

# Emerging opportunities in bioconjugates of Elastin-like polypeptides with synthetic or natural polymers.

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# Emerging opportunities in bioconjugates of Elastin-like Polypeptides with synthetic or natural polymers

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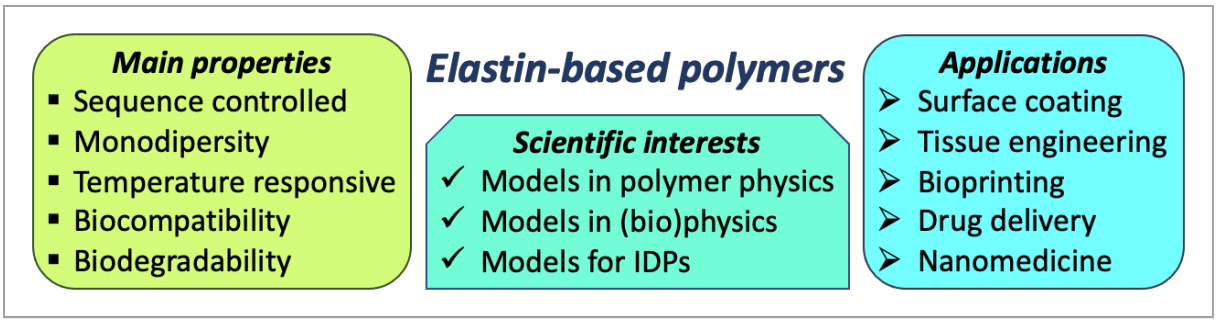
## ABSTRACT

Nature is an everlasting source of inspiration for chemical and polymer scientists seeking to develop ever more innovative materials with greater performances. Natural structural proteins are particularly scrutinized to design biomimetic materials. Often characterized by repeat peptide sequences, that together interact by inter- and intramolecular interactions and form a 3D skeleton, they contribute to the mechanical properties of individual cells, tissues, organs, and whole organisms. (Numata, K. *Polymer Journal* **2020**, *52*, 1043–1056) Among them elastin, and its main repeat sequences, have been a source of intense studies for more than 50 years resulting in the specific research field dedicated to elastin-like polypeptides (ELPs). These are currently widely investigated in different applications, namely protein purification, tissue engineering, and drug delivery, and some technologies based on ELPs are currently explored by several start-up companies. In the present review, we have summarized pioneering contributions on ELPs, progress made in their genetic engineering, and understanding of their thermal behavior and self-assembly properties. Considered as intrinsically disordered protein polymers, we have finally focused on the works where ELPs have been conjugated to other synthetic macromolecules as covalent hybrid, statistical, graft, or block copolymers, highlighting the huge opportunities that have still not been explored so far.

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## Graphical abstract



## 1. Introduction

Compared to other natural structural proteins, elastin presents unique properties that have been exploited in the design of novel biomaterials for various applications over the years. Indeed, elastin is a natural fibrous protein present in the extracellular matrix of higher vertebrates, especially in organs and connective tissues that need to stretch or contract repetitively and reversibly (*e.g.*, lungs, aorta, arteries, skin, elastic ligaments, tendons) providing them with elasticity and resilience properties.[1] Elastin is an extremely insoluble and degradation-resistant protein due to its high content in hydrophobic amino acids and to the high level of interchain crosslinks involving oxidized lysine residues. Elastin fibers are formed extracellularly around a fibrillin-rich microfibrillar structure upon enzymatic modification and aggregation of its soluble monomeric precursor, tropoelastin (70 kDa), synthesized and secreted by several types of cells such as smooth muscle cells and endothelial cells, fibroblasts and chondrocytes. The sequence and mechanical properties of elastin have been an inspiration for materials scientists, especially for biomedical applications in the last 2 decades. In the present review article, we have first briefly recalled the pioneering contributions that have led to the emergence of the specific research field dedicated to elastin-like polypeptides (ELPs). In a second part, we have highlighted the considerable progresses achieved in the genetic engineering and bacterial production of ELPs that have undeniably permitted these recombinant polypeptides to be considered realistically for the development of (bio)materials. Thermal responsiveness being a major defining property of ELPs, we have then highlighted the contributions that have enabled to understand this behavior and how the appropriate design of multi-block ELPs with different thermal responsiveness can lead to controlled and well-defined self-assembled morphologies. In the final and main section of this review article, we have addressed the field of ELP-polymer conjugates, and especially highlighted how ELPs have been cleverly incorporated into statistical, graft or block copolymer designs. Finally, in the concluding section, we provide the readers with our personal vision of the field and different possible perspectives.

## 2. Discovery of ELPs and pioneering contributions

### *2.1. From elastin fragments to the first synthetic ELPs*

In a first founding study from the 50's from S. M. Partridge *et al.*, partial hydrolysis of elastin and subsequent fractionation of the resulting products was found to yield two soluble proteins of similar amino acid compositions: a major protein component (α-fraction, 60-84 kDa) showing a characteristic property of

reversible heat precipitation, and a minor component (β-fraction, 5.5 kDa) that did not precipitate at any temperature up to 100 °C.[2–4] The α-fraction was observed to be water soluble below 25 °C, while phase separating and precipitating upon raising temperature to 30 °C, and readily redissolving on reducing back the temperature below 25 °C. This reversible temperature-elicited coacervation of α-elastin in aqueous buffer was found to be accompanied with a conformational change as demonstrated by circular dichroism (CD), from a disordered state in the soluble form below room temperature to a higher order state in which approximately 50 % of the protein was in α-helical conformation in the coacervate phase above 37 °C.[5] Later, J. A. Foster *et al.*, studying the amino acid composition of soluble elastin, described a protein containing two types of domains regularly spaced: Ala- and Lys-rich areas likely involved in crosslinks of natural elastin, and Gly, Pro and Val-rich domains presumably involved in the extensibility properties of elastin.[6] Peptide analyses of a tryptic digest of soluble (non-crosslinked) elastin, isolated from copper-deficient pigs' aorta, revealed three highly repeated motifs: a tetrapeptide (GGVP), a pentapeptide (PGVGV) and a hexapeptide (PGVGVA).[7] From molecular models, authors suggested the presence of a β-turn in -PGGV- motifs whose regular repetition shall lead to a helical conformation.[6]

Soon afterwards, D. W. Urry *et al.* reported the very first synthetic ELPs in the literature, namely penta-, deca- and pentadecapeptides $(VPGVG)_n$ with n= 1, 2 or 3, synthesized using a combination of solution and solid phase peptide synthesis and fragment condensation method.[8] Conformational studies of these peptides by $^1$H NMR confirmed the β-turn conformation stabilized by a non-covalent interaction between the carbonyl of $Val_1$ and amide group of $Val_4$. Higher molar mass ELPs $(VPGVG)_n$ with 10<n<15 were then obtained by pentafluorophenol-activated polycondensation and evidenced to show temperature-triggered coacervation and similar turbidity profile to α-elastin.[9] Urry postulated that intermolecular hydrophobic interactions were predominant in the coacervate phase. Urry also proposed the synthetic polytetrapeptide $(VPGG)_{40}$ as a polymer model to describe the CD signature for a β-turn conformation: a negative band at 224 nm, a positive band at 205 nm and a second negative band below 190 nm.[10] In 1980, a type II β-turn was evidenced from the crystal structure of a cyclic pentadecapeptide *cyclo*$(VPGVG)_3$ resolved from X-ray diffraction data.[11]

### *2.2. Characteristics of thermally-induced ELP coacervates*

In 1985, Urry *et al.* studied the effect of ELP concentration on the coacervation process and viscoelastic properties of a large average molar mass ELP (*ca*. 100

kDa) $(VPGVG)_{200}$ obtained by polycondensation. While concentration affected the temperature-response turbidity profiles (*i.e.*, sharper and lower temperature transition with increasing concentration), the conformational changes from random coil to β-turn were always observed.[12] Urry also correlated macromolecular order and elasticity: soluble polypeptide chains showed little elasticity, while the increase in type II β-turn secondary structures upon raising the temperature was accompanied with a decrease of chain mobility and increase of elastomeric forces, the latter being similar at high concentration of ELP to the one of γ-irradiated crosslinked ELP coacervate. These findings were opposite expectations based on the classical theory of rubber elasticity, but confirmed previous conclusions from thermodynamics and length-tension curves on elastin.[13] The coacervate/viscoelastic phase was also characterized by a high content of water (*ca*. 60 %) similar to fibrous elastin, consistent with the strong hydrocarbon-water interactions anticipated earlier.[6]

In 1988, Urry reported two seminal review articles on "entropic elastic processes in proteins" summarizing the findings of more than 20 years of research on ELPs and elastin.[14,15] Based on all previous physical characterizations, he correlated the reversible inverse temperature transition (ITT) of ELPs in water with macromolecular order, chain dynamics, and elastomeric force. He also postulated that these phenomena shall be tuned predictably by changing the hydrophobicity of the ELP, a hypothesis supported by previous results with ELPs containing hydroxylated proline residues and confirmed experimentally by differential scanning calorimetry (DSC).[16,17] Based on these data, Urry predicted the ITT for ELPs of various compositions,[18] (**Fig. 1**) and proposed a new hydrophobicity scale for amino acid residues.[18,19]

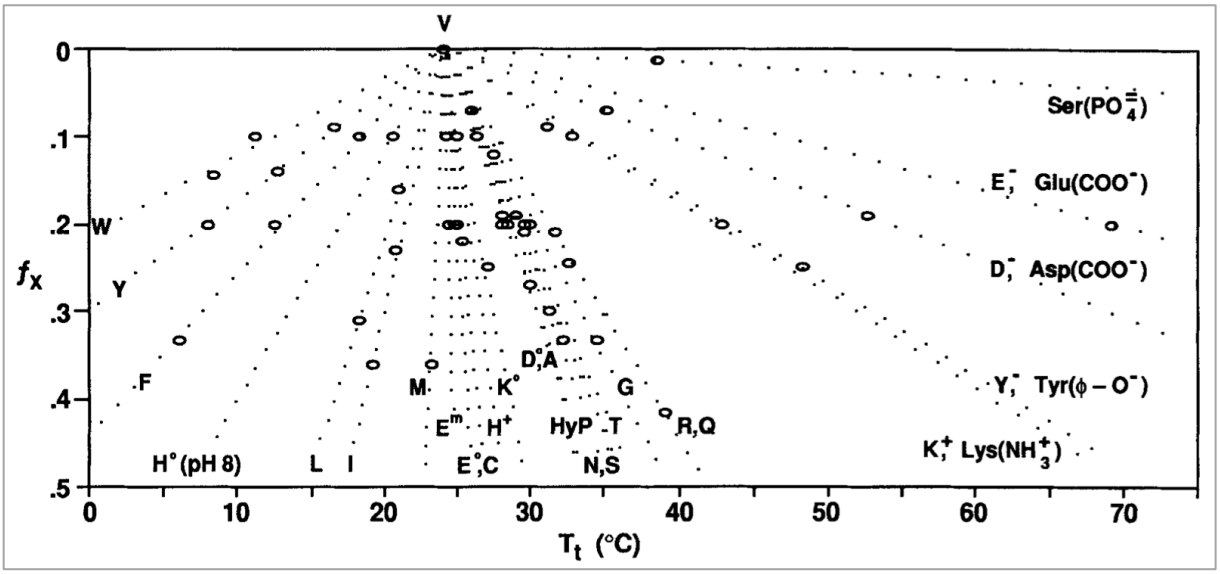



**Figure 1.** ITT dependance with varying composition of poly[$f_V$(VPGVG),$f_X$(VPGXG)] ELPs. Reproduced from Urry *et al.*[18]

### *2.3. Controlling the sequence and polydispersity of ELPs*

In 1992, Urry proposed the microbial synthesis of ELPs as "a more economical and practical means" than previously used peptide synthesis and polymerization methods.[20] The use of recombinant DNA technology was also a means to overcome the issues of macromolecular dispersity of ELPs obtained by polycondensation and of low amount of materials when prepared by peptide synthesis.[21] Urry *et al*. hence described the first recombinant production in *Escherichia coli* bacteria (*E. coli*) of an ELP by designing a recombinant gene containing the sequence G(VPGVG)$_{19}$VPGV fused to the gene of glutathione S-transferase (GST) allowing purification of the recombinant protein product from bacterial lysates by affinity chromatography. The monodisperse ELP (8.2 kDa, 13.8 mg from a 12 L culture) was recovered after proteolytic digestion and well characterized ($^{1}$H and $^{13}$C NMR, and turbidity profile with temperature). In 1995, Urry *et al*. described the hyperexpression of a synthetic gene in *E. coli* encoding for a higher molar mass ELP, G(VPGVG)$_{119}$VPGV, one of the first case reports of expression of a fully synthetic gene with no natural analog in *E. coli* bacteria.[22]
Last but not least, D. W. Urry published in 1996 a seminal work describing the genetic engineering of ELPs by concatemerization of a synthetic gene (encoding the sequence (GVGVP)$_{10}$) and the clever exploitation of their reversible phase transition properties to isolate and purify ELPs from bacterial lysates by simple rounds of cold and warm centrifugations,[23] a process later optimized and termed "inverse transition cycling" (ITC) by Chilkoti *et al*.[24] The process was successfully applied to obtain 5 different ELPs (GVGVP)$_n$ with n= 41, 121, 141, 251, with excellent purity and low endotoxin levels.[23]

Dan W. Urry pursued his intensive work on ELPs, that he qualified as "elastic protein-based polymers",[21] studying their physics as well as those of hydrogels thereof. In 2001, he acknowledged that what he termed the inverse transition temperature (noted as ITT or $T_t$) behavior of ELPs actually corresponded to the lower critical solution temperature (LCST) behavior of synthetic polymers.[25]

## 3. Development of recombinant ELPs

The most widely studied ELPs are based on (VPGXG) repeat units,[26] where the guest residue X can be any natural or non-natural alpha amino acid except for proline. Proline (P) is a forbidden guest residue since disrupting chain conformation and LCST properties.[18,26] As suggested by Chilkoti and collaborators, ELP constructs can be noted as ELP[$X_iY_jZ_k$-n], where X, Y, Z specify the guest residues (single letter amino acid code) in the ELP sequence, i, j, k written as subscripts their relative ratio, and n the total number of pentapeptide repeats.[27] This notation is however not specifying the precise positioning of the

different guest residues in the protein sequence, or indicating possible additional residues at the *N*- or *C*-terminal ends of the ELP.

Following Urry's pioneering work, A. Chilkoti strongly contributed to the research field on ELPs by optimizing the genetic engineering of ELPs, understanding the parameters influencing their LCST and pushing forward their developments towards biomedical applications. The difficult sequence control with polymerization reactions and the small scale and tedious syntheses *via* peptide synthesis has motivated the use of recombinant DNA and protein engineering techniques to produce ELPs. Such technique also allowed the fusion of ELP-encoding gene sequences with those of other proteins. However, the assembly of genes encoding ELP constructs using conventional cloning methods is still a tricky task for two main reasons: *i*) long and repetitive oligonucleotide sequences are complex to assemble synthetically, *ii*) the polymerase chain reaction (PCR) does not process efficiently repetitive genes. Different recombination techniques have therefore been developed. The construction of ELP encoding genes was initially achieved by concatemerization consisting in the random ligation of the gene encoding for an ELP monomer with sticky ends leading to longer ELPs by repeating several ligation cycles. Although rapid, this technique however does not ensure that the desired gene sizes will be obtained and does not permit the controlled assembly of different gene sequences. In 2002, Chilkoti *et al.* reported a method termed recursive directional ligation (RDL), to circumvent the limitations of concatemerization. RDL involved the stepwise oligomerization of short ELP genes derived from concatemerization in a plasmid vector by seamless cloning using type II restriction enzymes.[27] Later on, the group of Chilkoti perfected the technique by developing PRe-RDL (namely, recursive directional ligation by plasmid reconstruction), consisting in the dimerization of two moieties of a plasmid, each of them including the gene of an ELP. This technique prevents the recircularization of the vector or of the insert gene, and implies a limited number of cloning stages as compared to RDL.[28] These have allowed to assemble, in a controlled manner, genes encoding different polypeptide sequences and to access ELPs with more complex architectures such as multiple block-containing ELPs. RDL and Pre-RDL have also permitted the insertion of other peptide or protein sequences at the *N*- and *C*-terminal ends or within the ELP gene. Because, multiple cloning steps can be time consuming, Chilkoti *et al*. have also later developed a technique termed OERCA (overlap-extension rolling circle amplification) to rapidly access ELP genes with a high number of repetitions. The technique is based on the use of a circular single strand DNA encoding an ELP sequence which constitutes a template for thermal cycling using primers. The method is powerful to synthesize genes longer than the ones obtained by concatemerization, but solely permit to

obtain ELP homopolymers. Although there have been a few reports on the expression of ELP genes in heterologous hosts such as yeast, fungi, or plants, productions of ELPs have most of the time been achieved using *E. coli* bacteria regarding their rapid growth, inexpensive culture media, wide variety of strains and expression vectors available, as well as the decent production yields (> 50 mg ELP per L culture). Since classical strains of *E. coli* are devoid of excretion systems, ELPs are not secreted in culture media implying a cell lysis step for the recovery of ELPs. A simple centrifugation at cold temperature is therefore applied to discard cell residues in the pellet and retrieve the soluble ELPs in the supernatant. Isolation and purification of ELPs from other protein contaminants can finally be achieved by ITC exploiting their reversible thermo-responsive properties.[23,24]

## 4. Elucidation of the thermo-responsive properties of ELPs

Improvements in gene design techniques have allowed to constitute large libraries of ELPs and to perform intensive studies on their thermal responsiveness. As compared to the first ELPs described by Urry and synthesized by polycondensation, recombinant ELPs are monodisperse allowing the establishment of reliable structure-property relationships. While main parameters affecting the thermal response and cloud point temperature of ELPs had been previously described by Urry (*i.e.*, nature and mole fractions of the guest residues, total chain length, concentration, presence of salts, organic cosolutes or solvents, among others),[21] Chilkoti reported a seminal study where he established an empirical equation to quantitatively determine and predict the impact of chain length and concentration on the cloud point temperature ($T_{cp}$ or $T_t$) of a series of ELPs with identical residue composition.[29] The study was achieved using three libraries of ELPs: ELP[$V_1A_8G_7$-n] (most hydrophilic residue composition, n= 128, 160, 256, 320), ELP[$V_5A_2G_3$-n] (n= 60, 90, 120, 150, 180, 240, 330), and ELP[V-n] (most hydrophobic residue composition, n= 15, 30, 60, 90, 120). The cloud point temperatures (defined as the maximum of the first derivative of the turbidity profile versus temperature) of aqueous solutions of the 16 different ELPs were systematically measured by UV-Vis spectroscopy and plotted versus molar concentrations. The cloud point temperatures of a specific ELP were found to follow a linear law as a logarithmic function of molar concentration (*i.e*, $T_{cp}$ = A ln(C)+B) with a negative slope (A, *i.e.*, decreasing $T_{cp}$s with increasing concentrations), and the absolute value of the slope to be inversely correlated to the number of pentapeptide units (*i.e.*, chain length, n). (**Fig. 2A**)

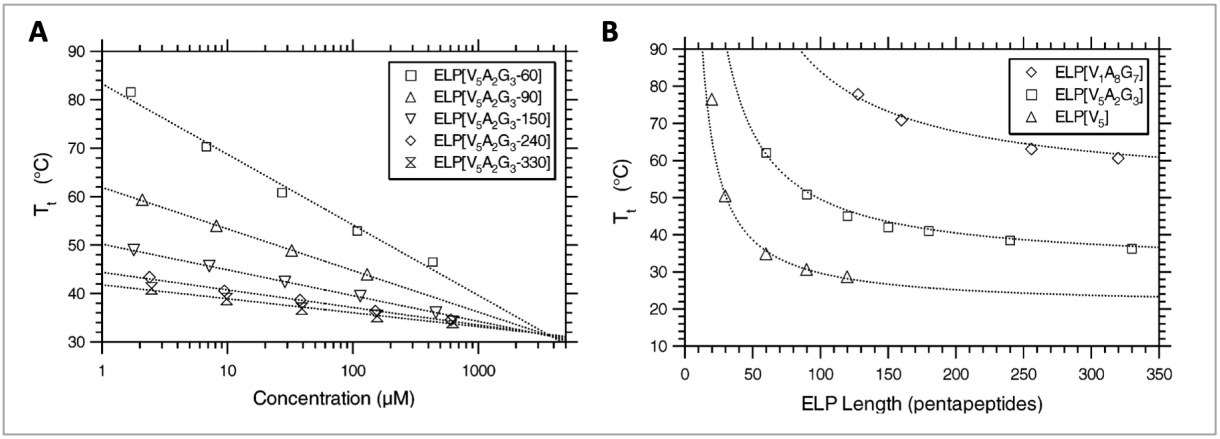



**Figure 2.** A/ $T_t$ (or $T_{cp}$) as a function of concentration for ELP[$V_5A_2G_3$-n] constructs (and logarithmic fits to the data); B/ $T_t$ as a function of ELP chain length for the three ELP libraries (25 *μ*M). Reproduced from Meyer *et al.*[29]

For each ELP library, the fits of $T_{cp}$ versus concentration for different ELP chain lengths appeared to converge to a single critical point defined by a sequence-specific concentration ($C_c$, μM) and temperature ($T_{cp,c}$, °C), which led to the empirical equation:

$$\mathbf{T_{cp} = T_{cp,c} + \frac{k}{n} \ln\left(\frac{C_C}{C}\right)}$$

$\mathbf{k}$ being a positive constant (°C) descriptive of a specific ELP composition, and $\mathbf{n}$ the number of pentapeptide units. This equation also writes as:

$$\mathbf{T_{cp} = -\frac{k}{n}\ln(C) + [T_{cp,c} + \frac{k}{n}\ln(Cc)]}$$

better reflecting the negative slope (A = $-\mathbf{k/n}$) of the curve $T_{cp}$=f(ln(C)) and y-intercept.

$\mathbf{T_{cp,c}}$ actually corresponds to the **LCST** which is **characteristic of a series of ELPs with identical amino acid composition** and **independent of chain length**. On the contrary, $T_{cp}$ does depend on chain length. Because the term LCST is sometimes used to design the cloud point temperature ($T_{cp}$), some confusion is frequently brought in the literature.

From this empirical equation, one understands that the slope approaches zero for infinite chain length-ELPs as illustrated on **Fig. 3**, implying a weaker effect of concentration on $T_{cp}$ for ELPs with long chain lengths. On the contrary, the slope approaches infinity as the chain length decreases to zero, traducing an enormous effect of concentration on $T_{cp}$ for ELPs with short chain lengths.

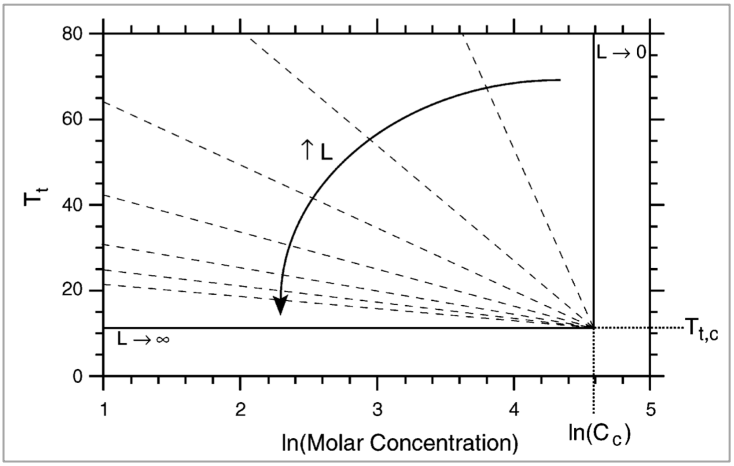



**Figure 3.** A schematic of the model that describes the variation of $T_t$ (or $T_{cp}$) as a function of ELP chain length (L or n) and ELP concentration. Reproduced from Meyer *et al*.[29]

Also, lower chain lengths are required for the most hydrophobic ELP sequences (characterized by low $k$ values) to reduce the influence of concentration on $T_{cp}$, in contrast to most hydrophilic ELP sequences (characterized by higher $k$ values).[30] At fixed concentration, Chilkoti *et al*. evidenced an excellent concordance between experimental and empirically predicted $T_{cp}$ for ELPs of fixed composition, but varying chain lengths.[29] (**Fig. 2B**)
In 2010, Chilkoti *et al*. adapted this model to incorporate the effects of pH on the $T_{cp}$ for ELPs that contain ionizable guest residues.[31] The model, allowing to predict the pH at which an ELP phase separates ($pH_{cp}$) and/or the cloud point temperature according to the concentration, chain length and pH, was validated with 2 libraries of pH-responsive ELPs that contained either acidic (Glu) or basic (His) residues.

Besides macromolecular parameters of an ELP and its concentration in aqueous solution, other parameters have been described to affect its thermal behavior. Some organic cosolvents (*e.g.*, dimethyl sulfoxide, dioxane, acetone and alcohols) were evidenced to have a biphasic effect on $T_{cp}$ (raising it at low content and decreasing it at high content).[32] Salts, such as NaCl, lower the $T_{cp}$ (explaining its use in the purification process of ELPs), while denaturants, such as urea and guanidinium chloride increase it. The influence of anions from the Hofmeister series at different concentrations has also been studied by Chilkoti.[33] The study was achieved on two ELPs, ELP[V-120] and ELP[$V_5A_2G_3$-120], and using sodium salts. (**Fig. 4**) In most cases, increasing salt concentrations decreased $T_{cp}$. This decrease was found to be linear for kosmotropes (strongly hydrated ions) and non-linear for chaotropes (more weakly hydrated ions).

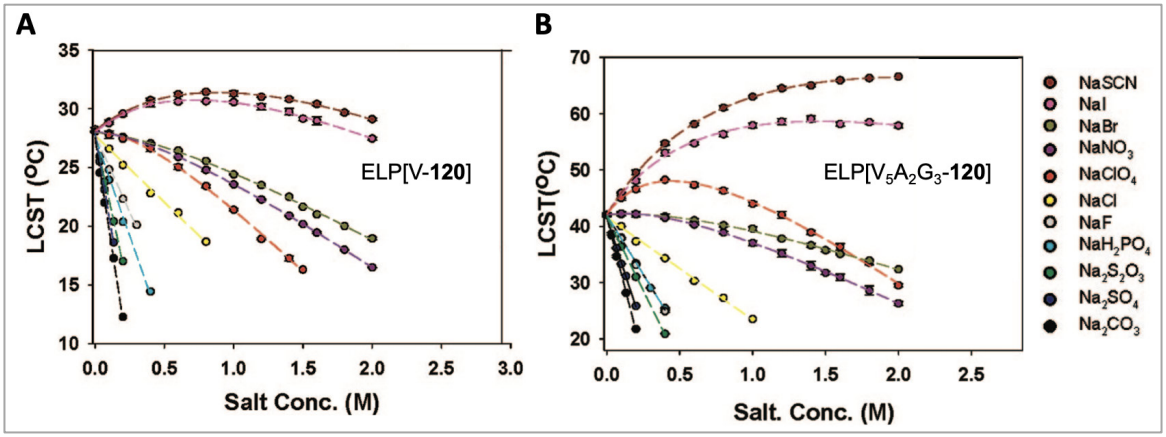



**Figure 4.** $T_{cp}$ as a function of salt concentration. Reproduced and adapted from Cho *et al*.[33]

## 5. Self-assembly properties of multi-block ELPs

An important consequence of ELPs' thermoresponsive properties is that they can lead to controlled and well-defined self-assemblies when appropriately incorporated into the design of macromolecules. In particular, ELP block copolymers result from the linear and covalent association of two or more ELP segments having different LCSTs, that can be obtained by playing with the sequence or length of each individual block. The first recombinant di- and triblock ELPs were described by Conticello *et al*. who evidenced the selective temperature-dependent segregation of the most hydrophobic block in aqueous solution and formation of nanoparticles,[34] (**Fig. 5**) and nano-textured hydrogels,[35] respectively. The presence of glutamic acid residues (E) in the more hydrophilic block also resulted in self-assembly behavior depending on pH.

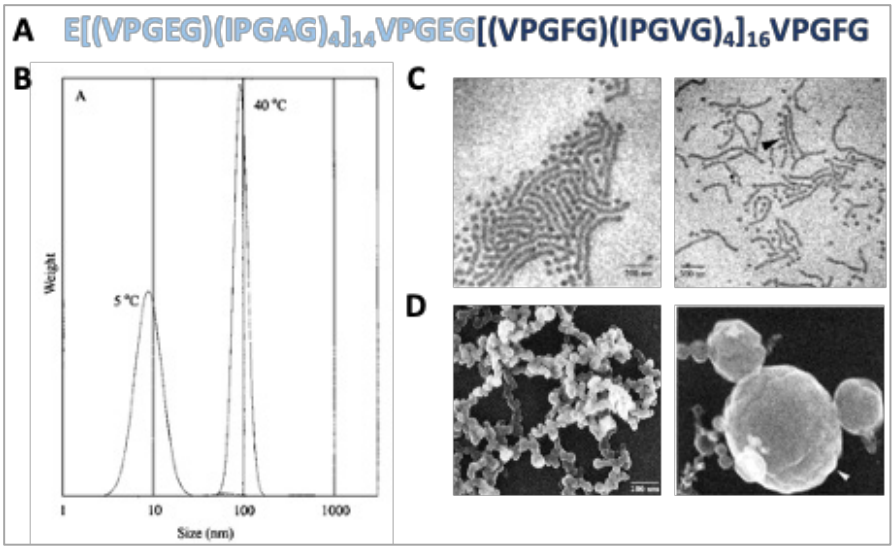



**Figure 5.** A/ Primary sequence of the first diblock ELP (more hydrophilic block written in light blue, more hydrophobic block in darker blue); B/ Weight distributions of particle sizes derived from DLS data (1 mg/mL in water); C/ TEM and D/ Cryo-HRSEM images. Reproduced and adapted from Lee *et al*.[34]

Chilkoti and coworkers also reported in 2002 the design (by RDL) and expression in *E. coli* of the diblock ELP[$V_1A_8G_7$-64]-[$V_5$-60]. They studied its thermal behavior by turbidimetry and dynamic light scattering and used, as control, a single block ELP of similar size (120 repeat units) and similar content in V, A, and G, but these guest residues were evenly distributed throughout the ELP chain.[27] The two blocks were selected regarding their high discrepancy in cloud point temperatures (*i.e.*, 35 °C for ELP[$V_5$-60] and > 90 °C for ELP[$V_1A_8G_7$-64] at 25 μM in PBS). When the single block ELP[$V_5A_2G_3$-120] in solution was heated, a sharp increase in turbidity was observed at approximately 45 °C. (**Fig. 6A**) DLS measurements indicated that this increase was related to the coacervation of soluble ELP chains ($R_h$ of 4.8 ± 1.1 nm) into micrometer-sized aggregates ($R_h$ of 1.2 ± 0.26 μm). (**Fig. 6B**) In contrast the turbidity profile of the block copolymer evidenced two major transitions (**Fig. 6A**): the first one at 40.0 °C corresponding to the transition from soluble ELP chains ($R_h$ of 4.4 ± 1.6 nm) to nanometer-sized particles ($R_h$ of 20.4 ± 5.8 nm up to 47 °C and 54.5 ± 20.3 nm above), the second one at 50.8 °C above which larger aggregates with a $R_h$ of 1.4 ± 0.35 μm were formed. (**Fig. 6C**) The doubling in nanoparticles size occurring at 47.5 °C was not explained at that time and rarely reported in following studies. Nevertheless, the results suggested the sequential and independent transitions of the two individual ELP blocks at two distinct temperatures respectively termed:

- **critical micelle temperature** (CMT) corresponding to the collapse of the most hydrophobic block ([$V_5$-60]), while the most hydrophilic block ([$V_1A_8G_7$-64]) remained solvated insuring colloidal stability to the self-assembled micelle-like nanoparticles,
- **bulk transition temperature** corresponding to the collapse of the most hydrophilic block ([$V_1A_8G_7$-64]) and destabilization of individual micelles into micrometer-sized aggregates.

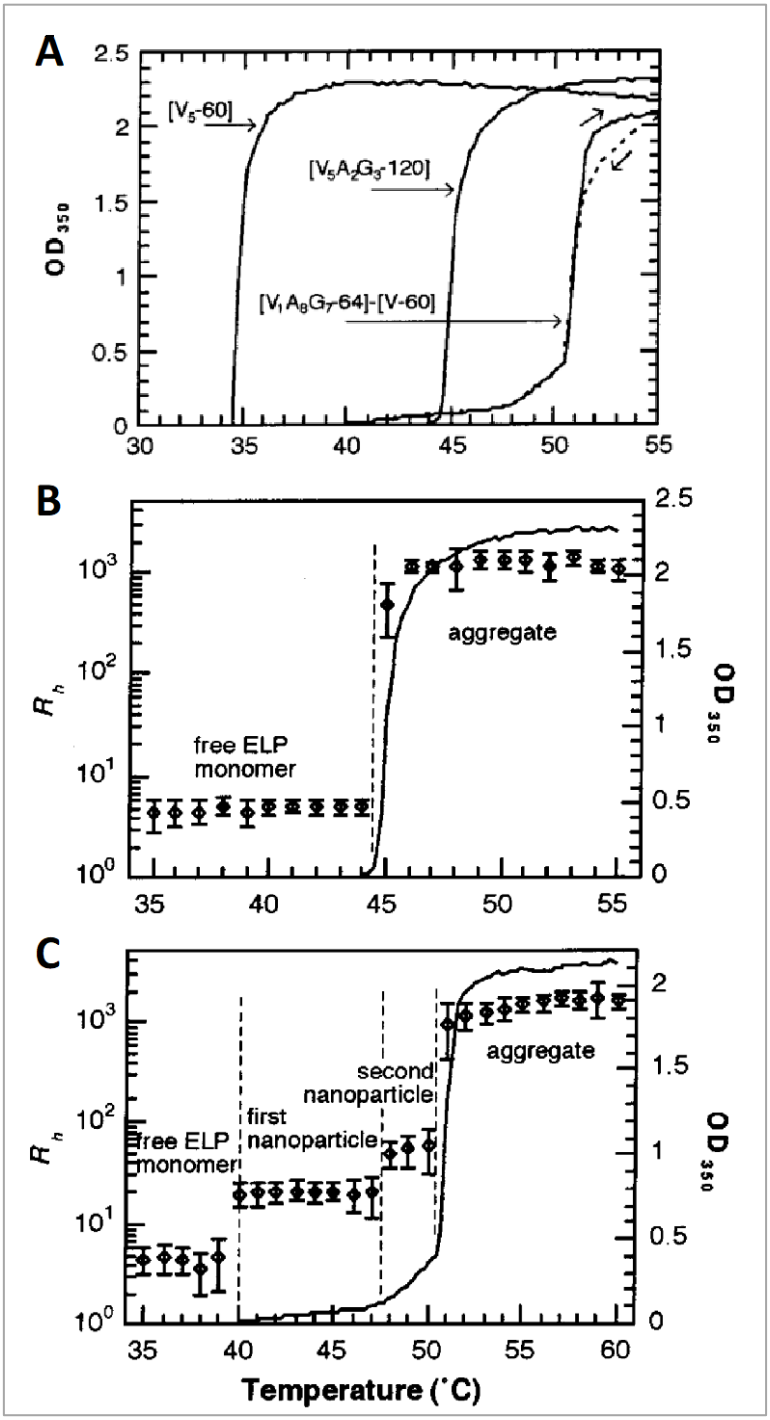



**Figure 6.** A/ Solution turbidity profile (OD 350 nm) as a function of temperature for the single blocks ELP[$V_5$-60] and ELP[$V_5A_2G_3$-120], and for the diblock ELP[$V_1A_8G_7$-64]-[$V_5$-60]; B/ and C/ Particle size (hydrodynamic radii) as a function of temperature measured by DLS for the single block ELP[$V_5A_2G_3$-120] and the diblock ELP[$V_1A_8G_7$-64]-[$V_5$-60], respectively. Corresponding turbidity profile were replotted for comparison. Reproduced and adapted from Meyer and Chilkoti.[27]

These finding were confirmed in a following study in which Chilkoti and coworkers engineered a series of 10 different diblock ELPs with different molar masses and hydrophilic-to-hydrophobic block ratios.[36] These consisted of a high cloud point temperature-ELP segment [$V_1A_8G_7$-n] (n= 64, 96, or 128), and

a low cloud point temperature-ELP segment [V-m] (m = 60, 90, or 120). All of the diblock ELPs with hydrophilic(Φi)-to-hydrophobic(Φo) block ratio (Φi/Φo) ranging in between 1:2 and 2:1 were shown to undergo two phase transitions as a function of solution temperature: a unimer-to-spherical micelle transition at an intermediate temperature (CMT) and a micelle-to-bulk aggregate transition at a higher temperature (bulk aggregation temperature), a 2-step self-assembly mechanism schematically represented by Hassouneh *et al.*[37] as shown on **Fig. 7**. The study also evidenced that the CMT was mainly controlled by the length of the hydrophobic block, and that the size of micelles was correlated to the total ELP length and to the hydrophilic-to-hydrophobic block ratio. In a later study, Chilkoti and coworkers qualified nanoparticles formed from diblock ELPs as "weak micelles" with dense cores and almost unstretched coronas due to low surface tension at the core−corona interface.[37]

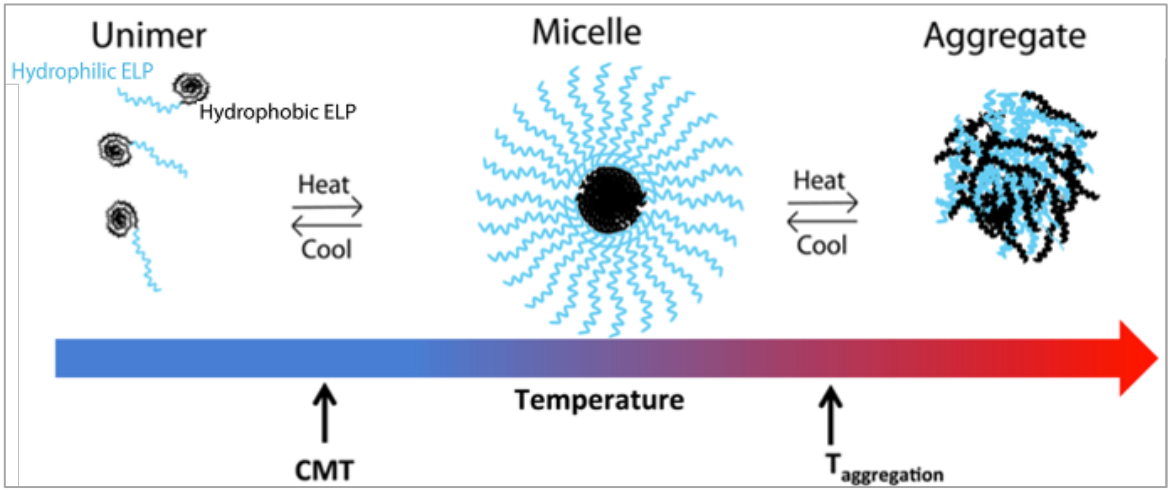



**Figure 7.** Schematic of self-assembly of ELP diblock copolymers. Reproduced and adapted from Hassouneh *et al.*[37]

In 2014, MacKay and coworkers working on two different libraries of diblock ELPs, namely ELP[I-n]-[S-n] and ELP[I-n]-[A-n] (n= 18, 24, 36, 48, or 96) established that a minimum of 48 repeat units in the most hydrophobic ELP block was required to form stable nanoparticles.[38] Authors also evidenced that for such diblock ELPs (n≥48) the CMT-concentration and $Tt_{bulk}$-concentration curves followed the same logarithmic trend as the one of the monoblock ELPs, while the CMT and $Tt_{bulk}$ were different from the $T_{cp}$s of their corresponding monoblocks:

$$CMT = T_{cp1} + \Delta T_{cp1}$$

$$Tt_{bulk} = T_{cp2} + \Delta T_{cp2}$$

where $T_{cp1}$ and $T_{cp2}$ are the corresponding cloud point temperatures of the hydrophobic and hydrophilic ELP monoblocks, and $\Delta T_{cp1}$ and $\Delta T_{cp2}$ are the corresponding differences when the two monoblocks are fused into a diblock. In other words, the CMT is almost entirely dependent on the hydrophobic core

ELP, while the bulk phase transition temperature ($T_{tbulk}$) depends predominantly on the hydrophilic block.

## 6. Bioconjugates of ELPs with natural or synthetic polymers

Recombinant ELPs have appeared as attractive protein-like polymers for biological and biomedical applications regarding several of their characteristic features, namely their exact and reproducible chemical structure (both in terms of monomer sequence and chain length), good production yields and possible production up-scalability using bioreactors, and stimuli-responsive properties allowing their non-chromatographic purification and self-assembly in absence of organic cosolvent. There are four main fields in which ELPs have been and are still being explored, namely (*i*) protein purification,[39–43] (*ii*) tissue engineering,[44–53] (*iii*) drug delivery,[54–58] and more recently (*iv*) protein vesicles, artificial cells, and membrane-less organels.[59–65] Noteworthily, the works cited here are far from being an exhaustive list. Some of these aspects shall be described in more details in this special issue dedicated to genetically engineered protein-based polymers, while others have already been addressed in dedicated review articles and book chapters available in the literature.

In this last section, we want to review the contributions where ELPs have been associated with natural or synthetic polymers. Because the design of such hybrid macromolecules containing a recombinantly produced protein segment and a synthetic or natural polymer backbone requires different expertises, there are actually a limited number of contributions available in the literature. A few of them were previously mentioned in a recent article from Ghazanfari and coworkers who reviewed the field of elastic materials for tissue engineering applications.[66] In the present review, we have attempted to distinguish ELP-bioconjugates based on the class of polymers to which they have been conjugated. Despite very interesting works have been achieved on the conjugation of ELPs with lipids,[67–69] or with oligonucleotides,[70] these will however not be described in the following as they do not enter in the scope of this review.

### *6.1. First reported hybrid ELP-polymer bioconjugates, i.e., polymer-g-ELP*

The first original contribution describing the use of an ELP-based sequence in a hybrid ELP-polymer macromolecule involved the design of a monomer containing the -VPGVG- pentapeptide sequence to be polymerized by atom transfer radical polymerization (ATRP) yielding a graft copolymer bearing pendant -VPGVG- motifs contrasting with previously described linear poly(VPGVG) and addressing the question of conservation of thermo-

responsive properties in such macromolecular design.[71] This was also a first case report of the use of ATRP to polymerize such oligopeptide-based monomers. To this end, J.C.M. van Hest and collaborators synthetized a methacrylate-functionalized -VGPGV-OH monomer (**Fig. 8A**) that was polymerized by ATRP either using a small molecule (ethyl-2-bromoisobutyrate, EBIB) as initiator yielding an homopolymer with pendant -VPGVG-OH groups, or a bifunctional poly(ethylene glycol) as macroinitiator therefore yielding a triblock ABA copolymer (the A block being poly(MMA-*g*-VPGVG) and the B block a 1,000 g/mol PEG). A well-controlled polymerization in each case resulted in polymers with relatively narrow macromolecular dispersities (<1.25 as determined by size exclusion chromatography). Both polymers showed a thermo-responsive behavior as studied by circular dichroism spectroscopy (characteristic transition from random coil to β-turn structures), while the ABA triblock copolymer was also found to form spherical aggregates upon raising the temperature and reversibly disassembling into soluble chains by decreasing back the temperature of the solution. Regarding the high content of carboxylic groups (at the free C-terminal end of each -VPGVG-OH peptide), these aggregation properties were also found pH-dependent. In a subsequent contribution, authors further investigated the stimulus responsive character of this class of elastin side chain block copolymers for comparison with linear poly(VPGVG).[72] A series of ABA triblock was therefore synthesized with varying degree of polymerization of the VPGVG side chain block, (**Fig. 8B** and **8C**) and their $T_{cp}$s investigated at different polymer concentrations (**Fig. 8D**) and pH values. Interestingly, the effects of these parameters on the LCST-behavior of these triblock copolymers were similar to those of linear poly(VPGVG). Nevertheless, deeper analyses by dynamic light scattering measurements revealed that the triblock copolymers did not self-assemble into spherical particles as initially thought, but rather formed a network, a hypothesis supported by cryo-SEM analyses. (**Fig. 8E**) These findings were the first demonstration that the incorporation of an elastin-based pentapeptide as pendant group into a polymer is possible and that it retains its physico-chemical properties, resulting in functional synthetic polymers which behave in a similar way as the original linear polypeptide it is derived from.

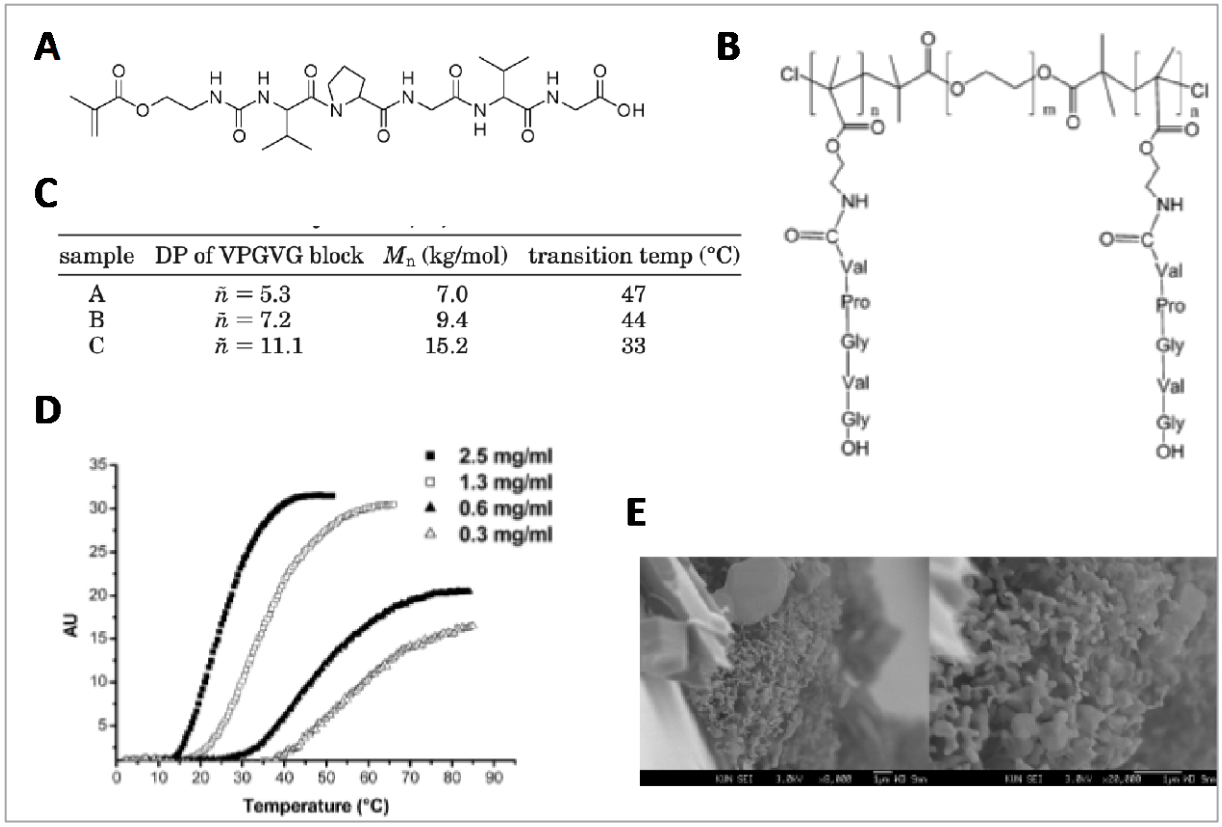



**Figure 8.** A/ Methacrylate-functionalized -VGPGV- monomer reproduced from Ayres *et al.*[71]; B/ Chemical structure and C/ Characteristics of ABA triblock copolymers (Molar mass determined by 1H NMR and $T_{cp}$ measured at pH 1); D/ Turbidity profile of triblock C at pH 1 and different concentrations. D/ Cryo-SEM images of 1mg/mL solution of triblock copolymer C, at pH 1, frozen in above its Tcp: (left image) 8,000 times enlargement; (right image) 20 000 times enlargement. Reproduced and adapted from Ayres *et al.*[72]

In 2007, N.R. Cameron and coworkers described similar elastin-based side-chain polymers from a methacrylate derivative of the -VPGVG- pentapeptide but using reversible addition-fragmentation chain transfer (RAFT) polymerization.[73] Homopolymers with higher degrees of polymerization (29-88 repeating units) and therefore larger molar mass could be obtained still with narrow molar mass distributions (dispersities 1.03-1.23). These elastin-mimicking polymers have been shown to have similar temperature-dependent behavior to their linear analogues, the cloud point temperatures decreased with increasing polymer concentration and molar mass. Not surprisingly however, a stronger dependence of the cloud point temperature on pH was observed due to higher amount of Cter carboxylic groups.

The same year, L.A. Setton in collaboration with A. Chilkoti applied the ring-opening metathesis polymerization (ROMP) technique to generate thermally responsive elastin-based oligopeptides of low molar mass and low dispersity by designing and synthesizing norbornyl elastin-based monomers.[74] The latter contained a dimeric repeat unit -(VPGVG-VPGKG)- attached to norbornene

monomers. Two oligomers with average degrees of polymerization of 5 and 12 were synthesized. Very interestingly authors observed the behavior of fibrochondrocytes for several weeks in culture slides coated with both oligomers. These cells were found to be viable, to adhere, proliferate and adopt a regular elongated and spread morphology evidencing no apparent toxicity of the poly(norbornene) backbone. A few years later, R.M. Conrad and R.H. Grubbs further explored ROMP to copolymerize norbornene-functionalized -GVPGVG-$_{OH}$ and norbornene-functionalized with oligo(ethylene glycol)$_5$ monomers.[75] Authors demonstrated the living character of the polymerization evidenced by the linear dependence of the molar mass of the copolymers on the ratio of monomer to initiator, as well as similar rates of incorporation of each of the monomers yielding statistical distribution of the two monomers in the copolymer. A small library of copolymers was synthesized with different content in elastin units and variable molar masses. The cloud point temperatures of these copolymers were found dependent on the number of elastin unit present in the copolymer and a classical dependance of the $T_{cp}$ with the molar mass of the copolymers.

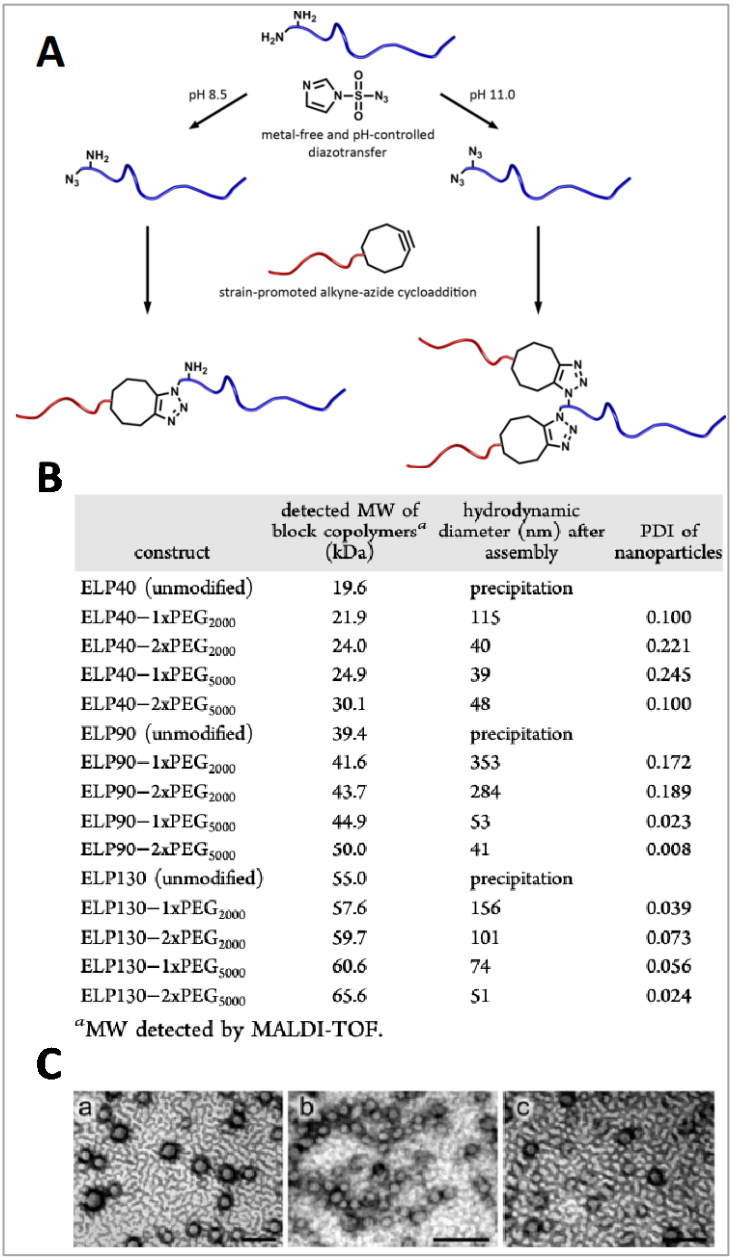
In 2013, combined efforts from the groups of A. Chilkoti and M. Schmidt successfully lead to the synthesis of cylindrical polymer brushes with relatively long ELP side chains.[76] These were obtained by radical polymerization of an ELP-based macromonomer (8.3 KDa), namely ELP[V-20] functionalized at the *N*-terminal chain end with a methacryloyl group. Interestingly, polymerization of the macromonomer was achieved in aqueous solution above its $T_{CP}$, therefore in a phase separated medium, and yielded polymers of high polymerization degree (~590, *i.e.*, of 4.9 MDa molar mass) adopting a cylindrical morphology as observed by AFM. The high density of ELP chains along the polymer backbone (and therefore the high local concentration of ELP) resulted in much lower $T_{cp}$s of the ELP cylindrical brush polymer as compared to the ELP[V-20] macromonomer, and almost no dependance of the $T_{cp}$ versus molar concentration of polymer. Very interestingly, authors observed an increase of the main chain flexibility with decreasing repulsion between the ELP side chains. The hydrodynamic size of the ELP cylindrical brush polymer was found to decrease upon increasing temperature, until a critical temperature was reached (36 °C) where aggregates of several hundred nm formed prior to phase separation.

The last class of polymer with pendant ELP groups we want to mention here are the one of dendrimers as reported by C. Kojima and his collaborators in 2013.[77,78] Dendritic structures of different generations and using different lengths of ELPs were synthesized and their thermal behavior studied. Interestingly, the

$T_{cp}$ of such star-like macromolecules was largely influenced by the peptide length but slightly influenced by the dendrimer generation.

### *6.2. Hybrid ELP-b-PEG bioconjugates*

Poly(ethylene oxide), PEO, more often called poly(ethylene glycol) (PEG) is a hydrophilic polyether commonly used for biological and biomedical applications due to its stealth and anti-fouling properties and low toxicity. With the goal of formulating stealth and stimuli-responsive nanoparticles, the group of J.C.M. van Hest designed a series of hybrid ELP-*b*-PEG copolymers.[79] These were synthesized by strain-promoted azide−alkyne cycloaddition (SPAAC) from PEG blocks of variable size (2,000 and 5,000 Da) functionalized at the chain end with a cyclooctyne group. ELPs of three different sizes were also used, namely ELP[$V_5L_2G_3$-n] with n= 40, 90, and 130. One or two azido groups were introduced by a pH-controlled diazo transfer reaction onto the *N*-terminal primary amine group and the ε-amine of a lysine residue present in the *N*-terminal domain of the ELPs. The subsequent SPAAC reaction therefore lead to either ELP-*b*-PEG diblock copolymers or to mikto-arm star polymers containing two PEG segments. (**Fig. 9A** and **9B**) Their self-assembly into micelles was achieved at room temperature by triggering the phase transition of the ELP by the addition of a high amount of sodium chloride (2.0-3.5 M). Hybrid macromolecules with the longest block (*i.e.*, PEG 5 KDa and n= 90 or 130 lead to uniform self-assemblies, while those obtained from the shorter 2 KDa PEG or from the ELP with n= 40 were larger and less well defined. (**Fig. 9B** and **9C**) Encapsulation of a hydrophobic fluorescent dye was achieved to illustrate the possibility for these micelles to serve as hydrophobic drug nanocarriers. This work was the first example of hybrid ELP-*b*-PEG copolymers exploiting the stimulus responsiveness of ELPs to trigger the formation of micelles composed of a hydrophilic and stealth PEG corona.




| construct | detected MW of block copolymers[a] (kDa) | hydrodynamic diameter (nm) after assembly | PDI of nanoparticles |
|---|---|---|---|
| ELP40 (unmodified) | 19.6 | precipitation | |
| ELP40−1xPEG$_{2000}$ | 21.9 | 115 | 0.100 |
| ELP40−2xPEG$_{2000}$ | 24.0 | 40 | 0.221 |
| ELP40−1xPEG$_{5000}$ | 24.9 | 39 | 0.245 |
| ELP40−2xPEG$_{5000}$ | 30.1 | 48 | 0.100 |
| ELP90 (unmodified) | 39.4 | precipitation | |
| ELP90−1xPEG$_{2000}$ | 41.6 | 353 | 0.172 |
| ELP90−2xPEG$_{2000}$ | 43.7 | 284 | 0.189 |
| ELP90−1xPEG$_{5000}$ | 44.9 | 53 | 0.023 |
| ELP90−2xPEG$_{5000}$ | 50.0 | 41 | 0.008 |
| ELP130 (unmodified) | 55.0 | precipitation | |
| ELP130−1xPEG$_{2000}$ | 57.6 | 156 | 0.039 |
| ELP130−2xPEG$_{2000}$ | 59.7 | 101 | 0.073 |
| ELP130−1xPEG$_{5000}$ | 60.6 | 74 | 0.056 |
| ELP130−2xPEG$_{5000}$ | 65.6 | 51 | 0.024 |

[a]MW detected by MALDI-TOF.

**Figure 9.** A/ Schematic representation of the synthesis of ELP-*b*-PEG copolymers; B/ Table summarizing the characteristics of copolymers synthesized and of their self-assemblies; C/ TEM micrographs (uranyl acetate stained) of self-assembled (a) ELP$_{130}$−1xPEG$_{5000}$, (b) ELP$_{90}$−2xPEG$_{5000}$, and (c) ELP$_{130}$− 2xPEG$_{5000}$. The scale bar indicates 200 nm. Reproduced and adapted from M.B. van Eldijk *et al.*[79]

With the goal of developing self-assembled nanoparticles for protein encapsulation and sustained delivery, Kim *et al.* also engineered hybrid protein-polymer conjugates and pegylated α-elastin derived from human adipose tissue.[80] A 5 KDa methoxy-PEG terminated with *N*-succinimidyl succinate was reacted to lysine residues of α-elastin chains. Hybrid bioconjugates self-assembled in water at 32 °C into 330 ± 33 nm spherical particles. Light scattering measurements and scanning electron micrographs tended to show that higher molar ratio in mPEG improved nanoparticles'

colloidal stability. Particles were loaded, upon simple mixing at low temperature in water and subsequent heating to physiological temperature, with a model protein such as BSA or with insulin. The release profiles evidenced a sustained release for 72 hrs and, similarly to free insulin, insulin-loaded particles induced the translocation of GLUT4 (insulin-regulated glucose transporter) in mouse skeletal muscle cells.

PEGylated-ELP nanoparticles were also explored by Z. Hua and coworkers as pH-responsive drug delivery systems.[81] To this aim, authors synthesized a hybrid PEG-*b*-ELP diblock copolymer from a 20 kDa PEG segment terminated by a maleimide group and a 30 kDa ELP[$V_1H_4$-70] containing a C-terminal cysteine residue. Histidine residues in the ELP were used to provide pH-responsive properties as well as to stabilize nanoparticles with $Zn^{2+}$ cations. Simultaneous self-assembly of the diblock copolymer and doxorubicin loading into micelle cores (around 114 nm) was achieved at physiological pH (7.4) upon addition of $Zn^{2+}$ cations. As the pH was dropped to 5.6, disassembly of nanoparticles was accompanied with drug release, consistent with doxorubicin release in the acidic conditions of lysosomes. Administration of drug-loaded particles in mice did not induce noticeable toxicity to murine liver cells, but elicited a significant tumor reduction.

With a slightly different goal, the group of S.C. Heilshorn has also incorporated PEG into a hybrid ELP-PEG hydrogel in order to increase the transparency of hydrogels solely composed of ELPs and therefore facilitate the observation of the morphology and behavior of encapsulated cells by optical imaging techniques.[82] ELP-based hydrogels are indeed attractive materials as 3D cell culture scaffolds, with tunable stiffness and allowing additional peptide sequence for specific cell adhesion to be incorporated.[83] However, coacervation or aggregates formed by ELPs in physiological media and at physiological temperature, as well as the necessary cross-linking of ELP chains induce some opacity and scattering effect that limits the imaging depth to approximately 100 μm using confocal microscopy.[48] To circumvent this pitfall, H. Wang *et al.* therefore designed an hybrid ELP-PEG hydrogel from a 3,400 g/mol homobifunctional PEG presenting primary amine at both chain ends and an ELP containing several lysine residues and subsequent cross-linking using tris(hydroxymethyl)phosphine in aqueous solution *via* a Mannich-type condensation. The resulting hydrogel presented improved light transmittance and smaller aggregates at 37 °C as evidenced by coherent anti-Stokes Raman scattering microscopy and was therefore explored to study the viability, spreading and morphology of encapsulated human fibroblasts.

Our group has also engineered hybrid ELP-*b*-PEG bioconjugates however in the completely different context of the design of synthetic artificial cells and

membrane-less compartments that result from liquid-liquid phase separation.[65] Membrane-less compartments, as first evidenced with P granules in embryo, have attracted significant attention in the past decade regarding their high content in eukaryotic cells and major roles in concentrating specific biomolecules and mediating biochemical reactions spatiotemporally. With the goal of mimicking and controlling the reversible assembly and disassembly of such architectures in space and time, our group designed an hybrid PEG-*b*-ELP diblock copolymer, using a 2 kDa PEG and a 17 kDa ELP[$M_1V_3$-40] as model of intrinsically disordered protein (IDP), this class of protein lacking ordered 3D structure being known to drive intracellular phase separation leading to liquid-like membrane-less bodies. The *C*-terminal end of the ELP was tagged with a fluorescent label (rhodamine) in order to follow the distribution of the $PEG_{2kDa}$-*b*-ELP[$M_1V_3$-40] bioconjugate within a cytoplasm mimicking system (binary phase of dextran and PEG, typically used as macromolecular crowding agents) entrapped into water-in-oil emulsion droplets obtained by a microfluidic process. (**Fig. 10A**-**C**) While the PEG-*b*-ELP bioconjugate distributed mainly and homogeneously within the PEG phase at 10 °C (below its $T_{cp}$), (**Fig. 10D**) it self-assembled into coacervate-core micelles distributing at the interface of the two distinct macromolecular crowding agents (PEG and dextran). (**Fig. 10D** and **10E**)

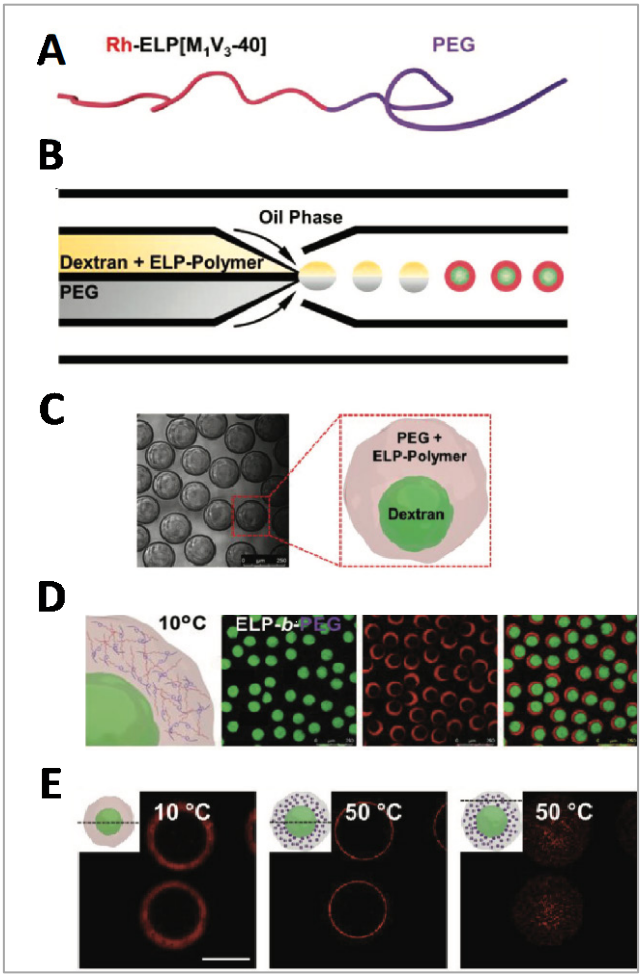



**Figure 10.** A/ Chemical structure of the rhodamine-labeled PEG-*b*-ELP diblock copolymer; B/ Illustration of the preparation of water-in-oil droplets containing the PEG-*b*-ELP diblock copolymer into the macromolecular crowding agents; C/ Optical image and schematic representation of the droplets; D/ Schematic representation and confocal images of rhodamine-labeled ELP-*b*-PEG conjugates (red)within microdroplets. The phase of fluorescein-labeled dextran shows in green. E/ Comparison of confocal images taken at 10 and 50°C. Adapted and reproduced from H. Zhao *et al.*[65]

### *6.3. ELP-polypeptides bioconjugates*

Polypeptide sequences were originally attached to ELP sequences by means of recombinant methods, namely by preparing a fusion protein to a polypeptide chain. A pioneer in this field is Eiry Kobatake who fist prepared a series of fusion proteins containing multiple repeats of $[(PGVGV)_{10}KI]_n$ (n = 4, 8, 12, or16) ELPs with polyaspartic acid chains $[D_{11}L]m$ (m = 2, 4, 8, 16].[84] Authors evidenced the formation of temperature-responsive monodisperse spherical particles with hydrodynamic diameters lower than 100 nm at 37 °C for most of the recombinant fusion products, except for the constructs with the largest ELP segment and shortest polyaspartic chain (*i.e.*, fusion protein with n = 16 and m = 2). Colloidal stability was provided by negatively charged polyaspartic

chains, and a model drug (*i.e.*, a fluorescent dye) could be encapsulated in the ELP core. In subsequent studies, a similar system was fused to the gene of the epidermal growth factor (EGF) to access EGF-receptor targeting nanoparticles,[85] or the two constructs were assembled together using coiled-coil structural motifs.[86]

While all these constructs required extensive molecular cloning steps, our group has explored a completely different approach to access hybrid ELP-polypeptide conjugates by using ELPs as macromolecular initiators for the ring opening polymerization (ROP) of *N*-carboxyanhydrides (NCA), a polymerization technique mastered in our lab. Using the primary amine group at the *N*-terminal chain end of a 17 kDa ELP[$M_1V_3$-40], Le Fer et *al.* optimized the ROP reaction of γ-benzyl-L-glutamate (γ-BLG NCA) to obtain a series of well-defined PBLG-*b*-ELP diblock copolypeptides with degrees of BLG polymerization from 25 to 180, and narrow macromolecular dispersity (below 1.04).[87] (**Fig. 11A** and **11B**) These amphiphilic hybrid synthetic/recombinant diblock copolypeptides were self-assembled in water using different self-assembly process (*i.e.*, dialysis, direct solubilization, solvent switch method) and the nanoparticles obtained were characterized by the combination of DLS and transmission electron microscopy (TEM). A variety of morphologies, namely polymersomes, inter-connected worm-like micelles and spherical micelles, were evidenced depending on the hydrophilic ratio of the diblocks as well as the self-assembly procedure. (**Fig. 11C**)

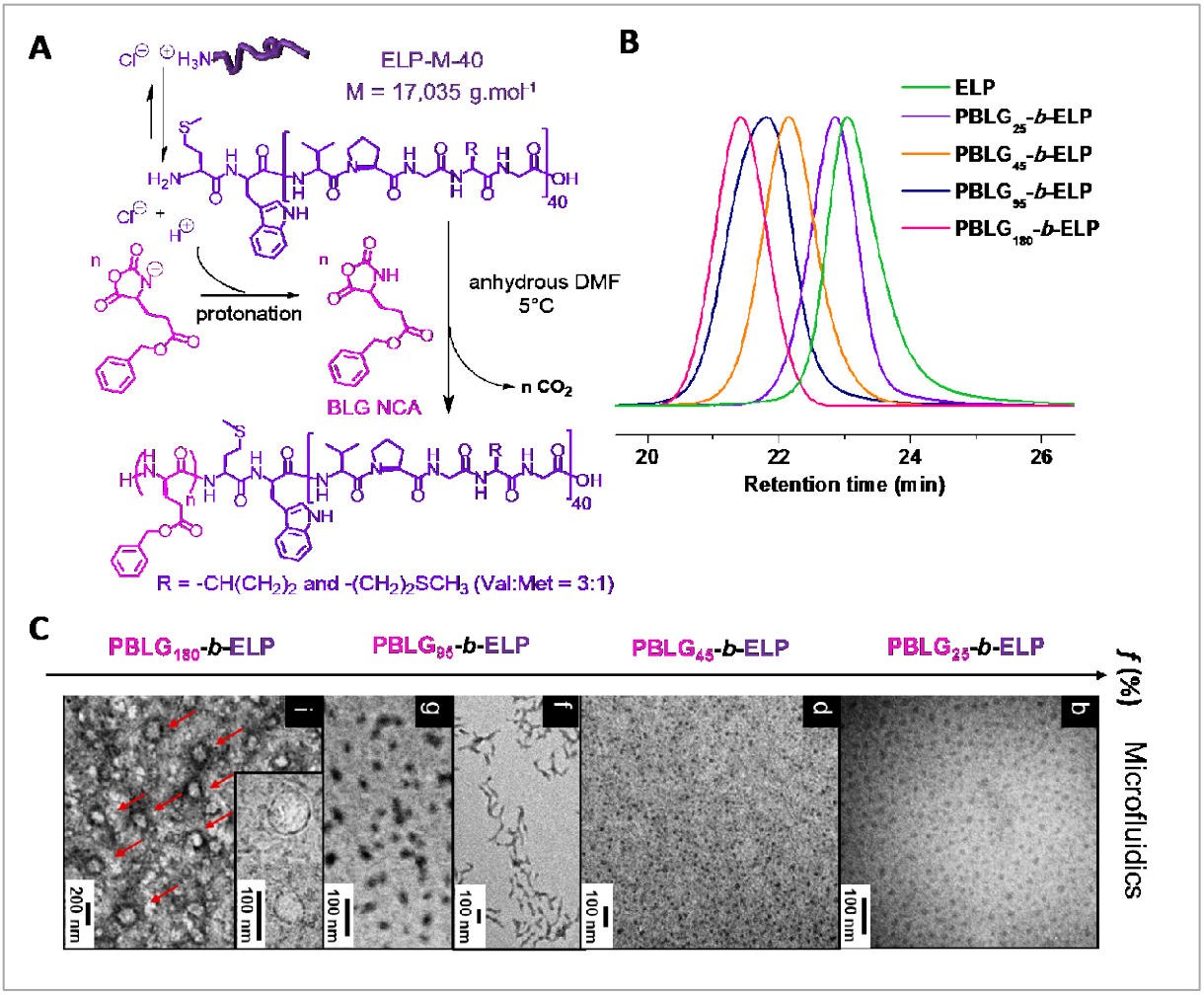



**Figure 11.** A/ Synthetic scheme of PBLG-*b*-ELP diblock copolypeptides; B/ Size exclusion chromatograms in DMF at 50 °C using a refractive index detector; C/ TEM micrographs of nano-sized objects obtained by solvent exchange using a microfluidic chip. Adapted and reproduced from G. Le Fer *et al.*[87]

In a subsequent study, the hydrophobic PBLG block was deprotected to obtain thermo-responsive double hydrophilic block copolypeptides, namely PGlu-*b*-ELP, from ELP[$M_1V_3$-*40*] and PGlu block polymerization degree from 23 to 61 (i.e., $PGlu_{23}$-*b*-ELP[$M_1V_3$-*40*], $PGlu_{45}$-*b*-ELP[$M_1V_3$-*40*] and $PGlu_{61}$-*b*-ELP[$M_1V_3$-*40*]).[88] Their temperature-triggered self-assembly in ultrapure water and in phosphate-buffered saline (PBS) was investigated at the macroscopic scale using complementary techniques such as turbidimetry, DLS and static light scattering (SLS), small-angle neutron scattering (SANS), and at the molecular scale by $^1$H NMR and circular dichroism (CD). In deionized water, PGlu-*b*-ELP copolypeptides showed one transition from free soluble chains below the $T_{CP}$ of the ELP block to macroscopic aggregates above the $T_{CP}$. Interestingly, in PBS, four successive regimes were observed upon increasing temperature: below the $T_{CP}$, copolypeptides were soluble, while above the $T_{CP}$, large aggregates appeared and fell apart into discrete and defined spherical nanoparticles at a specific temperature identified as the CMT, before reaching an equilibrium.

This phenomenon was attributed to lower content of type II β-turn secondary structures above the $T_{CP}$ as observed by CD as compared to the situation in water, yielding less cohesive aggregates able to reorganize into nanoparticles.

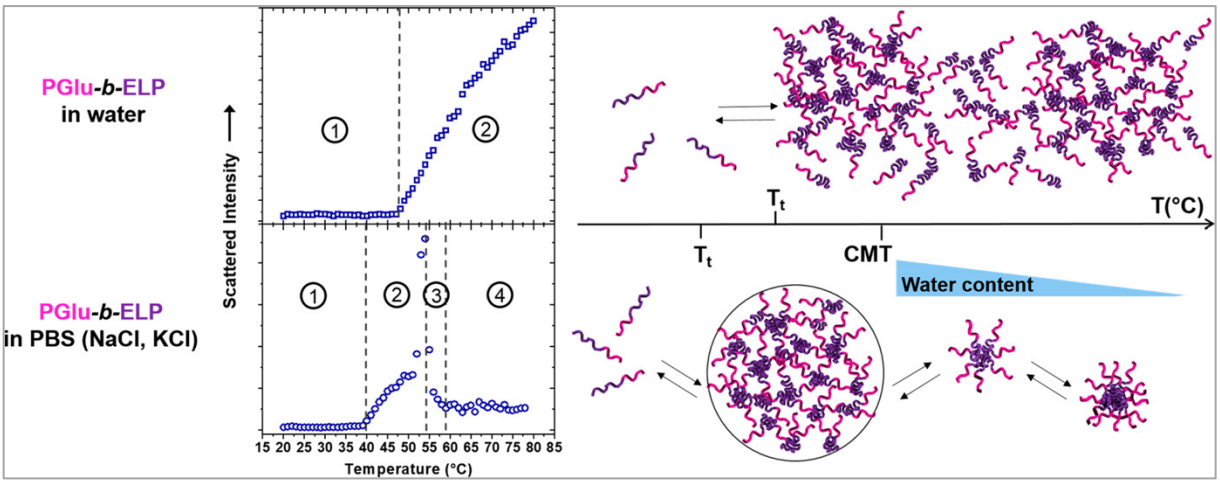



**Figure 12.** Illustration of the proposed self-assembly mechanisms of double hydrophilic block copolypeptides PGlu-*b*-ELP in water and in PBS. Reproduced form G. Le Fer *et al*.[88]

### *6.4. ELP-polysaccharides bioconjugates*

Polysaccharides are a wide class of natural polymers with different types of compositions (homo- or heteropolysaccharides), architectures (linear or ramified), structures (primary, secondary, tertiary), and biological functions (structural or mechanical roles, metabolic supply, receptor recognition, *etc*.) Amongst the wide variety of polysaccharides, hyaluronic acid (HA) has been the most often associated to ELPs. The first article mentioning this association is the work of S. Choi and J. Lee who synthesized ELP-*g*-HA conjugates through amide linkages.[89] We unfortunately cannot provide additional details on this study since published in a Korean journal. The same year, A.J.M. Yee and his collaborators described a composite material composed of a thiol-modified HA and an ELP that were combined in various ratios and cross-linked using a 3.4 kDa α,ω-diacrylate poly(ethylene glycol).[90] Authors mentioned the ELP to be "composed of five hydrophobic domains (exon 20 or 24) flanking four cross-linking domains (exon 21 and 23)", the latter containing the typical KA(A)AK motifs involved in elastin cross-linking, but no residue susceptible to react with acrylate reactive groups. The ELP, in this study, was therefore more likely added as filler in the material but not conjugated to HA cross-linked with PEG.

Therefore, the first bioconjugates involving ELPs and HA were described by the group of S.C. Heilshorn who designed an ELP-HA hydrogel by dynamic covalent chemistry through hydrazone bonds.[91] High molar mass HA (1,500-1,800 kDa) was oxidized by sodium periodate to reach 26% or 32% aldehyde groups. (**Fig. 13A**) The ELP was engineered recombinantly to comprise four repeats of a

sequence comprising a cell-binding domain (containing the- RGD- adhesion peptide) and an ELP[$I_4K_1$-15] domain. Lysine residues were reacted with Boc-protected hydrazinoacetic acid, which after acid deprotection yielded hydrazine moieties. (**Fig. 13B**) Hydrogels with comparable stiffness but variable HA contents (1.5, 3 and 5 %) were formed by simple mixing of the two polymers in phosphate buffer saline at 4 °C in various ratios. (**Fig. 13C**) These were evaluated for cartilage tissue regeneration purposes and were therefore loaded with chondrocytes which were found to express cartilage-marker genes in a dose-dependent manner with the HA content of the hydrogel. More importantly, authors evidenced increased deposition of cartilage-specific sulphated glycosaminoglycan but minimal undesirable fibrocartilage phenotype.

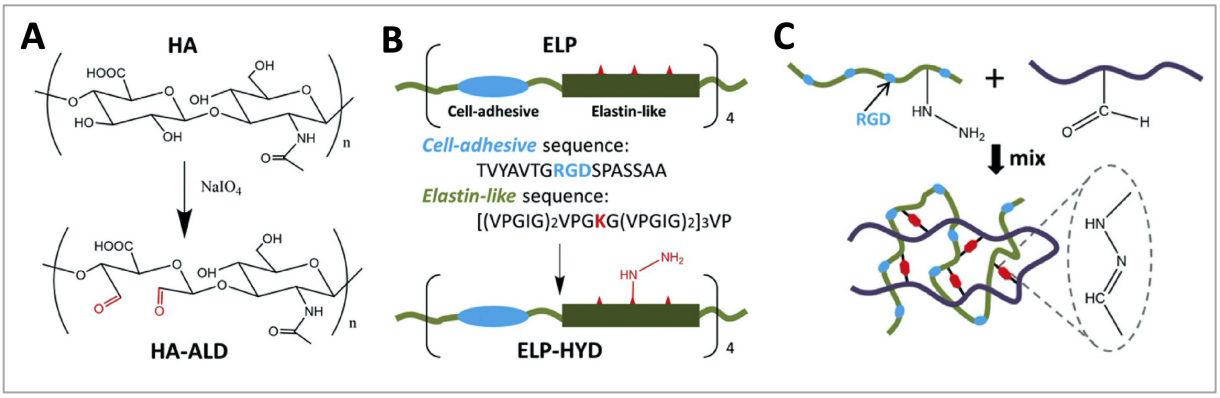



**Figure 13.** Three main steps of the ELP-HA hydrogel formation by dynamic covalent hydrazone bond formation. Adapted and reproduced from D. Zhu, H. Wang *et al.*[91]

Also in the context of tissue engineering applications and especially cartilage whose intrinsic regenerative capacity is limited, N. Annabi and coworkers fabricated hybrid ELP-HA hydrogels by photo-crosslinking a 1,600 kDa methacrylated HA with an ELP $[(VPGVG)_4(IPGVG)]_{14}$ containing a cysteine-containing tetrapeptide -KCTS- at both *N*- and *C*-terminal chain ends.[92] Zinc oxide nanoparticles were incorporated into the hydrogel to provide them with antimicrobial properties. The composite hydrogel, composed of 2 % HA, 10% ELP and 0.2% (w/v) ZnO nanoparticles, was found to indeed inhibit the growth of methicillin-resistant *Staphylococcus aureus* bacteria, but to support the growth, spreading and proliferation of human mesenchymal stem cells and NIH 3T3 fibroblast cells. No significant inflammatory response was observed upon subcutaneous implantation in healthy rats.

Also with the goal of forming hydrogels, first, second and third generations (G1, 2, and 3) of dendritic elastin-like polypeptides (constituted of -GLPGL- units) were constructed by the groups of R. Bitton and J.B. Matson.[93] (**Fig. 14A**) These were synthesized by microwave-assisted solid-phase peptide synthesis (SPPS), using lysine residues as branching units, and *N*-terminal and ε-primary amine

groups were involved in the grafting of 360 kDa HA by amide fond formation (with an average carboxylate to amine ratio of 5:1). (**Fig. 14B**) Hydrogels obtained from G2 and G3 ELPs were found to present similar mechanical properties, while the ones obtained from G1 were found rheologically weaker and had larger mesh sizes resulting in a poor absorption and rapid release of a model drug (*i.e.*, rhodamine). Interestingly, transparent hydrogels upon gelation were observed to present opaque regions after lyophilization and rehydration, a phenomenon that authors attributed to the formation of pre-coacervates in the gel-forming step as concluded from time-course SAXS studies. (**Fig. 14C** and **D**)

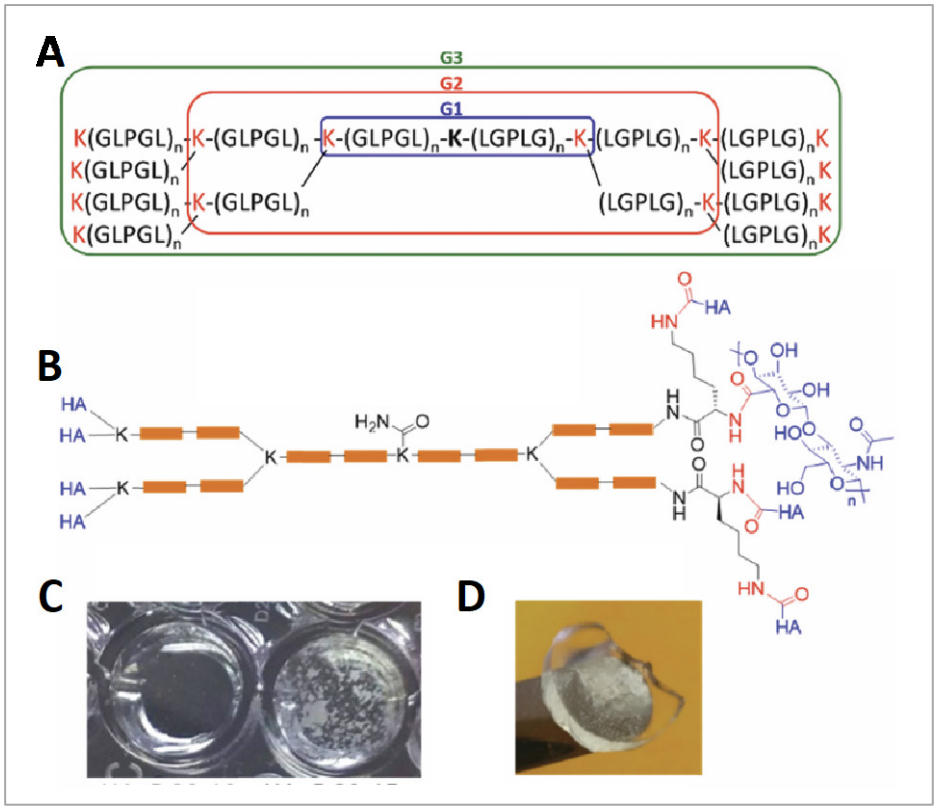



**Figure 14.** A/ General chemical structure of G1, G2, and G3 dendritic ELPs. The letter n in subscript indicates the number of -GLPGL- pentamers ; B/ Illustration of an hydrogel obtained from a G2 ELP with n = 2; C/ Hydrogels from G2 ELP with n = 2 (left) and G2 ELP with n = 3 before the feezing step; D/ Hydrogel from G2 ELP with n = 2 after lyophilization and rehydration. Adapted and reproduced from Y. Shmidov, M. Zhou *et al.*[93]

Our group has also contributed to the field of ELP-polysaccharides but not in the context of hydrogels and tissue-engineering applications, but rather with the aim of designing macromolecules featuring simultaneously self-assembly and bioactive properties to obtain intrinsically bioactive nanoparticles without the need for surface post-modification steps to introduce bioactive ligands. We therefore designed and synthesized a series of oligo- or polysaccharide-b-ELP diblock copolymer by copper-catalyzed azide-alkyne cycloaddition (CuAAC).[94] (**Fig. 15A**) A 17 kDa ELP[$M_1V_3$-*40*] was functionalized at the *N*-terminal chain end with an alkyne-containing linker and conjugated to three

different azido-terminated oligosaccharides with specific receptor binding ability, namely laminarihexaose,[95] low molar mass HA,[96,97] and dextran yielding three distinct bioconjugates that were characterized by NMR spectroscopy and size-exclusion chromatography (SEC) noted Lam-*b*-ELP, HA-*b*-ELP and Dex-*b*-ELP, respectively. (**Fig. 15B**) SEC analyses evidenced molar mass dispersities consistent with the distributions of the starting polysaccharides and elution times in agreement with the hydrodynamic volumes of the diblock copolymers. (**Fig. 15C**) Interestingly, the thermal behavior of all bioconjugates in aqueous solutions followed a similar trend that the ELP alone, namely the cloud point temperatures following a linear law with the log of their molar concentrations as described by Chilkoti and coworkers.[29] (**Fig. 15D**) Due to higher hydrophilicity, all bioconjugates presented higher $T_{cp}$ than the pristine ELP at a given concentration. In addition, conjugates with HA, that is well known for its strong water retention and hydration property, presented the highest dependance of $T_{cp}$ versus molar concentration. The thermal responsiveness of the resulting bioconjugates allowed the reversible formation of sub-micrometer-sized particles as observed by dynamic light scattering (DLS) and atomic force microscopy (AFM). (**Fig. 15E** and **F**)

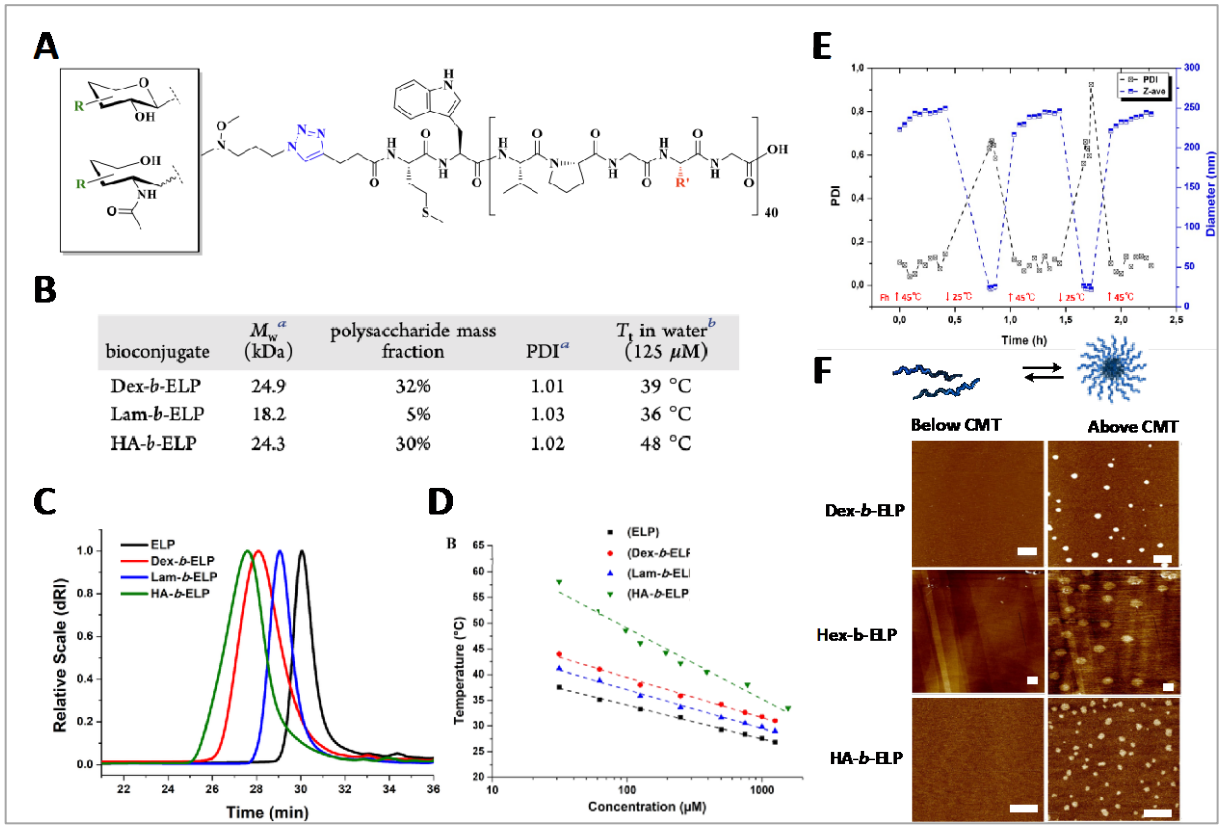



**Figure 15.** A/ General structure of the hybrid polysaccharide-*b*-ELP[$M_1V_3$-*40*] bioconjugates (R'=Val/Met 3:1); B/ Table summarizing their main characteristics; C/ SEC traces in aqueous buffer and D/ $T_{cp}$ values plotted as a function of concentration in water for the three bionconjugates as compared to the starting ELP; E/ DLS data (Z-average and polydispersity) showing the reversible self-assembly and disassembly of Dex-*b*-ELP upon repeating heating

(45°C) and cooling cycles (25°); E/ Liquid atomic force microscopy (AFM) images of the conjugates below and above the CMT. The scale bar corresponds to 1 μm. Adapted and reproduced from Y. Xioa *et al.*[94]

In a subsequent and very recent study, our group has focused on HA-*b*-ELP diblock copolymers and synthesized a series of 9 HA-*b*-ELP bioconjugates with different compositions and block lengths with the goal of establishing structure-property relationships.[98] The critical conditions for thermally-driven self-assembly upon temperature (CMT) and concentration (CMC) gradients was in particular investigated to access the phase diagrams for each individual HA-*b*-ELP bioconjugate. Understanding of these is critical to identify the composition of the best candidates for targeted biophysical and biological studies.

## 7. Future directions and conclusions

Elastin-like polypeptides, as pioneered by D.W. Urry, are synthetic thermo-responsive macromolecules defined by multiple repeat sequences of (VPGXG) pentapeptides (X ≠Pro). The first Val residue can actually be replaced for other amino acids (*e.g.*, Ile), while the Gly residue following the Pro residue can be replaced by a residue with a short side chain such as alanine. Initially synthesized by a combination of solid and solution phase peptide synthesis, polycondensation methods were rapidly considered to access polypeptides with higher degrees of polymerization and in larger amounts. Molar mass dispersity, inherent to the polymerization techniques, was however somehow a limiting factor to establish neat structure-property relationships. The use of recombinant DNA technology and protein-engineering techniques to overcome this issue, as well as the clever use of ELPs' reversible temperature-responsiveness to extract and purify them from bacterial lysates, allowing a chromatography-free purification, were real technical leverages for the field. Subsequent improvements in the cloning methods of highly repeated sequences were then a significant step forward enabling the recombinant production of ELPs in bacteria. ELPs of medium to large sizes (typically in the range of 20-200 pentapeptide repeats) and relevant $T_{cp}$ (10-60°C) can now most of the time be produced in very decent yields at the laboratory scale (100-500 mg/L culture in 5 L culture flasks), while upscaling of the production in 50-100 L fermenters is commonly achieved in biotech companies. These progresses undeniably permit these recombinant polypeptides to be nowadays considered realistically for the development of (bio)materials.

ELPs have a dual character: engineered at the gene level and produced recombinantly, they are monodisperse protein macromolecules, with a perfectly defined monomer sequence and chain length, but can also be

considered as polymers from their physico-chemical behavior in aqueous media, present as intrinsically disordered polymer chains in θ-solvent below their $T_{cp}$ and forming coacervates above their $T_{cp}$. As such, ELPs are therefore inert thermo-responsive polymers (*i.e.*, they have no intrinsic bioactivity) and are used as relevant models in (bio)physics. Actually, in 2009, J.L. Rodriguez-Cabello, an internationally recognized expert on the design and use of ELPs for biomedical devices, had termed them as "recombinamers" implying this dual behavior.[99]

Considerable design efforts in the last two decades have been devoted to confer specific bioactivity to ELPs for different purposes and target applications (mainly drug delivery and tissue engineering):

- recombinant methods have been explored to create bioactive fusions proteins;
- synthetic post-modifications at the chain end of ELPs have been used to access linear, sometimes amphiphilic, ELP-bioconjugates;
- introduction of natural or non-natural amino acids in the ELP sequences have been employed to permit subsequent chemoselective reactions, orthogonally to the other residues' side chain and *C*- and *N*-terminal end's groups;

and all these works constitute a significant body of literature.

The combination of ELPs with synthetic polymers or use of ELPs in polymerization reactions and processes have however been slower to evolve. First examples evidenced that ELPs can efficiently be incorporated in monomers for controlled radical polymerization (*e.g.*, ATRP, RAFT) for living chain-growth reaction such as Ring Opening Metathesis Polymerization. In few occasions, ELPs were used as macroinitiators for ring opening polymerization (ROP), especially of *N*-carboxyanhydrides (NCA) and one can easily envision more examples to come in the coming years with the use of other classes of monomers. One can also imagine that ELPs could be involved in emulsion polymerization processes, including PISA mechanisms. In a few more examples, ELPs have been covalently linked *via* bioconjugation reactions to synthetic polymers (*e.g.*, PEG, PLGA) or natural polymers, mainly polysaccharides and especially hyaluronic acid. Regarding the wide diversity of synthetic and natural polymers, one can anticipate the class of ELP-polymer conjugates to significantly enlarge in the next decade to access biomaterials with relevant mechanical properties and bioactivity especially for the field of regenerative medicine. More broadly, such approaches at the cross-roads of bioengineering and polymer science shall open new avenues in the near future.

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