complete mini EDTA-free protease inhibitors were purchased from Roche Diagnostics (Mannheim, D). Hydrogen peroxide and formic acid were purchased from Sigma Aldrich (St. Louis, MO, USA). Glacial acetic acid was obtained from Fisher Scientific (USA). Deionized water (18 M Ω -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 was designed. This 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 *EcoRI* and *HindIII*, and was ligated with the Quick ligation™ kit into similarly digested and dephosphorylated pUC19. After transformation into NEB 5a-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 was obtained by using a variation of the recursive directional ligation,³³ as previously described.³⁴ Cloning in the expression vector was as follows: The ELP sequence was extracted from pUC19- ELP-M-40 by a double digestion *NdeI* and *BamHI*, and ligated with the Quick ligation™ kit into similarly digested and dephosphorylated pET-44a(b) plasmid. The different ligation products was then used to transform BLR(DE3)-competent cells for production. The sequence of the resultant plasmid was confirmed by DNA sequencing. The sequences of the ELP-M-40 gene and of the corresponding protein are shown below.

```

atgtgggttccagggcgttgagtgccagggcatgggctaccaggtgtgggagttccaggt
M W V P G V G V P G M G V P G V G V P G
gttggggtaccgggctcggagttcctgggatgggagttccgggagttggtgtgccgggt
V G V P G V G V P G M G V P G V G V P G
gtcgggtgtgcctgggggtgggtgttccaggtatgggggttccggggtgtcggcgttccggc
V G V P G V G V P G M G V P G V G V P G
gttgggtgttccagggcgttaggtgtgccgggaatgggggttccgggagttggtgtacctggc
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V G V P G V G V P G M G V P G V G V P G
gttggggtaccgggctcggagttcctgggatgggagttccgggagttggtgtgccgggt
V G V P G V G V P G M G V P G V G V P G

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gtcgggtgtgcctgggggtgggtgttccagggtatgggggttccgggtgtcggcgttcccggc
V G V P G V G V P G M G V P G V G V P G
gttgggtgttccaggcgtagggtgtgccgggaatgggggttccgggagttggtgtacctggc
V G V P G V G V P G M G V P G V G V P G
gtgggagttacctggagtcggcgtgcctgggtatggggcgtgcctggcgtcggcgtacctggc
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-40.

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 µg·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 µg·mL⁻¹), and bacteria were cultivated at 37°C in 5 L flasks. When the optical density at 600 nm (OD₆₀₀) 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 OD₆₀₀ and SDS-PAGE analysis.

Isolation and purification of recombinant ELP-M-40.

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·g⁻¹ (wet weight) phosphate buffer (PBS; NaCl 137 mM, KCl 2.7 mM, Na₂HPO₄ 8 mM, KH₂PO₄ 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).²³ ELP-M-40 polypeptide 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, ELP-M-40-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-M-40 containing supernatant was subjected to an additional ITC round. Soluble ELP-M-40 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 ELP-M-40 were assessed by SDS-PAGE using 15% TRIS-glycine gels stained with colloidal blue G250.

Mass spectrometry analysis of ELP-M-40, ELP-M⁰-40 and ELP-M⁰²-40.

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: lyophilised ELPs were resuspended in water/acetonitrile (1/1, v/v) to obtain a final concentration lower than 100µM. Samples were then mixed with the matrix solution of sinapinic acid prepared at the concentration of 10mg/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 (10-20kDa).

NMR spectrometry analysis of ELP-M-40, ELP-M⁰-40 and ELP-M⁰²-40.

NMR spectra were acquired in D₂O at 283 K (ELP-M-40) or 298 K (ELP-M⁰-40 and ELP-M⁰²-40) 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 ($\delta = 4.70$ ppm). Data processing was performed using Topspin software. Chemical shifts of amino acids are well known in the literature.^{35,36} We have identified the α CH protons of proline and the initial valine in each repeat (VPGXG) at 4.5-4.4 ppm (60.5-57 ppm for ¹³C) and used these 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.

Oxidation of ELP-M-40 to yield ELP-M⁰-40.

ELP-M-40 was dissolved in 30% H₂O₂ and 1% AcOH in water and stirred at 0°C for 30 min.⁴ After quenching with a few drops of 1 M sodium thiosulfate aqueous solution, the reaction mixture was transferred to a 2,000 MWCO dialysis tubing and dialyzed against DI water for 48 h with water changes twice per day. The content of the dialysis bag was then lyophilized to yield ELP-M⁰-40 (95 % yield). ¹H NMR (400 MHz, D₂O, 25°C): δ 4.5-4.4 (m, 80 H, α CH VPGXG and α CH Pro), 4.2-4.15 (d, 30 H, α CH VPGVG), 3.05-2.9 (m, 22 H, CH₂S Met), 2.76-2.73 (d, 33 H, SCH₃ Met), 1.05-0.9 (br m, 420 H, CH₃ Val). MS MALDI: Theoretical MW = 17,212.3 Da, Experimental [M + H]⁺ = 17,216.8 Da

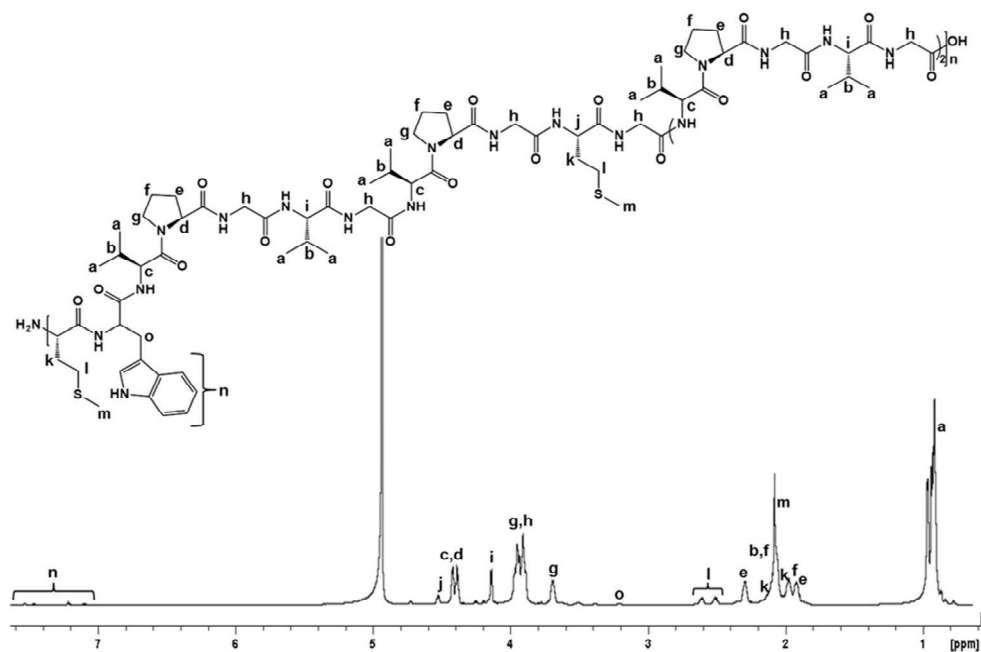
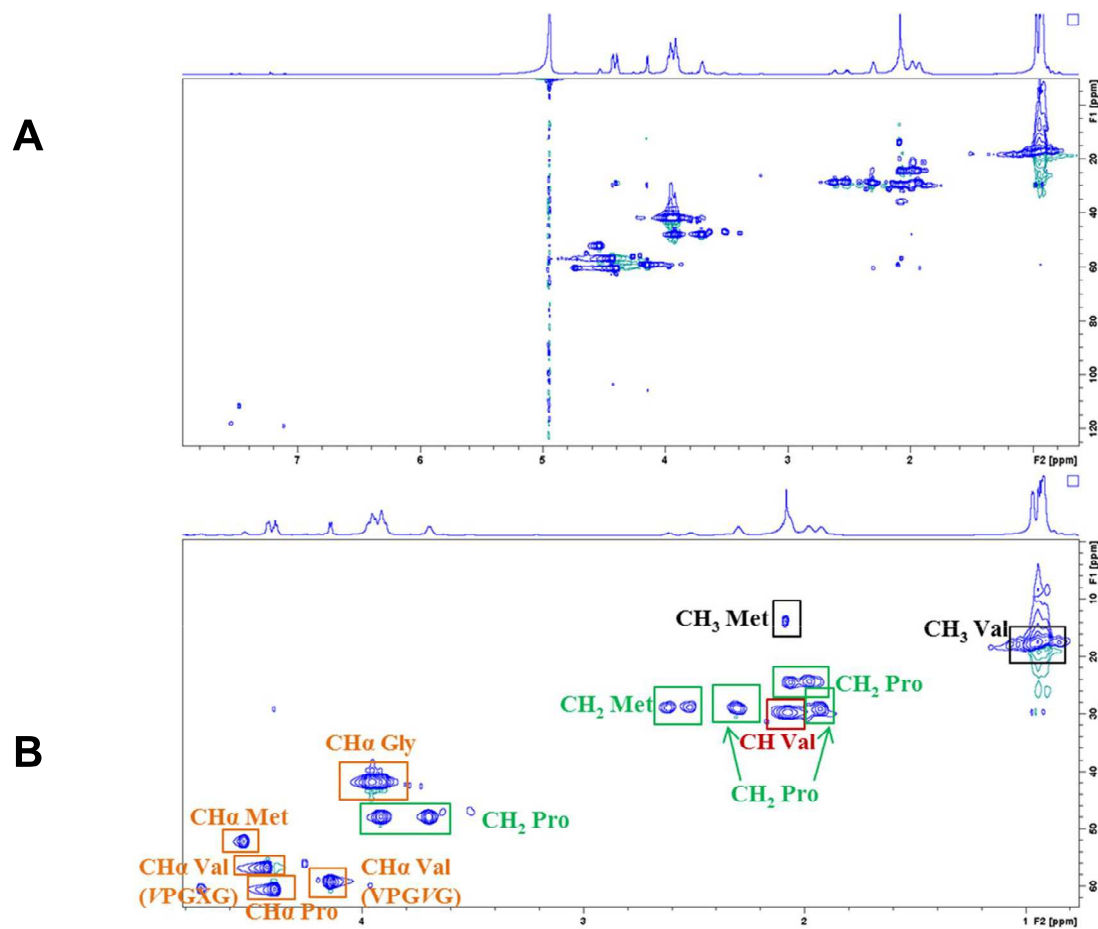
Oxidation of ELP-M-40 to yield ELP-M^{O2}-40.

ELP-M-40 was dissolved in 30% H₂O₂ and 1% HCOOH in water and stirred at room temperature for 6 h.⁴ After quenching with a few drops of 1 M sodium thiosulfate aqueous solution, the reaction mixture was transferred to a 2,000 MWCO dialysis tubing and dialyzed against DI water for 48 h with water changes twice per day. The content of the dialysis bag was then lyophilized to yield ELP-M^{O2}-40 (94 % yield). ¹H NMR (400 MHz, D₂O, 25°C): δ 4.5-4.4 (m, 80 H, αCH VPGXG and αCH Pro), 4.2-4.15 (d, 30 H, αCH VPGVG), 3.4-3.35 (t, 22 H, CH₂S Met), 3.15-3.1 (br s, 33 H, SCH₃ Met), 1.05-0.9 (br m, 420 H, CH₃ Val). MS MALDI: Theoretical MW = 17,388.2 Da, Experimental [M + H]⁺ = 17,383.1 Da

Transition temperature measurements.

Transition temperatures (T_t) were determined by measuring the turbidity at 600 nm between 20 and 80°C at a 1°C.min⁻¹ scan rate at several concentrations in DI water for ELP-M-40 (25, 50, 100, 200, 750, 1000 and 1250 μM), ELP-M^O-40 (200, 300, 400, 500, 750, 1000 and 1250 μM) and ELP-M^{O2}-40 (25, 50, 75, 100, 200, 250, 500, 750, 1000 and 1250 μM). Additional measurements were carried out at a 1 mM concentration in NaNO₃ and NaI 0.1 M solutions. 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 increasing on the absorbance versus temperature plot.

SUPPLEMENTARY FIGURES

Figure S1. ^1H NMR spectrum of ELP-M-40 in D_2O at 10°C .Figure S2. HSQC NMR of ELP-M-40 in D_2O at 10°C . (A) Full spectrum, (B) expanded region.

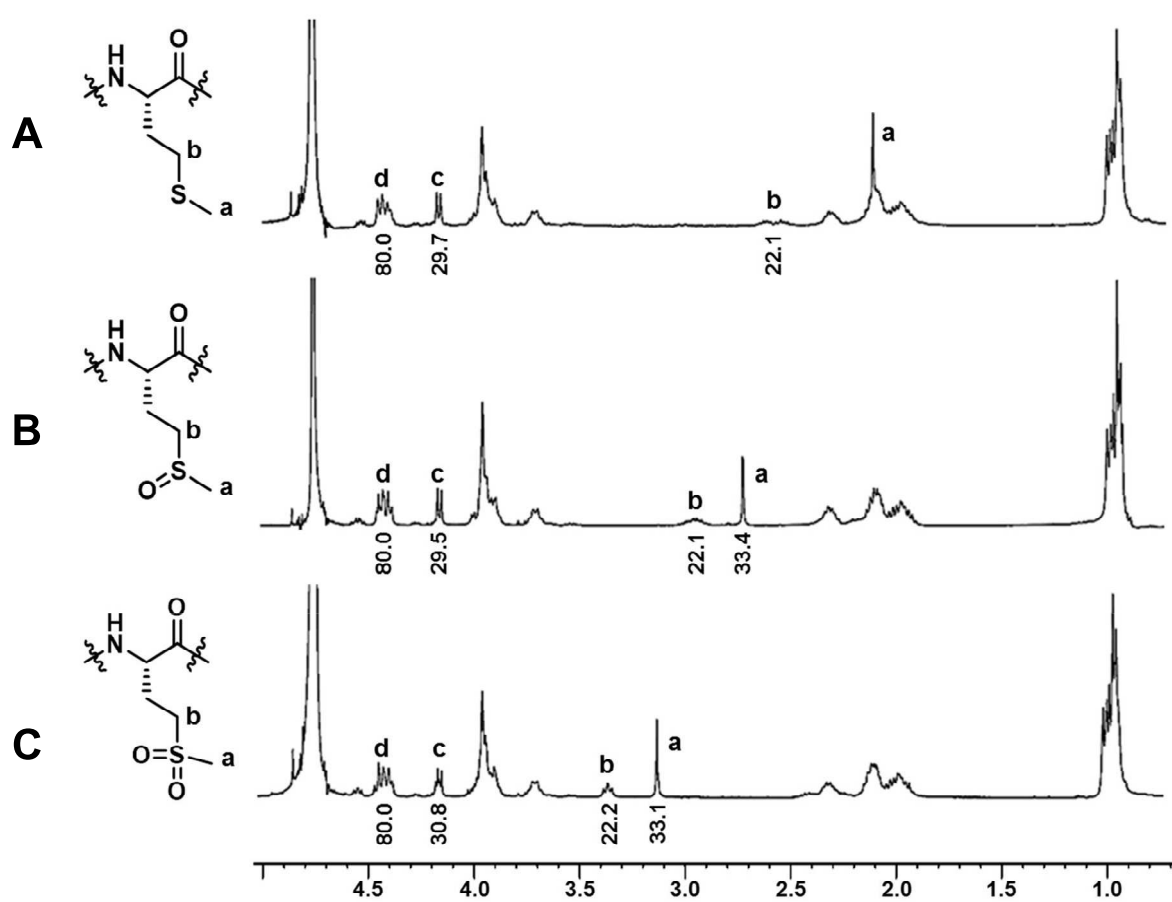
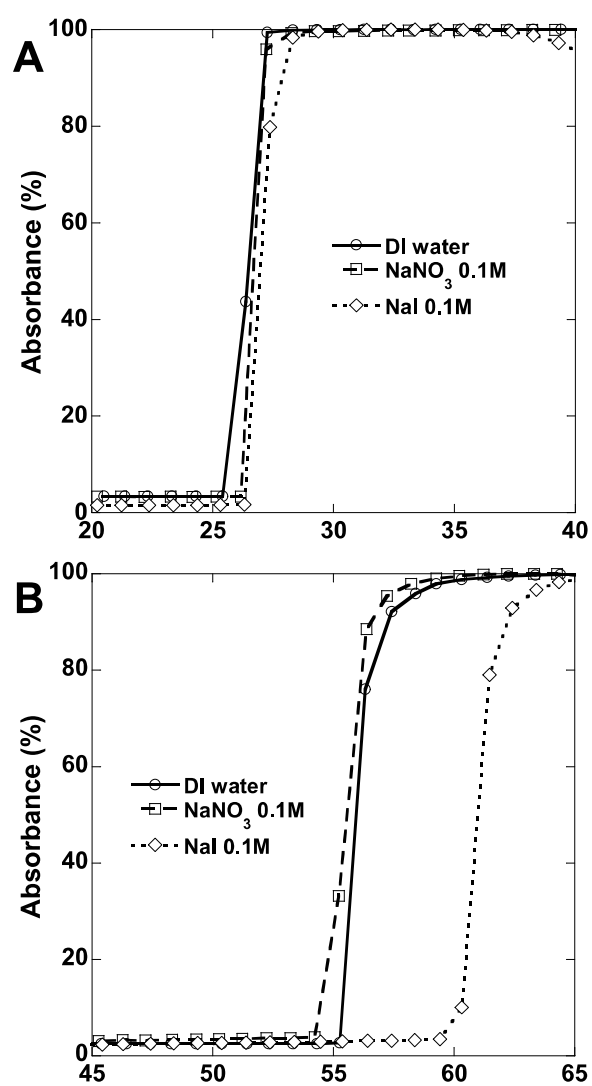


Figure S3. ^1H NMR spectra including integrals of key resonances for (A) ELP-M-40, (B) ELP-M⁰-40, (C) ELP-M^{O2}-40. c = α CH protons of valine guest residues (VPGVG), d = α CH protons of proline and the initial valine in each repeat (VPGXG).



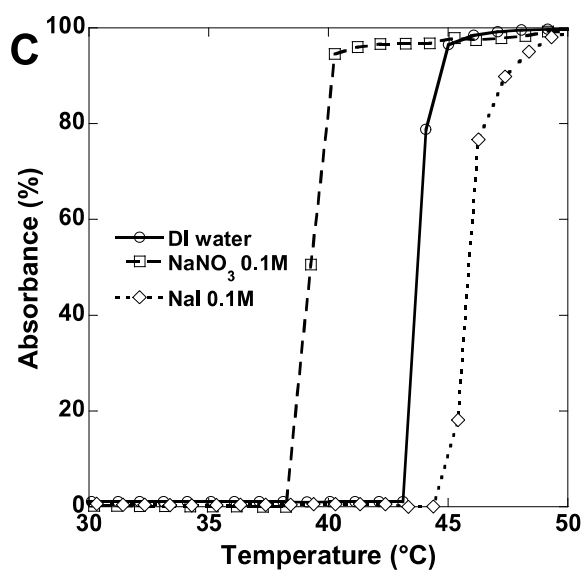


Figure S4. Effect of 2 Hofmeister salts, NaNO₃ and NaI, on the *T_t* values of ELP-M-40 (A), ELP-M⁰-40 (B) and ELP-M⁰²-40 (C). ELPs were dissolved at a 1 mM concentration in DI water, in a 0.1 M NaNO₃ solution, or in a 0.1 M NaI solution.

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CHAPTER 4

TUNING OF CATIONIC RECOMBINANT ELASTIN-LIKE POLYPEPTIDE THERMORESPONSIVE PROPERTIES BY VARYING COUNTERIONS AND SIDE-CHAINS

Abstract: We report 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 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, (VPGXG), where the guest residue *Xaa* can be any amino acid except proline.⁷ ELPs are characterized by a reversible LCST phase transition, where below a critical transition temperature (T_t), ELP chains are soluble in aqueous solution, while above the T_t they will desolvate and aggregate.^{8,9} Precise tuning of the T_t can be achieved mainly through adjustment of an ELP's primary sequence, including amino acid composition (i.e. the identity of *Xaa*) and chain length, 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 T_t 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 complete loss of thermoresponsive properties in the polysulfonium ELP. 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 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 *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.

I. DESIGN, PRODUCTION, PURIFICATION AND CHARACTERIZATION OF ELP-M-N

I.1. DESIGN OF ELP-M-N

For the present study, two recombinant methionine-containing ELPs with the primary structures MW[VPGVGVPGMG(VPGVG)₂]_{n/4}, where n = 20 or 40, thereafter 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 to enable UV-Vis detection, respectively.

The **ELP-M-40**-encoding gene was obtained by recursive directional ligation²¹ of the gene for **ELP-M-20**, which was obtained from a commercial source (See SI).

I.2. PRODUCTION, PURIFICATION AND CHARACTERIZATION OF ELP-M-N

After cloning and transfection, **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 $\mu\text{mol/L}$). The samples were also found to be of satisfactory purity as assessed by SDS-PAGE (Figure S1B). Their molecular weights of **ELP-M-20** and **ELP-M-40** (8,684.8 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).

II. TUNING THERMORESPONSIVE PROPERTIES BY VARYING COUNTERIONS

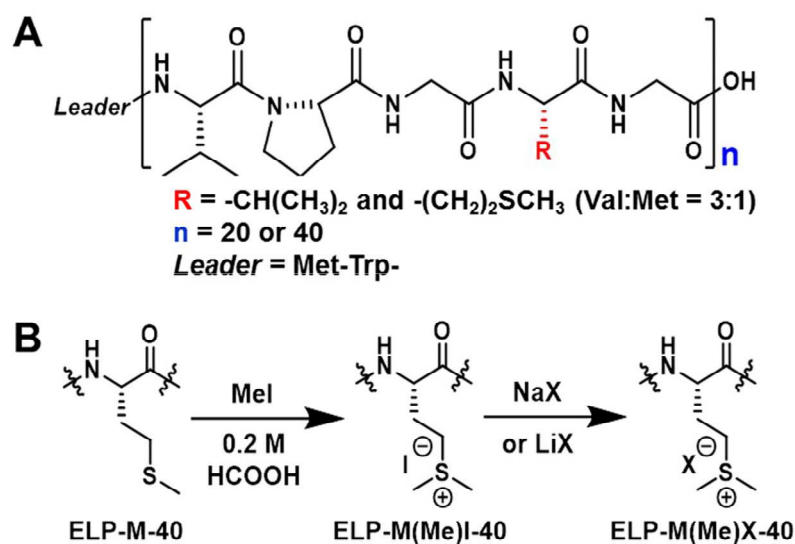


Figure 1. (A) General chemical structures of ELP-M-20 ($n=20$) and ELP-M-40 ($n=40$). (B) Scheme for methylation of ELP-M-40 and counterion exchange. Iodide was replaced by different anions using sodium or lithium salts.

With these new parent ELP samples in hand, we first sought to compare the methyl sulfonium derivatives of **ELP-M-20** and **ELP-M-40** with 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 that decrease with chain length, as well as with substitution of Met for Val (Figure S5). 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 charge density on the chains was significantly decreased. Because of their lower

charge density, we hypothesized that thermoresponsive behavior of 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}

II.1. COUNTERION EXCHANGE

II.1.1. Method of counterion exchange

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 procedures²⁰ 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 $(CF_3SO_2)_2N^-$ (NTf₂⁻), PF₆⁻, CF₃COO⁻ (Tfa⁻), CH₃COO⁻ (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 stirring overnight followed by desalting, **ELP-M(Me)X-40** derivatives were lyophilized and then analyzed using NMR spectroscopy to assess the degree of counterion exchange.

II.1.2. Proof of counterion exchange

For fluorinated anions, ¹⁹F 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 ¹H and ¹³C NMR spectroscopy in D₂O and CD₃OD, respectively (Figures S10-S15). Deuterated methanol was preferred to D₂O for ¹³C NMR spectra acquisition since it facilitated ion-pairing that led to shifted resonances for different counterions. For example, complete exchange from I⁻ to PF₆⁻ was confirmed by shifting resonances of the $-S^+(\underline{C}H_3)_2$ carbons in the ¹³C NMR spectra of **ELP-M(Me)I-40** (25.56 ppm and 25.38 ppm) and **ELP-M(Me)PF₆-40** (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 ^1H 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.

II.2. EFFECT OF SULFONIUM COUNTERIONS ON THE THERMORESPONSIVE BEHAVIOR

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.

Substitution of **ELP-M(Me)I-40** with Tfa $^-$ and Ac $^-$ counterions resulted in samples with weak thermoresponsive properties, where T_t s 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.

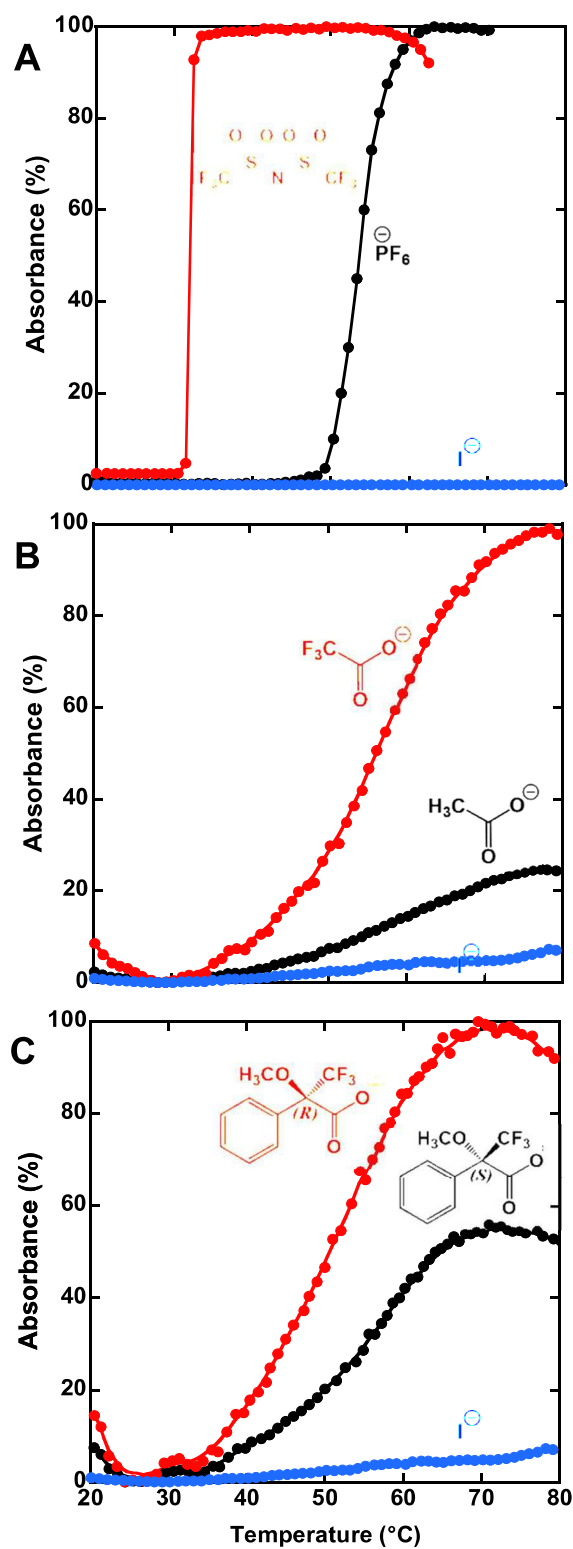


Figure 2. Normalized relative absorbance of 2.0 mM aqueous solutions of ELP-M(Me)X-40 derivatives as functions of temperature. (A) ELP-M(Me)NTf₂-40, ELP-M(Me)PF₆-40, and ELP-M(Me)I-40. (B) ELP-M(Me)Tfa-40, ELP-M(Me)Ac-40, and ELP-M(Me)I-40. (C) ELP-M(Me)RMC-40, ELP-M(Me)SMC-40, and ELP-M(Me)I-40.

Since no significant changes in ELP conformations (*i.e.*, relative content of random coils or type II β -turns) 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.

III. TUNING THERMORESPONSIVE PROPERTIES BY VARYING SIDE-CHAINS

A separate objective of this work was to further extend the variety of functional groups that can be introduced on to 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

III.1. CHEMOSELECTIVE ALKYLATION OF ELP-M-N USING EPOXIDES

III.1.1. Chemical strategy

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 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 N₂ 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).

III.1.2. Functionalization assessment by NMR and mass spectrometry

The extent of ELP alkylation from epoxide reactions was assessed by ¹H NMR spectroscopy. All spectra were calibrated using the unchanging resonances centered at 4.45 ppm, which correspond to the α CH protons of proline and the initial valine in each (**VPGXG**) 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.

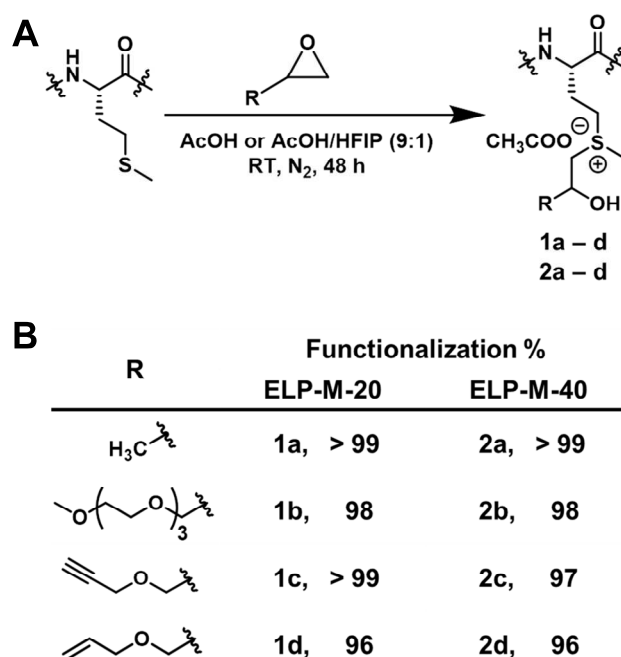


Figure 3. (A) General reaction scheme of ELP-M-n with different epoxides. (B) Table summarizing ELP-M-n derivatives and their percent functionalization.

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 functionalizations 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 ^1H NMR spectra, which correspond to $-\text{S}(\text{O})\text{CH}_3$ protons. Full ^1H 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).

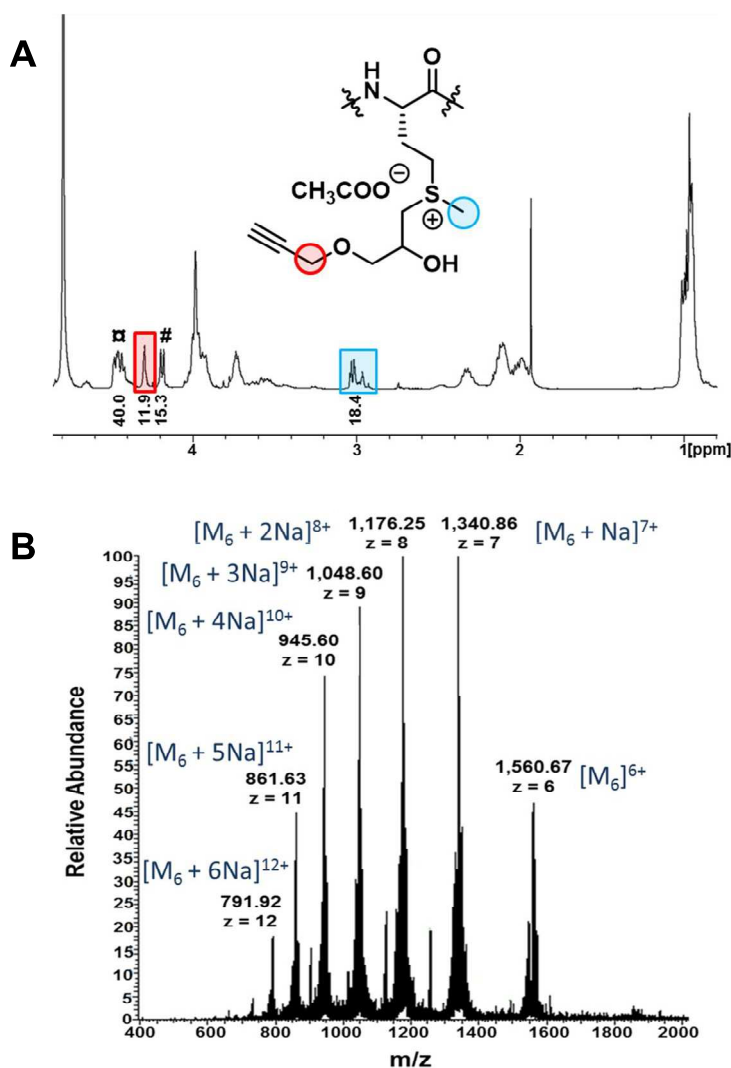


Figure 4. (A) Partial ¹H NMR spectrum of compound **1c** in D₂O. Resonance # corresponds to *Val* αCH in VPGVG repeat units, and resonance α corresponds to *Val* αCH and *Pro* αCH of VPGXG repeats. Resonance α was used to calibrate the integrals. Red and blue areas = protons used to calculate the degree of functionalization. (B) ESI mass spectrum (positive ion mode) of compound **1c**. Theoretical mass of **1c**, $[M_6]^+ = 9,363.8$ Da.

III.2. EFFECT OF SIDE CHAINS ON THE THERMORESPONSIVE BEHAVIOR

III.2.1. Dynamic light scattering

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 aggregation. Most samples showed a transition at increased temperatures, with a wide range in degree of scattering response. 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. 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)-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.

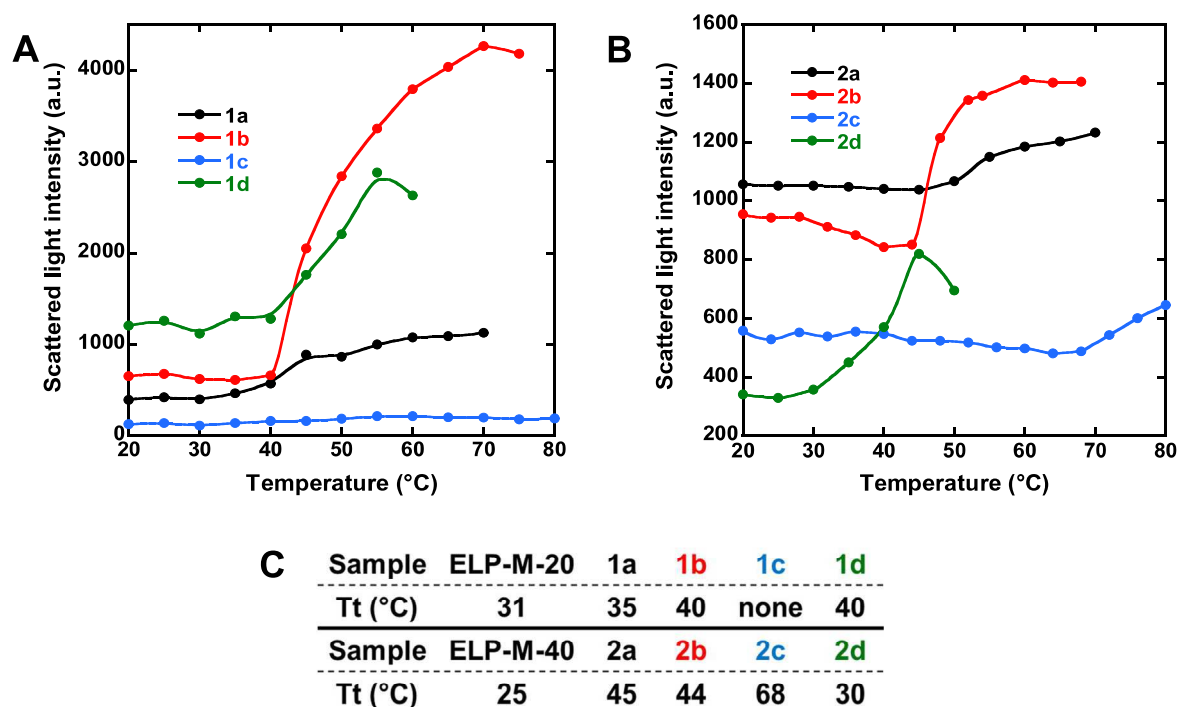


Figure 5. Scattered light intensity of (A) ELP-M-20 derivatives **1a-d**, and (B) ELP-M-40 derivatives **2a-d** as functions of temperature at 1 mM in ultrapure water. (C) Table summarizing the onset temperature of aggregation (T_t) of the different derivatives at 1 mM in water.

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 greater 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. This result can be attributed to the increased number of charges per chain in the **ELP-M-40** derivatives that appears to overwhelm the molar mass contribution.

III.2.2. Absorbance measurements

Samples **1b** and **2b** were found to give the greatest phase separation above T_t , and so the thermal transition of **2b** was quantified in more detail by absorbance measurements at 600 nm, 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 coworkers¹⁰ (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** (see Chapter 3), 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

$$T_t = T_{t,c} + k/L \ln(C_c/C) \quad (1)$$

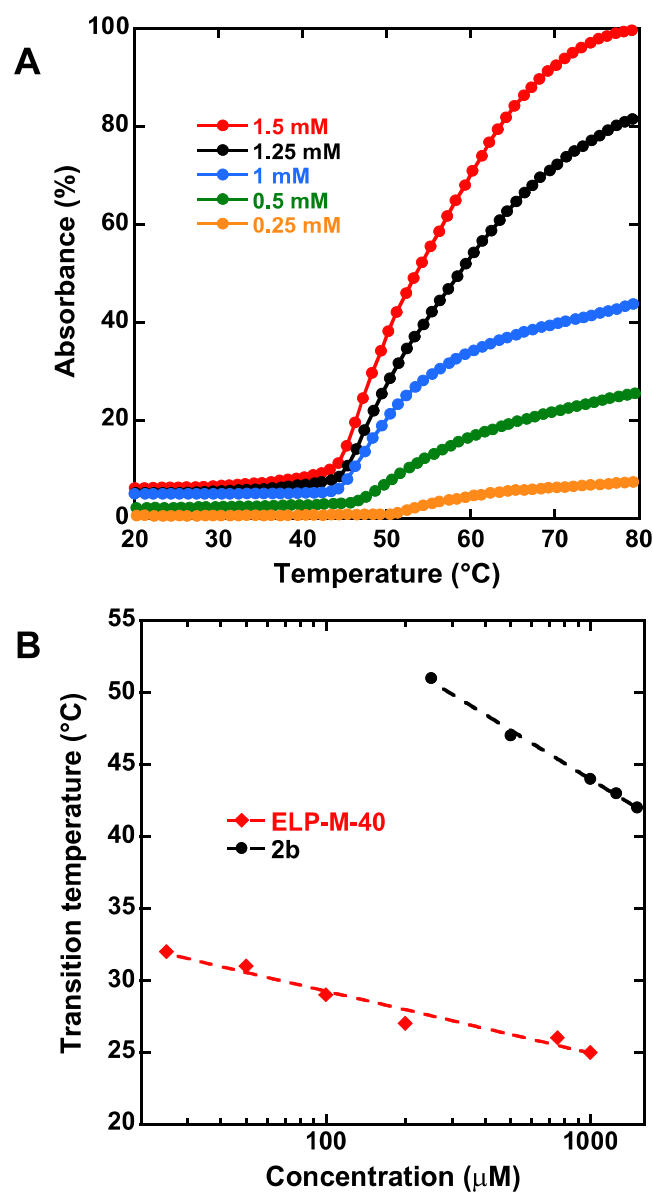
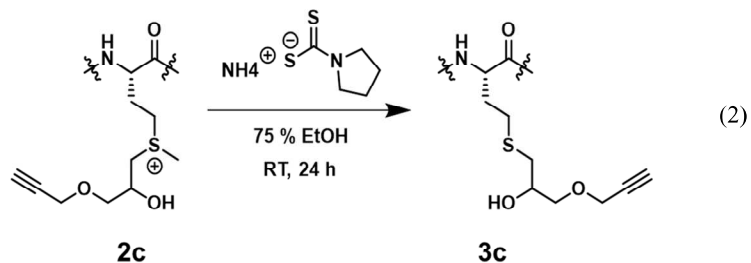


Figure 6. (A) Plots of absorbance at 600 nm for sample 2b at different concentrations in water. (B) T_t values of ELP-M-40 and sample 2b as functions of concentration. The dotted lines correspond to the fits of the data using equation 1.

III.2.3. Impact of the sulfonium charges

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).²⁹ 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.*²⁹ 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 ¹H 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.



CONCLUSION

New ELP sequences with periodically spaced 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 number of ELP derivatives, each with distinct properties, from single precursor sequences.

EXPERIMENTAL SECTION

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 (EG₃-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 M Ω -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 EcoRI and HindIII, and was ligated with the Quick ligation™ kit into similarly digested and dephosphorylated pUC19. After transformation into NEB 5 α -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.³⁰ Cloning in the expression vector was performed as follows: The ELP sequence was extracted from pUC19 by a double digestion with NdeI and BamHI, and ligated with the Quick ligation™ kit into similarly digested and dephosphorylated pET-44a(b) 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.

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 M W V P G V G V P G M G V P G V G V P G
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 V G V P G V G V P G M G V P G V G V P G
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 V G V P G V G V P G M G V P G V G V P G
gtagggttaa
 V G -

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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 µg·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 µg·mL⁻¹), and bacteria were cultivated at 37 °C in 5 L flasks. When the optical density at 600 nm (OD₆₀₀) 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 OD₆₀₀.

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·g⁻¹ (wet weight) phosphate buffer (PBS; NaCl 137 mM, KCl 2.7 mM, Na₂HPO₄ 8 mM, KH₂PO₄ 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 content of the cartridge was then lyophilized to provide the modified ELP as a white solid.

Counterion exchange (general procedure)

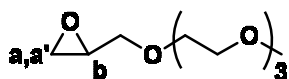
The methylated **ELP-M-40** previously synthesized was suspended in water (ca. 50 mg in 1 mL) and added dropwise to a solution of the desired salt (NaX or LiX, 5 equiv. per sulfonium, ca. 40 mg in 400 µL). The mixture was shaken overnight at room temperature, or for the most hydrophobic counterions at 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 content of the cartridge was 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 content of the cartridge was 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 N₂ into the solution for 1 hr and then stirring under N₂. The epoxide was added (10 equiv. per methionine residue) and the mixture was stirred for 48 hr under N₂ 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 content of the cartridge was 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 N₂ into the solution for 1 hr and then stirring under N₂. The epoxide was added (10 equiv. per methionine residue) and the mixture was stirred for 48 hr under N₂ 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 content of the cartridge was then lyophilized to provide the modified ELP as a white solid.

Synthesis of 2-(2,5,8,11-tetraoxadodecyl)oxirane (EG₃-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. H₂O (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 Na₂SO₄. The extracts were concentrated under reduced pressure. The residue was distilled under vacuum, providing EG₃-epoxide as a colorless liquid boiling at 110-117°C (0.1 mmHg). ¹H NMR (300 MHz, CDCl₃, 25°C): δ 3.80-3.44 (br m, 14 H, CH₂O(CH₂CH₂O)₃), 3.37 (s, 3 H, OCH₃), 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**.

^1H NMR (400 MHz, D_2O , 25°C): (main peaks): δ 4.5-4.4 (m, 40 H, $\alpha\text{CH VPGXG}$ and $\alpha\text{CH VPGXG}$), 4.2-4.15 (d, 15 H, $\alpha\text{CH VPGVG}$), 3.04-2.9 (m, 18 H, SCH_3), 1.4-1.35 (d, 18 H, CHCH_3 Met), 1.00-0.75 (br m, 210 H, CH_3 Val)

MS-ESI: Theoretical MW = 9,039.7 Da, Experimental $[\text{M}_6 + \text{Na} - \text{H}]^{6+} = 1,510.2$ Da

ELP-M(EG₃)-20, 1b

1b was prepared from **ELP-M-20** and EG_3 -epoxide using **Procedure A**.

^1H NMR (400 MHz, D_2O , 25°C): (main peaks): δ 4.5-4.4 (m, 40 H, $\alpha\text{CH VPGXG}$ and $\alpha\text{CH VPGXG}$), 4.2-4.15 (d, 15 H, $\alpha\text{CH VPGVG}$), 3.40 (s, 18 H, OCH_3 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 = 10,012.2 Da, Experimental $[\text{M}_6 + \text{Na} - \text{H}]^{6+} = 1,672.6$ Da

ELP-M(alkyne)-20, 1c

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

^1H NMR (400 MHz, D_2O , 25°C): (main peaks): δ 4.5-4.4 (m, 40 H, $\alpha\text{CH VPGXG}$ and $\alpha\text{CH VPGXG}$), 4.3 (s, 12 H, OCH_2CCH Met) 4.2-4.15 (d, 15 H, $\alpha\text{CH VPGVG}$), 3.06-2.9 (m, 18 H, SCH_3), 1.00-0.75 (br m, 210 H, CH_3 Val)

MS-ESI: Theoretical MW = 9,363.8 Da, Experimental $[\text{M}_6]^{6+} = 1,560.7$ Da

ELP-M(alkene)-20, 1d

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

^1H NMR (400 MHz, D_2O , 25°C): (main peaks): δ 6-5.9 (m, 6 H, $\text{OCH}_2\text{CHCH}_2$ Met), 5.4-5.2 (m, 12 H, $\text{SCH}_2\text{CHCH}_2$), 4.5-4.4 (m, 40 H, $\alpha\text{CH VPGXG}$ and $\alpha\text{CH VPGXG}$), 4.2-4.15 (d, 15 H, $\alpha\text{CH VPGVG}$), 4.15-4.05 (d, 12 H, $\text{OCH}_2\text{CHCH}_2$ 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 $[\text{M}_6 + \text{H}]^{7+} = 1,339.5$ Da

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

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

^1H NMR (400 MHz, D_2O , 25°C): (main peaks): δ 4.5-4.4 (m, 80 H, $\alpha\text{CH VPGXG}$ and $\alpha\text{CH VPGXG}$), 4.2-4.15 (d, 30 H, $\alpha\text{CH VPGVG}$), 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 $[\text{M}_{11}]^{11+} = 1,607.4$ Da

ELP-M(EG₃)-40, 2b

2b was prepared from **ELP-M-40** and EG₃-epoxide using **Procedure B**.

¹H NMR (400 MHz, D₂O, 25°C): (main peaks): δ 4.5-4.4 (m, 80 H, αCH VPGXG and αCH VPGXG), 4.2-4.15 (d, 30 H, αCH VPGVG), 3.40 (s, 33 H, OCH₃ Met), 3.06-2.9 (m, 33 H, SCH₃), 1.00-0.75 (br m, 420 H, CH₃ Val)

MS-ESI: Theoretical MW = 19,467.9 Da, Experimental [M₁₀ + 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**.

¹H NMR (400 MHz, D₂O, 25°C): (main peaks): δ 4.5-4.4 (m, 80 H, αCH VPGXG and αCH VPGXG), 4.3 (s, 22 H, OCH₂CCH Met) 4.2-4.15 (d, 30 H, αCH VPGVG), 3.06-2.9 (m, 33 H, SCH₃), 1.00-0.75 (br m, 420 H, CH₃ Val)

MS-ESI: Theoretical MW = 18,279.1 Da, Experimental [M₁₁]¹¹⁺ = 1,661.8 Da

ELP-M(alkene)-40, 2d

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

¹H NMR (400 MHz, D₂O, 25°C): (main peaks): δ 6-5.9 (m, 11 H, OCH₂CHCH₂ Met), 5.4-5.25 (m, 22 H, SCH₂CHCH₂), 4.5-4.4 (m, 80 H, αCH VPGXG and αCH VPGXG), 4.2-4.15 (d, 30 H, αCH VPGVG), 3.05-2.9 (m, 33 H, SCH₃), 1.05-0.9 (br m, 420 H, CH₃ Val)

MS-ESI: Theoretical MW = 18,301.3 Da, Experimental [M₁₀ - 6H]¹⁶⁺ = 1,137.3 Da

Demethylation of compound 2c.²⁹

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 N₂ and rapidly capped. The reaction mixture was vortexed until homogenous, 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 content was then lyophilized, to provide compound **3c**.

¹H NMR (400 MHz, D₂O, 25°C): (main peaks): δ 4.5-4.4 (m, 80 H, αCH VPGXG and αCH VPGXG), 4.25 (s, 22 H, OCH₂CCH Met) 4.2-4.15 (d, 30 H, αCH VPGVG), 2.8-2.55 (m, 44 H, CH₂SCH₂), 1.00-0.75 (br m, 420 H, CH₃ Val)

MS-ESI: Theoretical MW = 18,113.8 Da, Experimental [M₁₁ + 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 μ M. Samples were then mixed with the matrix solution of sinapinic acid prepared at the concentration of 10mg/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 (10-20kDa).

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 H₂O/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/ μ L and infused into the electrospray ionization source at a flow rate of 10 μ L/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/ μ L 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 D₂O 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.^{31,32} We have identified the CH α and of the proline (VPGXG) at 4.5-4.4 ppm (60.5-57 ppm for ¹³C) 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, PF₆, NTf₂).

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.

Circular dichroism (CD).

CD spectra were recorded on a Jasco J-815 spectropolarimeter equipped with a PFD-425S Peltier temperature control unit in 1 mm quartz cells. Spectra of ELP-M-40, ELP-M(Me)I-40, ELP-M(Me)SMC-40 and ELP-M(Me)RMC-40 in H₂O:TFE (5:95) at a concentration of 10 μM were recorded at 20°C and 10 nm·min⁻¹ scanning speed from 260 to 180 nm with 0.1 nm resolution, 1 sec DIT and high sensitivity. CD spectra represented the average of three measurements. CD data are reported as mean residue ellipticity [θ] (deg·cm²·dmol⁻¹).

SUPPLEMENTARY FIGURES

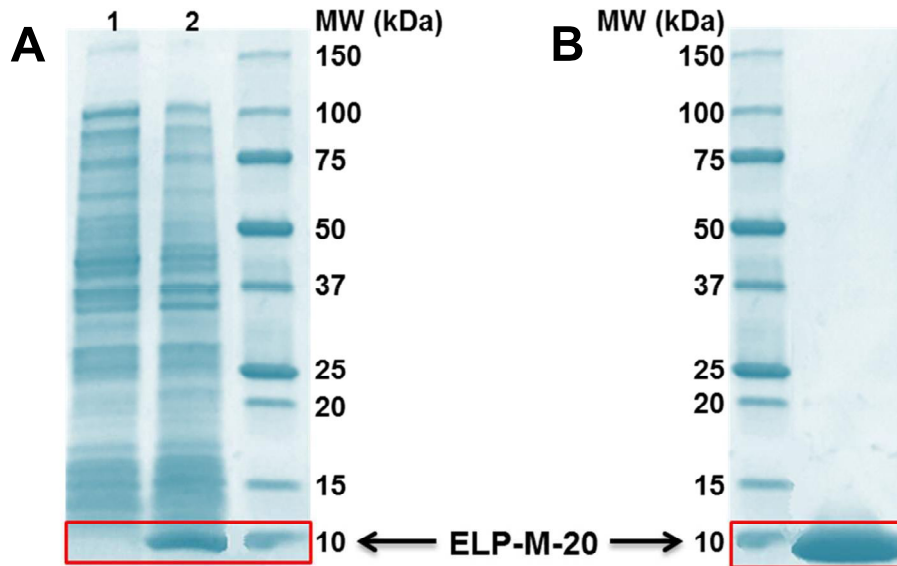


Figure S1. (A) Expression of recombinant ELP-M-20 during bacterial fermentation as analyzed by SDS-PAGE. Lane 1 = non-induced culture; lane 2 = culture 20 h after induction. M = molecular weight marker. (B) Purified ELP-M-20 as analyzed by SDS-PAGE. Data for ELP-M-40 were already provided in chapter 3.

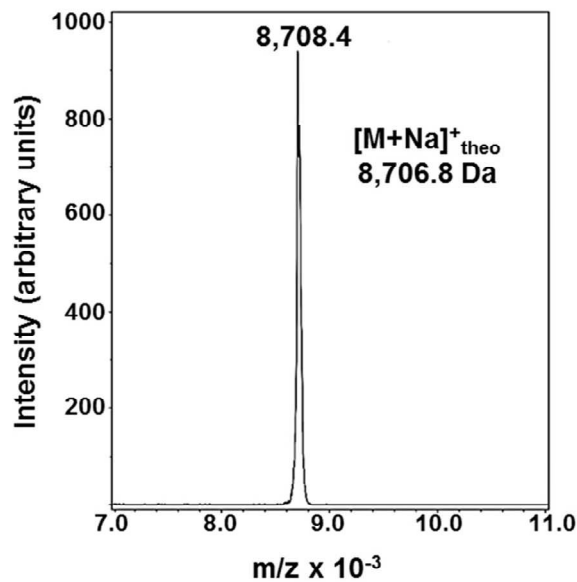


Figure S2. MALDI mass spectra of ELP-M-20. $[M+Na]^+_{theo}$ = theoretical mass of monocharged adduct. Data for ELP-M-40 were already provided in chapter 3.

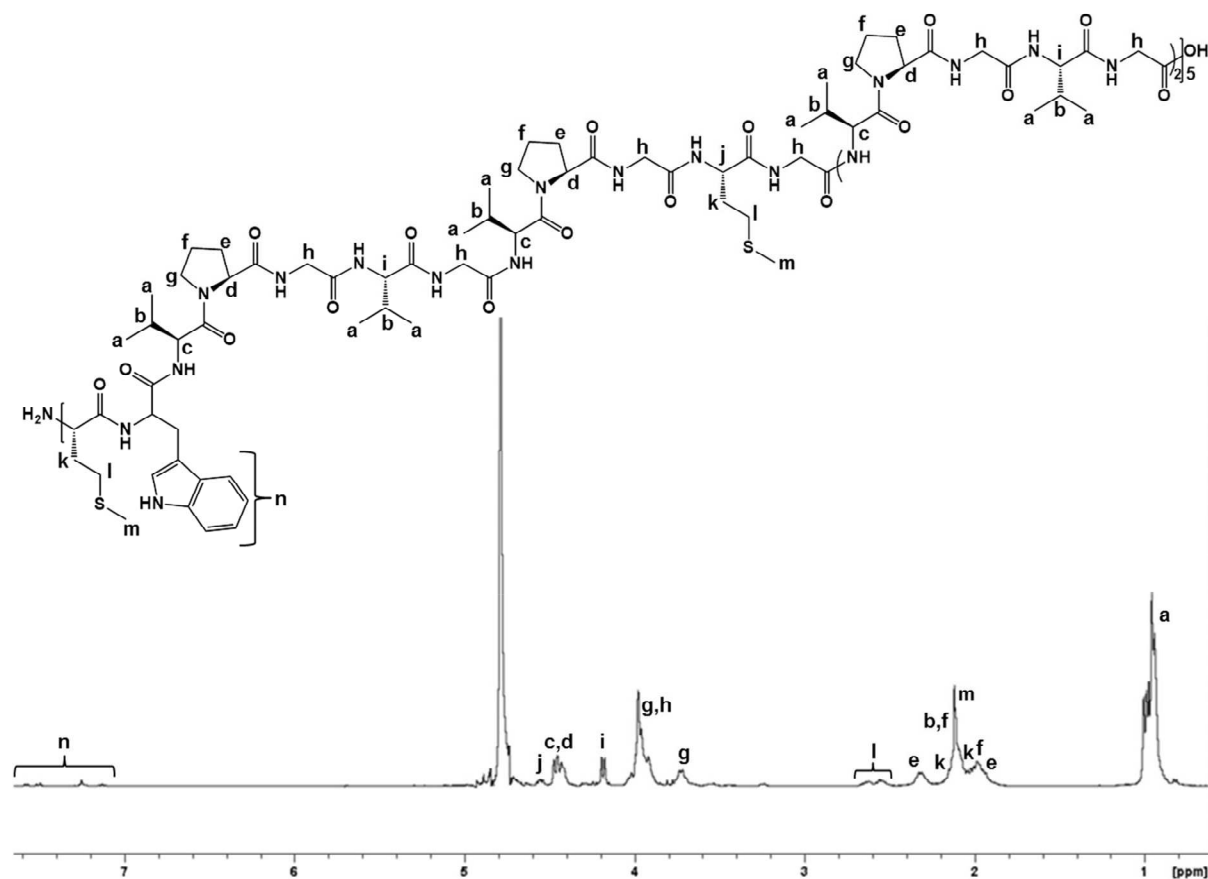


Figure S3. ^1H NMR spectrum of ELP-M-20 in D_2O . Data for ELP-M-40 were already provided in chapter 3.

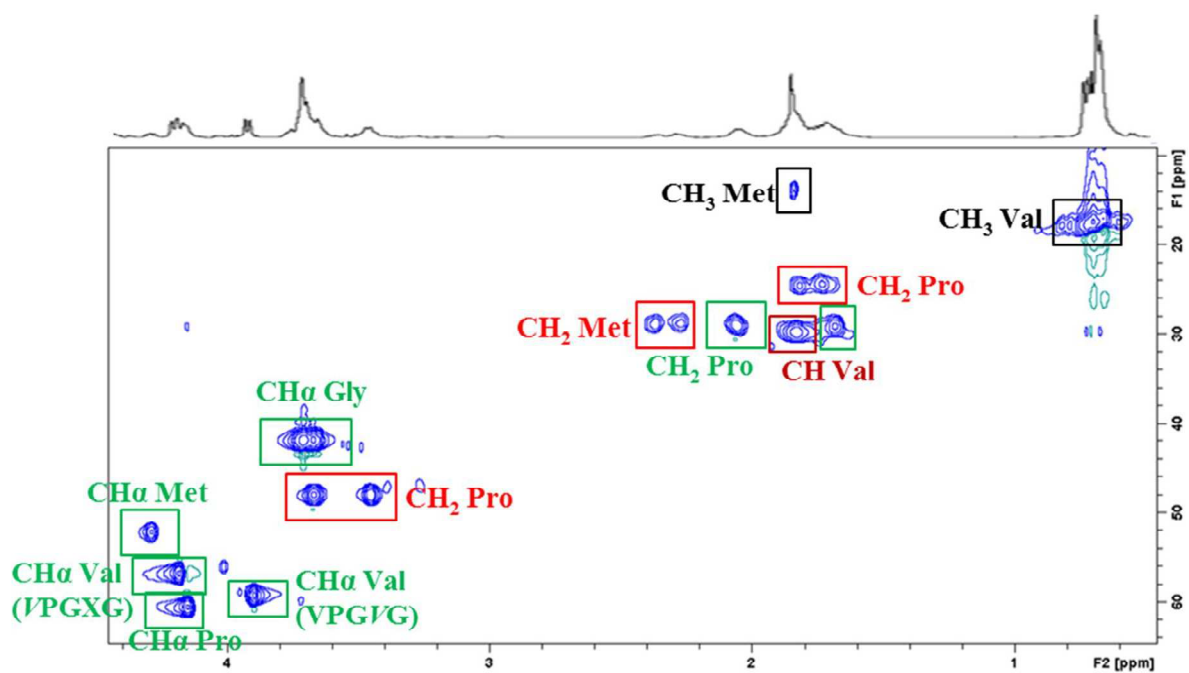


Figure S4. HSQC NMR of ELP-M-20 in D_2O . Data for ELP-M-40 were already provided in chapter 3.

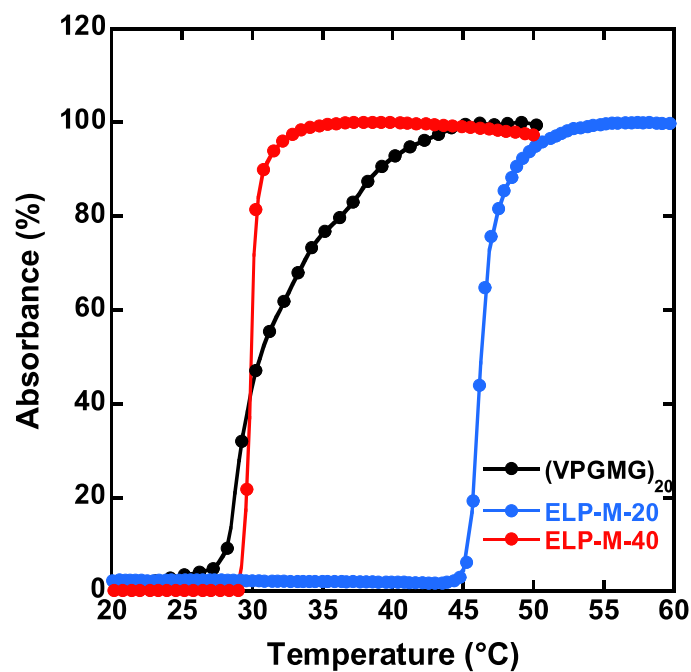


Figure S5. Normalized relative absorbance (measured at 600 nm) of 100 μM aqueous solutions of (VPGMG)₂₀, ELP-M-20 and ELP-M-40.

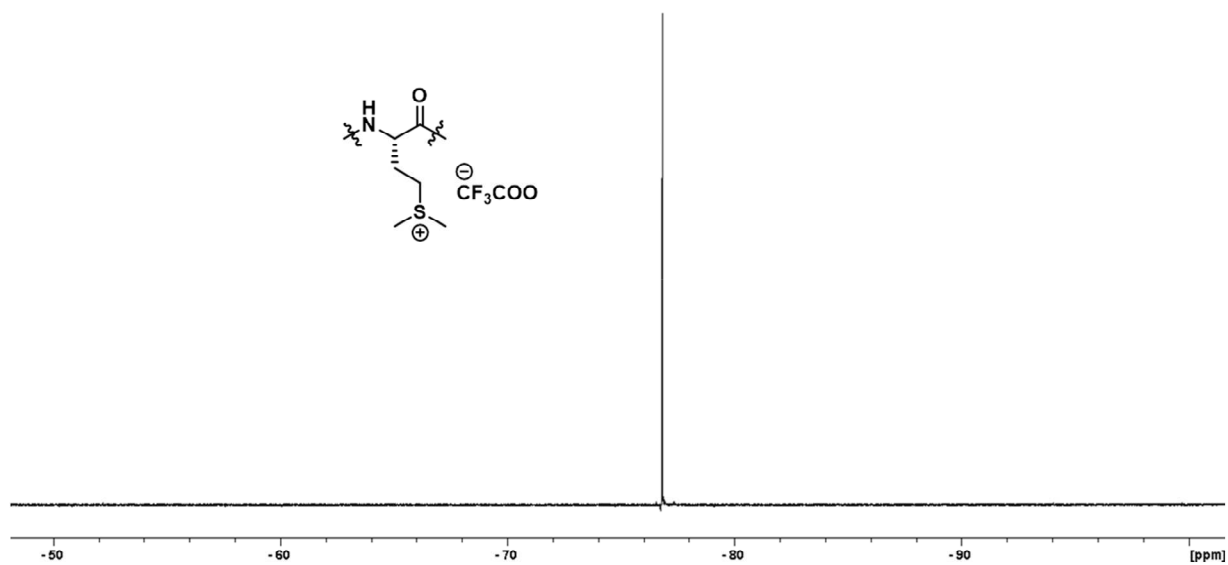


Figure S6. ¹⁹F NMR of ELP-M(Me)Tfa-40 in MeOD.

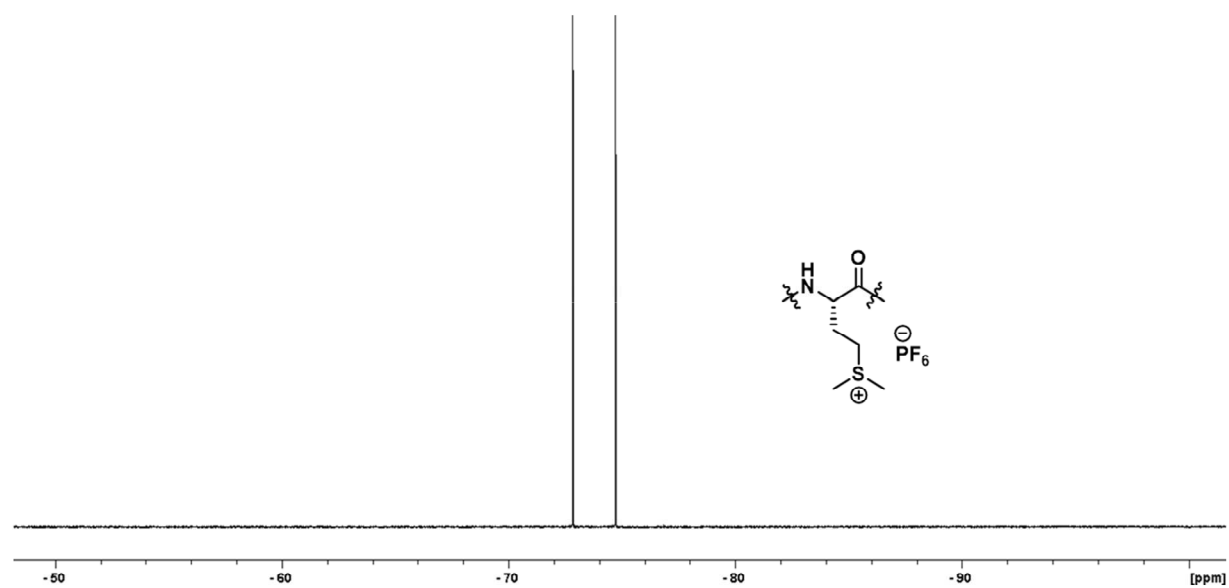


Figure S7. ^{19}F NMR of ELP-M(Me)PF₆-40 in MeOD.

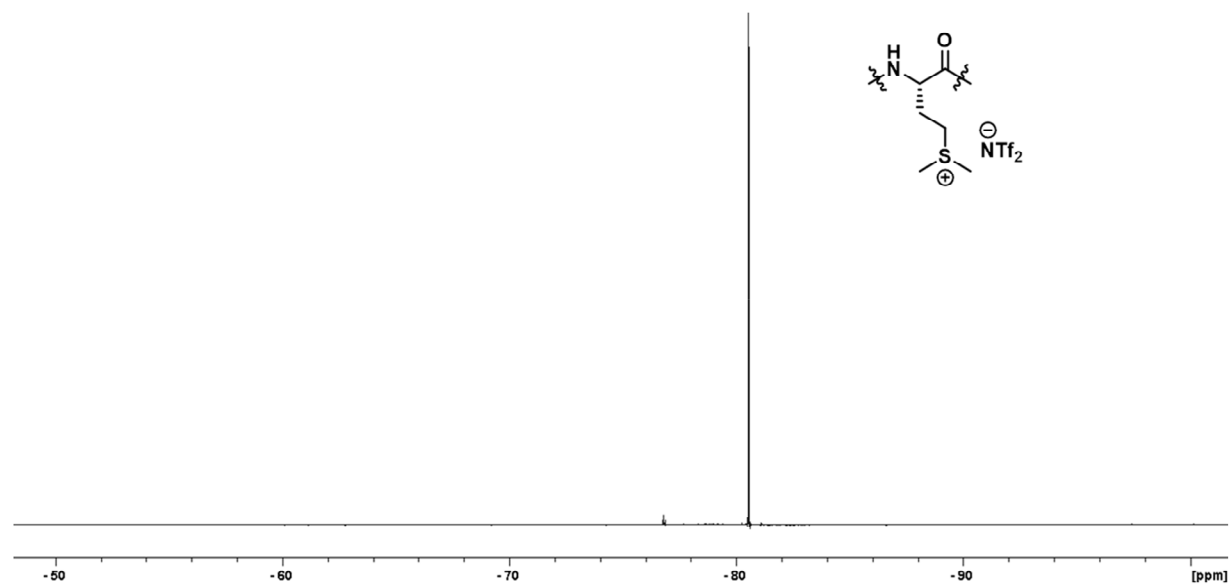


Figure S8. ^{19}F NMR of ELP-M(Me)NTf₂-40 in MeOD.

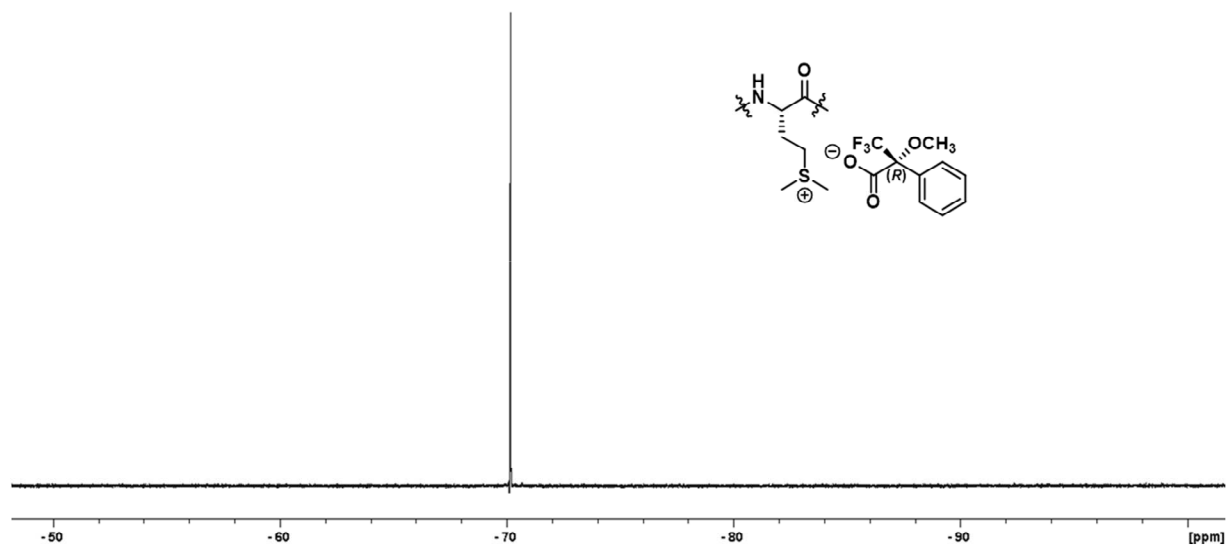


Figure S9. ^{19}F NMR of ELP-M(Me)RMC-40 in D_2O .

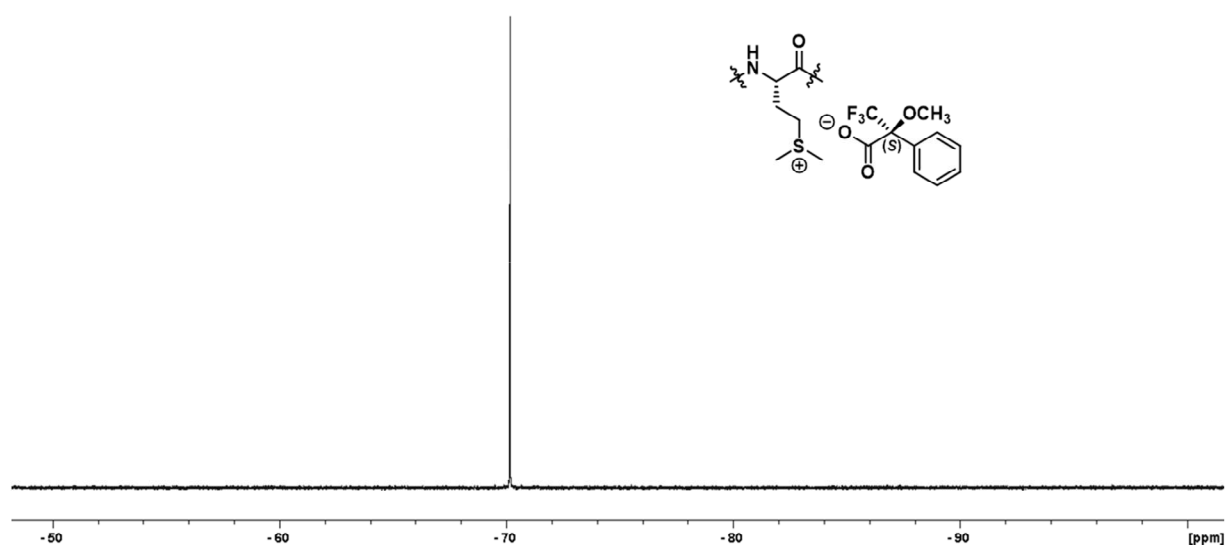


Figure S10. ^{19}F NMR of ELP-M(Me)SMC-40 in D_2O .

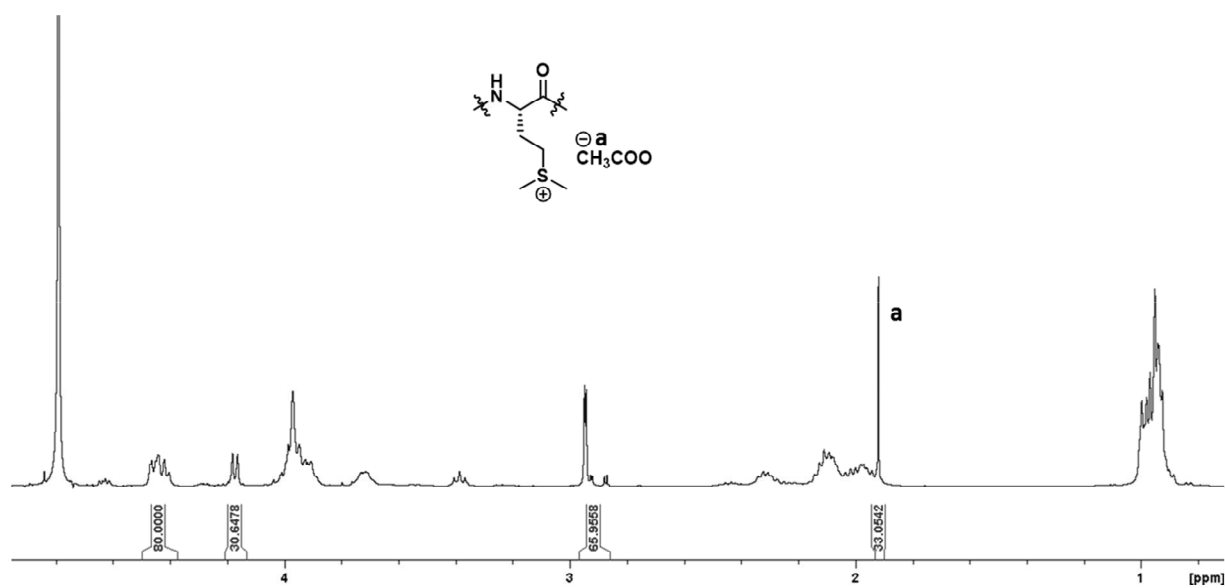


Figure S11. ^1H NMR of ELP-M(Me)Ac-40 in D_2O .

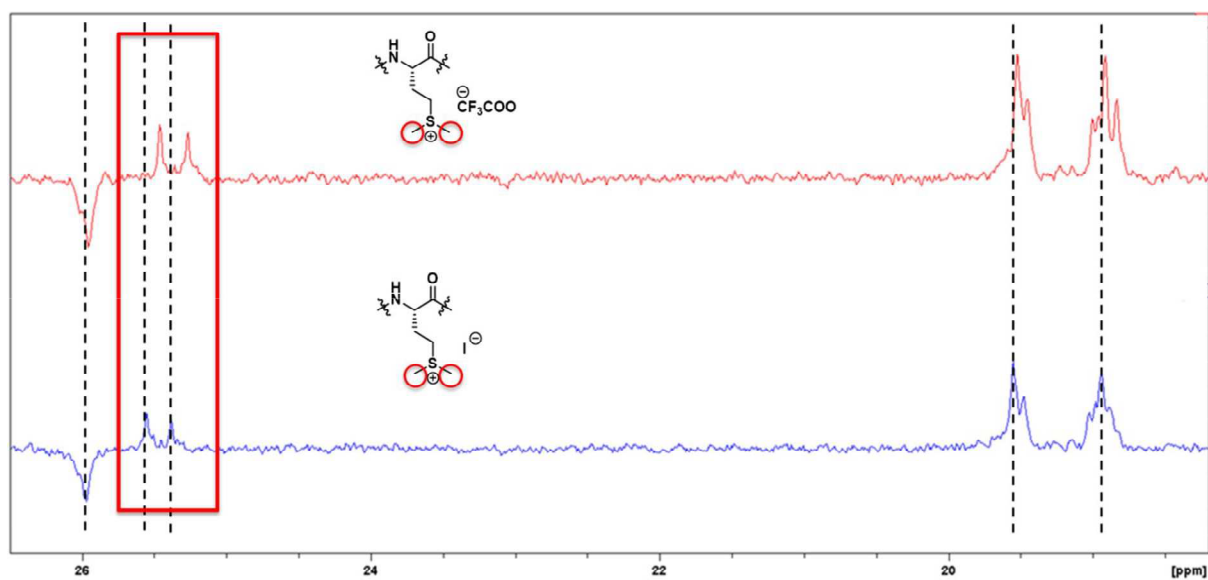


Figure S12. Stacked ^{13}C NMR of ELP-M(Me)Tfa-40 and ELP-M(Me)I-40 in MeOD.

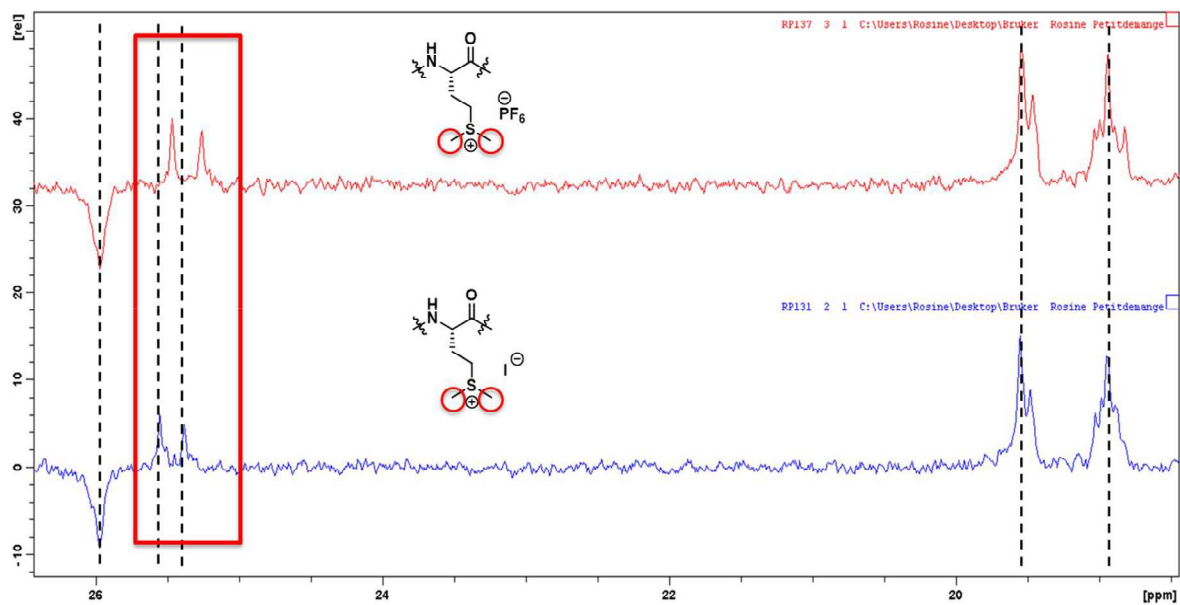


Figure S13. Stacked ^{13}C NMR of ELP-M(Me)PF₆-40 and ELP-M(Me)I-40 in MeOD.

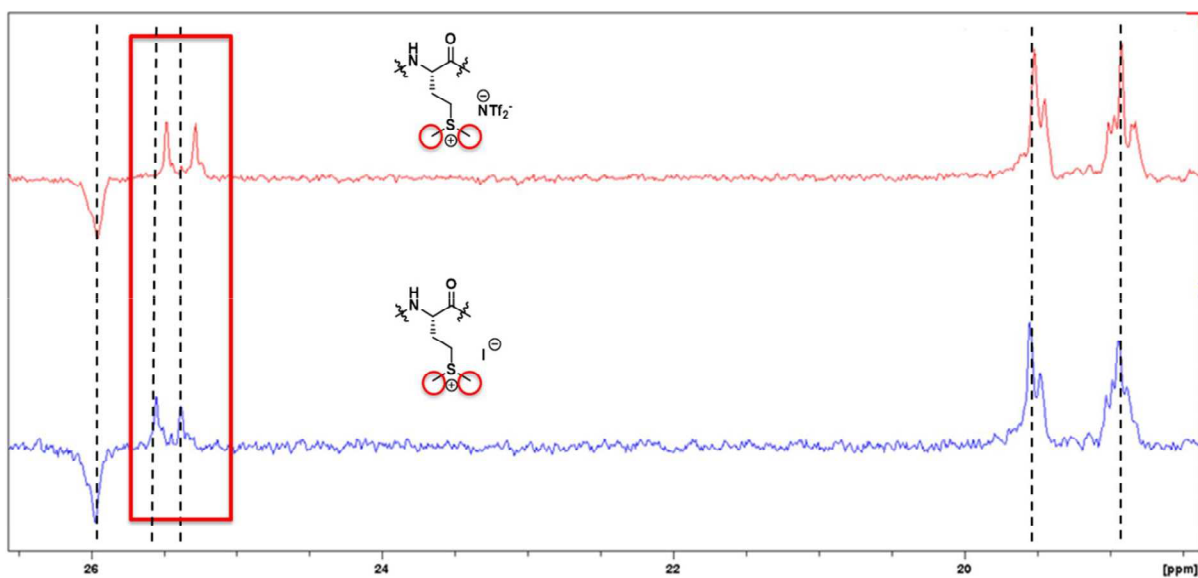


Figure S14. Stacked ^{13}C NMR of ELP-M(Me)NTf₂-40 and ELP-M(Me)I-40 in MeOD.

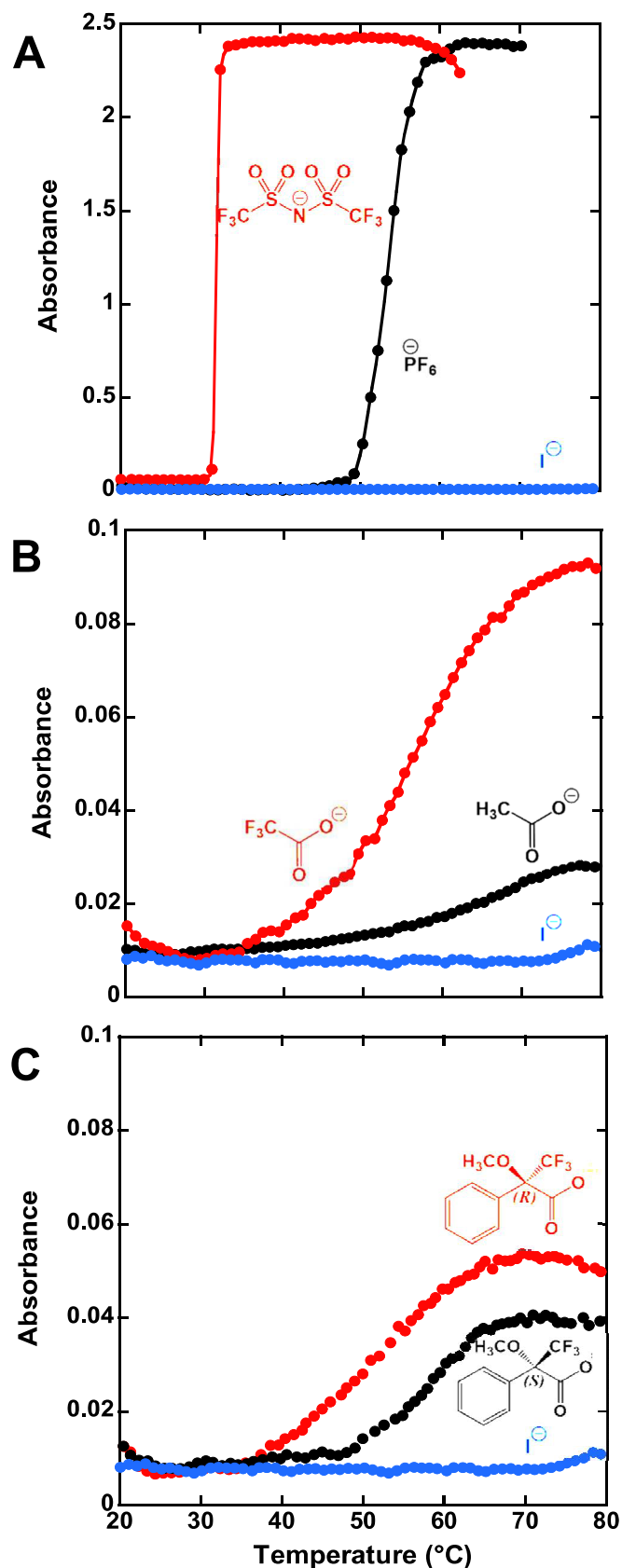


Figure S17. Absolute absorbance (measured at 600 nm) of 2 mM aqueous solutions of (A) ELP-M(Me)NTf₂-40, ELP-M(Me)PF₆-40 and ELP-M(Me)I-40, (B) ELP-M(Me)Tfa-40, ELP-M(Me)Ac-40 and ELP-M(Me)I-40, (C) ELP-M(Me)RMC-40, ELP-M(Me)SMC-40 and ELP-M(Me)I-40 in water.

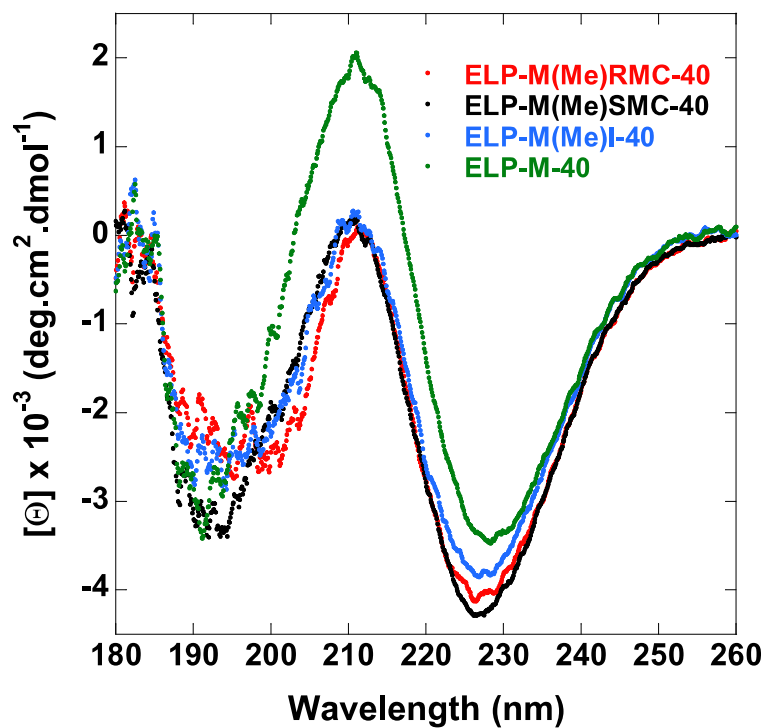


Figure S18. Superimposed CD spectra of ELP-M-40, ELP-M(Me)I-40, ELP-M(Me)SMC-40 and ELP-M(Me)RMC-40 in H₂O:TFE (5:95). Sample concentrations = 10 μ M.

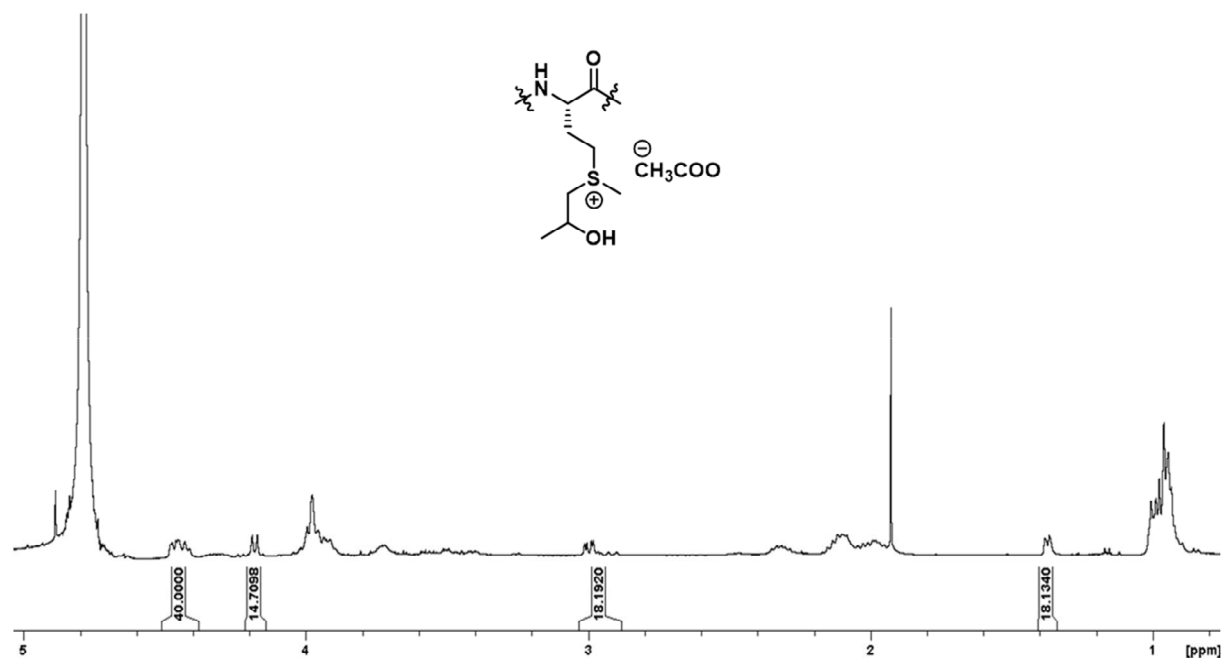


Figure S19. ¹H NMR of compound 1a in D₂O.

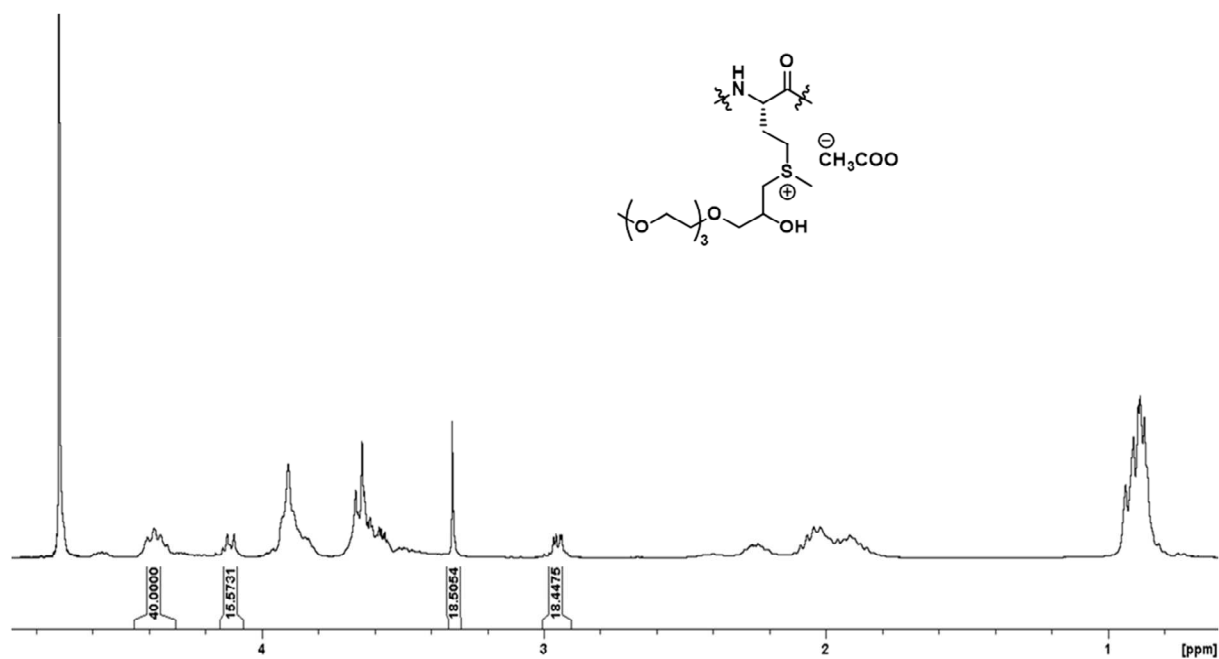


Figure S20. ¹H NMR of compound 1b in D₂O.

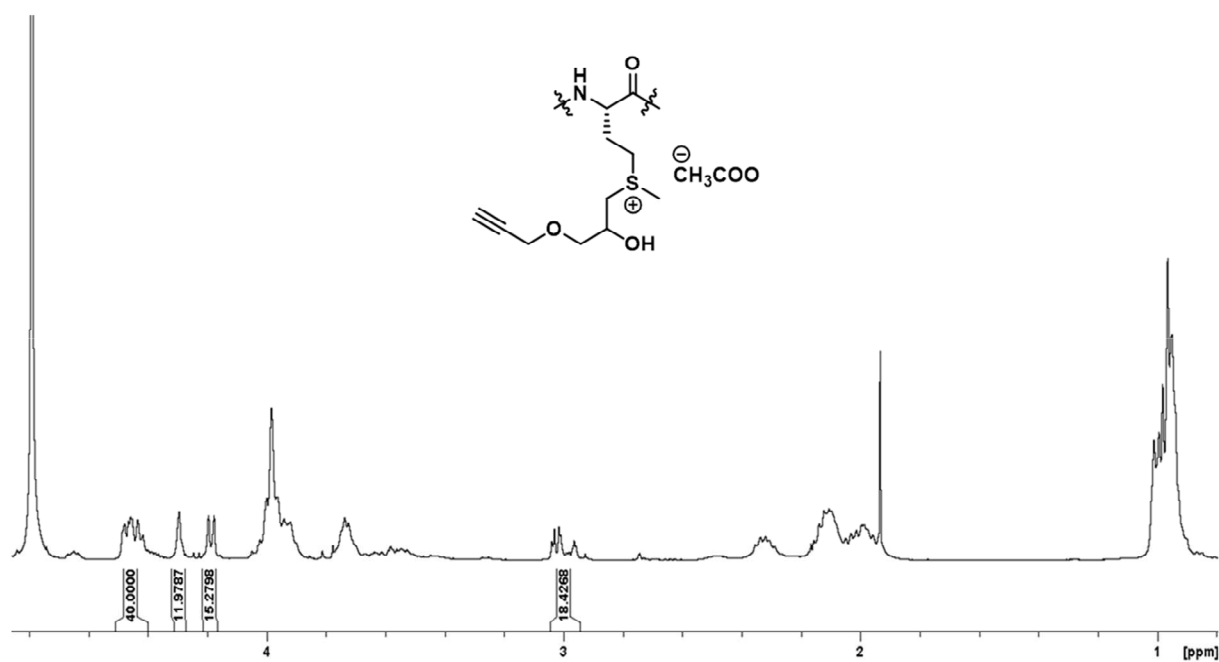
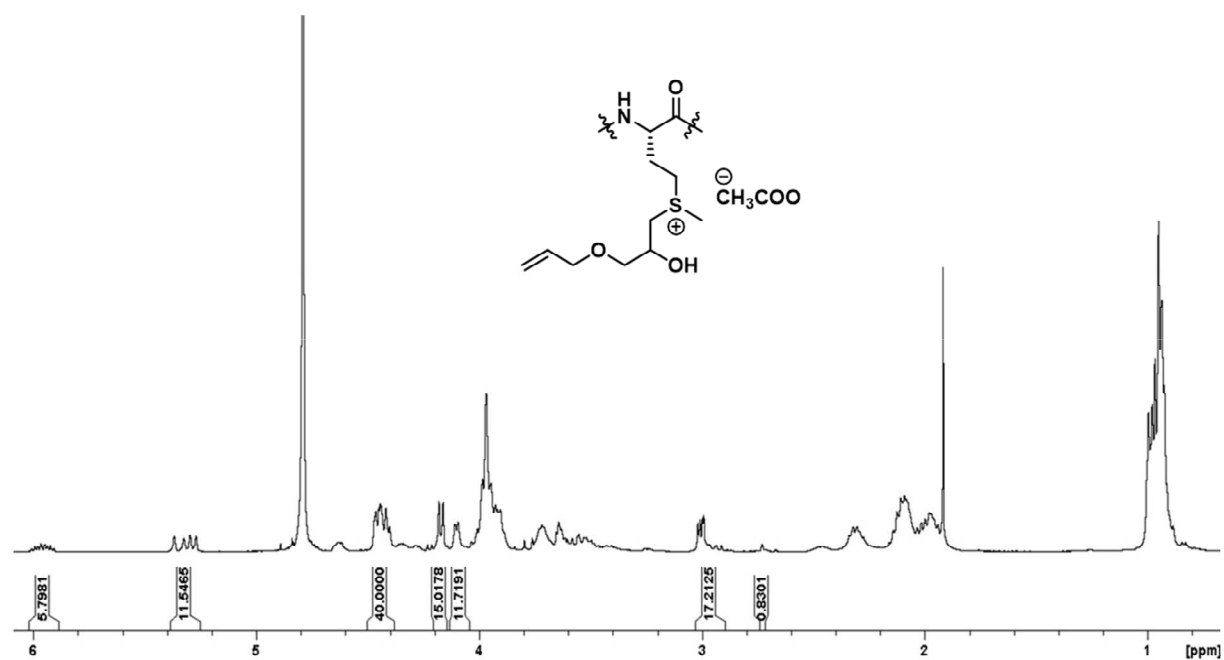
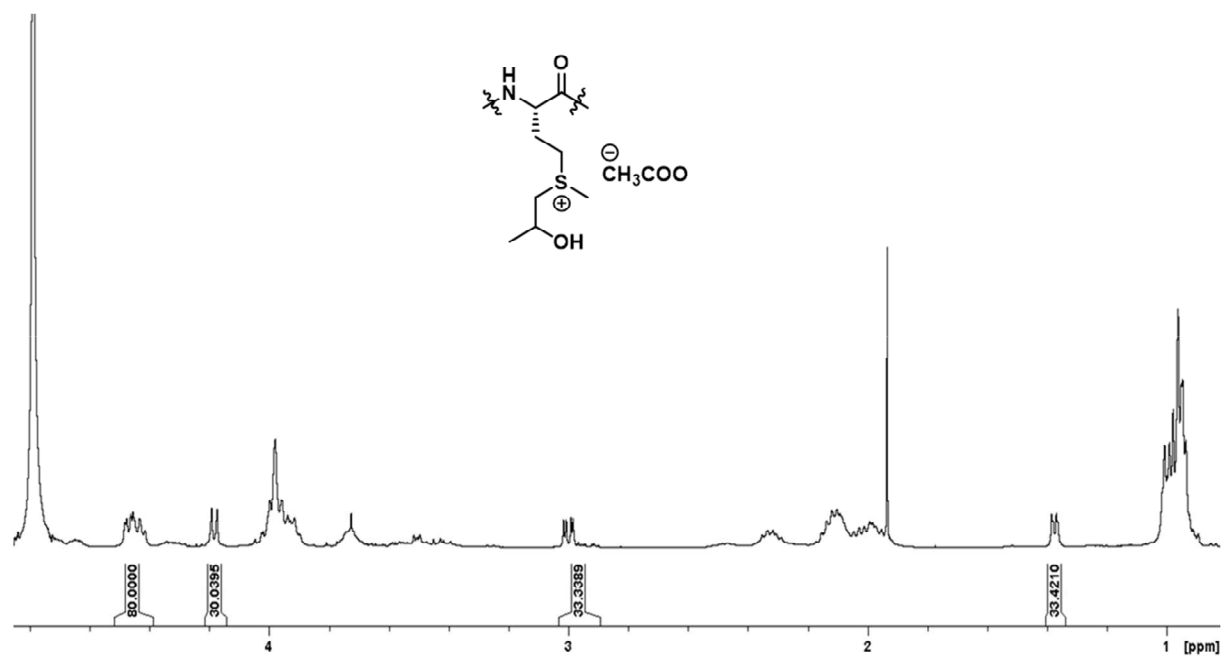


Figure S21. ¹H NMR of compound 1c in D₂O.

Figure S22. ^1H NMR of compound 1d in D_2O .Figure S23. ^1H NMR of compound 2a in D_2O .

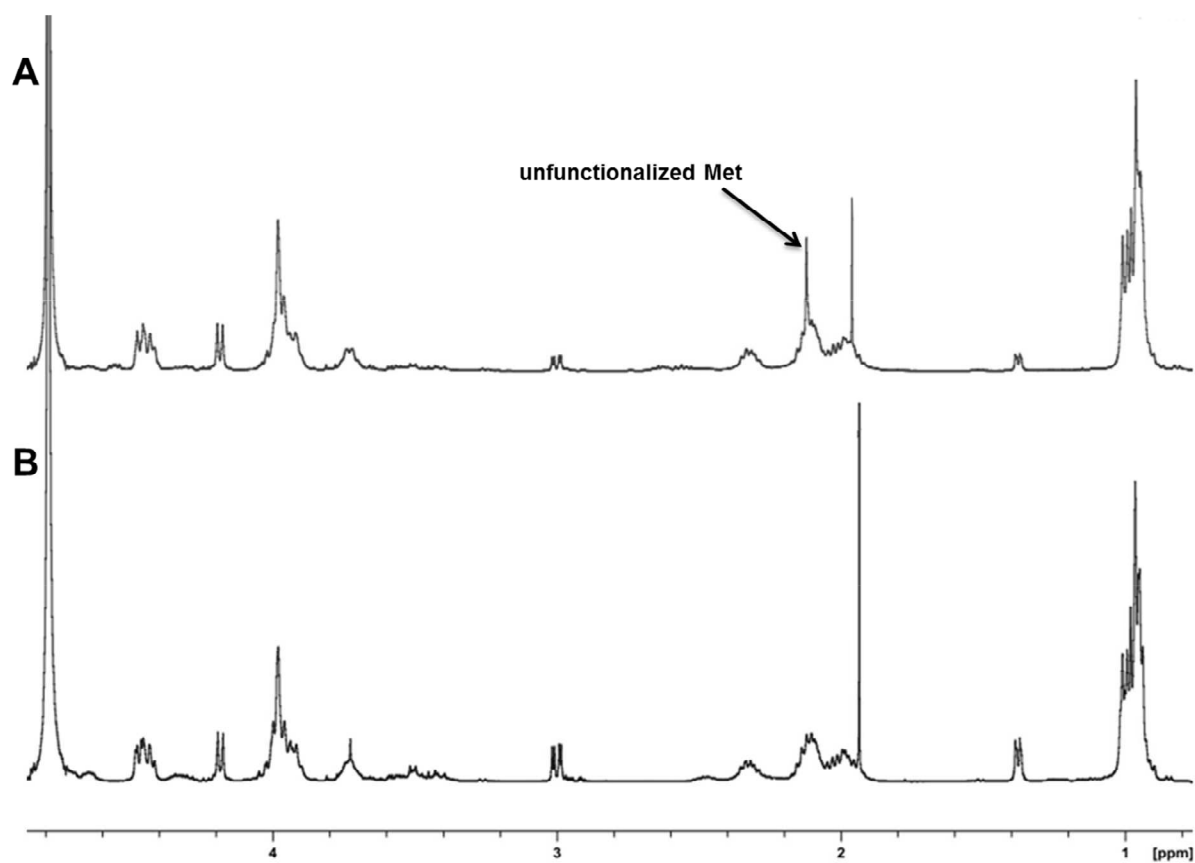


Figure S24. Stacked ^1H NMR spectra of compound 2a in D_2O . (A) Reaction run in AcOH, (B) reaction run in AcOH:HFIP (9:1).

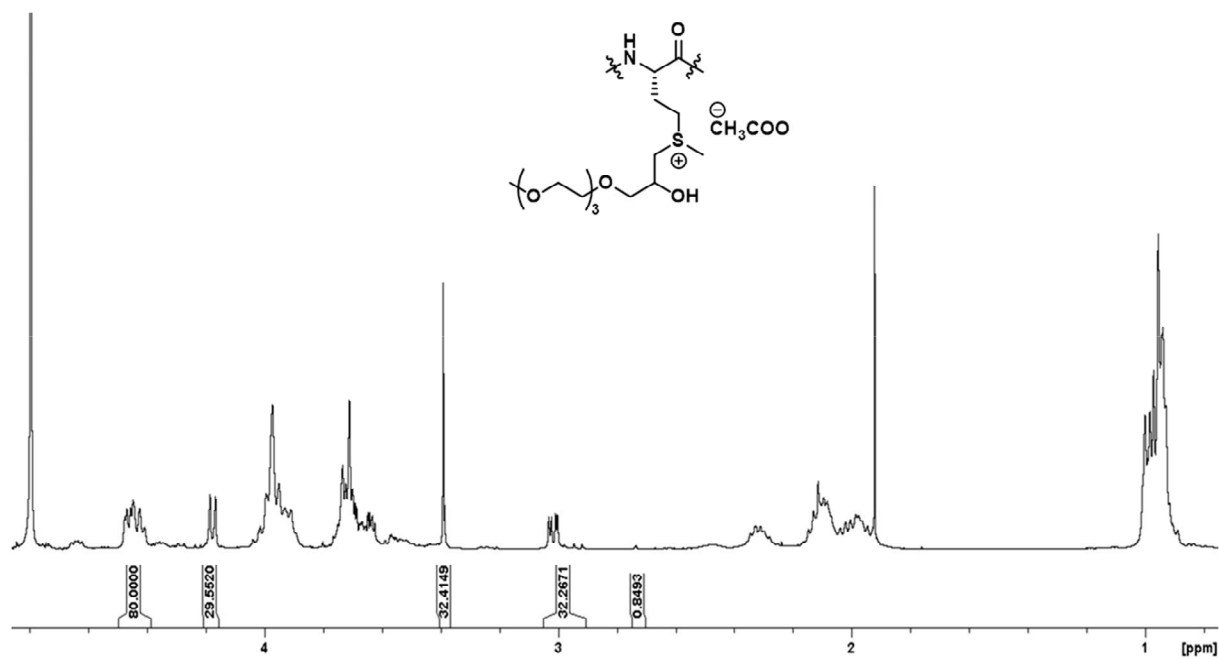
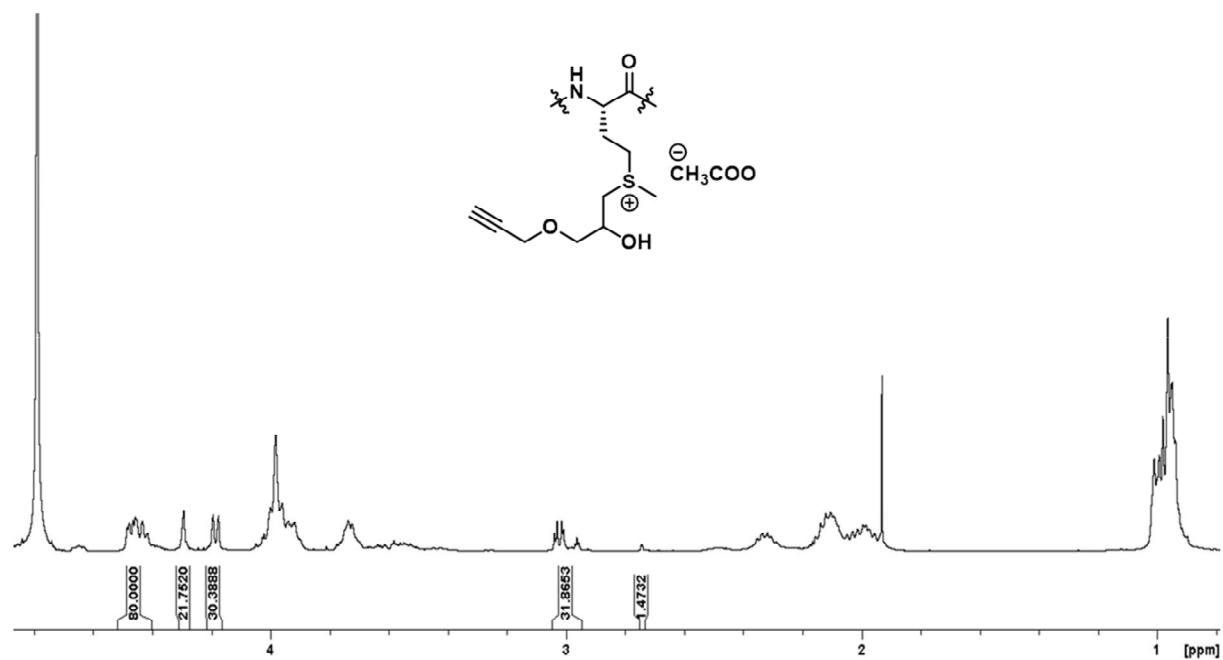
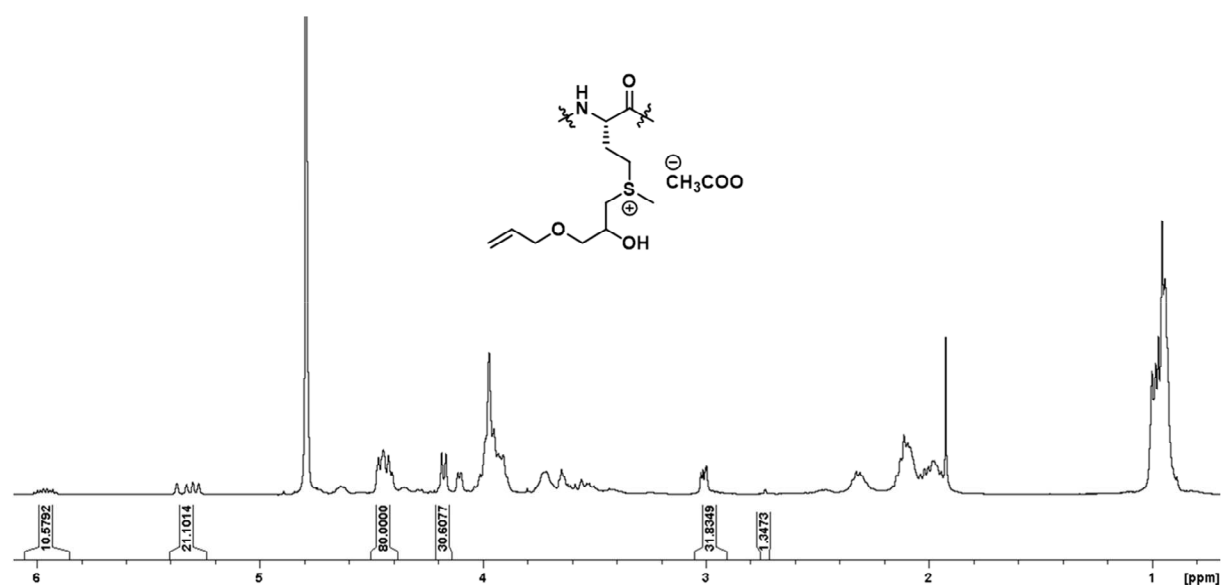


Figure S25. ^1H NMR of compound 2b in D_2O .

Figure S26. ¹H NMR of compound 2c in D₂O.Figure S27. ¹H NMR of compound 2d in D₂O.

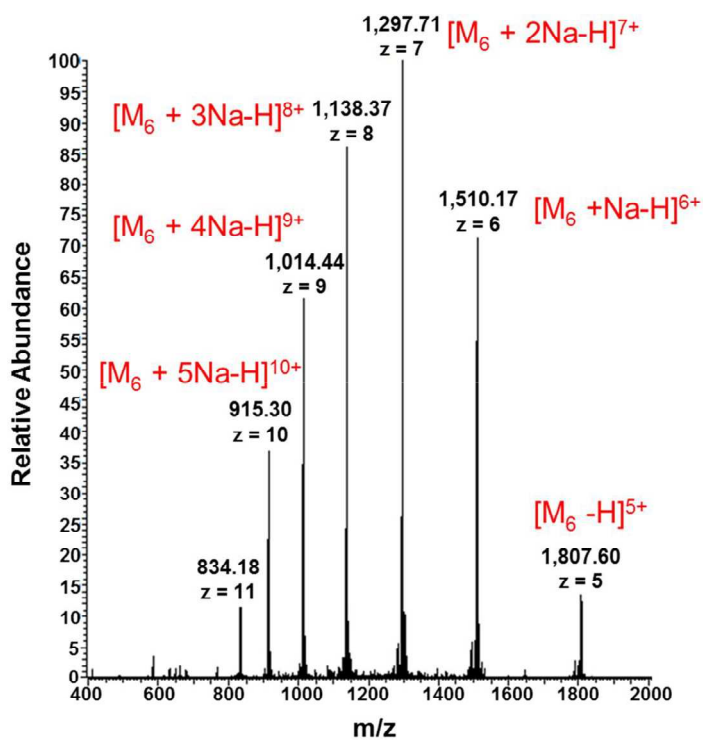


Figure S28. ESI mass spectrum of compound 1a.

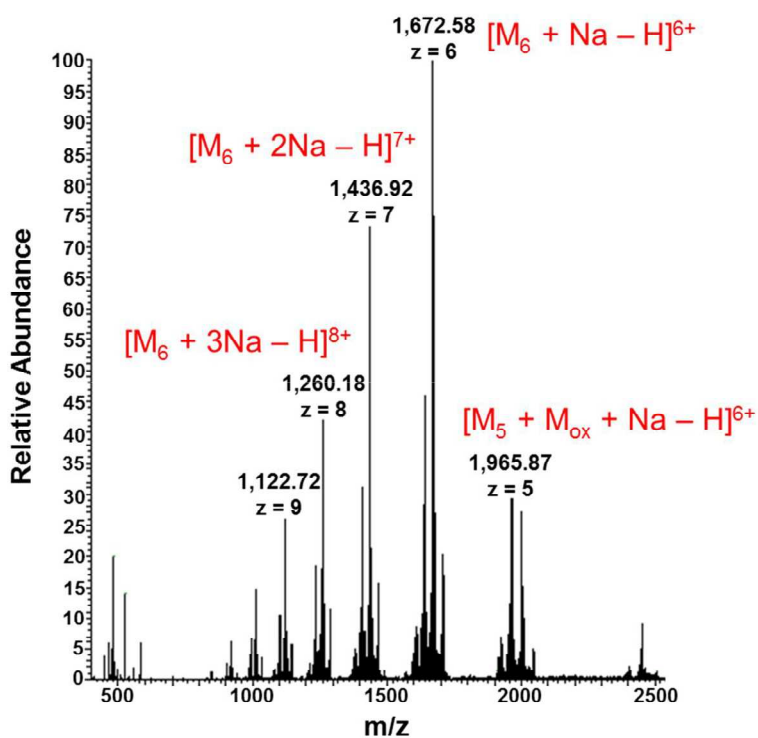


Figure S29. ESI mass spectrum of compound 1b.

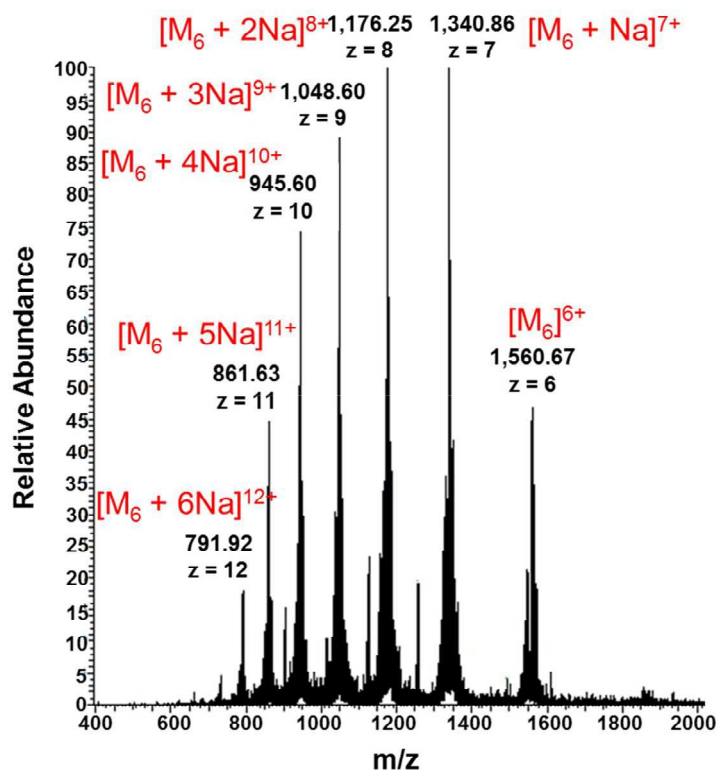


Figure S30. ESI mass spectrum of compound 1c.

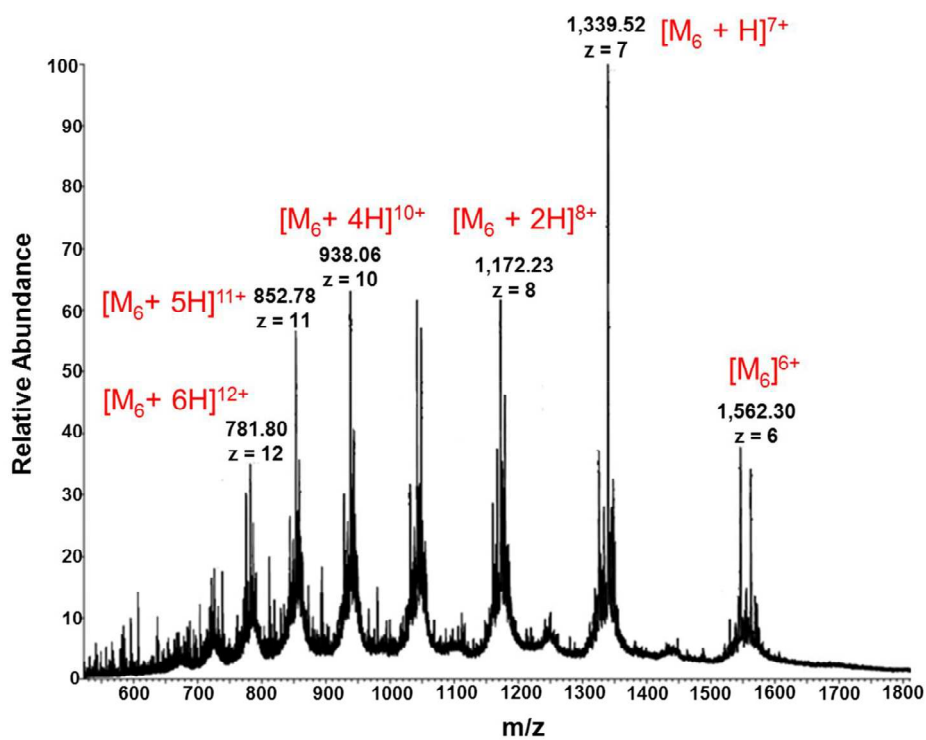


Figure S31. ESI mass spectrum of compound 1d.

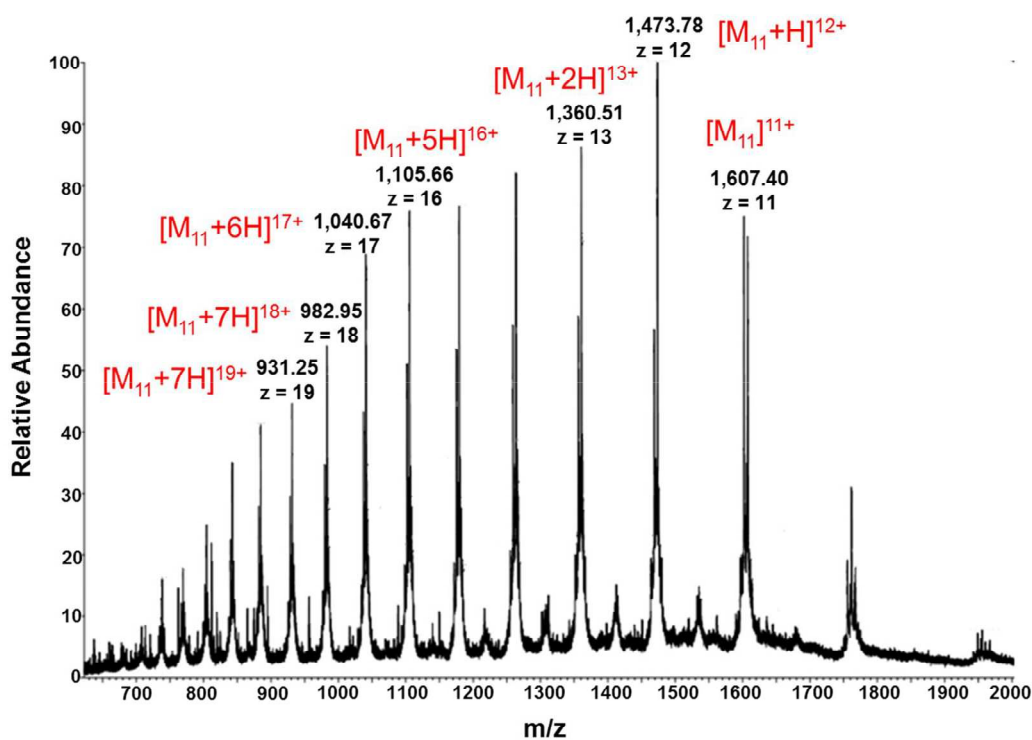


Figure S32. ESI mass spectrum of compound 2a.

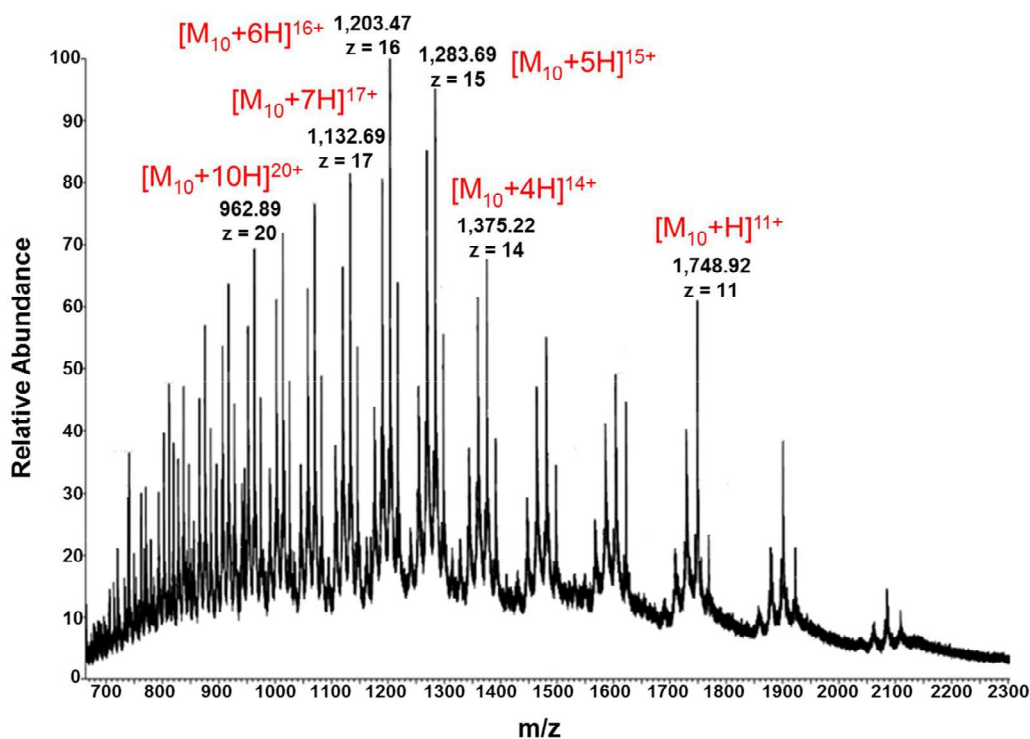


Figure S33. ESI mass spectrum of compound 2b.

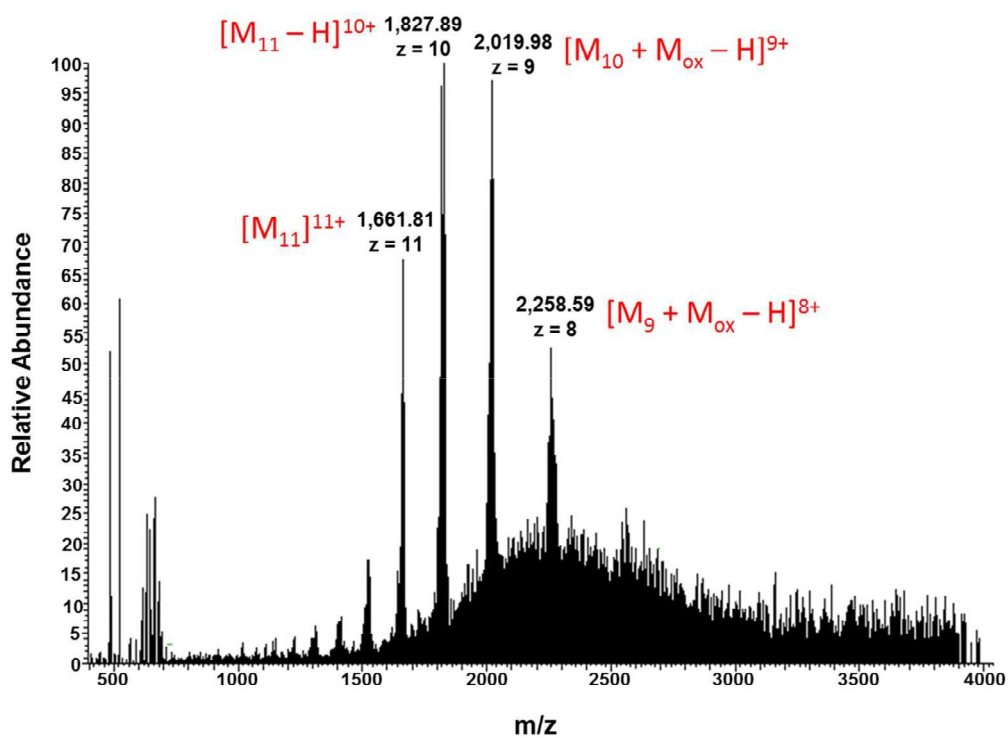


Figure S34. ESI mass spectrum of compound 2c.

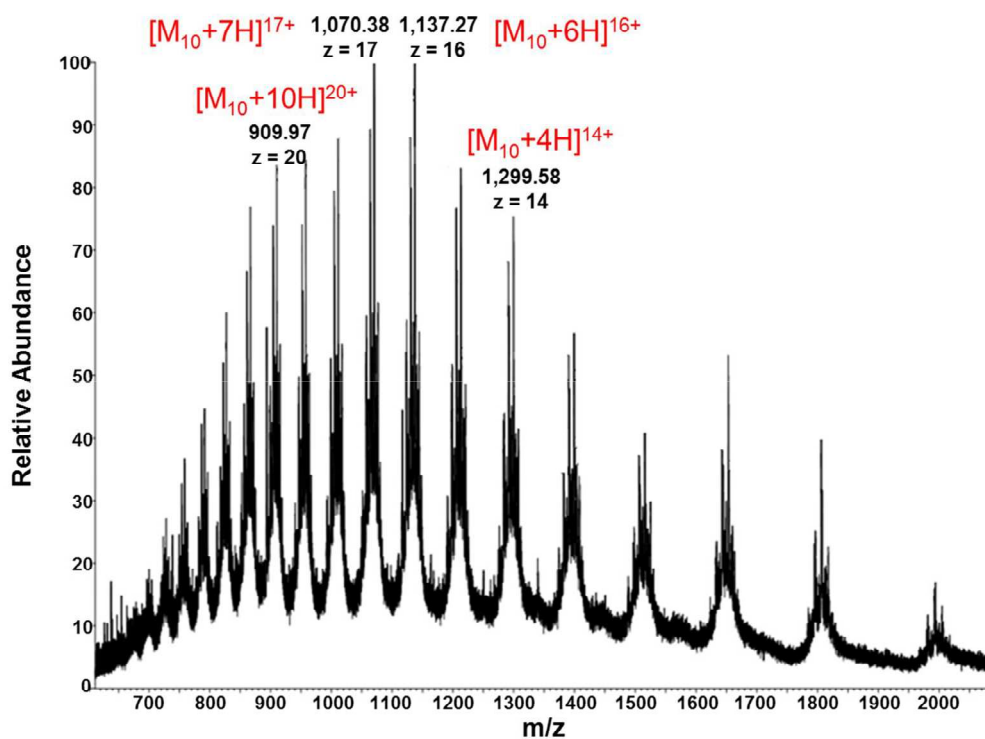


Figure S35. ESI mass spectrum of compound 2d.

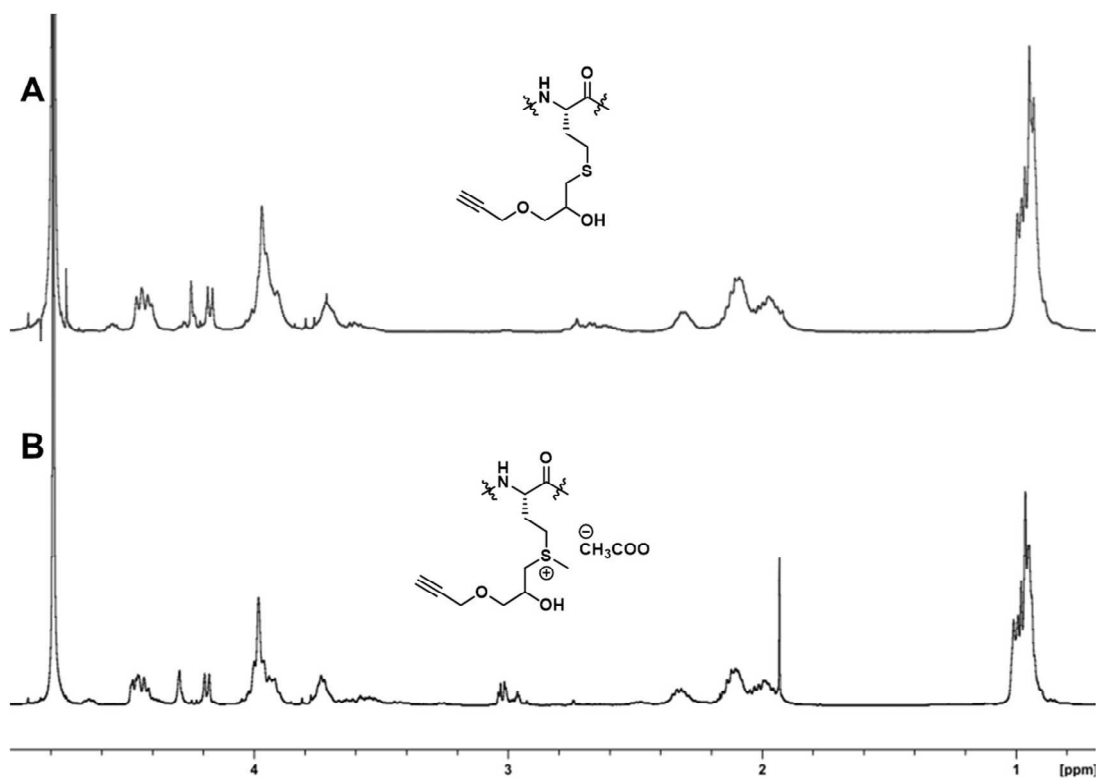


Figure S36. Stacked ^1H NMR spectra of compound (A) 3c and (B) 2c in D_2O .

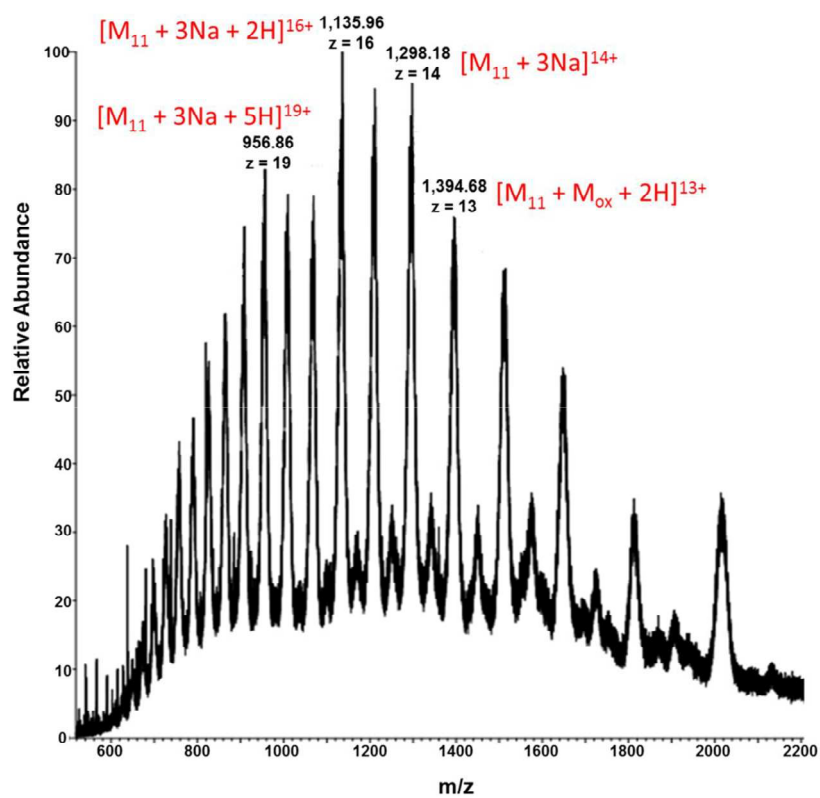


Figure S37. ESI mass spectrum of compound 3c.

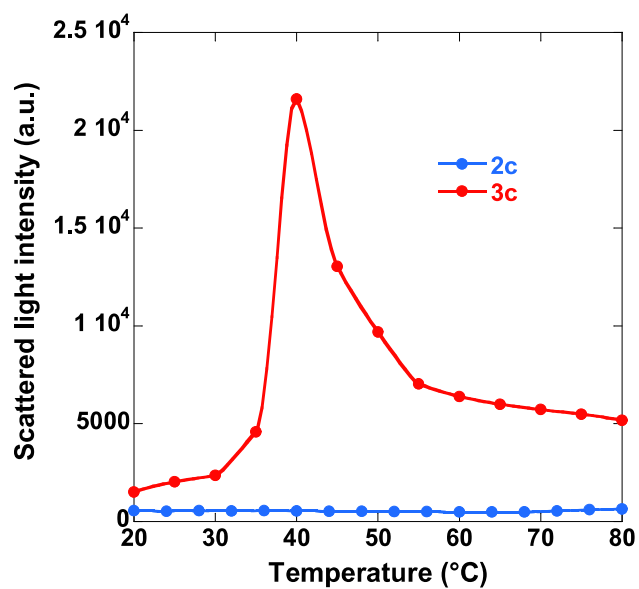


Figure S38. Scattered light intensity (arbitrary units) of compounds **2c** and **3c** as a function of temperature at 1 mM in DI water. Decrease in scattering intensity for **3c** above 40 °C was due to settling of phase separated **3c** from the suspension.

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CHAPTER 5

CHEMOSELECTIVE SUGAR-FUNCTIONALIZATION OF ELASTIN-LIKE POLYPEPTIDES SELECTIVE LECTIN BINDING AND SORTING

Abstract: Following our objective to chemoselectively modify elastin-like polypeptides and tune their thermo-responsive properties, we herein investigated the introduction of biofunctional groups onto ELP backbones. These biofunctional groups, namely glucose, galactose and mannose, were conjugated to alkyne-functionalized ELP derivatives. The impact of these modifications on T_t s was determined and preliminary experiments performed to assess the biological activity of these ELP bioconjugates for lectin binding and sorting.

INTRODUCTION

In nature, glycoproteins are an important class of biomolecules involved in fundamental biological processes such as neuronal development, hormone activities, immune surveillance, and inflammatory responses.^{1,2,3,4} Their structure and function are however not fully understood yet. Therefore synthetic glycoproteins and glycopolymers have been extensively studied over the past years.^{5,6,7,8,9,10} These synthetic analogs hold great promise for diagnostic or therapeutic applications. Glycopolymers have for instance been studied for potential use as drug delivery carriers,^{11,12,13,14,15,16,17,18,19,20,21} therapeutics,^{22,23,24,25,26,27} hydrogels,^{28,29,30} or cell culture scaffolds.^{31,32,33,34} Among glycopolymers, glycopolypeptides are particularly interesting candidates as glycoprotein analogs as their structure gives them the potential for reproducing glycoproteins' properties. The synthesis of glycopolypeptides has been achieved by several methods that can be divided into two main categories: the linear approach and the convergent approach. The linear strategy is based on the stepwise polymerization of glycosylated monomers, while in the convergent strategy the carbohydrates are introduced in a second step on a previously synthesized polypeptide backbone. Among these two categories there are several synthetic methods that all present advantages and disadvantages. The linear strategy is mostly performed by ring opening polymerization (ROP) of glycosylated NCA monomers, a technique pioneered by Okada and coworkers.³⁵ This method has the advantage of enabling the synthesis of fully glycosylated polypeptides but the disadvantage of requiring the synthesis and purification of many different monomers and the individual optimization of the reaction conditions. On the other hand, the ROP of NCA has also been used in a convergent manner in order to form the polypeptide backbone followed by post-polymerization glycosylation.^{36,37} This technique avoids tedious preparation of monomers but often leads to incomplete functionalization due to steric hindrance or inefficiency of the coupling reactions. Therefore these strategies often rely on "click" reactions. The convergent method can also be performed by solid phase peptide synthesis (SPPS) of the polypeptide backbone followed by a glycosylation step. This approach is especially efficient for producing sequence specific polypeptides leading to precise introduction of carbohydrates. However this technique does not allow the production of high molecular weight glycopolypeptides. The synthesis of high molecular weight glycopolypeptides with precise structures can however be achieved by glycosylation of recombinant produced polypeptides. This strategy was for instance used by Kiick and coworkers to prepare monodisperse glyconamidated polypeptides with controlled carbohydrate introduction.³⁸ It has been

scarcely used so far because of the multiple steps required for these preparations. However as the production of biomaterials with high sequence specificity is very important for biomedical applications we decided to explore this strategy using recombinant produced elastin-like polypeptides (ELPs). ELPs are composed of repeating sequences of [-Val-Pro-Gly-*Xaa*-Gly-] pentapeptides derived from the hydrophobic domain of tropoelastin, the guest residue *Xaa* being any amino acid except proline.³⁹ ELPs exhibit thermoresponsive properties: they are soluble in aqueous solution below a certain temperature and aggregate above a specific temperature called *T_t*.^{40,41} The latter can be precisely tuned by the initial design of the ELP, namely the amino acid composition and the ELP chain length,^{42,43,44} or by the subsequent chemical modification of the backbone (see Chapter 2-4).⁴⁵ Following the successful introduction of functional groups such as alkyne on our ELPs *via* methionine thioalkylation we decided to pursue the precise modification of these ELPs by introducing biofunctional groups. Therefore the objective of this study was to introduce monosaccharides on the ELP backbone by click chemistry, to investigate the thermoresponsive properties of the resulting glycoconjugates and perform preliminary lectin-binding experiments.

I. SUGAR-FUNCTIONALIZATION OF ELP-M-N

In the present work, we have focused on the synthesis of ELP glycoconjugates, featuring either glucose (Glc), galactose (Gal) or mannose (Man) units grafted at the methionine side chain of our previously described (see Chapter 4) ELP scaffolds, **ELP-M-n** (n=20,40) (Figure 1A). To this aim, **ELP-M-20** and **ELP-M-40** derivatives thioalkylated at each methionine side chain to present an alkyne handle (**1** and **2**, respectively) were used to conjugate the different azido-functionalized monosaccharides (Figure 1B) by copper-catalyzed azide-alkyne cycloaddition^{46,47} (CuAAC) (Figure 1C).

I.1. CLICK REACTION USING EG₃-N₃

The so-called “click reaction” was first optimized using the azido-containing triethylene glycol derivative EG₃-N₃ **a**. Similar reaction conditions (H₂O, Cu(II)SO₄, sodium ascorbate, PMDETA) to those described by Deming *et al.* for the modification of alkyne-functionalized statistical copolymers of Met and Lys,⁴⁸ were applied. In concordance with our previous observations (see Chapter 2, 4),⁴⁵ a longer reaction time (72 hrs) and a larger excess reagents (1.5 equiv azido-sugar per Met residue) was however necessary to achieve quantitative couplings onto ELPs as compared to synthetic polypeptides.

Complete functionalization was assessed by ^1H NMR spectroscopy by the disappearance of the resonance at 4.3 ppm corresponding to the methylene group in α position to the alkyne function and the appearance of a resonance at 8.2 ppm attributed to the proton of the triazole ring (Figure S1, panels B and C).

1.2. CLICK REACTION USING MONOSACCHARIDE- N_3

These reaction conditions were then used for the conjugation of Glc- N_3 **b**, Gal- N_3 **c**, and Man- N_3 **d** derivatives to obtain glucosylated, galactosylated and mannosylated versions of ELP-M-20 (**1b-d**) and ELP-M-40 (**2b-d**) (Figure 1D).

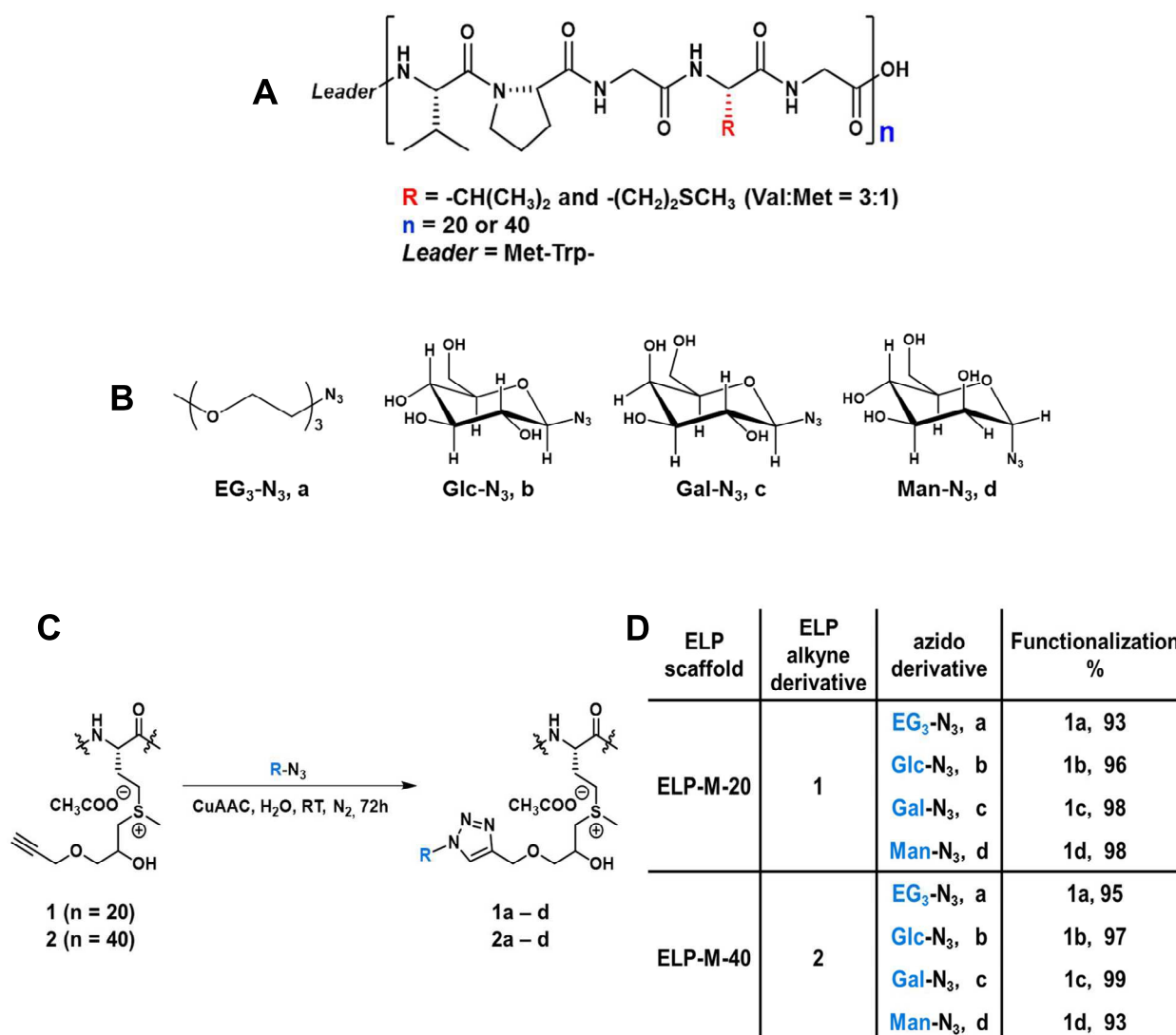


Figure 1. (A) Protein sequence of ELP-M-n (n=20,40) scaffolds. (B) Chemical structures of azido-containing derivatives a-d. (C) General reaction scheme of the synthesis of 1a-d and 2a-d ELP conjugates. (D) Table summarizing the series of ELP-M-n conjugates synthesized and functionalization rates. Glc = glucose, Gal = galactose, Man = mannose.

Chemical structures of ELP glycoconjugates and functionalization rates were determined by ^1H NMR spectrometry (Figures 2, S2-7). Spectra were calibrated using the resonance centered at 4.45 ppm corresponding to the αCH protons of proline and the initial valine in each (**VPGXG**) repeat, integrating for 40 protons in **ELP-M-20** and 80 protons in **ELP-M-40**. As highlighted on the ^1H NMR spectrum of **ELP-M(Man)-20** derivative **1d** as compared to the one of the starting ELP derivative **1** (Figure 2), complete functionalizations were evidenced by three main observations: *i*) disappearance of the resonance at 4.3 ppm corresponding to the methylene protons in α position to the alkyne function of derivative **1** (highlighted in blue); *ii*) appearance of the resonance at 6.18 ppm attributed to the anomeric proton of the monosaccharide (highlighted in red) integrating for 6 protons (11 protons in the case of **ELP-M-40** glycoconjugates); *iii*) appearance of the resonance at 8.2 ppm corresponding to the proton of the triazole ring (highlighted in green) integrating for 6 protons (11 protons in the case of **ELP-M-40** glycoconjugates). Quantitative functionalization was achieved for most glycopolypeptides (Figure 1D) except for **ELP-M(Man)-40 2d**, whose functionalization was limited to 93% despite the specific conditions used for this compound (SI, procedure B).

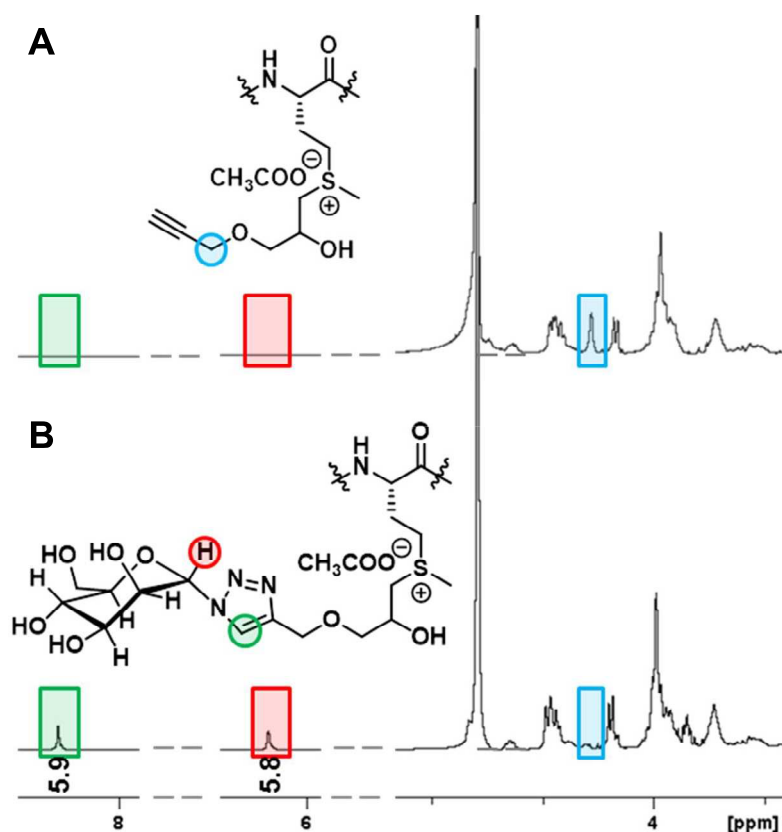


Figure 2. ^1H NMR spectra of (A) ELP derivative **1** and (B) ELP-M(Man)-20 glycoconjugate **1d**.

II. EFFECT OF SUGAR-FUNCTIONALIZATION ON THE THERMoresponsive BEHAVIOR

Because of the important effect afforded by pendant groups appended to the methionine side chain of Met-containing ELPs onto their temperature responsiveness, the temperature-induced aggregation of the glycoconjugates was monitored by dynamic light scattering (DLS) at a 90° angle. The scattered light intensity of 1 mM aqueous solutions of the different glycoconjugates was measured and plotted upon increasing temperature from 20°C to 80°C allowing to determine the onset temperature of aggregation (T_t) (Figure 3).

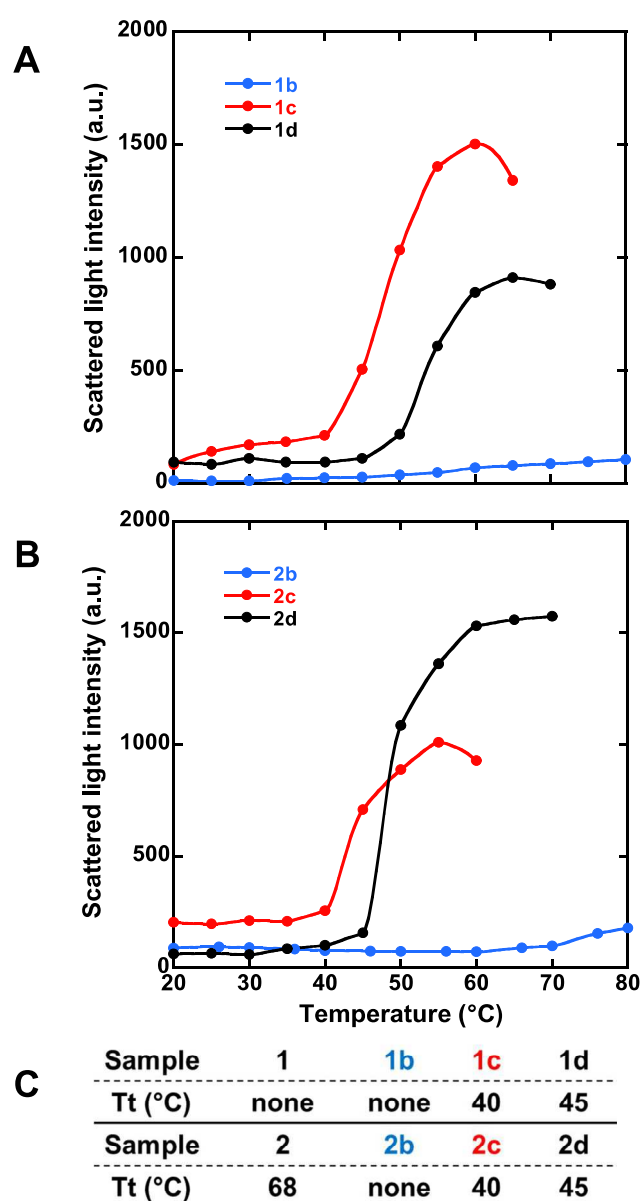


Figure 3. Scattered light intensity of (A) ELP-M-20 1b-d derivatives and (B) ELP-M-40 2b-d derivatives as functions of temperature at 1 mM in DI water. (C) Table summarizing the onset temperature of aggregation (T_t) of the different derivatives at 1 mM in water.

As already discussed in the previous chapter (see Chapter 4), **ELP-M-n** derivatives, **1** (n=20) and **2** (n=40), presenting alkyne handles show little thermo-responsiveness as compared to their parent ELPs, **ELP-M-20** ($T_t=31^\circ\text{C}$) and **ELP-M-40** ($T_t=25^\circ\text{C}$) respectively. This phenomenon was readily attributed to the increased hydrophilicity of the thioalkylated ELPs resulting from the positively charged sulfonium groups as well as the hydroxyl groups originating from the epoxide ring opening during the thioalkylation reaction. Glycopolypeptides showed significant different behaviors according to the monosaccharide unit appended to the ELP backbone, however the length of the ELP (n=20 or 40) was found to have very little impact (similar T_t s between individual compounds of the **1** or **2** glycoconjugate series). Regarding their respective molecular weight, glycoconjugates of **ELP-M-40** series (**2**) would have been expected to exhibit lower T_t s as compared to glycoconjugates of the **ELP-M-20** series (**1**). This is most probably due to the positive charges present on the ELP backbones overpowering the chain length effect, similarly to what was previously observed with other thioalkylated ELP compounds (see Chapter 4). In contrast, the nature of the monosaccharide appended to the ELP significantly affected the thermo-responsive behavior of the resulting glycoconjugate. Indeed, while glucosylated-ELPs **1b** (**ELP-M(Glc)-20**) and **2b** (**ELP-M(Glc)-40**) did not exhibit any aggregation behavior up to 80°C , galactosylated- (**1c**, **ELP-M(Gal)-20**, and **2c**, **ELP-M(Gal)-40**) and mannosylated-ELP derivatives (**1d**, **ELP-M(Man)-20**), and **2d**, **ELP-M(Man)-40**) presented strong shifts in T_t s lowered back to 40°C and 45°C , respectively. If solely considering the hydrophobic character of monosaccharide units, *i.e.* D-galactose < D-glucose < D-mannose, as measured by their partition coefficient K_{av} measured by polystyrene-gel water chromatography,⁴⁹ these results do not fit with the expected behavior for each individual glycoconjugate. As shown by Miyajima *et al.*,⁵⁰ the relative hydrophobic/hydrophilic character of saccharides however depends on multiple parameters (*e.g.*, dimension of hydrophobic/hydrophilic surface area, conformation, hydrogen bonding, *etc.*) and depending on which parameter is taken into account in the calculation of the hydrophobicity index, the ranking of individual saccharides is subject to variation. It is worth mentioning that the measured K_{av} and calculated hydrophobicity index mentioned above are based on isomeric mixtures of α and β conformations, while our ELP glycoconjugates were prepared from enantiomerically pure saccharide derivatives of β -D-glucose, β -D-galactose, and α -D-mannose., which likely affects their hydrophobic character.⁵⁰ The significant different behavior of Gal- and Man-ELP conjugates (**1c**, **2c**, **1d**, **2d**) as compared to Glc-ELPs (**1b**, **2b**) however let us think of specific interactions, such as hydrogen bondings or hydrophobic interactions,

between the clicked monosaccharides and the ELP backbone that strongly affect the global hydrophilicity and thermo-responsive properties of the glycoconjugates.

III. LECTIN-BINDING AND SORTING USING ELP GLYCOCONJUGATES

III.1. BIOACTIVITY ASSESSMENT BY MULTIANGLE LIGHT SCATTERING

Preliminary experiments were performed to test the biological activity of these glycoconjugates and assess the binding capacity of monosaccharides grafted onto ELP scaffolds to specific lectins. The **ELP-M(Gal)-40** conjugate **2c** was thus assayed for the binding to the galactose-binding lectin RCA120. Multiangle static light scattering analysis was used to monitor complex formation between the multivalent ELP glycoconjugate and the receptor. To this aim, aqueous solutions of **ELP-M(Gal)-40 2c** and RCA120 were mixed together at a final 5 mg/mL concentration (**2c**: 0.24 mM ; RCA120: 0.042 mM) and subjected to multiangle light scattering measurements. This concentration in ligand and receptor was necessary to provide sufficient light scattering signal and to ensure appropriate binding sites occupation. RCA120 indeed possessing two binding sites for galactose,⁵¹ the fraction of occupied receptors ($\frac{[RL]}{[R] + [RL]}$) is thus given by the equation:

$$\frac{[RL]}{[R] + [RL]} = \frac{[L]}{K_d + [L]}$$

with R (receptor) being RCA120 and L (ligand) galactose. The K_d value being of 227 μ M,⁵² the fraction of occupied receptors (0.97) is thus very close to 1, indicating favorable conditions for the binding of **ELP-M(Gal)-40 2c** to RCA120.

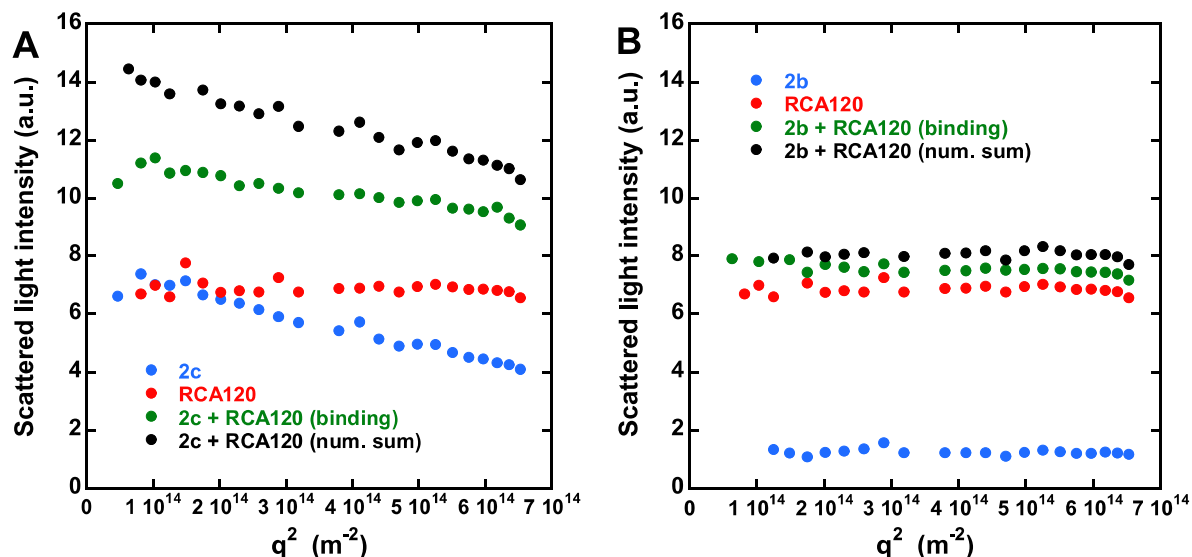


Figure 4. Binding study of (A) ELP-M(Gal)-40 2c (5 mg/mL) with RCA120 (5 mg/mL) and (B) ELP-M(Glc)-40 2b (5 mg/mL) with RCA120 (5 mg/mL), as measured by multiangle light scattering.

Multiangle static light scattering measurements enabled to plot the scattered light intensity as functions of the square of the wave vector (q^2) for all conditions studied, namely **ELP-M(Gal)-40 2c** or **ELP-M(Glc)-40 2b** used as control, RCA120, as well as their mixture (binding). The numerical sum of the scattered light intensity signals for the individual ligand and lectin alone was also plotted on the same graph for comparison with the binding condition. The scattered light intensity signal for **ELP-M(Gal)-40 2c** mixed with RCA120 was found significantly different from the numerical sum of the scattered light intensity of individual species, suggesting the formation of a complex rather than the two species just coexisting in solution (Figure 4A). In contrast, when using **ELP-M(Glc)-40 2b** as a non-binding ligand, the two curves almost superimposed suggesting that **ELP-M(Glc)-40 2b** does not bind and form a complex with RCA120 but just coexists with the lectin (Figure 4B). These first measurements suggested a specific binding of the **ELP-M(Gal)-40 2c** conjugate with RCA120.

III.2. BIOACTIVITY ASSESSMENT BY AGGREGATION-CENTRIFUGATION ASSAY

The specific binding of **ELP-M(Gal)-40 2c** to RCA120 was also assayed in temperature-triggered aggregation-centrifugation experiments. In this particular case, **ELP-M(Man)-40 2d** was preferred as control over or **ELP-M(Glc)-40 2b** regarding its similar thermo-responsiveness to **ELP-M(Gal)-40 2c**. In these experiments, glycoconjugates were incubated with fluorescein-labeled RCA120 at a final 1.2 mM concentration and the mixture was subjected to centrifugation at 40°C. The supernatant

was separated from the pellet and subjected to fluorescence measurements. When no ligand or the control **ELP-M(Man)-40 2d** was used, the soluble RCA120-fluorescein was recovered in the supernatant as evidenced by the high fluorescence signals in these two conditions (Figure 5). In contrast, when RCA120-fluorescein was incubated with **ELP-M(Gal)-40 2c**, a very weak fluorescence signal was recovered in the supernatant (Figure 5). This can readily be explained by the binding of RCA120 to the **ELP-M(Gal)-40 2c** derivative and the temperature-induced aggregation of the of RCA120/ELP-M(Gal)-40 complex upon heating at 40°C. This was confirmed by the high fluorescence signal recovered after resuspension of the pellet in cold buffer (Figure S9). This result is highly promising since it implies that ELP-glycoconjugates can be used to specifically aggregate and pull down specific lectins by simple temperature-triggered aggregation and centrifugation at reasonable temperature to maintain protein folding and activity. The next step is to attempt to fish out specific sugar-binding proteins from complex protein mixtures.

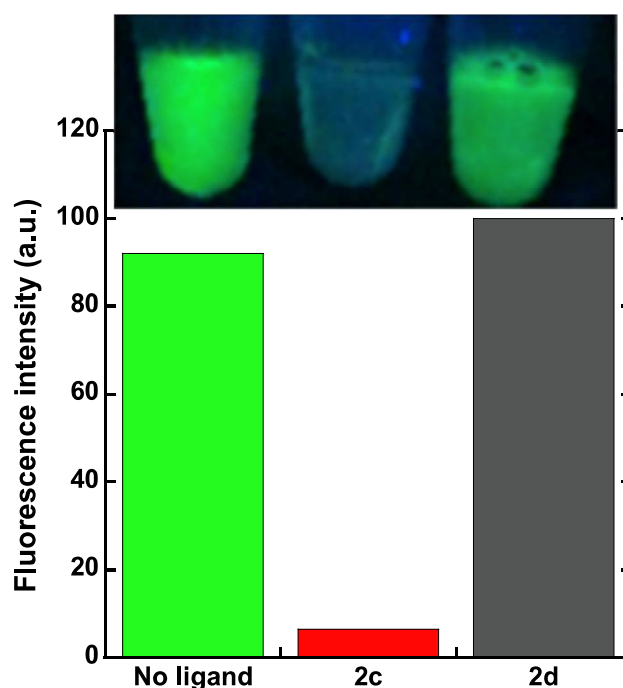


Figure 5. Lectin binding study. RCA120-Fluorescein (2 μ M) was incubated with either no ligand, ELP-M(Gal)-40 2c (1.2 mM) or ELP-M(Man)-40 2d (1.2 mM) and centrifuged at 40°C. Fluorescence intensity at 498 nm of supernatants were measured. From left to right: no ligand, ELP-M(Gal)-40 2c, ELP-M(Man)-40 2d.

CONCLUSION

We herein report the chemoselective and quantitative conjugation of monosaccharides onto alkyne-functionalized elastin-like polypeptide backbones by click chemistry. Thermo-responsive properties of the resulting ELP glycoconjugates were evaluated and showed that the glucose derivative, similarly to the starting alkyne-bearing ELP, did not present any measurable thermo-responsiveness, while galactose and mannose glycopolypeptides recovered a T_t in the 40-45°C range at relatively low concentration. The binding of ELP-M(Gal)-40 towards the lectin RCA120 was also investigated in two different experimental set-up confirming the ability of galactose units to specifically recognize and bind their lectin. These results are promising regarding the synthesis of glycopolypeptides with precise structure and should be pursued by the introduction of more complex saccharides.

EXPERIMENTAL SECTION

Materials.

Azide monosaccharides were obtained from Carbosynth (UK). RCA120 and RCA120-Fluorescein were purchased from Eurobio (FR). Triethylene glycol monomethyl ether and *N,N,N',N'',N''*-pentamethyldiethylenetriamine (PMDETA) were purchased from Sigma-Aldrich (FR). $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ was obtained from VWR (Fr). Sodium ascorbate was obtained from Fisher Scientific (FR). Deionized water (18 M Ω -cm) was obtained by passing in-house deionized water through a Millipore Milli-Q Biocel A10 purification unit.

Synthesis of EG₃-N₃

Step 1

A 100 mL round bottom flask was filled with triethylene glycol monomethyl ether (1 equiv.) and sodium hydroxide pellets (1.83 equiv.) in 10 mL water and cooled down to 3°C with rapid stirring. A solution of p-toluenesulfonyl chloride (1 equiv.) in 10 mL THF was then added to the reaction mixture and let warm up to room temperature overnight. After 15 hours, 20 mL diethyl ether were added, the organic layer removed and extracted with twice 50 mL diethyl ether. The combined organic layers were then washed three times with 25 mL H₂O. Organics were dried over magnesium sulfate and dried under vacuum to yield EG₃-OTs. Yield = 83.8 %

Step 1

A 100 mL round bottom flask was charged with EG₃-OTs (1 equiv.) and 50 mL of dry DMF were added *via* syringe. NaN₃ (2.5 equiv.) was added, the reaction placed under N₂ and heated at 70°C overnight. DMF was removed under high vacuum (while heated at 45°C). The product was then extracted by aqueous workup: five times 40 mL diethyl ether, three times 25 mL water. The combined organics were dried under vacuum to yield EG₃-N₃ as an oil. Yield = 50 %

Synthesis of ELP-M(EG₃)-n 1a and 2a.

The alkyne-bearing ELP derivative (**1** or **2**) was dissolved in water (5 mg/mL) and EG₃-N₃ (1.5 equiv. per alkyne) was added. The solution was degassed by bubbling N₂ for 2 hrs and then stirred under N₂. Separately, a solution of Cu(I) was prepared by addition of sodium ascorbate (0.65 equiv. per alkyne) to a degassed solution of Cu(II)SO₄ (0.13 equiv. per alkyne) and pentamethyldiethylenetriamine (0.13

equiv. per alkyne). The Cu(I) solution was transferred to the reaction mixture with a syringe. The reaction was stirred under N₂ at room temperature for 48 hrs, transferred to a 3,000 MWCO ultra-centrifugal filter tube and washed first with an aqueous solution of EDTA (0.15 M) and then with 40 mL DI water. The purified reaction mixture was then lyophilized to provide the ELP conjugate as a white solid.

ELP-M(EG₃)-20, 1a

Yield = 88 %

¹H NMR (400 MHz, D₂O, 25°C): (main peaks): δ 8.2-8.1 (br s, 6 H, triazole-*H*), 4.5-4.4 (m, 40 H, αCH VPGXG and αCH VPGXG), 4.2-4.15 (d, 15 H, αCH VPGVG), 3.8 (br s, 18 H, OCH₃), 3.05-2.9 (m, 18 H, SCH₃), 1.05-0.9 (br m, 210 H, CH₃ Val)

ELP-M(EG₃)-40, 2a

Yield = 87 %

¹H NMR (400 MHz, D₂O, 25°C): (main peaks): δ 8.2-8.1 (br s, 11 H, triazole-*H*), 4.5-4.4 (m, 80 H, αCH VPGXG and αCH VPGXG), 4.2-4.15 (d, 30 H, αCH VPGVG), 3.8 (br s, 18 H, OCH₃), 3.03-2.95 (m, 33 H, SCH₃), 1.05-0.9 (br m, 420 H, CH₃ Val)

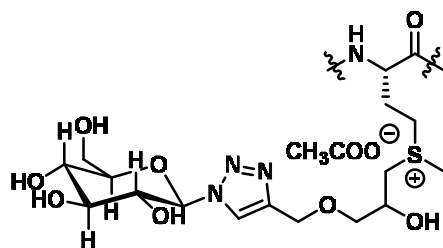
Synthesis of ELP glycoconjugates (general procedures).

Procedure A

The alkyne-bearing ELP derivative (**1** or **2**) was dissolved in water (5 mg/mL) and the desired azido-functionalized monosaccharide (powder, 1.5 equiv. per alkyne) was added. The solution was degassed by bubbling N₂ for 2 hrs and then stirred under N₂. Separately, a solution of Cu(I) was prepared by addition of sodium ascorbate (0.65 equiv. per alkyne) to a degassed solution of Cu(II)SO₄ (0.13 equiv. per alkyne) and pentamethyldiethylenetriamine (0.13 equiv. per alkyne). The Cu(I) solution was transferred to the reaction mixture with a syringe. The reaction was stirred under N₂ at room temperature for 72 hrs, transferred to a 3,000 MWCO ultra-centrifugal filter tube and washed first with an aqueous solution of EDTA (0.15 M) and then with 40 mL DI water. The purified reaction mixture was then lyophilized to provide the ELP glycoconjugate as a white solid.

Procedure B

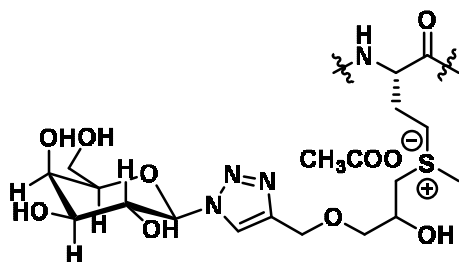
The alkyne-bearing ELP derivative **2** and α -D-mannopyranosyl azide (powder, 1.5 equiv. per alkyne) were dissolved in water (5 mg/mL) previously degassed under vacuum. Separately, a solution of Cu(I) was prepared by addition of sodium ascorbate (0.65 equiv. per alkyne) to a degassed solution of Cu(II)SO₄ (0.13 equiv. per alkyne) and pentamethyldiethylenetriamine (0.13 equiv. per alkyne). The Cu(I) solution was transferred onto the reaction mixture with a syringe. The reaction was stirred under N₂ at room temperature for 72 hrs, transferred to a 3,000 MWCO ultra-centrifugal filter tube and washed first with an aqueous solution of EDTA (0.15 M) and then with 40 mL DI water. The purified reaction mixture was then lyophilized to provide the **ELP-M(Man)-40 2d** as a white solid.

Details of ELP glycoconjugates.

ELP-M(Glc)-20 1b as prepared from ELP derivative **1** and β -D-glucopyranosyl azide (Glc-N₃) using **Procedure A**.

Yield = 86 %

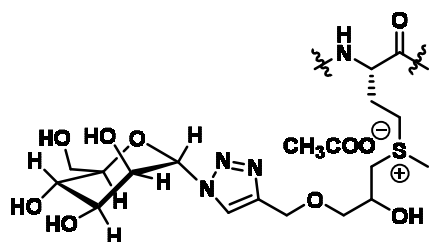
¹H NMR (400 MHz, D₂O, 25°C): (main peaks): δ 8.4-8.3 (br s, 6 H, triazole-H) 5.9-5.8 (br s, 6 H, anomeric-H), 4.5-4.4 (m, 40 H, α CH VPGXG and α CH VPGXG), 4.2-4.15 (d, 15 H, α CH VPGVG), 3.06-2.9 (m, 18 H, SCH₃), 1.00-0.75 (br m, 210 H, CH₃ Val)



ELP-M(Gal)-20 1c was prepared from ELP derivative **1** and β -D-galactopyranosyl azide (Gal-N₃) using **Procedure A**.

Yield = 85 %

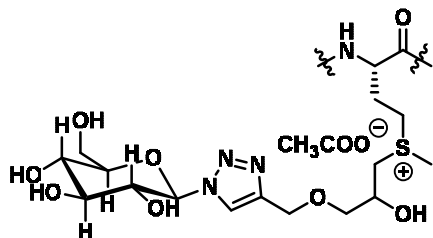
^1H NMR (400 MHz, D_2O , 25°C): (main peaks): δ 8.35-8.3 (br s, 6 H, triazole-*H*) 5.7-5.65 (br s, 6 H, anomeric-*H*), 4.5-4.4 (m, 40 H, αCH VPGXG and αCH VPGXG), 4.22-4.16 (d, 15 H, αCH VPGVG), 3.06-2.9 (m, 18 H, SCH_3), 1.00-0.75 (br m, 210 H, CH_3 Val)



ELP-M(Man)-20 **1d** was prepared from ELP derivative **1** and $\alpha\text{-D}$ -mannopyranosyl azide (Man- N_3) using *Procedure A*.

Yield = 87 %

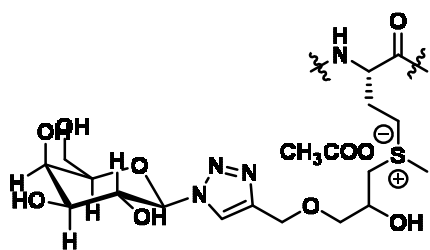
^1H NMR (400 MHz, D_2O , 25°C): (main peaks): δ 8.29-8.25 (br s, 6 H, triazole-*H*) 6.2-6.1 (br s, 6 H, anomeric-*H*), 4.5-4.4 (m, 40 H, αCH VPGXG and αCH VPGXG), 4.2-4.15 (d, 15 H, αCH VPGVG), 3.06-2.9 (m, 18 H, SCH_3), 1.00-0.75 (br m, 210 H, CH_3 Val)



ELP-M(Glc)-40 **2b** was prepared from ELP derivative **2** and $\beta\text{-D}$ -glucopyranosyl azide (Glc- N_3) using *Procedure A*.

Yield = 88 %

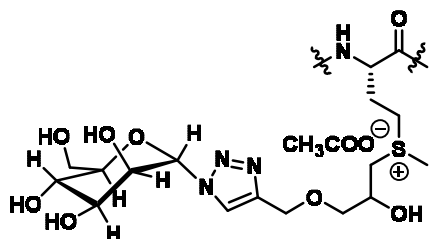
^1H NMR (400 MHz, D_2O , 25°C): (main peaks): δ 8.4-8.3 (br s, 11 H, triazole-*H*) 5.9-5.75 (br s, 11 H, anomeric-*H*), 4.5-4.4 (m, 80 H, αCH VPGXG and αCH VPGXG), 4.2-4.15 (d, 30 H, αCH VPGVG), 3.06-2.9 (m, 33 H, SCH_3), 1.00-0.75 (br m, 420 H, CH_3 Val)



ELP-M(Gal)-40 **2c** was prepared from ELP derivative **2** and **β -D-galactopyranosyl azide** (Gal-N₃) using **Procedure A**.

Yield = 84 %

¹H NMR (400 MHz, D₂O, 25°C): (main peaks): δ 8.4-8.3 (br s, 11 H, triazole-*H*) 5.85-5.7 (br s, 11 H, anomeric-*H*), 4.5-4.4 (m, 80 H, α CH VPGXG and α CH VPGXG), 4.2-4.15 (d, 30 H, α CH VPGVG), 3.06-2.9 (m, 33 H, SCH₃), 1.00-0.75 (br m, 420 H, CH₃ Val)



ELP-M(Man)-40 **2d** was prepared from ELP derivative **2** and **α -D-mannopyranosyl azide** (Man-N₃) using **Procedure B**.

Yield = 85 %

¹H NMR (300 MHz, D₂O, 25°C): (main peaks): δ 8.25-8.22 (br s, 11 H, triazole-*H*) 6.16-6.12 (br s, 11 H, anomeric-*H*), 4.5-4.4 (m, 80 H, α CH VPGXG and α CH VPGXG), 4.2-4.15 (d, 30 H, α CH VPGVG), 3.06-2.9 (m, 33 H, SCH₃), 1.00-0.75 (br m, 420 H, CH₃ Val)

NMR spectrometry analysis of ELP glycoconjugates.

NMR spectra were acquired in D₂O at 298 K either on a Bruker AV400 NMR spectrometer (UCLA) operating at 400 mHz. The solvent signal was used as the reference signal (δ = 4.79 ppm). Data processing was performed using Topspin software. Chemical shifts of amino acids are well known in the literature.^{53,54} We have identified the CH α and of the proline (VPGXG) at 4.5-4.4 ppm (60.5-57 ppm for ¹³C) and used it as reference for the calibration of integrations. Full assignments were done with the help of the COSY and HSQC spectra.

Transition temperature measurements of ELP glycoconjugates.

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 scattered light intensity was plotted against temperature and the Tt defined as the temperature onset temperature of aggregation on this plot.

Multiangle dynamic light scattering.

Multiangle light scattering measurements were performed using an ALV/CG6-8F goniometer, with a 35 mW red helium-neon linearly polarized laser ($\lambda = 632.8$ nm) and an ALV/LSE-5004 multiple tau digital correlator at 20 °C. The data were acquired with the ALV correlator software, the counting time was typically 15 s at each different scattering angles ranging from 30° to 150°, in 10° increments. The solutions were prepared by mixing solutions of **2b/2c** with RCA120 at final concentrations of 0.24 mM and 0.042 mM respectively in ultrapure water, filtered through 45 μ m filters.

Centrifugation and fluorescence measurements.

Solutions of **2c/2d** mixed with RCA-Fluorescein at final concentrations of 1.2 mM and 2 μ M in water respectively were prepared. A solution of RCA-Fluorescein at 2 μ M in water was prepared. The three solutions were centrifuge at 40°C for 20 minutes at 16,000 g. After separating the pellets and supernatants, the fluorescence spectra were obtained on a QM-4 SE spectrometer from Photon Technology International (PTI), equipped with double excitation and emission monochromators.

SUPPLEMENTARY FIGURES

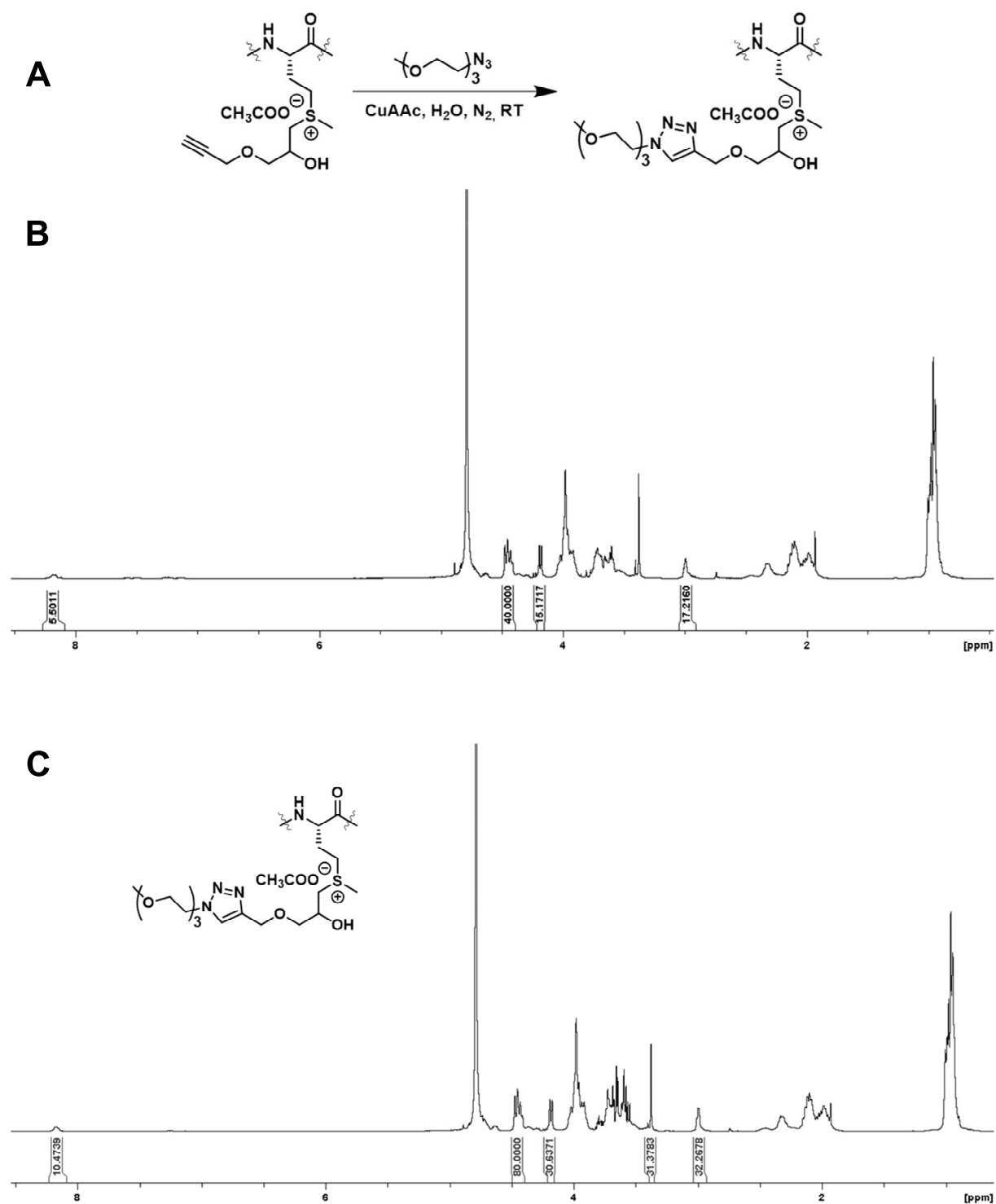


Figure S1. (A) Reaction scheme of with EG₃-N₃ with alkyne-functionalized ELP-M-20 and ELP-M-40 derivatives (1 and 2, respectively). (B) ¹H NMR spectrum of ELP-M(EG₃)-20 1a in D₂O. (C) ¹H NMR spectrum of ELP-M(EG₃)-40 2a in D₂O.

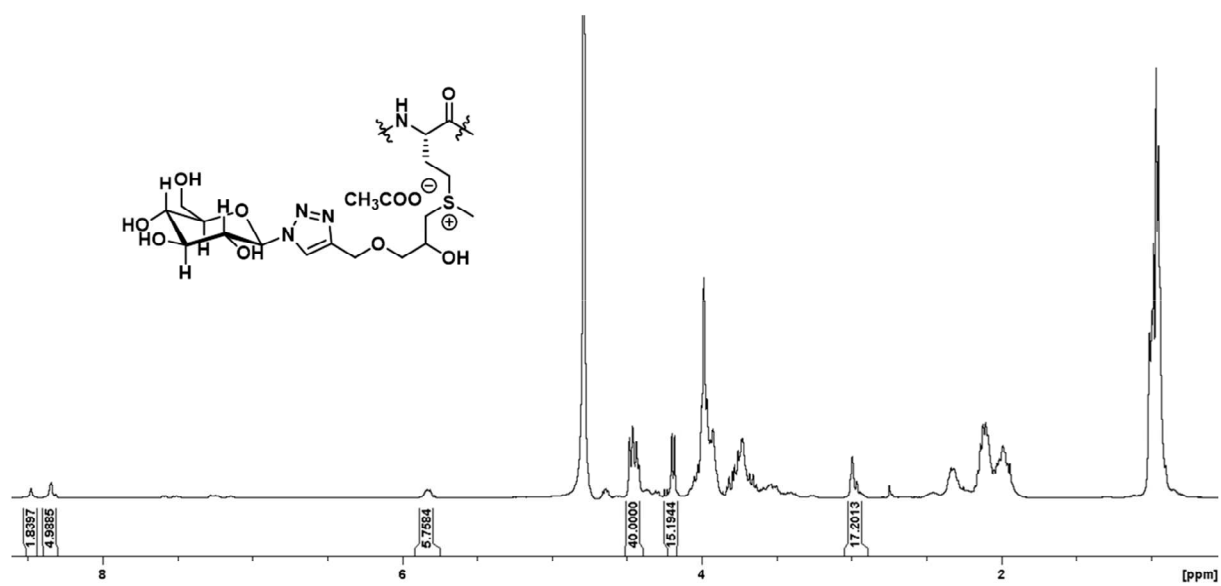


Figure S2. ¹H NMR spectrum of ELP-M(Glc)-20 1b in D₂O.

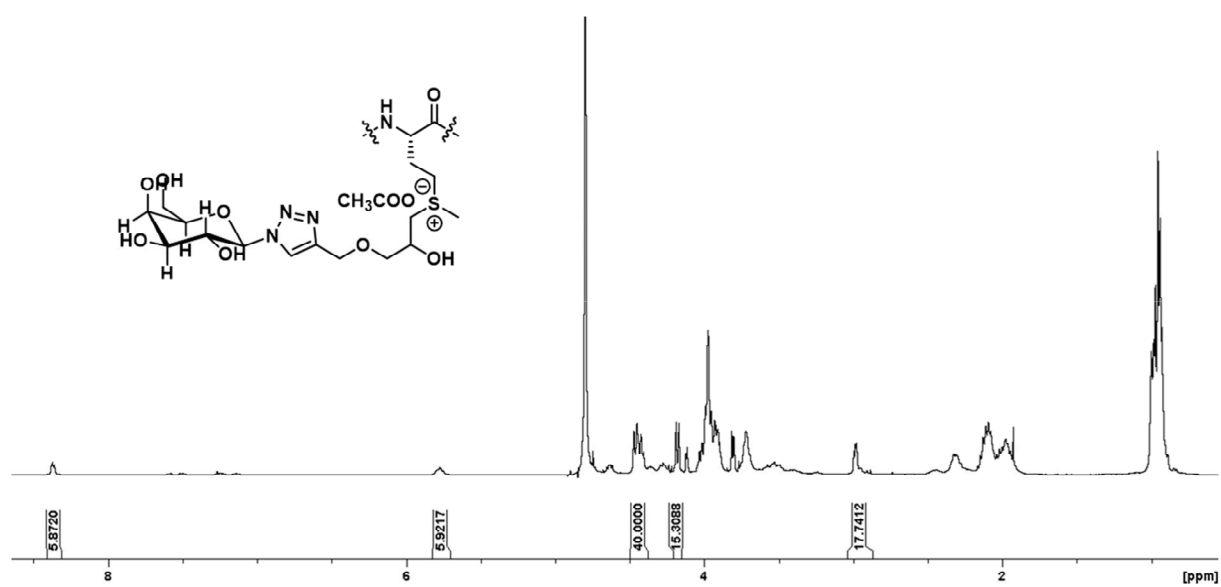


Figure S3. ¹H NMR spectrum of ELP-M(Gal)-20 1c in D₂O.

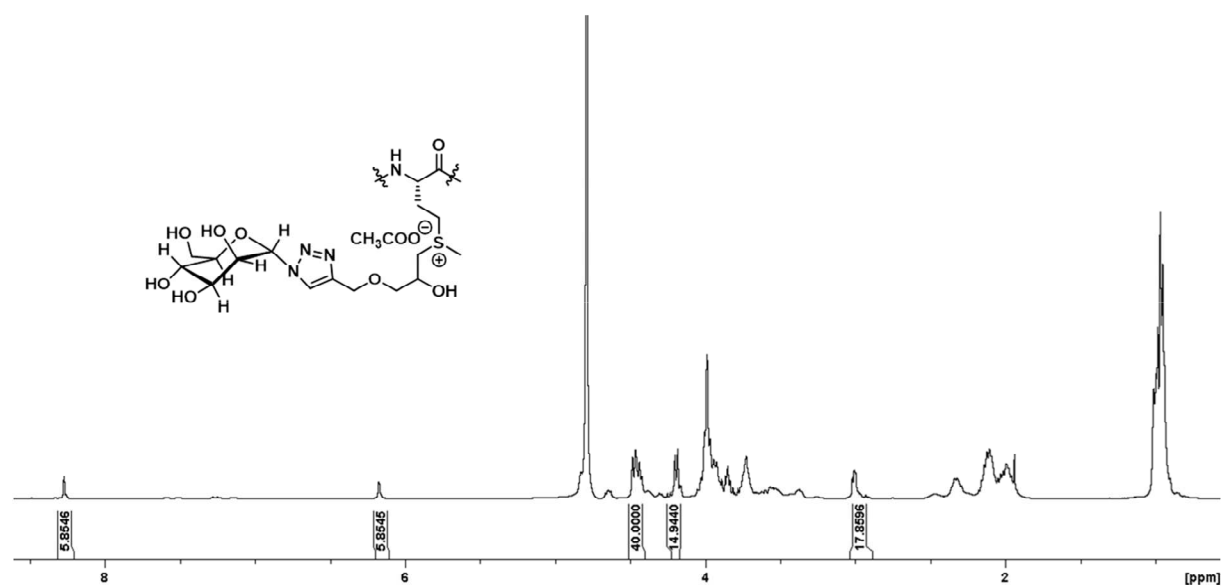


Figure S4. ¹H NMR spectrum of ELP-M(Man)-20 1d in D₂O.

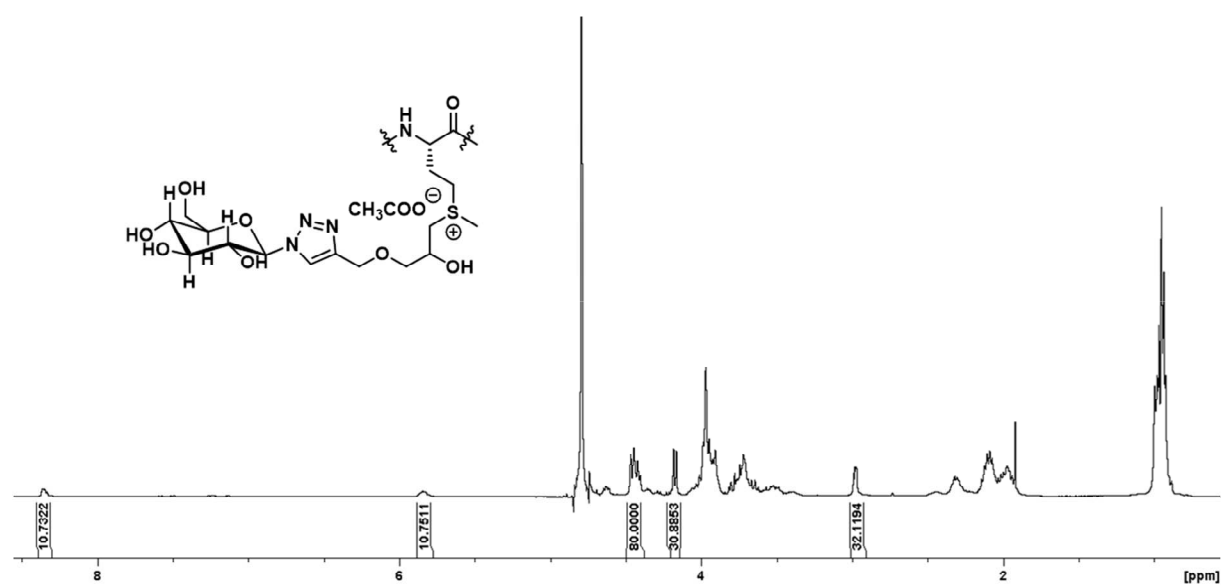


Figure S5. ¹H NMR spectrum of ELP-M(Glc)-40 2b in D₂O.

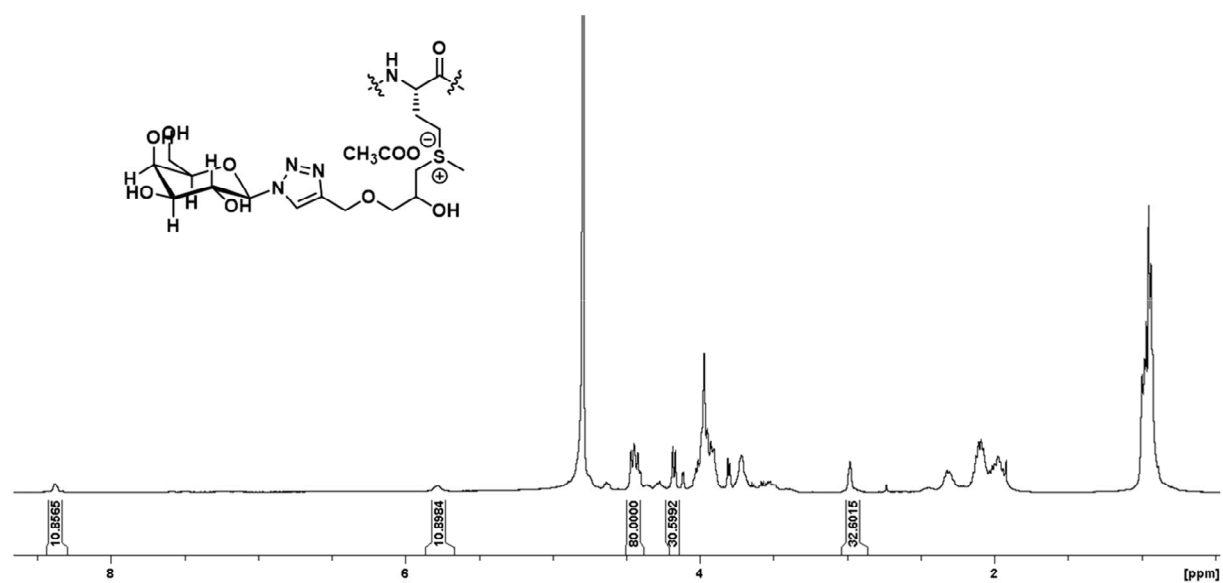


Figure S6. ¹H NMR spectrum of ELP-M(Gal)-40 2c in D₂O.

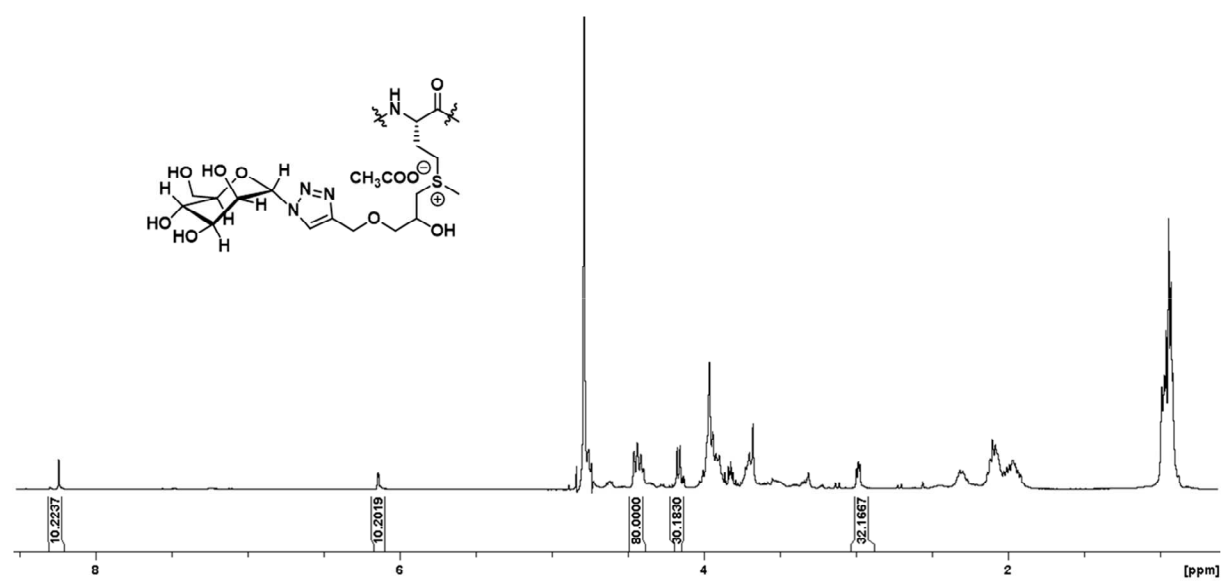


Figure S7. ¹H NMR spectrum of ELP-M(Man)-40 2d in D₂O.

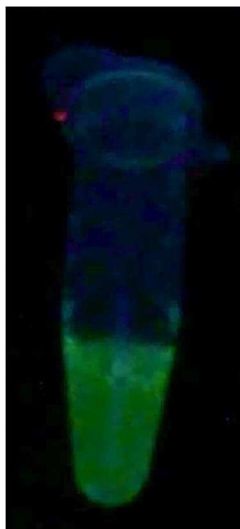


Figure S9. Picture of the pellet of RCA₁₂₀-fluorescein-bound ELP-M(Gal)-40 2c resuspended in water.

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GENERAL CONCLUSION AND OUTLOOK

Synthetic polypeptides are commonly used in our group as their synthesis by ring opening polymerization is particularly well known and well controlled. However in the context of biomedical applications synthetic polypeptides present some disadvantages. First of all, for now they cannot be synthesized with a perfect monodispersity and their synthesis is not perfectly reproducible from batch to batch. Moreover their structure lacks of precision and it is difficult to control their primary structure. In general 3 different type of structures can be reached: block copolymers, gradient copolymers and alternating copolymers. These structures remain very poor compare to natural biopolymers such as proteins. Therefore it is interesting to integrate concepts inspired from natural processes into macromolecular engineering. Recombinant DNA and protein engineering techniques are commonly used techniques in pharmaceutical industries to produce recombinant therapeutics. These techniques can be used to produce precision protein-like polymers.¹ Compared to synthetic polymers, recombinant polymers have the advantage to be genetically encoded which impart them an exact primary structure with a perfect control sequence and chain length.² The produced polymers are perfectly monodisperse and can reach higher molecular weights as compared to traditional peptide synthesis strategies. Finally productions of recombinant protein-like polymers are scalable to large batches with a perfect reproducibility. Among the protein-like polymers currently produced by recombinant DNA and protein engineering techniques, elastin-like polypeptides are one of the most versatile materials. They have for instance the interesting property to display a lower critical solubility temperature (*LCST*) behavior which means that they are soluble at low temperature and aggregate above a specific temperature called transition temperature (*T_t*). This behavior is very interesting as it facilitates isolation and purification of elastin-like polypeptides and it is a mean to control the self-assembly process. This transition temperature can be tuned by several means for example by designing different genes in order to obtain different sequences or different methods. However molecular biology protocols to obtain new genes are quite complicated. Therefore alternative methods have to be developed to tune ELPs thermoresponsive properties and also to confer them functional properties. In this context, the main objective of this thesis was to chemoselectively modify different recombinantly produced elastin-like polypeptides scaffolds, in order to tune their thermoresponsive properties and to introduce functional groups on their backbone.

Until now most chemical modifications on ELPs reported in the literature involved their chain ends while modifications of their backbone were more scarcely reported. Therefore, in order to mimic the diversity of post-translationally modified proteins, we decided to focus on the post-modification of ELP backbone by introducing methionine at the guest residue position and by functionalizing their side-chain. Instead of introducing unnatural amino acids which is a costly method and often leads to low yields we chose to focus on a natural amino acid. Among the natural amino acids methionine is a particularly interesting residue because of the unique reactivity of its thioether group. Indeed as it is not protonated at low pH methionine is the most reactive nucleophile present in peptides and proteins under acidic conditions thereby allowing orthogonal post-modifications.

Deming *et al.* recently developed versatile methods to modify methionine side-chain on synthetic polymethionine. We thus chose to apply and adapt these methods to the post-modification of methionine containing ELPs.

First of all we synthesized the full methionine containing ELP (VPGMG)₂₀, meaning that all guest residues in this ELP were methionine residues. We managed to quantitatively modify these residues using two different alkyl halides, although Met residues in ELPs turned out to be less reactive than the one in polymethionine thereby requiring more reagents and longer reaction times. This lower reactivity of methionine residues in (VPGMG)₂₀ might be due to steric hindrance by neighboring residues related to the conformations of ELPs. The two introduced methyl and benzyl groups, with different hydrophobicity, allowed us to start evaluating the impact of these thioalkylation reactions on ELP thermoresponsive properties. The resulting methylated ELP did not exhibit any transition temperature due the positive charges of the formed sulfonium groups that increased the overall hydrophilicity of the ELP. On the other hand, the benzylated ELP retained its LCST behavior, although the *T_t* was shifted to higher values, as the more hydrophobic benzyl groups could partly counterbalance the hydrophilic effect of the positive charges. We came to the conclusion that (VPGMG)₂₀ contained too many methionine residues leading to a high density of positive charges and a high hydrophilicity. Therefore decreasing the amount of methionine residues to one Met every 4 guest residues would allow us to reduce this effect and also to consider the introduction of bulkier groups that might be complicated in the case of (VPGMG)₂₀. Also this ELP scaffold present the other important limitation to be difficult to produce without its leader sequence while the presence of these 13 extra amino acids is not desirable for our studies.

After designing a new ELP scaffold containing less methionine residues, we then investigated another method for tuning methionine containing ELP properties *i.e.* by oxidation of its methionine residues. This method was inspired by the essential biological phenomenon of reversible methionine oxidation to sulfoxide. Therefore we synthesized the ELP named ELP-M-40, and quantitatively obtained its oxidized sulfoxide ELP-M⁰-40 and sulfone ELP-M^{O₂}-40 derivatives. This proved to be an efficient post-modification method that allowed us to significantly modify the transition temperature of ELP-M-40 in aqueous solution. Indeed significantly higher T_t values were obtained for ELP-M⁰-40 compared to ELP-M-40 likely due to increased water-solubility afforded by the sulfoxide groups as compared to thioether groups. On the other hand, the sulfone derivative ELP-M^{O₂}-40 exhibited T_t values greater than the one of ELP-M-40, but lower than the one of ELP-M⁰-40 likely due to the greater dipole moments of the sulfone groups compared to sulfoxides that can lead to sulfone-sulfone and sulfone-protein interactions and thereby decreased water solubility. We also observed that the empirical equation established by Chilkoti and coworkers enabled the accurate estimations of the T_t of ELP-M-40 as well as the ELP-M⁰-40 and ELP-M^{O₂}-40 derivatives at specific concentrations. The thermoresponsive properties of these three ELPs were further tuned by using Hofmeister salts in the aqueous solutions.

The next step of the present thesis involved the use of two ELPs, ELP-M-20 and ELP-M-40, containing fewer methionine residues than the first (VPGMG)₂₀. When using the full methionine containing ELP (VPGMG)₂₀ we observed that the obtained alkylated reagents were very hydrophilic due to the high density of positive charges introduced along the backbone by the formed sulfonium groups. Decreasing the amount of functionalizable residues was thus considered to retain the thermoresponsive properties of the alkylated derivatives while also allowing the introduction of bulkier functional groups. We then first synthesized the fully methylated derivative of ELP-M-40 using the previously established procedure and studied the effect of different sulfonium counteranions on its thermoresponsive properties. We observed that these counterions indeed influenced the transition temperature of the methylated ELP-M-40 and that a suitable choice of sulfonium counterions, for example NTf₂⁻ or PF₆⁻, could allow retaining its thermoresponsive properties. We also evaluated the influence of chiral counteranions on ELP conformation. In fact the presence of chiral counterions did not lead to significant changes in the relative content of random coils or type II β -turns of the ELP. This might be explained by the fact that sulfoniums and their counterions, are too separated from each other along the backbone to significantly affect the ELP chain conformation.

To obtain a wider range of side-chain functionality we also envisioned the use of epoxides instead of alkyl halides as reagents. Indeed many functional epoxides are commercially available or easily synthesized, they also proved to be efficient alkylating agents and are generally easier to handle than other alkylating agents such as triflates. We managed to quantitatively and selectively modify methionine residues in ELP-M-20 and ELP-M-40 using different epoxides thus leading to the synthesis of several derivatives. However Met residues were again less reactive than the one in polymethionine thereby requiring adapted alkylation procedures. We first thought of increasing both the amount of reagent and the reaction time but this was not enough to reach full functionalization. Indeed the reactions were carried out in acidic media thereby leading to the progressive hydrolysis of the epoxide which thus had to be added portionwise. While this allowed the full functionalization of ELP-M-20, ELP-M-40 on the other hand required further change in the procedure. In fact the larger molecular weight of this ELP probably prevented the access of epoxide to some of the Met residues. We thus had to use a more denaturing solvent to allow alkylation of all Met residues. By studying the thermoresponsive properties of the obtained alkylated derivatives we observed that the nature of the functional groups affected the transition temperature and the LCST behavior could be retained with some of these pendant groups, for instance the EG₃ or alkene derivatives. Surprisingly we also observed that two of the ELP-M-40 derivatives presented higher T_t values as compared to their ELP-M-20 counterparts, although higher molecular weights usually lead to lower T_t values. This is probably due to the higher number of charges per chain in ELP-M-40 derivatives that prevails over the molecular weight effect. We also evaluated more precisely the influence of the sulfonium positive charge on the thermoresponsivity by subsequent demethylation of methionine sulfoniums. The obtained S-alkyl-L-homocysteine containing ELP exhibited strong thermoresponsive properties compared to the corresponding polysulfonium derivative thus proving the strong effect of the sulfonium on thermoresponsivity of these ELPs.

We then decided to use the alkyne groups previously introduced along the ELPs backbone as a handle to introduce more relevant groups for potential biomedical applications. The synthesis of glycoprotein analogs has received much interest during the past years as these biomolecules present very interesting features in the scope of biomedical applications. Therefore we chose to investigate the preparation of saccharide-functionalized ELPs starting with monosaccharides. Glucose, galactose and mannose units were successfully introduced by click chemistry on the alkyne bearing ELPs. Similarly to the other modified ELPs the influence of the introduced groups on the transition temperatures of

ELPs was studied and showed that the galactose and mannose derivatives retained thermoresponsive properties while the glucose derivatives seemed to be more hydrophilic. Preliminary tests were then performed to assess the biological activity of the galactosylated ELP-M-40. Its binding towards the lectin RCA120 was investigated and confirmed the ability of the galactose residues to specifically recognize and bind this lectin. Further bioactivity tests would however be required to perfectly assess the biological relevance of these derivatives.

Finally efficient methods for the chemoselective and quantitative modification of three methionine containing ELPs were developed. These methods allowed tuning of the thermoresponsive properties of these ELPs. They also enabled the versatile functionalization of the ELPs backbone and the introduction of biologically relevant groups. This work should be pursued by investigating more deeply the biological activity of the saccharide-functionalized ELPs for instance by testing the selective binding of the galactose derivative ELP toward RCA120 in the presence of other proteins. The synthesis of glycopolypeptides with precise structures should also be pursued by the introduction of disaccharides or oligosaccharides leading to more complex scaffolds. The possibility to prepare synthetic glycoproteins analogs with precise and complex structures such as mucin mimics is very attractive in the field of biomedicine.^{3,4} Indeed mucins are a family of transmembrane glycoproteins involved in immunity and cancer. However their complex structures and biological functions are not fully known yet. Therefore synthetic analogs are interesting in order to better understand these features and could be used for diagnostics and therapeutics development. By designing different ELP scaffold with different methionine contents and by using the method presented here to introduce more complex saccharides, mucin-like structures could be created. Designing new ELP scaffolds with varied methionine density could also be interesting for different purposes. For instance by introducing more Met residues we might be able to control and change the ELP chain conformation with chiral counterions contrary to what was observed with a low methionine density ELP. Different spacing between the methionine residues could also allow us to obtain glycopolypeptides with varied density of saccharide functions along the backbone and to evaluate the influence of the saccharide spacing on the binding capacities of these glycopolypeptides.

Regarding the tuning of the thermoresponsive properties it would be interesting to pursue the study of ELP derivatives with demethylated Met sulfonium. Indeed we saw that the positive charges introduced by the sulfonium groups strongly affected the T_t of our ELPs. The obtained systems are in fact quite

complex with several parameters affecting the thermoresponsive: positive charges, OH groups, nature of the functional groups, density of Met residues, ELP molecular weights. Getting rid of these charges would simplify our systems thus allowing us to study more specifically the effect of the groups introduced on Met side-chain and to obtain materials with more robust thermoresponsive properties as seen with the demethylated alkyne bearing ELP. The T_t of our ELPs could also be tuned by modifying the OH group introduced on methionine side-chain following the epoxide ring opening, for instance by functionalization with more hydrophobic groups as recently shown by Deming and coworkers.⁵

We could also envision the conjugation of these functionalized ELPs, for instance the saccharide-functionalized ones, to more hydrophobic polymer or ELP blocks. This could allow the preparation of amphiphilic copolymers and the subsequent formation of glycosylated nanoparticles. For now the density of carbohydrate moieties along the ELPs is relatively low so the formation of nanoparticles would lead to higher density and multivalent binding, more relevant for bioactivity studies.

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Titre : Modifications chimiosélectives de polypeptides recombinant à base de motifs élastine : Modulation de la thermosensibilité et de l'activité biologique

Résumé : La thèse présentée porte sur la préparation de dérivés de polypeptides recombinant à base de motifs élastine (ELPs) ainsi que sur l'étude de leurs propriétés physicochimiques et biologiques. Des ELPs contenant des résidus méthionine ont été modifiés de manière chimiosélective soit en utilisant des halogénures d'alkyle ou différents époxydes, soit par oxydation des résidus méthionine. La caractérisation par RMN et par spectrométrie de masse des composés obtenus a permis de confirmer leur fonctionnalisation quantitative. L'étude de la réponse en température de ces dérivés d'ELP par des mesures de turbidité ou par des mesures de diffusion de la lumière a montré le fort impact des modifications entreprises sur la température de transition (T_t) des ELPs. Il a également été montré que la T_t peut être modifiée par échange des contre-ions des dérivés cationiques. Enfin, des monosaccharides ont été conjugué aux ELPs contenant des groupements alcyne par cycloaddition de Huisgen afin d'obtenir des glycopolypeptides. Les propriétés thermosensibles ainsi que les propriétés biologiques de ces conjugués ont été testées et ces dernières ont permis de montrer leur capacité à se lier sélectivement à des lectines. Leur utilisation pour trier des protéines d'intérêt et les redisperser est finalement évaluée de façon préliminaire.

Mots clés : élastine, polypeptides recombinants, méthionine, thermosensibilité, glycopolypeptides

Title: Chemoselective modifications of recombinant elastin-like polypeptides: Tuning thermosensitivity and bioactivity

Abstract: This thesis describes the preparation of elastin-like polypeptides (ELPs) derivatives and the study of their physico-chemical and biological properties. Methionine-containing ELPs were chemoselectively modified using either alkyl halides or epoxides or by oxidation of their methionine residues. The successful functionalization was assessed by NMR and mass spectrometry analysis of the resulting compounds. The thermoresponsive properties of these ELP derivatives were evaluated either by light scattering or by turbidity measurements showing the strong effect of these modifications on the ELPs transition temperature (T_t). The counterion effect on the thermosensitivity of the polycationic derivatives was also studied. The synthesis of ELP glycopolypeptides was finally achieved by conjugating monosaccharides to the ELP alkyne derivatives through Huisgens cycloaddition. Along with the thermoresponsive properties, the bioactivity of the ELP glycoconjugates was studied and proved their ability to specifically bind lectins. Their use for protein sorting and release was preliminary evidenced.

Keywords: elastin, recombinant polypeptides, methionine, thermosensitivity, glycopolypeptides