Developing functionality in elastin-like polymers by increasing their molecular complexity: the power of the genetic engineering approach

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Abstract

In spite of the enormous possibilities presented by macromolecules for the development of advanced materials with increased functionality, the achievement of functionality is often limited by the randomness associated with polymer synthesis and the exponential increase in technical difficulties encountered in attaining a desired degree of complexity in the molecular design. This paper describes an increasingly important approach to the design of complex and highly functional macromolecules, i.e. the genetic engineering of protein-based macromolecules. The exploitation of the efficient machinery of protein synthesis in living cells opens a route to precisely defined and complex macromolecules.

A series of molecular designs with increasing complexity are presented to show how this controlled increase yields materials with increasingly selective and sophisticated multifunctionality. The simplest designs already show interesting mechanical properties, but the adequate introduction of given chemical functions along the polymer chain provides an opportunity to expand the range of properties to smart behavior and self-assembly. Finally, examples are given where the molecular designs further incorporate selected bioactivities in order to develop materials for the most cutting-edge applications in f biomedicine and nanobiotechnology.

Keywords: Protein-based polymers, Genetic engineering, Smart polymers; Self-assembly, Nanobiotechnology, Tissue engineering, Drug delivery

Contents

1. Introduction

The extraordinary properties of biological materials have astonished several generations of materials scientists, since their synthetic materials fell far short of such extraordinary performance. Of course, this functionality is not restricted to more or less conventional mechanical properties. It also includes the smart nature of biological molecules, which sense their environment with extreme sensitivity and react in a precise manner (antibodies, cell surface receptors—including sensory receptors, membrane channels, enzymes with controlled activity, etc.), the highly efficient catalytic capability of enzymes, the extraordinary functioning of molecular machines (flagellar rotary motor, etc.), the self-assembling capabilities of enzymatic complexes and other proteins, such as those forming virus capsids, etc. Even more, in some cases, in an overwhelming example of the competence displayed by natural biomolecules, we can find all of these properties combined in one system of exquisite functioning. This is the case of the F_1 motor of ATP synthase, a system at the core of the metabolism of eukaryotic cells since it produces ATP, the 'metabolic coin'. This complex makes use of smart, self-assembling, and enzymatic capabilities while being an astonishing, highly efficient molecular stepper motor [1–5].

However, only very recently in the development of materials science, has biology started to make a decisive input in addition to the more classical contributions from chemistry and physics. Although material science has been learning from 'Mother Nature' for a long time, only recently is this knowledge starting to be widely applied to the design of advanced materials.

Nature usually makes use of large and very complex molecules containing diverse specific functional groups to generate and guide self-assembly and functionality. Nature makes use also of various physical processes that allow directed and controlled organization from the molecular to the macroscopic level. As a whole, both local organization through functional chemical groups and the physical properties giving rise to order up to the highest scales provide the properties and functions that biological systems require for their efficient functioning.

Natural biopolymers illustrate, as an impressive example, how all the properties displayed by biological materials and systems are exclusively determined by the physical–chemical properties of the monomers and their sequence. A well-defined molecular structure can lead to a rich complexity of structure and function on the mesoscale. Here, competing interactions, structural flexibility and functional properties are tailored by the succession of monomeric units taken from a rather limited set. Molecular organization on the mesoscale results in the formation of chemically or topographically structured interfaces and introduces new surface and bulk properties. Because macromolecules bridge the span of nanometers up to micrometers by virtue of their length and flexibility, they enable a unique control of hierarchical organization and long-range interactions. The growing ability to design higher structural organization in synthetic polymer materials provides the basis for a powerful new technology, which opens up new routes towards development of functional nanostructures and advanced materials with

a sophisticated control. In addition to chemical differences of the units forming the macromolecules, physical processes also govern structure formation of the molecules in various ways.

However, here resides also the main problem in our attempt to mimic nature to create materials with a high degree of complexity and functionality. 'Classical' methods of chemical synthesis are still not robust enough to provide control of the primary chemical composition and monomer sequence needed to achieve extraordinary functional macromolecular designs. Even in the simplest non-natural polymers, polymerization reactions usually result in a high degree of randomness. Generally speaking, polymerization products show, among other imperfections, molecular weight (M_W) polydispersity and randomness in the co-monomer disposition, which unavoidably frustrate our purpose of obtaining 'extremely functional' macromolecules. However, this task is routinely accomplished with exceptional high yield and efficiency by the cellular systems of protein biosynthesis. Protein biosynthesis is implemented with an absolute control of the amino-acid sequence, from the first amino acid to the last, with complete absence of randomness. Additionally, the protein biosynthesis machinery is able to process and produce any amino-acid sequence stored in the elements of information called genes, so its flexibility is absolute. If one controls the information that genes deliver into the machinery, one completely controls the biosynthesis process itself.

2. Genetic engineering of protein-based materials: the 'Gutenberg idea' in polymer design and production

Owing to the current developments in molecular biology, we have for the first time the ability to create almost any DNA duplex coding any amino-acid sequence at will. We also have the chance to introduce this synthetic gene in the genetic content of a microorganism, plant or other organisms and induce the production of its encoded protein-based polymer as a recombinant protein [6–14]. Therefore, as we now have all the required technology, the use of genetically modified cells as cellular factories to produce sophisticated polymers is extremely tempting. Many advantages can come from this approach.

First, genetically engineered protein-based polymers (GEPBPs) will, in principle, be able to show simple or complex properties present in natural proteins. In this sense, this method offers an opportunity to exploit the huge resources, in terms of functionality, hoarded and refined to the extreme by biology during the long process of natural selection. GEPBPs can easily make use of the vast amount of functional wealth present in the hundreds of thousands of different proteins existing in living organisms in the amplest sense, from the smallest prions or viruses to higher animals.

On the other hand, as we can construct coding gene, base by base, by following our own original designs and without being restricted to gene fragments found in living organisms, we can design and produce GEPBPs to obtain materials, systems and devices exhibiting functions of particular technological interest that are not displayed in living organisms.

Third, from the point of view of a polymer chemist, the degree of control and complexity attained by genetic engineering is clearly superior to that achieved by more conventional synthesis technologies. GEPBPs are characterized as being strictly monodisperse and can be obtained from a few hundred daltons to more than 200 kDa; and this upper limit is continuously increasing [15]. Among other things, this has provided the opportunity to study, in a simple and highly precise manner, the dependence of different material properties on the M_W [16,17] knowledge that opens possibility of fine tuning those properties in designer materials.

For conventional polymers, the increase in their complexity unavoidably means an exponential increase in time and cost of production. However, this rule is not fulfilled by GEPBPs. Paradoxically, experience constantly shows to those research groups obtaining GEPBPs that the molecular biology techniques used for the construction of the synthetic gene work better if the designed GEPBP is complex, at least to a certain extent. Furthermore, the repetitiveness of the sequence can cause gene deletions and recombination, and may also place an unbalanced demand on the aminoacyl-tRNA pool, which can make yields quite low. In all cases, all these biological systems are adapted to build complex

natural proteins, and they 'feel more comfortable' in an environment of complexity. Therefore, for GEPBPs, there is no a clear and direct relationship between time-money consumption and polymer complexity. In practice, usually complexity is more feasible than simplicity.

Fourth, the number of different combinations attainable by combining the twenty natural amino acids is practically infinite. In a simple calculation, if we consider how many different combinations are possible to obtain a small protein consisting of, for example, one hundred amino acids (their modest M_W would range in between 5.7 and 18.6 kDa), the figure is as high as 1.3×10^{130}. Even if we consider that in a living cell, a protein were biosynthesized at a rate as fast as 40 amino acids per second [18], the sequential biosynthesis of such number of different proteins would need 1×10^{123} years. If we compare this time with the estimated age of the universe, 12.1 Gyears [19], the needed time would be 113 orders of magnitude higher. In fact, we would need more than 1.2×10^{110} g of matter to produce just a single molecule of each different combination, but the observable universe is believed to contain 'only' around 3×10^{55} g of dark and luminous matter [20].

Fifth, the production cost of GEPBPs is not related to their complexity. The most consuming task in terms of time and money is the gene construction. However, once the genetically modified (micro)organism is obtained, the fast and cheap GEPBP production readily compensates for the costs associated with the molecular biology steps. In addition, conventional polymers, where the raw materials are the monomers, the raw materials employed in GEPBPs biosynthesis are not the amino acids themselves. We should not forget that the protein synthesis in a living cell occurs within a complex metabolic network, whereby many simple (and cheap) sources of carbon and nitrogen can be finally converted into the needed amino acids.

Somehow, this situation calls to mind the time when Johannes Gutenberg began building his press (in 1436). Rather than writing books one by one, Gutenberg found that the time spent in building the movable type and the press, even to print high quality and complex texts, was rapidly compensated by the reduced time in printing many identical copies. Therefore, Perhaps we are now in a situation to apply this concept to polymer production. If we want to obtain several identical batches of a complex polymer, we should direct our main effort not to building the polymer itself, but rather the gene that codes it. Then, the polymer can be produced by expressing the gene in a cellular 'factory' and thus these cells play the role of the press in book printing. At the time, Gutenberg was unlikely to have realized that he was giving birth to an art form that would take center stage in the social and industrial revolutions that followed. Perhaps, in the future the same will be said of GEPBPs.

Although this list does not intend to be exhaustive, the final advantage mentioned here relates to environmental considerations. GEPBPs are made from biomass and their production involves only renewable biomass and environmentally clean processes from raw materials to waste. In addition, no petroleum-based chemicals are used. GEPBPs are, evidently, biodegradable; and water is used exclusively as the solvent in most of the GEPBPs produced up to date. GEPBPS are obtained by an easily scalable technology, fermentation, which uses moderate amounts of energy at moderate temperatures.

There are, however, also some disadvantages that must be considered. Apart from the obvious ones that arise from the status of early infancy of the new field of GEPBP production, perhaps the first that might come to mind is that conventional polymer science has already produced thousands of different useful monomers. Therefore, the possibilities afforded by this large number of petroleum-based monomers, in terms of functionality, seems to be overwhelming if we assume that, in designing GEPBPs, we are restricted to just the 20 natural amino acids. However, perhaps this disadvantage looks worse that it really is if we pay attention to nature's lesson once more. It is unquestionable that no synthetic material matches the exquisite, very special functionality of enzymes or biological molecular machines, but let us set aside sophistication for now and restrict our comparison to simple mechanical properties.

We find in biology extraordinary proteins that show surprising mechanical properties. Indeed, we can find proteins that match and clearly outperform the mechanical properties of our best in both conventional and engineering petroleum-based polymers. For example, some spider silks, such as the *Nephila clavipes* dragline, exhibit superior strength

Table 1
Mechanical properties on some natural and artificial fibers

Fiber	Elongation at break (%)	Tensile strength (MPa)	Young's modulus (GPa)	Stress at break (GPa)	Absorbed energy at break (J/Kg)
N. Clavipes Dragline	10	4×10^3	60	2.9	1.2×10^5
B. mori Fibroin	15–35	650	5	0.6	7×10^4
Other silks	12–50	1×10^3	2–4	0.1–0.6	$3–6\times10^4$
Nylon	18–26	70	3	0.5	8×10^4
Cotton	5–7	4	6–11	0.3–0.7	$5–15\times10^3$
Kevlar	4	4×10^3	100	4	3×10^4
Steel	8	690	200	2	2×10^3

Data adapted from Refs. [21–24].

(see Table 1) [21–24]. *N. clavipes* dragline silk shows Young's modulus, tensile strength and stress at break of the same order as in Kevlar, which is itself a benchmark of modern polymer fiber technology; but it absorbs almost one order of magnitude more energy than Kevlar when breaking. In fact, the mechanical properties can be considered superior to those of steel. Its absorbed energy at break is almost two orders of magnitude higher while its tensile strength is almost six times higher, and the stresses at break are equivalent. Additionally, although the Young's modulus of steel is about three times higher than in spider-silk modulus, the latter material has a much lower density. Its ratio of tensile strength to density is perhaps five times better than that of steel. Therefore, per equal mass, the spider silk behaves much better than steel. In conclusion, spider-silk fibers are nearly as strong as several of the current synthetic fibers and can outperform them in many applications in which total energy absorption is important.

A final remark deserves to be made about spider silks. Again, they demonstrate that nature seems never to renounce sophistication, as if sophistication were an intrinsic part of the essence of natural materials. This is so even in apparently simple materials that nature has designed just to achieve a given mechanical performance. For example, spider silks show a highly efficient self-healing behavior that is now under intense scrutiny due to its evident technological potential [25].

Dragline spider silks are not the only impressive example. Within the elastic protein fibers, nature shows us examples covering a wide range of elastomeric properties. Again, we can find other kinds of spider silks, such as the flagelliform silks, that are capable of very high elastic strain; they can be extended up to ca. 200% without breaking, and show a high rate of energy dissipation [21,26]. This is well known in the case of flying insects that collide with a spider web, which, in spite of their kinetic energy, are very rarely able to break through the web: i.e. the impact energy is absorbed without catapulting the insect out of the web [21,26] and, once trapped, it finds that breaking the web is an exhausting task. On the contrary, other elastic protein show precisely the opposite property; i.e. they dissipate a negligible amount of work in a stress–strain cycle or, equivalently, they show a resilience value near 100% (100% of the elastic energy stored in the deformed sample is restored when released). This is so for resilin, the main elastic protein of jumping insects [27,28] and the abducting muscle of swimming bivalves. Also elastin has been claimed to show almost ideal elasticity [29]. All these elastic proteins are characterized by high resilience, large strains and low stiffness [27]. The nearly ideal elasticity of some proteins or some of their functional domains has been identified recently as being central part of a universal foundation of protein function: the coupled hydrophobic and elastic consilient mechanisms. This has been nicely described by Urry [30] who has made a profound study of the Gibbs free energy of hydrophobic hydration and the coupled hydrophobic and elastic consilient mechanisms in specially designed protein-based polymers. This mechanism has been postulated as the universal principle of functioning of the biological protein-based machines and has been identified with the biological 'vital force' (*élan vital*). A model for protein function based on this mechanism has been already postulated for key

molecular machines of the cell, such complex III in the mitochondrial electron transport chain that produces a proton gradient, the F1 motor of ATP synthase that uses the proton gradient to produce ATP, and the myosin II motor of muscle contraction that uses ATP to generate motion [30,31].

The list of proteins with superior mechanical performance also includes keratin. This protein shows a superior impact resistance with a Young's modulus of 2.50 GPa [21] and, appropriately, it is the main component of hooves, beaks and horns. Again, this protein shows sophisticated multifunctional character, as it is also the main component of feathers, prodigies of rigidity and lightness.

Although this list could be extended on and on with many other fascinating examples, collagen among others, just one more example will be mentioned here: mussel adhesive proteins. These are remarkable materials that display an extraordinary capability to adhere to almost any kind of natural or artificial substrates and, in addition, they do so under extreme conditions; i.e. underwater (in salt water) and in a continuously changing stresses (waves, tides, underwater flows, etc.). No one artificial adhesive is able to work, even minimally, under these circumstances. It is important to emphasize that this kind of environment is not far different from that within living tissues. For that reason, recent investigators from groups coming from quite diverse areas of expertise have made substantial progress in the identification of the genes and proteins that are involved in adhesive formation. These discoveries have led to the development of recombinant proteins and synthetic polypeptides that are able to reproduce the properties of mussel adhesives for applications in medicine and biotechnology [27].

In summary, the above examples show that a reduced set of 20 amino acids as the exclusive primary source to build polymers could well be enough to design materials with extraordinary properties, even in the least sophisticated sense of bulk materials. Therefore, the properties of GEBPs span a broad range in all directions, going from the simplest mechanical properties to the most sophisticated, smart and self-assembling characteristics. Many properties displayed by petroleum-based polymers lie within this range. Hence, from the technological point of view, the chance of obtaining different materials with properties spanning a very wide range and obtained by a single common basic technology, fermentation, which in addition shows clear environmental advantages, is a highly interesting scenario.

A final disadvantage that must be mentioned is production cost as the cost of GEPBP production is above that for petroleum-based polymers. This may be acceptable for commercial exploitation of, for example, sophisticated biomedical materials, but it is of extreme importance if we think in the production of commodity plastics or other mass consumption polymers. Nevertheless, significant efforts are being made to reduce the production cost, with efforts made to produce GEPBP in genetically engineered plants or certain yeasts examples.

3. Elastin-like polymers: a privileged family of GEPBPs

Presently, genetic engineering of PBPs has not reached its maturity. Since the methodology utilized to produce these polymers is new and radically different only a few of research groups and companies have as yet invested serious efforts in this new field. Among pioneer groups, interests have been mainly concentrated in two major polymer families: spider silk-like polymers and elastin-like polymers (ELPs). The first group is of evident interest in mimicking the extraordinary mechanical properties of spider silk fibers. However, the ELP family has also shown a more versatile and ample range of interesting properties that go well beyond mechanical performance. Certainly, the ELP family exhibit a set of properties that places them in an excellent position towards design of advanced polymers for many different applications, including the most cutting-edge biomedical uses, for which ELPs are particularly well suited, as discussed below. In addition, the deepening understanding of their function in terms of their molecular composition and behavior is shedding light on one of the most interesting basic problems in modern science, the understanding of protein folding and function in living organisms.

The basic structure of ELPs is a repeating sequence originating in the repeating sequences found in the mammalian elastic protein, elastin. Some of their main characteristics of these ELPs are derived from

the natural protein they are based on. For example, the cross-linked matrices of these polymers retain most of the striking mechanical properties of elastin [32], i.e. almost ideal elasticity with Young's modulus, elongation at break, etc. in the range of the natural elastin and outstanding resistance to fatigue [33,34].

This mechanical performance is accompanied by extraordinary biocompatibility, although, their most striking properties are perhaps their acute smart and self-assembling nature. These properties are based on a molecular transition of the polymer chain in the presence of water. This transition, the 'inverse temperature transition' (ITT), has become the key issue in the development of new peptide-based polymers as molecular machines and materials. The understanding of the macroscopic properties of these materials in terms of molecular processes taking place around the ITT has established a basis for their functional and rational design [35].

Although, in general terms, the phenomenology shown by these ELPs resembles that found in amphiphilic LCST polymers, such as PNIPAM, the presence of an ordered state in ELPs above the transition temperature, not present in the LCST polymers, has prevented the use of LCST as a descriptive term for the ITT of ELPs [36].

All these aspects of the ELP family are presented below in the context of the present state-of-the-art and some possible future outcomes.

4. Smart and self-assembling properties of ELPs

The most numerous family within ELPs is based on the pentapeptide VPGVG (or its permutations), with amino-acid side chains comprising simple aliphatic chains, without further functionalization. This polymer shows a strong thermo-responsive behavior, associated with the existence of the ITT. A wide variety of ELPs have been (bio)synthesized with the general formula (VPGXG), where X represents any natural or modified amino acid, except proline [37–39]. All the polymers with this general formula that can be found in the literature are functional; i.e. all show a sharp smart behavior. However, the achievement of functional ELPs by the substitution of any of the other amino acids in the pentamer is not so straightforward. For example, the first glycine cannot be substituted by any natural amino acid other than L-alanine [39].

All the functional ELPs exhibit this reversible phase transition in response to changes in temperature [39]. In aqueous solution below a certain transition temperature T_t, the free polymer chains remain disordered, random coils in solution [40] that are fully hydrated, mainly by hydrophobic hydration. This hydration is characterized by ordered clathrate-like water structures surrounding the apolar moieties of the polymer [41–43]: structures somewhat similar to that described for crystalline gas hydrates [43,44], although more heterogeneous, and of varying perfection and stability [42]. However, above T_t, the chain folds hydrophobically and assembles to form a phase-separated state 63% water and 37% polymer by weight [45], in which the polymer chains adopt a dynamic, regular, non-random structure, called a β-spiral, involving type II β-turns as the main secondary feature, and stabilized by intraspiral interturn and interspiral hydrophobic contacts [39]. This is the result of the ITT. In this folded and associated state, the chain loses essentially all of the ordered water structures of hydrophobic hydration [41]. During the initial stages of polymer dehydration, hydrophobic association of β-spirals takes on fibrillar form. This process starts from the formation of filaments composed of three-stranded dynamic polypeptide β-spirals, that grow to several hundred nm before settling into a visible phase separated state [39,46]. This folding is completely reversible on again lowering the sample temperature below T_t [39].

4.1. Basic molecular designs: thermal response

Poly(VPGVG) (or its permutations) may be considered among the simplest ELPs. The amino acids, excluding glycine, have aliphatic side chains without further functionalization. This gives rise to the straightforward thermal response shown in Fig. 1. As mentioned above, the transition can be easily followed either by turbidity measurements or by calorimetric methods measuring the heat flow during the transition. The first method is characterized by a turbidity profile showing a sharp step. T_t is taken as the temperature at 50% change in the relative

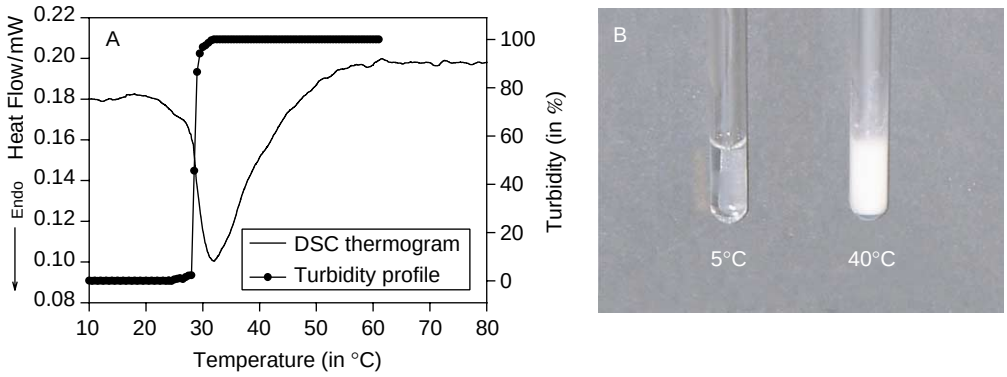

Fig. 1. (A) Turbidity profile as a function of temperature for a poly(VPGVG) 5 mg/L sample dissolved in water and DSC thermogram of a 50 mg/L aqueous solution of the same polymer (heating rate 5 °C/min). (B) Photographs of aqueous solution (5 mg/mL) of this poly(VPGVG) below (5 °C) and above (40 °C) its T_t.

turbidity. On the contrary, DSC measurements are always characterized by a broad peak, extending over 20 °C or more. In this case, T_t can be taken as either the onset or the peak temperature. Usually, T_t values obtained by these methods differ. Several factors may cause such differences. First, there is the dynamic nature of DSC and its associated thermal lags, which of course increase with higher heating rates. However, thermal lags can be eliminated by using different heating (or cooling) rates and extrapolating T_t values to zero heating rate [47]. Fig. 1(A) clearly shows the influence of this parameter; the peak temperature of the DSC obtained with a 10 °C/min heating rate is several degrees higher than the T_t. from turbidity. Other factor that can cause T_t differences between the two techniques is the different polymer concentration. It is well known that polymer folding is a cooperative process that is facilitated by the presence of other polymer chains and, accordingly, T_t can be several degrees higher for low concentrations [39]. There is a strong dependence of T_t on concentration in the range of 0.01 to 5–10 mg/mL. Above 5–10 mg/mL T_t does not show further significant change with increasing concentrations up to a limit of 150–200 mg/mL. Above this value, we find partly hydrated polymer chains and, due to the heterogeneity of the hydrophobic hydration structures, in water-deficient states, only the strongest structures are formed, which leads to a further increase in T_t as the polymer concentration increases [42]. Typical concentrations for turbidity experiments are in the range of 2–5 mg/mL while, for DSC, they usually are in the range of 50–150 mg/mL. Hence further differences in T_t due to concentration effects may be possible.

In addition, T_t also depends on the M_W: T_t decreasing as M_W increases [16,33]. Furthermore, the presence of ions, such us those of the buffer, and dissolved molecules can also affect the T_t value. All these factors make the comparison of T_t values among, not only different techniques but also different authors, a somewhat problematic matter.

The endothermic peak found in a DSC heating run is in fact the net result of a complex process reflecting different thermal contributions. Once a poly (VPGVG) solution reaches its T_t, there is first a destruction of the ordered hydrophobic hydration structures surrounding the polymer chain. This is further accompanied by an ordering of the polymer chain into the β-spiral structure. In their turn, these β-spirals further establish interchain hydrophobic contacts (van der Waals cohesive interactions) that cause the formation of nano- and micro-aggregates that separate from the solution. The first process must be endothermic while the second one must be exothermic. Although the events take place simultaneously, they are very different in nature. In particular, it is reasonable to expect that the two phenomena occur with different kinetics. In effect, previous kinetic studies made on poly(VPGVG) showed that the phase separation process is faster than re-dissolution [48]. This difference offers an opportunity to separate the different contributions to

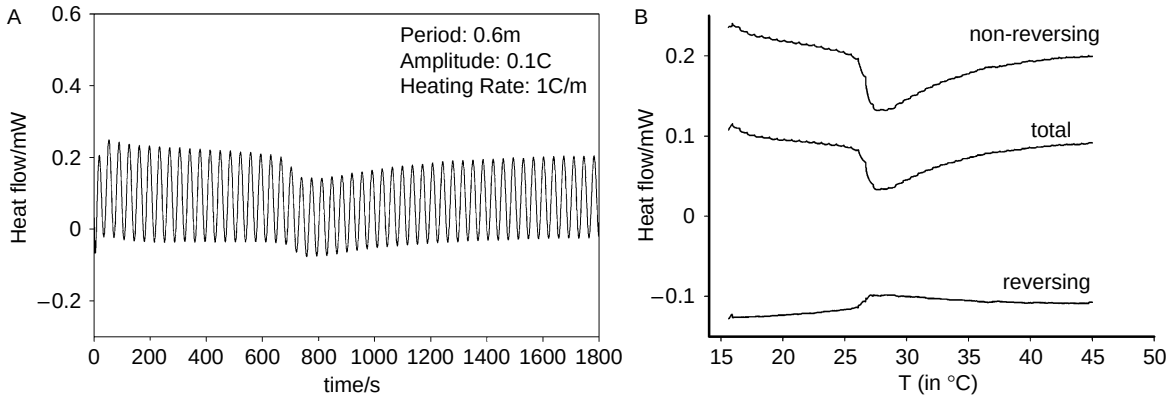

Fig. 2. (A) Heat flow vs time in TMDSC analysis of a 125 mg/mL aqueous solution of $(VPGVG)_{251}$. (B) Reversing, non-reversing and total thermograms. Reproduced with permission from Chem. Phys. Lett. 2004, 36, 8470–8476.

the ITT. This has been recently achieved for the first time using temperature modulated DSC (TMDSC) [49]. TMDSC is an improved DSC measurement that is able to separate thermally overlapping phenomena with different time dependences by using a heating program containing an alternating function of the temperature, such as a sine wave, superimposed on the constant heating rate ν [50–54]. In principle, TMDSC will provide a clear split of two overlapping processes when, under the particular dynamical conditions, one is reversible and the other is not. The processes can be split by finding a frequency for the periodic component low enough for the faster process to follow the oscillating temperature changes ('reversing') but high enough to impede alternating behavior of the slower ('non-reversing') one. This approach has been used to study the ITT of three ELPs: chemically synthesized poly(VPGVG), recombinant $(VPGVG)_{251}$ and recombinant $(IPGVG)_{320}$ [49]. Fig. 2(A) shows an example of the TMDSC thermogram found for $(VPGVG)_{251}$ while Fig. 2(B) shows the results of its analysis. Under the experimental conditions, the endothermic total curve ($\Delta H_{Tot} = -10.40$ J g^{-1}, $T_t = 27.72$ °C) is composed of a non-reversing endothermic component ($\Delta H_{non\text{-}rev} = -13.98$ J g^{-1}, $T_t = 27.63$ °C) and a reversing exotherm ($\Delta H_{rev} = 3.33$ J g^{-1}, $T_t = 27.30$ °C).

A detailed analysis has been carried out on reversing and non-reversing components as functions of ν and of amplitude A and period P of the alternating component. For the total contribution, the changes in ν (0.5–1.5 °C/min), A (0.1–1 °C) and P (0.1–1.0 min) did not significantly affect the enthalpy and T_t values, which are similar to those obtained by DSC. Also the reversing and non-reversing components were not affected by changes in ν and A (results not shown). However, P exerts a strong influence on the enthalpy values of both components.

ΔH_{rev} has been plotted in Fig. 3 as a function of P for the three polymers. In all cases, at low frequencies (high P), the reversing component shows an endothermic peak with enthalpy comparable to that of the endothermic peak of the non-reversing component. Thus, at these high P, values, the chain folding and dehydration contributions are not well

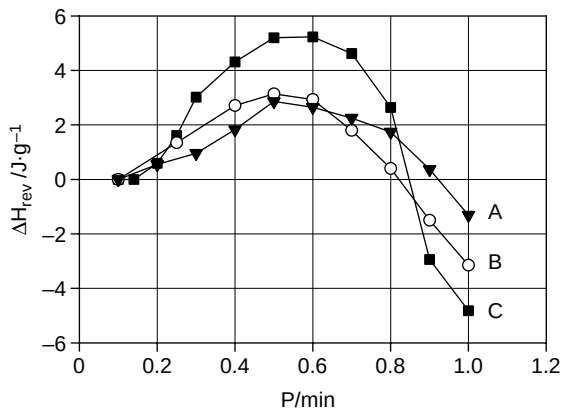

Fig. 3. ΔH_{rev} as a function of P for 125 mg/mL aqueous solution of (A) synthesized poly(VPGVG); (B) recombinant $(VPGVG)_{251}$; and (C) recombinant $(IPGVG)_{320}$ ($\nu = 1$ °C/min, and $A = 0.1$ °C). Reproduced with permission from Chem. Phys. Lett. 2004, 36, 8470–8476.

Table 2
Enthalpy values of the reversing, non-reversing and total components found at P_M

Polymer	ΔH_{rev} (J g^{-1})	$\Delta H_{non\text{-}rev}$ (J g^{-1})	ΔH_{tot} (J g^{-1})	P_M (min)
$(IPGVG)_{320}$	5.61	−22.82	−17.21	0.6
$(VPGVG)_{251}$	3.14	−11.34	−7.50	0.5
Poly(VPGVG)	2.96	−11.11	−8.79	0.5

separated. However, as P decreases, ΔH_{rev} undergoes a substantial increase. At P about 0.8–1 min, the reversing component turns into a positive exothermic peak, which reaches a maximum P_M at 0.5–0.6 min. Simultaneously, $\Delta H_{non\text{-}rev}$ suffers an equivalent decrease. Therefore, as P decreases, the reversing component is enriched in the exothermic component (chain folding), while the non-reversing component is enriched in the endothermic contribution (dehydration). The ΔH_{rev}, $\Delta H_{non\text{-}rev}$, ΔH_{Tot} values found at P_M can be seen in Table 2. Further decrease in P results in a progressive reduction in ΔH_{rev} to zero and an increase in $\Delta H_{non\text{-}rev}$ to the total enthalpy as a result of the complete overlap of both processes in the non-reversing component.

The maximum splitting was found at approximately the same P_M regardless of the polymer. Additionally, comparison of the data for $(VPGVG)_{251}$ and $(IPGVG)_{320}$ indicates that the reversing component at the maximum is higher for $(IPGVG)_{320}$. Owing to the higher hydrophobicity of I as compared to V, its chain folding has to show a higher exothermic ΔH_{rev} (see Table 2). Therefore, ΔH_{rev} values can be used as a quantitative measure of the amino-acid hydrophobicity. Additionally, the increased hydrophobicity of $(GVGIP)_{320}$ would also induce a greater extent of hydrophobic hydration; and hence its higher endothermic $\Delta H_{non\text{-}rev}$ is also reasonable.

There are no significant differences in data from $(VPGVG)_{251}$ and poly(VPGVG) (see Table 2). Since the only difference between these polymers is their M_W dispersity, the TMDSC results are practically the same, implying that the reversing and non-reversing TMDSC components depend mainly on the mean hydrophobicity of the monomer.

Therefore, TMDSC has been demonstrated to be an effective method to resolve the overlapping kinetic processes implicated in the ITT of elastic protein-based polymers. By tuning the frequency of the periodic component, a maximum split can be achieved which shows an exothermic contribution arising from the van der Waals contacts attending chain folding and assembly, and an endothermic contribution associated with loss of hydrophobic hydration, the former being about one fourth of the latter. To the best of our knowledge, TMDSC is the only method currently available to separate the two contributions. Accordingly, its utilization in future research to evaluate hydrophobicity of the full complement of naturally occurring amino acids (and relevant modifications thereof) is clear, as is its relevance to hydrophobic folding of polymers and natural proteins

4.2. Introducing additional chemical functions in the monomer: pH-responsive ELPs and the ΔT_t mechanism

In all ELPs, T_t depends on the mean polarity of the polymer, increasing as the hydrophobicity decreases. This is the origin of the so called 'ΔT_t mechanism' [39]; i.e. if a chemical group that can be present in two different states of polarity exits in the polymer chain, and these states are reversibly convertible by the action of an external stimulus, the polymer will show two different T_t values. This T_t shift ΔT_t opens a working temperature window in which the polymer isothermally and reversibly switches between the folded and unfolded states, following changes in the environment. This ΔT_t mechanism has been exploited to obtain many elastin-like smart derivatives [39,55–57].

This mechanism is also exploited in the model pH responsive polymer; $[(VPGVG)_2–VPGEG–(VPGVG)_2]_n$. In this ELP, the γ-carboxylic group of the glutamic acid (E) exhibits strong polarity changes between its protonated and deprotonated states as a consequence of pH changes around its effective pK_a.

Fig. 4 shows the folded chain content as a function of T at two different pH values for a genetically engineered polymer with the above general formula (with $n=45$). At pH = 2.5, in the protonated state, T_t of the polymer is 28 °C. Below this temperature, the polymer is unfolded and dissolved while above it, the polymer folds and segregates from the solution. However, at pH = 8.0 the increase in the polarity of

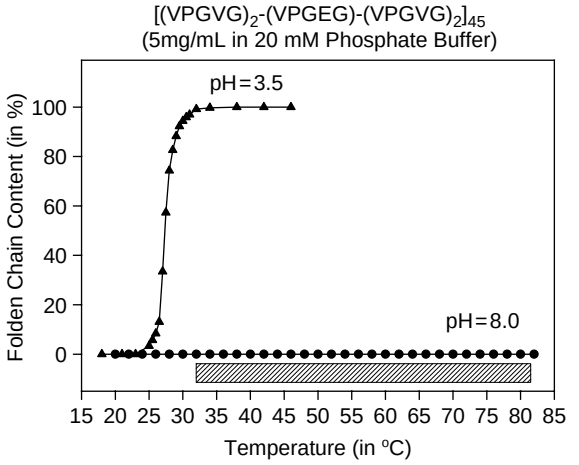

Fig. 4. Turbidity vs temperature profiles of model genetically engineered pH-responding ELP (see Ref. [16] for details about bioproduction of this polymer). Box at the bottom represents the window of working temperatures. Experimental conditions are given in the plot.

the γ-carboxyl groups, as they lose their protons to form carboxylate, is enough to increase T_t at values above 85 °C, opening a working temperature window wider than 50 °C. Therefore, at temperatures above 28 °C the polymer would fold at low pH and unfold at neutral or basic pH. These results reveal the extraordinary efficiency of ELPs as compared to other pH responsive polymers since this huge ΔT_t is achieved with just four E residues per 100 amino acids in the polymer backbone. This is of practical importance in using these polymers to design molecular machines and nanodevices such as nano-pumps or nanovalves because just a few protons are needed to trigger the two states of the system. In the literature, the creation of an electric charge in a side chain of ELP due to acid-basic equilibrium has been seen as a highly efficient way to achieve high ΔT_t. In the an ELPs designed and studied to date, the capability of the free carboxyl or amino groups of aspartic acid, glutamic acid or lysine to drive those the T_t shifts is only surpassed by the ΔT_t caused by the phosphorylation of serine [39].

Contrary to what happens with polydisperse synthetic polymers, the exquisite control of molecular architecture and the strictly monodisperse M_W attained by genetic engineering greatly facilitate study of the dependence of various polymer properties on M_W.

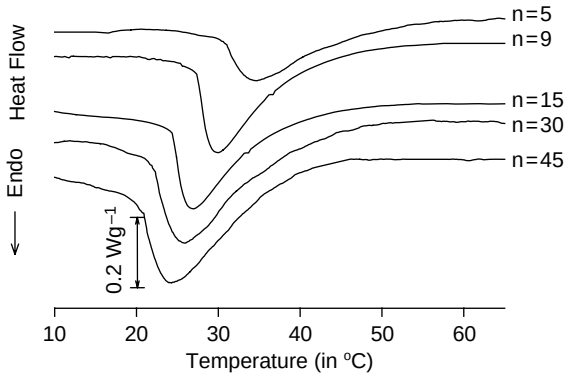

Fig. 5. DSC thermograms of 50 mg/mL phosphate buffered (0.1 M, pH 2.5) aqueous solutions of polymers. The polymerization degree (n) is shown on the right side of the plot. Heating rate 10 °C/min. Reproduced with permission from Macromolecules, 2004, 37, 3396–3400. Copyright 2004 Am. Chem. Soc.

A study of this kind has been done with the $[(VPGVG)_2–VPGEG–(VPGVG)_2]_n$ series. A series of monodisperse polymers was bioproduced, with $n=5$, 9, 15, 30, and 45. These polymers were designed to study the effects of M_W on the ITT and its dependence on pH. It was found that the transition temperature decreased and the transition enthalpy increased as M_W increased, especially for the lowest M_Ws. This can be seen qualitatively in Fig. 5, where a series of DSC thermograms has been plotted for a given polymer concentration and pH.

Quantitatively, these dependences can be seen in Fig. 6, in which true T_t values have been plotted vs M_W (By true T_t we denote the T_t value obtained by extrapolation of the DSC peak temperature to zero heating rate ($\nu=0$)). Moreover, we have observed that the pK_a of the free carboxyl of the glutamic side chain also depends on M_W. This striking fact can be seen in Fig. 7 where T_t has been followed as a function of pH for the different M_Ws. As shown in Fig. 7, the pH at which T_t starts to increase, following the first deprotonations of the free carboxyl groups, is lower for lower M_Ws. With the help of the enthalpy values found at different pHs and M_Ws, it is possible to estimate the apparent pK_a (pK_a') of this free carboxyl group as a function of M_W [16] (Fig. 8).

That behavior would imply that this carboxyl group is less acid for higher M_W and tends to remain in the protonated state, despite the fact that

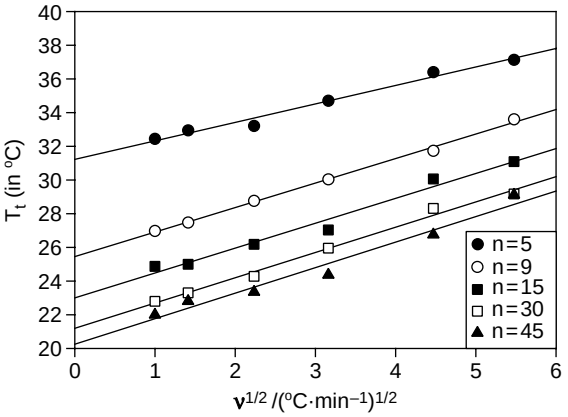

Fig. 6. Dependence of T_t on the square root of the heating rate for polymers. The corresponding polymerization degree (n) is indicated in the plot. The lines represent the least-squares linear regressions of the data for each n. Phosphate buffered samples (0.1 M, pH 2.5). Reproduced with permission from Macromolecules, 2004, 37, 3396–3400. Copyright 2004 Am. Chem. Soc.

the surroundings of this carboxyl are independent of M_W. This striking behavior could be partially explained by the influence of the polar chain-end groups, being this influence higher for lower M_Ws. However, the exclusive effect of the end-chain polarity seems not to be enough to account for the strong influence reported. We believe that a large part of the effect of M_W on the ITT is caused by the inter- and intrachain cooperativity of the hydrophobic self-assembly taking place during the ITT [40]. In this

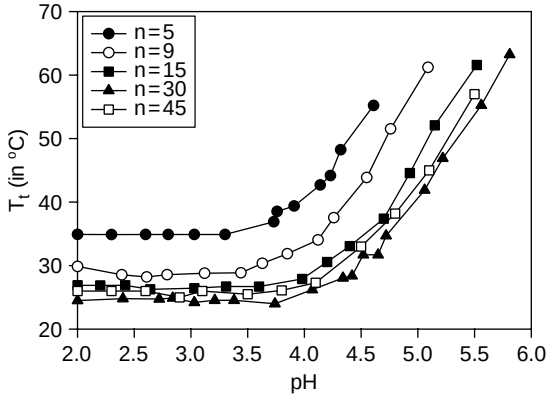

Fig. 7. Dependence of T_t on pH for polymers (as indicated in the plot). 0.1 M phosphate buffered samples. Reproduced with permission from Macromolecules, 2004, 37, 3396–3400. Copyright 2004 Am. Chem. Soc.

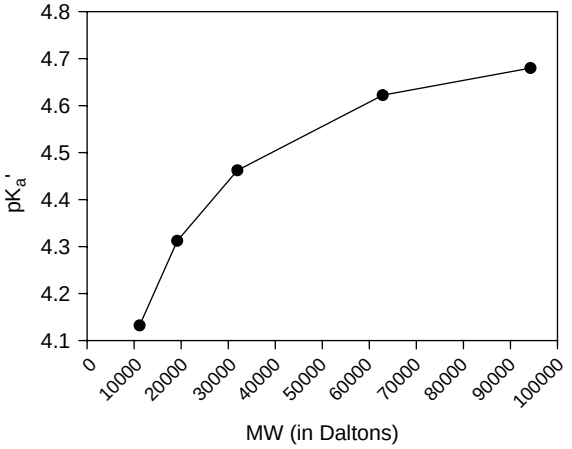

Fig. 8. Dependence of pK_a' for the glutamic acid γ-carboxyl group on M_W. Reproduced with permission from Macromolecules, 2004, 37, 3396–3400. Copyright 2004 Am. Chem. Soc.

sense, it is reasonable to think that short chains do not show an efficient cooperation so their self-assembly is hindered, while for high M_Ws the inter and intrachain cooperativity during folding is more efficient, which, at some degree, forces the carboxyl group to be in the protonated (less polar) state. This behavior is also in good agreement with the work of Urry's group [30,31] with certain Glu-containing ELPs. In these, the close vicinities of the γ-carboxyl groups are maintained in a highly hydrophobic environment by positioning Phe residues by precise nanometric design of the polymer sequence in accordance with the β-spiral structure of the folded state. Thus, once the polymer folds into the β-spiral structure, those Phe residues completely surround the free carboxyl group, creating a strong competition between the two mutually excluding forms of hydration; i.e. hydrophobic hydration of the Phe residues and the hydrophilic hydration of the carboxylate. The overwhelming presence of Phe residues causes extraordinary pK_a shifts of these γ-carboxyl groups toward higher values (the carboxyl group becomes less and less acid as the number of surrounding Phe increases).

4.3. Self-assembly of ELPs

In relation to self-assembly, natural elastin undergoes a self-aggregation process in its natural environment. It is produced from a water soluble

precursor, tropoelastin, which spontaneously aggregates yielding fibrillar structures that are finally stabilized by enzymatic interchain cross-links. This produces the well-known insoluble, elastic elastin fibers that can be found in abundance in the skin, lungs, arteries, and, in general, those organs subject to repeated stress–strain cycles.

The self-assembling ability of elastin seems to reside in certain relatively short amino-acid sequences, as has been recently proposed by Yang et al. [58] working on recombinant ELPs. Above their T_t, some of these polypeptides are able to form nanofibrils that further organize into hexagonally close-packed arrangements when the polymer is deposited onto a hydrophobic substrate [58].

However, in ELPs, this trend to self assemble to nanofibers can be extended to other topologies and nanostructural features [59–61]. With the potential afforded by genetic engineering in designing new polymers, the growing understanding in the molecular behavior of ELPs, and the enormous wealth of experimental and theoretical background gained during the last decade on the self-assembling characteristics of different types of block-copolymers, novel self-assembly properties are being unveiled within the ELP family. For example, Reguera et al. showed that the ELP $[(VPGVG)_2(VPGEG)(VPGVG)_2]_{15}$, previously found to be pH responsive, is able to form polymer sheets with self-assembled nanopores [61] (see Fig. 9). n AFM study of the topology of this Glu-containing ELP, deposited by spin coating on a Si hydrophobic substrate at temperatures below T_t showed that in acid conditions, the deposited polymer presents a flat surface without particular topological features (Fig. 9(A)).

However, from basic solutions the deposited polymer clearly has an aperiodic pattern of nanopores (ca. 70 nm wide and separated by ca. 150 nm) (Fig. 9(B)). This different behavior as a function of pH has been explained in terms of the polarity of the free γ-carboxyl group of the glutamic acid. In the carboxylate form, this moiety shows a markedly higher polarity than the rest of the polymer domains and the substrate itself. Under this condition, the charged carboxylates impede any hydrophobic contact in their surroundings, which is the predominant mode of assembly for this kind of polymers. The charged domains, along with their hydration sphere,

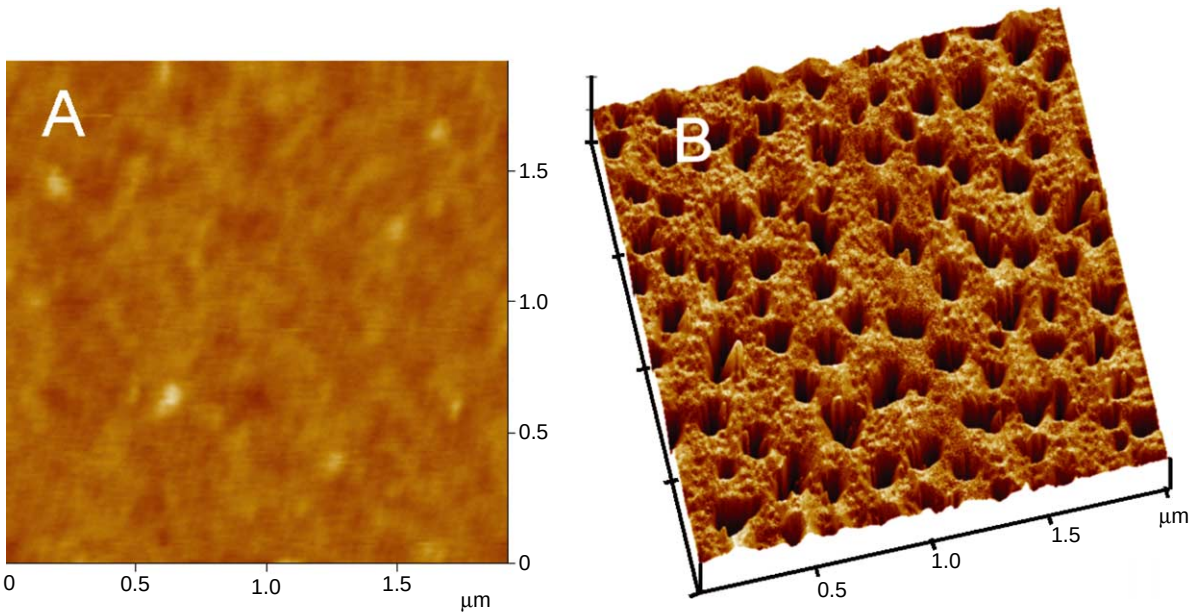

Fig. 9. Tapping mode AFM image of $[(VPGVG)_2–(VPGEG)–(VPGVG)_2]_{15}$ deposited from aqueous solution on Si hydrophobic substrate. Sample conditions: (A) 10 mg/mL in 0.02 M HCl solution (acid solution); and, (B) 10 mg/mL in 0.02 M NaOH solution (basic solution). Adapted from Ref. [61]. Reproduced with permission from J Am Chem. Soc, Copyright 2004 Am. Chem. Soc.

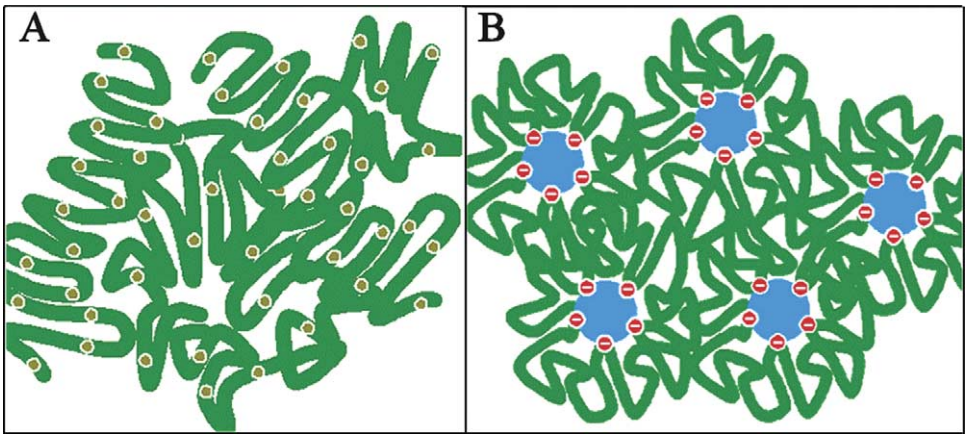

Fig. 10. Schematic cartoon of polymer distribution on hydrophobic substrate: (A) in acid medium, (B) in basic medium. For clarity counter-ions are not shown. Adapted from Ref. [6]. Reproduced with permission from J Am Chem. Soc. Copyright 2004 Am. Chem. Soc.

are then segregated from the hydrophobic surrounding giving rise to nanopore formation (Fig. 10).

The self-association of ELPs is starting to be employed to develop various applications. For example, Molina et al. [62] tested self-assembled nano- and micro-particles of poly(VPAVG), another version of ELP, as carriers of the model drug dexamethasone phosphate, in order to develop injectable systems for controlled drug release. In these particles, the drug is entrapped while the particles self-assemble as the temperature is raised above T_t.

In another remarkable example, as part of an effort to develop advanced biomaterials, regenerable biosensors and microfluidic bioanalytical devices [63–65], Chilkoti et al. obtained nanostructured surfaces, by combining ELPs and dip-pen nanolithography, that show reversible changes in physicochemical properties in response to changes in environmental conditions. In particular, these systems are able to capture and release proteins on a nanopatterned surface, by using the self-assembling characteristics of ELPs.

4.4. Further chemical functionalization of monomer: photo-responsive ELPs and the amplified ΔT_t mechanism

The range of stimuli that can exploit the ΔT_t mechanism is not limited to chemical reactions that take place on natural amino acid side chains. It is possible to modify certain side chains to achieve systems with extended properties. A good example of this are photo-responsive ELPs, which bear photochromic side chains, either coupled to functionalized side chains in the previously formed polymer (chemically or genetically engineered) or by using non-natural amino acids already containing functionalized with photochromic groups.

The first example is of the latter type. The polymer is an azobenzene derivative of poly(VPGVG), the copolymer poly[f_V(VPGVG), f_X(VPGXG)] (X = L-*p*-(phenylazo)-phenylalanine; f_V and f_X, are mole fractions). The *p*-phenylazobenzene group undergoes a photo-induced *cis–trans* isomerization. Dark adaptation or irradiation with visible light around 420 nm produces the trans isomer, the less polar isomer. On the contrary, UV irradiation (at around 348 nm) causes the appearance of high quantities of the *cis* isomer, which is slightly more polar than the trans. Although the polarity change is not great, it is enough to obtain functional polymers due to the sensitivity and efficiency of ELPs. Fig. 11 shows the photoresponse of poly[0.85(VPGVG), 0.15(VPGXG)] ($f_X = 0.15$). This mole fraction represents only three L-*p*-(phenylazo)phenylalanine groups per 100 amino acids in the polymer chain. In spite of the low polarity change and the exiguous presence of chromophores, a working temperature window at around 13 °C is evident (Fig. 11).

In another example, a different chromophore, a spiropyrane derivative, is attached at the free γ-carboxyl group of an E-containing ELP either

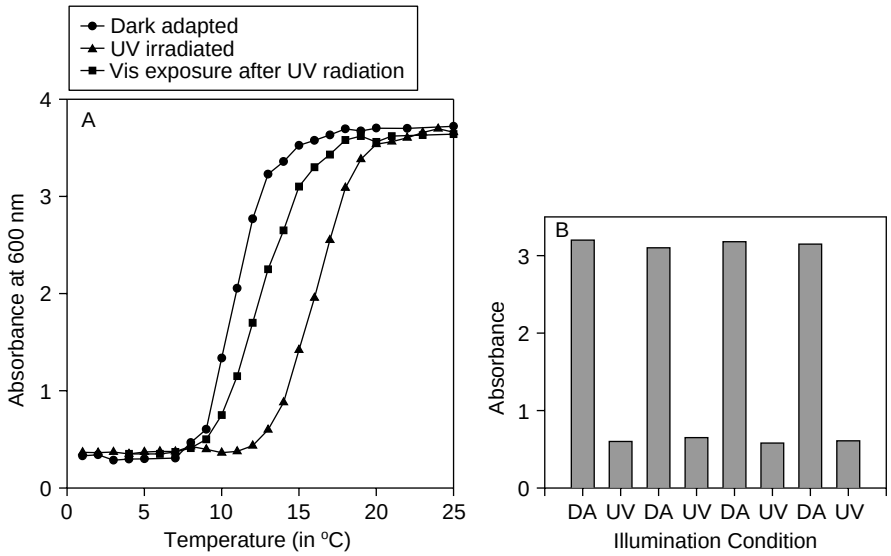

Fig. 11. (A) Temperature profiles of aggregation of 10 mg/mL water solutions of photoresponsive poly[0.85(VPGVG), 0.15(VPGXG)] (X = L-*p*-(phenylazo)-phenylalanine) under different illumination regimens. The correspondence between each profile and its illumination condition is indicated in the plot. Details on polymer synthesis and illumination conditions can be found in Ref. [30]. (B) Photomodulation of phase separation of 10 mg/mL aqueous solutions of poly[0.85(VPGVG), 0.15(VPGXG)] at 13 °C. The illumination conditions prior to measurements are indicated in the horizontal axis. DA, Dark adaptation; UV, UV irradiation. Reproduced with permission from Macromolecules, 2001, 34, 8072–8077. Copyright 2001 Am. Chem. Soc.

chemically synthesized or genetically engineered. Fig. 12 represents the photochromic reaction for this polymer [56]. As compared to *p*-phenylazobenzenes, spiropyrane compounds show a photoreaction that can be driven by natural sunlight–darkness cycles without the employment of UV sources, although UV irradiation causes the same effect as darkness but at a higher rate [66].

Again the difference in polarity between the spiro and merocyanine forms (Fig. 12) is enough to cause a significant T_t shift. Fig. 13 shows the turbidity profiles of the polymer in three illumination regimens (Fig. 13(A)) and the photomodulation of the polymer folding and unfolding (Fig. 13(B) and (C)).

Again, the efficiency of the polymer is outstanding since just 2.3 spiropyran chromophores per 100

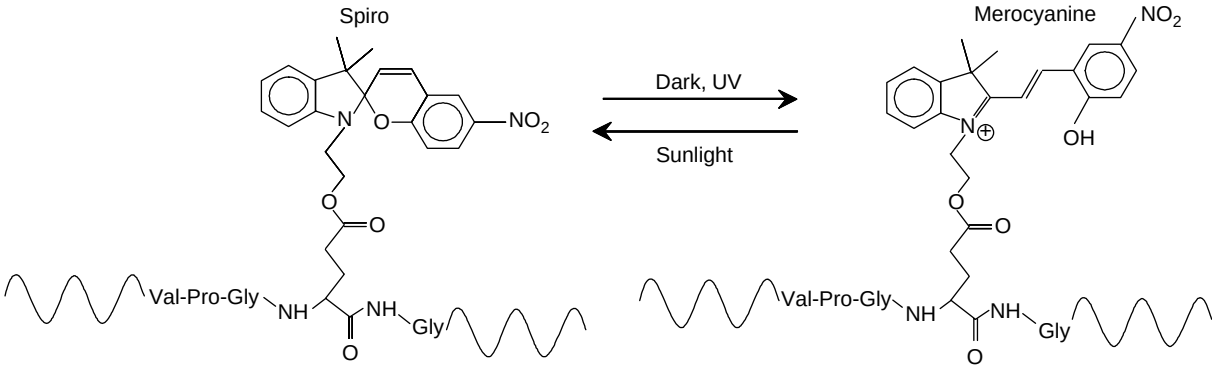

Fig. 12. Photochemical reaction responsible for photochromic behavior of spiropyrane-containing ELP. Reproduced with permission from Macromolecules, 2000, 33, 9480–9482. Copyright 2000 Am. Chem. Soc.

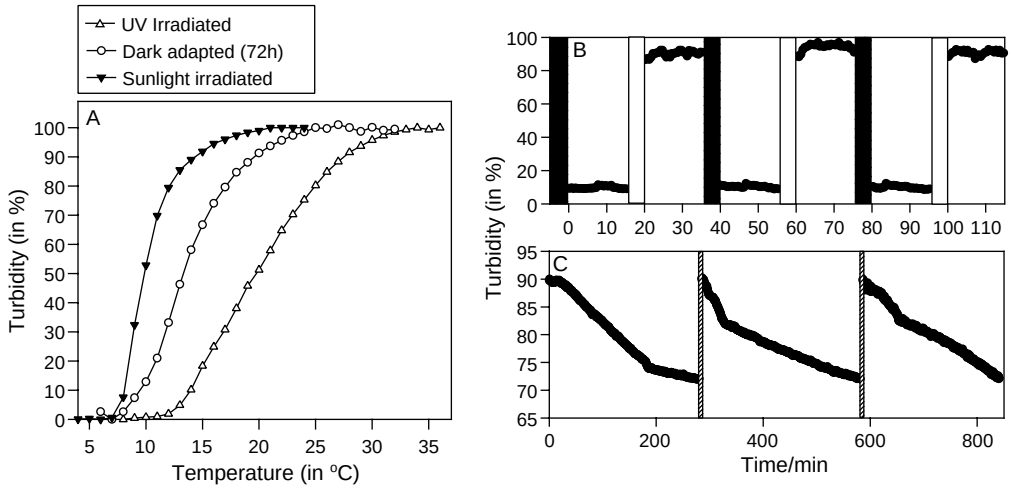

Fig. 13. (A) Temperature profiles of aggregation of 20 mg/mL phosphate-buffered (0.01 N, pH 3.5) aqueous solutions of the photoresponsive polymer under different illumination regimens. The correspondence between each profile and its illumination condition is indicated in the plot. Turbidity was calculated from absorbance values obtained at 600 nm on a Cary 50 UV–vis spectrophotometer equipped with a thermostatted sample chamber. (B) and (C) Photomodulation of phase separation of 5 mg/mL aqueous samples of photochromic polymer (T = 14 °C, 0.01 N phosphate buffer at pH = 3.5). (A) UV–sunlight cycles. Boxes in the subplot represent periods of irradiation: UV, black boxes; sunlight, white boxes. (B) Darkness–sunlight cycles. Boxes in the subplot represent periods of sunlight irradiation. Reproduced with permission from Macromolecules, 2000, 33, 9480–9482. Copyright 2000 Am. Chem. Soc.

amino acid residues in the polymer backbone are sufficient to render the clear photomodulation shown in Fig. 13.

Various ELP versions responsive to pH, light and other stimuli, such as an electrochemical potential or analyte concentration, can be found in the literature. Most of these were prepared by Urry's group by the exclusive use of chemical synthesis in a huge effort, over more than a decade, at a time when the use of genetic engineering to produce PBPs was scarcely developed (e.g. Ref. [39]). The smart response of ELPs has already found applications in different fields. For example, Chilkoti et al. have designed temperature- and pH-responsive ELPs for targeted drug delivery [67–71]. They have obtained responsive ELPs that conjugate to drugs and enable thermally targeted drug delivery to solid tumors having their T_t between body temperature and the temperature in a locally heated region [67]. In another example Kostal et al. have designed tunable ELPs for heavy metal removal [72]. These polymers have the general formula $MEF(VPGVG)_nH_6$. The presence of the histidine clusters enabled Cd^{2+} to bind strongly to the biopolymers. Recovery of biopolymer–Cd^{2+} complexes was easily achieved by triggering aggregation on increasing the temperature above T_t, e.g. by raising the temperature or by salt addition.

Another example is the use of an elastin-like polymer component in a block-copolymers, in which other component is a block with a special function. For example, Cappello et al. [73–78] developed Silk-ELP polymers with temperature and pH sensitivity for biomedical applications, and Dreher et al. showed the use of a ELP-Protein as a easy way to purified useful proteins [71,79–83].

In a different approach to the design of more efficient stimuli-responsive ELPs, it is possible to increase and further control the smart behavior of ELPs without increasing the number of sensitive moieties. This is possible if one of the states of that moiety is able to interact with a different compound, while the other state is not, and this interaction further increases the difference in polarity between both states. This is the basis of the so called 'amplified ΔT_t mechanism' and has been demonstrated for a *p*-phenylazobenzene-containing polymer poly[0.8 (VPGVG), 0.2(VPGXG)], of the kind shown above, in the presence of α-cyclodextrin (αCD) [55]. The αCD is able to form inclusion compounds with the trans isomer of the *p*-phenylazobenzene group but not

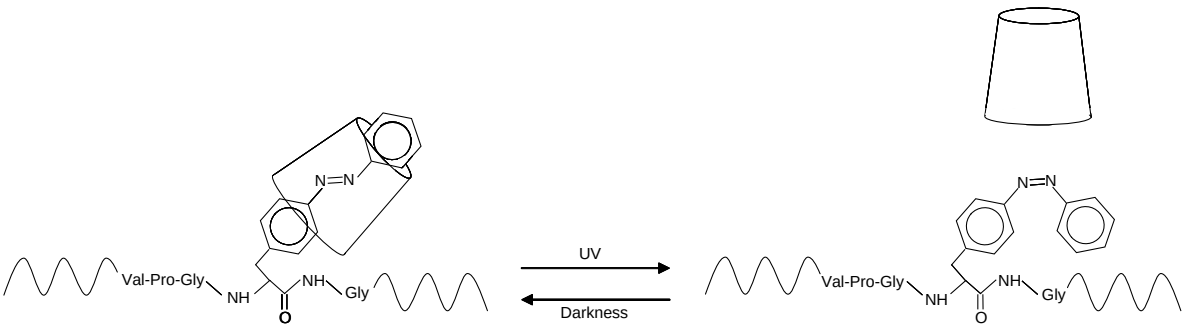

Fig. 14. Schematic diagram of proposed molecular mechanism for interaction between the *p*-phenylazobenzene pendant group and αCD. Reproduced with permission from Adv. Mater., 2002, 14, 1151–1154. Copyright 2002 Wiley-VCH.

with the *cis*, because of strong steric hindrance [55] (Fig. 14).

The αCD outer shell has a relatively high polarity, and is, of course, much more polar than the *p*-phenylazobenzene moiety in either the *trans* or *cis* states. The change in polarity between a dark-adapted sample (*trans* isomer buried inside the αCD) and a UV-irradiated one (*cis* isomer unable to form inclusion compounds) leads to an enhanced ΔT_t (Fig. 15). The magnitude of this effect is dependent on the αCD concentration, so it is possible to tune the width and position of the working temperature window just by changing [αCD].

As a result, in the αCD/poly[0.8(VPGVG), 0.2(VPGXG)] coupled photoresponsive system, αCD acts similarly to an amplifier in an electronic circuit. αCD promotes a tunable offset, gain and inversion of the photoresponse of the polymer (Fig. 15

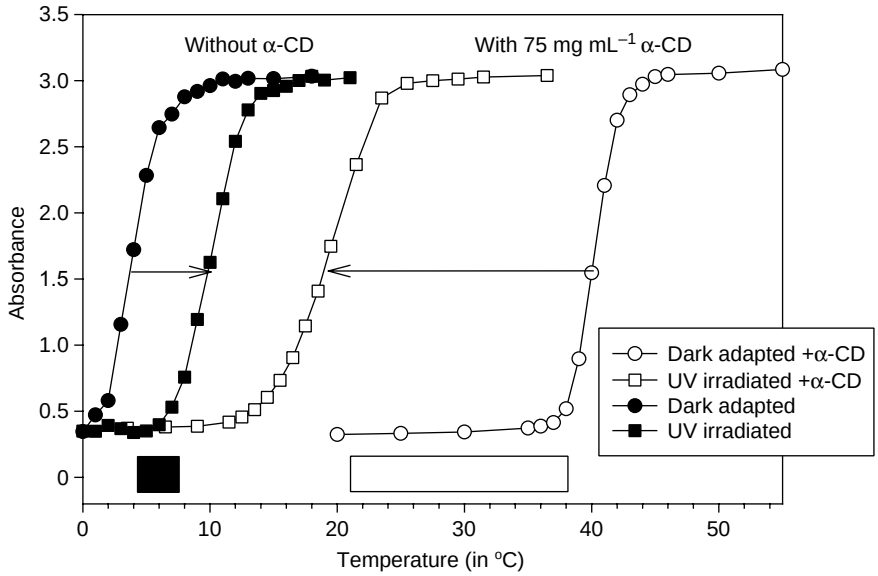

Fig. 15. Temperature profiles of aggregation of 10 mg/mL water solutions of photoresponsive ELP in absence and presence (75 mg mL^{-1}) of αCD under both illumination regimens. Circles represents dark-adapted samples and squares UV-irradiated samples. Hollow symbols, presence of αCD; filled symbols, absence of αCD. Arrows represents the sense of displacement of the turbidity profile caused by UV irradiation of the corresponding dark-adapted sample. Boxes at the bottom indicate the window of working temperatures open when the system is in the absence (filled box) and presence (hollow box) of αCD. Reproduced with permission from Adv. Mater., 2002, 14, 1151-1154. Copyright 2002 Wiley-VCH.

Table 3
Values of T_t, ΔT_t, offset and gain for a 10 mg mL^{-1} poly[0.8(VPGVG), 0.2 (VPGXG)] water solution in presence of different concentrations of α-CD. DA, dark adapted samples[a]; UV, UV irradiated samples[b]. Offset and gain as defined in the text

[α-CD] (mg mL^{-1})	DA T_t (in °C)	UV T_t (in °C)	ΔT_t (in °C)	Offset (in °C)	Gain
0	3.9	10.0	6.1	–	–
10	20.2	13.7	−6.5	16.3	−1.07
25	26.5	14.7	−11.8	22.6	−1.93
50	33.4	16.2	−17.2	29.5	−2.82
75	40.5	19.5	−21.0	36.6	−3.44

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[a] DA samples were samples kept as the final water solution in the dark for 24–48 h at 5 °C until a stationary transformation of the azo group to the trans isomer was obtained (assessed by UV–vis spectroscopy).

[b] UV samples were DA samples further irradiated with UV light. That was made in a standard spectrophotometer quartz cuvette with light from a 500 W Hg arc lamp (model 6285, Oriel corp.) mounted on a lamp housing with a F/1.5 UV grade fused silica condenser and rear reflector (model 66041, Oriel corp.). UV irradiation was achieved by the use of a band interference filter ($340 < \lambda < 360$ nm) from CVI Laser Corporation (F10-350.0-4-1.00) The irradiation time needed to obtain a photostationary state was 30 s. The exposure energy irradiation was ca. 4 mW cm^{-2}. Additional information on the irradiation setup can be found elsewhere [57].

and Table 3). In this way, the polymer photoresponse can be shifted to room or body temperature; and with a wider range of working temperatures, the need for precise temperature control can be avoided in most foreseeable applications covering a wide range that goes from photo-operated molecular machines to macroscopic devices (photoresponsive hydrogels, membranes, etc.) and to nano- and micro-devices (phototransducer particles, photo-operated pumps, etc.). Furthermore, the amplified ΔT_t mechanism is not restricted to photoresponsive ELPs and could be exploited in some other smart ELPs responding to stimuli of a different nature. It also adds a further possibility of control, since the ability of CDs to form inclusion compounds can be controlled by various stimuli in some modified CDs [84–86].

5. The outstanding biocompatibility of elastin-like polymers: three pillars of extraordinary biomaterial design

As discussed in the preceding, the existence of the ITT for ELPs is the basis of their remarkable smart and self-assembling properties. This is the first pillar of extraordinary biomaterials design.

The second pillar in the development of these extraordinary materials is, evidently, the power of genetic engineering to facilitate preparation of complex, well-defined polymers with controlled multiple (bio)functionality.

This ease of preparation is favored by simple purification protocols based on the ITT feature and to the high yields of bioproduction.

Additionally, ELPs show another property, which is highly relevant for possible use of these polymers in the most advanced biomedical applications, such as tissue engineering and controlled drug release. This third pillar is the extraordinary biocompatibility of ELPs. The complete series of ASTM generic biological tests for materials and devices in contact with tissues, tissue fluids and blood demonstrate unmatched biocompatibility [87]. In spite of the polypeptide nature of these polymers, it has not been possible to obtain monoclonal antibodies against most of them. Apparently, the immune system just ignores these polymers because it cannot distinguish them from natural elastin. Incidentally, nowadays it is believed that the high segmental mobility shown by the β-spiral, the common structural feature of ELPs, greatly helps in preventing the identification of foreign proteins by the immune system [30,31]. In addition, the secondary products of their biodegradation are just simple and natural amino acids.

Finally, the third pillar in the development of extraordinary materials is, evidently, the power of genetic engineering in promoting the facile preparation of complex and well-defined polymers with controlled and multiple (bio)functionality. This easiness is also favored by the simple purification protocols based on the ITT feature of this polymer, and the high yield of bioproduction [14].

With this nice set of properties is not surprising that biomedical application of ELPs seems to be the first area where they will enter the market. This is especially likely considering that the biomedical (and cosmetic) market shows a clear tendency to quickly adopt new developments that show superior performance. Additionally, this sector is not so conditioned by the cost associated with the materials used in their devices and developments, as happens in commodity manufacturing and other kind of applications. Hence companies producing ELPs will find the biomedical sector a good option to amortize the costs of development of know-how and technology for the production of ELPs.

5.1. ELPs for drug delivery: different strategies for molecular design

Different versions of ELPs designed for drug delivery can be found in the literature. However, they do not share a common basic strategy of design. As ELPs display many different properties that can be useful for drug delivery; i.e. smart behavior, self-assembly, biocompatibility, etc. the design strategy can be diverse. In fact, the different ELP-based drug delivery systems described to date mainly exploit a particular one of these properties.

The first ELP-based drug delivery systems were reported by Urry. They were based on simple devices in which γ-radiated cross-linked poly(VPGVG) hydrogels of various shapes (e.g. slabs) were loaded with a model water soluble drug (Biebrich Scarlet) [88]. The drug was then released by diffusion. In this simple design, just the extraordinary biocompatibility and the lack of pernicious compounds during the bioresorption of the device were exploited. Later designs were slightly more complicated. The basic VPGVG pentapeptide was functionalized by including some glutamic acid to provide free carboxyl groups for cross-linking purposes. The cross-linkers, of a type that forms caboxyamides, were selected because of their ability to hydrolyze at a controlled rate, releasing the polymer chains and, concurrently, any drug entrapped within the cross-linked slabs [89]. This was an apparently simple and conventional degradation-based drug delivery system. However, due to the use of ELPs, the displayed behavior was a little more complex and efficient. While the cross-links were intact, the carboxyl groups were amidated and, consequently, uncharged. This state of lower polarity yielded a cross-linked ELP material with a T_t below body temperature. At that temperature the chains were folded, the material contracted and deswelled, and the polymer chains essentially insoluble, entrapping the loaded drug quite efficiently in the models studied. When hydrolysis took place on the outer surface of the slab, charged carboxylates appeared, which strongly increased the T_t (well above body temperature) in this zone. The skin of the slab swelled and the loaded drug readily escaped from the outer layer of the pellet. Additionally, since the fully released chains were completely soluble, they soon diffused away and were reabsorbed. This insured the presence of an ever fresh surface on the slab, ready to release the more drug [89]. Accordingly, the kinetics of drug release were almost zero-order; and, hence the performance of the system was superior to others based on equivalent polymers but lacking the ΔT_t mechanism. In general, this statement is more precise as the size of the loaded drug hydrogel particle is increased, since, as no other particular functionality was added to the polymer chain, there was no substantial interaction between the drug and the polymer other than the constraint on movement of the drug within the polymer matrix, allowing some degree of uncontrolled diffusion and thus perturbing the kinetics of drug release.

In a different example, as mentioned in Section 4.3, the trend to form stable, drug-loaded, nano- and micro-particles by some of the ELPs, especially those based on the pentapeptide VPAPG, has facilitated the development of injectable systems for controlled release [62]. These polymers form stable particles with a size below 3 μm, encapsulating significant amounts of drug, as an aqueous or PBS polymer solution is warmed above its transition temperature (∼30 °C). Due to the peculiar composition of the monomer, the formation and dissolution of the self-assembled microparticles shows an interesting hysteresis behavior in which the particles formed do not redissolve until a strong undercooling of ∼12–15 °C is achieved. Therefore, the particles, once formed, are stable well below either room or body temperature, which greatly simplifies the handling of the loaded particles prior injection.

In addition, this hysteresis behavior has been explained by a combination of two factors, one

derived of the methyl group of Ala in hindering the bond rotation around the β-turns during folding and unfolding [48], as well as for the existence of a more perfect folding state of this polymer as compared to more conventional poly(VPGVG), as deduced from FTIR and Raman spectroscopies and ab initio calculations [90]. This last enhances the exclusion of water from the inner channel of the β-spiral in the folded chain. This minimizes the plastifying effect of water and its role in disrupting the intramolecular hydrogen bonds [90], greatly helping to stabilize the folded structure.

The slow diffusional release of the drug has been considered as the main mechanism of drug delivery for this simple model system, although more complex polymer designs based on this Ala-containing polymer could make use of other release mechanisms.

These examples are based on simple polymer formulations that still fall far short of the full potential of ELPs for drug delivery systems. Their smart and self-assembly properties, as well as deeper knowledge of the molecular basis of the ITT, are only marginally exploited. However, new systems are beginning to appear in the literature that already show a more decided bent toward exploiting the very special characteristics of ELPs and the powerful way they can be produced by genetic engineering. For example, Chilkoti's group has produced nice examples of ELPs specially designed for targeting and intracellular drug delivery. They exploited the soluble-insoluble transition of the ELPs to target a solid tumor by local hyperthermia, and then, in the most sophisticated versions an additional pH-response of these ELPs is used to mimic the membrane disruptive properties of viruses and toxins to cause effective intracellular drug delivery. Among the most evident advantages of this kind of advanced drug delivery system is the more efficient dosage of antitumor drugs, However, these polymers could serve also as alternatives to fusogenic peptides in gene therapy formulations and to enhance the intracellular delivery of protein therapeutics that function in the cytoplasm [68,71,72,91].

On the other hand, recent better understanding of the molecular characteristics of the ITT has allowed development of advanced systems for more general drug release that have achieved practically ideal zero-order release kinetics without the concerns caused by previous designs. The first examples are based on Glu-containing ELPs mentioned in the preceding, in which the microenvironment of the γ-carboxyl groups is maintained highly hydrophobic by positioning Phe residues in the polymer sequence. In neutral or basic pH (including physiological pH), those carboxylate moieties show a strong propensity to neutralize their charge by ion-coupling contacts with positively charged drugs if such coupling causes an effective decrease of the polarity of the carboxyl microenvironment. As a result these polymers, at neutral or basic pH and in the presence a drug of opposite sign, may form insoluble aggregates. These materials will be characterized by a high rate of drug loading and, upon implantation, will release the drug slowly as it is leached from its coupling on the outer surface of the aggregate. The release rate can be tuned by modifying the hydrophobic environment of the carboxyl with a properly chosen amino-acid sequence in the polymer [30,31]. Once the drug is released and the polymer-drug interaction is lost, and as a consequence of the charged state of the carboxyl group (carboxylate), the polymer unfolds and finally dissolves. At the same time, the interface between the remaining insoluble, still loaded, aggregate and body fluids is continuously renewing without changing physical–chemical properties for practically all the functional period of the system. This behavior results in near ideal zero-order release [30,31].

In the present situation, as demonstrated by the various examples shown above, the different alternatives afforded by the extraordinary set of ELP properties, as well as the power of genetic engineering, have shown a remarkable potential for future drug delivery developments. What is more, those independent approaches, exploiting different ELP properties, are not mutually exclusive. Hence the development of new ELPs combining various strategies of the kind depicted above is foreseeable. Obviously the basis exists for the development of drug delivery systems with unprecedented efficiency.

5.2. ELPs for tissue engineering: Introducing tailored biofunctionality

Any design of biomedical devices is a tremendous challenge for the materials developer. This has been shown above for drug delivery systems, but, the most demanding application is likely to be tissue

engineering (or as nowadays preferred, 'regenerative medicine'). When a mature or stem cell divides and spreads in growing tissue, that cell is passing through the most vulnerable stage of its life cycle. That is the reason why materials that work in biomedical uses may can fail when used for tissue engineering (the failure can be caused both by the material itself and by its biodegradation products).

Additionally, we have to keep in mind that when we design a matrix for tissue engineering, we are trying to substitute for the natural extracellular matrix (ECM), at least transiently. Therefore, many aspects have to be taken into consideration in designing an adequate artificial ECM. Initially, the materials developer must have a clear concern about the mechanical properties of the artificial scaffold. It is well know that, when properly attached to the ECM, cells sense the forces to which they are subjected via integrins (ubiquitous trans-membrane adhesion molecules that mediate the interaction of cells with the ECM). Integrins link cells to the ECM by interacting with the cell cytoskeleton. They couple the deformation of the ECM, in response to the applied forces, with the deformation of the cytoskeleton. The deformed cytoskeleton triggers an intracellular signal transduction cascade that finally causes the expression of the genes related to the rebuilding of the ECM [92]. In this way, cells continuously sense their mechanical environment and respond by producing an ECM that adequately withstands the forces. In this sense, cells are very efficient force transducers. Therefore, any artificial ECM has to properly transmit forces from the environment to the growing tissue. Only in this way, can the new tissue build an adequate natural ECM that will eventually replace the artificial ECM. However, a stronger or too weak artificial ECM will cause its substitution by a too weak or too dense natural ECM, respectively, which can seriously compromise the success of the tissue regeneration.

We now know, however, that the ECM is not merely a scaffold showing certain mechanical properties to which the cells attach just to achieve the necessary tissue consistency and shape. Far from that, the proteins of the natural ECM (fibronectin, collagen, elastin, etc.) contain in their sequences a huge number of bioactive peptides that are of crucial importance in the natural processes of wound healing. Those sequences include, of course, not only the well-known cell attachment sequences. In the natural ECM we find target domains for specific protease activity. Proteases, such as the metalloproteinases of the ECM, are only expressed and secreted to the extracellular medium when the tissue needs to remodel its ECM [93]. They act on specific sequences that are present only in the proteins of the ECM, so they cannot cause damage to other proteins in their vicinity. It is also know that some fragments of these hydrolyzed ECM proteins are not just mere debris; once released they show strong bioactivity, which includes the promotion of cell differentiation, spreading and angiogenesis, among other activities. Finally, growing tissue is delicately controlled by a well-performed symphony of growth factors and other bioactive substances that are segregated by the cells. Incidentally, these factors are mainly peptides.

This is the scenario that tissue requires when is passing through the difficult processes of growing and regenerating. Therefore, this is the situation that we have to (or try to) mimic with our artificial scaffolds designed for tissue engineering. This picture looks quite disheartening; and, in fact, one can hardly think of a petroleum-based polymer that fulfills the minimum requirements of being bioreabsorbable, sufficiently biocompatible, non-toxic (the polymer itself and/or its biodegradation products), having adequate mechanical properties, and able to display or induce a minimum number of needed biofunctionalities. In spite of the expectations raised by tissue engineering, it has achieved up to now, only moderate success. Among the first properties that seem to be unattainable by conventional polymers is complexity. The set of minimum requirements listed above clearly points to the need for a very complex material that may be beyond the practical reach of our synthesis technology. This should not surprise us. We are trying to mimic a natural ECM that is intrinsically complex to a level that, in fact, we have not yet fully uncovered. It is hardly imaginable that such a variety of specific properties and biofunctionalities could be achieved by one of our petroleum-based polymers, despite the fact that can choose functionalities among an impressive set of different monomers developed by organic chemistry during recent decades. In spite of the discouraging scenario depicted above, we could be in a position with some options to come to our help;

GEPBPs may represent such a clear breakthrough alternative.

Soon after the extraordinary biocompatibility of the (VPGVG)-based ELPs was known [87], their capabilities for tissue engineering were tested. The first candidates were simple VPGVG polymers and their cross-linked matrices. Surprisingly, tests of cross-linked poly(VPGVG) matrices showed that cells do not adhere to this matrix and no fibrous capsule forms around it when it is implanted [94]. Accordingly, this matrix and other states of the material have potential for use in prevention of post-operative, post-trauma adhesions [94] but, in principle, they do not seem to be adequate candidates for tissue engineering. Nonetheless, this absolute lack of cell adherence is not a drawback; on the contrary, it is ideal as a starting material since it provides adequate mechanical properties and biocompatibility and lacks unspecific bioactivity. Very soon these simple molecules were enriched with short peptides having specific bioactivities. Due to the polypeptide nature of the ELPs, those active short sequences were easily inserted within the polymer sequence even though, at the time, chemical synthesis was still the only option to obtain these polymers. The first active peptides inserted in the polymer chain were the well-known general-purpose cell adhesion peptide RGD (R = L-arginine, G = glycine, D = L-aspartic acid) and REDV (E = L-glutamic acid, V = L-valine), which is specific for endothelial cells. The results were clear; the bioactivated VPGVG derivatives showed a high capacity to promote cell attachment, specially those based on RGD, which had a cell attachment ability almost equivalent to that of the human fibronectin [95]. Once genetic engineering was finally adopted as the production method, new molecular designs increased in complexity. Different ELPs compositions were tested as base polymers. Additionally, the cell attachment domains were not restricted exclusively to the active, short peptide domain; and they were increased in size as more amino-acids were placed surrounding the central active REDV or RGD domains as a way to obtain more active cell-binding sites [96]. For example, Panitch et al. showed that by using the longer CS5 region of the human fibronectin, which is an eicosapeptide having the REDV sequence in its central part, cell adhesion was more effective than with the short REDV inserts [97]. They present a great advance in the obtaining of artificial extracellular matrix proteins with a polymer that contains periodically spaced CS5 regions and the elastin-like sequences $(GIGVP)_n$. These still simple GEPBPs, which cannot be considered much different of their chemically synthesized precursors, have been complicated by adding different functionalities such as cross-linking domains [34,98–102]. Welsh et al. [98] showed that the introduction of the cross-linking domains would also be useful for controlling mechanical properties. They present a polymer containing CS5 domains, elastin-like sequences $(GIGVP)_n$, with lysines at the end of the sequence so they can control the cross-link density and, accordingly, the final mechanical properties of the cross-linked hydrogels by varying the molecular weight.

The designs of GEPBPs continue, including more functionalities and bioactivities in an effort to mimic the complex composition and function of the natural ECM extracellular matrix.

Girotti et al. bioproduced the ELP polymer depicted in Fig. 16 [103]. This last ELP is made from an 87 amino-acid monomer and has been obtained with $n = 10$ ($M_W = 80695$ Da). The monomer contains four different functional domains in order to achieve an adequate balance of mechanical and bioactive responses. First, the final matrix is designed to show a mechanical response comparable to the natural ECM, so it is formed over the base of an ELP

$[(VPGIG)_2–(VPGKG)–(VPGIG)_2–(EEIQIGHIPREDVDYHLYP)–(VPGIG)_2–(VPGKG)–(VPGIG)_2–(VGVAPG)_3]_n$

Fig. 16. Schematic composition of monomer used in the ELP design described in the text. The scheme shows the several functional domains of the monomer, which can be easily identified with their corresponding peptide sequences.

of the $(VPGIG)_n$ type. This sequence assures the desired mechanical behavior and outstanding biocompatibility, as discussed above. In addition, this basic composition endows the final polymer with smart and self-assembling capabilities, which are of high interest in the most advanced tissue engineering developments. The second building block is a variation of the first one. It has a lysine substituting for the isoleucine. The lysine γ-amino group can be used for cross-linking while retaining the properties of elastin-like polymers. The third group is the CS5 human fibronectin domain. This contains the well-known endothelial cell attachment sequence, REDV, immersed in its natural sequence to retain its efficiency. Finally, the polymer also contains elastase target sequences to favor its bioprocessability by natural routes. The elastase target sequence is the hexapeptide VGVAPG, which is found in natural elastin. This sequence is a target for specific proteases of the natural ECM. The leitmotif is that those proteases are only produced and excreted to the extracellular medium when the tissue requires that the natural ECM to be remodeled. In this sense, the presence of these specific sequences in the artificial polymer guarantees that the polymer is bioprocessed only when the growing tissue needs to substitute it by natural ECM, while, in practice, it remains fully functional until that moment. In addition, the activity of this domain is not restricted to being an inert target of protease activity. It is well known that these hexapeptides, as they are released by the protease action, have strong cell proliferation activity and other bioactivities related to tissue repair and healing [104].

Although we are sill far from exploiting the full potential of genetic engineering, this last example impressively shows that we now have the ability to create materials for tissue engineering with composition and (bio)functionality unprecedently closer to the rich complexity in functionality and bioactivity of the natural ECM. This polymer is also a good example that shows the potential of genetic engineering in producing complex polymers in general, since one can hardly imagine how to obtain polymers of the complex composition displayed in this last example by chemical methodologies, that in addition, would have to be as clean, cheap and easily scalable.

6. Conclusions

Although genetic engineering of protein-based materials has not reached maturity, it has already shown extraordinary potential. By this technique, really complex, well defined and tailored polymers can be obtained with properties covering a wide range. Examples can be found from bulk materials and fibers with extraordinary mechanical performance to the most advanced, functional, self-assembling and smart materials for biomedical uses and nano(bio) technology. The degree of complexity achievable, and the concurrent development of function, are really unparalleled by other techniques. Complexity can be carried to a limit where the concept of polymer itself vanishes, with the design and bioproduction of materials in which the monomer is getting bigger and more complex from design to design. We are approaching the protein concept in GEPBPs where, rather than a polymer made by the repetition of single relatively short monomers or a combination of them, we have a macromolecule without unwanted repetition. In that molecule the single amino acids are grouped within functional domains, which, in their turn, are arranged along the polymer chain in a well-defined molecular architecture, in which there is no space for randomness. In this way we can obtain an advanced material of which an unprecedented set of structural, physicochemical and biological functionalities are required. In addition the flexibility of bioproduction is so high that we can surely say that the achievable complexity of the GEPBPs, in terms of macromolecular sequence, is for the first time, not limited by any technological constraint but only by our imagination.

All the above is accomplished by a technology that, in the near future, could be a serious alternative to conventional polymer chemistry, particularly if we take into consideration environmental concerns.

Now that there is an intense debate on the way that oil will be progressively replaced as the main energy source, we must not forget that more than 200 millions Tm of crude are used yearly to produce ‘plastics’. Therefore, we will have to be ready to substitute for oil as the raw material for plastics and rubbers. Genetic engineering of protein-based materials is one of the most promising alternatives. By this clean procedure, we can produce cheap, complex materials

that could even outperform the efficiency of the existing petroleum-based polymers. GEPBPs are expanding the limits of macromolecular functionality to territories never before glimpsed.

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References

[1] Soong RK, Bachand GD, Neves HP, Olkhovets AG, Craighead HG, Montemagno CD. Powering an inorganic nanodevice with a biomolecular motor. Science 2000;24: 1555–8.

[2] Noji H, Yasuda R, Yoshida M, Kinosita K. Direct observation of the rotation of F1-ATPase. Nature 1997;386: 299–302.

[3] Yasuda R, Noji H, Kinosita K, Yoshida M. F1-ATPase is a highly efficient molecular motor that rotates with discrete 120 degrees steps. Cell 1998;93:1117–24.

[4] Yasuda R, Noji H, Yoshida M, Kinosita K, Itoh H. Resolution of distinct rotational substeps by submillisecond kinetic analysis of F1-ATPase. Nature 2001;410:898–904.

[5] Liu H, Schmidt JJ, Bachand GD, Rizk SS, Looger LL, Hellinga HW, et al. Control of a biomolecular motorpowered nanodevice with an engineered chemical switch. Nat Mater 2002;1:173–7.

[6] Cappello J. Genetic production of synthetic protein polymers. MRS Bull 1992;17:48–53.

[7] McGrath K, Kaplan DS, editors. Protein-based materials. Boston: Birkhaüser; 1997.

[8] Krejchi MT, Atkins EDT, Waddon AJ, Fournier MJ, Mason TL, Tirrell DA. Chemical sequence control of beta-sheet assembly in macromolecular crystals of periodic polypeptides. Science 1994;265:1427–32.

[9] Cappello J, Ferrari F. In: Mobley DP, editor. Plastics from microbes. Cincinnati, OH: Hanser/Gardner; 1994. p. 35.

[10] McMillan RA, Lee TAT, Conticello VP. Rapid assembly of synthetic genes encoding protein polymers. Macromolecules 1999;32:3643–8.

[11] Meyer DE, Chilkoti A. Genetically encoded synthesis of protein-based polymers with precisely specified molecular weight and sequence by recursive directional ligation: examples from the elastin-like polypeptide system. Biomacromolecules 2002;3:357–67.

[12] McPherson DT, Morrow C, Minehan DS, Wu JG, Hunter E, Urry DW. Production and purification of a recombinant elastomeric polypeptide, G-(VPGVG)19-VPGV, from Escherichia coli. Biotechnol Prog 1992;8:347–52.

[13] Prince JT, McGrath KP, Digirolamo CM, Kaplan DL. Construction, cloning, and expression of synthetic genes encoding spider dragline silk. Biochemistry 1995;34: 10879–85.

[14] Guda C, Zhang X, McPherson DT, Xu J, Cherry JH, Urry DW, et al. Hyperexpression of an environmentally friendly synthetic polymer gene. Biotechnol Lett 1995;17: 745–50.

[15] Lee J, Macosko CW, Urry DW. Elastomeric polypentapeptides cross-linked into matrixes and fibers. Biomacromolecules 2001;2:170–9.

[16] Girotti A, Reguera J, Arias FJ, Alonso M, Testera AM, Rodríguez-Cabello JC. Influence of the molecular weight on the inverse temperature transition of a model genetically engineered elastin-like pH-responsive polymer. Macromolecules 2004;37:3396–400.

[17] Meyer DE, Chilkoti A. Quantification of the effects of chain length and concentration on the thermal behavior of elastin-like polypeptides. Biomacromolecules 2004;5:846–51.

[18] Berg JM, Tymoczko JL, Stryer L. In: Biochemistry. 5th ed. New York: W.H. Freeman; 2002.

[19] Hansen BMS, Brewer J, Fahlman GG, Gibson BK, Ibata R, Limongi M, et al. The white dwarf cooling sequence of the globular cluster messier 4. Astrophys J 2002;574:155–8.

[20] Guth AH, Lightman AP. The inflationary universe: the quest for a new theory of cosmic origins. New York: Wesley; 1997.

[21] Elices M. Structural biological materials: design and structure-property pelationships. London: Elsevier Science; 2000.

[22] Hinman MB, Jones JA, Lewis RV. Synthetic spider silk: a modular fiber. Tibtech 2000;18:374–9.

[23] Vollrath F, Knight DP. Liquid crystalline spinning of spider silk. Nature 2001;410:541–8.

[24] Shao Z, Vollrath F. Surprising strength of silkworm silk. Nature 2002;418:741.

[25] Becker N, Oroudjev E, Mutz S, Cleveland JP, Hansma PK, Hayashi CY, et al. Molecular nanosprings in spider capture-silk threads. Nat Mater 2003;2:278–83.

[26] Tatham AS, Shewry PR. Elastomeric proteins: biological roles, structures and mechanisms. Tibs 2000;25:567–71.

[27] Gosline J, Lillie M, Carrington E, Guerette P, Ortlepp C, Savage K. Elastic proteins: biological roles and mechanical properties. Philos Trans R Soc B 2002;357:121–32.

[28] Lombardi EC, Kaplan DL. Preliminary characterization of resilin isolated from the cockroach, periplaneta americana. Mater Res Soc Symp Proc 1993;292:3–7.

[29] Urry DW, Hugel T, Seitz M, Gaub HE, Sheiba L, Dea J, et al. Elastin: a representative ideal protein elastomer. Philos Trans R Soc Lond B 2002;357:169–84.

[30] Urry DW. What sustains life? consilient mechanisms for protein-based machines and materials. New York: Springer; 2005.

[31] Urry DW. Deciphering engineering principles for the design of protein-based nanomachines, in protein-based nanotechnology. In: Renugopalakrishnan, V. Lewis, R., editors. Dordecht: Kluwer Academic Publishers, in press.
[32] Ayad S, Humphries M, Boot-Handford R, Kadler K, Shuttleworth A. The extracellular matrix facts book, facts book series. San Diego, CA: Academic Press; 1994.
[33] Urry DW, Luan C-H, Harris CM, Parker T. Protein-based materials with a profound range of properties and applications: the elastin ΔT_t hydrophobic paradigm. In: McGrath Kevin, Kaplan David, editors. Proteins and modified proteins as polymeric materials. Boston: Birkhäuser Press; 1997. p. 133–77.
[34] Tirrell DA. Mechanical properties of artificial protein matrices engineered for control of cell and tissue behavior. Macromolecules 2003;36:1553–8.
[35] Urry DW. Five axioms for the functional design of peptide-based polymers as molecular machines and materials—principle for macromolecular assemblies. Biopolymers 1998; 47:167–78.
[36] Lee J, Macosko CW, Urry DW. Phase transition and elasticity of protein-based hydrogels. J Biomater Sci Polym Ed 2001;12:229–42.
[37] Gowda DC, Parker TM, Harris RD, Urry DW. Synthesis, characterization and medical applications of bioelastic materials. In: Basava C, Anantharamaiah GM, editors. Peptides: desing, synthesis and biological activity. Boston: Birkhäuser; 1994. p. 81–111.
[38] Martino M, Perri T, Tamburro AM. Biopolymers and biomaterials based on elastomeric proteins. Macromol Biosci 2002;2:319–28.
[39] Urry DW. Molecular machines: how motion and other functions of living organism can result from reversible chemical changes. Angew Chem Int Edit Eng 1993;32: 819–41.
[40] San Biagio PL, Madonia F, Trapane TL, Urry DW. The overlap of elastomeric polypeptide coils in solution required for single-phase initiation of elastogenesis. Chem Phys Lett 1988;145:571–4.
[41] Urry DW. Physical-chemistry of biological free-energy transduction as demonstrated by elastic protein-based polymers. J Phys Chem B 1997;101:11007–28.
[42] Rodríguez-Cabello JC, Alonso M, Pérez T, Herguedas MM. Differential scanning calorimetry study of the hydrophobic hydration of the elastin-based polypentapeptide, poly(VPGVG), from deficiency to excess of water. Biopolymers 2000;54:282–8.
[43] Tanford C. The hydrophobic effect: formation of micelles and biological membranes. New York: Wiley; 1973.
[44] Pauling L, Marsh E. The structure of chlorine hydrate. Proc Natl Acad Sci USA 1952;38:112–6.
[45] Urry DW, Trapane TL, Prasad KU. Phase-structure transitions of the elastin polypentapeptide-water system within the framework of composition-temperature studies. Biopolymers 1985;24:2345–56.
[46] Manno M, Emanuele A, Martorana V, San Biagio PL, Bulone D, Palma-Vittorelli MB, et al. Interaction of processes on different length scales in a bioelastomer capable of performing energy conversion. Biopolymers 2001;59: 51–64.
[47] Alonso M, Arranz D, Reboto V, Rodríguez-Cabello JC. Effect of α-, β- and γ-cyclodextrins on the inverse temperature transition of the bioelastic thermo-responsive polymer poly(VPGVG). Macromol Chem Phys 2001;202: 3027–34.
[48] Reguera J, Lagaron JM, Alonso M, Reboto V, Calvo B, Rodriguez-Cabello JC. Thermal behavior and kinetic analysis of the chain unfolding and refolding and of the concomitant nonpolar solvation and desolvation of two elastin-like polymers. Macromolecules 2003;36:8470–6.
[49] Rodríguez-Cabello JC, Reguera J, Alonso M, Parker TM, McPherson DT, Urry DW. Endothermic and exothermic components of an inverse temperature transition for hydrophobic association by TMDSC. Chem Phys Lett 2004;388:127–31.
[50] Reading M. Modulated differential scanning calorimetry—a new way forward in materials characterization. Trends Polym Sci 1993;1:248–53.
[51] Wunderlich B, Androsch R, Pyda M, Kwon YK. Heat capacity by multifrequencies sawtooth modulation. Thermochim Acta 2000;348:181–90.
[52] Gill PS, Sauerbrunn SR, Reading M. Modulated differential scanning calorimetry. J Therm Anal 1993;40:931–9.
[53] Jorimann U, Widmann G, Riesen R. Temperature modulated DSC (TMDSC) applications and limits of phase information, cp determination and effect separation. J Therm Anal Calorim 1999;56:639–47.
[54] Menczel JD, Judovist L. Literature status on temperature-modulated differential scanning calorimetry. J Therm Anal 1998;54:419–36.
[55] Rodríguez-Cabello JC, Alonso M, Guiscardo L, Reboto V, Girotti A. Amplified photoresponse of a p-Phenylazobenzene derivate of elastin-like polymer by a-Cyclodextrin: the amplified ΔT_t Mechanism. Adv Mater 2002;14:1151–4.
[56] Alonso M, Reboto V, Guiscardo L, Martín AS, Rodríguez-Cabello JC. Spiropyran derivative of an elastin-like bioelastic polymer: photoresponsive molecular machine to convert sunlight into mechanical work. Macromolecules 2000;33: 9480–2.
[57] Alonso M, Reboto V, Guiscardo L, Mate V, Rodríguez-Cabello JC. Novel Photoresponsive p-Phenylazobenzene Derivative of an Elastin-like polymer with enhanced control of azobenzene content and without pH sensitiveness. Macromolecules 2001;34:8072–7.
[58] Yang G, Woodhouse KA, Yip CM. Substrate-facilitated assembly of elastin-like peptides: studies by variable-temperature in situ atomic force microscopy. J Am Chem Soc 2002;124:10648–9.
[59] Wright ER, Conticello VP. Self-assembly of block copolymers derived from elastin-mimetic polypeptide sequences. Adv Drug Deliver Rev 2002;54:1057–73.
[60] Lee TAT, Cooper A, Apkarian RP, Conticello VP. Thermoreversible self-assembly of nanoparticles derived from elastin-mimetic polypeptides. Adv Mater 2000;12:1105–10.

[61] Reguera J, Fahmi A, Moriarty P, Girotti A, Rodríguez-Cabello JC. Nanopore formation by self-assembly of the model genetically engineered elastin-like polymer $[(VPGVG)_2(VPGEG)(VPGVG)_2]_{15}$. J Am Chem Soc 2004; 126:13212–3.

[62] Herrero-Vanrell R, Rincón A, Alonso M, Reboto V, Molina-Martinez I, Rodríguez-Cabello JC. Self-assembled particles of an elastin-like polymer as vehicles for controlled drug release. J Controlled Release 2005;102:113–22.

[63] Nath N, Chilkoti A. Fabrication of a reversible protein array directly from cell lysate using a stimuli-responsive polypeptide. Anal Chem 2003;75:709–15.

[64] Nath N, Chilkoti A. Interfacial phase transition of an environmentally responsive elastin biopolymer adsorbed on functionalized gold nanoparticles studied by colloidal surface plasmon resonance. J Am Chem Soc 2001;123:8197–202.

[65] Nath N, Chilkoti A. Creating smart surfaces using stimuli responsive polymers. Adv Mater 2002;14:1243–7.

[66] Ciardelli F, Fabbri D, Pieroni O, Fissi A. Photomodulation of polypeptide conformation by sunlight in spiropyran-containing poly(L-glutamic acid). J Am Chem Soc 1989;111: 3470–2.

[67] Meyer DE, Kong GA, Dewhirst MW, Zalutsky MR, Chilkoti A. Targeting a genetically engineered elastin-like polypeptide to solid tumors by local hyperthermia. Cancer Res 2001;61:1548.

[68] Stayton PS, Hoffman AS, Murthy N, Lackey C, Cheung C, Tan P, et al. Molecular engineering of proteins and polymers for targeting and intracellular delivery of therapeutics. J Controlled Release 2000;65:203–20.

[69] Chilkoti A, Dreher MR, Meyer DE, Raucher D. Targeted drug delivery by thermally responsive polymers. Adv Drug Deliver Rev 2002;54:613–30.

[70] Meyer DE, Shin BC, Kong GA, Dewhirst MW, Chilkoti A. Drug targeting using thermally responsive polymers and local hyperthermia. J Controlled Release 2001;74:213–24.

[71] Dreher MR, Raucher D, Balu N, Colvin OM, Ludeman SM, Chilkoti A. Evaluation of an elastin-like polypeptide-doxorubicin conjugate for cancer therapy. J Controlled Release 2003;91:31–43.

[72] Kostal J, Mulchandani A, Chen W. Tunable biopolymers for heavy metal removal. Macromolecules 2001;34:2257–61.

[73] Cappello J, Crissman JW, Crissman M, Ferrari FA, Textor G, Wallis O, et al. In-situ self-assembling protein polymer gel systems for administration, delivery, and release of drugs. J Controlled Release 1998;53:105–17.

[74] Dinerman AA, Cappello J, Ghandehari H, Hoag SW. Solute diffusion in genetically engineered silk-elastinlike protein polymer hydrogels. J Controlled Release 2002;82:277–87.

[75] Dinerman AA, Cappello J, Ghandehari H, Hoag SW. Swelling behavior of a genetically engineered silk-elastinlike protein polymer hydrogel. Biomaterials 2002;23:4203–10.

[76] Nagarsekar A, Crissman J, Crissman M, Ferrari F, Cappello J, Ghandehari H. Genetic engineering of stimuli-sensitive silkelastin-like protein block copolymers. Biomacromolecules 2003;4:602–7.

[77] Megeed Z, Haider M, Li D, O'Malley BW, Cappello J, Ghandehari H. In vitro and in vivo evaluation of recombinant silk-elastinlike hydrogels for cancer gene therapy. J Controlled Release 2004;94:433.

[78] Ohgo K, Kurano TL, Kumashiro KK, Asakura T. Structure of the model peptides of bombyx mori silk-elastin like protein studied with solid state NMR. Biomacromolecules 2004;5: 744–50.

[79] Hyun J, Lee WK, Nath N, Chilkoti A, Zauscher S. Capture and release of proteins on the nanoscale by stimuli-responsive elastin-Like polypeptide 'Switches'. J Am Chem Soc 2004;126:7330–5.

[80] Trabbic-Carlson K, Liu L, Kim B, Chilkoti A. Expression and purification of recombinant proteins from Escherichia coli: comparison of an elastin-like polypeptide fusion with an oligohistidine fusion. Protein Sci 2004;13:3274–84.

[81] Meyer DE, Chilkoti A. Purification of recombinant proteins by fusion with thermally-responsive polypeptides. Nat Biotechnol 1999;17:1112–5.

[82] Frey W, Meyer DE, Chilkoti A. Dynamic addressing of a surface pattern by a stimuli-responsive fusion protein. Adv Mater 2003;15:248–51.

[83] Nath N, Hyun J, Ma H, Chilkoti A. Surface engineering strategies for control of protein and cell interactions. Surf Sci 2004;570:98–110.

[84] Kuwabara T, Nakamura A, Ueno A, Toda F. Inclusion complexes and guest-induced color changes of pH-indicator-modified.beta.-cyclodextrins. J Phys Chem 1994;98: 6297–308.

[85] Chokchainarong S, Fennema OR, Connors KA. Binding constants for complexes of α-cyclodextrin with L-phenylalanine and some related substrates. Carbohydr Res 1992; 232:161–9.

[86] Reguera J, Alonso M, Testera AM, López IM, Martín S, Rodríguez-Cabello JC. Effect of modified α, β, and γ-cyclodextrins on the thermo-responsive behavior of the elastin-like polymer, poly(VPGVG). Carbohydr Polym 2004; 57:293–7.

[87] Urry DW, Parker TM, Reid MC, Gowda DC. Biocompatibility of the bioelastic materials, poly(GVGVP) and its gamma-irradiation cross-linked matrix: summary of generic biological test results. J Bioact Compat Polym 1991;6: 263–83.

[88] Urry DW, Gowda DC, Harris CM, Harris RD. Bioelastic materials and the ΔT_t-mechanism in drug delivery. ACS 1994.

[89] Urry DW. Preprogrammed drug delivery systems using chemical triggers for drug release by mechanochemical coupling. Polym Mater Sci Eng 1990;63:329–36.

[90] Schmidt P, Dybal J, Rodriguez-Cabello JC, Reboto V. The role of water in the structural changes of poly(AVGVP) and poly(GVGVP) studied by FTIR and raman spectroscopy and *ab initio* calculations. Biomacromolecules 2005;6:697–706.

[91] Chilkoti A, Dreher MR, Meyer DE. Design of thermally responsive, recombinant polypeptide carriers for targeted drug delivery. Adv Drug Deliver Rev 2002;54:1093–111.

[92] Jiuliano RL. Signal transduction by cell adhesion receptors and the cytoskeleton: functions of integrins, cadherins, selectins, and immunoglobulin-superfamily members. Annu Rev Pharmacol Toxicol 2002;42:283–323.
[93] Sternlicht MD, Werb Z. How matrix metalloproteinases regulate cell behavior. Annu Rev Cell Dev Biol 2001;17: 463–516.
[94] Urry DW, Nicol A, Gowda DC, Hoban LD, McKee A, Williams T, et al. Medical applications of bioelastic materials. In: Gebelein CG, editor. Biotechnological polymers: medical, pharmaceutical and industrial applications. Atlanta, GA: Technomic Publishing Co. Inc.; 1993. p. 82–103.
[95] Nicol A, Gowda DC, Parker TM, Urry DW. Cell adhesive properties of bioelastic materials containing cell attachment sequences. In: Gebelein C, Carraher C, editors. Biotechnology and bioactive polymers. New York: Plenum Press; 1994.
[96] Urry DW, Pattanaik A, Xu J, Woods TC, McPherson DT, Parker TM. Elastic protein-based polymers in soft tissue augmentation and generation. J Biomater Sci Polym Ed 1998;9:1015–48.
[97] Panitch A, Yamaoka T, Fournier MJ, Mason TL, Tirrell DA. Design and biosynthesis of elastin-like artificial extracellular matrix proteins containing periodically spaced fibronectin CS5 domains. Macromolecules 1999;32:1701–3.
[98] Welsh ER, Tirrell DA. Engineering the extracellular matrix: a novel approach to polymeric biomaterials. I. Control of the physical properties of artificial protein matrices designed to support adhesion of vascular endothelial cells. Biomacromolecules 2000;1:23–30.
[99] Nowatzki PJ, Tirrell DA. Physical properties of artificial extracellular matrix protein films prepared by isocyanate crosslinking. Biomaterials 2004;25:1261–7.
[100] Liu JC, Heilshorn SC, Tirrell DA. Comparative cell response to artificial extracellular matrix proteins containing the RGD and CS5 cell-binding domains. Biomacromolecules 2004;5: 497–504.
[101] Richman GP, Tirrell DA, Asthagiri AR. Quantitatively distinct requirements for signaling-competent cell spreading on engineered versus natural adhesion ligands. J Controlled Release 2005;101:3–12.
[102] Heilshorn SC, Liu JC, Tirrell DA. Cell-binding domain context affects cell behavior on engineered proteins. Biomacromolecules 2005;6:318–23.
[103] Girotti A, Reguera J, Rodríguez-Cabello JC, Arias FJ, Alonso M, Testera AM. Design and bioproduction of a recombinant multi(bio)functional elastin-like protein polymer containing cell adhesion sequences for tissue engineering purposes. J Mater Sci Mater Med 2004;15: 479–84.
[104] Alix AJ. A turning point in the knowledge of the structure-function-activity relations of elastin. J Soc Biol 2001;195: 181–93.