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Recombinant Technology In The Development Of Materials And Systems For Soft-Tissue Repair

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The field of biomedicine is constantly investing significant research efforts in order to gain a more in-depth understanding of the mechanisms that govern the function of body compartments and to develop creative solutions for the repair and regeneration of damaged tissues.The main overall goal is to develop relatively simple systems that are able to mimic naturally occurring constructs and can therefore be used in regenerative medicine. Recombinant technology, which is widely used to obtain new tailored synthetic genes that express polymeric protein-based structures, now offers a broad range of advantages for that purpose by permitting the tuning of biological and mechanical properties depending on the

intended application while simultaneously ensuring adequate biocompatibility and biodegradability of the scaffold formed by the polymers. This review is focused on recombinant protein-based materials that resemble naturally occurring proteins of interest for use in soft tissue repair. An overview of recombinant biomaterials derived from elastin, silk, collagen and resilin will be given, along with a description of their characteristics and suggested applications. Current endeavors in this field are continuously providing more-improved materials in comparison with conventional ones. As such, a great effort is being made to put these materials through clinical trials in order to favor their future use.

Introduction

The field of biomedicine is nowadays increasingly focusing on the development of new approaches for the creation of materials and devices that could act as biological substitutes for the rehabilitation, preservation or enhancement of tissue function. The present review will focus on such systems obtained using recombinant technology and intended for use in soft-tissue regeneration.

Tissue engineering, which was first specifically discussed in the late 1980s[1], combines knowledge from the fields of medicine, biology, chemistry and engineering in order to improve the quality of life of patients who have suffered specific tissue damage. The development of new materials and systems for application in this context tends to occur synergically with the acquisition of a better understanding of human genetics, which permits the use of advanced approaches such as the use of human embryonic stem cells (hESCs), patient-specific cell lines, induced pluripotent stem cells, and gene therapy[2]. Progress in this field is occurring in response to the increasing gap between the number of patients in need of an organ and the quantity of suitable donor organs available. The common requirements for all materials employed in biomedicine are biocompatibility, biodegradability, appropriate mechanical characteristics, scaffold architecture and suitable manufacturing techniques[3].

Systems for tissue repair can be synthetic or natural and, depending on the application, degradable or not. Protein-derived synthetic biomaterials are inspired by naturally occurring proteins in the extracellular matrix (ECM), and are presented as enhanced substitutes for the more widely used chemically synthesized non-protein based materials. Generally speaking, the latter ensure adequate mechanical characteristics but are disadvantaged from a biocompatibility and biodegradability point of view. Protein-derived scaffolds can be obtained using different approaches, ranging from extraction from animal and human source tissue to more modern advanced techniques, such as the recombinant production of protein-based constructs. The lower immunogenicity of protein-based structures (collagen, elastin, silk, resilin, gelatin, fibrinogen, amongst others) due to their resemblance to natural amino acid sequences in the human body confers a significant advantage in comparison with proteins derived from animal tissue, the use of which should be avoided, if at all possible, due to their heterogeneous composition, the possible presence of infectious agents or their ability to cause hypersensitivity reactions. The biodegradability of these materials permits their temporary use, when necessary, and gradual replacement with newly formed tissue[4].

Recombinant technology involves the formation of a completely new and tailored synthetic gene, given by a DNA sequence, which leads to the production of a recombinant protein polymer when transcribed and translated into heterologous hosts (microorganisms, plants or mammalian cells). The overall process has been described in detail elsewhere[5] and involves two synthetic strategies for expression of the protein (random oligomerization or controlled-multimerization methods)[6]. The progress of the procedure can be seen in **(Figure 1)**. After production, various purification protocols can be used (inverse transition cycling[7], chromatography methods[8], combination of heating/acidification/ammonium sulfate precipitation[9] etc.).

Recent advances in the synthesis of genes encoding any repeating unit offer practically inexhaustible possibilities for the design of innovative polymeric materials with unique functionalities. The striking feature of recombinant technology is its potential to match the complex characteristics of natural proteins with technological functionalities not found in natural structures by allowing specific and highly controlled changes in the amino acid sequence, thereby conferring adequate mechanical and structural characteristics on the product and the modulation of cell behavior. In addition, new protein-based polymers can be obtained using non-natural amino acids or by favoring the assembly of *de novo* peptide sequences. The main features of the recombinant technology are summarized in **(Figure 2)**. Collagen, elastin, resilin, silk-elastin and other naturally occurring proteins have served as a source of inspiration for the production of recombinant polymers by carefully identifying and selecting the sequences that confer the desired properties required to guarantee crucial structural characteristics on the new synthetic materials. Indeed, modifications to the amino acid sequence and its length influence biological, thermodynamic and mechanical features, and permit the addition of specific functionalities.

In the past, biomedical devices were considered to be simply supports for the healing process in a damaged region of the body, whereas currently they interact actively with mechanisms of the body, via different pathways, in order to favor the natural healing mechanism[3]. The main role of these advanced scaffolds is to mimic the whole range of complexity of the extracellular matrix and tissue components found in healthy organs as closely as possible. The most frequent approach in this regard is to combine porous platforms made up of biomaterials and cells which, together, promote the regeneration of damaged tissues or organs. As hydrogels and porous 3D networks are the most widely used scaffolds for regeneration and replacement when emulating natural soft tissue, they will be the main focus of this review.

One of the major issues to be overcome in tissue repair is the inadequate vascularization of the constructs, which leads to cell death and improper waste elimination~~,~~. In this regard, it is important to point out that, in the case of implanted platforms, high porosity and interconnection between the pores of the structure guarantees a good platform for cell colonization and entry of the nutrients required by the cells and the extracellular matrix surrounding them. In addition, the fragments formed upon gradual degradation of the scaffold should be able to easily exit the body without affecting body structures, and any bioactive molecule loaded onto the scaffold should diffuse out at an appropriate delivery rate. Pore size must also be adapted to the tissue in which the scaffold is applied and the cell type with which it is to be colonized. Various techniques, such as electrospinning[10], casting/porogen leaching[11], salt leaching/gas foaming[12], emulsion freeze-drying[13], 3D printing[14], high pressure CO_2[15], amongst others, permit pore size to be adapted to the specific application. Sophisticated biomaterials are engineered to ensure that they contain multi-functional domains which confer smart behavior, sensitivity to specific stimuli (responsiveness to a specific temperature and pH value[16] of the environment, ionic strength, light, electric or magnetic fields) or bioactivity (to introduce sensitivity to enzymatic cleavage[17], to achieve the desired cell behavior,…) and an ability to produce regenerative signals in the surrounding tissue in order to avoid long *in vitro* culture times before application. As far as network structured hydrogels are concerned, crosslinking of the scaffold is crucial to ensure stability and resilience and therefore avoid easy rupture under stress. Physical crosslinking relies primarily on hydrophobic interactions, whereas chemical crosslinking leads to the formation of strong covalent bonds that can influence physical features. These techniques involve the use, among others, of tannic acid, hexamethylenediisocyanate (HDMI), glutaraldehyde, tetrakis(hydroxymethyl)phosphonium chloride (THPC)[18] or, more recently, (catalyst-free) click chemistry[19]. The physical characteristics of the system, in combination with the activity

of extracellular matrix components, are converted by the cells into information that governs subsequent specific cell behaviors.

The materials employed as platforms for cell growth, including both preformed scaffolds for implantation or injectable systems, must ensure spatial control of the scaffold at a tissue damage level. In all cases, and in order to ensure interaction of the scaffold with elements of the host organism, natural sequence motifs inspired by signaling molecules have been inserted into the structures to modulate cell behavior, inflammatory responses and remodelling. These signaling motifs can be applied through protein adsorption (immobilization) by blending, co-polymerization, network formation or chemical/physical treatment[20] or, in the case of recombinant materials, incorporated into the overall structure of the biomaterial.

In conclusion, protein-based biomaterials are already playing a crucial role in the field of soft tissue engineering, and it is expected that their future uses will only increase and reach widespread clinical utility. Although, from a commercial point of view, the development of new biomaterials is expensive due to the long regulatory process required to gain approval for clinical use, the potential market is very broad and, once functionality and safety is assured, the improvement in efficiency with respect to conventional materials makes them one of the most promising products for biomedical implants. Recombinant protein-based polymers are of high quality and purity and guarantee predictable performance. Moreover, as they allow problems related to scaling up to be overcome, their design and optimization will be one of the main issues for biomedical applications in the near future.

1. Elastin

Elastin is the dominant component of mature elastic fibers, providing strength, prolonged elasticity and structural integrity to a wide range of tissues that undergo repetitive and

reversible deformations, such as lungs, skin, blood vessels or elastic ligaments[21]. Elastin has the unique property of being able to completely recover after thousands of elastic deformations without losing its mechanical properties, such as in the walls of the aorta for example, where it deforms more than 65 times per minute over more than 70 years without failure (excluding diseases)[22]. It is important to note that elastin is mainly produced in the early stages of development (late fetal and early neonatal periods) or when tissue is damaged, with very little being secreted in adults, thus indicating the stability and durability of elastin[23]. After injury, the production of elastin is triggered by a set of exogenous factors such as interleukin 1-beta[24], insulin-like growth factor[25], transforming growth factors[22] and fibulins 4 and 5[26].

Elastin is important for both its durability and its wide range of mechanical properties, with modulus values ranging from 200 kPa for the elastin found in pulmonary arteries to 1.72 MPa for that found in the aorta walls. Elastin also plays a key role in cellular signaling and regulation. Indeed, it contains bioactive domains that are able to modify cell behavior in wound healing and under healthy conditions. Thus, in wound healing, the hydrophobic hexapeptide VGVAPG (valine glycine valine alanine proline glycine) is known to serve as a chemotactic molecule for recruiting inflammatory and remodeling cells, whereas under healthy conditions it influences cell adhesion and proliferation[27]. Other amino acid sequences present in elastin improve cell adhesion, such as those with the general motif GXXPG, which are known to be a binding site for the elastin/laminin receptor[28], as well as the C-terminus of tropoelastin, which is known to interact with glycosaminoglycans present on the cell surface and with αvβ3 integrins via the pentapeptide GRKRK[29].

These unique properties of elastin make it a desirable material for tissue engineering. Moreover, as elastin is a natural material present in the human body, it is invisible to the

immune system, thereby avoiding one of the major problems that arise when using other materials for tissue engineering[30].

The determination and publication of the complete DNA sequence encoding elastin, and the development of recombinant expression techniques that allow expression of either the complete gene[31] [32] or the parts of interest, resulted in a major leap forward as regards the possibility of using elastin as a material for tissue engineering.

Based on this recombinant approach, many different constructs have been designed, ranging from use of the complete sequence of the precursor monomer of elastin (tropoelastin) to the use of different exons, or combinations of exons, present in the monomer sequence.

2.1 Tropoelastin.

The tropoelastin gene encodes a polypeptide of about 60-70 kDa, depending on protein maturation. The resulting protein is characterized by being composed of alternating hydrophilic and hydrophobic domains. The hydrophobic regions are composed of non-polar amino acids, such as valine, proline, glycine and leucine arranged in repetitive sequences, whereas the hydrophilic domains consist of alanine- and lysine-rich regions positioned within **K**xx**K** or **K**xxx**K** spacings[33], with the lysines being involved in intermolecular crosslinking. This crosslinking is carried out naturally by the enzyme lysyl oxidase[34] and leads to the formation of insoluble elastin **(Figure 3)**.

Studies on the molecular conformation of tropoelastin have confirmed a high proportion of disordered regions, mainly related to hydrophobic domains[35]. This lack of order allows tropoelastin to adopt several conformations and jump from one to the other with no input of energy. These different conformations include beta-turns, beta-strands[36] and polyprolineII structures[37]. All these conformational changes result in the hydrophobic groups becoming exposed to, and in direct contact with, water, which is an important issue as this fact is crucial to explaining tropoelastin's spontaneous recoil[38].

Tropoelastin has been widely used in tissue engineering due to its unique mechanical and cell-signaling properties[31].

2.1.1 Recombinant Tropoelastin for Skin Regeneration

As the largest organ, skin covers the entire outer surface of the body and accounts for around 8% of total body mass [39]. As such, it plays a crucial role in maintaining many physiological functions, such as body temperature, and also has a barrier function, providing a water-, electrolyte- and bacteria-proof barrier to the outside world [40].

Skin damage or loss can occur for many reasons, including disorders, acute trauma, chronic wounds, burns or even surgical interventions. Depending on the extent and depth of the injury, skin loss can present a mayor problem, with approximately 20,000 burn-related hospitalizations per year in the U.S. alone[41]. Tissue engineering has approached this problem in different ways and using different biomaterials, which can be either natural, synthetic, a mixture of both or autologous implantations, with the latter currently being the gold standard in skin reconstruction[42]. A perfect biomaterial for skin regeneration should assist successful engraftment of the tissue-engineered skin and promote granulation tissue formation, fibroblast-driven remodeling, angiogenesis and re-epithelialization[43].

As elastin is one of the main components in the crosslinked fibrillar network of the dermis, the use of elastin or any of its derivatives as a biomaterial for skin tissue engineering has been extensively assessed. For example, Mithieux *et al*. developed a recombinant tropoelastin-based material crosslinked with bis(sulfosuccinimidyl) suberate (BS3) that allows the construction of elastic sponge-like sheets and tubes with characteristics similar to those of natural elastin hydrogels. *In vitro* studies with murine and human epithelial and human fibrosarcoma cells cultured on both flat and smooth casting surfaces and on open-porous surfaces demonstrated that this tropoelastin-based crosslinked material also resembles natural

elastin when cells are cultured on it. Cell proliferation and migration along the thickness of the tridimensional scaffolds confirmed these results. *In vivo* studies performed with male guinea pigs for 13 weeks, with collagen as control, elicited a similar low foreign-body response[44].

Great effort has been dedicated to developing a skin scaffold based on crosslinked tropoelastin. Thus, Kovacina *et al*. electrospun recombinant tropoelastin dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol using different flow rates and studied the mechanical properties and porosity of the scaffold formed. Higher flow rates resulted in an increase in fiber width, pore size and porosity of the network, along with improved mechanical properties. Crosslinking was performed with glutaraldehyde vapors in order to avoid dissolution of the fibers when placed in water. Dermal fibroblasts were cultured in both high and low porosity scaffolds and, wherease cell spreading was achieved in both cases, cell proliferation and infiltration was greater in the scaffold with larger pores, which was therefore chosen for further long-term studies *in vitro*. Cells were able to secrete their own extracellular matrix proteins, such as collagen I and fibronectin, after 14 days, with levels increasing progressively over time. Scaffolds were tested *in vivo* by implanting them subcutaneously in mice for 6 weeks, with moderate scaffold degradation and the absence of neutrophils and monocytes being observed. In addition, high levels of fibroblast were able to infiltrate the scaffolds, thereby demonstrating that they did not provoke a marked immunological response. Moreover, small capillaries were formed at the edges of the scaffolds, thus indicating the onset of endothelialization of the artificial skin [45]. Similar comparative studies were carried out by the same group with electrospun fibers and 3D hydrogels crosslinked with glutaraldehyde and BS3, respectively. The porosity of 3D hydrogels was insufficient to allow cell migration, whereas electrospun 3D scaffolds allowed skin fibroblast infiltration, with the scaffold with higher pore size again being the one that allowed deeper infiltration [46].

Weiss group overcame the need of using chemical crosslinkers for the formation of insoluble tropoelastin structures when they discovered that, under alkaline conditions and when heated upon a certain temperature, tropoelastin proceeds through an irreversible sol-gel transition that leads to the formation of a hydrogel, a process that does not occur at neutral pH. The resulting material is stable, insoluble, elastic and flexible and is able to support human skin fibroblasts *in vitro*. Moreover, *in vivo* studies demonstrated that subcutaneous injection of a 200 mg/ml solution and subsequent gelation was feasible. The so formed scaffold persisted at least 2 weeks and promoted collagen deposition, although a mild foreign-body response and encapsulation were observed [47].

Different strategies, as for example crosslinking a mixture of α-elastin and tropoelastin with glutaraldehyde in high pressure CO_2, have been developed to improve the pore size of 3D tropoelastin hydrogels. High-pressure CO_2 treatment eliminates the top skin of hydrogels, thereby increasing the pore size and porosity of the hydrogels, reducing the swelling ratios and enhancing the mechanical properties (compression modulus and tensile properties) of the hydrogels formed. This enhancement in mechanical properties is related to a higher degree of crosslinking. The CO_2 depressurization rate was also found to have a marked impact on interconnection and pore size, with higher rates inducing larger pore sizes and increased interconnectivity. Human skin fibroblasts cultured in the resulting high porosity hydrogels were able to grow, proliferate and colonize to a depth of more than 300 μm in the scaffold[48].

Another approach for increasing pore size and facilitating cell migration into the scaffold was developed by co-blending tropoelastin with other polymers. Thus, blending of tropoelastin with heparin or dermatan sulfate and crosslinking with BS3 resulted in a scaffold with increased pore size. Indeed, the heparin blend had a three-times larger pore size and a lower swelling ratio, but similar mechanical properties. *In vitro* cell-proliferation studies showed that human dermal fibroblasts could penetrate deeper into the heparin-tropoelastin blend,

penetrating more than 300 μm in just 2 days, which is considered to be the maximum penetration depth in static cell culture due to limitations to oxygen and nutrient diffusion[49].

Other tropoelastin blends, such as tropoelastin with ovine type I collagen, have been used in different proportions to create crosslinked electrospun fibers. Under the same electrospinning conditions, the tropoelastin-collagen blend induced the formation of larger pores and a higher porosity fibrilar network in comparison with electrospun tropoelastin scaffolds. No significant variations in mechanical properties were observed between tropoelastin and its blends until the ratio of collagen to tropoelastin reached 50% w/w, when mechanical properties increased sixfold compared to pure tropoelastin. In order to assess the viability of these scaffolds, human dermal fibroblasts were cultured for 14 days. After the first day, cells had already formed a monolayer, thus indicating good cell adhesion. Scaffolds comprising by tropoelastin and collagen promoted the proliferation and colonization of these cells. Indeed, after 3 days of culture the fibroblasts had penetrated 50% deeper into the blended scaffold, achieving a depth of 150 μm. By day 8 fibroblasts were present on the unseeded surface, and complete cell infiltration was observed by day 14. Scaffolds were subcutaneously implanted in mice to assess *in vivo* biocompatibility and persisted at least 6 weeks, with a moderate immune response to both type of scaffolds (pure tropoelastin and blended tropoelastin) being observed, with monocytes and multinucleated cells infiltrating from the edges. Moreover, blended scaffolds recruited fibroblasts, which were able to deposit new collagen [50].

Other blends have been used to improve the mechanical properties of tropoelastin scaffolds. In one of these approaches, tropoelastin was blended with *Bobyx mori* silkworm silk in different proportions and the resulting mixtures electrospun and crosslinked. Human dermal fibroblasts were seeded to assess cell viability, demonstrating higher proliferation rates in the blend when compared to pure silk scaffolds. *In vivo* tests were conducted by subcutaneously implanting the scaffolds into IL-1b luciferase mice in order to measure the expression of IL-

1β in real-time. Incorporation of tropoelastin into the silk scaffold reduced the acute and chronic inflammatory response after 3 weeks of implantation, lowering the number of inflammatory cells, the levels of pro-inflammatory IL-1beta cytokine expression, and the levels of anti-inflammatory IL-10 production and pro-inflammatory IL-6 expression. The authors concluded that tropoelastin-silk blends improve the biocompatibility of silk fibroin, at least from an inflammatory point of view[51].

The fact that the hydrophobicity of the surface tailors the binding of tropoelastin in a cell-adhesive or non-adhesive manner without affecting the amount of bound tropoelastin[52] allows the functionalization of many surfaces with the ability to tailor cell adhesion. For example, this technique has been used to modify the surface properties of polytetrafluoroethylene by plasma immersion ion implantation and subsequent coating with tropoelastin upon immersion in a tropoelastin solution, thereby achieving covalent binding of tropoelastin to the surface. Human dermal fibroblasts were used for cell culture. When polytetrafluoroethylene was treated with plasma, cell adhesion and proliferation was enhanced when compared with the untreated surface. Moreover, tropoelastin bound to the plasma-untreated polytetrafluoroethylene surfaces showed high cell-adhesive properties, whereas tropoelastin on plasma-treated polytetrafluoroethylene surface showed no cell-adhesive properties. This was explained as being due to the different conformations adopted by tropoelastin depending on the hydrophobicity of the surface: the cell-adhesive C-terminus of tropoelastin is exposed when the underlying surface is hydrophobic, whereas it is hidden when surface hydrophilicity is enhanced. The authors therefore developed a method to convert a highly hydrophobic surface into a high cell-binding surface[52]. These *in vitro* results were confirmed *in vivo* by subcutaneously implanting samples, with no inflammatory response being observed[53]. A similar biofunctionalization approach was used to improve cell adhesion to other surfaces, such as silicon[54] or polyurethane[55]. Furthermore, this technique

has also been applied to functionalize a conducting polymer (polypyrrole), thereby demonstrating an improved ability to electrically stimulate cells while spatially controlling their attachment[56].

Significant effort has been dedicated to the field of severe skin injury regeneration when autografts are not available. Thus, a tropoelastin-based dermal regeneration template was obtained by blending tropoelastin and collagen in a 90/10% weight ratio with a type I collagen/chondroitin-6-sulfate matrix. An extensive *in vivo* study using a mouse and pig animal model was performed to compare this mixture with the INTEGRA® Dermal Regeneration Template, the leading commercial synthetic skin substitute. Although both scaffolds possess similar porosity, pore size and mechanical properties, the tropoelastin-collagen mixture exhibited enhanced *in vitro* fibroblast migration into the scaffold. In the mouse model, INTEGRA® and the blend showed similar performances, with both scaffolds greatly reducing contraction, and similar fibroblast infiltration was observed when compared with the untreated injury. Moreover, the tropoelastin-collagen scaffold significantly stimulated angiogenesis with respect to the INTEGRA® scaffold. These results were further confirmed in the *in vivo* pig model. Thus, contraction in both systems was indistinguishable, with similar degrees of fibroblast infiltration. Interestingly, vascularization was enhanced in the tropoelastin-blended scaffold, which showed better performance in terms of dermal regeneration. In addition, a histological analysis confirmed the better performance of tropoelastin-collagen scaffolds, with 88% of the sections examined showing epidermal ridges, compared with 19% for the INTEGRA® scaffold [57].

2.1.2 Recombinant Tropoelastin for Vascular Tissue Regeneration

Vascular grafts are in great demand nowadays, especially for coronary and peripheral bypass surgeries. Although autologous replacements are the most commonly used substitutes, this

approach is not always possible. As replacement of vessels with pure synthetic grafts often results in failure due to the formation of thrombosis, stenosis or calcium deposits, amongst others[58], the combination of scaffolds with cells is the most promising approach as it avoids some of these undesired side effects. The vascular wall mainly comprises three cell types, namely endothelial cells, smooth muscle cells and scattered fibroblasts, with the former two playing the most important role. Endothelial cells line the internal surface of the vessel, provide a continuous selective barrier and avoid the formation of thrombus, whereas smooth muscle cells provide elasticity and tone to the vessel[59]. As such, fully functional blood vessel scaffolds should be non-thrombogenic, invisible to the immune system, blood compatible and have similar mechanical properties to natural tissue[60]. These highly specific requirements cannot be achieved without incorporating endothelial cells and smooth muscle cells into the scaffold[59]. The elastin produced by endothelial and smooth muscle cells has been shown to produce minimal platelet adhesion and very low thrombogenicity[61]. Moreover, tropoelastin has been shown to prevent calcification in smooth muscle cells[62] and to play a key role in blood vessel growth[63]. These unique properties of elastin, and its precursor tropoelastin, makes them suitable candidates for blood vessel tissue engineering[64].

A proof of concept for developing a biomaterial in which vascular related cells (smooth muscle and endothelial cells) could be cultured was carried out by manufacturing a crosslinked electrospun support based on recombinant tropoelastin. Human fibroblasts, human umbilical vein endothelial cell (HUVECs) and human carotid artery smooth muscle cells (HCASMCs) attached to and grew on the scaffold, acquiring normal morphology and proliferation rates[65]. In a similar approach, electrospun recombinant tropoelastin was crosslinked with BS3 to form flat[66] and tubular scaffolds[67] on which smooth muscle cells[66] and endothelial cells[67] were cultured. These cells were able to proliferate and differentiate, as indicated by the positive staining for endothelial-cell-specific von Wildebrand factor (vWF)

and the formation of a confluent monolayer with the typical endothelial cell cobblestone morphology, although the mechanical properties of these scaffolds were not as good as those of natural tissue[67].

Tissues are well-organized structures, and some of them contain fibers aligned in a specific direction with cells following the same structure. When developing a scaffold, is important to take this fact into account as substrate orientation strongly influences cell organization in tissues[68]. Following this concept, Nivison-Smith *et al*. have developed an electrospun synthetic-elastin parallel-fiber scaffold to orientate smooth muscle cells. Thus, electrospun tropoelastin was crosslinked to form insoluble fibers with mechanical properties similar to those of native elastin. *In vitro* cell cultures demonstrated the ability of cells to grow and form a continuous monolayer over random and parallel-oriented scaffolds, with similar proliferation rates. Fiber orientation had a significant impact on the orientation of smooth muscular cells, which were more elongated on aligned fibers, spreading parallel to the fiber direction and developing a morphology similar to that of native smooth muscle cells[69].

Tropoelastin has been also used to modify the surfaces of other polymers used in vascular tissue engineering. Bax *et al*., for example, modified a polyurethane co-polymer's surface by tailoring the adhesive or non-adhesive properties of tropoelastin. This treatment enhanced adhesion, cytoskeletal assembly and the number of cell-cell junctions of HUVEC when compared to controls, all of which are good signs of cell differentiation. Moreover, tropoelastin coating over treated or untreated samples resulted in a dramatic decrease in surface thrombogenicity, thus making the coated polyurethane a high blood-compatible material[55].

This technique was also used to modify metallic vascular implants, such as coronary stents. Waterhouse *et al*. were able to reduce the thrombogenicity and enhance endothelial cell proliferation of metal alloys used in coronary stents by covalently binding tropoelastin to the

stent's surface. These results suggest that this surface functionalization enhances the biocompatibility of stents by avoiding thrombus formation and promoting integration of the stent into the vessel[70]. A similar surface functionalization was obtained by covalently binding tropoelastin to metallic surfaces via an intermediate polymerized acetylene layer[71].

Other groups demonstrated that these effects were reproducible with shorter tropoelastin-based sequences. Thus, two shorter sequences corresponding to the first 10 and 18 N-terminal exons of tropoelastin, respectively, were grafted onto the surface, where they proved their ability to retain the same cell-adhesive and non-thrombogenic properties as the full sequence while displaying a greater resistance to proteolytic degradation[72].

In order to match the mechanical requirements and easy handling of small vascular grafts, Wise *et al*. co-blended tropoelastin with polycaprolactone. After demonstrating the ability of HUVECs to attach and proliferate in the electrospun scaffolds and their low thrombogenecity, they performed an *in vivo* experiment implanting the grafts into a rabbit carotid interposition model. The grafts remained physically intact one month post-implantation, thereby demonstrating the suturability and mechanical durability of these small vascular grafts[73].

In addition to being used in blends, tropoelastin has also been used to develop a new elastic tissue equivalent by its methacrylation, thus creating a new material named MeTro. This new material presents elastomeric properties that can be tuned by changing the degree of methacrylation and protein concentration, thus allowing desired mechanical properties to be selected for different applications. This material is able to photocrosslink upon exposure to UV for a short time, using an aqueous solution as precursor. To assess the *in vitro* biocompatibility of the hydrogels formed, MeTro gels with a 10% w/v protein concentration and 31% degree of methacrylation were used for cell cultures. Immortalized green fluorescent protein (GFP)-expressing endothelial cells and fibroblasts were used in 2D and 3D cultures. In 2D cultures, the cells showed more than 92% viability and actively spread over the surface

to form a monolayer. Only fibroblasts were used for the 3D cell-encapsulation tests with MeTro gels, remaining viable for at least a week in culture[74].

In addition to blood vessel scaffolds, functional cardiac tissue has also been developed using methacrylated tropoelastin (MeTro) by forming micropatterned hydrogels with parallel grooves. The parallel micropatterned grooves in MeTro modulated cell-material interactions in terms of promoting the attachment, spread, alignment, function, differentiation and intercellular communication of cardiomyocytes, thus providing an elastic mechanical support that mimics the anisotropic organization and mechanical properties of native tissue. Cardiomyocytes spread and proliferated more rapidly over the MeTro surfaces than in GelMA (methacrylated gelatin), achieving surface confluence in less than half the time. The authors of this study also demonstrated that micropatterning of the surface led to an orientation parallel to the grooves of actin filaments in just 8 hours post-seeding. Moreover, immunostaining of cardiomyocytes was positive for different differentiation markers, such as troponin I, thus indicating a well-developed contractile apparatus, sarcomeric α actinin, thus demonstrating the development of a well-defined sarcomeric structure, and connexin 43, thereby suggesting a good cell-cell interconnection. Cells seeded in MeTro maintained their contractile activities for at least 2 weeks, as demonstrated by electrically stimulating cells and observing a cell-contractile response in synchrony with the electrical stimulation[75].

The same group developed a method for coating microfluidic channels with a cell-compatible photocrosslinkable hydrogel layer using MeTro gel. The thickness of the coating could be controlled by changing the injection flow rate or UV irradiation intensity. This hydrogel layer facilitated the attachment, spread, organization and beating behavior of cardiomyocytes inside the microfluidic channels, and primary cardiomyocytes were able to differentiate into adult cardiomiocytes, thus developing a system that could be used as a heart-on-a-chip device[76].

2.1.3 Recombinant Tropoelastin for Other Tissues *Regeneration*

Recombinant tropoelastin has been used in other tissue-engineering approaches. For example, Xiao Hu *et al*. developed a blend of tropoelastin with natural silk fibroin in which charge attraction was the main force of interaction. These blends were crosslinked into 3D scaffolds by autoclaving the mixture, as wet high temperature and pressure treatment causes silk to form insoluble β-sheets. Moreover, a different net charge in the blend, ranging from -36 for pure elastin to +37 for pure silk, was obtained by varying the proportions of silk and tropoelastin. The charge on extracellular matrices plays a critical role in cell behavior by modifying cell growth, differentiation and migration, especially for charge-sensitive cells such as neurons. To study how charge modulated the cell response, rat brain cortical neurons were seeded on scaffolds containing different silk and tropoelastin ratios for 10 days. The results demonstrated that a negatively charged surface did not allow neurons to adhere, where highly positively charged surfaces enhanced neuron adhesion in the first few days but resulted in neuronal death after 7 days. The blend with a weakly positive charge (+15.6) provided the best neuronal growth and development, with neurons acquiring a normal morphology and normal expression levels of β-III tubulin, glial fibrillary acidic protein and developing cell-cell connections[77].

(Table 1)

(Table 2)

2.2 Tropoelastin-derived materials

Tropoelastin is composed of 34 exons[78]. As such, several authors have broken tropoelastin apart to isolate and biosynthesize certain functional domains, subsequently working with these smaller sequences encoded in the tropoelastin gen. Herein we present the more relevant studies from the past few years.

Bandiera *et al*. developed two short tropoelastin-exon derived peptides, named HELP and HELP1, comprising the second half of exon 23 and most of exon 24 from human tropoelastin. As can be seen in reference, HELP1 differs from HELP by lacking the crosslinking domain AAAAKAAAKAAQF [79] **(Figure 4).**

These authors developed 3D matrices by crosslinking HELP1 using the enzyme transglutaminase and assessed the cytotoxicity of the structures formed by culturing endothelial cells on them. The results showed that these scaffolds were not cytotoxic, were able to swell to more than 20 times their original weight, depending on the concentration of the precursor solution, and had similar properties to those reported in the literature. [80]. In another study, the same group developed a system to coat surfaces with HELP1 using a simple evaporation method, which unexpectedly formed concentric grooves. Cell cultures were conducted with the HUVEC derivative Eahy926 as an endothelial cell model. These cells are derived from the fusion of human umbilical vein endothelial cells with the human cell line A549 of epithelial origin. Eahy926 cells displayed a clear response towards the topography of the surface, aligning within the concentric grooves. In order to study this behavior further, human epithelioid MCF-7 cells were seeded and, curiously, did not behave like the HUVEC-derived cells. A third cell culture with the A549 cell line again displayed the same pattern as Eahy926 cells. The authors concluded that the cell response is related to features that are present in both A549 phenotypes rather than to the endothelial or epithelial origin. It should be mentioned that all cell types adhered perfectly to the substrate and did not show any signs of cytotoxicity[81]. Further studies were conducted with HELP and HELP1 in order to assess the cell biocompatibility of these recombinant polypeptides with other cell types, such as SH-SY5Y, a human neuroblastic subclone widely used as a neuronal cell model[82], or a human hepatoma cell line[83].

The development of 3D scaffolds using HELP was a great boost to the possible use of this polypeptide for tissue engineering. Thus, a 3D HELP-based hydrogel was crosslinked using the microbial transglutaminase from *Streptomyces mobaraensisas,* which is a mild-condition crosslinker. Human hepatoblastoma and breast cancer cells were used in the subsequent *in vitro* experiments. After 72 hours of encapsulation, islets of proliferating cells were visible. Cell viability was assessed by transferring the islets into a new, standard, plastic tissue-culture vessel, where cells proliferated for more than a week. Because of the mild reaction conditions of the method, the enzymatic crosslinking of HELP for preparation of matrices represents a very attractive opportunity for cell encapsulation[84]. Rat myoblasts cultured in these HELP scaffolds also exhibited an improved behavior in comparison with those cultured on collagen. Thus, myoblasts cultured over HELP had longer and more aligned f-actin filaments and HELP increased vinculin expression, showing a significant 15% increase in metabolic activity with respect to controls. Myotube formation was monitored for 1 week, with a marked increase in both length and width for cells cultured over HELP being observed, thus demonstrating an increased differentiation capability[79].

An elegant stud reported by Boccafoschi *et al*. involved the use of co-blended collagen and HELP to encapsulate murine myoblasts. In this case, the embedded cells are one of the most important components of the artificial tissue and also act as a crosslinker agent by secreting the enzymes that crosslink the 3D scaffolds. Blended scaffolds resulted in better mechanical properties than collagen and improved cell activity, as confirmed by the increased expression and secretion of endogenous elastin and collagen type III in addition to the synthesis of matrix metalloproteinase-2 (MMP2). Myoblasts were able to actively reorganize their cytoskeleton, developing a spindle-shaped morphology and acquiring an enhanced contractile phenotype when compared to controls[85].

Other tropoelastin-derived exons have been used to mimic some properties of the complete tropoelastin molecule. For example, Woodhouse *et al*. demonstrated the utility of these shorter exon-based constructs by coating commercially available cardiovascular devises made out of different materials, such as polyethylene terephthalate, poly(tetrafluoroethylene/ethylene) copolymer, polycarbonate and polycarbonate polyurethane, with an elastin-based polypeptide named EP-20-24-24. This engineered elastin-derived construct is derived from fusion of exons 20, 21, 23 and 24 of tropoelastin into a new single molecule. All four coated materials demonstrated reduced platelet activation *in vitro*, as demonstrated by microparticle release and P-selectin expression, and reduced adhesion *in vitro* and *in vivo*[86].

Another family of tropoelastin-derived recombinamers with different numbers of repeating hydrophobic and crosslinking domains were absorbed onto a commercially available polyethylene terephthalate surface by simply immersing it in an aqueous solution containing these constructs **(Figure 5)**. This modification demonstrated the ability of these polypeptides to inhibit platelet adhesion and fibrinogen adsorption in a size-dependent manner, with the longest construct being the most efficient[87].

Blit *et al.* made further progress in the use of these elastin-exon based polypeptides by covalently modifying a polycarbonate urethane surface to enhance its blood compatibility in a three-step procedure. A fluorinated surface modifier precursor was initially constructed and coupled to the surface. A synthetic elastin crosslinking peptide was then coupled to this precursor in a controlled reaction that left unreacted lysines, which were subsequently used to react further with the elastin-exon based polypeptide, named ELP4. Linkage and crosslinking of ELP4 was performed using genipin. This surface modification led to low platelet adhesion and low bulk platelet activation, while promoting endothelial cell adhesion, for at least one week[88]. This methodology was also used to covalently biofunctionalize other commercial

surfaces, such as LDPE, with similar results[89]. Flat and electrospun polyurethane scaffolds were also coated with ELP4. This coating demonstrated enhanced human umbilical vein smooth muscle cell (HUV-SMCs) adhesion in both types of scaffold, exhibiting a spindle-like morphology and actin filament organization in the direction of the fibers, thereby suggesting the development of a contractile phenotype [90].

Other commercially available materials have been biofunctionalized by coating with ELP4. Thus, Hughes *et al*. developed an oriented and random electrospun Tecoflex® scaffold that was further modified by reacting with ELP4. Fiber orientation was found to have a marked impact on both the mechanical properties of the scaffolds formed and cell morphology. Human vocal fold fibroblasts were seeded into these scaffolds. Cells seeded onto ELP4-covered scaffolds upregulated elastin and collagen III synthesis at day 7 of culture, and remained viable for up to 14 days. These results suggest that this scaffold might be of use for reconstructing vocal fold *lamina propria*[91].

(Table 3)

(Table 4)

2.3. Elastin-Like Recombinamers

The synthetic proteins known as elastin-like recombinamers (ELRs) are recombinant elastin-mimetic polymers derived from natural elastin that have been widely investigated as candidates for restoring the structural and biological functions of damaged tissues or organs[92].

Natural elastin is the most abundant elastic component of the ECM in many vertebrate tissues in which elasticity is required. Thus, elastin is responsible for the compliance and recoil of the aortic vessels[93], as well as the recoil after stress/deformation of the skin[94] and lungs[95]. Elastin also shows other remarkable physical and mechanical properties, such as fatigue

resistance and extreme durability. These properties arise due to the primary sequence of the soluble elastin precursor, tropoelastin, in which recurrent elastomeric domains containing repetitive motifs such as (VPGVG, IPGVG,VPGG, VGVAPG) are found[96]. ELPs, or their recombinant version (elastin-like recombinamers (ELRs)), are "elastin mimetics" based on the very same recurring amino acid sequences as found in tropoelastin. These modular protein polymers are engineered biomaterials produced using recombinant DNA techniques, biotechnological methods and *Escherichia coli* biosynthesis[97]. Biosynthesized ELRs are obtained with absolute control over the amino acid composition, with high reproducibility and monodispersity, and, with respect to animal-derived proteins, avoid the potential risk of infection and immunogenic response. One of the most widely studied ELR types is based on the elastomeric pentapeptide sequence Val-Pro-Gly-Xaa-Gly, where Xaa is any natural amino acid except proline. ELRs retain the most relevant characteristics of elastin, especially the aforementioned mechanical properties, in conjunction with other properties, such as a stimuli-responsive behavior[98], the ability to self-assemble[99] and an excellent biocompatibility. The biocompatibility and stealth properties retained in ELRs for the model pentapeptide, as well as in more complex structures, when implanted *in vivo* show long-term stability without inducing any inflammatory response[100].

All VPGXG-based ELRs present temperature responsiveness in aqueous solution. This behaviour, which involves a reversible phase transition in response to changes in temperature, is based on the inverse temperature transition (ITT), which is itself defined by the temperature transition (T_t) specific for each ELR. In aqueous solution, and below the transition temperature T_t, the free polymer chains remain disordered with a random coil conformation, whereas when the temperature increases above T_t, the chain folds hydrophobically and assembles to form a separated phase[101]. In the folded state, the polymer chains adopt a dynamic, regular, non-random structure, called a β-spiral, with the polymer transforming

thermal energy into mechanical work as the molecule folds[102]. The folded molecules associate themselves into micro- and macro-aggregates, which separate from the solution to form a precipitate, thus meaning that the overall process is a reversible phase transition from a soluble to an insoluble state. Modifications to the primary sequence of ELRs allow additional responsiveness[103] to other external stimuli that affect ELR performance, such as pH[104], UV-vis light[105] or ion concentration[106], to be added. The temperature-dependent inverse transition state in ELRs is also routinely employed for polymer purification and is also a simple and economic alternative to conventional purification strategies for ELR-based fusion proteins[107]. The recombinant protein technology employed for ELR synthesis allows their sequence to be designed by combining different functional peptide units in a single recombinant molecule. Studies performed to identify the relationship between the modifications introduced into the sequence and the new features acquired, and subsequent application of these technologies to engineer protein-based biomaterials, has made it possible to modulate different characteristics that allow several properties of ELRs to be refined. One example of the improvement obtained by way of such rational design is the synthesis of amphiphilic block co-polymers that spontaneously self-assemble to form continuous hydrogels for use as tissue-engineering scaffolds that have been cast with a defined and controlled topography[108]. In this way, the physical properties, stimuli-responsiveness[109] and mechanical behavior[110] are tuned and additional biofunctionalities[111] included, thereby resulting in customized materials to control specific functions or to elicit tissue regeneration[112]. This tissue regeneration is guided by the association between cells and their ECM, a bi-directional and dynamic interaction that activates intracellular signaling pathways[113]. Extracellular structural proteins are molecules containing several cell-signaling motifs that strongly influence a cell's response and fate. Similarly, tissue rearrangement in both healthy tissues and during tissue repair occurs as a result of a dynamic bidirectional

process between cell receptors and specific regulated motifs of the ECM proteins secreted by cells themselves. In addition, the presence of integrin binding peptides in the scaffolds limits another common problem that occurs during tissue regeneration, namely wound contraction due to a reduction in cell-cell attachment[114]. Several molecular signals in the form of short amino acid motifs have been included in the sequence of ELRs to transform these regenerative scaffolds from being merely a three-dimensional support for the formation of new tissues to a cell-material communicator during reconstitution of the new host tissue[10, 115].

2.3.1 ELRs for Vascular Tissue Regeneration

The integration of motifs that elicit cell-material interactions into ELR sequences is one of the modifications required to produce the most advanced biomaterials, especially those that promote colonization of these artificial ECMs by the host's own specific cells. Indeed, although the simple "backbone" of ELRs possesses excellent biocompatibility, it is not able to stimulate the adhesion of various types of cells, such as human endothelial cells (HUVECs) for example[116]. To overcome this problem, ELR scaffolds have been coated or grafted with natural structural proteins such as fibronectin that increase endothelial cell anchorage and proliferation. An example of this approach is the composite of ELR and natural collagen that was utilized to form scaffolds for vascular tissue engineering. Thus, artificial vessels were constructed by combining ELR layers crosslinked and fortified with collagen microfibers to produce scaffolds with improved strength and mechanical properties [117]. The integration of natural proteins enhances the adhesion but doesn't eliminate the potential risks of using natural proteins[118]. In a pioneering study in this field, the sequence of ELRs was engineered to include a motif that induces cell adhesion. This biofunctionalization was achieved by including a pre-determined proportion of the well-known RGD[119] (arginine glycine-aspartic acid) integrin-mediated cell-adhesion peptide into the ELR sequence, which resulted in a

marked improvement in endothelial cell adhesion. An increase in RGD density lead to more extensive cell adhesion and spreading[120]. A similar enhancement was observed when ELR-RGD was cast into 3D cell supports, with its density seeming to regulate cell behavior or differentiation[121].

More complex ELRs for vascular tissue engineering were designed by Tirrell and coworkers, who inserted a lysine at the X position of the model pentapeptide on the assumption that the free epsilon amino group of this amino acid could subsequently beused for crosslinking or modification purposes. Moreover, bioactive fragments from human fibronectin, namely the CS5 cell-binding domain (EEIQIGHI-PREDVDYHLYPG)[122] containing the tetrapeptide REDV (arginine-glutamic acid-aspartic acid-valine), which binds the $\alpha 4\beta 1$ integrin[123] specifically expressed in the endothelial lineage, and a loop including the peptide RGD[124], were included in the ELR backbone. The *in vitro* cell-adhesion properties of these ELR scaffolds were compared and both motifs found to enhance the attachment[125] of endothelial cells, with RGD being more efficient at promoting adhesion and spreading and REDV being more specific for the endothelium[126]. To ensure the other mandatory requirement of a temporary scaffold, namely biodegradability and reabsorption, a bioactive functional fragment from elastin was included in the REDV-ELR sequence to mimic natural ECM remodeling[127]. This fragment is an elastase target sequence comprising three reiterations of the hexapeptide VGVAPG, which is also known to be a member of the family of “matrikines”, a group of peptides that strongly influence cell response during the regeneration process, such as the promotion of cell migration, differentiation and endothelial tube formation. Endothelial cells are able to adhere, infiltrate and colonize scaffolds when seeded in highly porous hydrogels obtained by chemical crosslinking[128]. The specificity of the adhesion motif of this recombinamer was analyzed in a study in which ELR-REDV was mixed and crosslinked with collagen to form scaffolds for both endothelial and fibroblast cultures. A cellular phenotype

and proliferation analysis demonstrated that the REDV motif of this recombinamer provides cell selectivity for the scaffolds[126b, 129].

This ELR-REDV version was also employed to biofunctionalize metal-based stents and so to prevent restenosis and late thrombosis diseases. The recombinamer was efficiently immobilized on CoCr alloy surfaces determining the improvement of HUVEC adhesion and spreading and so enhancing their endothelialization [130].

In a recent study, Gonzalez de Torre *et al*. described the performance of vascular stents can be improved by coating them with RGD and REDV-ELR **(Figure 6).** To fabricate the ELR-based bioactive stents by injection molding, the authors utilized a bi-component system in which the two components rapidly crosslink to form a hydrogel in a specific, efficient and fast manner, in a catalyst-free click chemistry reaction, when mixed together under physiological conditions[131]. The bare metal stents were fully endothelialized under dynamic pressure and flow conditions in 15 days when coated with ELR-RGD, which was found to elicit a higher adhesion and proliferation rate of endothelial cells with respect to the stents coated with REDV-ELR. *In vitro* endothelialization of the devices prior to *in vivo* implantation resulted in improved mechanical stability, physiological hemocompatibility and a reduced risk of thrombosis as a result of minimal platelet adhesion and fibrin adsorption [132].

A similar bi-component system was developed to obtain a 3D scaffold with embedded cells. Thus, cell-containing ELR solutions were cast simultaneously and the 3D scaffolds obtained found to support long-term cultures of different cell types, such as human primary endothelial, mesenchymal or fibroblastic cells, which were able to proliferate throughout the thickness of the gels. These hydrogels were found to be non-cytotoxic and highly biocompatible, and could therefore be employed as injectable implants in *in situ* tissue-regeneration procedures[133].

Other types of cell behavior can be significantly influenced by selecting the appropriate signal peptides for integration into the artificial ELR-based scaffolds. Thus, tailor-made elastin recombinamers were found to retain the bioactivity selected from the regulatory domains of natural proteins. For example, incorporation of YIGSR (tyrosine-isoleucine-glycine-serine-aspartic acid), a motif derived from the ECM protein laminin[134], into the ELR-RGD sequence has been found to enhance cell anchorage and inhibit endothelial cell migration[135]. Similarly, pro-angiogenic activity has been attained in endothelial cell cultures by integrating the peptide (V2) from heparin-binding protein, a matrix protein that regulates vascular tissue regeneration and wound healing. The biofunctionalized V2 ELR triblock copolymer has been shown to enhance endothelial adhesion, proliferation, migration, and tubule formation[136]. In order to promote the proliferation and differentiation of embryonic stem cells into adult cardiomyocytes, an ELR with the receptor-binding domain of insulin-like growth factor binding protein 4 was produced and the efficiency of cardiomyocyte differentiation was found to be improved in comparison with standard methods[137].

Although the integration of functional peptide modules into the sequence of recombinamers has proven to be a successful strategy for modulating cell response by way of cell-material interactions, the insertion of sequences for larger molecules, such as those derived from regulatory factors, can perturb the mechanical functionality and stimuli-responsiveness of ELRs mostly due to the balance of the hydrophobic amino acid composition. Three different innovative strategies have been proposed to solve this difficulty. The first such approach, which involved immobilizing growth factors in ELRs vascular scaffolds, was developed by Cai *et al*., who grafted a bioactive angiogenic peptide called QK, which simulates the receptor-binding domain of VEGF and increases endothelial cell attachment and proliferation, onto 2D/3D ELR-RGD scaffolds. In the same work, the authors also obtained a three-dimensional scaffold in which HUVEC cells were encapsulated and the QK peptide

covalently immobilized by making use of a biocompatible crosslinker. Cell behavior was analyzed in both 2D and 3D ELR-RGD-QK scaffolds and endothelial cells found to be able to adhere, expand and proliferate, whereas although the viability of the cells in the artificial 3D matrix was close to 100%, they were not able to degrade and remodel the artificial scaffolds, thus confirming that proteolytic target sites must be included to allow cell-mediated matrix degradation, migration and colonization [138]

The second approach was developed by the Kobatake group, who genetically engineered both ELR and growth factors with the aim of non-covalently binding molecules. The leucine zipper dimerization domains of two proteins (Fos/Jun) that spontaneously form a heterodimer that can be incorporated into artificial extracellular matrix proteins were selected for this purpose. One of these heterodimerization domains was included into the sequence of a multifunctional ELR containing two cell-adhesion domains, namely RGD and IKVAV[139] (isoleucine-lysine-valine-alanine-valine) from laminin, and a collagen-binding domain, whereas the other heterodimerization domain was used to produce three fusion proteins with an epidermal growth factor (EGF), a fibroblast growth factor (bFGF), or a vascular endothelial growth factor (scVEGF121). All these growth factors influence endothelial cell behavior. Indeed, it has been reported that simultaneous incubation with scVEGF121 and bFGF is able to increase angiogenesis and blood vessel maturation, whereas EGF possesses pro-angiogenic activity. Endothelial cells were cultured on multifunctional scaffolds dimerized with growth factors to amplify the synergistic effects in promoting angiogenesis, and it was found that cell proliferation and capillary tube-like formation were achieved[140].

The third approach involved the inclusion of protein fragments that determine intermolecular associations into ELR sequences to control the macromolecular conformations and form a more complex matrix component[141]. A pair of binding partners (SpyTag and SpyCatcher) derived from the bacterial protein CnaB2 of *Streptococcus pyogenes* were selected as tags in

this work. Under physiological conditions, the Spy protein partners bind together and spontaneously form an irreversible link via isopeptide bonds. The presence of the "Spy" pair allowed the ELRs to form soft hydrogels and encapsulate cells even though the ELR sequences were designed to be complemented with a full-length globular protein that may generate the cell-differentiation state. The authors demonstrated that embedded mouse embryonic stem cells cultivated in an injectable ELR-based soft hydrogel whose sequence included leukemia inhibitory factor maintained their pluripotent nature, and that the addition of a soluble factor to modulate cell behavior was not required under these conditions[142].

2.3.2 ELRs for other tissue regeneration

Similar approaches have been followed to obtain tailor-made ELR-based biomaterials for the substitution of different soft tissues. To achieve a more specific material for skin regeneration, genetically engineered ELRs for use as scaffolds were designed and biosynthesized. Thus, three functional cell-binding domains from essential natural human skin components were chosen for inclusion into the sequence of different ELRs. All these domains are the target of the most representative integrins of epidermal keratinocytes, and their inclusion could therefore lead to more effective scaffolds for human skin epidermal keratinocyte cultures. The laminin peptide PPFLMLLKGSTR, the PHSRN RGD domains of fibronectine or the GEFYFYDLRLKGDK peptide of type IV collagen were incorporated into the elastin-like backbone. Several adhesion, proliferation, colony forming ability and competition assays were carried out to analyze the efficiency of these different ELRs in comparison with each other or with the native proteins from which they were derived. The results demonstrated that the ELRs adapted for keratinocyte culture determined a specific cell-binding domain response in both keratinocyte and keratinocyte stem cells, with no significant differences being

observed in comparison with their respective native controls. The new materials resulted in improved keratinocyte viability, proliferation and even the growth of epidermal stem cells[143]. The application of elastin-like recombinamers as cell-growth scaffolds for other particularly complex cells, such as some of those that comprise the nervous system, has shown promising results, as described by Heilshorn's group. Thus, by controlling the bioactive domain density, it proved possible to modulate the adhesion and proliferation of neuronal-like[144] or neural progenitor cells[145], while scaffold rigidity, which could be modulated by using higher crosslinker/ELR stoichiometric ratios, had a significant effect in terms of stimulating neurite growth[146].

Other ELRs have been designed and adapted in an attempt to improve the performance of devices for ocular tissue engineering. These adaptations resulted in the adhesion and growth of conjunctival epithelial cells[147] and retinal pigment epithelial cells on ELR surfaces with sequences containing integrin-mediated signals[148]. Although complementation of the primary ELR sequence with peptide motifs that stimulate cell adhesion resulted in a marked improvement in their features, more improvements are required to mimic the functionality of tissues, such as their microstructure and the organization of cells and the ECM. The stroma of human corneas, for example, is composed of highly structured layers of collagen fibers and specific corneal cells known as keranocytes. These structured and organized layers ensure that the cornea is sufficiently transparent and resistant to function correctly. To mimic the natural structure of the corneal stroma, Kilic *et al*. assembled micropatterned collagen-ELR films possessing micron-scale grooves that cells can sense and align with into 3D scaffolds **(Figure 7)**. The ELR utilized in this work comprises a cell-adhesion sequence, namely the YIGSR peptide present in human laminin, blended with natural collagen. The films, which were crosslinked by way of a controlled dehydrothermal treatment, were stacked orthogonally with respect to the latter to obtain a 3D multilayer scaffold. Human keranocytes were cultured in

the micropatterned 3D scaffolds and their performance analyzed *in vitro*. The keranocytes cultivated in the stroma substitute were able to grow well, following the surface alignment of the corneal replacement, for a period of at least three weeks. Moreover, other crucial properties of this substitute, such as their transparency and light transmittance, were improved in the cultivated micropatterned scaffolds. Indeed, it was clear how all factors, namely composition, microstructure and keratocyte alignment, contribute to this enhancement of the properties of the collagen-ELRs cornea replacements[149]. Interesting results have also been obtained upon the assembly of multi-layers of collagen and ELR blends to produce abdominal wall substitutes for ventral hernia repair. To this end, ELR films reinforced with collagen microfibers were stacked to form a multilamellar flexible composite. Control of both the component content and fiber organization resulted in multi-layer scaffolds with tunable mechanical properties in terms of elongation to breaking, Young's modulus and tensile strength, with values that exceeded those of native human tissue being obtained in some cases. An *in vivo* study was carried out in a rat model with abdominal wall defects and tissue repair was observed after application of the collagen-ELR patches. Indeed, these patches prevented hernia recurrence in the animal model. Moreover, a histological study showed how the collagen-ELR scaffolds stimulated new tissue formation and substantial replacement of the elastin-like recombinamer by infiltration of new host tissue[150].

Three-dimensional collagen-ELR scaffolds have also been fabricated for epithelial oral tissue, the growth of which was promoted over long culture times with respect to conventional artificial 3D scaffolds. An ELR-RGD/collagen blend was chosen to enhance cell adhesion, and the 3D porous supports manufactured by electrospinning were subsequently chemically crosslinked using genipin[151]. Co-culture of human fibroblasts and oral epithelial cells resulted in the formation of an oral epithelial mucosal equivalent with multilayered features.

These cells were able to proliferate and colonize the scaffold, which was able to sustain the culture for at least six weeks and to express specific oral epithelial markers[152].

ELR-based biomaterials have also been described to be excellent substrates for stimulating regenerative processes in pancreatic tissues. Thus, the combination of ELR and polyethyleneimine copolymer (PEI), for example, was described to be a suitable substrate for hepatocyte culture, resulting in the formation of size-controlled, self-assembled hepatic spheroids. In this work, the ELR was chemically conjugated to PEI using carbodiimide chemistry, and hepatoma cells were subsequently cultured on surfaces coated with the ELR-PEI conjugate. Under these culture conditions, cells organized themselves into spheroidal features and continued to grow throughout the experimental time, forming organized structures. Moreover, the authors described the potential of this ELR-PEI system for controlling the final spheroid dimensions, with spheroid size being inversely proportional to polyelectrolyte conjugation[153].

Other versions of this bio-funcionalized ELR-RGD has been utilized as a support for culturing pancreatic cells, thereby resulting in the formation of islet-like structures. The pancreatic cells cultured on coated ELR surfaces were more viable and were able to grow into spheroids with an islet-like structure and to secrete insulin in response to glucose[154].

3. Recombinant Silk Proteins

3.1 *Bombyx mori* Silkworm Silk

Silk is an ancient material, the product of millions of years of evolution, and has recently become an important material in the field of tissue engineering. It was first used as a weaving thread in China thousands of years ago after having been obtained from silkworm *Bombyx mori* cocoons, and the Chinese kept the secret of its production for many centuries due to its enormous impact in early commercial exchanges between the Western World and imperial China along the so-called Silk Road.[155] Silk subsequently attracted the attention of surgeons

as a potential suture material, thus starting a long history of use in the medical field. The unique mechanical properties of silk and silk-like fibroin, especially their high strength-to-weight ratios and wide range of moduli (3-10 GPa), ranging from rubber-like to extremely rigid, make these proteins potential biomaterials for the development of biomedical devices.[156] Two main groups of silk protein producing insects can be differentiated: larvae of the order *Lepidoptera*, mainly silkworms of the species *Bombyx mori*, and members of the *Arachnida* class (spiders) **(Figure 8)**, both of them having extremely good properties for their use in soft-tissue engineering applications, and being extensively produced by recombinant techniques, which makes them more affordable and a promising material in the next years in the field of biomedicine.

The silk protein produced by silkworms consists of fibroin and sericin, two different proteins with diverse functions that have been strongly preserved during evolution due to their essential properties, which have allowed the survival of the *Bombyx* species. The former accounts for the majority of the cocoon, forming insoluble fibers, and is composed of nonpolar and hydrophobic residues that interact via hydrogen bonds, self-assembling into a semi-crystalline, well-ordered β-sheet structure that confers high strength and toughness. In contrast, sericin is a more hydrophilic protein that acts as a glue between fibroin fibers in order to maintain their microstructure. Due to the immunogenicity of the latter, it has to be removed to obtain 99% pure fibroin when used in biomedical applications, in a process known as degumming.[157]

Nowadays, silk biomaterials are mostly obtained from the cocoons of *B. mori* silkworm, with a worldwide cocoon production of 1 million tons (fresh weight) in 2002.[158] This approach has obvious requirements, namely the amount of space required for storage of the larvae, the food needed to raise silkworms, and the subsequent cost of the purification process. Nevertheless, the most important problem is probably time as it takes a long time to reach the

final product. To address this issue, recombinant DNA techniques can be implemented for the large-scale bioproduction of silk biomaterials, thereby reducing costs in a less time-consuming strategy. *E. coli* is the host chosen for the expression of many recombinant proteins as it offers a well-known expression system (plasmid vectors and bacterial strains) and scalable production and purification methods with lower costs than when using other hosts. Silk biomaterials have been recombinantly conjugated to elastin and collagen to give rise to silk-elastin-like and silk-collagen-like proteins.[159]

Recombinant silk biomaterials, known as silk-like proteins (SLPs), are based on the repetition of the hexapeptide GAGAGS motif found in silk fibroin[160], which is known to assemble into anti-parallel β-sheet secondary structures that lead to crystallization and stabilization of the fibrous structures formed by self-assembly, thereby conferring high strength, toughness and ductile elongation.[161] However, this crystallization phenomenon is also responsible for the low solubility of the protein, which makes subsequent purification steps expensive and very time-consuming due to the need for use of organic acids, in a similar manner to the way in which natural silk fibroin is obtained, or high volumes of aqueous solutions for purification of the protein. To solve this problem, other polymeric protein sequences can be fused to the SLP to obtain a more soluble product and could also contribute to improving the properties of the material. For example, fusion to elastin-like polypeptides (a co-polymer known as silk-elastin-like protein, SELP) makes the SLP more soluble and also confers elasticity to the biomaterial.[161a]

Various recombinant silk-like polymers have been synthesized in the past decade. Thus, *Bombyx mori* silk-like polypeptides derived from repetition of the hexapeptide GAGAGS have been fused, in different ratios, to the poly-alanine (polyAla) crystalline region from *Samia cynthia ricini* $(Ala)_{12}$, to the crystalline region from spider dragline spidroin $(Ala)_6$ and to the glycine-rich region of spider silk fibroin $(GGA)_4$. Three diverse recombinant silks were

designed by changing the number of repetitions of different “structural-blocks”, and secondary structure formation was studied by ^{13}C NMR spectroscopy. The results showed stable structures that could be self-assembled into different scaffolds for tissue engineering.[162] Furthermore, in subsequent work, the amino acid sequence for the hydrophilic region of spider dragline silk, YGGLGSQGAGRG, which is considered to be the origin of supercontraction of the natural protein, was combined with a $(GAGAGS)_6$ polypeptide to form fibers by electrospinning in order to obtain a mesh that could be used in biomedical applications.[163] Additionally, RGD cell-adhesion sequences derived from fibronectin have been included to provide attachment of epithelial kidney cells (VERO) and normal human dermal fibroblasts (NHDF), thereby suggesting potential uses in tissue regeneration.[164]

Transgenic *B. mori* silkworms have also been engineered to produce modified silk fibroin including either RGD cell-adhesion motifs or carrying partial sequences from different types of collagen (Collagen-Fusion, or Coll-F, sequence). Purification was performed as in the case of natural silk, and the improvement in cell-adhesion properties was confirmed using mouse fibroblast cultures on films in both cases, with the silk-RGD material being the most cell-adherent.[165]

In another work, it was hypothesized that *B. mori* silkworms could be modified using transgenic techniques to introduce RGD and YIGSR cell-adhesive peptides into the gene coding for the Heavy or Light Chain (H- and L-chain, respectively) of silk fibroin in silkworms to obtain a bioactive material liable to be used in the development of vascular grafts. RGD is a cell-adhesion motif with specificity towards cell membrane integrins as described above, while YIGSR, a motif derived from the laminin B1 chain, supports binding to the laminin binding protein. The latter has also been shown to have an effect in inhibiting tumour growth and metastasis when found in a multimeric form.[166] Genetic transformation

of silkworms was achieved and the resulting recombinant silks were found to perform better than wild-type fibroin, allowing adhesion and spreading of mouse fibroblasts and endothelial cells on the materials. Transformation of the H-chain with the laminin YIGSR-derived sequence was found to be more suitable for the development of vascular grafts according to the results obtained from *in vitro* cell culture. This biomedical device was successfully engineered and wild-type silk fibroin coated with the recombinant silk tested by implantation into rat abdominal aorta, giving satisfactory results in terms of improved cell migration and colonization *in vivo* when compared to wild-type silk. However, further investigation of this recombinant material itself as a vascular graft is still required to determine whether the recombinant silk is mechanically able to sustain stresses when implanted.[167]

As can be seen, relatively few recombinant *B. mori* silk fibroin matrices and scaffolds have been produced (**Table 5**), especially when compared with the number of devices developed from natural protein. This is mainly due to the facile and robust extraction of natural silk fibroin from farmed silkworms. Nonetheless, recombinant technology offers many advantages as regards the engineering of copolymers made out of silk and other proteins found in nature, such as elastin or collagen, thereby combining their properties. These copolymers have mostly been obtained by bioproduction in different hosts and some of them have enormous potential in the field of soft tissue repair. Below we focus on some examples of these co-recombinamers.

3.2 Silk-Elastin-Like Proteins

Silk-elastin-like proteins (SELPs) are block co-recombinamers composed of elastin and silk blocks, both of which contribute to the final properties of the material. Thus, elastin provides high elasticity and high solubility in aqueous media at low temperatures (below its lower critical solution temperature, LCST), thereby improving the purification method of single recombinant silk. Silk, on the other hand, confers stability on the structures formed as a result

of the crystalline β-sheet regions, which also strengthen and stiffen the material, complementing the elasticity. The combination of these motifs has led to the generation of novel recombinant biomaterials with dual mechanical properties, thereby notably increasing their potential uses in comparison to silk- or elastin-like polypeptides alone (**Table 6**).[168]

The silk block (S) is usually composed of the hexapeptide GAGAGS from the amino acid sequence of *B. mori* silkworm fibroin, whereas the elastin block comprises the model VPGXG sequence. The first SELP was designed and bioproduced in 1990 by Cappello et al., who studied crystallization governed by the antiparallel β-sheets and compared it to SLPs, showing that the ELP region influenced the self-assembly of the silk domain by interrupting this crystallization, hence increasing solubility, although the system exhibited a tendency to form such crystals after some time.[161a] Despite this early study, it was only recently when experiments concerning the generation of a library of SELPs were performed to gain an understanding of the changes observed in the mechanical and physicochemical properties depending on the guest amino acid in the elastin block and the silk:elastin ratio.[169] This study involved expression of the recombinant SELPs in 96-well plates and purification *in situ* by inverse transition temperature cycles. Temperature- and pH-induced responses were subsequently evaluated along with the adhesive properties of 64 SELPs constructed from 12 different monomers after an initial selection from over 2000 colonies by absorbance measurement and SDS-PAGE screening to select those with the best features. All in all, this new technique shows significant potential for the high throughput screening of novel SELP materials on the basis of their responsiveness and adhesive properties.

In order to demonstrate the potential of *E. coli*-based recombinant SELP bioproduction, a fed-batch strategy was chosen for the production of SELP-59-A, a copolymer with the VPAVG pentapeptide in the elastin block. Very high yields of up to 4.3 g/L following purification, which is 50-fold higher than the yields reported by other groups, were obtained. As this

process is scalable, it can be implemented in an industrial scale bioreactor, which could encourage the development of novel SELPs with different bioactivities for soft-tissue engineering.[170]

A novel SELP in which the elastin block comprises an amphiphilic di-block, a hydrophilic block in which the guest residue is a glutamic acid (VPGEG) and a hydrophobic one which possess an isoleucine at the fourth amino acid position (VPGIG), has also been designed. The silk domain includes repetitions of the GAGAGS hexapeptide. This construct is able to undergo a thermal transition triggered by the hydrophobic interaction between the non-polar side chains in the hydrophobic isoleucine-containing block, which collapses into a hydrogel at high concentrations. However, this gel is unstable due to the weak forces that support the structure, thus meaning that other interactions are necessary to stabilize the hydrogel. In this case stabilisation is provided by the silk domains and their crystallization via β-sheet folding. This approach results in a dual gelation mechanism, and long-term stable networks with an elastic modulus as high as 10 kPa are formed in the “annealed” (matured or stable) hydrogel. This value is higher than that for the counterpart with no silk motifs, in which only hydrophobic interactions permit hydrogel formation. Furthermore, this SELP was shown to be able to self-assemble into fibers, which were studied by TEM and AFM, thus adding a new characteristic to the original elastin-like recombinamer.[171] This biomaterial offers numerous possibilities in the field of biomedicine, since bioactive sequences can be genetically fused to it to perform more specific functions required for tissue regeneration.

A novel SELP with lysine-containing elastin blocks (SELP-47K) has been bioproduced and the mechanical properties of physically and chemically cross-linked hydrogels were measured. The results showed an excellent elasticity, as evidenced by tensile stress-strain, creep, and stress-relaxation analysis, comparable to that for elastin, with an elastic modulus of 1.7 MPa, a strain to failure of 190% and resilience of 86%. Nevertheless, this was only

achieved with physical gels treated with methanol to promote β-sheet formation. Although this approach has major drawbacks due to the impossibility of embedding cells within the scaffold, the mechanical properties and the possibilities of further cross-linking via lysine modification suggest that this material may find a wide range of tissue-engineering applications.[172] Similarly, wet-spun SELP-47K fibers were prepared in a methanol bath to finally obtain chemically cross-linked fibers with a tensile strength of up to 20 MPa and large strains to failure of 200-700%, thereby confirming the potential of SELP-47K for use in biomedical applications.[173]

SELP-47K has also been evaluated as an injectable matrix for cell-based therapeutics by encapsulation inside the hydrogel of human mesenchymal stem cells (hMSCs) in a high concentration (12% wt) aqueous solution of the recombinamer. In this study, differentiation and cartilage matrix accumulation were examined. Cell-viability assays revealed no cytotoxicity for the material and chondrogenesis was achieved upon addition of the chondrogenic growth factor TGF-β3 to the culture medium. This is therefore a good example of how the use of recombinant techniques to precisely control SELP structure permits the development of scaffolds suitable for soft-tissue engineering.[174]

Electrospinning has been used to produce mats of SELP fibers from the polymers SELP-1020-A and SELP-59-A, which were obtained by combining the GAGAGS silk hexapeptide and the VPAVG elastin sequence. The numbers in the names refer to the quantity of repetitions of the silk motif (first digit) and of the elastin pentapeptide (last one). The resulting fiber network was studied *in vitro* in cell culture, and it was found that human skin fibroblasts were able to adhere and spread on its surface, whereas indirect cytotoxicity studies revealed a good cytocompatibility and promotion of cell proliferation, thus making this material suitable for skin regeneration.[175]

Two different SELPs have been proposed for ophthalmic applications due to their optical properties. The first study involved SELP-47K and thin films of this biopolymer with a thickness of about 100 µm were formed by either no (by treatment with methanol) or covalent (via glutaraldehyde) cross-linking. A subsequent transmittance study showed the first film to have 95% light transmittance in the range 350-800 nm, with the chemically cross-linked film having a value of 77% at close to 800 nm. These results, combined with the cell viability assay, led to a significant amount of interest in the development of SELP-47K films for ophthalmic applications, such as contact lenses, synthetic corneas or intraocular lenses.[176] In addition, a recombinant SELP named PS2E8K was synthesized and chemically modified via the lysine residues present in the elastin block as guest residue with the biocompatible moiety retinal protonated Schiff base (RPSB) to generate a material for light-induced dynamic changes. Birefringence was induced when the recombinamer was irradiated with linearly polarized 488 nm laser light, thereby suggesting its application in eye tissue engineering.[177]

In a similar study, recombinant DNA techniques were used to include a matrix-metalloproteinase-2 and -9 (MMP-2 and MMP-9) responsive sequence as a new domain in the copolymer SELP-815K with the aim of improving the biodegradability of the SELPs. Addition of proteases to the SELP-815K solution led to complete degradation of the polymer, while no effect was observed in the negative control (silk-elastin-like protein lacking the MMP responsive sequence). These results suggest that, since the hydrogels displayed sensitivity to proteases, their use *in vivo* for soft-tissue engineering would lead to biodegradation of the scaffold by the MMPs secreted into the extracellular matrix by the cells involved in the regeneration of the damaged tissue.[178]

3.3 Silk-collagen-like proteins

These block copolymer proteins comprise silk and collagen domains fused by genetic engineering and expressed in a heterologous host to obtain a recombinant material with the

benefits of both natural proteins, namely the stiffness and strength of silk crystallites and the mechanical properties and cell-interaction ability of collagen. All these characteristics are accompanied by perhaps the most important ones: the proven biocompatibility and biodegradability of these domains. See **Table 7** for summarized information about silk-collagen-like proteins.

Within this type of material, pH-responsive hydrogels have been obtained by self-assembly of a tri-block copolymer as there is a central region surrounded by two ends. One block (S) is a pH-responsive silk-like octapeptide repeat GAGAGAGE, which self-assembles into antiparallel β-sheets capable of forming crystalline structures, combined with sequence repetitions of glycine and glutamic acid, the latter of which confers this responsiveness. The other block (C) has the same amino acid ratio composition as collagen, namely G-Xaa-Yaa, except for hydroxyproline in the Yaa position. The absence of this residue in the sequence prevents it from self-assembling, thus meaning that it remains disordered. However, stable hydrogels have been formed when building either a CSSC or a SCCS polypeptide, thereby suggesting a potential application of this material in tissue engineering when the possibility of adding bioactive sequences to the molecule is taken into account.[179] Furthermore, the elastic properties of these gels under dilute conditions, and at an acidic pH solution, were measured in a rheological study, with a high storage modulus of as much as 10 kPa being obtained at a concentration of 8 g/L for both CSSC and SCCS. However, as only SCCS gels are formed within a few minutes, this particular material is the one that is likely to be the most suitable for *in situ* tissue-engineering applications. Indeed, to this end, the pH needed for the formation of the hydrogels should be closer to the physiological value, which could be accomplished, for example, by replacing the glutamic acid residues with lysines.[180]

In another recent experiment, and similarly to the above studies, a silk-collagen-like protein with a central block of 48 repetitions of the silk octapeptide GAGAGAGAX, with histidine

instead of glutamic acid in the X position, and collagen-like sequences in the end blocks was designed. This construct was able to form nanofibers and stable, stiff and self-healing fiber hydrogels at physiological pH, which was one of the major drawbacks of the previous gel system. The cytotoxicity of this material was ruled out in a viability assay with primary rat bone mesenchymal stem cells (MSCs), although cells did not spread as readily as on collagen matrices and consequently did not proliferate to form a confluent layer. To address this issue, RGD cell-adhesion sequences could be included in future studies using recombinant DNA techniques. Thus, this material has good perspectives for the development of tissue-engineering systems.[159b]

To summarize, even if synthetic silk has not been extensively bioproduced, recombinant techniques allow natural motifs to be combined to form novel biomaterials with dual or multiple intrinsic properties and the inclusion of bioactive sequences. Among such materials derived from the repetitive hexapeptide GAGAGS and the octapeptide GAGAGAGX found in silkworm fibroin, the most extensively used are those that combine amino acid sequences from different structural proteins, such as silk-elastin-like (SELP) and silk-collagen-like proteins. It is to be expected that the development of new different copolymers will lead to an increased use of these biomaterials in the field of soft-tissue regeneration in the following years.

3.4 Spider Silk

Although similar to the silk proteins produced by insects, such as *Bombyx mori* silkworm, spider silk has evolved differently since it performs different functions. As a result, this has led to differences in the protein's primary sequence and, subsequently, different self-assembly and mechanical properties. This latter characteristic makes this protein very valuable as it possesses high tensile and compressive strength, thus making it a perfect candidate for different applications, such as the fabrication of structural components and design of

protective clothing, as it exceeds the properties of one of the hardest man-made fiber-based materials known, namely kevlar.[181] It is also biocompatible and biodegradable and is therefore an ideal material for the development of biomedical devices.[182] Within this field, natural spider silk has been used as a suture for wounds as it has better mechanical properties than nylon, the gold-standard in this application.[183]

One major difference between silkworms and spiders is the possibility of farming. The former are very easy to farm and natural silk fibroin can be obtained after purification, whereas spiders are typically aggressive and often cannibalistic, so large-scale extraction of spider silk by farming is not possible.[184] In this case, recombinant DNA technology is very valuable for the production of spider silk-based materials as it is the only means of achieving the industrial-scale batches required for commercial purposes.

The DNA sequences used in the recombinant production of silk spidroin are those that translate into amino acid sequences which have been found to be relevant in conferring the desired mechanical properties on natural spider silk. The first kind of silk spidroin to be recombinantly expressed was the one produced by the major ampulla (MA), also known as dragline silk, which is used by the spider to weave the radii of its web because of its unique tensile strength. The genes that have been identified to codify the amino acid sequences of these spidroins are ADF3 and ADF4 (*Araneus diadematus* Fibroin 3 and 4, respectively) found in *A. diadematus* and MaSp1 and MaSp2 (Major ampullate Spidroin) in *Nephila clavipes*. The primary amino acid sequence in every case is composed of poly-alanine blocks or stretches in combination with motifs containing $(GGX)_n$ or GPGXX (X = tyrosine, glutamine, leucine). Poly-alanine regions fold into crystallized β-sheets, thereby conferring high tensile strength on spider silk fibers, while the repetitive motifs assembly into β-turns because of their high glycine content, thus conferring elasticity and flexibility on the fibers. Numerous studies aimed at identifying the amino acid composition that determines the

specific function of each motif in the final secondary structure has led to the production of synthetic spider silk genes, which are the repetition of the exact domains that confer the extraordinary mechanical properties on these proteins but lacking other regions of the coding sequence. This has led to better yields than when the whole genes are expressed.

Recombinant production of silk spidroin has been successfully performed in diverse host organisms with different yields. The first host used was *E. coli* due to the wide-ranging experience with this model host. Transformation of this bacterium to express the MaSp1-derived gene from *N. clavipes* resulted in a yield of 1.2 g/L of a high molecular weight spidroin in a metabolically engineered *E. coli* strain.[185] However, similar high yields in the production of low molecular weight spider silk could not be obtained when using a range of common bacterial strains.[186] In related studies, synthetic ADF3 and ADF4 genes gave rise to moderate yields of recombinant spidroin.[187] One of the major issues affecting low yields is the low intracellular solubility of the protein,[188] which leads to the formation of inclusion bodies inside the bacteria.[189] Recombinant spidroin has also been obtained in eukaryotic hosts, such as *Pichia pastoris* yeast,[190] *Nicotiana tabacum* tobacco plants,[191] insect cells and transgenic *Bombyx mori* silkworm.[192] It has also been expressed in transgenic mice and transgenic goats, which were used as mammary gland bioreactors to obtain milk containing silk spidroin (15-20 g per week in the latter case).[193] However, various issues still need to be addressed for large-scale production in these hosts, such as yield and purification processes required to produce a biocompatible material.

One of the most interesting attempts to introduce bioactive sequences into the sequence of spider silk using recombinant DNA techniques involved fusion of this protein to an RGD-containing cell-adhesion sequence (GRGDSPG peptide). The effect of this fusion protein on the cell attachment of BALB/3T3 mouse fibroblasts was assessed and compared to a chemically modified recombinant spidroin containing a cyclic RGD peptide. Adhesion

appeared to be even better in the recombinantly modified spider silk, suggesting that this spidroin could be a potential biomaterial for future skin tissue engineering applications that require the inclusion of cells.[194] Surprisingly, it was also possible to reproduce the effect of the RGD sequence on cell adhesion by simply changing the topography of the substrate manufactured from spider silk.[195] Another functionality was attained by addition of the peptide IKVAV from the ECM protein laminin. Thus, Schwann cells isolated from human spinal nerves were shown to adhere to, and achieve a spread morphology on, matrices made of recombinant spider silk carrying this cell-adhesion motif, thus making this material potentially suitable for the treatment of glial cell related diseases.[196]

It has also been proved possible to generate three-dimensional scaffolds from recombinant spider silk using a spidroin named rS1/9 that is analogous to *N. clavipes* spidroin 1 (MaSp1). This biomaterial shows good biocompatibility when used in cell culture and provides efficient cell adhesion and proliferation over a long time period, thereby suggesting its likely use as an implant material.[197] When used for this purpose, its good biocompatibility properties were confirmed and it was also found to promote the ingrowth of fibrous, nerve and adipose tissue elements, as well as angiogenesis, thus making it completely suitable for biomedical applications.[198] In addition, rS1/9 spidroin was able to regenerate injured bone in rats by inducing the growth of soft connective tissue. It was subsequently concluded that the porous inner structure of the rS1/9 scaffolds provided a better microenvironment for tissue regeneration than *B. mori* fibroin, thus making it a highly promising biomaterial in regenerative medicine.[199]

Likewise, 4RepCT, another recombinant spidroin fibrous material derived from *Euprosthenops australis* MaSp1, has been implanted subcutaneously in rats for seven days to study systemic and local reactions in comparison with commercial Mersilk. No abnormal reactions were observed by either macroscopic visualization or histological staining. In

contrast, fibers were able to support the ingrowth of fibroblasts and newly formed capillaries. These results show that 4RepCT is biocompatible and suitable for stimulating host cell colonization and angiogenesis when implanted subcutaneously *in vivo*, although further studies regarding its suitability in tissue engineering and regenerative medicine are still required.[200]

4RepCT was also chosen to develop matrices for neural stem cell (NSC) cultures. The stimulating effect of this material on the differentiation of NSCs into neurons, astrocytes and oligodendrocytes was studied and compared to conventional poly-L-ornithine and fibronectin (P + F) coated polystyrene plates, which is the gold-standard for the culture of this cell type. The results showed that this biomaterial provides a novel substrate for efficient culturing of NSCs with no negative effects, except for a slightly lower oligodendrocyte differentiation compared to P + F coated plates, and is therefore an alternative to this culture technique. Further optimization could be possible by the fusing of different factors and tailoring of the matrix structure using recombinant DNA techniques.[201]

4RepCT was also fused to binding domains directed towards different receptors found in blood. Albumin binding domain (ABD), Z and C2 IgG binding motifs and the biotin-binding domain M4 were chosen and showed a correct folding and good stability in both the soluble state and as films, as assessed by infrared spectroscopy. These functionalized spidroin films were able to selectively bind the intended target molecules from complex samples such as rabbit and human plasma. Furthermore, when these diversely functionalized silk spidroins were combined in order to build films, it also proved possible to select all the target molecules, thus indicating that multifunctional materials could be obtained. Additionally, target proteins could be released from the film by proteolytic cleavage. The above results suggest that this material is a good candidate for different applications in the field of tissue engineering.[202]

In conclusion, different spider silk motifs have been used in the recombinant expression of spidroin or spider silk fibroin (**Table 8**). This method offers the possibility of large-scale production, thereby overcoming issues regarding the farming of spider species. Two major biomaterials, namely rS1/9 and 4RepCT, have been obtained using this approach and both have shown good biocompatibility. In addition, they have both been modified to add different bioactivities, heralding a promising future in the field of tissue engineering for soft tissue substitution and regeneration.

In general, not only referring to spider silk but to every type of silk biomaterial, there are many *in vitro* studies regarding mechanical properties, such as high strength-to-weight ratios and toughness, and many biofunctionalization studies, with different bioactive domains, that propose several potential applications as biomedical devices. However, there are some restrictions in their use, because of the lack of features such as elasticity or resilience. This issue could be addressed with the combination of different materials by recombinant technology, which is a field with a lot of future, even when it has been explored yet. Although little evidence of *in vivo* applications has been shown yet, the literature suggests an evolution of these silk biomaterials towards smarter scaffolds and the development of more complex and adaptable biomedical devices to be used in soft-tissue engineering, since new approaches are needed to overcome several limitations in tissue regeneration. Hence, the next steps should be focused on the verification *in vivo* of the results observed *in vitro*.

4. Collagen

Collagen is considered to be the most abundant and important structural protein found in the ECM of vertebrate tissues as it confers great flexibility and tensile strength due to its ability to withstand large forces between body compartments. Moreover, several bioactive functions, including an ability to modulate adhesion, migration, proliferation and cell fate, have been attributed to collagen[203]. Its monomeric structure contains a repetitive building block

comprising glycine-X-Y, where X is usually a proline and Y is a 4-hydroxyproline; these repeating sequences fold around one another in order to form triple helices comprising three left-handed helical chains forming a right-handed supercoil. Protein formation can be seen in **(Figure 9)**. Collagen also contains non triple-helical domains, known as non-collagenous domains (NCDs), that are occasionally found in other modular proteins as building blocks[204]. These NCDs are able to interact with ECM components, and favor triple-helical assembly and antiparallel dimer formation in procollagen[205]. More than 20 genetically different collagen types have been found in various animal tissues, thus suggesting that the use of any particular type for biomedical purposes should be based on its natural occurrence in a specific tissue. In the case of collagen I, which is the most abundant type (principally found in bone, cornea, dermis and tendon), the triple-helical conformation is assumed after a hundred hydroxylated proline residues have formed in the helix, starting from the carboxy-terminus and reaching the amino-terminus. These triple helices are subsequently intermolecularly crosslinked into parallel self-assembled collagen fibrils with a diameter of 100-500 nm[206]. Several post-translational modifications are required to attain a correct conformation that determines the functionality of the protein. Two of these, which have frequently been mentioned in literature, are hydroxylation of the proline residue in a reaction mediated by prolyl-4-hydroxylase (P4H)[207], an $\alpha_2\beta_2$ tetramer in vertebrates[207], and hydroxylation of a few lysine residues by lysyl hydroxylase 3 (LH3) followed by glycosylation[208]. Recombinant production of collagen is made difficult by the need for these post-translational modifications to achieve the appropriate higher order structure since prokaryotic heterologous hosts are not able to perform them[209]. Some host organisms, such as transgenic plants, are also unsuitable for expression of recombinant collagen as some modification enzymes in plants, such as P4Hs, differ from their animal counterparts in terms of substrate specificity, often leading to inefficient hydroxylation[207]. The production of functional recombinant collagen is currently only

possible by co-expressing the genes that code for the human enzymes necessary for post-transcriptional modification (P4H, LH3 and others) in the host, along with the expression of collagen-codifying genes. These bioproduced materials appear to be resistant to thermal denaturation and unfolding at normal body temperature[210].

The main reason underlying the development of recombinant collagen production is the need to obtain a pure product. In the case of collagen I, this is important due to the fact that, in addition to potentially containing infectious agents, all animal-derived batches may also be contaminated with other collagen types. Similarly, the expression of other collagen types is much lower than that for collagen I, therefore recombinant production may address the need for larger quantities of material[203]. For example, Stephan *et al.*[211] have reported a procedure for obtaining homotrimers of non-fibrillar recombinant α1(VIII) and α2(VIII) collagen, co-expressed with P4H in transfected cells, in order to ensure the stability of the pepsin-resistant triple-helical domains. Further optimization studies or scaffolds made of collagen VIII are, however, required, since this collagen type is present in various tissues, such as cornea, optic nerve, endothelial/smooth muscle/mast cells and macrophages, thus meaning that constructs containing this collagen type could be used to treat various types of tissue damage.

Recombinantly expressed human collagens and collagen-like proteins have been reported in yeasts such as *Pichia pastoris*[212], bacterial organisms such as *Escherichia coli*[213], insect cells[214] and plants[215].

The overall achievement of recombinantly produced collagen proteins is the high grade of stability of the fibril-forming structure, which is assured by a post-translational extent of hydroxylation similar to that for the natural protein[210]. The fields in which recombinant collagen has been applied mainly comprise bone grafting, skin replacement and cartilage and corneal repair.

4.1. Recombinant collagen for skin repair

Epidermal regeneration does not occur spontaneously since the epidermis needs a dermis layer on which to migrate. Although collagen I is involved in epidermal adherence as it constitutes 80-85% of the dermal matrix[216], other types of collagen also play a role in skin integrity. Consequently, the use of collagen-based materials appears to be a suitable approach for the treatment of cutaneous injuries and diseases provided that the specific collagen type used is adapted to the peculiar context.

An example in which recombinant collagen has been employed for skin regeneration is the work of Willard *et al.*, who constructed two types of scaffolds from plant-derived human collagen type I (PDHC): an electrospun nonwoven platform and a lyophilized sponge[216]. Both PHDC scaffolds were compared to a bovine-derived collagen obtained in a similar manner, and with a similar architecture and mechanical properties to the recombinant version, with the aim of presenting the former as suitable engineered skin-substitutes that overcome the immunogenicity issues of the bovine material. The choice of scaffold architecture was intended to offer two alternative solutions for skin engineering, namely a platform for *in vitro* cell adhesion and growth and a raw substrate scaffold for skin engineering. It was observed that both PDHC scaffolds favored human primary cell (dermal fibroblasts, endothelial and epidermal keratinocytes) adhesion and proliferation and resulted in an appreciable maturation and growth of engineered skin. In comparison to the bovine-derivede control, the PDHC scaffolds presented a faster rate of cell adhesion and proliferation and a lower risk of allergic reaction or disease transmission.

Similarly, a flowable gel formed from recombinant human collagen type I (rhCI), obtained from a transgenic tobacco plant, has been used for the *in vivo* treatment of acute, chronic and tunneled cutaneous lesions by Shilo *et al.*[217] The gel formulation was obtained by applying a

saline solution to the dry collagen sample. Application of the rhCI gel to a full-thickness cutaneous wound healing rat and pig model resulted in faster lesion closure, an enhanced healing process and an earlier angiogenic effect in comparison to controls (from a bovine and a human source), although the use of rhC provoked a mild inflammatory response.

Recombinant collagen has also been used to treat a cutaneous disease in the work presented by Woodley *et al.*[205, 218] In these examples, recessive dystrophic epidermolysis bullosa (RDEB), a heritable illness presenting mutations in the type VII collagen gene, was treated. Collagen VII is responsible for the anchoring of fibrils, and consequently, the disease presents with skin fragility, blisters, scars and a deficiency of normally anchored fibrils. Since collagen VII represents only 0.001% of human skin, recombinant production was required. When transfected into human embryonic kidney cells (HEK 293) carrying specific mutations observed in RDEB, this recombinantly obtained product was found to to adhere to human foreskin fibroblasts (HFFs). The intradermal and intravenous injection of recombinant collagen VII into mice transplanted with an RDEB human skin equivalent resulted in the formation of adequate anchoring fibrils and reversal of the RDEB skin phenotype, and wound healing by re-epithelization of the epidermis was also confirmed *in vivo*.

4.2. Recombinant collagen applied for cartilage repair

As cartilage tissue does not have an intrinsic capability to regenerate when damaged[219], its repair requires a long-term tissue engineering approach with strict control over the mechanical features of the scaffold inserted into the defect void and a guaranteed interaction between the repaired tissue and the native one. In this regard, cartilage repair using recombinant human collagen II (rhCII) has been evaluated by Pulkkinen *et al.*[220] In this study, *in vivo* tests with nude mice were performed over a six-week period using an rhCII gel scaffold for bovine chondrocyte growth. Gelation of the scaffold was performed simply by leaving the

chondrocyte-containing collagen solution to stand at 37 °C for an hour. The study compared a rhCII construct carrying chondrocytes (rhCII-cell) with two controls: a cell-free rhCII gel (rhCII-gel) and chondrocytes without the scaffold. The results after *in vivo* implantation of the scaffolds showed an adequate expansion and support for the formation of new cartilage tissue in the case of rhCII-cell, while no tissue formation was found in the rhCII-gel samples. Interestingly, chondrocytes alone also resulted in tissue construction under both cell-containing conditions, with a chondrocytic phenotype being observed along with *lacunae*-like formations, whereas rhCII-cell samples had a regular and oval final appearance. The chondrocytes alone acquired a flattened layer-type morphology. A potential use of this collagen gel for the treatment of chondral lesions was presented.

4.3. Recombinant collagen applied for corneal repair

Corneal damage or blindness, which is noted as an irreversible decrease in optical clarity, tends to be treated using donated human corneas. However, since there is a notable shortage of donor tissue, and this approach can lead to transplant rejection and therefore long-term steroid-based immunosuppression, alternative pathways are needed. In this regard, Fagerholm's group has presented various publications concerning the development of an innovative artificial corneal substitute made of recombinant collagen type III[221]. In a two-years follow-up study[222], acellular recombinant collagen I and III matrices crosslinked with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) were used as artificial corneas to assess their benefits in a pig model when compared to allografts. As no remarkable difference was observed when comparing the collagen type I and collagen type III constructs, thereby suggesting that nerve regeneration was successful, the work moved on to a clinical phase I study to assess the effectiveness of corneal re-epithelization and nerve regeneration without the need for long-term immunosuppression. The same group confirmed the durability of the implant after four years post-application in patients[223].

4.4. Non-human recombinant collagen materials

Interestingly, recombinant bacterial collagen-like proteins have also been studied and presented in recent publications[213, 224] and found to be able to form triple-helical structures. Thus, An *et al.*[225] focused on production of the specific bacterial collagen-like protein Scl2, produced by the gram-positive bacterium *Streptococcus pyrogens*, which was expressed and produced in *E.coli* and purified chromatographically. The authors postulated the hypothesis that it may be better to optimize the production of collagen-based materials that differ from human collagen, by avoiding the co-expression of P4H, the expression of which depends on conditions that cannot be fully controlled. In contrast, the stability of bacterial collagens may be questioned since most bacteria lack prolyl hydroxylase. However, it has been shown that it is possible to achieve a suitable conformation as a result of electrostatic interactions[226]. Subsequently, the Scl2 gene was genetically fused to gene sequences promoting specific bioactivities such as cell adhesion, heparin binding or proteolytic enzymes, etc.[225] This suggests a possible "plug and play" approach for the design of such systems. Although only a few examples of their use in the biomedical field have since been proposed, perhaps one of the most important is the study by Browning *et al.*[227]. The authors of this study used Scl2 to construct a vascular graft bearing photoreactive crosslink sites, which was bioconjugated with poly(ethylene)glycol (PEG) via the lysine residues and reinforced with a polyurethane mesh. Grafts were obtained by electrospinning, and subsequent mechanical characterization showed adequate resistance to physiological blood flow. The adhesion and spreading of endothelial cells was also confirmed, along with a higher rate of migration in comparison with controls lacking the Scl2 protein. However, more studies of the potential use of bacterial collagen-based materials must be performed before clinical uses can be considered. Examples of recombinant collagen materials are summarized in **(Table 9)**.

5. RESILIN

Resilin is an elastomeric insect protein found in a specialized region of the insect cuticle that favors typical insect behaviors, such as leg movement in arachnids and the extraordinary ability of some species to jump (flea), vocalize (cicadas) and fly (desert locusts and dragonflies)[228]. Resilin is characterized by high elasticity, low stiffness (2 kPa), a high energy-storage capacity, high fatigue time and resilience. It has been observed that resilin appears to be insoluble in media that do not contain peptide-bond degrading agents and resists temperatures as high as 140 °C. The difficulty in obtaining large quantities of recombinant resilin is due to the fact that early attempts at recombinant production led to production of the protein in inclusion bodies[229]. As reported by Tamburro's group[230], the structure of soluble pro-resilin, the precursor of resilin, in *Drosophila melanogaster* presents glycine-rich repeat sequences, which organize in three dimensions to form β-turns and almost extended structures. When crosslinked, these sequences are interconnected via tyrosine residues and appear to be quite independent from the rest of the molecule when observing the supramolecular arrangement. Tamburro's group also studied the similarity between the amino acid compositions of resilin and elastin and highlighted the lower hydrophobicity of the overall structure of resilin (due to the higher percentage of acid residues and lower number of apolar ones), which consequently affects its features as a material **(Figure10)**.

The first work to reflect resilin's high resilience was published by Elvin *et al.*[231], who constructed the recombinant protein (Rec1-resilin) inspired by the first exon of *D. melanogaster* CG15920 gene, which codes for the elastic $(GGRPSDSYGAPGGGN)_{17}$ sequence, and expressed it in *E.coli*. Said protein was used for network structure formation by Ru(II)-mediated photo-crosslinking. Scanning probe microscopy (SPM) tests showed a 92%

resilience for the Rec1-resilin scaffold and a negligible hysteresis upon compression, while in a tensile test, viscoelasticity showed material deformation at about 5 Hz and 10^{-3} Hz**.** The remarkable softness of the scaffold appears to be a result of its 80% water content at swollen equilibrium, with a stress/strain modulus at 100% of about 2.5 kPa. The resemblance between resilin's contraction/extension behavior and the function of human arteries suggests blood vessel repair as a biomedical field in which resilin-based materials may find a use.

Subsequent studies by the same group[232] showed that Rec1-resilin appeared to be pH-responsive and to exhibit a dual phase-transition behavior (DPB; having a lower and upper critical temperature). Differential scanning calorimetry (DSC) and Fourier-transform infrared spectroscopy (FTIR) results suggested that the secondary structure of the protein contains α-helices and a random coil conformation. In addition to the peculiar DPB, Rec1-resilin exhibits photophysical properties due to the presence of 20 Tyr fluorophore residues. The set of Rec-resilin characteristics replicate resilin's features well and make purification and production scale-up easier.

In a subsequent study, Lyons *et al.*[228] produced two alternative resilin-like proteins containing the repeat sequence $(AQTPSSQYGAP)_{16}$ from the mosquito *Anopheles gambiae* (An16) and the sequence $(GGRPSDSYGAPGGGN)_{15}$ from *D. melanogaster* (Dros16), respectively, in *E.coli*. In both cases, the polymers were crosslinked by Ru(II)-mediated photo-crosslinking in order to obtain hydrogels by formation of dityrosines. Mechanical characterization was focused on a comparison with the Rec-resilin mentioned above. Despite having a different amino acid composition, the results highlighted a structural disorder in both the An16 and Rec-resilin proteins, which present a low helix content and some contribution from sheets, turns and polyproline II structures. Although Dros16 appeared to be more ordered, it nevertheless showed similar mechanical characteristics to An16. SPM showed high resilience and suggested good elasticity for both materials. Even though this method is not

usually employed to evaluate the latter, elasticity is thought to be inversely proportional to the number of di-tyrosines. The crosslinked structure of An16 was the softest of the three structures studied and had the highest resilience of about 98%. These characteristics potentially offer new solutions for resilin-inspired biomaterials as their mechanical features are comparable to those of other resilin-based scaffolds.

Renner *et al.*[233] evaluated the compressive modulus cell viability and cell spreading within hydrogels obtained by fast crosslinking with tris(hydroxymethyl)phosphine and containing resilin repeat sequences from *A. gambiae* (composing 65% of the overall structure) and cell adhesion motifs (RGD). The protein was expressed in *E.coli* and purified by salting out and freeze-thaw cycles. Although the aggregation temperature behavior of this material has not yet been studied in detail, it appears to be potentially tunable by varying the amino acid sequence, as is the case for elastin-based recombinamers. Mechanical characterization showed a storage modulus of about 2.4 MPa, which is twice the value for other resilin-based materials and comparable to the modulus of human cartilage. This feature, along with the spreading of cultured mesenchymal stem cells (MSCs) on the material, which showed a viability of 95% after three days, suggest the possible use of this platform to direct chondrogenesis in cartilage repair.

Various groups have studied the development of systems with the remarkable mechanical features of hydrogels formed from resilin-like polypeptides. Thus, Charati *et al.* designed a polymer containing the repeating structural resilin consensus motif from *D. melanogaster* (GGRPSDSYGAPGGGN) fused to bioactive domains, thereby conferring cell-adhesion, heparin-binding capacity and enzymatic cleavage susceptibility (RLP12)[234]. In addition, the tyrosine residues were substituted with phenylalanines to permit possible future photo-crosslinking. The proteins were expressed and produced in *E. coli* and an increase or decrease of specific bioactivity was observed upon changing the percentage of each bioactive domain.

The lysines contained in the sequence were to perform an efficient gelation by way of a Mannich type reaction using THPP (β-(tris(hydroxymethyl)phosphine propionic acid). Determination of the secondary structure of RLP12 in solution after crosslinking showed similar conformations, with a minor quantity of β-turns and majority disordered structures, thereby favoring the chain mobility required for the expected elastomeric behavior. The ability of these hydrogels to support cell anchorage and proliferation was confirmed by culturing NIH 3T3 mouse fibroblasts. In addition, the mechanical characteristics and non-degradability (evaluated for 21 days) of the films (storage shear modulus of 0.5-10 kPa; Young's modulus 15-35 kPa) suggest their possible use for the regeneration of mechanically demanding tissues, such as vocal fold tissue.

Li *et al.* also used RLP12 in another work[235] in which the target body compartment was also vocal fold tissue. This latter study also confirmed the tunable mechanical stability and deformation response of resilin-based constructs. A mechanical evaluation of the material showed reversible elasticity, negligible hysteresis and notable resilience, and the storage modulus of the material could be adjusted over the wide range of values (from 500 Pa to 10 kPa) simply by changing the polymer concentration and crosslinking ratio. In addition, the gelation time was found to decrease as the polymer concentration and crosslinker ratio increased, with the crosslinking temperature also increasing under these conditions. The evaluation of G' at high frequencies (similar to those of human phonation) using a torsional wave apparatus (TWA) revealed values of about 200 to 2500 Pa, similar to those of human and porcine vocal folds. The high resilience of these materials is noteworthy as phonation is influenced by vocal fold tissue transient response[236], thereby supporting the hypothesis that the time-dependent mechanical characteristics of tissues are directly connected to their function. Given its mechanical and biological features, this resilin-derived polymer could also

be used to manufacture scaffolds for other tissues that require particular elastic properties, such as the cardiovascular system.

A different crosslinking approach has been used by Qin *et al.*[237] to produce resilin-like protein hydrogels derived from the sequence of the *D. melanogaster* gene CG15920, which contains the naturally occurring chitin-binding domain. The purpose of this study was to determine the interaction between resilin and the cuticle polysaccharide chitin in these insects as a better understanding of the natural structure and function of this composite may lead to findings of interest for applications in the biomedical field. In this case, horseradish peroxidase-mediated network formation was employed and subsequently compared with hydrogels obtained using a photo-catalyzed Fenton-reaction. The unusual internal organization of the scaffold observed, which mainly arises as a result of random coils, suggests that order is not conferred by the di-tyrosine crosslinks present. The elastomeric characteristics of the scaffold suggest possible use in tissues such as blood vessels and skin, which undergo a constant pulsating movement that can be supported by resilin-like structures.

Tamburro's group designed a chimeric biomaterial, known as REC polypeptide, with the aim of combining features exhibited by elastin, resilin and collagen into a single construct[18]. The inclusion of elastin-like sequences (LGGVG) provides the overall structure with an ability to self-assemble, the resilin-like repeating domain (SDTYGAPGGGNGGRP) increases resilience and provides a notable fatigue lifetime, while the insertion of collagen-like sequences (GPY; Y=G,A) guarantees satisfactory mechanical characteristics by limiting scaffold deformation. Lysines are spread throughout the overall polymer structure to allow future crosslinking. The use of recombinant technology in this context reflects the whole range of advantages of this technique since it permits a strikingly tunable biomaterial with characteristics conferred by three different natural proteins to be obtained. From a mechanical point of view, the Young's modulus for this chimera is in the range 0.1-3 MPa when

measured at 37 °C. The conformation of the structure, as analyzed by atomic force microscopy, shows its tendency to self-assemble into a fibrous composition. Although the authors did not suggest a specific field of application for the material, its tunable characteristics make it a suitable candidate for optimization and application in the field of tissue regeneration.

In summary, resilin-based materials are very promising due to their mechanical and biological features, and these findings are a great boost for future studies in this field.

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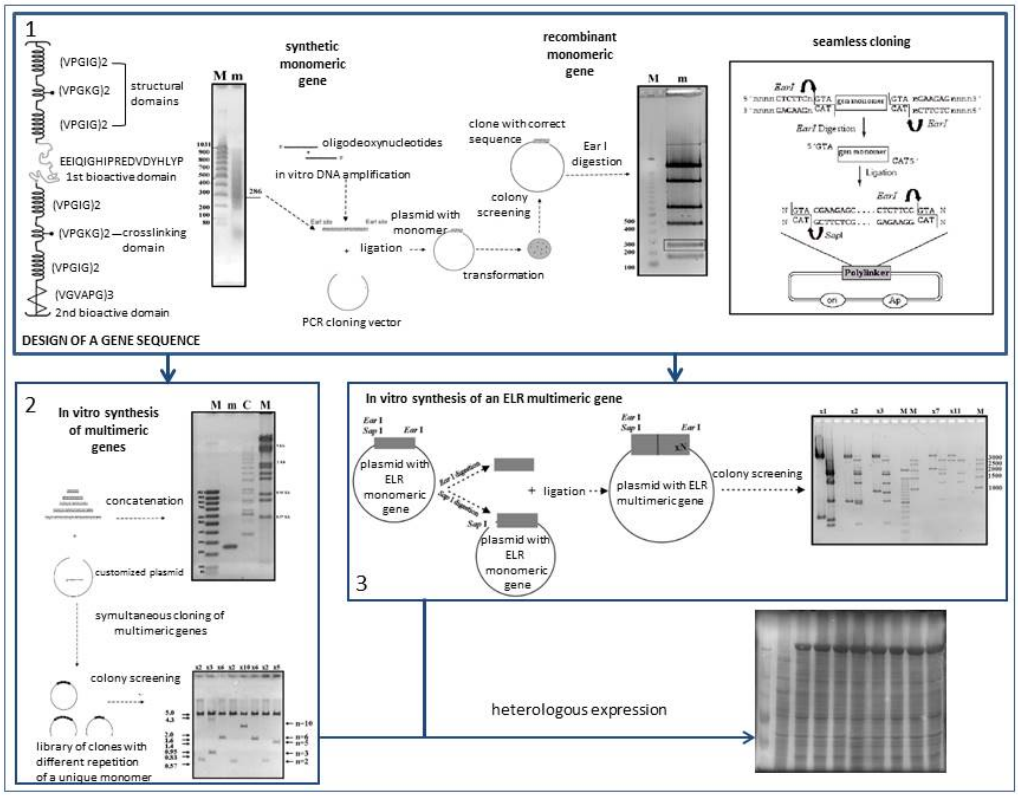

Figure1. Scheme showing the design and biosynthesis of an elastin-like recombinamer: (1) design of the recombinamer amino acid sequence and construction of the gene that codifies the monomeric gene by seamless cloning employing recombinant DNA techniques; (2) multimeric gene synthesis by random concatemerization method; (3) multimeric gene synthesis using the step-by-step iterative-recursive method

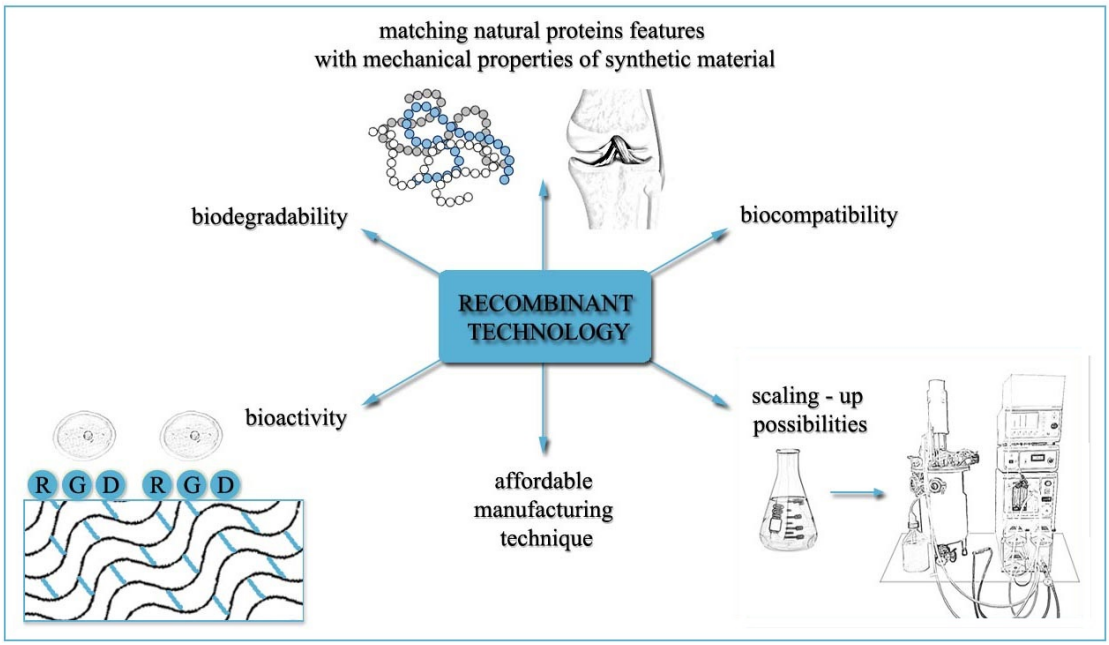

Figure2. Recombinant technology features

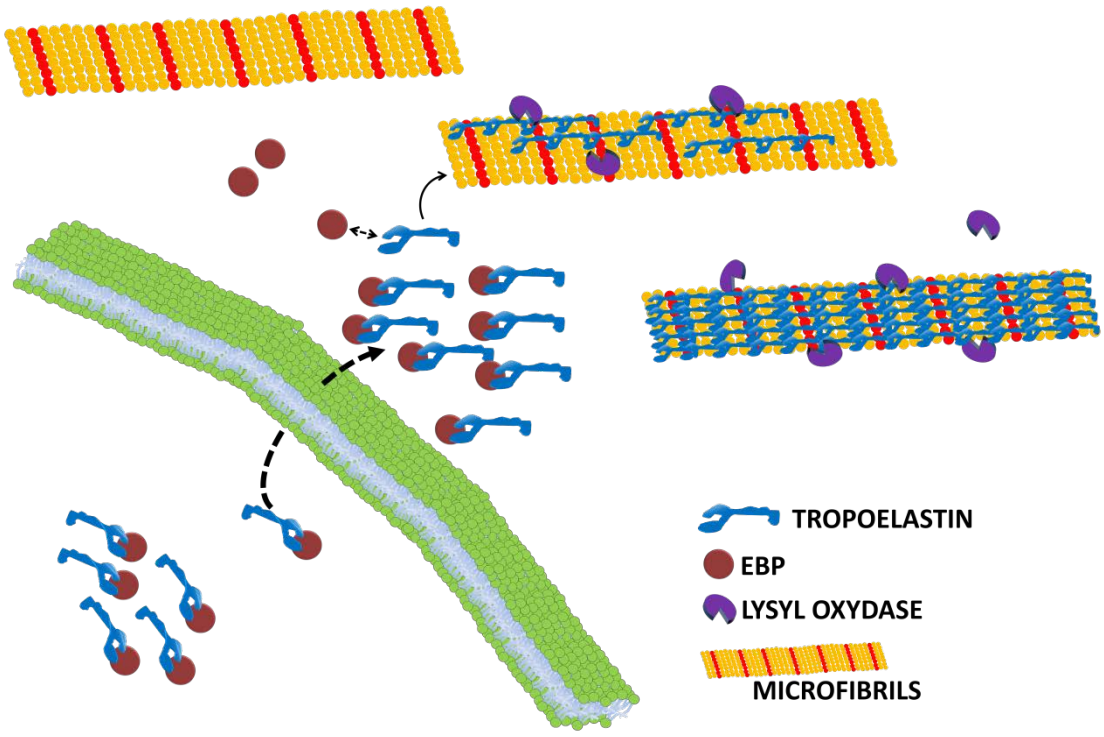

Figure 3. Tropoelastin is transcribed and transported to the plasma membrane associated with elastin-binding protein (EBP). Once released, tropoelastin and EBP dissociate. Tropoelastin aggregates are oxidized by lysyl oxidase on microfibrils to create mature oriented elastin fibers.

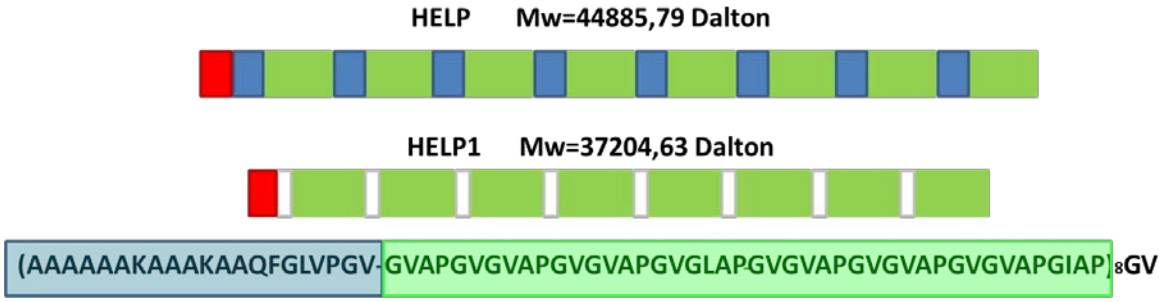

Figure 4. Schematic representation of HELP and HELP1 tropoelastin-exon derived. Adapted from ref[79]

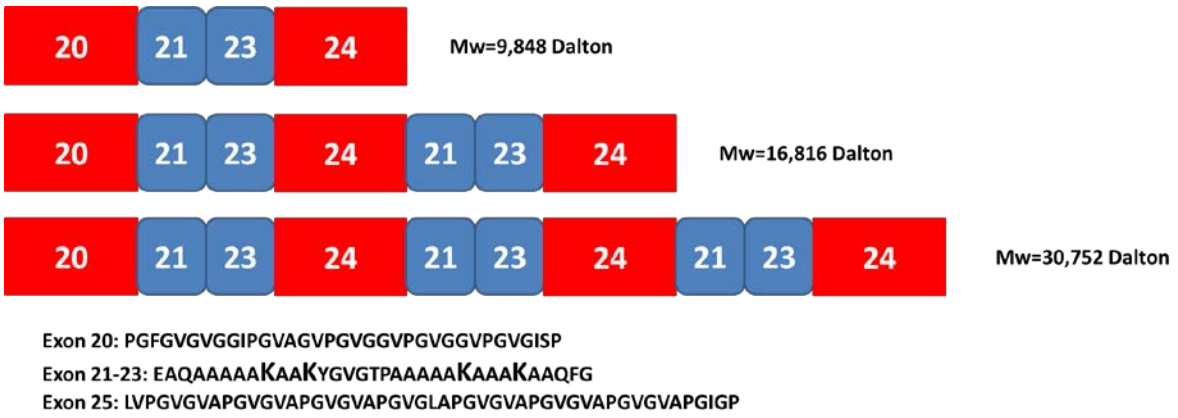

Figure 5. Schematic representation of the constructs used by Srokowsky *et al.* for developing a blood compatible coating for vascular devices. Adapted from ref[87]

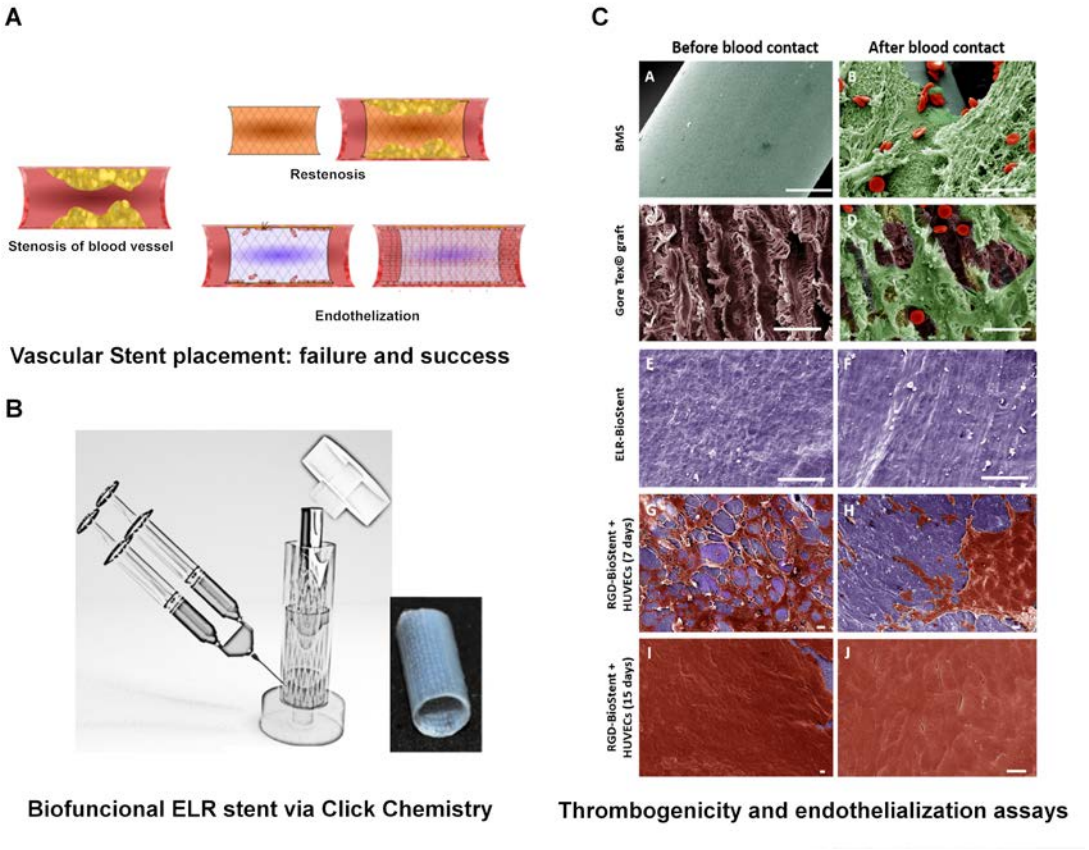

Figure 6. Schematic representation of the produced Click ELR-covered stents. A**:** scheme of the stenosis of vascular vessel and of the fail or success of stent implantation. B: Representation and image of the Click ELR covered stent obtantion. C: SEM images of the results of the thrombogenicity and endothelialization assays of Click ELR. Adapted from ref[132]

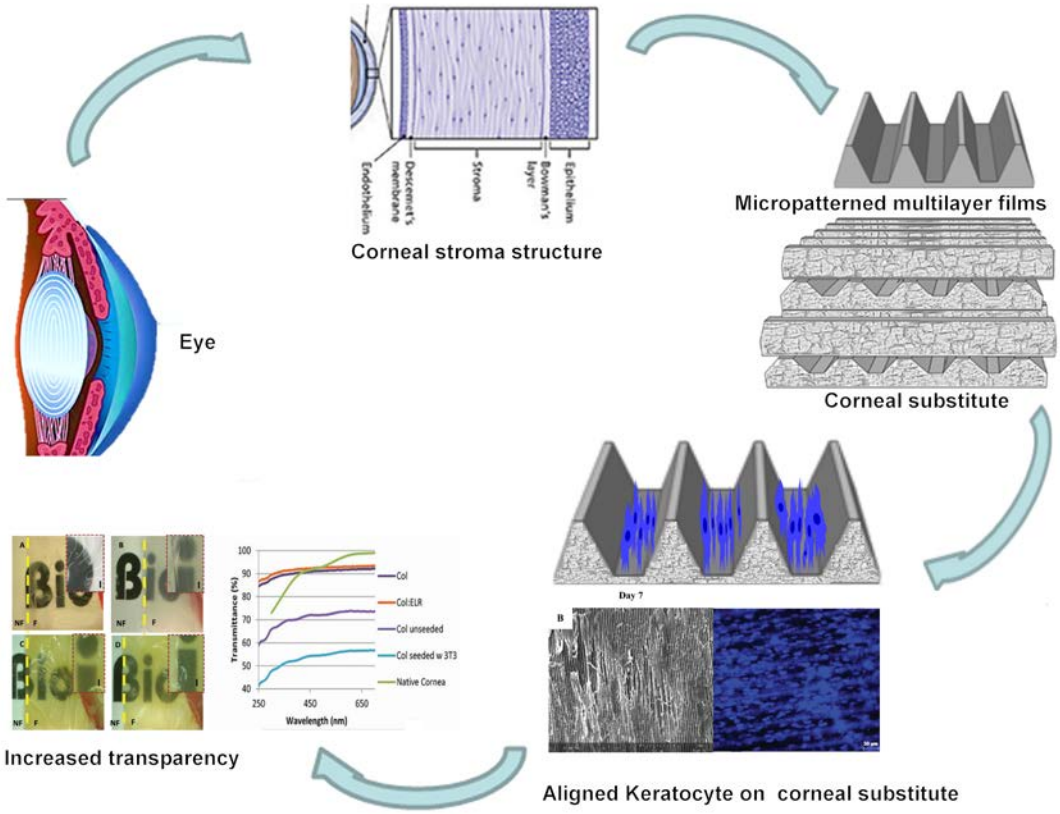

Figure 7. Schematic representation of the ELR-collagen corneal substitute design, production and biofunctional assays. Adapted from ref[149]

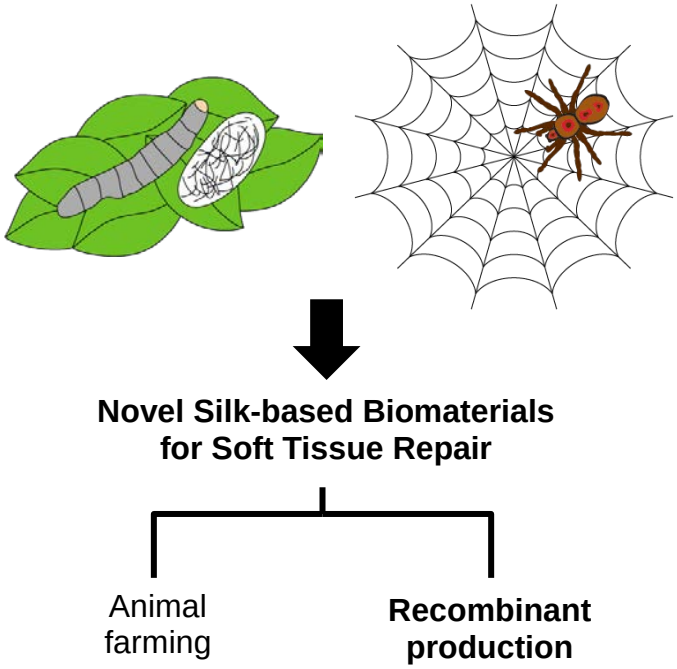

Figure 8. Schematic representation of the engineering novel silk-based biomaterials.

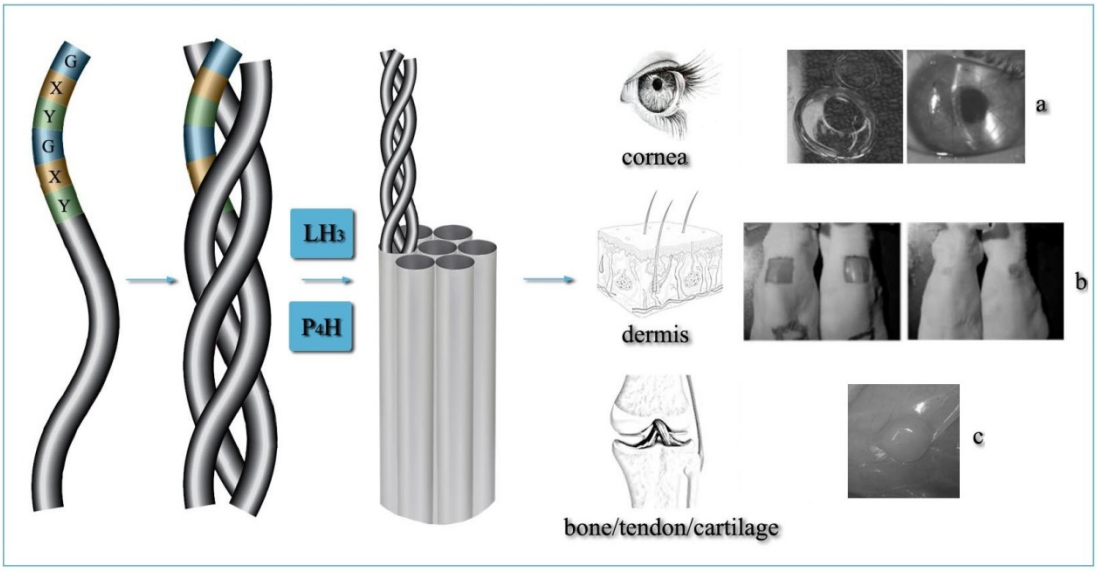

Figure 9. Collagen formation from a single α-chain given by G-X-Y amino acid sequences, to the attainment of stable fibrils, conferring various tissues flexibility and great tensile strength; examples of collagen-based scaffolds (a) Reproduced with permission[222]; (b) Reproduced with permission[218a]; (c) Reproduced with permission[220]

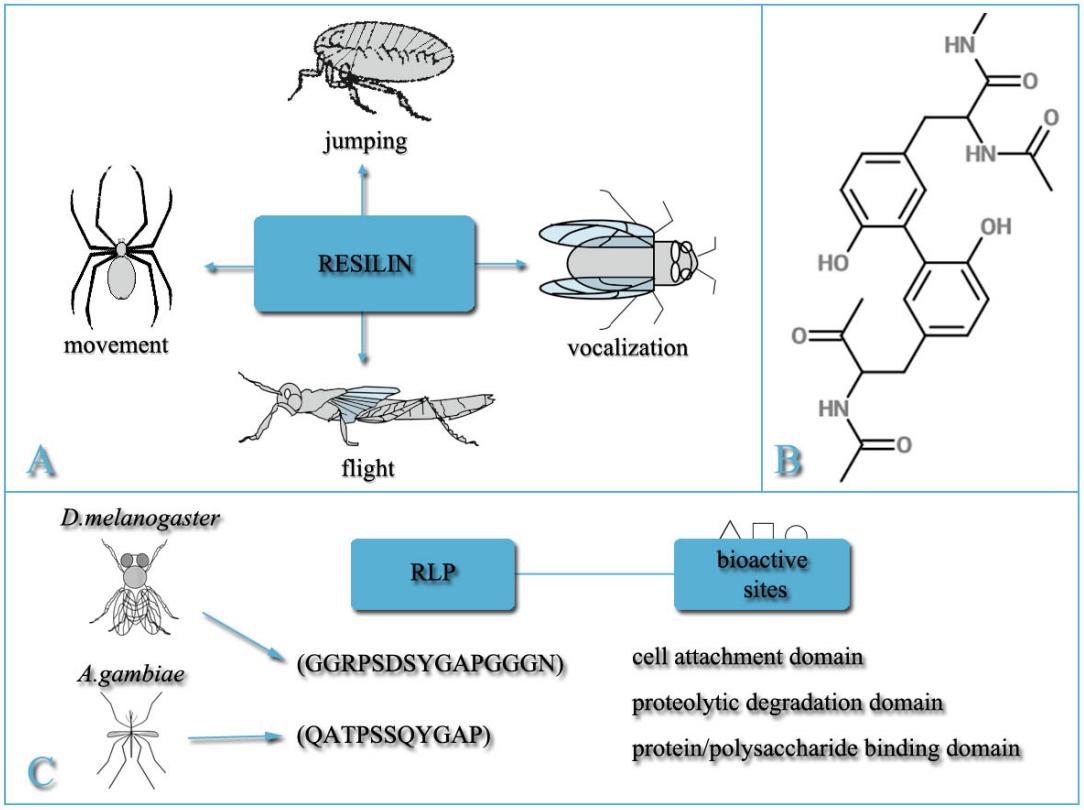

Figure 10. (a) Animal behaviors needing high resilience and elasticity are favored by the presence of resilin in a specific region of the cuticle; (B) di-tyrosine bonds present in the supramolecular arrangement; (C) frequent resilin-like domains present in RLPs, inspired by sequences found in *D. melanogaster* and *A. gambiae*, combined with various bioactive domains

Table 1. Recombinant tropoelastin devices fabricated for soft tissue regeneration. Chemical and physical methods for crosslinking tropoelastin and tropoelastin´s blends and biological *in vitro* and *in vivo* assays performed.

CROSSLINKING METHODS FOR TROPOELASTIN AND TROPOELASTINS´ BLENDS

CHEMICAL METHODS				
MATERIAL	**CROSSLINKING METHOD**	***In vitro* cell type**	***In vivo* aninal model**	**REF.**
TROPOELASTIN	Bis(sulfosuccinimidyl) suberate	Human dermal fibroblasts	Male guinea pigs	[44]
		Primary human dermal fibroblast	-	[46]
		Baboon carotid artery smooth muscle cells	-	[66]
		Porcine bone marrow derived endothelial outgrowth cells	-	[67]
	Glutaraldehyde/glutaraldehyde vapors	Human skin fibroblasts	Female & male BALB/c mice	[45]
		Primary human dermal fibroblast	-	[46]
		Human fibroblasts and Human Umbilical Vein Endothelial Cells	-	[65]
	1,6-diisohexanecyanate (HDMI)	Human fibroblasts and Human Umbilical Vein Endothelial Cells	-	[65]
		Vascular smooth muscle cells	-	[69]
TROPOELASTIN BLENDS				
α-elastin	Glutaraldehyde	Human skin fibroblast cells	-	[48]
Heparin	Bis(sulfosuccinimidyl)suberate	Human dermal fibroblasts	-	[49]

Dermatan sulfate	Bis(sulfosuccinimidyl)suberate	Human dermal fibroblasts	-	[49]
Ovine type I collagen	Glutaraldehyde	Human dermal fibroblasts	-	[50]
Bobyx mori silkworm silk	Glutaraldehyde	Human dermal fibroblast	Male IL-1b mice	[51]
Type I collagen-chondroitin-6-sulfate	Glutaraldehyde	Primary human dermal fibroblasts	Female & male Balb/c mice Female pig	[57]
Polycaprolactone	Glutaraldehyde	Human umbilical vein endothelial cells	Male New Zealand White Rabbits	[73]
PHYSICAL METHODS				
MATERIAL	**CROSSLINKING METHOD**	***In vitro* cell type**	***In vivo* aninal model**	**REF.**
TROPOELASTIN TROPOELASTIN BLENDS	Temperature under alkaline conditions	Human dermal fibroblast	Female Sprague–Dawley rats	[47]
Methacrylate	UV irradiation	Immortalized green fluorescent protein (GFP)-expressing human umbilical vein endothelial cells NIH 3T3 mouse embryonic fibroblast cells	-	[74]
		Sprague Dawley rats cardiomyocyte	-	[75]
		Sprague Dawley rats primary cardiomyocyte	-	[76]
Bombyx mori silkworm	Autoclaving	Primary cortical neurons from embryonic Sprague Dawley rats	-	[77]

Table 2 Recombinant tropoelastin devices for soft tissue regeneration obtained by surface modification. Surfaces are modified and biofunctionalized with tropoelastin using different activation methods and biological *In vitro* and *in vivo* experiments are listed.

SURFACE	SURFACE TREATMENT	*In vitro* cell type	*In vivo* animal model	REF.
Polytetrafluoroethylene	With/without plasma treatment	Human dermal fibroblast	Male & female BALB/c mice	[52]
		Human dermal fibroblast	-	[53]
Silicon	With/without plasma treatment	Human dermal fibroblast	-	[54]
Polyurethane co-polymer	With/without plasma treatment	Human dermal fibroblast	-	[55]
		Heparinized whole blood		
Polypyrrole	With/without plasma treatment	Human dermal fibroblast	-	[56]
Metal coronary stents	Plasma treatment	Human umbilical vein endothelial cells	-	[70]
		Human non-smoker whole blood		
Metallic surface	Acetylene intermediate	Human umbilical vein endothelial cells	-	[71]
Polystyrene	Plasma treatment	Human dermal fibroblasts	-	[72]
		Male human non-smoker whole blood		

Table 3. Tropoelastin-derived exons used to develop soft tissue scaffolds, *in vitro* and *in vivo* experiments performed with each material are listed.

TROPOELASTIN-DERIVED EXONS

EXON SEQUENCE	CROSSLINKING METHOD	*In vitro* cell culture	*In vivo* animal model	REF.
Exon 23, 24	Bacterial Transglutaminase	Human umbilical vein endothelial cells	-	[80]
Exon 24	-	EaHy926 (Human endothelial) A549 (Adenocarcinomic human alveolar basal epithelial cells) MCF-7 (Human breast adenocarcinoma cell)	-	[81a]
Exon 23 and 24	-	EA.Hy926 (Human endothelial)	-	[81b]
Exon 23 and 24	-	SH-SY5Y cells (Human neuroblastoma)	-	[82]
Exon 23 and 24	-	HepG2 cells (Hepatocellular carcinoma)	-	[83]
Exon 23 and 24	Microbial transglutaminase from	HepG2 cells (Hepatocellular carcinoma)	-	[84]

	Streptomyces mobaraensisas	MCF-7 (Human breast adenocarcinoma cell)			
Exon 23 and 24	-	H9c2 cells (Rat myoblast)	-	[79]	
Exon 23 and 24 blended with collagen	-	Murine myoblast	-	[85]	

Table 4. Surface modification with tropoelastin-derived exons and *in vitro* and *in vivo experiments* performed with coated materials.

EXON SEQUENCE	MODIFIED SURFACE	BINDING METHOD	*In vitro* cell culture	*In vivo* animal model	REF.
Exons 20, 21, 23 and 24	Tefzel™ (Ethylene TetrafluoroEthylene) Mylar™ (Polyester) Corethane™ (Polyurethanes)	Physical adsorption	Human whole blood	Male New Zeeland rabbits	[86]
Exon 20, 21, 23 and 24	Mylar™ (Polyester)	Physical adsorption	Human whole blood	-	[87]
Exon 20, 21, 23 and 24	Polycarbonate urethane	Chemical linking Physical adsorption	Human whole blood Human umbilical vein endothelial cell	-	[88]
Exon 20, 21, 23 and 24	Mylar™ (Polyester) Low Density Polyestyrene	Physical adsorption	Human whole blood	-	[89]
Exon 20, 21, 23 and 24	Polycarbonate-urethane base polymer	Chemical linking	Human umbilical vein smooth muscle cells	-	[90]
Exon 20, 21, 23 and 24	Tecoflex™ (Polyether polyurethanes)	Physical adsorption	Human vocal fold fibroblast	-	[91]

Table 5. Development of recombinant silk-like proteins (SLPs) and their potential use in soft tissue repair.

Silk amino acid sequence	**Natural protein**	**Transgenic organism**	**Bioactivity**	***In vitro* results**	***In vivo* applications**	**Ref.**
$(GAGAGS)_n$	*B. mori* silk fibroin	No	None	Study of secondary	None	[162]
$(Ala)_{6-12}$	*S. cynthia ricini*			structure formarion		
$(GGA)_4$	dragline spidroin			by C^{13} NMR.		
	S. cynthia ricini			Stable structures		
	silk fibroin			found		
$(GAGAGS)_6$	*B. mori* silk fibroin	No	None	None	Potential use as a	[163]
YGGLGSQG	*N. clavipes*				mesh in biomedical	
AGRG	dragline spidroin				applications	
$(GAGAGS)_6$	*B. mori silk* fibroin	No	RGD	VERO, NHDF	Potential use in	[164]
YGGLGSQG	*N. clavipes*			cells. Enhanced	tissue regeneration	
AGRG	dragline spidroin			cell adhesion		
Natural silk	*B. mori* silk fibroin	Yes	RGD and	3T3 mouse	None	[165]
			Coll-F	fibroblasts. Better		
				cell adhesion in		
				RGD-silk		

Natural silk	*B. mori* silk fibroin	Yes	RGD and YIGSR	3T3, TDK2 cells. Best cell adhesion and migration in YIGSR	Coating of natural silk vascular grafts implanted in rat abdominal aorta. Enhanced cell migration in YIGSR	[167]

Table 6. Experimental results of the application of different recombinant silk-elastin-like proteins (SELPs).

Elastin domain	Application	Results	Ref.
12 different pentapeptides	Screening of thermoresponsive and adhesive properties	Several SELPs suitable for tissue engineering and development of a high-throughput SELP screening strategy	[169]
VPAVG (SELP-59A)	Yield production enhancement	4.3 g/L yield (50-fold higher than previous works)	[170]
Hydrophilic VPGEG Hydrophobic VPGIG	Physical hydrogel formation	Fibers observed by TEM and AFM and 10kPa storage modulus hydrogels	[171]
VPGKG (SELP-47K)	Physical and chemical hydrogels formation (methanol and glutaraldehyde treated, respectively)	High tensile strength and resilience	[172]
	Electrospun fibers (methanol and glutaraldehyde treated)	High tensile strength	[173]
	In vitro encapsulation of hMSCs	No cytotoxicity and *in vitro* chondrogenesis in combination with TGF-β3	[174]
	Physical and chemical thin films (methanol and glutaraldehyde treated, respectively) for ophtalmic applications	Highly transparent films suitable for lenses development	[176]
VPAVG (SELP-1020A and 59A)	Electrospun fibers for *in vitro* cell cultures	Good cytocompatibility and cell proliferation	[175]

VPGKG (PS2E8K)	Chemically cross-linked to RPSB, a light inducible moiety	Birefringence induction by laser irradiation, suggesting light response and potential ophtalmic applications	[177]
VPGKG (SELP-815K)	Addition of protease (MMPs) sensitive domains to enhance biodegradation	Complete degradation of the SELP in solution after addition of MMPs	[178]

Table 7. Experimental results of the application of different recombinant silk-collagen-like proteins (SCLPs).

Building blocks	Application	Results	Ref.
CSSC SCCS Silk domain GAGAGAGE	Physical hydrogel and fiber formation	Stable hydrogels of 10kPa storage modulus and fiber observation	[179] [180]
$C_2S_{48}C_2$ Silk domain GAGAGAGH	Physical hydrogel and fiber formation	Stable hydrogels. No cytotoxicity but low cell spreading of MSCs cultured on hydrogels	[159b]

Table 8. Applications of recombinant spidroin derived proteins

Spidroin	Bioactivity	*In vitro* results	*In vivo* applications	Ref.
eADF4(C16) derived from MaSp of *A. diadematus*	RGD fibronectin derived cell adhesion sequence	Enhanced cell attachment of 3T3 mouse fibroblasts	Potential use in skin tissue engineering applications	[194]
rS1/9 derived from MaSp1 of *N. clavipes*	None	Good biocompatibility, cell adhesion and migration of 3T3 cells in 3-D scaffolds	Promotes ingrowth of fibrous, nerve and adipose tissue and angiogenesis. Induced growth of soft connective tissue in rat injured bone	[197] [198] [199]

4RepCT derived from MaSp1 of *E. australis*	IKVAV laminin derived neural cell adhesion sequence	Enhanced cell attachment and spreading of human Schwann cells	Potential use in the treatment of glial cell related diseases	[196]
	None	None	Fibroblast ingrowth and angiogenesis when implanted subcutaneusly in rats	[200]
		Differentiation of neural stem cells (NSCs) to neurons, astrocytes and oligodendrocytes	None	[201]
	None Albumin Binding Domain (ABD) IgG binding motifs Biotin binding domain (M4)	Selective binding of the target molecules from plasma and target release after proteolytic cleavage	Potential addition of bioactivities to a previously reported biocompatible material	[202]

Table 9. Summary of recombinant collagen-based materials recently developed

MATERIAL	CROSSLINKING METHOD	*In vitro* cell type	*In vivo* animal model	REFERENCE
CHEMICAL METHODS				
COLLAGEN TYPE I	EDC (1-ethyl-3-3-dimethylaminopropyl carbodiimide hydrochloride)	human dermal fibroblasts primary human endothelial cells human epidermal keratinocytes THP-1 cells	-	[216]
COLLAGEN TYPE I COLLAGEN TYPE III	EDC (1-ethyl-3-3-dimethylaminopropyl carbodiimide hydrochloride)	human corneal epithelial cells dorsal root ganglia from chick embryos	Male Sprague-Dawley rat pig human	[221a] [221b] [221c] [223]
BACTERIAL COLLAGEN (from *S.pyrogens*)	GA (glutaraldehyde)	endothelial cells mouse and human lung fibroblasts	SJL/J mice Arc albino mice	[224]
PHYSICAL METHODS				
COLLAGEN TYPE I	Temperature	-	Male Sprague-Dowley Rat Pig	[217]
COLLAGEN TYPE II	Temperature	Bovine chondrocytes	Nude mice	[220]
COLLAGEN TYPE VII	Gene delivery	human foreskin fibroblasts human dermal fibroblasts human keratinocytes	Athymic nude mice	[218a] [218b] [218c]