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Review

Fibrous protein-based hydrogels for cell encapsulation

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ABSTRACT

Tissue scaffolds play a vital role in tissue engineering by providing a native tissue-mimicking environment for cells, with the aim to promote cell proliferation, proper cell differentiation, and regeneration. To better mimic the microenvironment of native tissues, novel techniques and materials have emerged in recent years. Among them, hydrogels formed from self-assembled biopolymer networks are particularly interesting. This paper reviews the fabrication and use of fibrous protein-based hydrogels, with an emphasis on silk, keratin elastin and resilin proteins. Hydrogels formed by these proteins show close structural, chemical and mechanical similarities with the extracellular matrix, typically good biological compatibility, and they can trigger specific cellular responses. In addition, these hydrogels can be degraded in the body by proteolytic enzymes. For these reasons, fibrous protein hydrogels are one of the most versatile materials for tissue engineering.

1. Introduction

Tissue engineering aims to restore or regenerate damaged tissue by combining cells derived from a patient biopsy with engineered biomaterial scaffolds that provide a temporary extracellular matrix for the cells to attach and to proliferate [1]. At the same time, these scaffolds may also serve as carriers for growth factors, other enzymes, or drugs [1–3]. Two main strategies for combining cells with biomaterial scaffolds can be distinguished: cells are either seeded onto prefabricated porous scaffolds, or cells are encapsulated during scaffold formation [4,5] (Fig. 1). In both cases, tissue engineering aims to replicate the anatomical structure and function of the specific tissue or organ to be replaced or repaired [6]. To do so, different types of materials have been develop or used, including foam structures, microsphere scaffold, hydrogels, fibrous structures, and polymer-bioceramic composite scaffold.

In recent years, fibrous protein-based hydrogels have become popular [7,8] due to their structural and mechanical similarity with the native extracellular matrix (ECM) and their relatively simple processability under mild, cell-compatible conditions [9]. These hydrogels consist of networks of hydrophilic biopolymers that have the ability to bind large quantities of water, which together with osmotic forces prevents the network from collapsing [10]. The 3-dimensional structure of the biopolymer networks is stabilized either by chemical crosslinking (covalent and ionic) or physical crosslinking (entanglements, crystallites, and hydrogen bonds). Hydrogels are attractive as a cell matrix and as connective tissue substitutes due to their ability to form mechanically stable, porous, hydrated 3D polymer networks [11–13] that facilitate the transport of nutrients and metabolic waste products [10,14–18]. Moreover, hydrogels can be formed *in vivo* and are therefore compatible with minimally invasive surgery methods: a liquid precursor solution together with suspended cells can be injected at the site of interest, and the polymerization process leading to the hydrogel formation takes place in the body [10,18–22].

Nature offers an abundance of structural building blocks for hydrogel fabrication that can be derived from mechanically stable protein biopolymers: silk fibroin from spider webs; collagen from skin, bone and tendons; keratin from wool or hair; elastin from elastic tissues; fibrin from blood clots; resilin from insect tendons. Each of these biological materials shows unique properties unmatched by known technical materials.

The present paper will give an overview of the basic fabrication principles and properties of biopolymer-based hydrogels for tissue engineering. In particular silk fibroin, keratin, elastin and resilin will be explored in detail. However, we will not discuss collagen and fibrin here as several excellent reviews are already available [9,23–34].

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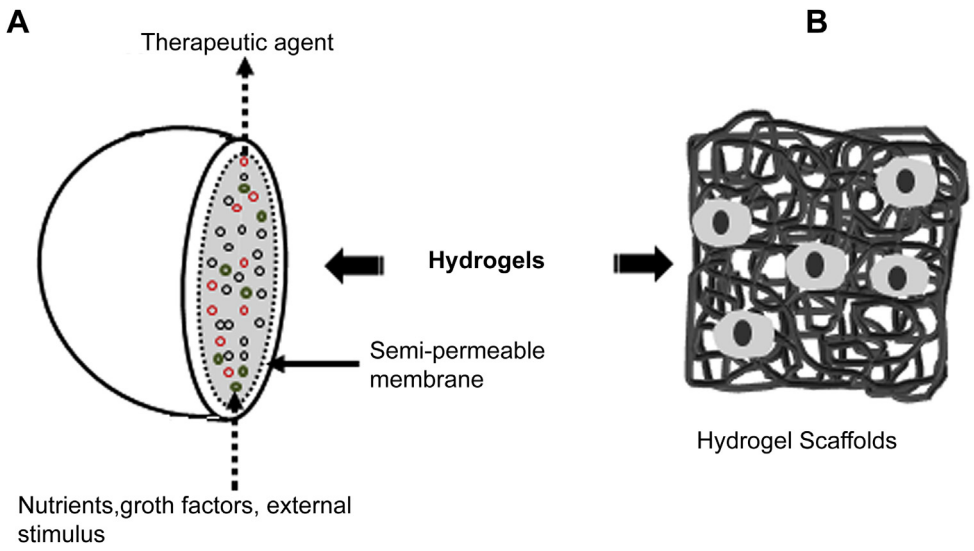

Fig. 1. Hydrogels and tissue engineering. Schematic diagram of the use of hydrogels in microencapsulation (A) and in tissue-engineering scaffold (B).

2. Biopolymer-gels based on fibrous proteins: general considerations

A wide range of natural materials can form non-cytotoxic polymeric hydrogels [21,35]. These natural polymers can be classified into proteins (i.e., silk, collagen, gelatin, fibrinogen, elastin, keratin, actin, and myosin), polysaccharides (i.e., cellulose, amylose, dextran, chitin, and glycosaminoglycan's), or polynucleotides (i.e., DNA, RNA) [36]. In particular protein-based hydrogels can mimic features of the extracellular matrix and thus have the potential to promote the migration, growth and organization of cells during tissue regeneration and wound healing. Protein-based hydrogels are therefore also often suitable materials for cell encapsulation [37–39].

Fibrous proteins, such as collagens, elastins, silks, and keratins are characterized by highly repetitive amino acid sequences that give these proteins unique mechanical and architectural properties. These repetitive amino acid sequences result in the formation of relatively homogeneous secondary structures (e.g. β-pleated sheets, coiled coils, or triple helices), which in turn promote the spontaneous polymerization of protein monomers that self-assemble into structurally interesting hierarchical materials [40]. Furthermore, fibrous proteins are attractive materials for designing bioactive scaffolds, because cells can recognize and bind to specific sites within proteins, as well as secrete enzymes that may degrade specific amino acid sequences [41]. Table 1 summarizes the use of protein-based hydrogel materials for tissue engineering applications and as cell culture scaffolds.

3. Silk fibroin

Silks are naturally occurring protein polymers that can be found in a wide diversity of insects and spiders. The most widely used and characterized silks are from the domesticated silkworm (*Bombyx mori*) and from some spiders (*Nephila clavipes* and *Araneus diadematus*) [42,43]. Silk proteins are usually produced within

Table 1
Protein-based hydrogels and applications.

Protein	Tissue engineering application	Encapsulated/seeded cell types	Animal model	References
Silk fibroin (SF)	Bone regeneration	Osteoblasts (MG63)	Rabbit distal femurs	[89,93]
	Bone/cartilage	Human bone marrow derived mesenchymal cells	–	[91,92]
	Bone regeneration	–	Rabbits with 6 maxillary sinuses	[94]
	Bone regeneration	Human peripheral blood mononuclear cells	–	[95,96]
Keratin	Wound healing/tissue regeneration	Microvascular endothelial cells and keratinocytes	Rats with wounded on either side of the dorsal midline	[154,155]
	Regeneration of peripheral nerves	Schwann cells	Mice and rats with peripheral nerve injury or critical size nerve defect	[156,158]
	Soft tissue regeneration	L929 murine fibroblasts	–	[160]
	Regeneration of sciatic nerve injury	Schwann cells	Rats with sciatic nerve injury	[159]
	Wound healing/tissue regeneration	L929 murine fibroblasts and vascular smooth muscle cells	–	[261]
	Parkinson's disease	Hepatocytes neurospheres forming cells		[163]
Elastin	Elastic tissue	M1 murine epithelial cells and human fibrosarcoma cells	Guinea pigs	[207]
	Vascular tissue	Porcine vascular smooth muscle cells	–	[216]
	Cartilage	NIH-3T3 fibroblasts	–	[219]
	Topical/dermal application	Human skin fibroblasts	Sprague–Dawley rats	[220]
Resilin	Vocal cord	NHT-3T3 fibroblast	–	[255]
	Cartilage	Human mesenchymal stem cells	–	[257]
	Cartilage	Primary human mesenchymal cells from bone marrow	–	[258]
	Cardiovascular tissue	Human aortic adventitial fibroblasts	–	[259]

specialized glands in these animals after biosynthesis in epithelial cells that line the glands, followed by secretion into the lumen of the gland prior to spinning into fibers [44–46]. Spider silk is lightweight, extremely strong and elastic, and exhibits mechanical properties comparable to the best synthetic fibers produced by modern technology [47,48]. Spider silk is spun near ambient temperatures and pressures using water as the solvent, which makes them also environmentally safe and non-cytotoxic [48]. However, it is not possible to maintain domesticated spiders to produce economically meaningful amounts of silk with standardized properties. Therefore, the attention has been turned to silk fibroin from the silkworm *B. mori*, which has been used commercially as biomedical sutures for decades and in textile production for centuries [49].

The protein from *B. mori* is characterized by two main protein components, sericins, the water-soluble glue-like proteins that bind the fibroin fibers together, and fibroin, the structural protein of silk fibers that is normally used to produce biomaterials. Fibroin is typically a dimer composed of a light chain (≈26 kDa) and a heavy chain (≈390 kDa), which are present in a 1:1 ratio and are linked by a single disulfide bond [50]. In a silk fiber, the fibroins are coated with a family of hydrophilic sericins (20–310 kDa) that account for 25% of the silk cocoons mass [50–52]. Sericins consist mainly of glycine (44%), alanine (29%) and serine (11%) [53].

The crystalline domains of silk fibroin contains glycine-X repeats, with X being alanine, serine, threonine and valine [54]. The protein conformation of silk fibroin in the solid state can assume two polymorphs, the glandular state prior to crystallization/spinning (silk I), and the spin silk state with a β-sheet secondary structure (silk II) [55,56]. The silk I structure is water soluble and, upon exposure to heat, organic solvents, or physical treatments, can be easily converted to silk II structure, which is water insoluble but can be dissolved by several chaotropes [57,58]. When the methyl groups and the hydrogen groups from the opposing β-sheets are interacting, they form anti-parallel β-sheet stacks (silk II). Strong hydrogen bonds (inter- and intra-chain) and van der Waals interactions generate a structure that is thermodynamically stable (Fig. 2) [50,59,60]. Such structural changes of the fibroin from a disordered state in solution to a β-sheet-rich conformation has been confirmed by FTIR and circular dichroism measurements over a range of fibroin concentrations, temperatures, and pH values [61].

Fibroin has been widely used to produce materials for medical applications. Typically, the fibroin is extracted from the silkworm cocoon by removal of the sericin [62] and is then purified. There are several methods to extract and purify silk fibroin protein. One of the most widely used procedures for the removal of sericin ("degumming") is sodium carbonate boiling and/or autoclaving. The fibroin is then extracted from the degummed silk by dissolving it in a concentrated solution of lithium bromide [63–71] or sometimes in a ternary solvent system of calcium chloride/ethanol/water [72–78]. After evaporation of the solvent, the fibroin can be further purified by dissolving in 1,1,1,3,3,3-hexafluoro-2-propanol [75,79–81] and formic acid [53,73,74,82]. The relative ease with which silk proteins can be processed in water or various solvents to form gels, fibers or sponges with different chemical functionalization, together with the excellent biocompatibility, enzymatic degradability and mechanical resilience, makes these proteins interesting candidates for many biomedical applications [83,84].

Hydrogels can be formed from reconstituted silk fibroin (SF) solution by a sol–gel transition in the presence of acid, ions, or other additives [85–90]. Other factors such as temperature, SF concentration, and pH significantly affect the gelation process. Generally, gelation time decreases with an increase in SF concentration, temperature, and concentration of additives like Ca^{2+}, glycerol and poly(ethylene oxide), or with a decrease in pH [87,88]. The pore size of the hydrogel decreases, and the mechanical strength and stiffness increases, with higher SF concentration or gelation temperature [87].

To better control SF gelation, high energy ultrasonication has been used [91]. Depending on the sonication parameters, including power output and time, along with silk fibroin concentration, gelation time can be controlled from minutes to hours, allowing for the post-sonication addition of cells prior to the final gelation. For example, human bone marrow derived mesenchymal stem cells (hMSCs) have been mixed into 4% SF solutions after sonication, followed by rapid gelation. Subsequently, the cells proliferated in the gels over 21 days [91].

Ultrasonication has been shown to initiate the formation of β-sheets by altering the hydrophobic hydration, thus accelerating the formation of physical crosslinks for better gel stabilization. By contrast, vortexing SF hydrogels slows down the gelation kinetics from minutes to hours. Vortexing low-viscosity silk solutions lead

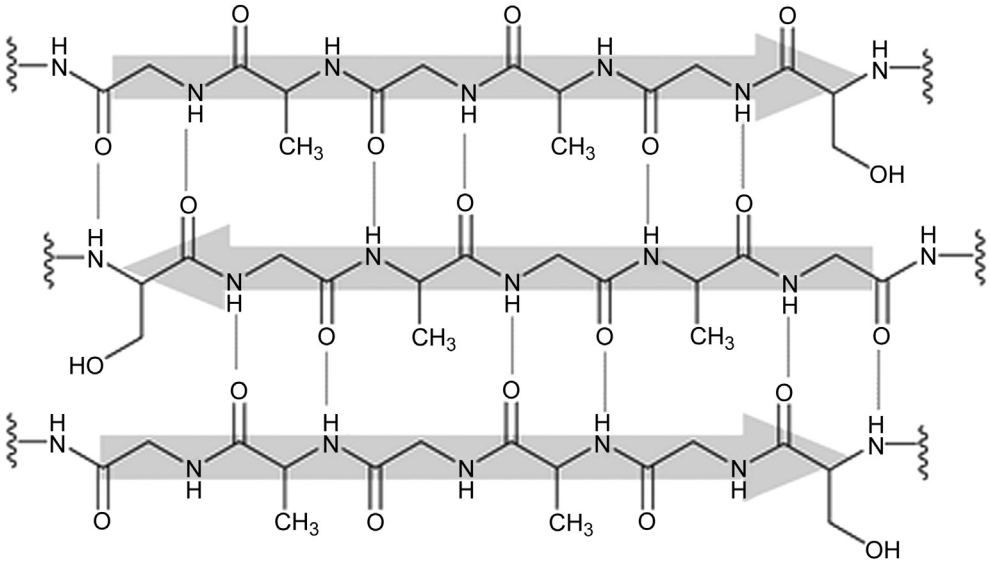

Fig. 2. Silk amino acid repeat units that self-assemble into an anti-parallel β-sheet structure. Reprinted from ref. [60]; Copyright 2010, with permission from Royal Society of Chemistry.

to an orders-of-magnitude increase in the complex shear modulus, G*, and the formation of rigid hydrogels ($G^* \approx 70$ kPa for 5.2 wt% protein concentration). Changing the vortex time, assembly temperature and/or protein concentration thus gives great flexibility to optimize the timeframe for cell encapsulation [92]. In addition, the stiffness of preformed hydrogels recovered quickly following injection through a needle, allowing these hydrogels to be used for injectable cell delivery scaffolds.

SF hydrogels have been tested for their potential to promote tissue repair [89]. For example, the repair of confined, critical-sized cancelous bone defects has been demonstrated in a rabbit model using SF hydrogels obtained by treating a fibroin-water solution with glycerol (Glygel), or keeping the solution at 4 °C (Thermgel). The data showed that the Glycel substrate promoted osteoblast proliferation, whereas the Thermgel substrate mainly favored osteoblast activity and differentiation [89].

An osteoblast cell line (MG63) cultured *in vitro* in an injectable SF hydrogel matrix showed increased TGF β1 production, a cytokine that controls cell proliferation and differentiation [93]. The same injectable SF hydrogel matrix was also studied *in vivo* in a rabbit femur model and was shown to promote the healing of critical size defects of the trabecular bone through enhanced bone remodeling and maturation [93].

In order to further enhance the osteogenic features of sonication-induced SF hydrogels, vascular endothelial growth factor ($VEGF_{165}$) and bone morphogenic protein-2 (BMP-2) have been added, which are key regulators of angiogenesis and osteogenesis during bone regeneration [94]. Such functionalized gels were shown to promote bone regeneration and height maintenance in a rabbit sinus floor elevation model [94].

In another *in vitro* study, injectable scaffolds were prepared by lyophilizing hydrogels obtained from a 12% (w/v) SF aqueous solution at 4 °C (thermgel). The cytocompatibility of the hydrogel was confirmed by good adhesion, survival and proliferation of a 3D culture of human peripheral blood mononuclear cells (hMNCs) over a time course of 3 weeks [95].

A cytocompatible method for *in situ* human hMSC encapsulation in charged or neutral SF-based hydrogels has recently been reported. Silk aqueous solutions were mixed with silk-poly-L-lysine or silk-poly-L-glutamate, and hydrogels formation was controlled by ultrasonication [96]. Interestingly, silk ionomers exhibited different effects on stem cells differentiation: fibroin/poly-L-lysine hydrogels enhanced osteogenesis of hMSCs, inducing differentiation towards an osteogenic lineage even in the absence of osteogenic induction supplements, while also inhibiting adipogenesis. By contrast, fibroin/poly-L-glutamate hydrogels supported the osteogenic and adipogenic differentiation of hMSCs only when the cells were cultured in the presence of the respective induction supplements [96].

Another interesting approach for bone tissue engineering is the mineralization of fibroin-derived polypeptides (FDPs) [97]. FDPs can be obtained through the chymotryptic separation of the hydrophobic crystalline (Cp) fractions and the hydrophilic electronegative amorphous (Cs) fractions of fibroin [97]. Mineralization of SF can be achieved by immersion in simulated body fluid (SBF) and is dictated exclusively by the electronegative amino-acidic sequences of the Cs fractions. Cs fractions of fibroin up to 10 wt% have also been incorporated into dense collagen gels, and within 6 h of SBF treatment, apatite was formed in these collagen-Cs hybrid gels. After 7 days of SBF treatment, carbonated hydroxyapatite crystals formed, resulting in a nine-fold increase in the compressive modulus of the hydrogel [97].

Among genetically engineered biomaterials, the family of silk-elastin like protein polymers (SELPs) [98] is particularly promising. By combining the silk-like and elastin-like blocks in various ratios and sequences, it is possible to produce a variety of biomaterials with diverse properties. Structurally, SELPs consist of repeats of silk-like (Gly–Ala–Gly–Ala–Gly–Ser) and elastin-like (Gly–Val–Gly–Val–Pro) peptide blocks, and with the appropriate sequence and composition, they undergo an irreversible sol–gel transition, which is accelerated at body temperature compared to room temperature [99–103]. The formation of hydrogen bonds between the silk-like blocks is thought to be the primary driving force behind gelation of SELPs that serve as points of contact (crosslinks) between the polymer chains. By contrast, the periodic inclusion of elastin-like blocks increases the flexibility and water solubility of the polymer. The polymeric solutions are liquid at room temperature and form a firm yet pliable hydrogel *in situ* within minutes after injection. Genetic SELP hydrogels have been explored for the controlled release of non-viral and viral genes for cancer gene therapy in a murine model of tumor breast cancer [104].

Reconstituted silk fibroin can also be blended with other biopolymers [105] like gelatin [106–108], chitosan [85,109,110], alginate [111,112] hyaluronic acid [113,114] and cellulose [115], to form hydrogels with a large range of material properties for specific tissue engineering applications.

4. Keratins

Keratins are the main constituents of skin, fur, hair, wool, claws, nails, hooves, horns, scales, beaks and feathers [116]. To generate hair, keratin proteins self-assemble into fibers in the hair follicle. Keratin production is controlled by more than 30 growth factors and cytokines [117–121]. Prior to extrusion through the skin, the keratin fiber is formed into a highly stable structure by covalent bonds, oxygen-catalyzed disulfide crosslinks, and non-covalent interactions. Crosslinking can occur between separate polypeptide chains (intermolecular) but also between different points of the same polypeptide chain (intramolecular) [122,123].

Generally, keratin fibers consist of two major morphological parts: a cortex (the inner part of the fiber) and the cuticle outer layer. The cortex comprises spindle-shaped fibrils that are separated from each other by a membrane (Fig. 3), which consists of non-keratinous proteins and lipids [124–126].

The cuticle layer comprise 10% of the total weight and is composed of overlapping, nearly rectangular scales, or sheets, that protect the cortex [124,125]. They are composed of three distinct layers: the epicuticle, which is the outer resistant surface

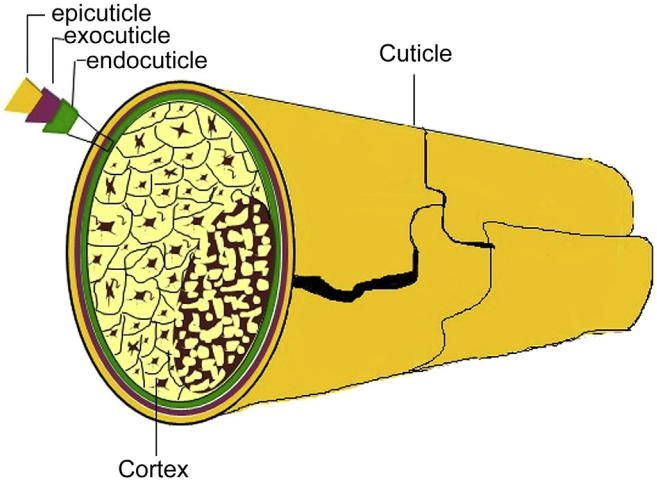

Fig. 3. Structure of the α-keratin fiber.

membrane; the exocuticle, which is subdivided into two main layers that differ in their cysteine content; and the endocuticle, which is the cuticular layer nearest to the cortex [122] (Fig. 3).

Keratins can be subdivided into α, β and γ-keratins. α-keratins are intermediate filaments that are located mainly in the fiber cortex. They constitute approximately 50 wt% of the total protein of a fiber, have an average molecular mass in the range of 40–60 kDa, are low in sulfur, partly crystalline, and form an α-helical secondary structure [122]. α-keratins self-assemble into long filamentous fibers that can be stretched considerably without rupturing [127].

γ-keratins form the matrix in between the α-keratin filaments. They constitute approximately 25 wt% of the total protein of a fiber, have a low molecular mass of around 15 kDa, they are globular and are noted for their high content in cysteine, glycine and tyrosine residues. γ-keratins function primarily as disulfide crosslinkers that hold the α-keratin fibers together and give rise to the high mechanical strength, inertness and rigidity of the cortical superstructure of hair and wool [126]. γ-keratins can be further subdivided into 3 groups: (i) high sulfur proteins (HSPs) and (ii) ultra-high sulfur proteins (UHSPs), depending on their cysteine content, have a molecular mass in the range of 11–26 kDa, whereas (iii) high glycine–tyrosine proteins (HGTPs) have a molecular mass in the range of 6–9 kDa [128–130].

Finally, β-keratins are found in the cuticle. They protect the cortical filaments from physical and chemical damage and are difficult to extract [131].

Over the last years, several methods to extract keratins have been reported. Oxidative [132,133] and reductive [134–136] solvents have been used to break the disulfide crosslinks between the cysteines, which converts the keratins into their non-crosslinked form [137]. The protein mixture obtained with oxidative solvents, which convert the cysteines to cysteic acid, are referred to as keratose, whereas the protein mixture obtained with reductive solvents, which leave the cysteines intact and thus ready to form new crossbridges, are referred to as kerateines.

The keratose obtained from oxidative extraction [132,133] with peracetic acid or hydrogen peroxide is hygroscopic, water-soluble, non-disulfide crosslinkable, and at extreme pH values is susceptible to hydrolytic degradation [138]. Biomaterials generated with keratose degrade relatively fast *in vivo*, on the order of days to weeks [138]. Conversely, biomaterials generated with kerateines from a reductive extraction procedure can persist *in vivo* for weeks to months. This is because the kerateines can be re-crosslinked through oxidative coupling of cysteine groups, and as a consequence are less soluble in water and more stable at extreme pH. The reductive extraction [134–136] is usually performed with dithiothreitol (DTT), 2-mercaptoethanol [139], or with sodium disulfite [140–142].

One of the most extensively studied properties of keratin solutions, both at the microscale [143,144] and the macroscale [145], is the ability to spontaneously self-assemble, resulting in a 3-dimensional fiber network with a reproducible fiber architecture and porosity [137]. In addition, keratin, in particular acidic α-keratin, contains cell-binding motifs, such as arginine-glycine-aspartic acid (RGD) and leucine-aspartic acid-valine (LDV). These motives are similar to those found on ECM proteins such as collagen or fibronectin, and also interact with integrins to support cellular attachment, proliferation and migration [146–150]. Like other intermediate filaments, keratins are also believed to participate in some regulatory functions that mediate cellular behavior. In the case of malignant melanoma and breast carcinoma, for instance, there is a strong indication that intracellular vimentin and keratin intermediate filaments are overexpressed, but it is currently unknown if a keratin extracellular matrix also converse specific regulatory signals e.g. for epithelial to mesenchymal transition [151,152].

Keratin-based biomaterials come in different morphologies, including films, sponges and hydrogels. The processing of keratin protein solutions into gels, films and scaffolds has first been attempted in the early 1970's [153]. The use of human hair-based keratin hydrogels as wound healing promoters was patented by Blanchard and co-workers in 1999 [154]. To produce such hydrogels, the hair is first oxidized with peracetic acid. To cleave part of the disulfide linkages, the protein suspension is heated to about 60 °C for 4 h and then cooled to room temperature, where the cleaved disulfide linkages are reduced to form cysteine groups, which solubilizes the protein even further. Therefore, a hydrogel of pure keratin can be formed by disulfide and hydrogen bonds alone, with no need of additional crosslinker agents. The authors reported that keratin hydrogels promoted the proliferation of microvascular endothelial cells, keratinocytes and fibroblasts [154]. In an *in vivo* skin wound healing assay in rats, keratin treatment accelerated the epithelialization and maturation process [154]. Since then, much work has been done to fabricate and characterize keratin-based hydrogels materials and to demonstrate their cytocompatibility and biodegradation.

Sierpinski et al. [155] and Apel et al. [156] demonstrated that keratin-based hydrogels were neuroinductive and capable of facilitating regeneration in a peripheral nerve injury model in mice. They showed that a keratin gel derived from human hair enhanced the *in vitro* activity of Schwann cells by inducing cell proliferation and migration, and by up-regulating the expression of specific genes required for important neuronal functions. When translated into a mouse tibial nerve injury model, keratin gel-filled conduits served as a neuroinductive provisional matrix that mediated axon regeneration and improved functional recovery compared to traditional nerve autografts [155]. In another study, the time course of peripheral nerve regeneration was evaluated with respect to neuromuscular recovery and nerve histomorphometry [156]. Again, keratin-based hydrogel scaffolds facilitated peripheral nerve regeneration and promoted neuromuscular recovery that was equivalent to the gold standard, sensory nerve autografts [156]. The same group has also shown that keratin hydrogels from human hair can act as a hemostatic agent in a rabbit model of lethal liver injury [157]. In comparison to other commonly used hemostats (QuickClot and HemCon bandages), the keratin hemostatic gel improved 24 h survival and performed as well, if not better, than conventional hemostats in terms of total blood loss and shock index. The keratin gel used in these experiments acted on the injury site by instigating thrombus formation and by forming a physical seal of the wound site that acted as a porous scaffold to allow cellular infiltration and granulose tissue formation [157]. Later, to support the finding that keratin hydrogel fillers have the potential to be used clinically to improve nerve repair, the authors developed a rabbit peripheral nerve defect model and assessed the effectiveness of a keratin hydrogel conduit filler [158]. They found that the use of keratin resulted in a significant improvement of electrical conduction speed compared to both empty conduits and autografts, as well as a significant improvement in amplitude recovery compared to empty conduits. Furthermore, nerves in keratin-treated conduits had a significantly greater myelin thickness than nerves in empty conduits. More recently, Van Dyke and co-workers [159] showed that keratin hydrogels provide a permissive matrix that is well tolerated and rapidly infiltrated by cells of the peripheral nervous system. In a 1 cm sciatic nerve injury model in rats at an early stage of regeneration, they studied conduits filled with keratin hydrogels and compared the cell responses to those in conduits filled with Matrigel or saline solution. Keratin hydrogels facilitated earlier migration of dedifferentiated Schwann cells from the proximal

nerve end, a faster Schwann cells differentiation, better myelin debris clearance, and decreased macrophage infiltration of the distal nerve tissue [159].

Ng and co-workers [160] showed that keratins extracted from human hair can form hydrogels by inducing polymerization with Ca^{2+}; these hydrogels exhibit highly branched and porous microarchitectures. Moreover, such keratin hydrogels are comparable to collagen hydrogels as regards fibroblast cell adhesion, proliferation and cell viability [160].

Cardamone et al. [161] performed a study involving keratin sponge/hydrogel formation from reduction versus oxidation hydrolysis of wool, and they found that the produced materials were distinctly different in appearance and behavior. Those formed by reduction hydrolysis appeared smooth and homogeneous. Those formed by oxidation hydrolysis appeared rough and inhomogeneous. Regardless of oxidation or reduction hydrolysis, keratin sponge/hydrogels showed swelling in a simulated gastric fluid but maintained their structural integrity over time. This behavior can be exploited for drug release with a dual-control kinetics for delivering higher dosages immediately after immersion, and lower dosages over prolonged time periods [162].

The use of keratin fragments in combination with other biopolymers has been reported by Iwata and co-workers [163]. The poor survival of neural stem/progenitor cells following transplantation into the brain is the major problem limiting the effect of cell-based therapies for Parkinson's disease. In this study, the authors proposed the use of a keratin-based hydrogel that can serve as a physical barrier to prevent the infiltration of inflammatory cells. These keratin-based hydrogels contain a polypeptide that promotes integrin-mediated cell adhesion. For that, they used a chimeric protein consisting of an R-helical polypeptide and a globular domain derived from recombinant laminin. This protein co-assembled with extracted keratins to form hydrogels through intermolecular coiled-coil association of R-helical segments [163]. It was found that neurosphere-forming cells adhered specifically to the keratin-based hydrogel and proliferated with a high survival rate, suggesting that this material provides a microenvironment suitable for the survival and proliferation of neural progenitor cells [163].

5. Elastin

Elastin is a protein component of the ECM and is abundant in organs that need to stretch and recoil, like blood vessels, elastic ligaments, lungs and skin [164–166]. Its total amount varies between different tissues, depending on the tissue structure and the required elastic properties [167]. For example, elastin in the elastic lamina of the arterial wall is mostly responsible for the elastic recoil after vessel expansion, and is therefore important for the regulation of blood flow and maintenance of blood pressure during the diastole [168]. In the lung, elastin is arranged as a lattice that supports the shape stability of the alveoli during tidal breathing [169]. In skin, elastin fibers are enriched in the dermis where they impart skin flexibility and extensibility [164,170].

Elastin is a protein comprised of approximately 800 amino acid residues [171,172]. It is synthesized from a ≈72 kDa precursor, tropoelastin, that is water soluble, non-glycosylated and highly hydrophobic. Tropoelastin can be further converted into the insoluble elastin polymer [173–175]. The amino acid sequence of tropoelastins from various sources (human, chick, bovine and rat) has been determined, and all were found to have a close homology at both DNA and amino acid levels [176]. The tropoelastin molecule consists of two types of domains encoded by separate exons: hydrophobic domains rich in nonpolar amino acids (glycine, valine, alanine and proline residues, which often occur in repeats of tetra-, penta-, and hexapeptides, like Gly–Val–Gly–Val–Pro, Gly–Val–Pro–Gly–Val and Gly–Val–Gly–Val–Ala–Pro), and hydrophilic domains (mainly lysine and alanine residues, which are potential involved in crosslinking domains of tropoelastin). Desmosine and isodesmosine are the two predominant crosslinks of native elastin, each involving four lysine residues, that are crosslinked by lysyl oxidase (Fig. 4) [177].

The assembly of tropoelastin into a polymeric matrix is facilitated by the elastin binding protein EBP (67 kDa) that assembles tropoelastin into a microfibrillar network seed, which serves as a scaffold for additional tropoelastin deposition [178–181]. The lysine residues are then crosslinked by lysyl oxidase and stabilize the mature insoluble fiber [167]. In the absence of lysyl oxidase, tropoelastin tends to associate with glycosaminoglycan (GAG, with negative charge) due to the presence of α-amino groups in the elastin lysine residues, which have a positive charge [182–184].

A number of biophysical properties are crucial for the biochemical/physiological role of elastin in the body, such as elasticity [185–187], glass transition temperature [188] and coacervation [176]. The elasticity of elastin is entropy-driven, thus stretching decreases the entropy of the system. Elastic recoil is induced by spontaneous return to the maximum level of entropy [189–191]. The glass transition temperature (Tg) of elastin is highly dependent on its water content [192,193]. When dehydrated, Tg is about 200 °C, and at 30% of hydration, Tg is around 30 °C [194]. Upon raising the temperature above Tg, elastin and elastin-based materials, unlike other proteins that become denatured, form ordered structures and undergo a second phase transition (coacervation, droplet formation). It has been suggested that the nonpolar

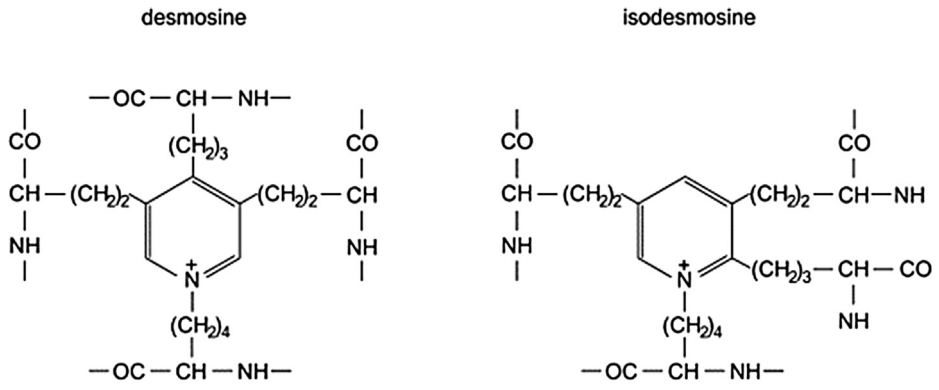

Fig. 4. Specific elastin intermolecular crosslinks include the tetrafunctional desmosine and isodesmosine formed from four Lys residues from two different tropoelastin molecules. Reprinted from ref. [177]; Copyright 2007, with permission from Elsevier.

domains of elastin are responsible for the coacervation process through an entropic mechanism of hydrophobic association due to the loss of entropy from the protein chains that is compensated by the release of water [195,196]. Thus, the coacervation temperature can be affected by protein concentration, hydrophobicity (amino acid composition and distribution), pH and ionic strength of the solvent [195,197–199]. Elastin-based polymers lacking hydrophobic domains, e.g. those based on crosslinking domains, cannot coacervate [200,201]. It was reported that synthetic peptides based on hydrophobic sequences of elastin form fibrillar structures following coacervation [202]. Similarly, tropoelastin forms an open, string-like network of fibrils upon coacervation [203]. After overnight incubation above the coacervation temperature, the loose network becomes a compact aligned fibrilar structure [203].

The use of elastin in biomaterials is particularly advantageous when its superior elasticity can be exploited. To fabricate elastin-based biomaterials, the insoluble elastin from animal tissue must first be hydrolyzed to obtain a soluble elastin protein. Soluble elastin can be obtained by hydrolysis of insoluble elastin using oxalic acid or potassium hydroxide to break the peptide bonds [177,204]. Alternatively, repeated elastin-like sequences can be produced by synthetic or recombinant methods to obtain elastin-like polypeptides (ELPs). This gives a better control over the physical and chemical properties of the resulting material. For instance, Urry [205] showed that the glass transition temperature of polymers based on the elastin-like sequence $(Val–Pro–Gly–X_{aa}–Gly)_n$ can be manipulated by the choice of the amino acid X_{aa}. More hydrophobic amino acids resulted in a lower glass transition temperature [205]. Depending on the phase transition temperature and the self-assembly behavior of elastin and ELPs, vastly different biomaterials like hydrogels [206,207], films [177], nanoparticles [208], sponges [209] and nanoporous materials, each with different mechanical properties, can be obtained [210].

To increase the mechanical strength elastin hydrogels, the protein molecules can be chemically crosslinked. Several crosslinkers such as glutaraldehyde (GA) [211,212] disuccinimidyl glutarate (DSG) [212], bis(sulfosuccinimidyl) suberate (BS3) [207,213–215], copper sulfate and pyrroloquinoline quinone (PPQ) [209], ethylene glycol diglycidyl ether (EGDE) [216], hexamethylene diisocyanate (HMDI) [217], tris-succinimidyl aminotriacetate (TSAT) [218], disuccinimidyl suberate (DSS) [214,215], and β-[tris(hydroxymethyl) phosphino] propionic acid (THPP) [219] have been used to crosslink genetically engineered ELPs [211–215,217–219], tropoelastin [207] and α-elastin [216].

However, only few of these systems were tested in combination with cells. Weiss and co-workers described the production and properties of synthetic elastin formed by chemically crosslinking recombinant human tropoelastin with BS3, allowing for the construction of elastic sponges, sheets and tubes [207]. *In vitro*, these materials support the growth and proliferation of different cell types (adherent epithelial cells including M-1 murine cells and human fibrosarcoma cells). *In vivo*, subcutaneously inserted implants in guinea pig were well tolerated [207]. Furthermore, EGDE was used to crosslink elastin based-materials from α-elastin [216]. These cross-linked materials supported vascular smooth muscle cell (porcine VSMC) adhesion but, compared to polystyrene controls, they exhibited a decreased proliferation rate [216]. In another study, lysine-containing elastin-like polypeptide hydrogels were formed in aqueous solution by crosslinking with THPP under physiological conditions in the presence of mouse NIH-3T3 fibroblasts [219]. These cells survived the crosslinking process and were viable after *in vitro* culture for 3 days [219].

High pressure CO_2 has been used as a foaming agent to produce porous elastin hydrogels [220,221] or tropoelastin/elastin hydrogels followed by chemical crosslinking [222]. Higher pressures resulted in an increase in the porosity, improvement of mechanical properties, and swelling ratio. Additionally, due to the formation of large pores in the hydrogels, cell proliferation and growth were substantially enhanced. Alternatively, it is also possible to prepare elastic biomaterials from tropoelastin without crosslinking [223]. Under alkaline conditions, tropoelastin proceeds through a sol–gel transition leading to the formation of a stable elastic hydrogel that has been shown to support the growth of human skin fibroblast. Further, *in vivo* studies with recombinant human elastin hydrogels implanted in female Sprague–Dawley rats revealed its potential as a cell support biomaterial [223].

Also hybrids of ELPs with other proteins have been studied. Particularly promising are genetically engineered silk-elastin like polymers (SELPs) composed of amino acid motifs from *B. mori* silk and elastin [98]. By suitable recombinant techniques, functional motifs can be introduced into SELPS that provide control over gelation, crosslinking, biodegradation, biorecognition and cell adhesiveness [224], with applications in drug delivery [99,102] and gene delivery systems [100,103,104]. In another study, elastin hydrogels combined with glycosaminoglycans (GAGs) were investigated [225]. GAGs were co-blended into synthetic elastin hydrogels to increase their porosity. Finally, thiolated hyaluronic acid hydrogels crosslinked with new bioinspired crosslinkers based on desmosine, the crosslinker in natural elastin, have been described [226].

6. Resilin

Resilin is an elastomeric structural protein found in insect cuticles [227,228]. This protein is one of the most stretchable elastomeric proteins currently known [229,230]. Resilin from dragonfly tendons has an elastic modulus of 600–700 kPa and can be stretched to three times its original length before breaking (resilience) [231]. However, it has been difficult to identify the primary sequence and molecular structure of resilin due to the reduced stability during purification [232]. The predicted 620 amino acid sequence of the *Drosophila* gene product CG15920 (a precursor of resilin) has a tripartite structure: the first exon with 323 amino acids (exon I reported as “pro-resilin”) consists a so-called signal peptide sequence with 17 amino acids followed by 18 pentadecapeptide repeats (GGRPSDSYGAPGGGN) [233,234]; the second exon with 62 amino acids (exon II) contains a typical cuticular chitin-binding domain [234,235]; the third exon with 235 amino acids (exon III) contains 11 tridecapeptide repeats (GYSGGRPGGQDLG) [235] (Fig. 5). Both, exon I and exon III contain tyrosine residues, which are probably involved in crosslinking, and also have a high content of continuous glycine residues, which are thought to be responsible for the high flexibility of the polypeptide chains [236,237].

Resilin behaves as an entropic elastomer consisting of unordered chains linked through stable crosslinks. Its restoring elastic force arises due to the loss in conformational entropy upon stretching [236,238,239]. Unlike elastin, the resilin sequence is dominated by hydrophilic residues, suggesting that hydrophobic interactions are minimal [240]. Exon I consists of more hydrophilic blocks and has a more flexible structure that promotes self-aggregation to fibrillar structures in water, compared to exon III, which is composed of hydrophobic and hydrophilic regions that tend to form micelles in water [241]. Exon II, which is relatively hydrophobic, forms micelles of different sizes in water [241].

Resilin has a similar upper critical solution temperature (UCST, the temperature above which coacervates disappear when heated) in water as elastin [242,243]. When heated further, however, resilin-based proteins exhibit a lower critical solution temperature

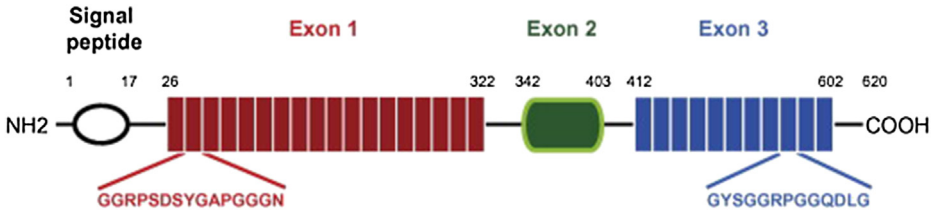

Fig. 5. The putatative resilin sequence from the Drosophila melanogaster CG15290 gene product. The sequence consists of three exons (exons I–III). Exons I and III include 18 repeats of GGRPSDSYGAPGGGN and 11 copies of GYSGGRPGGQDLG, respectively. The sequence in exon II is involved in binding of chitin. Reprinted from ref. [260]; Copyright 2013, with permission from Elsevier.

(LCST, the temperatures above which the protein becomes immiscible).

Following the identification of a resilin-like motif sequence [235], a range of resilin-like biomaterials were generated by genetic engineering of resilin-encoding genes [234,237,244,245] for applications as pH sensors and as hydrogel reservoirs for drugs, nanoparticles, enzymes, and catalysts [246]. The first genetically engineered resilin-based material that exhibited the high resilience of natural resilin was a crosslinked recombinant protein comprising exon I of the CG15920 gene (rec1-resilin) [233]. Rheological studies showed that crosslinked rec1-resilin hydrogels exhibit outstanding elasticity and a resilience (yield strain) of 92%, which exceeds that of most other polymer hydrogels [247]. Charati et al. [248] modified the resilin repeat by replacing the tyrosine with phenylalanine, in order to facilitate a photochemical crosslinking of the polypeptide [249–251]. Lysine residues have also been included outside the putative resilin repeat as additional crosslinking sites, as well as the cell-adhesion ligand RGDSP, derived from the fibronectin subunit module FN-III10 [252]. Furthermore, a matrix metalloproteinase (MMP)-sensitive sequence, (GPQG↓IWGQ, derived from the human α1(I) collagen chain and readily cleaved by MMPs), was included to promote proteolytic degradation [253]. In addition, the heparin-binding domain (CKAAKRPKAAKDKQTK) was included in the sequence for the noncovalent immobilization of heparin to allow for the sequestration and controlled release of growth factors [254]. The combination of these approaches for the generation of a resilin-like hydrogel induced cell adhesion and proliferation of mouse NIH 3T3 fibroblasts. The stiffness of this hydrogel was tuned within the range of 500 Pa to 10 kPa by changing the polypeptide concentration and crosslink ratio [255].

Qin et al. produced hydrogels from two different exons (I and III) of the resilin protein via horseradish peroxidase-mediated crosslinking [256]. They cloned the two exons (I and III) from the resilin gene in *Drosophila melanogaster*, and expressed the encoded proteins in *Escherichia coli*. The protein derived from exon I exhibited a resilience (yield strain) of 90%, in comparison to 63% for the protein derived from exon III, and therefore exon 1 is probably the more important domain for materials mimicking native resilin [256].

Consensus sequences derived from *Anopheles gambiae* (mosquito) genes have also been identified, and it was found that proteins obtained from the mosquito sequences display similar properties to rec1-resilin [238,242]. Based on these studies, a new modular protein containing repeating motifs derived from *A. gambiae* and a cell-binding domain derived from fibronectin was designed [257]. When crosslinked with tris(hydroxymethyl)phosphine, the hydrogels had a complex modulus of 22 kPa, a yield strain of 63%, and a compression modulus of 2.4 MPa, which is on the same order of magnitude as human cartilage. Human mesenchymal stem cells cultured on such resilin-based hydrogels showed good spreading behavior and had a viability of 95% after three days of culture [257].

Recently, new resilin-like polypeptides (RLPs), containing 12 repeats of the putative resilin consensus sequence and an MMP-1-sensitive domain were produced [258]. These RLP-based polypeptides exhibit largely random-coil conformation, both in solution and in hydrogels crosslinked with tris(hydroxymethyl) phosphine. Primary human mesenchymal stem cells (hMSCs) encapsulated in RLP hydrogels were viable over extended time periods [258]. Additionally, RLP–PEG hybrid hydrogels were investigated. These hydrogels are cross-linked through a Michael-type addition reaction between cysteine residues on the polypeptide and a vinyl sulfone-terminated PEG [259]. These RLP–PEG hydrogels form stable networks upon mixing of the two components. Human aortic adventitial fibroblasts were successfully encapsulated in such hydrogels [259].

7. Final remarks and future perspectives

In this paper, we reviewed the properties of biomaterials based on naturally occurring fibrous proteins. Their degradability, biocompatibility, availability, and similarity with extracellular matrix proteins make them attractive for numerous biomedical applications, in particular in tissue engineering and regenerative medicine. In addition, one of the major advantages of these proteins is that they can be specifically modified and enhanced by genetic engineering strategies to add functionality, for instance to facilitate cell adhesiveness and controlled degradability by proteases. These proteins self-assemble under specific conditions, which render them compatible with emerging technologies such as rapid prototyping and biofabrication approaches. The importance of biomaterials based on naturally occurring fibrous proteins is bound to grow substantially in the future.

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