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Genetic Engineering of Protein-Based Polymers: The Example of Elastinlike Polymers

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Abstract In spite of the enormous possibilities of macromolecules as key elements in developing advanced materials with increased functionality and complexity, the success in this development is often limited by the randomness associated with polymer synthesis and the exponential increase in technical difficulties caused by the attempt to reach a sufficiently high degree of complexity in the molecular design. This paper describes a new approach in the design of complex and highly functional macromolecules, the genetic engineering of protein-based macromolecules. The exploitation of the efficient machinery of protein synthesis in living cells opens a path to obtain extremely well-defined and complex macromolecules.

Different molecular designs are presented, with increasing degree of complexity, showing how the controlled increase in their complexity yields (multi)functional materials with more select and sophisticated properties. The simplest designs show interesting properties already, but the adequate introduction of given chemical functions along the polymer chain presents an opportunity to expand the range of properties to enhanced smart behavior and self-assembly. Finally, examples are given where those molecular designs further incorporate selected bioactivities in order to develop materials for the most cutting-edge applications in the field of biomedicine and nano(bio)technology.

Keywords Elastinlike polymers · Genetic engineering · Protein-based polymers · Self-assembly · Smart polymers

1 Introduction

1.1 The Present and Future Global Challenges of Polymer Science

In recent decades, polymer science has definitively shown that macromolecules can be excellent candidates to create highly functional materials. With the availability of thousands of different monomers and the possibilities opened by their different combinations, polymer science has succeeded on many occasions when a material was needed for a particular application, from the simplest uses as bulk commodities to the most sophisticated and special biomedical, engineering, or nanotechnological ones. Very few other technical developments in history have shown both the rapid development and deep societal impact of polymer science. Currently, the number of different technologies enabled by the existence of the adequate polymer is amazing, and the crucial role of polymer science in the current stage of societal development and well-being is beyond question.

Up till now, when a new development was required from polymer science, it has been possible to design and obtain a new polymer fitting that particular requirement. The most challenging tasks for polymer science were rather of a more logistical nature than scientific. For example, a reduction in the number of different polymers used in practice to cover the whole range of consumer demands (in order to simplify and make more profitable their manufacture) has been and still is the cause of important research efforts. Additionally, environmental and other related matters, such as sustainability, have also been addressed, but they have always remained in the background and have not significantly limited the development of polymer science.

However, this situation started to change a couple decades ago. At that time, the concepts of self-assembly and hierarchical organization, as well as others such as "smartness," began to awake extended interest within the polymer science community and boosted expectations for new applications. The

deeper knowledge on the physical-chemical basis of the high functionality of those pioneer polymers triggered a rapid scaleup in the complexity of new designs as well as the need for controlling their composition.

However, all methodologies of polymer synthesis are characterized by an unavoidable component of randomness and lack of control. This is especially true for the classical radical, cationic, or ring-opening polymerizations, where, even in the simplest polymers, it is not possible to control parameters such as the degree of polymerization. We are used to considering this as a mean value bearing a statistical meaning. Generally, the information given by the mean molecular weight needs to be completed by a polydispersity index in order to quantify how broad or narrow is the molecular weight distribution of our polymer. In the case of copolymers, we are also used to dealing with random copolymers, although, with some effort, we can prepare alternating or block copolymers.

However, the design of highly functional polymers unavoidably means the design of complex molecules and a tight control in their synthesis.

New discoveries in the area of catalysis and controlled polymerizations with the work of Matyjaszewski, Hawker, Waymouth, Coates, Deming, and many more [1–5] have allowed us to keep pace, but with the impression that the demands are growing faster than the achievements. This could be a signal that in the future conventional polymer science could reach one of its most critical limits; polymer chemistry could be overwhelmed by the demands of the new polymer designs. Additionally, in the existing technologies of polymer synthesis, unavoidably, there is an exponential relation between the cost (money and time) needed to synthesize a polymer and the complexity of its primary structure. But that is a matter not only of costs. As the complexity increases, the synthesis methods and protocols become less and less robust and more and more difficult to scale up, preventing, to a large extent, its commercial exploitation. Presently, although we already have the knowledge needed to design advanced polymers envisaged as possessors of extraordinary properties, the frustrating fact is that they are very difficult to synthesize in practice (Fig. 1).

In addition, perhaps this is not the only limiting condition in the current state of development of polymer science. The field could be facing an additional crucial problem in the middle or long term. Most synthesis methodologies and the polymers we currently produce are based exclusively on petroleum-derived chemicals. It is estimated that more than 200 million Tm of crude are used yearly as raw material to produce plastics and rubbers, while an equivalent amount of oil is burned to generate the energy needed for their synthesis. As a source of materials, oil is not renewable. Although there is no consensus about the level of oil reserves, it is clear that this resource is not infinite and that its price will likely continue to increase if we keep our increasing rate of demand. Additionally, perhaps we do not have to wait until the imminent exhaustion of oil reserves to reduce oil's use as a source of en-

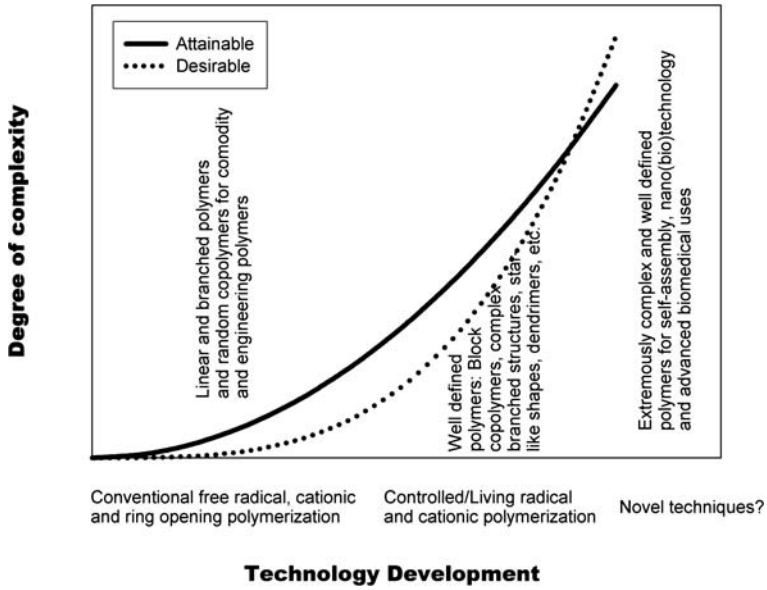

Fig. 1 Evolution of polymer synthesis methodologies and of demand by polymer designers

ergy and plastics. The growing evidence that the increase in the atmospheric CO_2 level is causing a palpable modification of the global climate [6] could lead, in the middle or long term, to abandon, or at least reduce drastically, oil as our main source of raw materials for plastics.

The above-described scenario is, obviously, unpleasant. However, as has happened before, when a technology arrives at a bottleneck, there could exist an alternative way to break through the impasse. This paper is devoted to gathering arguments in favor of one of those possible alternative routes: the genetic engineering of protein-based polymers (GEPBPs). By this approach, evidences on the possibility of obtaining very complex and highly functional polymers, well beyond the reach of the present chemical methods of synthesis and from exclusively renewable sources, will be presented.

1.2 Biological Macromolecules: The Lesson from Nature

Biology discovered long ago that macromolecules are the best option for obtaining highly functional materials. Novel concepts in materials science such as hierarchical organization, mesoscale self-assembly, or smartness are common to many natural macromolecules such as proteins, nucleic acids, and polysaccharides (or combinations thereof). In fact, the slow but implacable process of natural selection has produced materials showing a level of functionality that is really much higher than the level we have reached in our syn-

thetic materials. One of the best (and nicest) examples is proteins. Proteins in living cells show an amazing set of capabilities in terms of functionality. From structural proteins, all of them showing acute self-assembly capabilities, to extraordinary enzymes, with their superior catalytic performance and highly efficient molecular machines (flagellar rotary motor, etc.), examples abound. Natural proteins are usually large and very complex molecules containing diverse specific functional groups to generate and direct self-assembly and function. Nature makes use also of different physical processes that allow for 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.

Nevertheless, all of this amazing functionality displayed by natural proteins seems to be based on a simple fact: a complex and completely defined primary structure. In living cells, protein biosynthesis is carried out with an absolute control of the amino acid sequence, from the first amino acid to the last with a complete absence of randomness. In fact, the need for this absolute control is dramatically clear in some genetic disorders in which the lack or a substitution of a single amino acid in the whole protein leads to a complete loss of the original function, which can have dramatic consequences in some cases such as falciform anemia (sickle cell anemia), phenylketonuria, and cystic fibrosis [7].

Herein lies the lesson that, if we want to create really functional materials, we must find a way to synthesize complex and completely defined macromolecules. This task, which completely overwhelms our most sophisticated chemical methods, is taking place incessantly in all living cells. One more characteristic of protein biosynthesis deserves mention. The protein biosynthesis machinery is extraordinarily flexible. Ribosomes are able to process and produce practically any amino acid sequence stored in the holders of information called genes, so its flexibility is nearly absolute. Therefore, for practical purposes, it is interesting to realize that if one controls the information that genes deliver to the machinery, then one completely controls the biosynthesis process itself.

2
Genetic Engineering of Protein-Based Polymers: The "Gutenberg Method" in Polymer Design and Production

Due to current developments in molecular biology, we have for the first time the ability to create almost any DNA duplex codifying 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 codified protein-based polymer (PBP) as a recombinant protein [8–16]. 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. This approach has many advantages.

First, as our knowledge of the protein-function relationship continues to grow, GEPBPs will be able to show any function or property, simple or complex, present in natural proteins. In this sense, this method opens the opportunity for exploiting the huge amounts of resources, in terms of functionality, hoarded and refined in a very efficient manner over the long course of natural selection. GEPBPs easily make use of the vast amounts of functional wealth present in the hundreds of thousands of different proteins in living organisms in the widest sense, from the smallest prions or viruses to highly complex animals.

On the other hand, as we can construct the codifying gene base by base following our own original designs and without being restricted to genes of fragments found in living organisms, we can design and produce GEPBPs to obtain materials, systems, and devices exhibiting a function not displayed in living organisms but of a particular technological interest [17].

Third, from the point of view of a polymer chemist, the degree of control and complexity attained by genetic engineering is clearly superior to those achieved by any present chemical synthesis technologies. GEPBPs are strictly monodisperse, while they can be obtained from a few hundred daltons to more than 200 kDa, and these limits are continuously expanding [18]. Among other things, this has opened the possibility of studying, in a simple and highly precise manner, the dependence of different material properties on the molecular weight (MW) [19, 20], and this knowledge also opens the possibility of finely tuning those properties in the designed materials. In addition, although monodispersity is not an important requirement for bulk polymers—it is even desirable in some cases—it clearly enhances the chances of success in designing materials with self-assembling and smart behavior [21].

Although, as discussed above, the increase in complexity of conventional polymers unavoidably means an exponential increase in time and cost of production, this relationship is not fulfilled by GEPBPs. Paradoxically, experience constantly shows that the enzymes and all other techniques of molecular biology that are used for the construction of synthetic genes as well as all the molecular machinery implicated in protein biosynthesis work better with complex GEPBPs than with simple and highly repetitive GEPBPs. Biological systems are adapted to build complex natural proteins, so they feel more comfortable in an environment of complexity. Therefore, for GEPBPs, there is not a clear and direct relationship between production yield and polymer complexity. In practice, usually complexity is more feasible than simplicity. In addition, the cost of production of GEPBPs is not related to their complexity. By this approach, the most costly task in terms of time and money is gene

construction. However, once the genetically modified (micro)organism is obtained, the fast, robust, and cheap GEPBP production readily compensates the costs associated with the molecular biology steps.

In addition, in contrast to conventional polymers, where the raw materials are the monomers, the raw materials employed in GEPBP biosynthesis are not the amino acids themselves. Recall that protein synthesis in living cells is inserted within a dense and complex metabolic network, by which many simple, renewable, and cheap sources of carbon and nitrogen can be finally converted into the needed amino acids and, finally, to the desired GEPBP.

Fourth, the number of different combinations attainable by combining the 20 natural amino acids is virtually infinite, so the number of different GEPBPs that can be obtained seems to be more than enough.

Somehow, this situation recalls the time when Johannes Gutenberg began building his press (in 1436). At that time, 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 position to apply this concept to polymer production (the "Gutenberg Method"). If we want to obtain several identical batches of a sophisticated and complex polymer, we should not direct our main effort to building the polymer itself but to building the gene that codifies it. Then, polymer production can be done by expressing the gene in a cellular factory. Thus, these cells play the role of the press in book printing.

Although this list does not pretend to be exhaustive, the final advantage mentioned here stresses 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 as the exclusive solvent in most GEPBPs produced to date. GEPBPs are obtained by an easily scalable technology, fermentation, that uses moderate amounts of energy and temperatures. Additionally, a main goal in the production of GEPBPs is their production in genetically modified plants. In this way, there is even no need for fermentation facilities, which reduce significantly the productions costs, while this could be a way to help revitalize the agriculture sectors in many countries.

It is not easy to imagine clear disadvantages of GEPBPs vs. petroleum-based polymers because even the differential in production costs is decreasing rapidly on one hand due to the progressive increase in bioproduction yields and the possibilities opened by using genetically modified plants instead of microorganisms, and the continuous increase in oil prices on the other hand. Perhaps, as polymer scientists, the first thing to come to mind would be that conventional polymer science has produced thousands of different useful monomers. Therefore, the possibilities opened by this high number of petroleum-based monomers, in terms of availability of function,

seems to be overwhelming if we consider that, in designing GEPBPs, we must restrict ourselves to just the 20 natural amino acids. However, this reasoning could also be fallacious if we paid attention to nature once more. It is unquestionable that no synthetic material matches the exquisite and 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 find proteins that match and clearly outperform the mechanical properties of our best petroleum-based polymers. For example, some kinds of spider silks, such as the *Nephila clavipes* dragline, show a superior strength [22, 23]. An *N. clavipes* dragline silk shows a Young's modulus, tensile strength, and stress at break of the same order of Kevlar, which is a benchmark of modern polymer fiber technology but absorbs almost one order of magnitude more energy than Kevlar when breaking [22–24]. In fact, their mechanical properties can be considered above those of steel itself. Its absorbed energy at breaking point is almost two orders of magnitude higher, while its tensile strength is almost six times higher and the stresses at breaking point are equivalent [22–24]. Additionally, although the Young's modulus of steel is about three times higher than the spider-silk modulus, this last material has a much lower density. Its ratio of tensile strength to density is perhaps five times better than steel. Therefore, at 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.

Spider silks deserve additional commentary. Again, this example shows as that Nature never gives up to complexity, as if complexity were an intrinsic part of natural materials, and this is so even in these apparently simple materials that Nature has designed just to reach a given mechanical performance. 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. Among elastic protein fibers, Nature shows us examples covering a wide range of elastomeric properties. Again, we find other kinds of spider silks, such as flagelliform silks, that show elastomeric behavior with the ability to withstand high levels of elastic strain; such silks can be extended up to $\sim 200\%$ without breaking, but they also show a high rate of energy dissipation [22, 26]. This is well known in flying insects that collide with spider webs; the insects, in spite of their high kinetic energy, very rarely are able to break through the webs. On the contrary, this impact energy is absorbed without catapulting the insect out of the web [22, 26]. In addition, once trapped, they find that breaking the web is a very exhausting and hopeless task.

In contrast, other elastic proteins show precisely the opposite property, i.e., they dissipate a negligible amount of energy in a stress-strain cycle or, equiv-

alently, 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 of the swimming bivalves. Also, elastin has been claimed to show and almost ideal elasticity [29]. All these elastic proteins are characterized by high resilience, large strain, and low stiffness [27].

The nearly ideal elasticity of some proteins or some of their functional domains has been identified recently as being a 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 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 being the universal principle of functioning of biological protein-based machines and has been identified with biology's vital force (*élan vital*). The model for protein function based on this mechanism has already been postulated for key molecular machines of the cell, such as the complex III in the mitochondrial electron transport chain that produces a proton gradient, the F1 motor of the 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 can also include keratins. This protein shows a superior impact resistance with a Young's modulus of 2.50 GPa [22]; not for nothing is it the main component of hoofs, beaks, and horns. Again, this protein shows multifunctional character and complexity because keratin is also the main component of feathers, a prodigy of rigidity and lightness.

Although this list could be extended ad infinitum with many other fascinating examples, such as collagen and others, just one more example will be mentioned: mussel adhesives. Mussel adhesive proteins are remarkable materials that display an extraordinary ability to adhere to almost any kind of natural or artificial substrate, and, in addition, they do so in extreme conditions. The environments where these proteins show their functionality are underwater (in salty water, for instance) and standing continuous and changing stresses (waves, tides, underwater flows, etc.). No artificial adhesive is able to work, even minimally, under those circumstances. It is important to emphasize that this kind of environment is not much different than the one found, for example, inside living tissues. For that reason, recent investigations 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 exclusive primary source to build polymers could be enough to de-

sign materials with extraordinary properties, even in the less complex sense of bulk materials. It could be even extended with the recent progress in the development of methods for incorporating nonnatural amino acids into recombinant proteins that can be an alternative strategy for extending GEPBPs with diverse chemical, physical, and biological properties [32]. Therefore, the properties of GEPBPs span a broad range in all directions, from the simplest mechanical properties to the most complex, smart, and self-assembling characteristics. Practically all the properties displayed by petroleum-based polymers are within this range. Thus from the technological point of view, the possibility of obtaining many different materials with a wide range of properties that outperform existing polymers, are obtained by only one common basic technology, and in addition show clear environmental advantages, is a highly interesting scenario.

3
State of the Art in GEPBPs

Presently, genetic engineering of PBPs is still in its early infancy. The radically different approach in the methodology used to produce these polymers has resulted in the fact that, even now, a limited number of research groups and companies have made the effort to make this transition. Among these pioneer groups, the main interest has been mainly concentrated in two major polymer families: spider-silk-like polymers and elastin-like polymers ("ELPs"), although some other interesting protein polymers have also been researched. Those include coiled-coil motifs and their related leucine zippers [33–36], β-sheet-forming polymers [37], poly(alylglicine) [38], and homopolypeptides such as poly(glutamic acid) [39].

The different strategies and methodologies for gene construction, iterative, random, and recursive ligations, have been summarized recently [13, 40, 41].

4
Silklike Polymers

Silks are fibrous proteins produced by spiders and insects such as the silk worm (*Bombyx mori*). There are an astonishing variety in different mechanical properties and compositions of the different silks naturally produced. Many spiders and insects have a varied tool kit of task-specific silks with divergent mechanical properties [42–49]. Those silks seem to have evolved to match a very particular need for the creature that produces them. Furthermore, although some spiders may use silk sparingly, most make rather elaborate nests, traps, and cocoons typically using more than one type of finely tuned and specialized silk. Those different silks are

produced by a wide and diverse range of glands, ducts, and spigots. However, in spite of the extraordinary physical properties of spider silks as well as the enormous variety there is only limited information on the composition of the various silks produced by different spiders. Among the different types of spider silks, draglines from the golden orb weaver *Nephila clavipes* and the garden cross spider *Araneus diadematus* are most intensely studied.

Based on DNA analysis it could be shown that all spider silk proteins are chains of iterated peptide motifs ("repeating units"). The small peptide motifs can be grouped into four major categories: GPGXX (with X often representing Q), alanine-rich stretches [An or (GA)n], GGX, and spacers. A fifth category is represented by nonrepetitive (NR) regions at the amino and carboxyl termini of the proteins, often representing polypeptide chains of 100 or more amino acids [48–56].

On the basis of several studies, the major categories of peptide motifs in spider silk proteins have been assigned structural roles [57–61]. It has been suggested that the GPGXX motif is involved in a β-turn spiral, probably providing elasticity, based on structures of comparable proteins [62–65]. If elasticity is due to GPGXX β-spirals, then this motif should be found in the more elastic silks. Flagelliform silks, which show the highest elasticity with more than 200%, consist of contiguous repeats of this motif at least 43 times in each repeating unit. Alanine-rich motifs typically contain 6–9 alanine residues and have been found to form crystalline β-sheet stacks leading to tensile strength [23, 57, 58]. The major and minor ampullate silks are both very strong, and at least one protein in each silk (there are always pairs) contains the $(A)_n$ or $(GA)_n$ motif. Interestingly, this motif is not found in flagelliform silks. A glycine-rich 31-helix is adopted by the GGX motif forming an amorphous matrix that connects crystalline regions and that provides elasticity [49, 66, 67]. The postulated GGX motif is widely distributed and this motif can be found in major and minor ampullate and flagelliform silks. Several groups have suggested that the GPGXX and GGX motifs might be involved in forming an amorphous matrix, which would provide the elasticity of the fiber. The spacers contain charged groups and separate the iterated peptide motifs into clusters. NR termini are common to all sequenced major and minor ampullate and flagelliform silks belonging to the Araneoidea family with highly conserved carboxyl-terminal sequences [53, 68, 69].

Regarding genetically engineered silklike polymers (GESLPs), they have been mainly restricted to those designed on the repetition of the sequences [GGAGQGGYGGLGSQ-GAGRGGLGGQGGAG] and [GPGGYGGPGQQGPGGY APGQQPSGPGS] from the silk produced by the *N. clavipes* major ampullate glands 1 and 2, respectively. Some modifications of those base sequences have also been explored. In the first instance, some of them were used to control the degree of crystallinity as a way to improve the processability of those polymers. However, some other modifications have been

Table 1 Selected examples of GEPBPs

Polymer structure	Type	Properties/Uses	Ref.
$(GVGVP)_n$	ELP	Model polymer, prevention of adhesions	[73]
$(GVGVAP)_n$	ELP	Model polymer	[73]
$(GVGIP)_n$	ELP	Model polymer	[74]
$(GGIP)_n$	ELP	Model polymer	[75]
$(GGAP)_n$	ELP	Model polymer	[75]
$(GVGVP\ GVGVP\ GKGVP\ GVGVP\ GVGVP\ GVGVP)_n$	ELP	Model polymer	[76]
$(GVGVP\ GVGFP\ GKGFP\ GVGVP\ GVGVP\ GVGVP)_n$	ELP	Model polymer, drug delivery	[76]
$(GVGVP\ GVGVP\ GKGVP\ GVGVP\ GVGFP\ GFGFP)_n$	ELP	Model polymer, drug delivery	[76]
$(GVGVP\ GVGFP\ GKGFP\ GVGVP\ GVGFP\ GVGFP)_n$	ELP	Model polymer, drug delivery	[76]
$(GVGVP\ GVGFP\ GKGFP\ GVGVP\ GVGFP\ GFGFP)_n$	ELP	Model polymer, drug delivery	[76]
$(GVGVP\ GVGVP\ GEGVP\ GVGVP\ GVGVP\ GVGVP)_n$	ELP	Model polymer, drug delivery	[31]
$(GVGVP\ GVGFP\ GEGFP\ GVGVP\ GVGVP\ GVGVP)_n$	ELP	Model polymer, drug delivery	[31]
$(GVGVP\ GVGVP\ GEGVP\ GVGVP\ GVGFP\ GFGFP)_n$	ELP	Model polymer, drug delivery	[31]
$(GVGVP\ GVGFP\ GEGFP\ GVGVP\ GVGFP\ GVGFP)_n$	ELP	Model polymer, drug delivery	[31]
$(GVGVP\ GVGFP\ GEGFP\ GVGVP\ GVGFP\ GFGFP)_n$	ELP	Model polymer, drug delivery	[31]
$[(GVGVP)_2\ GGGVP\ GAGVP\ (GVGVP)_3\ GGGVP\ GAGVP\ GGGVP]_n$	ELP	Model polymer	[77]
$[(GVGVP)\ (GAGVP\ GGGVP)_7\ GAGVP]_n$	ELP	Model polymer	[77]
$[VPGKG(VPGVG)_m]_n$	ELP	Model polymer	[78]
$[VPGQG(VPGVG)_6]_n$	ELP	Model polymer	[79]
$[(GVGVP)\ (GAGVP\ GGGVP)_7\ GAGVP]_4$-$(GVGVP)_{60}$	ELP	Model polymer	[40]

Table 1 (continued)

Polymer structure	Type	Properties/Uses	Ref.
$[(GVGVP)_2$ GGGVP GAGVP $(GVGVP)_3$ GGGVP GAGVP GGGVP]-doxorubicin	ELP-Protein	Purification of recombinant proteins and cancer therapy	[80]
$[(GVGVP)_2$ GGGVP GAGVP $(GVGVP)_3$ GGGVP GAGVP GGGVP$]_n$-Trx (*Trx: thioredoxin*)	ELP-Protein	Purification of proteins, and capture release of proteins by stimuli responsive switches	[81]
$[(GVGVP)_2$ GGGVP GAGVP $(GVGVP)_3$ GGGVP GAGVP GGGVP$]_n$-CAT (*CAT: acetyltransferase*)	ELP-Protein	Purification of proteins	[82]
$[(GVGVP)_2$ GGGVP GAGVP $(GVGVP)_3$ GGGVP GAGVP GGGVP$]_n$-BFP (*BFP: blue fluorescent protein*)	ELP-Protein	Purification of proteins	[82]
$[(GVGVP)_2$ GGGVP GAGVP $(GVGVP)_3$ GGGVP GAGVP GGGVP$]_n$-CalM (*CalM: calmodulin*)	ELP-Protein	Purification of proteins	[82]
$[(GA)_nGE]_m$	Silklike	High performance fibers and biomaterials	[83]
$[(GEEIQIGHIPREDVDYHLYP)(GVPGI)_m]_n$	ELP	Tissue engineering	[84]
$[LD(GEEIQIGHIPREDVDYHLYP)G((VPGIG)_2VPGKG(VPGIG)_2)_4VP]_n$	ELP	Tissue engineering	[85]
$[(AG)_3PEG]_n$	alanylglycine-rich	Biomaterials	[86]
$[(AG)_4$ PEG$]_n$	alanylglycine-rich	Biomaterials	[86]
$[E_{17}D]_n$	Rodlike-polymer	Biomaterials	[39]
MRGSHHHHHHGSDDDDKWA-Helix-IGDHVAPRDTSMGGC (Helix: SGDLENEVAQLEREVRSLEDEAAELEQKVSR LKNEIEDLKAE)	Leucine zipper	Biomaterials and reversible hydrogels	[87]

Table 1 (continued)

Polymer structure	Type	Properties/Uses	Ref.
MRGSHHHHHHGSDDDDKASYRDPMG-$[(AG)_3PEG]_{10}$-ARMPTSW	alanylglycine-rich	Biomaterials	[87]
MRGSHHHHHHGSDDDDKWA-Helix-IGKHVAPRDTSYRDPMG-$[(AG)_3PEG]_{10}$- ARMPTSD-Helix- IGDHVAPRDTSMGGC	Leucine zipper-alanylglycine-rich	Biomaterials and reversible hydrogels	[87]
$[(PGVGV)_2(PGEGV)(PGVGV)_2]_n$	ELP	Model polymer	[19]
$[(VPGIG)_2(VPGKG)(VPGIG)_2$(EEIQIGHIPREDVDYHLYP)$(VPGIG)_2(VPGKG)$ $(VPGIG)_2(GVGVAP)_3]_n$	ELP	Tissue Engineering	[88]
$[(GVGVP)_4GKGVP(GVGVP)_3(GAGAGS)_4]_n$	Silk-ELP	High performance fibres and biomaterials	[89]
$[(GVGVP)_4GEGVP(GVGVP)_3GAGAGS]_n$	Silk-ELP	High performance fibres and biomaterials	[90]
$[(GVGVP)_8GAGAGS]_n$	Silk-ELP	High performance fibres and biomaterials	[90]
$[(GAGAGS)_9GAAVTGRGDSPASAAGY]$	Pronectin F^{TM}	Tissue engineering	[70]
$[(GAGAGS)_2(GVGVP)_4(GKGVP)(GVGVP)_3]_n$	Silk-ELP	High performance fibres and biomaterials	[91]
$[(GAGAGS)_3(GVGVP)_4(GKGVP)(GVGVP)_3]_n$	Silk-ELP	High performance fibres and biomaterials	[91]
$[(GAGAGS)_4(GVGVP)_4(GKGVP)(GVGVP)_3]_n$	Silk-ELP	High performance fibres and biomaterials	[91]
$[(GAGAGS)_4$ $(GVGVP)_8]_n$	Silk-ELP	High performance fibres and biomaterials	[91]
$[(GAGAGS)_8(GVGVP)_{16}]_n$	Silk-ELP	High performance fibres and biomaterials	[91]
$[(VPGVG)_4(VPGKG)]_n$	ELP	Model Polymer	[12]
$[(VPGVG)_4(VPGIG)]_n$	ELP	Model Polymer	[12]
$[(GPGGSGPGGY)_2GPGGK]_n$	Silk	High performance fibres and biomaterials	[92]
$[VPGEG(IPGAG)_4]_{14}$-$[VPGFG(IPGVG)_4]_{14}$	ELP (Block Copolymer)	Drug Delivery	[93]

Table 1 (continued)

Polymer structure	Type	Properties/Uses	Ref.
$[(APGGVPGGAPGG)_2]_x$-$[VPAVG(IPAVG)_4]_{16}$	ELP (Block Copolymer)	Drug Delivery	[93]
$[VPAVG(IPAVG)_4]_{16}$-$[VPGVG(IPGVGVPGVG)_2]_{19}$-$[VPAVG(IPAVG)_4]_{16}$	ELP (Block Copolymer)	Drug Delivery	[93]
$[VPAVG(IPAVG)_4]_{16}$-$[VPGEG(VPGVG)_4]_{30}$-$[VPAVG(IPAVG)_4]_{16}$	ELP (Block Copolymer)	Drug Delivery	[93]
$[VPAVG(IPAVG)_4]_{16}$-$[VPGEG(VPGVG)_4]_{48}$-$[VPAVG(IPAVG)_4]_{16}$	ELP (Block Copolymer)	Drug Delivery	[93]
$[VPAVG(IPAVG)_4]_{16}$-$[(APGGVPGGAPGG)_2]_{22}$-$[VPAVG(IPAVG)_4]_{16}$	ELP (Block Copolymer)	Drug Delivery	[93]
$[VPAVG(IPAVG)_4]_{16}$-$[(VPGMG)_5]_x$-$[VPAVG(IPAVG)_4]_{16}$	ELP (Block Copolymer)	Drug Delivery	[93]
$\{VPAVG[(IPAVG)_4(VPAVG)]_{16}IPAVG\}$ - $VPGVG[(VPGVG)_2VPGEG[(VPGVG)_2]_{48}VPGVG$ - $\{VPAVG[(IPAVG)_4(VPAVG)]_{16}IPAVG\}$	ELP (Block Copolymer)	Drug Delivery	[94]
$\{VPAVG[(IPAVG)_4(VPAVG)]_{16}IPAVG\}$ - VPGVG VPGVG - $\{VPAVG[(IPAVG)_4(VPAVG)]_{16}IPAVG\}$	ELP (Block Copolymer)	Drug Delivery	[94]
$(AG)_6(VPGVG)(AG)_7$	Silk-ELP	High performance fibres and biomaterials	[95]
$(AG)_5(VPGVG)_2(AG)_5$	Silk-ELP	High performance fibres and biomaterials	[95]
$(AG)_n$	Silk	High performance fibres and biomaterials	[95]
$(GGAGSGYGGGYGHGYGSDGG(GAGAGS)_3)_4$	Silkworm	High performance fibres and biomaterials	[96]
$AS(A)_{18}TS(GVGAGYGAGAGYGVGAGYGAGVGYG$ AGAGY)TS	Silkworm	High performance fibres and biomaterials	[96]

Table 1 (continued)

Polymer structure	Type	Properties/Uses	Ref.
$[(GPGGYGPGQQ)_2GPSGPGSA_8]_n$	Silk	High performance fibres and biomaterials	[23]
$[GGAGQGGYGGLGGQGAGRGGLGGQ(GA)_2A_5]_n$	Silk	High performance fibres and biomaterials	[23]
[(A GQG GYS GLS SQG)(A GQG GYG GLG SQH A GRG GLG GQG A GAAAAAAAGG) (A GQG GLG SQH A GQG A GAAAAAA GG) (A GQG GYG GLG SQH A GRG GQG A GAAAAAA GG)]$_n$	Silk	High performance fibres and biomaterials	[97]
[(GPGGY GPGQQ GPGGY GPGQQ GPGGY GPQGG GPSGPGS AAAAAA)(GPGQQ GPGGY GPGQQ GPGGY GPQGG GPSGPGS AAAAAAAA)(GPGGY GPGQQ GPGGY GPQGG GPSGPGS AAAAAAAAAA)]$_n$	Silk	High performance fibres and biomaterials	[97]
$(AEAEAKAKAEAEAKAK)_9$	β –sheet fibres	High performance fibres and biomaterials	[37]
$(GGP)_n$-collagen	Fusion polymer	Tissue engineering	[98]
$(VPGVG)_{78}$-OPH (organophosphorus hydrolase:OPH)	ELP-protein	Biotechnology	[99]
[GGAGQGGYGGLGSQGAGRGGLGGQGGAG]	Silk	High performance fibres and biomaterials	[15]
[GPGGYGGPGQQGPGGYAPGQQPSGPGS]	Silk	High performance fibres and biomaterials	[15]

added to further functionalize the polymers, such as the incorporation of RGD cell attachment sequences (Pronectin) [70] or the creation of block copolymers combining silk and elastin motifs [71, 72]. Some of the representative examples of GEPBPs produced to date have been summarized in Table 1.

5
Elastinlike Polymers: A Privileged Family of GEPBPs

5.1
Introducing ELPs

The ELP family has shown a versatile and ample range of interesting properties that go well beyond their simple mechanical performance. Certainly, ELPs show a set of properties that places them in an excellent position towards designing advanced polymers for many different applications, including the most cutting edge biomedical uses, for which ELPs are particularly well suited, as will be discussed later. 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 still faced in modern science, the understanding of protein folding and function in living organisms.

The basic structure of ELPs is a repeating sequence having its origin in the repeating sequences found in the mammalian elastic protein, elastin. Regarding their properties, some of their main characteristics 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 [100], i.e., an almost ideal elasticity with Young's modulus, elongation at break, etc. in the range of natural elastin and an outstanding resistance to fatigue [85, 101].

Interestingly, this mechanical performance is accompanied by an extraordinary biocompatibility, although, however, the 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 when their temperature is increased above a certain level. This transition, called 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 the molecular processes taking place around the ITT has established a basis for their functional and rational design [102].

All these aspects of the ELP family will be presented below in the context of the present state of the art and the foreseeable future outcomes.

5.2 Smart and Self-assembling Properties of ELPs

The most numerous members of the ELP family are those based on the pentapeptide VPGVG (or its permutations). A wide variety of polymers have been (bio)synthesized with a general formula (VPGXG), where X represents any natural or modified amino acid [103–105] with the exception of L-proline. All the polymers with that 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 other natural amino acid different from L-alanine [105].

The model poly(VPGVG), whose amino acid side chains are simple aliphatic chains without further functionalization, shows an acute thermoresponsive behavior associated to the existence of the ITT.

All of the functional ELPs exhibit this reversible phase transitional behavior [105]. In aqueous solution and below a certain transition temperature (T_t), the free polymer chains remain disordered, random coils in solution [106] that are fully hydrated, mainly by hydrophobic hydration. This hydration is characterized by the existence of ordered clathratelike water structures surrounding the apolar moieties of the polymer [107–109] with a structure somehow similar to that described for crystalline gas hydrates [109, 110], although showing a more heterogeneous structure with structures varying in perfection and stability [108]. In contrast, above T_t, the chain hydrophobically folds and assembles to form a phase-separated state of 63% water and 37% polymer by weight [111] in which the polymer chains adopt a dynamic, regular, nonrandom structure, called β-spiral, involving type II β-turns as the main secondary feature, and stabilized by intraspiral, interturn, and interspiral hydrophobic contacts [105]. This is the product of the ITT. In this folded and associated state, the chain loses essentially all of the ordered water structures of hydrophobic hydration [107]. 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 a several-hundred-nanometer particle before settling into a visible phase-separated state [105, 112]. This folding is completely reversible upon lowering again the sample temperature below T_t [105].

Although, generally speaking, the phenomenology shown by these ELPs resembles that found in amphiphilic LCST polymers, such as poly-(*N*-isopropylacrylamides) (PNIPAM), the presence of an ordered state in ELPs above the transition temperature, which is not present in the LCST polymers, has prevented the use of LCST as a descriptive term for the ITT of ELPs [74].

5.3 Basic Molecular Designs: Thermal Responsiveness

Poly(VPGVG) (or its permutations) can be considered one of the simplest ELPs. The nonexistence of further functionalization, apart from the hydrophobic nature of valine and proline side chains, gives rise to a straightforward thermal response as shown in Fig. 2. 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 considered the temperature showing a 50% change in the relative turbidity change. In contrast, DSC measurements are always characterized by a broad peak, expanding 20 °C or more. In this case, T_t can be considered either as the onset or peak temperature. Usually, T_t values obtained by these methods differ among each other. Different factors cause such differences. The first one is the dynamic nature of the DSC and its associated thermal lags; those thermal lags being, of course, higher for higher heating rates. However, those thermal lags can be eliminated using different heating (or cooling) rates and obtaining an extrapolated T_t value to a heating rate equal to zero [113]. Figure 2a clearly shows the influence of this parameter; the DSC peak temperature for a 10 °C/min heating rate is several degrees higher than the turbidity T_t.

Another factor that can cause T_t differences between the two techniques is the different polymer concentrations. 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 [20, 105]. There is a strong dependence of T_t on concentration in the range of 0.01 to 5–10 mg/mL. Above this concentration, T_t does not show further significant changes with increasing concentrations up to a limit of 150–200 mg/mL. Above this value, we find deficiently hydrated polymer chains and, due to the heterogeneity of the hydrophobic hydration structures, in water deficiency states only the strongest structures are formed, which leads to a new increase in T_t as the polymer concentration increases [108]. Typical concentrations for turbidity experiments are in the range of 2–5 mg/mL, while those for DSC usually are in the range of 50–150 mg/mL, so further differences in T_t caused by concentration effects could be possible.

In addition, T_t also depends on the MW. T_t decreases as the MW increases [19, 20, 101]. Furthermore, the presence of other ions, such us those of the buffer, and molecules also changes the T_t value. In conclusion, all these factors make the comparison of T_t values among not only different techniques but also different authors a delicate matter.

The endothermic peak found in a DSC heating run is in fact the net result of a complex process containing different thermal contributions. Once

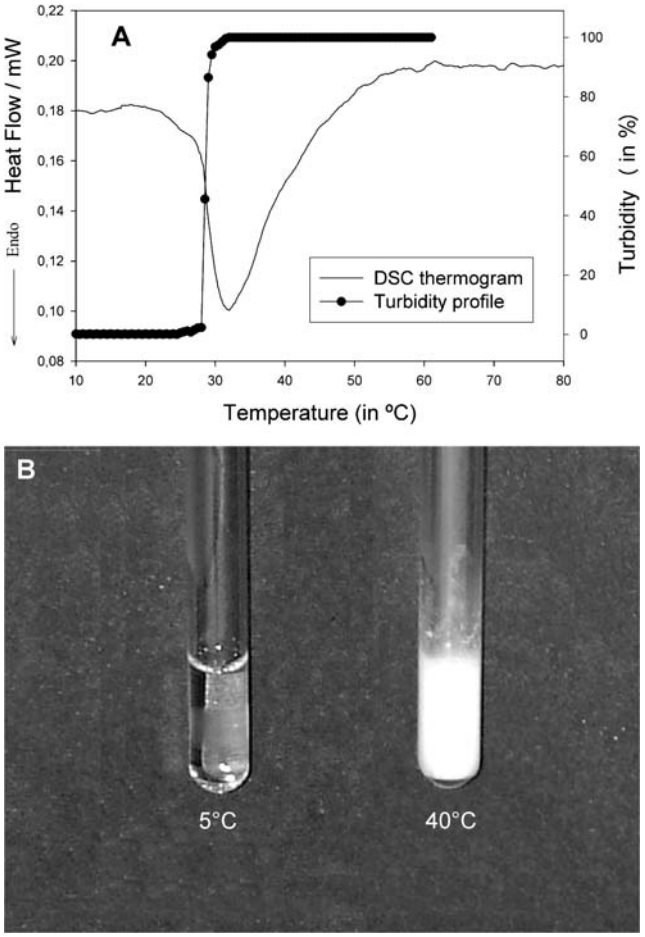

Fig. 2 **A** Turbidity profile as a function of temperature for a poly(VPGVG) 5-mg/L sample dissolved in water and DSC thermogam of a 50-mg/L water solution of the same polymer (heating rate 5 °C/min). **B** photographs of a water solution (5 mg/mL) of this poly(VPGVG) *below* (5 °C) and *above* (40 °C) its T_t

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 turn, these β-spirals further establish interchain hydrophobic contacts (Van der Waals cohesive interactions) that caused the formation of nano- and microaggregates segregating from the solution. The first process must be considered endothermic while the second one must be exothermic. Although both events take place simultaneously, they are very different in nature. In particular, it is reasonable to consider that both phenomena occur with different kinetics. In effect, previous

kinetic studies made on poly(VPGVG) showed that the process of phase separation is faster than the process of redissolution [114]. This difference creates a chance to split the different contributions of the ITT. This has been recently achieved for the first time using temperature-modulated DSC (TMDSC) [115]. 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 sinus, superimposed on the constant heating rate (ν) [116–120]. In principle, TMDSC will provide a clear split of two overlapping phenomena when, under the particular dynamical conditions, one is reversible and the other is not. Therefore, by this experimental approach, both phenomena could be split by finding a frequency for the periodic component low enough for the faster phenomenon to follow the oscillating temperature changes ("reversing") while high enough to impede this alternating behavior of the slower one ("nonreversing"). This approach has been used to study the ITT of three different ELPs chemically synthesized poly(VPGVG), recombinant $(VPGVG)_{251}$, and recombinant $(IPGVG)_{320}$ [115]. Figure 3a shows an example of the TMDSC thermogram found for $(VPGVG)_{251}$, while Fig. 3b shows the results of its analysis. Under those experimental conditions, the endothermic total curve ($\Delta H_{tot} = -10.40\ Jg^{-1}$, $T_t = 27.72\ °C$) is composed by a nonreversing endothermic component ($\Delta H_{non\text{-}rev} = -13.98\ Jg^{-1}$, $T_t = 27.63\ °C$) and a reversing exotherm ($\Delta H_{rev} = 3.33\ Jg^{-1}$, $T_t = 27.30\ °C$).

A detailed analysis has been carried out to study the dependence of the reversing and nonreversing components as a function of ν and amplitude (A) and period (P) of the alternating component. For the total contribution, the changes in ν (0.5 to 1.5 °C/min), A (0.1 to 1 °C), and P (0.1 to 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 nonreversing components were not affected by changes in ν and A. However, P exhibits a strong influence on the enthalpy values of both components.

ΔH_{rev} is plotted in Fig. 4 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 an enthalpy comparable to the one shown by the endothermic peak of the nonreversing component. Thus, at these high P, the chain-folding and dehydration contributions were not well separated. However, as P decreases, ΔH_{rev} undergoes a substantial increase. At $P = 0.8–1$ min, the reversing component turns into a positive exothermic peak which reaches a maximum at $P = 0.5–0.6$ min (P_M). Parallelly, $\Delta H_{non\text{-}rev}$ suffers an equivalent decrease. Therefore, as P decreases, the reversing component is being enriched in the exothermic component (chain folding), while the non-reversing is being 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

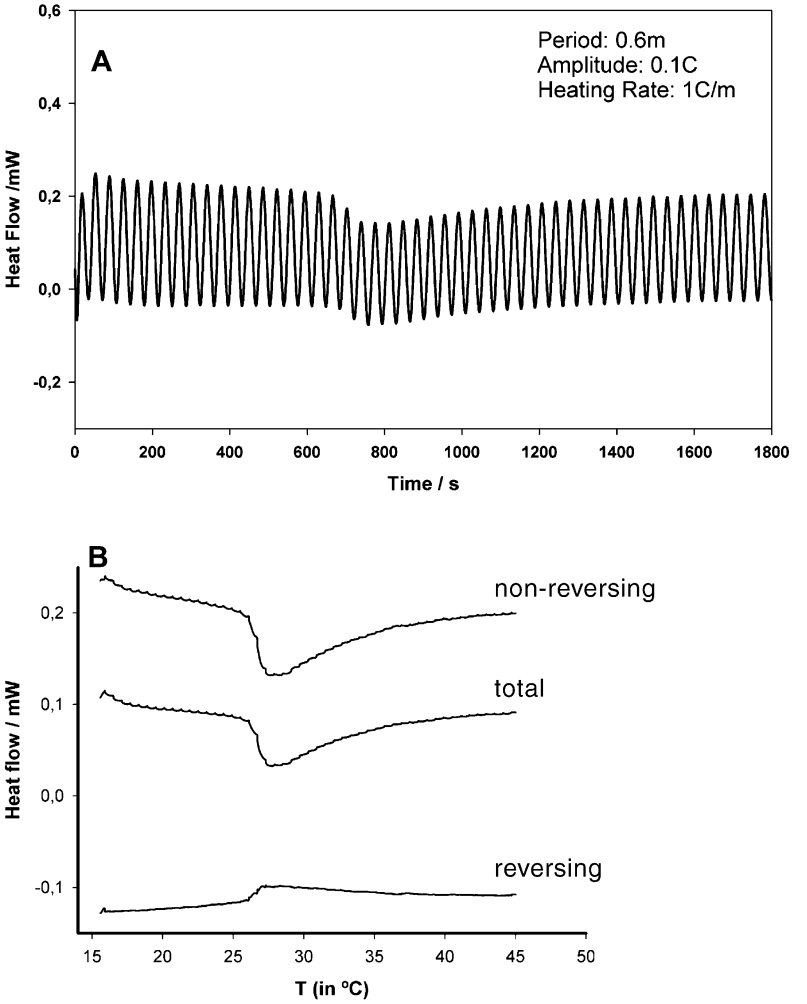

Fig. 3 **A** Heat flow vs. time in a TMDSC analysis of a 125-mg mL^{-1} water solution of $(VPGVG)_{251}$. **B** Reversing, nonreversing, and total thermograms. Reproduced with permission from Elsevier

an increase in $\Delta H_{\text{non-rev}}$ to the total enthalpy as a result of the complete overlap of both phenomena in the nonreversing component.

The maximum splitting was found at approximately the same P_M regardless of the polymer. Additionally, a comparison of the data found for $(VPGVG)_{251}$ and $(IPGVG)_{320}$ indicates that the reversing component at maximum is higher for $(IPGVG)_{320}$. Due to the higher hydrophobicity of I as compared to V, its chain folding has to show a higher exothermic ΔH_{rev} (Table 2). Therefore, ΔH_{rev} values could then be used as a quantitative measurement of the amino acid hydrophobicity. Additionally, the increased hydrophobicity of

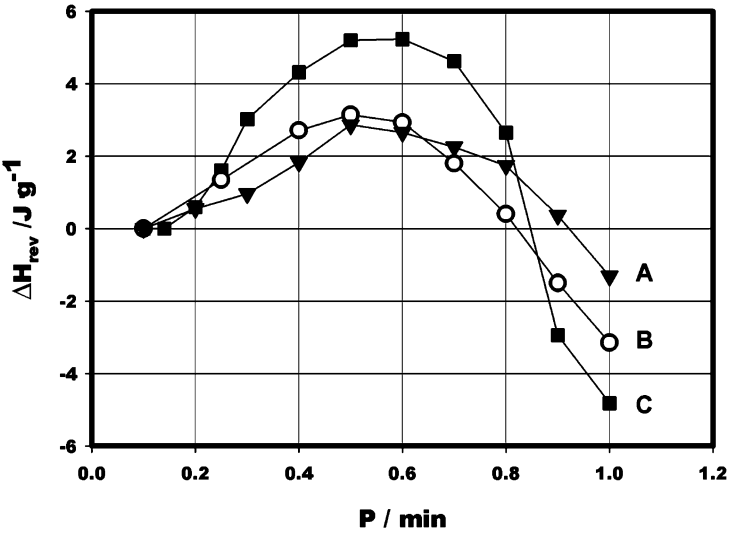

Fig. 4 ΔH_{rev} as a function of P for 125 mg mL^{-1} water solution of **A** synthesized poly(VPGVG), **B** recombinant $(VPGVG)_{251}$, and **C** recombinant $(IPGVG)_{320}$ ($\nu = 1\,^{\circ}C\,min^{-1}$, and $A = 0.1\,^{\circ}C$). Reproduced with permission from Elsevier

$(GVGIP)_{320}$ would also induce a higher extension of hydrophobic hydration, so its higher endothermic $\Delta H_{non\text{-}rev}$ is also reasonable.

There are no significant differences when comparing data from $(VPGVG)_{251}$ and poly(VPGVG) (Table 2). Since the only difference between these two polymers is their MW dispersity, their TMDSC results are practically the same, which would imply that the reversing and nonreversing TMDSC components depend mainly on the mean hydrophobicity of the monomer.

Therefore, TMDSC has been demonstrated to be an effective method to split the overlapping phenomena present in the ITT of elastic protein-based polymers. By tuning the frequency of the periodic component, a maximum split can be achieved that 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

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

Polymer	ΔH_{rev}/Jg^{-1}	$\Delta H_{non\text{-}rev}$/Jg^{-1}	ΔH_{tot}/Jg^{-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

former being about one fourth of the latter, in absolute values. To the best of our knowledge, TMDSC is the only method currently available to separate both contributions. Accordingly, its utility for evaluating the hydrophobicity of the full compliment of naturally occurring amino acids and relevant modifications thereof is clear, and its relevance to hydrophobic folding of polymers and natural proteins is noteworthy.

5.4 Introducing Further Chemical Functions in the Monomer: pH-responding 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" [105]; i.e., if a chemical group that can be present in two different states of polarity exists 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 the changes in the environmental stimulus. This ΔT_t mechanism has been exploited to obtain many elastinlike smart derivatives [105, 121–124].

This mechanism is also exploited in the following model pH-responding polymer: $[(VPGVG)_2\text{-}VPGEG\text{-}(VPGVG)_2]_n$. In this ELP, the γ-carboxylic group of the glutamic acid (E) suffers strong polarity changes between its protonated and deprotonated states as a consequence of pH changes around its effective pK_a.

Figure 5 shows the folded chain content as a function of T at two different pHs for a genetically engineered polymer with the above general formula ($n = 45$). At pH = 2.5, in the protonated state, the T_t shown by 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 the γ-carboxyl groups, as they lose their protons, becoming carboxylate, is enough to cause T_t to rise to 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 pHs and unfold at neutral or basic pHs. In addition, this fact reveals the extraordinary efficiency of ELPs as compared to other pH-responding polymers since this huge ΔT_t is achieved with just 4 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 nanopumps or nanovalves because just a low number of protons is needed to trigger the two states of the system.

The materialization of an electric charge in a side chain of a given ELP due to acid-basic equilibrium has been considered in the literature as a highly ef-

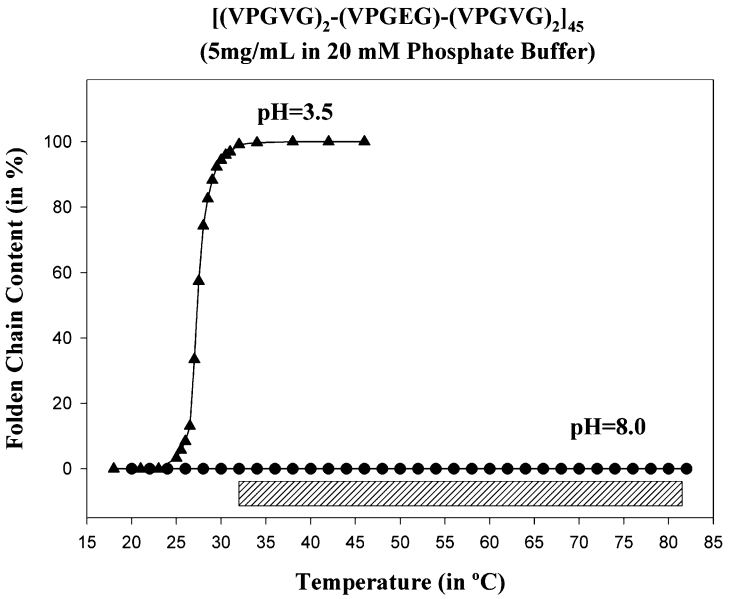

Fig. 5 Turbidity temperature profiles of a model genetically engineered pH responding ELP (see [19] for details on bioproduction of this polymer). *Box at bottom*: window of working temperatures. Experimental conditions are given in plot

ficient way to achieve high ΔT_t. In the number of 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 T_t shifts is only surpassed by the ΔT_t caused by the phosphorylation of serine [31].

Contrary to what happens with polydisperse synthetic polymers, the exquisite control on the molecular architecture and the strict monodisperse MW attained by genetic engineering make easy the study of the dependence of the different polymer properties vs. MW.

This has been done in the $[(VPGVG)_2\text{-}VPGEG\text{-}(VPGVG)_2]_n$ series for pH-responding ELPs. A set of different monodisperse versions of polymers has been bioproduced, with $n = 5$, 9, 15, 30, and 45. These were set to study the effects of MW on the properties of their ITT and its dependence on pH. As a result, the transition temperature decreased and the transition enthalpy increased as MW increased, especially for the lowest MWs. This can be qualitatively seen in Fig. 6, where a series of DSC thermograms has been plotted for a given polymer concentration and pH.

Quantitatively, these dependences can be seen in Fig. 7, in which enthalpy and true T_t values have been plotted vs. MW. True T_t is the term used to describe the T_t value obtained by extrapolation to zero heating rate ($\nu = 0$) of the DSC peak temperature.

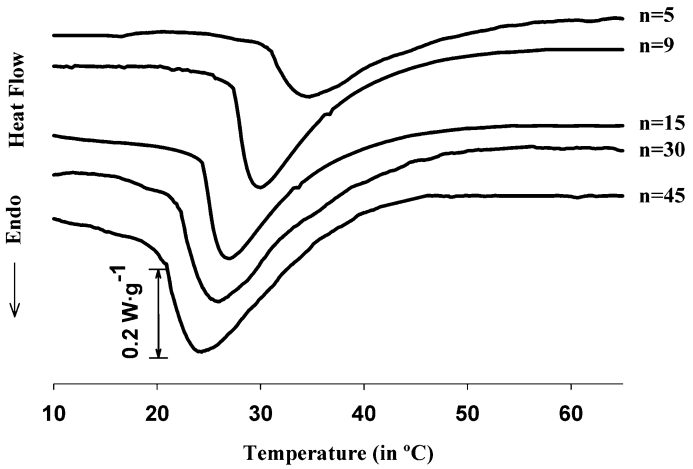

Fig. 6 DSC thermograms of 50 mg mL^{-1} phosphate buffered (0.1 M, pH 2.5) water solutions of studied polymers. Their polymerization degree (*n*) is shown on the *right-hand side* of the plot. Heating rate 10 °C min^{-1}. Reproduced with permission from American Chemical Society

Moreover, we have observed that the pK_a of the free carboxyl of the glutamic side chain also depends on MW. This striking fact can be seen in Fig. 8, where T_t has been followed as a function of pH for the different MWs.

As shown in that figure, the pH at which T_t starts to increase, following the first deprotonations of the free carboxyl groups, is lower for lower MWs. With the help of the enthalpy values found at different pHs and MWs, it has been possible to estimate the apparent pK_a (pK_a') of this free carboxyl group as a function of MW [19] (Fig. 9).

That behavior would imply that for higher MWs this carboxyl group is less acidic and shows a greater tendency to remain in the protonated state, and this despite the fact that the surroundings of this carboxyl are equivalent in all MWs. This striking behavior could be partially explained by the influence of the polar chain-end groups, as this influence is higher for lower MWs. However, the exclusive effect of the end-chain polarity seems insufficient to account for the strong influence reported. We believe that a large part of the effect of MW on the ITT is caused by the inter- and intrachain cooperativity of the hydrophobic self-assembly taking place during the ITT [106]. In this sense, it is reasonable to think that short chains do not show an efficient cooperation so their self-assembly is hindered, while for high MWs the inter- and intrachain cooperativity during folding is more efficient, which, to some degree, forces the carboxyl group to be in the protonated (less polar) state.

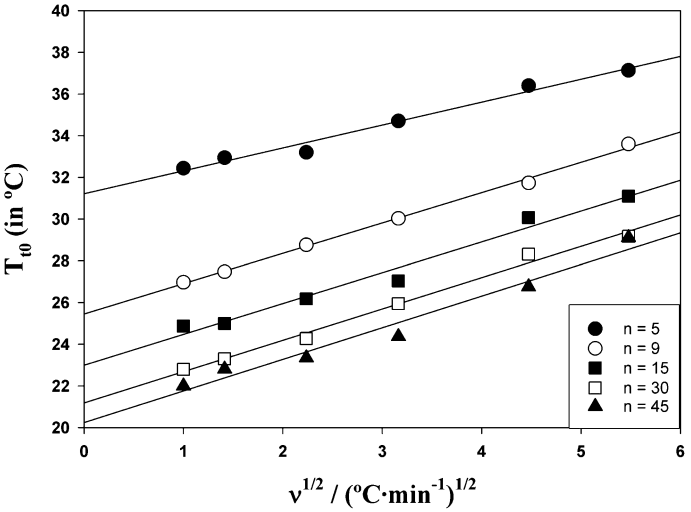

Fig. 7 Dependence of T_t on square root of heating rate for studied polymers. The corresponding polymerization degree (n) is indicated in plot. *Lines*: least square linear regressions of data for each n. Phosphate-buffered samples (0.1 M, pH 2.5). Reproduced with permission from American Chemical Society

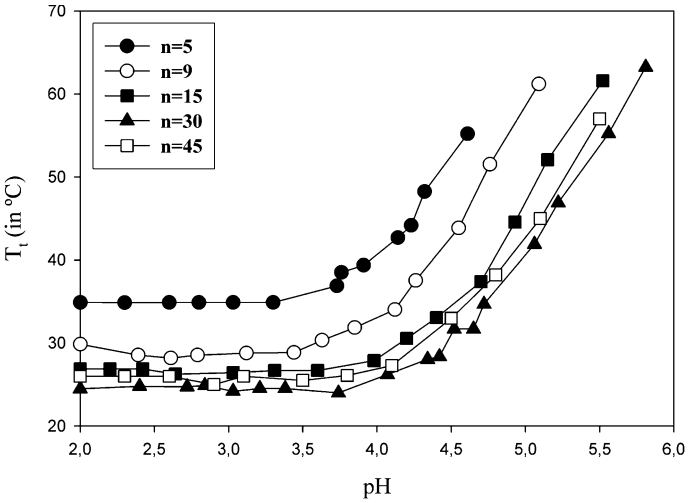

Fig. 8 Dependence of T_t on pH for studied polymers (as indicated in plot). 0.1 M phosphate-buffered samples. Reproduced with permission from American Chemical Society

As shown in Fig. 9, the pH at which T_t starts to increase, following the first deprotonations of the free carboxyl groups, is lower for lower MWs. With the help of the enthalpy values found at different pHs and MWs, it has been

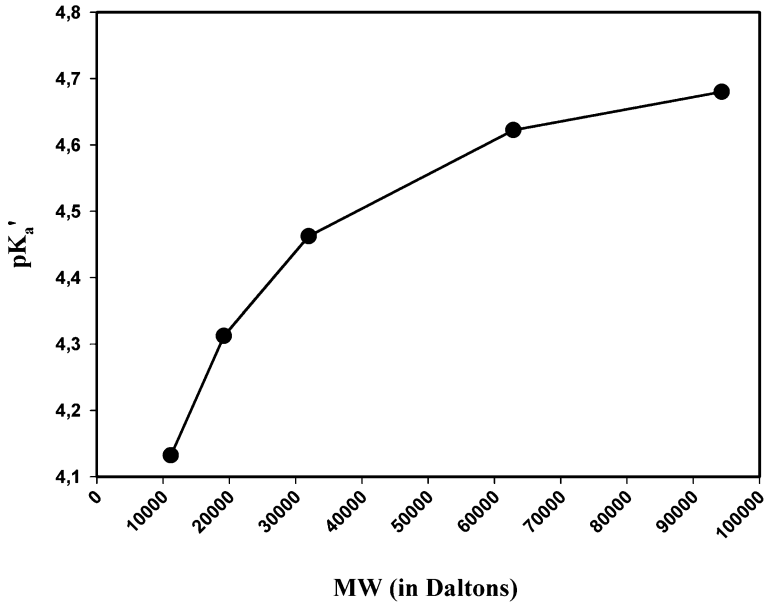

Fig. 9 Dependence of pK'_a for the γ-carboxyl group of glutamic acid on MW. Reproduced with permission from American Chemical Society

possible to estimate the apparent pK_a (pK'_a) of this free carboxyl group as a function of MW [19] (Fig. 9).

That behavior would imply that for higher MWs this carboxyl group is less acidic and shows a greater tendency to remain in the protonated state, and this despite the fact that the surroundings of this carboxyl are equivalent in all MWs. This striking behavior could be partially explained by the influence of the polar chain-end groups, as this influence is higher for lower MWs. However, the exclusive effect of the end-chain polarity seems insufficient to account for the strong influence reported. We believe that a large part of the effect of MW on the ITT is caused by the inter- and intrachain cooperativity of the hydrophobic self-assembly taking place during the ITT [106]. In this sense, it is reasonable to think that short chains would not show an efficient cooperation so their self-assembly is hindered, while for high MWs the inter- and intrachain cooperativity during folding is more efficient, which, to some degree, forces the carboxyl group to be in the protonated (less polar) state.

5.5 Self-Assembling Capabilities of ELPs

In relation to self-assembling, natural elastin suffers a self-aggregation process in its natural environment. Elastin is produced from a water-soluble precursor, tropoelastin, which spontaneously aggregates yielding fibrilar struc-

tures that are finally stabilized by enzymatic interchain cross links. This produces the well-known insoluble and elastic elastin fibers that can be found in abundance in the skin, lungs, arteries, and, in general, those parts of the body undergoing repeated cycles of stress-strain.

The self-assembling ability of elastin seems to reside in certain relatively short amino acid sequences, as has been recently probed by Yang et al. [124] working in recombinant ELPs. Some of these polypeptides have shown that, above their T_t, they are able to form nanofibrils that further organize into hexagonally close-packed arrangements when the polymer was deposited onto a hydrophobic substrate [124].

However, in ELPs, this tendency to self-assemble in nanofibers can be expanded to other topologies and nanostructured features [93, 125, 126]. Taking advantage of the opportunities and potential given by genetic engineering in designing new polymers, the growing understanding in the molecular behavior of ELPs and the enormous wealth of experimental and theoretical experience gained in recent decades on the self-assembling characteristics of different types of block copolymers, different self-assembling properties are starting to be unveiled within the ELP family. For example, Reguera et al. have shown that the ELP previously shown as a pH-responding polymer, $[(VPGVG)_2(VPGEG)(VPGVG)_2]_{15}$, was able to form polymer sheets showing self-assembled nanopores [126] (Fig. 10).

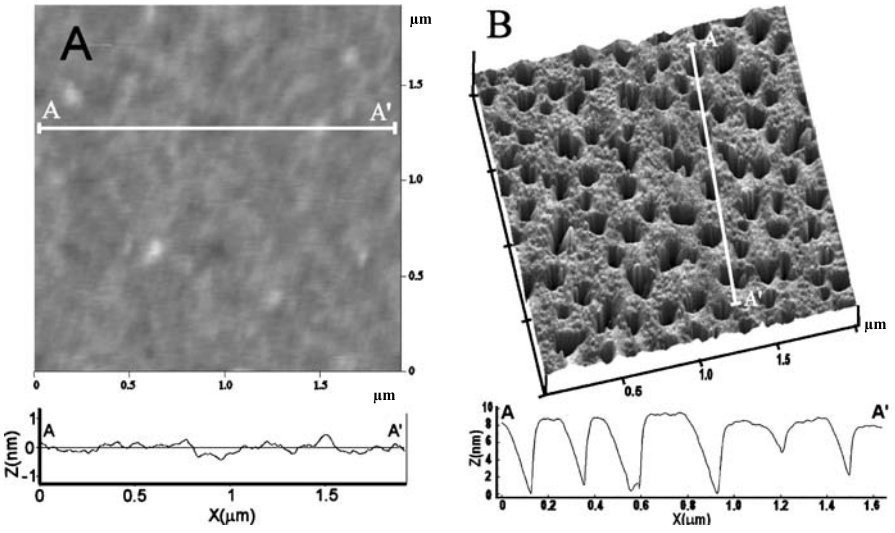

Fig. 10 Tapping Mode AFM image of $[(VPGVG)_2\text{-}(VPGEG)\text{-}(VPGVG)_2]_{15}$ deposited from a water solution on a Si hydrophobic substrate. Sample conditions: **A** 10 mg mL^{-1} in 0.02 M HCl water solution (acid solution); and, **B** 10 mg mL^{-1} in 0.02 M NaOH water solution (basic solution). Adapted from [126]. Reproduced with permission from American Chemical Society

An AFM study of the topology of polymer spin-coated depositions of Glu-containing ELPs, from acid and basic solutions, on a Si hydrophobic substrate at temperatures below T_t has shown that in acidic conditions, the polymer deposition just shows a flat surface without particular topological features (Fig. 10a).

However, from basic solutions the polymer deposition clearly shows an aperiodic pattern of nanopores ($\sim$ 70 nm width and separated by about 150 nm) (Fig. 10b). This different behavior as a function of pH has been explained in terms of the different polarity shown by the free γ-carboxyl group of glutamic acid. In the carboxylate form, this moiety shows a markedly higher polarity than the other polymer domains and the substrate itself. Under this condition, the charged carboxylates impede any hydrophobic contact with their surroundings, which is the predominant way of assembling for this kind of polymer. These charged domains, along with their hydration sphere, are then segregated from the hydrophobic surroundings, giving rise to nanopore formation (Fig. 11).

The self-association of ELPs is starting to be employed to develop different applications. For example, Molina el al. [127] have tested self-assembled nano- and microparticles 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 rises above its T_t.

In another remarkable example, Chilkoti et al. have developed nanostructured surfaces by combining ELPs and dip-pen nanolithography that show reversible changes in their physicochemical properties in response to changes in their environmental conditions. In particular, these systems are able to capture and release proteins on nanopatterned surfaces by using the self-assembling characteristics of ELPs in an effort to develop advanced biomaterials, regenerable biosensors, and microfluidic bioanalytical devices [127–130].

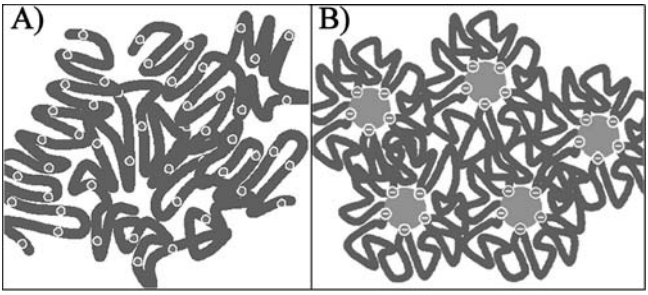

Fig. 11 Schematic cartoon of polymer distribution on hydrophobic substrate. **A** In a acid medium. **B** In a basic medium. Counterions have been not drawn for clarity. Adapted from J Am Chem Soc. Reproduced with permission from American Chemical Society

However, the exploitation of the huge potential of ELPs in producing self-assembling polymers is still very poor. In recent decades, the development achieved in the design of self-assembling polymers, especially block copolymers, has been enormous in spite of the difficulties found in the synthesis of these polymers [21]. The different blocks show different compositions and physicochemical properties, so in an adequate environment those blocks segregate in various immiscible phases that with the combination of adequate external fields are able to self-organize into different highly interesting nanostructures [21]. Among the different physicochemical properties that can be used to trigger phase segregation among the different blocks is their hydrophobic-hydrophilic nature. This opens an interesting possibility for using ELP blocks to construct self-assembling block copolymers. The tendency of these ELPs to show controlled hydrophobic association can be exploited to obtain advanced multiblock copolymers with the advantage given by three salient facts. First, the hydrophobic association of ELPs can be externally controlled since it is associated to the ITT, and this is stimulus triggered (temperature, pH canges, etc.). Second, currently and as a consequence of extensive and deep work on tens of different model ELPs of the type $(VPGXG)_n$ carried out by Urry's group in recent decades, there is a deep and quantitative body of knowledge on the degree of hydrophobicity of the different amino acid side chains [30, 31, 131]. We now have a precise classification of the hydrophobicity of amino acids. The parameter used to precisely quantify the hydrophobic character is based on the direct experimental measurement of the Gibbs free energy of hydrophobic association. Therefore, for the first time, the hydrophobic character has been evaluated from the origin of the hydrophobicity itself and not from its indirect effects, such as the distribution coefficient between solvents, etc. The available data include the 20 natural amino acids and some derivatives. For those amino acids with polarizable side chains, such as glutamic acid, lysine, or phosphorilated and unphosphorilated serine, this datum has been evaluated in the two states. These values have been summarized in Fig. 12.

As can be observed, the hydrophobic character of the different amino acids covers a broad range between the most hydrophobic, tryptophan, to the most hydrophilic, phosphorilated serine. The energy gap between these two extremes is as high as 15 kcal per mol of VPGXG. A gradual transition between those extreme values can be used to adjust the hydrophobicity of the designed blocks with unprecedented precision.

The third relevant fact is the unparalleled capacity to achieve complex and completely controlled PBPs given by genetic engineering. The block length, hydrophobicity, composition, and position can be engineered at will with absolute precision. Additionally, genetically engineered elastinlike block copolymers can easily incorporate any other structural feature of interest for self-assembly and function such as β-sheet-forming domains, leucine zippers, binding of domains to different substrates, and any biofunctionality

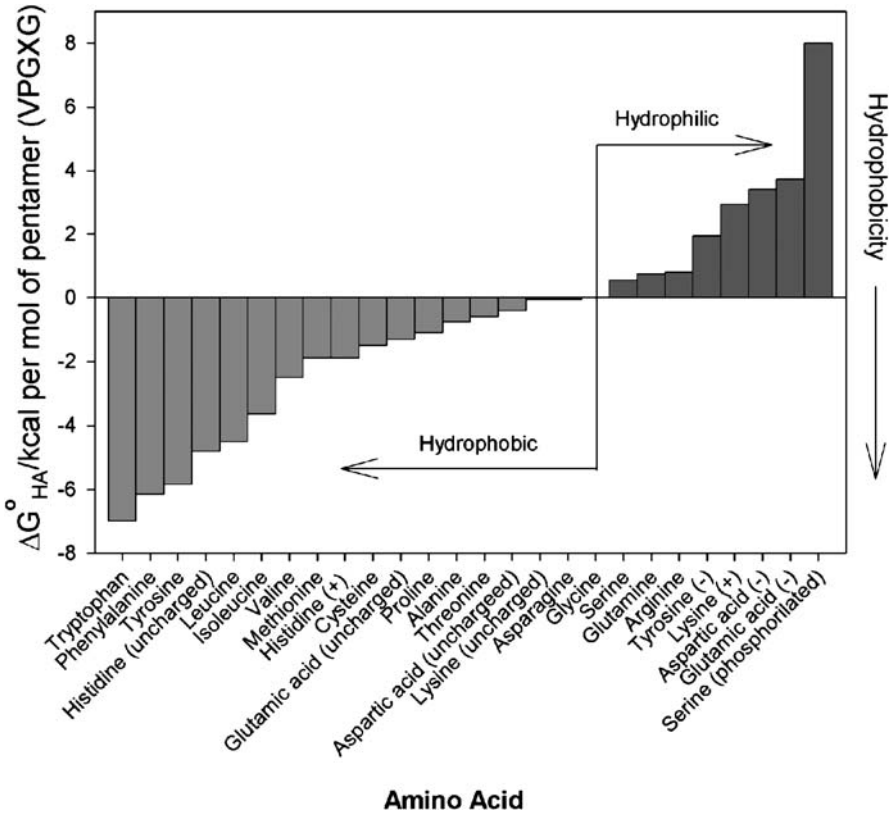

Fig. 12 Hydrophobicity scale of 20 natural amino acids in their different polarizated state. Adapted from data taken from [30, 31, 131]

imparted by bioactive peptides (cell attachment sequences, etc.). All three of these characteristics will certainly open new ways of creating advanced multiblock copolymers with applications spreading to many technological fields.

5.6 Further Chemical Functionalization of the Monomer: Photoresponding ELPs and the Amplified ΔT_t Mechanism

The range of stimuli that can exploit the ΔT_t mechanism is not limited to those chemical reactions taking 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 photoresponding ELPs, which bear photochromic side chains either coupled to functionalized side chains in the previously formed polymer (chemically or genetically engineered) or by

using nonnatural amino acids that were already photochromic prior to chemical polymerization.

The first example corresponds to this last kind. 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 suffers a photo-induced cis-trans isomerization. Dark adaptation or irradiation with visible light around 420 nm induces the presence of the trans isomer, the most unpolar isomer. In contrast, UV irradiation (at around 348 nm) causes the appearance of high quantities of the cis isomer, which is slightly more polar than the trans isomer. Although the polarity change is not high, it is enough to obtain functional polymers due to the sensitivity and efficiency of ELPs. Figure 13 shows the photoresponse of one of these polymers with $f_X = 0.15$. That mole fraction represents only 3 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, the existence (Fig. 13a) of a working temperature window at around 13 °C is evident (Fig. 13b).

In another example, a different chromophore, a spiropyrane derivative, is attached at the free γ-carboxyl group of an E-containing ELP either chemically synthesized or genetically engineered. Figure 14 represents the photochromic

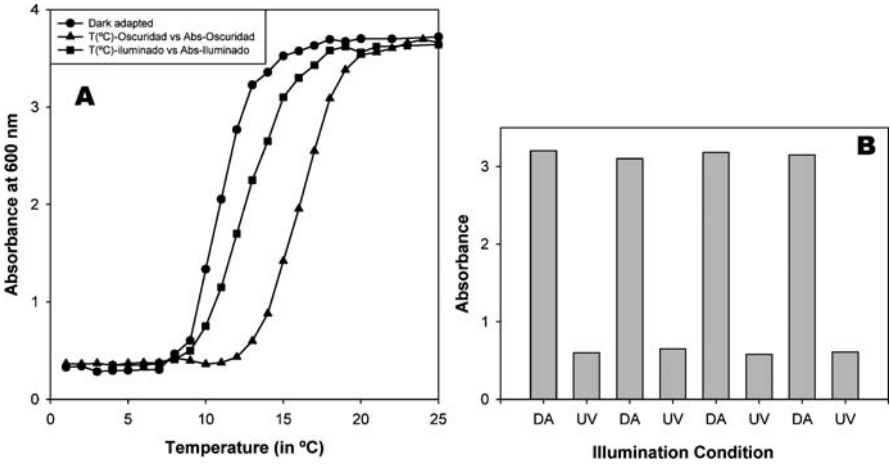

Fig. 13 **A** Temperature profiles of aggregation of 10-mg mL^{-1} water solutions of photoresponsive poly[0.85(VPGVG), 0.15(VPGXG)] (X ≡ L-*p*-(phenylazo)-phenylalanine) under different illumination regimens. The correspondence between each profile and its illumination condition is indicated in plot. Details on polymer synthesis and illumination conditions can be found in [30]. **B** Photomodulation of phase separation of 10-mg mL^{-1} aqueous samples of poly[0.85(VPGVG), 0.15(VPGXG)] at 13 °C. The prior measurements of the illumination conditions are indicated by the horizontal axis. DA, dark adaptation; UV, UV irradiation. Reproduced with permission from American Chemical Society

reaction for this polymer [122]. As compared to *p*-phenylazobenzenes, spiropyrane compounds show a photoreaction that can be driven by natural cycles of sunlight-darkness without the employment of UV sources, although UV irradiation causes the same effect as darkness but at a higher rate [132].

Again, the difference in polarity between the spiro and merocyanine forms (Fig. 14) is enough to cause a significant T_t shift. Figure 15 shows the turbidity profiles of the polymer in different illumination regimens (Fig. 15a) and the photomodulation of polymer folding and unfolding (Fig. 15b,c).

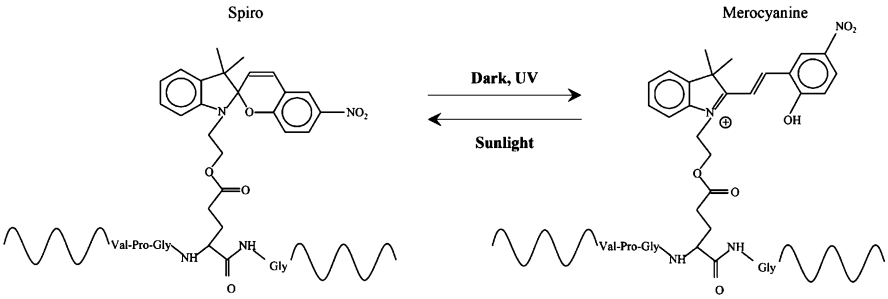

Fig. 14 Photochemical reaction responsible for photochromic behavior of spiropyrane-containing ELP. Reproduced with permission from American Chemical Society

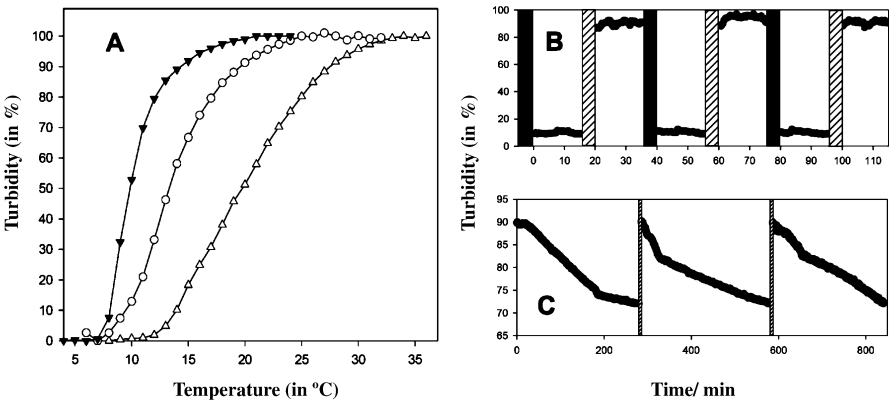

Fig. 15 **A** Temperature profiles of aggregation of 20-mg mL^{-1} phosphate-buffered (0.01 N, pH 3.5) water solutions of photoresponsive polymer under different illumination regimens. The correspondence between each profile and its illumination condition are indicated in plot. Turbidity was calculated from absorbance values obtained at 600 nm on Cary 50 UV-Vis spectrophotometer equipped with a thermostatized sample chamber. **B** and **C** Photomodulation of phase separation of 5-mg mL^{-1} aqueous samples of photochromic polymer (T = 14 °C, 0.01 N phosphate buffer at pH = 3.5). **A** UV-sunlight *cycles*. *Boxes* in subplot: periods of irradiation: UV, *black boxes*; sunlight, *white boxes*. **B** Darkness-sunlight *cycles*. *Boxes* in subplot: periods of sunlight irradiation. Reproduced with permission from American Chemical Society

The efficiency of the polymer is again outstanding, since just 2.3 spiropyran chromophores per 100 amino acid residues in the polymer backbone were sufficient to render the clear photomodulation shown in Fig. 15.

Different ELP versions responding to pH, light, and other stimuli, such as electrochemical potential or analyte concentrations, can be found in the literature. Most of them were produced by the exclusive use of chemical synthesis in a huge effort, lasting more than a decade, by Prof. Urry's group in a time when the use of genetic engineering to produce PBPs was not sufficiently developed (see, for example, [105]). In some cases, this smart response of the ELPs has already found applications in different fields. For example, Chilkoti et al. have designed thermally and pH-responsive ELPs for targeted drug delivery [77, 80, 133–136], and Kostal et al. have designed tunable ELPs for heavy metal removal [137].

In a different approach in the design of more efficient stimulus-responding 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 causes additional increases in the difference in polarity between both states. This is the basis of the so-called "amplified ΔT_t mechanism", and this has been proved for a *p*-phenylazobenzene-containing polymer of the kind shown above, poly[0.8(VPGVG), 0.2(VPGXG)], in the presence of α-cyclodextrin (αCD) [121]. The αCD is able to form inclusion compounds with the trans isomer of the *p*-phenylazobenzene group and not with the cis isomer due to a strong steric hindrance [121] (Fig. 16).

The αCD outer shell has a relatively high polarity, which, of course, is much more polar than the *p*-phenylazobenzene moiety both in the trans or cis states. The change in polarity between the dark adapted sample (trans isomer buried inside the αCD) and the UV irradiated one (cis isomer unable to form inclusion compounds) led to an enhanced ΔT_t (Fig. 17). Of course, the magnitude of this effect is [αCD] dependent, so it is possible to tune the width and position of the working temperature window just by changing the [αCD].

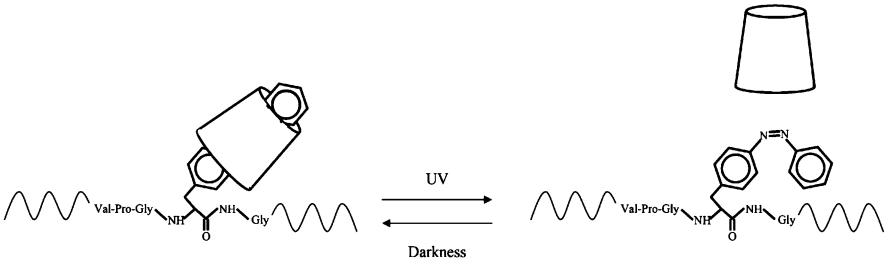

Fig. 16 Schematic diagram of proposed molecular mechanism on interaction between *p*-phenylazobenzene pendant group and αCD. Reproduced with permission from Wiley

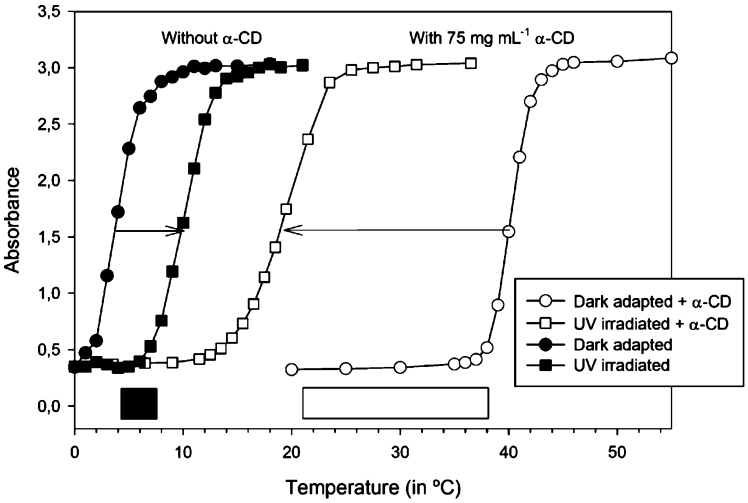

Fig. 17 Temperature profiles of aggregation of 10-mg mL^{-1} water solutions of photoresponsive ELP in absence and presence (75 mg mL^{-1}) of αCD under both illumination regimens. *Circles*: dark-adapted samples; *squares*: UV-irradiated samples. *hollow symbols*: presence of αCD; *filled symbols*, absence of αCD. *Arrows*: sense of displacement of turbidity profile caused by UV irradiation of corresponding dark-adapted sample. *Boxes at bottom*: window of working temperatures open when system is in absence (*filled box*) and presence (*hollow box*) of αCD. Reproduced with permission from Wiley

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*; UV, UV-irradiated samples**. Offset and gain as defined in text. Reproduced with permission from Wiley

[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

* 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). **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) mounted on a lamp housing with an F/1.5-UV-grade fused silica condenser and rear reflector (model 66041, Oriel). UV irradiation was achieved by the use of a band interference filter ($340 < \lambda < 360$ nm) from CVI Laser (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 [123].

As a result, in the αCD/poly[0.8(VPGVG), 0.2(VPGXG)] coupled photoresponsive system, αCD acts much like an amplifier acts on an electronic circuit. αCD promoted a tunable offset, gain, and inversion of the photoresponse of the polymer (Fig. 17 and Table 3). In this way, the polymer photoresponsiveness could be shifted to room or body temperature and with a wider range of working temperatures. Therefore, the use of precise temperature control can be avoided in most conceivable applications, as these applications have a wide range of uses, from photo-operated molecular machines to macroscopic devices (photoresponsive hydrogels, membranes, etc.) and nano- and microdevices (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 different stimuli in some modified CDs [138–140].

5.7 The Outstanding Biocompatibility of Elastinlike Polymers: The Third Pillar for Extraordinary Biomaterial Designs

The existence of an ITT for ELPs is the base of their remarkable smart and self-assembling properties. A second pillar in the development of extraordinary materials is, evidently, the power of genetic engineering in promoting the easy obtaining of complex and well-defined polymers with controlled and multiple (bio)functionality. Additionally, ELPs show a third property, which is highly relevant when planning the use of these polymers in the most advanced biomedical applications, such us tissue engineering and controlled drug release. This third pillar is the tremendous biocompatibility shown by ELPs.

The complete series of the ASTM-recommended generic biological tests for materials and devices in contact with tissues and tissue fluids and blood demonstrate an unmatched biocompatibility [141]. 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, it is now believed that the high segmental mobility shown by the β-spiral, the common structural feature of ELPs, greatly helps in preventing the identification of these foreign proteins by the immune system [30, 31]. In addition, the secondary products of their bioabsorption are just simple and natural amino acids.

With this nice set of properties, it is not surprising that the biomedical uses of ELPs seem to be the first area where ELPs will disembark in the market. This is especially true considering that the biomedical (and cosmetic) market shows a clear disposition to quickly adopt those 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 applications, so the companies producing ELPs will find the biomedical sector a good option for amortizing the cost previously used in the development of all know-how and technology around the production of ELPs.

5.7.1 ELPs for Drug Delivery Purposes: Different Strategies for Molecular Designs

Different versions of ELPs designed for drug delivery purposes can be found in the literature. However, they do not share a common basic strategy on design. On the contrary, ELPs display many different properties that can be useful for drug delivery purposes, i.e., smart behavior (sensitivity to certain stimuli), self-assembly, biocompatibility, etc., so design strategies can be diverse. In fact, the different ELP-based drug delivery systems described to date emphasize exploiting a particular one of those properties.

The first ELP-based drug delivery systems were reported by Urry. They were quite simple devices in which γ-radiated cross-linked poly(VPGVG) hydrogels of different shapes were loaded with a model water-soluble drug (Biebrich Scarlet) [142]. This 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. The designs then became slightly more complicated. The basic VPGVG pentapeptide was functionalized by including some glutamic acids whose free carboxyl groups were used for cross-linking purposes. The cross-linker was of the type that forms caboxyamides, which were selected because of their ability to hydrolyze at a given and controlled rate releasing the polymer chains and, concurrently, any drug entrapped within the cross-linked slabs [143]. This was an apparently simple and conventional degradation-based drug delivery system. However, due to the use of ELPs, the displayed behavior was slightly more complex and efficient. While the cross-link was intact, the carboxyl groups were amidated and, consequently, uncharged. This state of lower polarity yielded a cross-linked ELP material showing a T_t below body temperature. Therefore, the chains were folded at that temperature, the material contracted and deswelled and the polymer chains essentially became insoluble, entrapping the loaded drug quite efficiently in the model drugs studied. When hydrolysis took place on the outer surface of the slab, charged carboxylates appeared, which strongly increased the T_t in this zone (well above body temperature). The skin of the slab became swelled and the loaded drug readily escaped from the outer layer of the device. Additionally, the fully released chains were completely soluble, so they soon diffused and were reabsorbed. This caused the presence of an always fresh surface on the slab and the readiness in the release of the loaded drug within the hydrolyzed surface [143].

For this reason, the kinetics of drug release were almost of the zero-order type and, accordingly, the performance of the system was superior to those made of other equivalent polymers but without showing the ΔT_t mechanism. In general, this statement is more reliable as the size of the loaded drug is higher, since, as no other particular functionalities were added to the polymer chain, in practice, there is no substantial interaction between the drug and the polymer other than the movement constraint of the loaded drug within the polymer matrix. Therefore, a certain degree of uncontrolled diffusion can take place perturbing the kinetics of drug release.

In a different example, and as mentioned in Sect. 5.5, the tendency to form stable, drug-loaded, and nano- and microparticles by some ELPs, especially those based on the (VPAVG) pentapeptide, has facilitated the development of injectable systems for controlled release [127].

Nonetheless, those examples are based on simple polymer formulations that are still far from reaching the full potential of ELPs in developing drug delivery systems. Their smart and self-assembling properties, as well as the deeper knowledge on the molecular basis of the ITT, are only marginally exploited. However, new systems are starting to be published in the literature that already show a more decided bet on exploiting the very special characteristics of ELPs and the powerful way they can be produced, that is, through 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 of these ELPs, an additional pH responsiveness of these ELPs was used to mimic the membrane disruptive properties of viruses and toxins to cause effective intracellular drug delivery. Among the most evident uses of this kind of advanced drug delivery systems is the more efficient dosage of antitumoral drugs, but 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 [40, 80, 133, 135, 136, 144].

On the other hand, the recent deepening knowledge about the molecular characteristics of the ITT has allowed the development of advanced systems for more general drug release that have achieved a practically ideal zero-order drug release kinetics without the concerns caused by previous designs. The first examples are based on Glu-containing ELPs, in which the close vicinities of the γ-carboxyl groups are maintained in a highly hydrophobic environment by positioning phe residues by a precise nanometric design of the polymer sequence in accordance with the β-spiral structure of the folded state [30, 31] in the sense that, once the polymer folds into the β-spiral structure, those phe residues completely surround the free carboxyl group, creating a well-defined battleground where there is a strong competition between the two mutually exclusive forms of hydration, i.e., hydrophobic hydration of the phe residues and 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 acidic as the number of surrounding phes increases).

Therefore, in neutral or basic pH (including physiological pH), those carboxylate moieties show a strong propensity to neutralize their charge by ion-coupling, i.e., by establishing contacts with positively charged drugs, if this coupling causes an effective decrease in the polarity of the carboxyl vicinities. As a result these polymers, at this neutral or basic pH and in the presence of an adequate oppositely signed drug, form strong insoluble aggregates, which are characterized by a high rate of drug loading and, as implanted, 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 by properly choosing the 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 that moment, the interface between the remaining insoluble, still loaded, aggregate and the body fluids are continuously renewing without changing their physicochemical properties for practically the entire functional period of the system. This behavior causes a practically near ideal zero-order release [30, 31].

In the present situation, as demonstrated by the various examples shown above, the different alternatives presented 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, so the development of new ELPs combining various strategies of the kind depicted above is foreseeable. As can be easily understood, this could set basis for the development of drug delivery systems with unprecedented efficiency.

5.7.2 ELPs for Tissue Engineering: Introducing Tailored Biofunctionality

Designing a biomedical device is always a tremendous challenge for the material developer. This has been shown above for drug delivery systems, but the most demanding application is likely that of tissue engineering (or as is now preferred, regenerative medicine). When a mature or stem cell divides and spreads in a growing tissue, that cell is passing through the most vulnerable and difficult stage of its life cycle. This is the reason why materials that efficiently work in different biomedical applications can fail when used in for tissue engineering purposes (the failure can be caused both by the material itself and by its biodegradation products).

Additionally, we have to keep in mind that when designing a matrix for tissue engineering, we are trying to substitute the natural extracellular matrix

(ECM), at least transiently. Therefore, many aspects have to be taken into consideration upon designing an adequate artificial ECM. Initially, the material developer must have a decided concern regarding the mechanical properties of the artificial scaffold. It is well known that, when properly attached to the ECM, cells sense the forces to which they are subjected via integrins. Integrins are ubiquitous transmembrane adhesion molecules that mediate the interaction of cells with the ECM. Integrins link cells to the ECM by interacting with the cell cytoskeleton. By this means they couple the deformation of the ECM, as a consequence of the applied forces, with the deformation of the cytoskeleton. The deformed cytoskeleton triggers an intracellular signal transduction cascade that finally causes the expression of those genes related to the rebuilding of the ECM [145]. In this way, the cells are continuously sensing their mechanical environment and responding by producing an ECM that withstands those forces in an adequate manner. In this sense, cells are very efficient force transducers. Therefore, all artificial ECMs have to properly transmit forces from the environment to the growing tissue. Only in this way will the new tissue build the adequate natural ECM that eventually will replace the artificial ECM. In contrast, a stronger or too weak artificial ECM will cause its substitution by a too weak or too dense natural ECM, respectively, which can really compromise the success of the regenerated tissue.

Additionally, we know that the ECM is not just a scaffold showing certain mechanical properties in which the cells attach simply to achieve the necessary tissue consistency and shape. Far from that, the proteins of the natural ECM (fibronectin, collagen, elastin, etc.) contain in their sequence a huge number of bioactive peptides that are of crucial importance in the natural processes of wound healing. Those sequences include, of course, the well-known cell attachment sequences. In natural ECMs we find target domains for specific protease activity. Those proteases, such as the metalloproteinases of the ECM, are only expressed and secreted to the extracellular medium when the tissue wants to remodel its ECM [146]. 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 vicinities. It is also known 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, a growing tissue is delicately controlled by a well orchestrated symphony of growth factors and other bioactive substances segregated by the cells. Incidentally, these factors are mainly of a peptide nature.

This is the scenario that a growing tissue expects to find when passing through the difficult circumstances of growth and regeneration. 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 would be hard-pressed to think of a petroleum-based polymer that fulfills a minimum requirement of being reabsorbable, sufficiently

biocompatible, and nontoxic (the polymer itself and/or its biodegradation products), having adequate mechanical properties and being able to display or induce a minimum number of needed biofunctionalities. One must not be surprised by the fact that, in spite of the expectations caused by tissue engineering, it has achieved a quite moderate success to date. Among the first properties that seem to be unachievable by conventional polymers is complexity. The set of minimum requirements listed above clearly points to the need for a very complex material that could be well beyond the practical reach of our synthesis technology. This must not surprise us. We are trying to mimic an intrinsically complex natural ECM 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 can be achieved by one of our petroleum-based polymers and in spite of the fact that we really can choose functionality from among an impressive set of different monomers developed by organic chemistry in recent decades.

In spite of the discouraging scenario depicted above, we could be in a position where different options could come to our aid; GEPBPs could represent one of these clear breakthrough alternatives.

Soon after the finding of the extraordinary biocompatibility of the (VPGVG)-based ELPs [141], the capabilities of ELPs for tissue engineering were tested. The first candidates were the simple polymers like poly(VPGVG)s and their cross-linked matrices. Surprisingly, the cross-linked matrices of poly(VPGVG)s when tested for cell adhesion showed that cells do not adhere at all to this matrix and no fibrous capsule forms around it when implanted [147]. Of course, this matrix and other states of the material have a potential use in the prevention of postoperative, posttrauma adhesions [147], but in principle they do not seem to be realistic candidates for tissue engineering.

Nonetheless, this absolute lack of cell adherence is not a drawback; on the contrary, it is highly desirable since it provides us with a starting material with the adequate mechanical properties and biocompatibility and lacks unspecific bioactivities. Very soon those 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 in the polymer sequence even though, at that time, chemical synthesis was still the only option for obtaining 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, and D = L-aspartic acid) and REDV (E = L-glutamic acid and V = L-valine), which is specific to endothelial cells. The results were clear: the bioacivated (VPGVG) derivatives showed a high capacity to promote cell attachment, especially those based on RGD, which showed a cell attachment capability almost equivalent to that of the human fibronectin [148]. Once genetic engineering was finally adopted as the production method, the molecular designs started to increase in complexity.

Different ELP compositions were tested as base polymers. Additionally, the cell attachment domains were not restricted to the exclusive short peptide active domain and were increased in size as more amino acids were placed in such a way as to surround the central active REDV or RGD domains as a way of obtaining a more active cell-binding site [73]. For example, Panitch et al. have shown that by using the longer CS5 region of the human fibronectin, which is an eicosapeptide having the REDV sequence in its central part, the achieved cell adhesion was more effective than the short REDV inserts [84].

However, those still simple GEPBPs were made more complex by the addition of different functionalities such as cross-linking domains [85, 149, 150].

There are now examples based on more complex designs that include various bioactivities and other functionalities in an effort to mimic the complex composition and function of the natural ECM extracellular matrix. Girotti et al. have bioproduced the ELP polymer depicted in Fig. 18 [88].

This last ELP is made from a monomer 87 amino acids in length and has been produced with $n = 10$ (MW = 80 695 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 that the matrix is produced over a base of an ELP of the type $(VPGIG)_n$. This basic 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. It has a lysine substituting the isoleucine so the lysine γ-amino group can be used for cross-linking purposes while retaining the properties of elastinlike 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 chosen 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 once

Fig. 18 Schematic composition of monomer used in ELP design described in text. The scheme shows the different functional domains of monomer, which can be easily identified with their corresponding peptide sequences

the tissue decides that the natural ECM must be remodeled. In this sense, the presence of this specific sequence in the artificial polymer guarantees that the polymer will be bioprocessed only when the growing tissue decides that it is time to substitute it by a natural ECM, while in practice it remains fully functional until that time. 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 repairing and healing [151].

Although we are sill far from exploiting the full potential of genetic engineering, this last example impressively shows that we are now able to create materials for tissue engineering purposes whose composition and (bio)functionality are unprecedently closer to the rich complexity in functionality and bioactivity of the natural ECM. This polymer also shows the potential of genetic engineering in producing complex polymers in general, since one can hardly imagine obtaining polymers of the complex composition displayed by this last example by chemical methodologies that, in addition, will likely never be so robust, clean, cheap, and easily scalable.

6 Conclusions

Although the creation by genetic engineering of protein-based materials is still in its infancy, it has already shown extraordinary potential. Very complex, well-defined, and tailored polymers can be obtained by this technique, with a wide range of properties. Examples can be found in bulk materials and fibers with extraordinary mechanical performance as well as the most advanced, functional, self-assembling, and smart materials for biomedical uses and nano(bio)technology. The achievable degree of complexity and the concurrent development of function are unparalleled by other techniques. Complexity can be carried to a limit where the concept of the 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 a concept of the protein in our GEPBPs where, rather than having a polymer made by the repetition of a relatively short monomer or a combination of them, a macromolecule without excessive or no repetition is obtained. In that molecule, the single amino acids are grouped within functional domains. In their turn, those domains are arranged along the polymer chain in a well-defined molecular architecture in which there is no space for randomness. All of this to obtain a material in which an unprecedented given set of structural, physicochemical, and biological functionalities are required and must be fulfilled. 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 in practice by technological constraints but only by our imagination.

All the above examples were accomplished by a robust and relatively easy technology that, in the near future, could be a serious alternative to conventional polymer chemistry, especially if we take into consideration environmental concerns. By this clean procedure, we can produce economical and complex materials that would outperform the efficiency of the existing petroleum-based polymers. GEPBPs are expanding the limits of macromolecular functionality to territories never before imagined.

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