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Feature Article

"Recombinamers" as advanced materials for the post-oil age

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This work is dedicated to the memory of Prof. Antonio M. "Tony" Tamburro. His passion for science was only matched by his passion for life.

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ABSTRACT

Biotechnology offers powerful solutions to the challenges that arise during the design and development of new complex biomimetic materials to achieve specific biological responses. Recombinant DNA technologies, in particular, provide unique solutions in the biomaterials field, especially regarding the control of macromolecular architectures involving protein sequences with the aim of addressing the multiple functional requirements needed for biomaterials' applications. Here, elastin-like recombinamers are presented as an example of an extraordinary convergence of different properties that is not found in any other polymer system. These materials are highly biocompatible, stimuli-responsive, show unusual self-assembly properties and can include bioactive domains along the polypeptide chain. Applications of these engineered biomimetic polymers in nanotechnological systems, stimuli-responsive biosurfaces and tissue engineering will be discussed.

1. Introduction

Polymer science has clearly shown over several decades that macromolecules are excellent candidates for the creation of highly functional materials. As a result of the availability of thousands of different monomers and the multiple possibilities arising from their different combinations, polymer science has succeeded in producing a specific material for a particular application on many occasions, ranging from very simple materials for use as bulk commodities to highly sophisticated ones with special biomedical, engineering or nanotechnological uses. Very few other technical developments in history have shown the same rapid development and had the same deep impact on society as polymer science. The number of different technologies enabled by the existence of the appropriate polymer is amazing, and the crucial role of polymer science in the development of modern society and human well-being is unquestionable.

Most of the synthetic methodologies and the polymers we produce nowadays are, however, based exclusively on petroleum-derived chemicals. Although there is no consensus regarding how many oil reserves remain, it is clear that this resource is finite and that its price will continue to increase if we maintain our increasing rate of demand. Additionally, we would be well advised not to wait until the imminent exhaustion of our planet's oil reserves to reduce its use as a source of energy and plastics. Growing evidence that the recent increase in atmospheric CO_2 levels is causing a measurable modification of the global climate could, in the mid- to long-term, lead us to abandon, or at least drastically reduce, oil as our main source of raw materials for plastics [1]. Polymer science will therefore soon face a similar reduction in its dependence on oil to that currently being experienced by the energy sector.

Our current state of technological development and well-being cannot be maintained by sacrificing the expectations of future generations-sustainability must therefore also be a key objective in polymer science. However, we must be fully aware of the actual meaning of "sustainability". We are obliged to develop sustainable technologies that fulfill the needs of future generations. This does not mean that we must search for alternative and sustainable technological solutions, simply to maintain our current level of development. Therefore, with the degree of technical development that our grandparents enjoyed, our grandchildren will not be satisfied by a world in which polymers produced from renewable sources "only" match the performance of the "old" oil-derived plastics.

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The challenge that polymer science is currently facing must therefore be tackled from all sides. We, as polymer scientists, must develop technologies to change our current oil-dependence and unsustainability. This challenge must also be considered an opportunity to create a new polymer science which, in addition to being sustainable, will launch a technological revolution that will lead to new concepts, materials and products which will be more efficient, functional and than those we have today. Part of the aim of the present manuscript is to present evidence that this purpose is not just a utopian yearning for a better world and that some signs that this is possible may already be present.

Our level of technological development has been supported by a progressive abandonment of natural products and the extensive use of "synthetic" elements which, in terms of composition and concept, are far from being natural substances. Paradoxically, one of the most promising strategies for solving current problems is to reconsider natural products, or rather to introduce concepts imported from nature in our future synthetic materials and systems, and not only for the sake of sustainability. Thus, taking polymer science as an example, biology discovered long ago that macromolecules are the best option for obtaining highly functional materials. Relatively novel concepts in materials science such as hierarchical organization, mesoscale self-assembly or stimuli-responsiveness are common to many natural macromolecules such as proteins, nucleic acids or polysaccharides (or combinations of them). In fact, the slow but relentless process of natural selection has produced materials that show a level of functionality significantly more complex than that reached by synthetic materials. One of the best (and nicest) examples of this is the proteins. The proteins in living cells show an amazing set of capabilities in terms of functionality, ranging from the structural proteins, all of which show a significant ability to self-assemble, to the extraordinary enzymes, with their superior catalytic performance, and highly efficient molecular machines such as the flagellar rotary motor, etc. Natural proteins are usually large and very complex molecules which contain diverse specific functional groups that generate and promote self-assembly and function. Nature also makes use of different physical processes that allow directed and controlled organization from the molecular to the macroscopic level. In general, both local organization through functional chemical groups and the physical properties that give rise to order on larger scales provide the properties and functions that the biological systems require to function efficiently.

Nevertheless, the basis for all of this amazing functionality displayed by natural proteins seems to be based on one simple concept, namely a complex and completely defined primary structure. Protein biosynthesis in living cells occurs with an absolute control of the amino-acid sequence, from the first amino acid to the last, with a complete absence of randomness. Indeed, the need for such absolute control becomes dramatically apparent in some genetic disorders where the lack or substitution of just one amino acid in the protein leads to a complete loss of the original function, which can have dramatic consequences in patients with sickle cell anemia, phenylketonuria and cystic fibrosis [2]. If we want to create truly functional materials, we must therefore find a way to synthesise complex and completely defined macromolecules. This task, which is currently too difficult for even our most sophisticated chemical methods, occurs routinely in all living cells. One further characteristic of protein biosynthesis should be highlighted at this point, namely that the machinery for protein biosynthesis is extraordinarily flexible. Ribosomes are able to process and produce practically any amino-acid sequence stored in the information elements known as genes, which means that the flexibility of this process is essentially absolute. From a practical point of view, if we can therefore somehow control the information that genes deliver into the machinery, we can also control the biosynthesis process itself.

This idea also has remarkable precedents. In the last few years, significant effort has been dedicated to replacing petrochemical-based chemical processes with biological methods using renewable resources. Thus, fermentation processes for the production of biological monomers have been improved by numerous studies involving the metabolic engineering of microorganisms and the directed development of enzymes. Such microorganisms have been widely exploited for medical, agricultural, food and industrial applications. In addition, they have been engineered to produce recombinant proteins, amino acids and chemicals for use as drugs and biofuels [3,4]. For example, various monomers have been produced via different biological pathways, depending on the microorganism, from substrates such as succinic acid, lactic acid or some diols [5]. This process involves the whole metabolic and regulatory network together with fermentation, recuperation and subsequent purification processes.

The development of new technologies has made it possible to follow protein expression in cells and tissues through proteomics and it has allowed researchers to engineer proteins with new functions that lead to extraordinary technical applications. Nature has designed proteins with specific functional properties, such as the ability to self-assemble, recognition specificity or monodispersity, and scientists are now starting to exploit and enhance these properties in protein-based materials. Genetic and protein engineering provide us with the tools to precisely produce numerous protein-based polymers far above the current capabilities of synthetic polymer chemistry. These techniques allow us to synthesize protein chains with absolute control over their molecular mass, composition, sequence and stereochemistry. This is a key drawback of conventional chemical synthesis, where any increase in the complexity of the final molecule unavoidably leads to an almost exponential increase in the time and cost of the synthesis.

The use of recombinant DNA technologies to obtain protein-based polymers with total control of the randomness of the polymer sequence permits us to design the required functionalities of the final biomaterial in a highly precise manner. The success of engineered protein polymers in material applications will, however, depend on being able to obtain materials with specified physical and chemical properties. As an example of these approaches, we show here how elastin-like polymers (ELPs) play an important role in the synthesis of advanced materials, with a particular emphasis on biomedical and nanotechnological uses.

1.1. Genetic engineering of protein-based macromolecules

In the last few years, the application of powerful molecular biological methods has allowed the design and synthesis of new advanced materials almost at will. The use of the 20 naturally occurring amino acids in the design and production of genetically engineered functional protein-based macromolecules with specific or multifunctional properties offers practically infinite possibilities and a significant number of advantages. First, DNA technologies allow the introduction of tailored synthetic genes into the genetic make-up of a microorganism, plant or other organisms which induces the production of its encoded protein-based polymer as a recombinant protein [6,7]. These macromolecules offer the possibility to obtain materials with some of the complex properties found in natural proteins in combination with functions of particular technological interest that are not displayed in living organisms. Secondly, the degree of control and complexity attained by genetic engineering is clearly superior to that achieved by conventional chemical synthesis. These polymers, for example, are strictly monodisperse and can be obtained with molecular weights

ranging from a few hundred Daltons to more than 200 kDa [8]. This has enabled the study of the dependence of different material properties on molecular mass in a simple and highly precise manner [9]. Thirdly, the production cost of those materials is not related to their complexity as the most costly task in terms of both time and money is the gene construction. However, once the genetically modified (micro)organism is obtained, its fast and cheap production rapidly compensates for the costs associated with the molecular biology steps. This intrinsic advantage also has environmental benefits as recombinant protein-based materials are obtained by an easily scalable technology fermentation that uses only moderate amounts of energy and temperatures, with water as the only solvent. Finally, the genetic engineering of protein-based polymers is a relatively new methodology and only a limited number of research groups and companies have adopted this approach for their production. Despite the fact that interest in these materials has mainly concentrated on two major polymer families, namely spider-silk polymers [10,11] and ELPs [12–16], and their combinations [17,18], other interesting protein polymers, such as those based on resilin [19], abductin [20] or gluten [21], among others, have also been studied.

1.1.1. Some considerations regarding nomenclature

There is no consensus on how those materials should be named. The term most often used in the literature is "Recombinant Protein Polymers", although this term is unsuitable for many reasons. First of all is too long to be of practical use and, more importantly, it is confusing and does not describe what it tries to. "Protein polymer" can be understood as a polymer made of proteins, which of course is not the case. These materials are proteins only because they are produced as recombinant proteins, although their composition is the result of an engineered design step and the creation of a synthetic gene which is often totally unrelated to the composition of any natural protein. In addition, the term "polymer" is not adequate in most cases. The recombinant materials obtained from an artificial gene are normally macromolecules with a molecular mass comparable to that of conventional polymers. However, these recombinant materials are in many cases made from huge and complex monomers that are only repeated a few times (or do not repeat at all) in the final molecule so, despite their high molecular mass, they should strictly speaking be termed oligomers. In light of these considerations, we propose the use of a specific term that can be specifically associated with this new kind of macromolecules and which is sufficiently informative to clearly describe the main characteristics of this emerging class of materials. This term is "recombinamer", which clearly indicates the oligomeric nature of these compounds and their production as recombinant proteins. In addition, recombinamer strongly resembles the term polymer, thereby suggesting the macromolecular nature of these materials but without requiring that they involve a continual repetition of small and simple monomers. The term recombinamer also prevents the reader from automatically identifying these molecules with natural proteins, or some modification thereof, but rather suggests a molecule whose composition is defined strictly by engineering design. This term will therefore be used throughout this manuscript.

1.1.2. The example of elastin-like recombinamers (ELRs)

ELRs are a promising model of biocompatible protein-based polymers. The basic structure of ELRs is a repeat sequence found in the mammalian elastic protein elastin, or a modification thereof [22]. Some of their main characteristics are derived from those of the natural protein. For example, the cross-linked matrices of these polymers retain most of the striking mechanical properties of elastin [23], which becomes important when this behavior is accompanied by other interesting properties, such as biocompatibility [24], stimuli-responsive behavior, and the ability to self-assemble. These properties are based on a molecular transition of the polymer chains called the inverse temperature transition (ITT). This transition is the key to the development of new peptide-based polymers as molecular devices and materials.

The expansion of molecular biology has allowed the design of complex bioengineered ELRs as well-defined polymers [9,15,25,26]. The most well known members within the ELR family are based on the pentapeptide VPGVG (or its permutations), and a wide variety of polymers with the general formula (VPGXG), where X represents any natural amino acid except proline, have been (bio)synthesized [15,27,28]. All the polymers with this general formula found in the literature display functional properties such as acute stimuli-responsive behavior. The substitution of any of the other amino acids in the pentamer is not so simple. For example, the first glycine can only be substituted by L-alanine [27].

All functional ELRs exhibit a reversible phase transition in response to changes in temperature [29]. In aqueous solution, and below a certain transition temperature (T_t), the free polymer chains remain disordered, random coils [30] that are fully hydrated, mainly by hydrophobic hydration. This hydration is characterized by ordered clathrate-like water structures surrounding the apolar moieties of the polymer with a structure somewhat similar to that described for crystalline gas hydrates, although with a more heterogeneous structure that varies in terms of perfection and stability [31,32]. Above T_t, however, the chain folds hydrophobically and assembles to form a phase-separated state containing 63% water and 37% polymer by weight in which the polymer chains adopt a dynamic, regular, non-random structure known as a β-spiral, which involves type II β-turns as the main secondary feature and is stabilized by intra-spiral, inter-turn, and inter-spiral hydrophobic contacts [27]. This is an effect of the ITT. In this folded and associated state, the chain loses essentially all of the ordered water structures arising from hydrophobic hydration. During the initial stages of polymer dehydration, hydrophobic association of the β-spirals means they take on a fibrillar form. This process starts with the formation of filaments composed of three-stranded dynamic polypeptide β-spirals which grow to form particles several hundred nanometers in diameter before settling into a visible phase-separated state. This folding is completely reversible on lowering the sample temperature below T_t [27].

However, coacervation can be a complex process that is strongly influenced by the composition of the ELP. This is evident, for example, by examining the molecular and microscopical phenomena taking place in the coacervation of tropoelastin. Recent studies suggests the presence of complex structures involved in the coacervation of the that natural protein. Circular dichroism has showed the importance of α-helices and subsequent helical side chain interactions that limit the conformation of tropoelastin during coacervation [33]. Further, studies support a role for domain 26 [34], a junction between 10, 19, and 25 [35] and more recently for domain 30 [36].

2. Stimuli-responsiveness and self-assembly properties of ELRs

The responsive behavior of peptide-based materials has been defined as their ability to respond to external stimuli. This behaviour is even more interesting when the materials show reversibility at either the structural or functional levels, thereby offering obvious advantages as stimuli-responsive materials.

The T_t of the ITT can be measured by different techniques, the most widely used being turbidity measurements and calorimetric methods that measure the heat flow during the transition. The turbidity profile and heat flow from a differential scanning

calorimetry (DSC) measurement are plotted against the temperature in Fig. 1.

The T_t values obtained by these methods often differ depending on the method, with several factors likely to be responsible for these differences [16]. In addition, T_t also depends on the molecular mass, the mean polarity of the polymer [9], and the presence of other ions and molecules [37–39]. In conclusion, all these factors make the comparison of T_t values a very delicate matter.

As regards self-assembly, many synthetic strategies have been developed to obtain advanced devices in an attempt to mimic the behavior of biology in nature. These techniques have been based on the concept of self-organization and self-assembly in order to arrange hierarchically ordered complexes. Both of these mechanisms are widely used by nature and can be exploited in synthetic devices. Peptides and proteins are useful building blocks to obtain ordered nanostructures via self-assembly due to their well-stabilized folding, stability, and protein–protein interactions [40].

Natural elastin undergoes a self-aggregation process in its natural environment, leading to the formation of nanofibrils from a water-soluble precursor called tropoelastin [27,41]. This ability resides in certain relatively short amino acid sequences, which are known to coacervate and form fibrillar aggregates with a high degree of β-turn structure [42]. The development of genetic engineering techniques has allowed tailored molecular designs of ELRs with wide-ranging possibilities of being able to form other topologies and nanostructures to be obtained. Thus, the pH-responsive ELR $[(VPGVG)_2(VPGEG)(VPGVG)_2]_{15}$ is able to form polymer sheets containing self-assembled nanopores (see Fig. 2) [43].

An atomic force microscopy (AFM) study of the topology of polymer spin-coated films of the Glu-containing ELR, from acid and basic solutions, onto a hydrophobic Si substrate at temperatures below T_t has shown that, under acidic conditions, the polymer film shows only a flat surface with no outstanding topological features (Fig. 2A). When deposited from basic solution, however, the polymer film clearly shows an aperiodic pattern of nanopores (width of approximately 70 nm and separated by approximately 150 nm; Fig. 2B). This different behavior as a function of pH has been explained in terms of the different polarity shown by the γ-carboxyl group of the glutamic acid. In the carboxylate form, this moiety has a markedly higher polarity than the rest of the polymer domains and the substrate itself. The charged carboxylate groups therefore impede any hydrophobic contacts in their surroundings, which is the predominant assembly method for this kind of polymer. These charged domains, along with their hydration sphere,

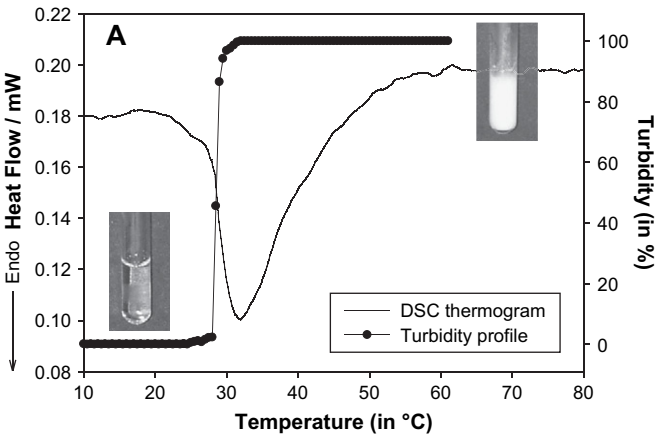

Fig. 1. DSC thermogram of poly(VPGVG) (50 mg/ml) in water (heating rate: 5 °C/min) and turbidity profile as a function of temperature for a 5 mg/ml aqueous solution of the same polymer. The two photographs are taken below (4 °C) and above (40 °C) the T_t [16].

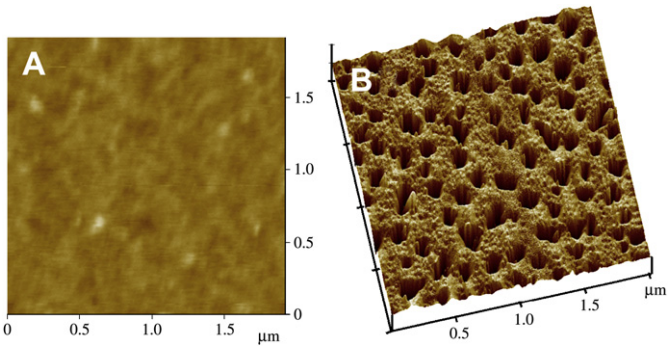

Fig. 2. AFM image of $[(VPGVG)_2(VPGEG)(VPGVG)_2]_{15}$ deposited from a water solution onto a hydrophobic Si substrate. Sample conditions: (A) 10 mg/ml in aqueous 0.02 M HCl solution (acid solution). (B) 10 mg/ml in aqueous 0.02 M NaOH solution (basic solution) [43].

therefore become segregated from the hydrophobic surrounding, thus leading to nanopore formation.

3. ELRs as advanced materials for biomedical applications

ELRs show an additional property which is highly relevant for the use of these polymers as advanced materials for biomedical applications, namely their extraordinary biocompatibility [24]. In addition, their biodegradability is obvious as the secondary products of their bioabsorption are simply natural amino acids.

3.1. Nanotechnological systems

The increasing need for drug-delivery systems that improve specificity and activity whilst at the same time reducing toxicity to ensure maximum treatment safety has led to the development of a wide variety of new materials, many of which have been employed to control the release of drugs and other active agents. Polymeric systems are, however, often the system of choice because of their desirable physical properties [44].

Recombinant polymers, such as thermo-responsive ELRs, represent one of the possible next steps in the development of drug carriers beyond traditional, synthetic polymers. Elastin biopolymers respond to external stimuli by undergoing a reversible phase transition where, at temperatures above T_t, the ELR hydrophobically self-assembles into an insoluble aggregate, thus forming nano- and microparticles which could be loaded with a drug. The first ELR-based drug-delivery system was a simple device in which γ-radiated cross-linked poly(VPGVG) hydrogels of different shapes were loaded with a model water-soluble drug (Biebrich Scarlet), which was released by diffusion. Additionally, the inclusion of some glutamic acids along the polymer chains was used to control the hydrolyzable cross-linking. The cross-linking was of the carboxy-amide type and the drug was released as these cross-links were hydrolyzed at a given rate [45]. Poly(VPAVG) nano- and microparticles have also been tested as carriers of the model drug dexamethasone phosphate in order to develop injectable systems for controlled drug release [46]. Nanoparticles with a diameter of around 300–400 nm have recently been obtained from ELRs by a novel application of the electrospray technique to encapsulate drugs. The morphology and size of these polymer nanoparticles can be controlled by varying the composition, molecular mass, and solvent, amongst others [47]. In other recently study, nanoscale protein particles with less than 100 nm in diameter were constructed using temperature-sensitive ELR and polyaspartic acid chain under physiological conditions. The critical temperature of formation of the particle can be adjusted by the lengths of the

aspartic acid chain and the ELR. Therefore if a lower critical temperature is required, it is only necessary to elongate the ELR [48].

ELRs are particularly attractive for the synthesis of block copolymers that self-assemble into polymer nanostructures such as micelles. The first work in this area involved an elastin-mimetic di-block copolymer containing VPGEG–$(IPGAG)_4$ and VPGFG–$(IPGVG)_4$ as the hydrophilic and hydrophobic blocks, respectively [49]. The resulting micelles were studied by dynamic light scattering (DLS) and DSC was used to measure the enthalpy of self-assembly. A tri-block copolymer was subsequently synthesized and the TEM images of this polymer showed that it formed spherical aggregates [50]. Other multivalent spherical micelles have been obtained from linear elastin-like AB di-block copolymers in the temperature range 37–42 °C with the aim of targeting cancer cells [51]. Bidwell et al. have also exploited the ELRs for its ability to serve as macromolecular carriers for thermally targeted delivery of drugs. Attachment of doxorubicin to ELR-based system showed enhanced cytotoxicity in uterine sarcoma cells when aggregation was induced with hyperthermia [52].

We have also synthesized amphiphilic di- and tri-block copolymers to study the micelle self-assembly process. These block copolymers contain two different blocks: one with the monomer $[(VPGVG)_2$-(VPGEG)-$(VPGVG)_2]$ (E-block) and the second with the monomer $[(VPGAVG)_m]$ (A-block). Both these blocks are thermo-responsive and the E-block is also pH-responsive [53]. The spontaneous formation of nanostructures can therefore be controlled by changing both the pH and the temperature, and vesicles with different sizes have been obtained [54] (Fig. 3).

3.2. Biosurface engineering

Surface engineering is an important tool for understanding the molecular mechanisms involved in protein adsorption and cell–surface interactions. The design and control of these features is a key challenge for diverse specific biological applications [55,56]. Multiple approaches involving physical and chemical modifications, such as coatings and grafts or the introduction of small biological ligands (peptides or proteins), have been developed in the surface engineering of biomaterials [55–59]. These approaches allow surfaces to be functionalized with fouling-anti fouling features, specific groups for cell–material interactions, responsive behavior (stimuli or environmentally sensitive), or with micro- and nano-patterns.

3.2.1. Functionalized surfaces with ELRs

The modification of surfaces with stimuli-responsive polymers that vary their physical and chemical properties in response to changes in their environment or external stimuli makes these polymers excellent candidates for the development of stimuli-responsive surfaces [60–62]. In most cases, the surface is grafted with derivatives of poly(*N*-isopropylacrylamide), a well-known thermo-sensitive polymer. ELRs exhibit some additional advantages that make them excellent candidates for the development of responsive surfaces. Therefore, Ozturk et al. have prepared recently micropatterned pNIPAM films as thermo-responsive cell carriers. They were chemically modified by ELR adsorption containing RGD amino acid sequence to promote cell adhesion. They have studied the thermal responsiveness to apply mechanical stress on cells under *in vitro* conditions to induce bone formation showing that ELR is crucial for maintaining the cells attached on the surface in dynamic culturing [63]. For instance, genetic engineering allows them to be designed with extraordinary control of the sequence and with desirable properties, which means that in addition to their thermo-responsive behavior they can also respond to other stimuli such as pH, light, or ionic strength, amongst others. On the other hand, biosynthesis enables precise control of the reactive sites on the polypeptide chain for use in surface grafting. For example, the nanometric control of their position leads to a tremendous potential for self-assembly and other functionalities displayed by these systems. Biosensing surfaces can take advantage of the reversible phase-transition behavior of ELRs to obtain an active surface whose properties such as hydrophobicity and functionality can be quickly modulated by a simple temperature change. However, in spite of the enormous potential of these biopolymers, there are only a few examples of surfaces functionalized with ELRs, one of which makes use of the above-described topologically modified self-assembly ability of the recombinant ELR $[(VPGVG)_2$-(VPGEG)-$(VPGVG)_2]_{15}$. Thus, by covalently micropatterning ELRs onto glass surfaces, Chilkoti's group has created what they have called "thermodynamically reversible addressing of proteins" (TRAP) [55,61]. This allows the reversible, spatio-temporal modulation of protein binding at a solid-liquid interface and can be applied in different systems for bio-analytical devices.

Other techniques used for ELRs include layer-by-layer deposition of alternating ELR-polyelectrolytes, which is a simple technique to generate bioactive surfaces [64]. These ultra-thin nanoscale coatings promote cell adhesion and proliferation and the results show that the thickness and mechanical integrity of the multilayer assembly modulates the cell response. Costa et al., for example, have developed thermo-responsive thin coatings using electrostatic self-assembly (ESA). A recombinant ELR containing the cell attachment sequence RGD has been deposited onto chitosan and has been found to show enhanced cell adhesion in comparison

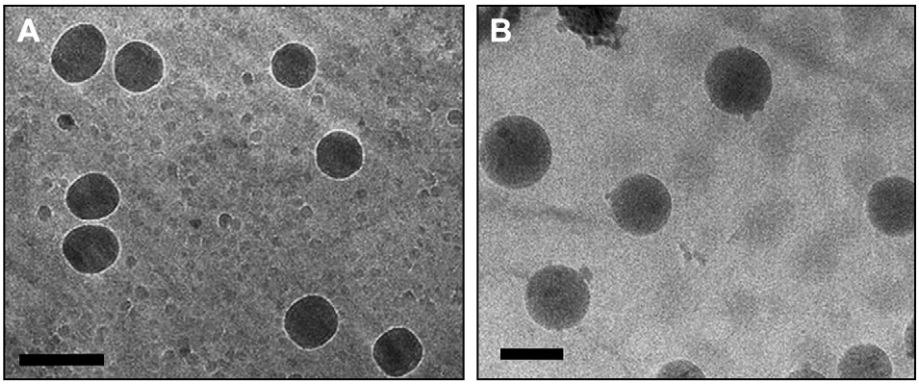

Fig. 3. Cryo-TEM images in aqueous solution: (A) E50A60E50, (B) E50A60. Scale bars: 200 nm.

with the original chitosan monolayers or glass substrates [65]. These examples open up the field of polymeric coatings that include specific biofunctional responses.

3.2.2. Nano- and microtopographical surfaces

In the last few years the combination of surface chemistry with microfabrication techniques has provided new tools to study the interactions of cells with their environment. Using lithography and patterning techniques, peptides and proteins can be deposited with complete spatial control on specific regions of a surface [66,67]. The ability to obtain nano- and micrometer-sized patterns of biological macromolecules is of great importance for several applications, including biological assays, miniaturized biosensors, and biomedical diagnostics.

ELRs have been employed in the design and development of regenerable biosensors and microfluidic bioanalytical devices, as reported by Chilkoti et al. [68]. Thus, nanostructured surfaces that are able to capture and release proteins using the self-assembly properties of ELRs have been obtained by combining ELRs and dip-pen nanolithography.

In our group, we have adapted the simple method of replica molding to obtain 3D microstructured thermo-responsive hydrogels [69]. Replica molding is a fast, flexible, and straightforward micropatterning technique that can be carried out routinely and consists of only a few steps: dispensing of the polymer on the mold, cross-linking, and release of the replica ELR hydrogel. In this study, which aimed to test the thermally responsive behavior of macroscopic and micropatterned features, we obtained hydrogels with micropatterns such as lines or pillars with different dimensions and spacings (see Fig. 4).

The dimensions of the microfeatures with micropatterned lines were tested with respect to the water temperature, with a 30–35% decrease in dimensions being observed for both patterns at a temperature above the transition temperature of the hydrogels (20 °C). This thermo-responsive behavior does not modify the topography and can be used to change the dimensions of the micropatterned features during cell culture. Furthermore, these systems permit controlled topography to be added as a further factor for studying cell behavior and cell-surface interactions, thereby improving the extraordinary properties of ELR hydrogels, particularly their bioactivity, biocompatibility, and the "tunability" of their mechanical properties.

The ability to generate micro/nanoscale features with synthetic and natural polymers has been improved by using a simple fabrication technology known as electrospinning [70,71]. This process has been widely used in the textile industry and organic polymer science and has now reappeared as a novel tool for fabricating biopolymer scaffolds [72–74]. The electrospinning process involves applying a high voltage to create an electrically charged jet of polymer solution, which dries to leave a polymer nanofiber mesh. The fibers produced by this process usually have diameters on the order of a few micrometers down to less than a hundred nanometers. Their structural properties depend on processing parameters such as polymer concentration and viscosity, flow rate, and applied voltage, amongst others [75]. The ability to vary fiber size in the nanometer range opens up the possibility of mimicking the size scale of fibrous proteins found in the natural extracellular matrix. Indeed, fibers made from different proteins such as fibrinogen [76], gelatin [73], collagen–elastin mixtures [77], or silk-like proteins [78] have been obtained. The first elastin-mimetic protein fibers were produced from a genetically engineered ELR [79]. Different morphological patterns, such as beaded fibers, thin filaments, or broad with a ribbon-like appearance, were obtained by varying the solution concentration. To date, electrospun elastin analogs have only played a key role in modulating the viscoelastic properties of the resulting blended material the inclusion of bioactive domains in their structure has not been reported. We have also exploited this technique to obtain nanofibers from an aqueous solution of multifunctional recombinant ELRs containing cell-attachment sequences [80]. These fibers were chemically cross-linked after deposition and immersed in water to study their morphology. Fig. 5 shows the SEM micrographs of our ELR nanofibers.

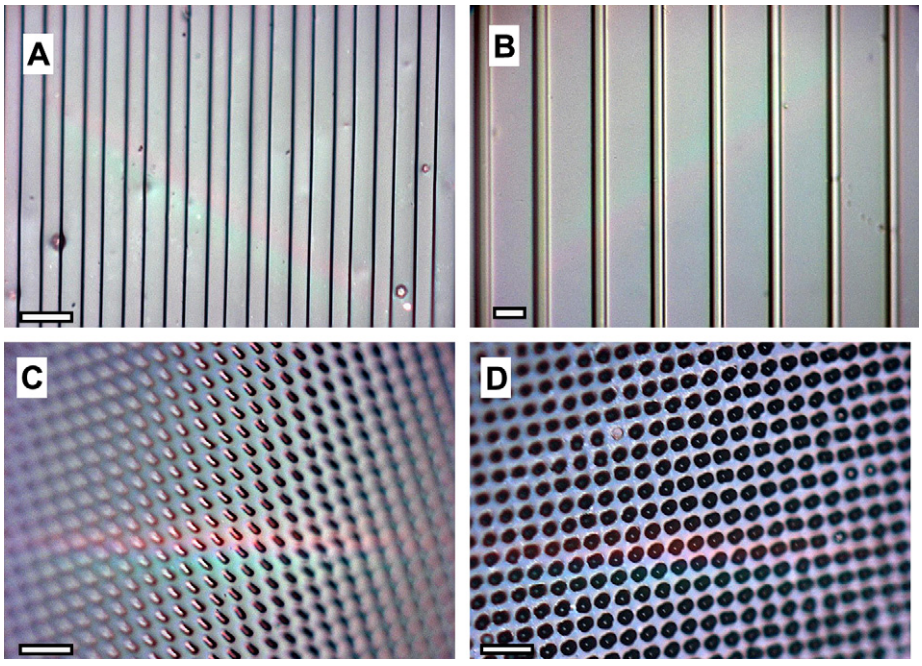

Fig. 4. Optical micrographs of different micropatterned hydrogels. Scale bars: 50 μm [69].

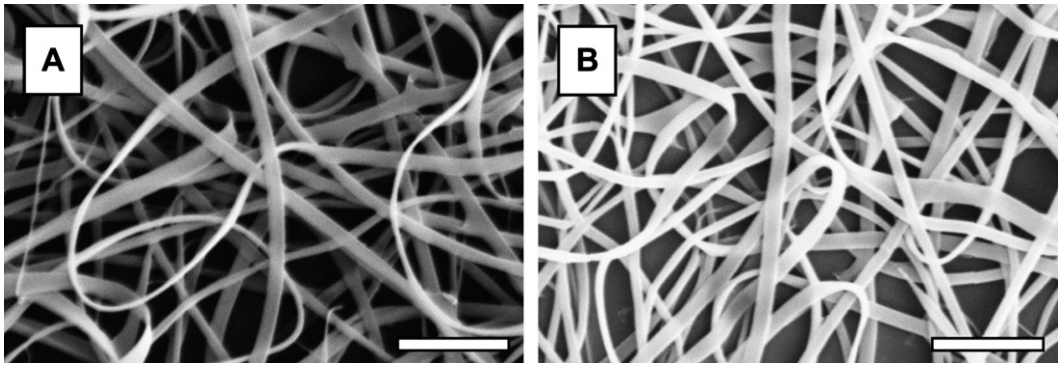

Fig. 5. SEM micrographs of ELR nanofibers: A) as-deposited fibers; B) cross-linked fibers. Scale bars: 5 μm.

The average size indicated a diameter increase of about 15–20% after the cross-linking reaction. These substrates were tested in different cell human lines and showed interesting properties in terms of cell adhesion. An ELR with a similar composition but lacking bioactive domains has shown non-fouling properties, thereby suggesting future applications where nonspecific adhesion could be desirable [80].

4. Tissue engineering

The design of functional biomaterials that induce a specific cellular response is a major challenge in the field of materials science. Methods for fabricating stimuli-responsive biomaterials have allowed these materials to play a more interactive role in tissue engineering [81,82]. However, there are several requirements that a biocompatible material should provide to promote cell attachment, differentiation, and proliferation. Thus, the scaffold must be biocompatible and biodegradable and it should show properties that support tissue morphogenesis. This generally requires an artificial extracellular matrix that can supply temporary mechanical support until the engineered tissue has sufficient mechanical integrity to support itself.

The extracellular matrix (ECM), which contains a complex composition of fibrous proteins and heteropolysaccharides, is an important model for biomaterials' design. Recombinant DNA technologies allow the design and expression of artificial genes to prepare artificial analogues of ECM proteins with controlled mechanical properties that incorporate domains to modulate cellular behavior [83]. Future advances in tissue engineering will depend on the development of biomimetic materials that actively participate in the formation of functional tissue.

The first candidate ELRs for tissue engineering were poly-(VPGVG) and their cross-linked matrices [12]. These materials were tested for cell adhesion and it was found that cells did not adhere to them. This provided a starting point to obtain key biomaterials which maintain their biocompatibility and adequate mechanical properties but lack nonspecific bioactivity. The subsequent incorporation of active peptides as cell-adhesion ligands resulted in a high capacity to promote cell attachment. These bioactive (VPGVG) derivatives containing the general adhesion peptides RGD and REDV, the latter of which is specific for endothelial cells, showed similar cell attachment behaviors to human fibronectin [84]. Once genetic engineering was adopted as the production method of choice, the molecular designs started to increase in complexity. The addition of different functionalities as cross-linking domains facilitates the attainment of more uniform substrates, which are usually based on lysine residues incorporated in the elastin-based repeat unit (VPGXG) [23,26,85]. ELR hydrogels produced by photoinitiation [86], irradiation [87,88], amine reactivity [13,23,26,85,89–93], and enzymatic cross-linking by tissue transglutaminase [94] show interesting properties as stimuli-responsive substrates. These hydrogels are a new class of soft materials which, in response to a small change in temperature, light, or other environmental stimulus, swell to several times their original volume or shrink to the same degree [81,95]. These materials have proved extremely useful in biomedical and pharmaceutical applications due to their high water content and rubbery nature, which is similar to that of natural tissue.

Chemical cross-linking in organic solvents leads to the formation of more uniform hydrogels as the ELR molecules exhibit no inverse phase transition. McMillan et al., for example, have used a bis(sulfosuccinimidyl) suberate cross-linker to join ELRs containing lysine residues in phosphate buffer at pH 8.5 or in dimethyl sulfoxide. These authors studied the effect of the solvent on the cross-linking density and the gel structures [91,92]. The physical properties of chemically cross-linked hydrogels can be modulated by varying the ELR's molecular mass, concentration, and lysine content. This ability to prepare "tunable" hydrogels allows these ELRs to be used in a wide range of applications [96]. Although there are many examples of ELR hydrogels cross-linked in organic solvents, the application of in situ cross-linking in an aqueous medium is limited by factors such as the toxicity of the reagents and byproducts and slow gelation kinetics. Lim et al. have reported that the chemical gelation of ELRs in physiological conditions provides a biocompatible and injectable biomaterial for support-tissue regeneration [93].

In our group, Girotti et al. have bioproduced the ELR polymer $[(VPGIG)_2$-VPGKG-$(VPGIG)_2$-(EEIQIGHIPREDVDYHLPY)-$(VPGIG)_2$-VPGKG-$(VPGIG)_2$-$(VGVAPG)_3]_n$ ($n = 10$; MW = 80925 Da) [7]. The monomer unit contains four different functional domains in order to achieve an adequate balance between mechanical and bioactive responses. The $(VPGIG)_n$ sequence in this material confers its excellent mechanical properties, extreme biocompatibility, and stimuli-responsive nature. The second building block is a modification of the first, with a lysine instead of isoleucine, which means that the lysine ϵ-amino groups can be used for cross-linking and other chemical modifications whilst retaining the properties of ELRs. The third group contains the (REDV) peptide sequence found within the alternatively spliced CS5 fibronectin domain, which is specifically recognized by the integrin α1β4 [97]. This integrin is present in a few cell lines and its specificity for REDV tetrapeptide has been confirmed in endothelial cells, which selectively bind to REDV-coated surfaces [98]. Finally, the polymer possesses another functional block, in this case a recurring hexapeptide derived from the human elastin exon 24-encoded product $(VGVAPG)_3$ [99]. This sequence was introduced to drive enzymatic hydrolysis of the synthetic scaffold by the same

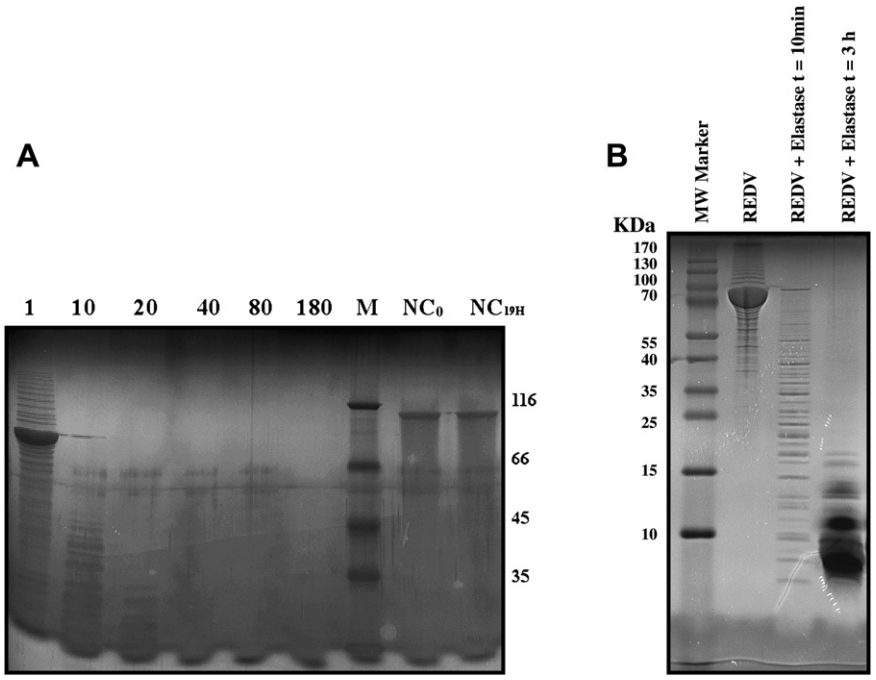

Fig. 6. SDS-PAGE analysis of ELR proteolysis: A) digested fragments obtained at different incubation times; B) tricine SDS-PAGE is used to separate the resulting total digestion fragments. The numbers at the side of the images indicate the position and size of the molecular mass protein marker.

physiological pathways as natural elastin during ECM remodeling as it is a target for certain proteolytic enzyme elastases [99].

REDV–ELR biopolymer biodegradation was tested with a specific protease, namely human leukocyte elastase I (Fig. 6). When recombinant ELRs were incubated under optimal enzymatic conditions the elastase was found to quickly and completely digest the REDV biopolymer and no significant degradation of the control biopolymer (lacking the target hexapeptide) was observed even when extending the experimental time (Fig. 6A).

The molecular mass of the bands produced in a complete digestion was analyzed in tricine SDS-PAGEs [100]. This electrophoresis method allows us to separate bands corresponding to proteins and/or peptides with a molecular mass of less than 9000 Da. The bands of the ELR proteolytic fragments resulting from complete enzymatic hydrolysis were found to possess a similar molecular mass to that of the theoretical fragments produced by specific elastase proteolysis (Fig. 6B).

We have also tested the adhesion of human umbilical vein endothelial cells (HUVECs) to chemically cross-linked ELR film scaffolds containing REDV adhesion sequences and to a negative control (lacking the bioactive sequences) [101]. On REDV–ELR scaffolds the cells showed a spread morphology with the cytoskeleton actin filaments (stained green) well organized into stress fibers, which is indicative of strong adhesion (Fig. 7A). The cell number and morphology of the HUVECs seeded on the ELR-negative control were completely different from those seeded on the REDV scaffold, with few smaller and rounded cells with minor lamelopodia extensions, thus indicating that passive adhesion was the main cell–scaffold interaction (Fig. 7B).

This ELR-containing REDV cell-adhesion sequence has also been used to prepare hybrid scaffolds [102] as the introduction of ELR as an elastic element in collagen-based scaffolds enhances their mechanical properties. In this study, enzymatic cross-linked ELR-collagen scaffolds were tested *in vitro* as substrates to study cell viability with different cell lines. An increasing ELR fraction in the scaffold was found to have an antifouling effect in fibroblasts, whereas endothelial cells displayed normal behavior and proliferation in the hybrid scaffolds. Varying the proportion of both materials should allow us to design the optimal requirements for future applications, such as vascular tissue or skin wound healing.

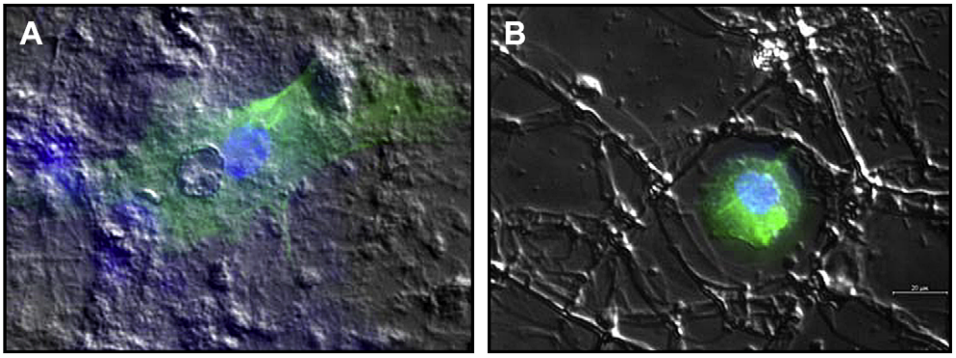

Fig. 7. Microscopy images of HUVECs seeded on ELR films after 16 hours of incubation: A) REDV film; B) negative control.

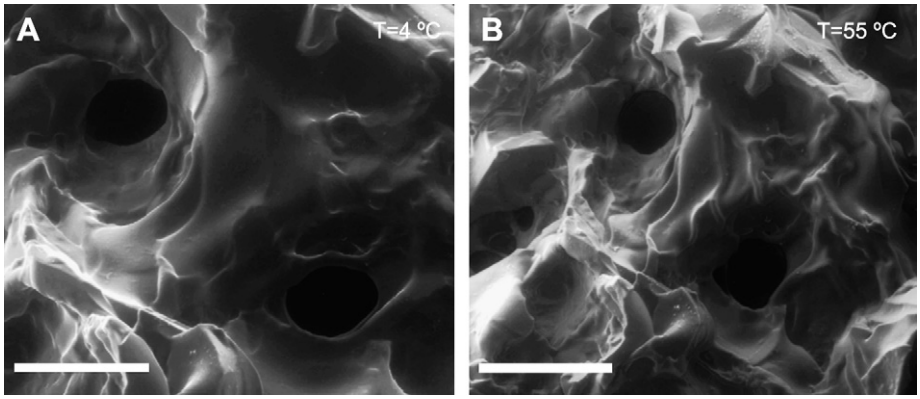

Fig. 8. ESEM micrographs showing the change in pore-size with temperature in swollen hydrogels in water: A (4 °C); B (55 °C). Scale bars: 100 μm.

Highly porous hydrogels formed from recombinant elastin-like polymers chemically cross-linked with hexamethylene diisocyanate have been obtained by the salt leaching/gas foaming technique for use in 3D cell culture [103]. The pore size of these gels can be controlled by varying the size of the salt particle incorporated during the cross-linking reaction. Physical properties such as the porosity, swelling ratio, and mechanical properties are also influenced by the salt/polymer weight ratio. The thermal behaviour of these gels was also studied in terms of the physical properties. Thus, the swollen hydrogels were heated to test the effect on the pore size (see Fig. 8). The collapse observed due to the phase transition above T_t decreased the mean pore size by about 30%. This technique should provide a simple approach to the fabrication of advanced scaffolds with "tunable" biological and physical properties in which the elasticity and thermo-responsive behaviour expand the range of potential applications of these materials.

Reverse thermosensitive polymers are very promising base materials for "in situ generated implants". The ability to produce low viscosity physiological solutions at room temperature which form a gel at higher temperature opens up numerous possibilities, although these are normally directed in two main directions: hydrophobic materials which acquire desired mechanical properties or water-based systems for the controlled release of hydrophilic macromolecules. The structural complexity of ELRs with specific mechanical, chemical, and biological properties allows us to design specific features that make it possible to acquire some or all of these properties. The self-assembly behavior of ELRs, for example, has been triggered by the addition of different main peptide blocks in the structure. Thus, hydrophilic blocks provide conformationally elastic properties whereas hydrophobic blocks form physical cross-links through hydrophobic aggregation (see Fig. 9).

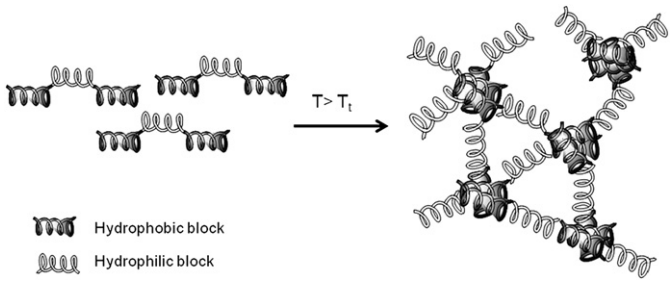

Fig. 9. Hydrophobic aggregation scheme of ELR tri-block copolymers in aqueous systems.

In a recent example, ELR tri-block copolymers with different hydrophobic architecture were found to form gels with a complex shear modulus ranging from 4.5 to 10.5 kPa and which can be increased by changing the hydrophilicity of the inner block [104]. This elastic modulus has been enhanced by including additional chemical cross-linking sites in the polymers' composition [105], thus making them excellent candidates for biomedical applications.

5. Conclusions

The goal of replacing current oil-based chemical processes with biological methods has resulted in the development of a method to produce complex recombinamers with a well-defined sequence and complete control of the molecular architecture. In this review we have summarized some examples that demonstrate the versatility of ELRs for a wide range of applications. The potential of ELRs to self-assemble in response to environmental changes makes them attractive for the construction of nano-devices for use as controlled delivery systems, stimuli-responsive biosurfaces, or advanced nanobiotechnological applications. The tailored introduction of cross-linking groups, cell-binding domains, and enzymatic biodegradation along the polypeptide chain makes these materials excellent substrates which can be used to mimic some of the most important characteristics of the ECM for tissue engineering. Thus, ELR hydrogels are promising candidates in terms of microstructure, "tunable" mechanical properties, and topography for the study of cell behavior and cell–surface interactions, which is an important step towards the development of cell-based biomedical systems.

Acknowledgments

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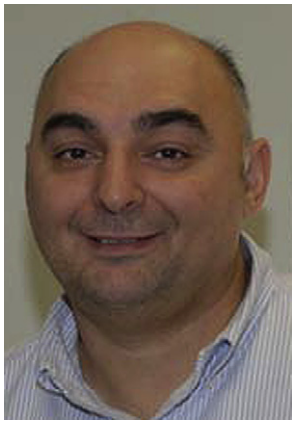

Prof. J. Carlos Rodríguez-Cabello was born in 1963. He got a degree in chemistry and a second degree in physics at the University of Valladolid in Spain. His PhD was carried out in the same University examining the relationships between macroscopic properties and molecular state in polymers, making special emphasis at the deformation processes. He got postdoctoral formation in Essen University (Germany) and the Institute of Macromolecular Chemistry in Prague (Czech Republic). After that, his interest moved to the field of biomacromolecules, and in particular to recombinamers (recombinant protein polymers). Nowadays, from his Professor position in the Department of Condensed Matter Physics in the University of Valladolid, he is leading a multidisciplinary research group, BIOFORGE, dealing with the design, production and characterization of recombinamer-based materials and systems in areas such as regenerative medicine, nanomedicine and nanotechnology. Complexity and multi(bio)functionality are the characteristics of the systems developed by his group. He has published more that 70 research papers, 4 patents and 10 book chapters.

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Prof. Matilde Alonso got a Degree in Chemistry by the University of Valladolid (Valladolid, Spain). Later she got a PhD degree in the same University. Her thesis dealt with the molecular structure and its relation with macroscopical properties of polymer composites, specially those formed by polyolefins and mineral (nano)particles. Her mentor was Prof. J.A. de Saja. After that period, she cofunded the BIOFORGE research group with Prof. Rodríguez-Cabello. She is the leader of the unit of Chemical Technology in BIOFORGE, coordinating the efforts of chemical processing and surface chemistry of recombinamers. She is now Professor in the Analytical Chemistry Department of the University of Valladolid. Her research production is summarized in more than 50 original research papers, 2 patents and 5 book chapters.

Dr. F. Javier Arias was born in 1966. He studied chemistry at the University of Valladolid in Spain and carried out his doctoral studies under supervision of Prof. T. Girbés examining the Ribosome-Inactivating Proteins (RIPs) present in several plant species. In 1993 he received his PhD degree in Biochemistry and in 1994 he joined as postdoc Dr. E. Benvenuto at the ENEA of Rome (Italy) with a Spanish fellow first and a Biotechnology programme fellow (European Community) later. There he worked on the molecular biology of Single-Chain Antibody Fragments (scFv) and their bioproduction in bacterial and plant expression systems. In 1998 he returned to the University of Valladolid as assistant professor and since 2002 he holds a position as associate professor for biochemistry and molecular biology. He has more than 50 scientific publications in different journals and more than 10 patents of invention. Present research topics are recombinant biopolymer design and synthesis for biomaterials, drug delivery and functionalized nanoparticles issues at Bioforge group as head of the Biotechnology Unit.

Dr. Ana María Testera was born in 1969 and obtained her PhD degree in Chemistry from University of Valladolid (Spain) in 1997. In this year, she joined the group of Prof. Dr. V. Gotor in the Organic and Inorganic Department, Faculty of Chemistry, University of Oviedo, (Spain) as postdoc. Her work was concerned to the field of biotransformations with microorganisms or enzymes. Since 1992 she holds a position as assistant professor in the Organic Chemistry Department of the University of Valladolid. In 2001, she joined Dr. José Carlos Rodríguez-Cabello research group BIOFORGE, where she works on elastin like proteins bioproduction, characterization and their chemical modification in order to obtain, functional polymers of great interest in tissue engineering, drug delivery or nanotechnology, with additional sensibility towards different factors as pH, electromagnetic radiation or concentration of some chemical species such as glucose.