Advanced Materials for Biomedical Engineering Applications

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Advanced Materials for Biomedical Engineering Applications

Guokui Qin[1,*] and *Xin Kai*[2]

ABSTRACT

This chapter describes mainly the smart design, structural formation, remarkable mechanical behavior and potential biomedical applications of selected natural protein-based advanced biomaterials including silk/silk-like polymers (SLPs), elastin/elastin-like polymers (ELPs), resilin/resilin-like polymers (RLPs) and other natural protein-based biopolymers. The reader will gain insight into the remarkable mechanical properties of the advanced biomaterials, the use of biotechnology to engineer the proteins and specific biomedical applications of these unique protein-based advanced biomaterials. The genetic manipulation and surface modification of these protein-based materials also reveal key relationships between structure and function in advanced biomaterials. The chapter will be involved in the interdisciplinary studies of protein-based advanced biomaterials for many potential

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applications and will be of interest to many—from graduate students getting started in their research to materials scientists and engineers. The interdisciplinary interchange is at the center of studies on protein-based natural advanced biomaterials. The information provided here including descriptions of advances in the biology, material properties, processing and biomedical applications of natural protein advanced biomaterials should be of interest to researchers in areas relevant to biomedical engineering, mechanical engineering, biology, physics, chemistry and clinical medicine.

1. Introduction

Biological materials or biomaterials can be defined as materials that are non-immunogenic, biocompatible and biodegradable, which can be functionalized with bioactive proteins and chemicals and serve the stated medical and surgical purposes (Cao and Wang 2009). Biopolymers, one group of polymeric biological materials are produced by living organisms for various functions such as information or energy storage, biocatalysts, stabilization and protection (Heim et al. 2010b). Polypeptides or proteins, polynucleic acids and polysaccharides are the examples of biopolymers with complex three-dimensional structures that are responsible for their highly specialized properties (Vendrely et al. 2008). For example, cellulose and chitin are the most abundant polysaccharides on earth and serve as important structural elements in plant cell walls and animal exoskeletons; collagen and elastin, the sequence-specific polypeptides, are synthesized by the DNA-directed templates as the main components of blood vessels, connective tissues and skins in animals and humans (Baier 1988, Eiras et al. 2010, Rusling et al. 2014). Biological synthesis and processing of biopolymers can provide important information on fundamental interactions involved in molecular recognition, self-assembly, and formation of biomaterials with well-defined architectures, features that are relevant for advanced biomaterial needs, such as for drug delivery and tissue engineering (Gagner et al. 2014, Kim 2013, Maskarinec and Tirrell 2005, Romano et al. 2011, Vendrely et al. 2008).

Proteins (known as polypeptides) are essential for all biological systems and contain the prominent secondary structures including α-helices, β-sheets, β-turns and random coils due to supramolecular interactions between side chains of amino acids, that functions in catalysis, binding, signal transduction, protection, and more (Heim et al. 2010b, Krishna and Kiick 2010, Maskarinec and Tirrell 2005, Vendrely et al. 2008). Natural structural proteins are the most versatile representatives of advanced biomaterials, such as silks (Omenetto and Kaplan 2010), elastins (Rodriguez-Cabello 2004), collagens (Chattopadhyay and Raines 2014) and keratins

(Mogosanu et al. 2014), are synthesized in higher organisms from combinations of up to 20 amino acid monomers and characterized by highly ordered domains in the materials formed from these protein polymers. Structural proteins are used for producing natural materials such as hair, connective tissue and silk, all of which show incredible and unique physical properties (Grove and Regan 2012). Because of their impressive mechanical properties, slow degradation *in vivo*, biocompatibility and versatile processing into many material formats, natural structural proteins are particularly suited for advanced biomaterials needs especially for biomedical engineering applications (Grove and Regan 2012).

This chapter describes the smart design, structural formation, remarkable mechanical behavior and potential biomedical applications for selected natural protein-based advanced biomaterials including silk/silk-like polymers (SLPs), elastin/elastin-like polymers (ELPs), resilin/resilin-like polymers (RLPs) and other natural protein-based biopolymers. The reader will gain insight into the remarkable mechanical properties of advanced biomaterials, the use of biotechnology to engineer the proteins, and specific biomedical applications of these unique protein-based advanced biomaterials. The genetic manipulation and surface modification of these protein-based materials also reveal key relationships between structure and function in advanced biomaterials. The chapter will look at the interdisciplinary studies on protein-based advanced biomaterials for many potential applications and will be of interest to many—from graduate students getting started in their research to materials scientists and engineers. The interdisciplinary interchange is at the center of studies on protein-based natural advanced biomaterials. The information provided here including descriptions of advances in the biology, material properties, processing and biomedical applications of natural advanced biomaterials should be of interest to researchers in areas relevant to biomedical engineering, mechanical engineering, biology, physics, chemistry and clinical medicine.

2. Natural Protein-Based Advanced Biomaterials

Structural proteins have been created in nature through billions of years of evolution for a wide variety of biological functions, and the translation of natural structural concepts into bio-inspired materials requires the combining of amino acid sequences and their associated folding patterns that can produce advanced biomaterials with elastic, rigid or tough behaviors (Annabi et al. 2013, Gagner et al. 2014, Main et al. 2013, Maskarinec and Tirrell 2005, Smeenk et al. 2005, van Hest and Tirrell 2001). Natural protein-based biomaterials exhibit desirable mechanical responses or behaviors, such as elasticity – undergo high deformation under stress without rupture,

to recover the original state, once stress is removed. A growing number of advanced biomaterials based on silk, elastin and resilin biopolymers provide the challenging examples in materials design for material scientists and are considered as an alternative to conventional synthetic polymers, presenting a promising class of next generation advanced biomaterials for biomedical applications (Desai and Lee 2015).

In natural protein-based biopolymers discussed in this section, resilin and elastin have relatively high extensibility and resilience, but lack stiffness and strength when compared with the collagen and the silks (Su et al. 2014). Collagen and dragline silk are much stiffer materials, but lack the extensibility that is characteristic of the rubber-like proteins (Chattopadhyay and Raines 2014, Omenetto and Kaplan 2010, Su et al. 2014).The molecular origins of the remarkable physical/mechanical properties for protein biomaterials have not been completely understood. However, the primary amino acid sequences of these structural proteins have revealed the critical features that related to their unique structural and functional properties, that is, they are largely comprised of distinct tandem repeats of oligopeptide domains with a well-defined secondary structure, containing short amino acid sequences as protein polymer building blocks (of the order 5–20 residues) which tend to be rich in glycine residues and 'above average' fraction of proline residues (Kim 2013). Three-dimensional architectures are formed further through physically cross-linked networks via self-assembly or with chemical cross-linking to achieve desirable physical and mechanical characteristics (Lu et al. 2010, Lu et al. 2012, Murphy and Kaplan 2009).

2.1 Silk and Silk-Like Polymers (SLPs)

As one of the most ancient insect-derived advanced biomaterials due to its utility in the textile world, silk is originally produced by many insects for different purposes, such as cocoons for survival by silkworms, orb webs for prey capture by spiders, and nest construction by the Hymenoptera (bees, wasps, and ants) (Bellas et al. 2015, Fu et al. 2009, Sutherland et al. 2010, Veldtman et al. 2007). To date, the best-characterized silks include mainly the cocoon silk of the mulberry silkworm *Bombyx mori* and the dragline silk of spiders (*Nephila clavipes* and *Araneus diadematus*) and their astonishing properties have been studied heavily (Altman et al. 2003, Asakura et al. 2003, Fu et al. 2009, Jin and Kaplan 2003, Valluzzi et al. 2002). The high strength and elasticity of silks are the key to their potential utility in advanced biomaterials applications, influenced by temperature, state of hydration and extension rate (Madsen and Vollrath 2000, Shao and Vollrath 2002, Vollrath et al. 2001). In fact, silk is a remarkable advanced biomaterial as strong as aramid filaments such as Kevlar and superior to high-grade steel (Altman et al. 2003). Some spider silks can especially stretch to 140%

of their original length without breaking and hold their strength up to –140°C (Heim et al. 2009, Lewis 2006). However, spider silk is extreme light. A single strand of spider silk long enough to circle the Earth would weigh less than 500 g (Lewis 2006).

The primary amino acid sequences and thus the structure of silk are different for different species of silkworms and spiders with corresponding differences in molecular organization (Jin and Kaplan 2003, Omenetto and Kaplan 2010, Valluzzi et al. 2002). Generally, silks contain a high level of the amino acids glycine, alanine and serine, and have been characterized as natural block copolymers including hydrophobic blocks with short side-chain amino acids such as glycine and alanine, and hydrophilic blocks with larger side-chain amino acids, as well as charged amino acids. They are semicrystalline materials with either ordered molecular structures or β-sheets (crystallites), determining the mechanical properties of silks. Compared with globular proteins, the enhanced environmental stability of silk materials is attributed to the extensive hydrogen bonding and the hydrophobic nature of the protein, which leads to the formation of β-sheets or crystals (Fig. 1) (Keten et al. 2010). For example, an antiparallel β-sheet structure having extended polypeptide chains has been characterized for spider dragline silks and cocoon fibroin silks, with hydrogen bonds formed between the carbonyl oxygen atoms and amide hydrogen atoms from adjacent peptide chains, resulting in a pleated structure along the backbone of the peptide chain (Matsumoto et al. 2008, Rabotyagova et al. 2010, Valluzzi et al. 2002, Vepari and Kaplan 2007, Xu et al. 2014).

The *B. mori* cocoon silk is the most prominent silk production due to its use as a raw material in textiles and as medical sutures that are approved by the U.S. Food and Drug Administration (FDA) (Heim et al. 2010a, Heim et al. 2009, Heim et al. 2010b). They comprise of highly organized

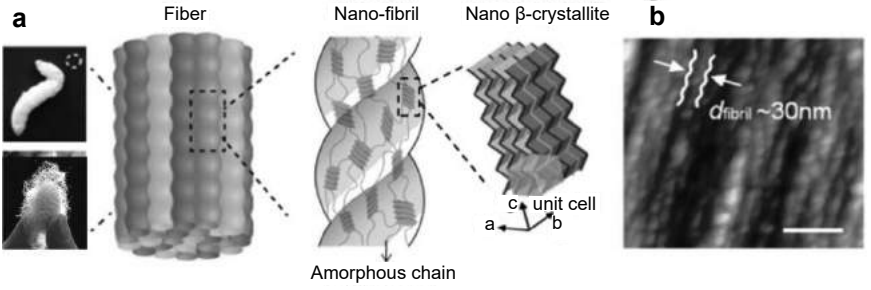

Figure 1. Hierarchical structure of *Bombyx mori* silkworm silk. **(a)** Silk fibers are composed of numerous interlocking nano-fibrils. **(b)** AFM image of the nano-fibrillar structure in silkworm silk with a sequence of linked segments (scale bar: 100 nm). Reproduced with permission from Ref. (Shao and Vollrath 2002), Copyright 2002 Nature Publishing Group 2002 (the cocoon image at lower left adapted), and Ref. (Xu et al. 2014), Copyright 2014 The Royal Society of Chemistry (the rest panels).

β-sheet regions (about 55% of the total structure), including two types of proteins, fibroin and sericin. Sericin is a family of antigenic glue like proteins, helping with the formation of the composite cocoon fibers in nature. Sericin must be removed for biomedical applications by degumming that is a typical process by boiling silk fibers in an aqueous solution of sodium carbonate (Gasperini et al. 2014). Separation is required for silk purification as the sericin proteins might cause an inflammatory response, and solubilizing the degummed silk fibers can be further fabricated and processed into various advanced material formats for a range of biomedical applications, including porous silk sponges, silk films, nano- or micro- scale coatings, hydrogels and nano- and micro- particles (Fig. 2) (Rockwood et al. 2011). The core filaments of cocoon or silkworm silk have at least two major fibroin proteins, a light chain (25 kDa) and a heavy chain (350 kDa) linked by disulfide bonds (Altman et al. 2003). The complete sequence of the fibroin heavy chain contains repetitive amino acids (-Gly-Ala-Gly-Ala-Gly-Ser-) along its sequence, forming a β-sheet secondary structure so that the methyl groups and hydrogen groups of opposing sheets interact. The hydrophobic domains play an important role in the final

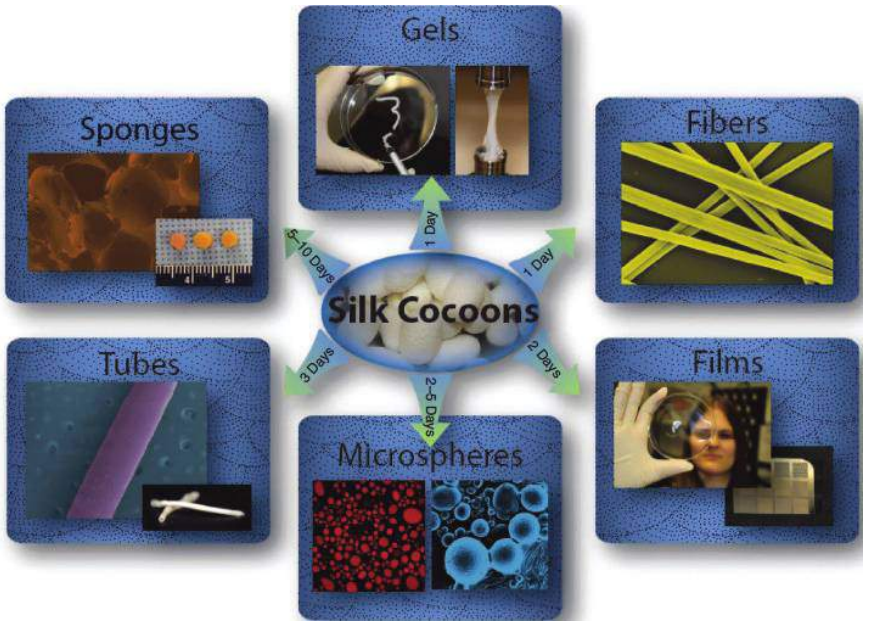

Figure 2. Schematic of material forms fabricated from silk fibroin using both organic solvent- and aqueous-based processing approaches. Overall, the silk fibroin extraction process takes 4 d and the time within the arrows indicates the time required to process the silk fibroin solution into the material of choice. Reprinted with permission from Ref. (Rockwood et al. 2011). Copyright 2011 Nature Publishing Group.

molecular assembly of the proteins into silk fibers, which are responsible for insolubility, leading to the high strength and thermal stability of the silk fibers (Fu et al. 2009). The materials properties of silk fibroins are determined by their special molecular structures which include mainly three different morphologies: (1) silk I, water soluble structure containing random coils and amorphous regions; (2) silk II, insoluble in several solvents (mild acid and alkaline conditions) with antiparallel β-sheets; (3) silk III, which consists of threefold polyglycine II-like helices (Valluzzi et al. 2002). In regenerated silk fibroins, the silk I structure easily converts to a β-sheet structure by chemical methods such as methanol treatment and silk II structure is more stable where the sheets are arranged back to back in alternation (Wilson et al. 2000). Silk II is insoluble and stabilized by strong hydrogen bonds and van der Waals forces that can be broken down by solvents with high ionic strength and high concentration of salts such as lithium bromide to obtain a water-soluble silk I random-coil conformation (Lu et al. 2010). Silk hydrogels can be further processed from silk I fibroin solutions in water with mild conditions, which can be influenced by mechanical stresses, protein concentration, temperature, pH and salt concentration in solution (Bellas et al. 2015, Wu et al. 2012, Yao et al. 2012, Yucel et al. 2009).

Spider silks are remarkable natural polymers and their molecular weights vary from 70–700 kDa with various protein sequences depending on the different spider species (Vepari and Kaplan 2007). The protein sequences with consensus repeat units have been identified (Fig. 3), including three main domains in natural sequences of spider silk: (1) a repetitive middle core domain where two basic sequences, crystalline (rigid) [poly(A) or poly(GA)] and less crystalline (highly elastic) (GGX or GPGXX) polypeptides alternate; (2) and (3) nonrepetitive N-terminal and C-terminal domains which are critical for pH-responsive fiber spinning in insect glands. Moreover, the polyalanine blocks can self-assemble into tightly packed β-sheets that are embedded in an amorphous matrix, leading to the extraordinary mechanical properties of the silk (Jin and Kaplan 2003, Rabotyagova et al. 2010, Valluzzi et al. 2002, Wilson et al. 2000). The dragline silks, major ampullate spidroin 1 and 2 (MaSp1 and MaSp2) have been investigated dramatically for recombinant expression because they can form the toughest fibers (Tokareva et al. 2014, Tokareva et al. 2013). Recent studies of chimeric silks with only one fifth of the native protein length, combining MaSp2 and flagelliform silk containing the elastic 'GPGGX' repeats, have demonstrated the ability to create a highly extensible yet strong silk-like polypeptide (SLP), providing a route for making light-weight advanced materials with high toughness and strength (Lewis 2006). In addition, minor ampullate silk possesses mechanical properties almost similar to major ampullate silks, but does not supercontract in water (Heim et al. 2009).

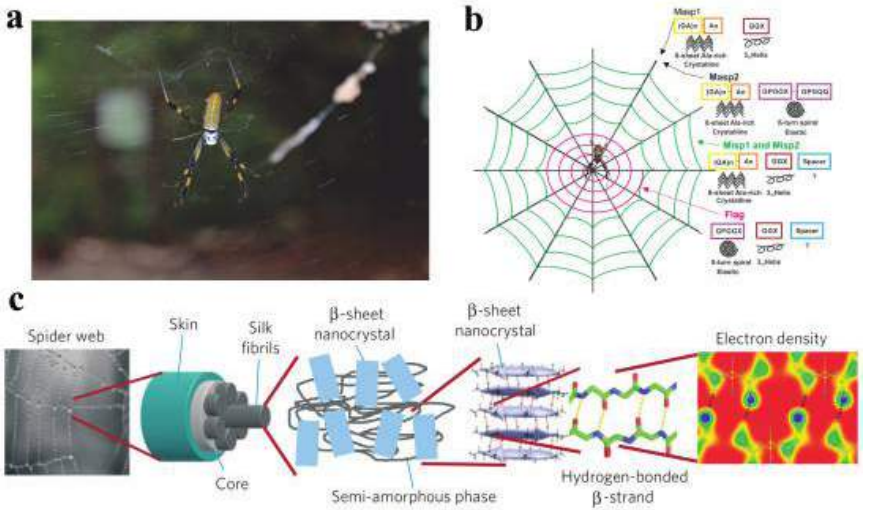

Figure 3. Hierarchical structure of spider silk. **(a)** An adult female orb weaver spider *Nephila clavipes* and her web. **(b)** Schematic overview of *N. clavipes* web composed of three different spider silk proteins and their structures. The coloured boxes indicate the structural motifs in silk proteins. An empty box marked '?' indicates that the secondary structure of the 'spacer' region is unknown. Note: MaSp1 or MaSp2: major ampullate spidroin 1 or 2; MiSp1 and 2: minor ampullate spidroin 1 and 2; Flag: flagelliform protein. **(c)** Schematic of the hierarchical spider silk structure that ranges from nano to macro, including the electron density at the Angstrom scale. Reproduced with permission from Ref. (Tokareva et al. 2013) (panel a & b adapted in an open access article under the terms of the Creative Commons Attribution License), and Ref. (Keten et al. 2010). Copyright 2010 Macmillan Publishers Limited (panel c adapted).

2.2 Elastin and Elastin-Like Polymers (ELPs)

Elastin is one of the main components of the extracellular matrix proteins present in blood vessels, lung epithelium, skin and other tissues where stretch and relax more than a billion times during life, providing structural integrity, high elasticity and resilience (Desai and Lee 2015). Elastin is a heavily cross-linked biopolymer with highly repetitive sequences that formed in the elastogenesis process. The cross-linked elastin is fibrous and hydrophobic, making it insoluble and difficult to isolate. Tropoelastin is a ~ 72 kDa soluble precursor of elastin and a highly repetitive protein with alternating elastic hydrophobic and lysine-rich hydrophilic peptide domains. The elastin protein sequence and genes have been identified for its biochemistry and structure, increasing our understanding of the role of elastin and its potential to biomedical applications (van Eldijk et al. 2012). The biodegradation sequence and specific cell adhesion motifs can be

further added to the elastin polymer chain, enhancing the ability of elastin biopolymer for biomedical engineering applications.

In tropoelastin, lysine residues interspersed with alanine are mainly found in the hydrophilic domains, while the hydrophobic domains are composed of repetitive sequence units such as the tetra-, penta-, and hexa- peptides, containing 'VPGG', 'VPGVG', and 'VAPGVG', respectively (Annabi et al. 2013, Grove and Regan 2012, van Eldijk et al. 2012). The hydrophobic domains of tropoelastin are the source of elasticity and intrigue the unique thermal responsiveness, which is critical for mature elastin formation. This phenomenon occurs as an inverse temperature transition (ITT), which is also known as lower critical solution temperature (LCST) behavior, inducing the aggregation of tropoelastin. Tropoelastin is soluble with random-coil conformation in aqueous solutions under the transition temperature (T_t); upon increasing the temperature above its characteristic transition temperature (T_t), the tropoelastin molecule chain aggregates and folds, and its phase separates into a coacervated state, hydrophobically forming a regular, ordered beta-spiral structure stabilized by the interactions between their hydrophobic domains. This phenomenon is fully reversible by heating and cooling and thermodynamically controlled between room and body temperature, which can be influenced by amino acid composition/hydrophilicity, protein length/molecular weight (MW) and protein concentration, as well as ionic strength (salt concentration) and pH in the environment (Krishna and Kiick 2010, Li et al. 2014, van Eldijk et al. 2012) Most notably, it has been found the T_t of ELPs is inversely related to the ELP molecular weight and concentration, indicating the increase in ELP molecular weight or concentration results in a lower T_t of ELPs. In addition, the local pH can also influence the T_t of ELPs by influencing the amino acid sequences (Hassouneh et al. 2010, Rusling et al. 2014, Thapa et al. 2013).

Self-assembly is the transition process from the spontaneous organization of molecules under thermodynamical equilibrium conditions into structurally well-defined and rather stable arrangements through a number of non-covalent interactions (Daamen et al. 2007). Self-assembly is another important property of elastin and ELPs, leading to alignment of elastin molecules to intermolecular cross-linking under physiological conditions. The coacervation based on the LCST behavior of tropoelastin will induce the formation of ordered structure because raising the temperature and the release of water result in dehydration of the hydrophobic side chains, leading to the alignment of tropoelastin molecules or self-assembly (Li et al. 2014, Pinedo-Martin et al. 2014, van Eldijk et al. 2014). The self-assembly behavior of elastin-based biomaterials may be extremely valuable to obtain nano-scale advanced biomaterials with defined structure and mechanical properties, including nanotubes, nanofibres, nanoporous films and nanoparticles, providing the emerging and promising applications

for cellular orientation and small-diameter blood vessels in soft tissue regeneration and for drug delivery or growth factor delivery devices (Daamen et al. 2007).

Previous studies have shown the possibility of creating self-assembled advanced systems with thermal responses by combining elastin-like polypeptides (ELPs) and globular proteins (Li and Kiick 2013a, Lv et al. 2010b, Xia et al. 2011b). For example, when introduced with ELPs, the new mCherry-ELPs protein fusion system can self-assemble into micelles or aggregated nanoparticles in solution, and further investigations demonstrated the behavior for order-disorder transition at high concentrations above T_t of ELPs (Qin et al. 2015). In addition, the feasibility of purification of specifically designed fusion proteins has been demonstrated on large scale. For example, proteins can be purified as fusions with ELPs by inverse transition cycling (ITC) (Fig. 4), where the thermo-sensitive solubility imparted by the ELP tag allows for large scale purification of fusion proteins at low cost (Bellucci et al. 2013, Meyer and

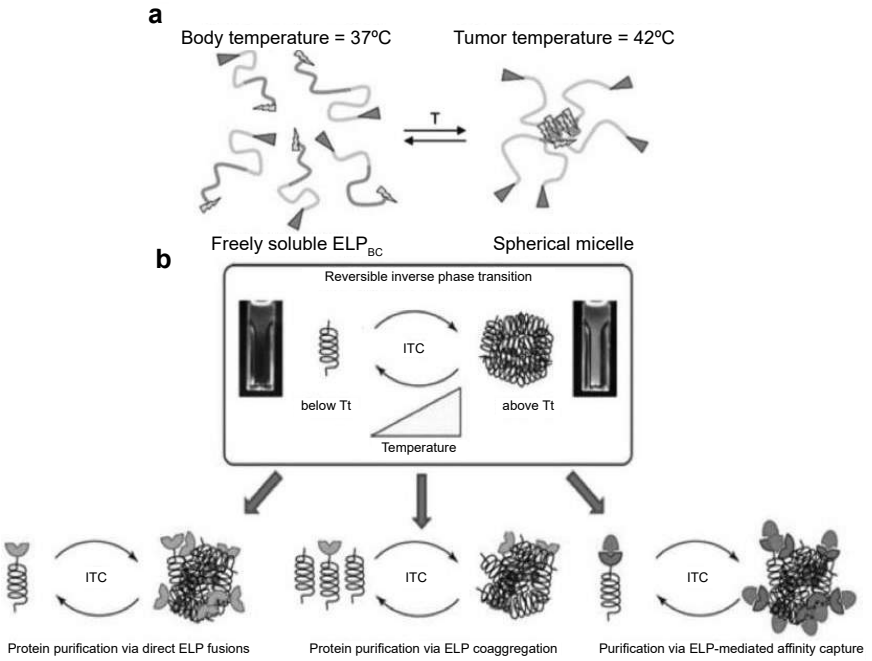

Figure 4. Thermal transition and self-assembly of ELPs. **(a)** Hyperthermia-triggered multivalency. Block copolymers consisting of two ELP blocks, a hydrophilic block and a hydrophobic block were designed. **(b)** Purification of ELPs by ITC is based on the reversible inverse phase transition. Reproduced with permission from Ref. (van Eldijk et al. 2012). Copyright 2011 Springer-Verlag Berlin Heidelberg.

Chilkoti 1999, Trabbic-Carlson et al. 2004a, Trabbic-Carlson et al. 2004b). The fusion protein containing an ELP fused with green fluorescent protein (GFP) was expressed successfully in *E. coli* with nutrient-rich medium without IPTG induction and purified at large scale, and the yield of resulting GFP/ELP fusion was extremely high up to 1.6 g/L of bacterial culture (Chow et al. 2006).

2.3 Resilin and Resilin-Like Polymers (RLPs)

Resilin was discovered in 1960 and it is a highly resilient protein that is a critical component within structures where energy storage and long-range elasticity are needed, such as the flight system of locusts, the jumping mechanism of fleas and the sound producing organ of cicadas (Qin et al. 2009). Resilin is a polymeric rubber-like protein with outstanding mechanical properties. For example, resilin could be stretched up to 3–4 times of its original length before breaking, demonstrating a remarkable capacity for stretching and immediately snap back to its resting length upon release of the tensile force, showing no deformation and great extensibility (Charati et al. 2009, Li and Kiick 2013b, Qin et al. 2012). Resilin is stable up to 140°C, and possesses high resilience (92% or more) and a very high fatigue lifetime, due to the covalent cross-linking between tyrosine residues, generating di- and tri- tyrosines, that is mediated through the action of peroxidases (Andersen 2010, Qin et al. 2011, Su et al. 2014). In the specialized cuticle regions of insects, resilin binds to the cuticle polysaccharide chitin via a chitin binding domain and is further polymerized through oxidation of the tyrosine residues resulting in the formation of di-tyrosine bridges and assembly of a high-performance protein – carbohydrate composite advanced materials (Qin et al. 2012, Qin et al. 2009, Qin et al. 2011).

The investigation of resilin advanced biopolymers have been increased dramatically since the gene CG15920 in *Drosophila melanogaster* was found to encode a resilin precursor due to its amino acid composition and an isoelectric point that resembled resilin closely, as well as the presence of an N-terminal signal peptide sequence for secretion (Andersen 2010, Li and Kiick 2013b, Su et al. 2014). Further sequence analysis showed that resilin protein was 620 amino acids long with highly conserved repeat sequences containing a greater percentage of acidic residues than collagen, elastin and silk fibroin and fewer non-polar residues than silk fibroin and elastin, which might be the reason of resilin's hydrophilicity as well as its low isoelectric point. Resilin also contains more tyrosine residues (~ 5% of the total weight) than the other structural proteins except silk fibroin. Chemical stability of the di- and tri- tyrosine cross-links indicates that resilin might be an ideal network and a variety of cross-linking strategies have been employed to introduce covalent cross-links in resilin (Li and Kiick 2013b,

Qin et al. 2012, Su et al. 2014). The soluble resilin protein from exon 1, rec1-resilin can create cross-linked hydrogels by reacting tyrosines using a peroxidase or through Ru (II) – mediated photo – cross-linking (Elvin et al. 2005). Other RLP (RLP_{12}) incorporating bioactive motif (GRGDSP) could be cross-linked through lysine residues and via THPP ((tris(hydroxymethyl)-phosphino)propionic acid), exhibiting the ability of stretching to average 180% before breaking and cell adhesion and NIH-3T3 proliferation (Desai and Lee 2015, Li and Kiick 2013b, Li and Kiick 2014, Su et al. 2014). The photo-cross-linked GB1-resilin biomaterials were also generated to mimic the unstructured and elastic features of the muscle behavior and served as a molecular spring where resilin was fused for unordered structures, demonstrating high resilience of > 99% and the stretching of 135% without breaking (Lv et al. 2010a).

Resilin protein has been identified for three exons (Fig. 5), including exon 1 (hydrophilic N-terminal segment being highly elastic), exon 2 (hydrophobic mid-segment containing chitin-binding domain (ChBD)) and exon 3 (hydrophilic C-terminal segment that can reversibly undergo conformational changes indicating energy storage). Exon 2 containing

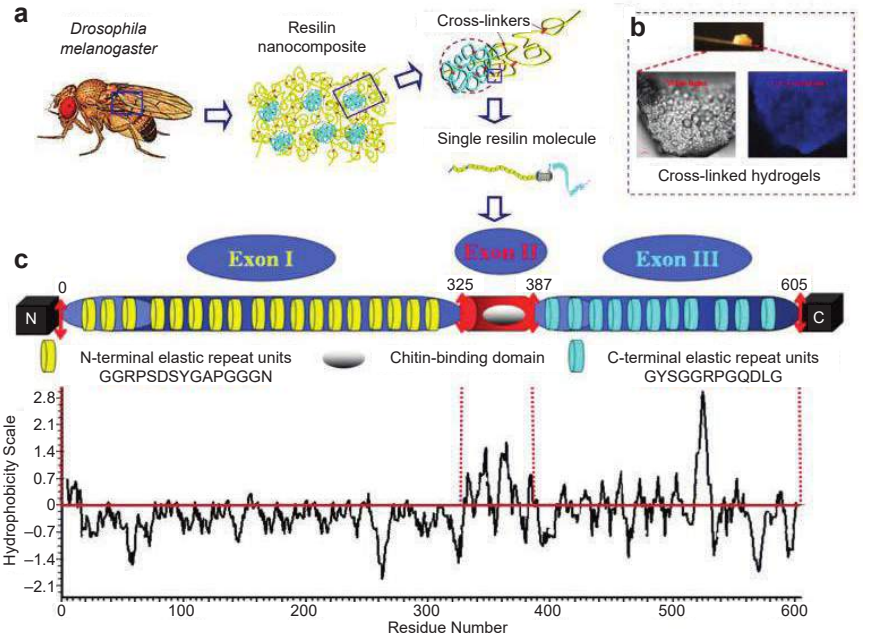

Figure 5. Primary sequence and structure model of resilin in *Drosophila melanogaster*. **(a)** Hierarchical structure of fruit fly resilin. The resilin fibrils with crosslinking consist of two major unstructured peptides derived from exon I and III of full-length resilin. **(b)** Cross-linked resilin hydrogels under ultraviolet. **(c)** Amino-acid sequence scheme of three exons in the full-length resilin protein and hydrophobicity index of the full-length resilin.

62 amino acids showed consensus to a Rebers – Riddiford sequence, and convinced the high affinity to chitin via the ChBD, providing evidences for its role in the formation of the resilin – chitin composites in the cuticle (Andersen 2010, Ardell and Andersen 2001, Qin et al. 2009). A N-terminal region for exon 1 is composed 18 pentadecapeptide repeats (GGRPSDSYGAPGGGN), while a C-terminal region comprising 11 tridecapeptide repeats (GYSGGRPGGQDLG) dominates exon 3, providing the basis for the development of recombinant resilin-like polypeptides (RLPs) that attempt to mimic and recreate the long-range elasticity of natural resilin (Li and Kiick 2013b, Qin et al. 2012, Qin et al. 2011, Su et al. 2014). Both these exons have a high content of glycine and proline, and lack sulfur-containing amino acids or tryptophan. Recent findings have shown the mainly unstructured and flexible chains of resilin and might form β-turns as well as more extended poly-proline II (PPII) secondary structures. Based on scanning probe microscopy (SPM) and tensile testing, the resilin exon 1 (the N-terminal segment) had up to 92% resilience and could be stretched to over 300% of its original length before breaking, exhibiting the near-ideal rubber elasticity, which is potentially useful in the design of advanced hydrogel structures with controlled morphology from resilin proteins that could be exploited as a reservoir for drugs, nanoparticles, enzymes, catalysts and sensor applications (Elvin et al. 2005). The resilin exon 3 (the C-terminal segment) has been studied for energy storage through a reversible conformation transition observed from random coil to β-turns by energy inputs including mechanical stretching and thermal treatments, explaining the molecular elasticity mechanisms for resilin in insects and enabling insects to jump and/or fly with great efficiency (Qin et al. 2012).

Recombinant resilin-like polypeptides (RLPs) have been shown to closely match native resilin in both physical and mechanical properties, composing of tandem repeats of consensus sequences from the N-terminal segment of resilin. For example, the nano-indentation studies by SPM or AFM for RLPs including rec1-resilin, An16 ($(AQTPSSQYGAP)_{16}$) and Dros 16 ($(GGRPSDSYGAPGGGN)_{16}$) confirmed the negligible hysteresis and resilience of 97%, 98% and 91%, respectively (Balu et al. 2014, Lyons et al. 2009, Nairn et al. 2008, Su et al. 2014, Truong et al. 2010). In addition, similar to elastin and ELP, resilin and RLP are also thermo-responsive and behavior as LCST with a sharp transition from hydrophilic to hydrophobic above a transition temperature, and the LCST of the protein occurs at a relatively high temperature of ~ 70ºC due to the presence of many hydrophilic residues in RLP sequences (Desai and Lee 2015). Furthermore, the properties of the dual phase transition behavior and pH-responsiveness make resilin and resilin-mimetic advanced biomaterials the good candidates, allowing the control of cell adhesion and migration for biomedical engineering and the creation of a functional surface for biosensors (Liu et al. 2015, Su et al. 2014, Truong et al. 2010).

2.4 Other Natural Protein-Based Advanced Biomaterials

Collagens are the major proteins in the extracellular matrix (ECM) and characterized by their triple-helical molecular structure composed of the $(Gly\text{-}Xaa\text{-}Yaa)_n$ repeating amino acid sequence. They have high content of proline and require the post-translationally modified hydroxyproline (Hyp) to promote stabilization (An et al. 2013, Chattopadhyay and Raines 2014, Faraj et al. 2007). The most abundant collagens are used widely to form axially periodic fibrils in tendon, bone, cartilage and other tissues, playing an important role in cell signaling and development (Chattopadhyay and Raines 2014, Faraj et al. 2007, Nillesen et al. 2007). Collagen molecular structure in fibrils is characterized by the formation of right-handed triple helix, extending to 300 nm in length with 1.5 nm in diameter, and then self-assembled into higher-level supramolecular structures within the fibril. Collagen, one of the versatile structural proteins with triple helices, has mechanical properties and biological functions, providing building block for design of self-assembled advanced biomaterials and other applications. Currently available commercial collagen is derived mostly from animal sources and an alternative biosynthetic methods using genetic and protein engineering can be developed to overcome the potential problem such as immunological responses (Kim 2013). Collagen-like polypeptides (CLPs) can be designed and produced to enhance molecular organization and biological properties by recombinant DNA techniques, based on the most frequently used tripeptide sequences in natural collagen (Grove and Regan 2012, Kim 2013, Main et al. 2013). Recently a bacterial collagen domain was fused with a repetitive cocoon silk consensus sequence to generate the advanced collagen-silk chimeric proteins, allowing more rapid cell interactions with silk-based biomaterials and improving regulation of stem cell growth and differentiation and formation of artificial extracellular matrices useful for tissue engineering applications (An et al. 2013). In addition, collagen can be engineered to develop advanced functionalized collagens with new functional motifs such as repetitive cell binding domains, showing the ability to promote cell adhesion for drug delivery, tissue engineering and wound healing (Chattopadhyay and Raines 2014, Faraj et al. 2007, Nillesen et al. 2007).

Camouflage and signaling/communication are the natural optical features or coloration patterns widespread across the animal kingdom, from the most exotic iridescent patterns of butterfly species to the feathers of peacocks and other birds. Cephalopods can also rapidly alternate the color and reflectance of their skin in response to the environmental or external stimuli, such as specific light pulses, enzymatic reactions, or relative humidity (Grove and Regan 2012, Kim 2013, Krishna and Kiick 2010). Reflectins are a unique group of structural proteins involved in

dynamic optical systems in cephalopods and function in camouflage by modulating incident light or bioluminescence. The specialized reflectin architectures have been found as the major component in flat, structural platelets in reflective tissues of the Hawaiian bobtail squid, *Euprymna scolopes* (Cephalopoda: Sepiolidae) (Crookes et al. 2004), producing structural color for camouflage that may have potential applications in the fields of advanced materials science and optical nanotechnology. Hawaiian bobtail squid reflectin proteins possess five repeating domains containing a highly conserved core subdomain, defined by the repeating motif ($M/FD(X)_5MD(X)_5MD(X)_{3/4}$), and could exhibit diverse morphologies, unusual solubility and self-organizing properties. The reflectin proteins can be further processed into thin films, diffraction grating structures, and fibers under various conditions (Crookes et al. 2004, Ghoshal et al. 2014, Izumi et al. 2010, Kramer et al. 2007). Previous studies on native incident light in the Loliginid squids have demonstrated that the dynamic, responsive and tunable optical function of iridophore cells was facilitated by the hierarchical supramolecular assembly of nanoscale reflectin protein particles that elicited large volume changes upon condensation (Tao et al. 2010). Furthermore, thin films created from the recombinant reflectin protein refCBA that reduced complexity compared to native reflectins display interesting optical features and diffraction patterns after self-assembly (Qin et al. 2013). Although little has been reported for the reflectin-mimetic biomaterials, biosynthesis of reflectin-like polypeptides based on repetitive sequences and multilayered thin films generated by bottom-up fabrication provide the opportunity in a range of camouflage and nanostructured advanced devices potentially for optical nanotechnology (Kim 2013).

3. Advanced Biomaterials for Biomedical Engineering Applications

Natural protein-based biopolymers like silk, elastin and collagen have promising advantages over synthetic polymers, providing an important set of advanced material options for biomaterials and scaffolds in biomedical and pharmaceutical applications. Diverse and unique biomechanical properties together with good biocompatibility and controllable biodegradability make natural protein-based biopolymers excellent candidates as advanced biomaterials for drug delivery, tissue engineering scaffolds and wound-healing matrices (House et al. 2010, Park et al. 2010, Wang et al. 2010, Wharram et al. 2010). The synthesized protein-based biopolymers can be further processed into various formats such as films, fibers, scaffolds and hydrogels to expand their applications as advanced biomaterials.

Among these natural polymers, silk-based biomaterials from silkworm cocoon silk have been used for sutures and the core fibroin fibers are comparable to most of the commonly used biomaterials in terms of biocompatibility after sericin is removed (Leal-Egana and Scheibel 2010). Natural spider silks have also demonstrated non-cytotoxicity, low antigenicity and non-inflammatory characteristics. Silk is classified as a non-biodegradable advanced biomaterial as a result of the wax coatings processed on silk fibers. However, recent studies of enzymatic degradation have shown that silk is susceptible to proteolytic degradation and slowly breaks down into smaller polypeptides and free amino acids over time with the adding of α-chymotrypsin that cleaves the less crystalline regions of the silk protein into peptides and protease XIV that degrades the antiparallel β-sheet structures of silks into nanofibrils and subsequently into nanofilaments (Horan et al. 2005, Numata and Kaplan 2010, Numata and Kaplan 2011). The degradation rate relies heavily on the beta-sheet content and is related to the preparation process of silk films or hydrogels. In addition, natural degradation products of silk crystals are not cytotoxicity, compared to cross-beta sheet crystals associated with cytotoxicity and amyloid-like deposits in Alzheimer's and related diseases (Horan et al. 2005, Numata and Kaplan 2010, Numata and Kaplan 2011). Several protein-degrading enzymes can also degrade elastin, including elastases, matrix metalloproteinases (MMPs) and cathepsins, and then interact with other ECM proteins to induce a broad range of biological activities (van Eldijk et al. 2012).

3.1 Sustained Drug Delivery and Controlled Drug Release

The goal of sustained drug delivery is to delivery the drug to the target therapeutic range, continuously maintain the constant drug concentrations within the therapeutically desirable range without peaks and valleys, and extend the inter-dose duration for chronic use medications, providing the potential clinical benefits such as reduction or elimination of unwanted side effects, low toxic thresholds, increased patient convenience and compliance, and enhanced efficacy and cost-efficiency (Pritchard et al. 2013, Pritchard and Kaplan 2011, Yucel et al. 2014b). Controlled degradation and release of drugs from the drug delivery system after accumulation at a specific site are the most important properties as required for regenerative medicine and drug delivery applications, and can be triggered by physiological stimuli such as pH, temperature and ionic strengths to release the encapsulated drugs. Particularly, the difference of extracellular pH of normal tissue (pH 7.2–7.4) and many solid tumors (pH 6.2–6.9) can be used to design a pH-sensitive advanced delivery system, improving the efficiency for the drug delivery application (Nitta and Numata 2013).

Protein-based natural polymers including silk, elastin and collagen have been explored as an advanced vehicle to deliver a wide range of bioactive molecules including genes, small molecules and biological drugs (Pritchard et al. 2013, Pritchard and Kaplan 2011, Yucel et al. 2014b). They are applied as drug carriers for cancer therapy (Yucel et al. 2014a), cartilage repair (Yodmuang et al. 2015) and vascular grafts (Liu et al. 2013) because of their biocompatibility, low toxicity, non-antigenicity, biodegradability, and tunable drug loading and release properties, as well as the abilities of emulsification, gelation, forming and water binding capacity (Numata and Kaplan 2010, Numata and Kaplan 2011, Yucel et al. 2014b). To match the needs of controlled drug delivery well, the combination of material synthesis, processing conditions, drug compounds used and finally drug release kinetics and mechanisms are needed to consider for any future advanced drug delivery. In protein biopolymer-based nanoparticle delivery systems, the design of specific sizes for drugs-loaded nanoparticles is one of the most important criteria to cross epithelial barriers, circulate in the blood vessels before reaching the target site and avoid the inflammatory or immunological responses (Nitta and Numata 2013). The sizes, shapes, solubility, biodegradability and surface properties of biopolymer-based nanoparticles need to be considered for cellular internalization via endocytosis to achieve the site-specific delivery and bioactive drug release at required rate and quantity (Nitta and Numata 2013). Furthermore, protein-based biomaterials can be engineered and incorporated directly with additional features, such as cell-specific targeting, to produce more efficient advanced drug delivery systems.

Silk protein-based materials have been considered for advanced drug delivery systems because of their unique mechanical properties, controlled biodegradation into non-inflammatory by-products, aqueous-based ambient purification and processing options, biocompatibility with sterilization methods and utility in drug stabilization (Pritchard et al. 2013, Yucel et al. 2014a, Yucel et al. 2014b). Various formats based on silk fibroins have been explored from the aqueous silk fibroin solution, processing into materials for advanced drug delivery such as hydrogels, films, tubes, nano/microspheres and transdermal micro-needles (Pritchard et al. 2013, Rabotyagova et al. 2010, Valluzzi et al. 2002). Silk micro- and nano- spheres with controllable sizes have been investigated for the studies of distribution and loading efficiency of drug molecules, resulting in different drug release behaviors in silks used with different hydrophobicity and charge (Wang et al. 2007). Silk film coating was also explored for small molecule drug delivery and the subsequent drug release was regulated by controlled drying and silk film treatment, with drug release profiles lasting (drug retention time) from a few hours to 10+ days, respectively. Further incorporation of protease inhibitors may enhance the ability to control local degradation

rates of silk fibroin, improving the efficacy for controlled localization of drug release. Silk fibroins were blended with chitosan polymers to form < 100 nm nanoparticles for local and sustained therapeutic curcumin delivery to cancer cells (Kasoju and Bora 2012). The *in vitro* stability and half-life of insulin were also efficiently improved by conjugating with silk nanoparticles via covalent cross-linking (Humenik and Scheibel 2014, Klok et al. 2004, Lin et al. 2013).

Due to the most powerful property of its tunable ITT, elastin-like polypeptides have been used extensively for therapeutic drug delivery and targeting applications. These thermally associating advanced materials can also be applied for drug loading and release with desirable thermal response properties (Koria et al. 2011, Saxena and Nanjan 2015, Smits et al. 2015). The ITT of a desired ELP relies on hydrophobicity and the molecular weight of the ELP, showing different transition properties of ELPs. The transition temperature of an ELP designed by Chilkoti and coworkers can be tuned to about 41°C, allowing for the localization and remarkable accumulation of the ELP peptides in tumors through the induction of mild hyperthermia without any tags (Christensen et al. 2013, MacEwan and Chilkoti 2014, Rusling et al. 2014). The hydrophobic drug within ELP-drug conjugates might lower ELP ITT to 37°C to allow for physical gelation upon injection, improve residence time of the drug and enable efficient drug release over time to minimize side effects (Kimmerling et al. 2015, MacEwan and Chilkoti 2010, McDaniel et al. 2010, Wu et al. 2009). Recombinant ELPs can also be functionalized with specific targeting sequences or internalization peptides, enhancing the accumulation or intracellular delivery of drug carriers at the disease sites. The chimeric ELP developed with a tumor-homing AP1 peptide that targets cell surface interleukin-4 (IL-4) (an overexpressed cell surface marker in solid tumors) was shown to accumulate preferentially in tumors and significantly enhance the tissue localization (Sarangthem et al. 2013). The functionalization of ELPs fused with cell penetrating peptides (CPPs) has been evaluated to improve efficiency of cellular uptake and targets inside eukaryotic cells by non-specific, receptor-independent mechanisms (Bidwell and Raucher 2010, Massodi et al. 2005, Ryu et al. 2014). The delivery of kinase inhibitor peptide p21 drug using CPP-functionalized ELP cargo demonstrated the enhancement of the interaction of drug cargo with intracellular therapeutic targets and thereby increased drug efficacy (Bidwell and Raucher 2010, Massodi et al. 2005, Ryu et al. 2014). A different strategy to deliver drugs to a specific site rather than diffusing to all tissues and affecting normal cells is to use amphiphilic diblock ELP chains with a self-associating hydrophobic block fused with a hydrophilic block. The relatively hydrophobic domain in diblock ELPs may coacervate at a lower temperature, resulting in self-association while the other block still remains soluble, that is temperature-dependent amphiphilicity. Amphiphilic ELPs

have demonstrated the high tumor vasculature retention once fused with tumor-targeting sequences NGR to target CD-13 receptors in tumor vasculature, providing the potential of these ELP-based nanoparticles for advanced targeted drug delivery (MacEwan and Chilkoti 2014, McDaniel et al. 2010, Wu et al. 2009).

The self-assembly investigation of silk-elastin-like polymers (SELPs) demonstrated the formation of the core of micelle-like nanoparticles by adding hydrophobic molecules with the size range from 20 to 150 nm in diameter, which is enough to cross the endothelial barrier, making them the good candidates as advanced drug delivery vehicles (Xia et al. 2014). Furthermore, protein-based biomaterials can be designed and synthesized by recombinant DNA techniques, expanding the versatility of protein-based advanced biomaterials with tightly controlled drug delivery capabilities (Numata et al. 2012). Silk copolymers have been engineered and incorporated with a specific peptide sequence for targeting and localization. For example, the combination of bioengineered silks with tumor homing peptides (THPs) would offer the opportunity to enable functionalization for targeted drug delivery, enhancing significantly the target specificity of the resulting nanoscale drug-loaded spheres to tumor cells with low toxicity (Numata and Kaplan 2010, Numata and Kaplan 2011, Numata et al. 2012).

3.2 Gene Delivery and Gene Therapy

It is believed the human disease can be cured by the transfer of genetic materials into specific patient cells to supply defective genes, and this strategy of gene therapy has been applied for many diseases including cancer, AIDS and cardiovascular diseases (Nitta and Numata 2013). The delivery of therapeutic genes into target cells in the patient is a promising approach for the treatment of various diseases, with either naked DNA or a viral vector used. To do so, the advanced gene delivery system with gene encapsulation must be small enough to internalize into cells and passage to the nucleus, escape endosome-lysosome processing and following endocytosis and finally protect the gene until it reaches its target site. To improve the safety and efficacy of gene delivery, current studies attempt to localize the gene delivery to particular tissue, protect the DNA from degradation, and control gene release profiles (Numata et al. 2010, Numata and Kaplan 2010, Numata et al. 2011, Numata et al. 2009).

Compared to other gene delivery vehicles such as liposomes and synthetic polymers, protein-based advanced biomaterials are used commonly to deliver plasmid DNA or adenoviral vectors due to their ability to be functionalized (Yucel et al. 2014b). An example from the amphiphilic diblock copolymer complexes composed of silk repetitive oligopeptide

block and poly(L-lysine) block (pLL) shows the non-cytotoxicity ability to deliver plasmid DNA for non-viral gene therapy, where the anionic plasmid DNA (pDNA) can form ionic pairs with the cationic pLL block via electrostatic interactions. The resulting silk-pDNA complexes may then be further functionalized with cell-binding motif to enhance cell binding and modified with cell penetrating and cell membrane destabilizing peptides to improve transfection efficiency, allowing for cell-specific targeting and efficient gene transfer (Numata and Kaplan 2010, Numata et al. 2009, Yucel et al. 2014b). In addition, the transfection efficiency of silk-pDNA complexes modified with RGD motif might be determined by the number of RGD domain if applied the silk-pLL-RGD fusion block copolymers for gene delivery to several cell types (Kim et al. 2014, Kim 2013, Numata et al. 2010, Numata et al. 2009, Wu et al. 2012).

The stimuli-responsive ELPs and SELPs have potential to be served as advanced polymeric matrices for gene delivery, enabling the hydrogel formation once injected in the body while being liquid at room temperature, an attractive feature for any injectable system application. For example, recombinant SELP-47K hydrogels have been reported the controlled gene delivery with adenoviral vector delivery, showing the inverse DNA release and diffusivity related to the molecular weight of the plasmids used (Kim et al. 2012, Megeed et al. 2006, Swierczewska et al. 2008). The binding/releasing mechanism of DNA to SELPs was further explored by the influence of different factors such as ionic strength, DNA concentration, SELP concentration and molecular weight. The results have shown the increased release of DNA and adenovirus bound within polymeric matrices if increasing the ionic strength in buffer or lower concentration of the polymers (Kim et al. 2012, Megeed et al. 2006, Swierczewska et al. 2008). Particularly SELPs hydrogels loaded with adenovirus in a mouse model demonstrated greater reduction in tumor volume as compared to control injections of adenovirus in saline solution, providing the effective route for adenoviral gene therapy for cancer treatment (Numata and Kaplan 2010, Numata et al. 2009, Yucel et al. 2014b).

3.3 Tissue Regeneration and Tissue Engineering

The treatment of organ failure is heavily limited by donor supply and increasing morbidity, and the regeneration of functional tissue is still challenging to closely mimic the *in vivo* physiological microenvironment for desired cellular responses. Good communication between the host and implanted system is critical for substitution of a human body part with a material. The goal of tissue engineering is to regenerate tissue within suitable scaffold for implanting the constructed tissue at the target site. Using cells, scaffolds and appropriate growth factors in

tissue engineering is a key approach in the treatments of tissue or organ failure. Advanced biomaterials 3D tissue engineering scaffold may provide a suitable microenvironment, acting as an architectural template (Kundu et al. 2013, Kundu et al. 2014, Kundu et al. 2010). Structural protein, being a component of natural tissues, is a rational choice to be used as porous 3D tissue scaffolds in tissue engineering, including silk, elastin and collagen. Functional requirements in tissue repair, regeneration and implantation for biomaterial scaffolds include providing support, surface topography and charge for cell attachment, mitogenesis and cell differentiation. Biomaterial scaffolds that mimic native extracellular matrix (ECM) have been studied to match the functional requirements for specific tissues, providing the potential to produce a functional tissue and organ (Annabi et al. 2013, Desai and Lee 2015, Gagner et al. 2014, Keatch et al. 2012, Khaing and Schmidt 2012, Kim 2013).

Silk protein fibroin is an attractive advanced biomaterial for tissue engineering because of the unique combination of elasticity and strength along with mammalian cell compatibility, which can be used effectively to produce a scaffolding material for development of advanced biomedical device (Desai and Lee 2015, Gagner et al. 2014, Keatch et al. 2012). Silk porous 3D sponges are ideal structures for tissue engineering scaffolds, which can be prepared by freeze drying, porogenic leaching and solid free form fabrication techniques with a good control over porosity and pore sizes. The resulting sponges possess the range of pore sizes from 60 to 250 μm, relying on the freezing temperature, pH and organic solvents. Due to the favorable tensile strength and their ability to be sterilized, silk-engineered scaffolds have been produced as substrates that mimic nanoscale properties of native ECM for cell attachment, cell proliferation and tissue regeneration (Bellas et al. 2015, Mandal et al. 2011, Park et al. 2012, Preda et al. 2013, Yodmuang et al. 2015) including tissue bone, ligaments, tendons, blood vessels and skin and cartilage (Dinis et al. 2013, Dinis et al. 2015, Elia et al. 2014, Hronik-Tupaj et al. 2013, Kimmerling et al. 2015, Liu et al. 2013, Lovett et al. 2015, Seib et al. 2013, Yodmuang et al. 2015). The mechanical and biological functions of protein-based biomaterials may be tailored by genetic engineering and surface chemical modification to produce advanced hybrid and composite systems and thus match tissue-specific needs (Vepari et al. 2010). For example, spider silks have been genetically engineered with various functionalities including the mineralizing domain R5 to perform bone like properties and dentin matrix protein 1 to mineralize calcium phosphate (CaP) (Wong Po Foo et al. 2006), and silk protein properties may be further enhanced through binding and delivery of cell signaling factors such as RGD-functionalized SLP to improve cell adhesion (Bini et al. 2006, Gil et al. 2010, Morgan et al. 2008). In addition, composite silk 3D scaffolds can be prepared to obtain good mechanical

and biological outcomes by combining inorganic or organic fillers and by bio-mimicking approaches with other natural extracellular materials since the complex structure of native tissue requires a composite scaffolding material. The successful example is the using of composites of silk fibroin and human-like-collagen for the development of vascular constructs. Furthermore, silk layering with collagen-I could enhance the cell attachment and dispersion of keratinocytes cells, while silk coating with fibronectin might improve the cell adhesion and dispersion within the matrix for both keratinocytes and fibroblasts cells (An et al. 2013, Bhardwaj et al. 2015, Vasconcelos et al. 2008). Other silk composites successfully used include nano-fibrous silk-chitin, silk-collagen and silk fibroin-alginate blended scaffolds. All the findings suggest that the blending of silk fibroins with other natural materials may offer better prospects than pure silk fibroin for tissue regeneration.

Elastin is a main component of the extracellular matrix with non-immunogenic, biocompatible and biodegradable properties, providing the attractive advanced materials from elastin-derived biomaterials for tissue engineering. Easy purification by exploiting their ITT from bacteria with high yields of ELP protein production make ELPs the good candidate for cartilaginous, vascular, ocular and liver tissue regeneration. ELPs can be processed further to form various advanced material formats such as hydrogels, films and fibers to match the application needs. For example, ELP hydrogels were used to mimic ECM-like 3D environments for cell encapsulation in tissue engineering. The enzymatically cross-linked ELPs via lysine results in the encapsulation of chondrocytes and the formation of hyaline cartilage-like substrate rich in collagen II (McHale et al. 2005). By using cysteine-based disulfide bridge cross-links, the ELP hydrogels can be engineered further with gelation rate and gel stiffness controlled by H_2O_2 and protein concentration, enabling cell encapsulation and *in situ* formation of soft gels useful in tissue engineering (McHale et al. 2005, Trabbic-Carlson et al. 2003, Xu et al. 2012). ELP cross-linked hydrogels can also be cast into elastic films, performed the further cross-linking via lysine residues linking using chemicals, and fused with cell-adhesive motifs and related functional modules. For example, the simple surface coatings of ELPs fused with RGD and CS5 domain have been developed to investigate their biochemical effects on cells, generally improving cell-interactive properties of ELP scaffold surfaces (Heilshorn et al. 2003, Liu et al. 2004, Rodriguez-Cabello 2004). Additional studies on higher surface area of ELP fibers from concentrated protein solutions have demonstrated a better display of cell signaling modules to the interacting cells (Benitez et al. 2013).

Resilin and RLPs have also been proposed as the promising advanced biomaterials for tissue engineering due to their outstanding mechanical properties. For example, Resilin-based biomaterials (RZ10) has shown

an unconfined compressive modulus similar to that of human cartilage, and the investigation of the chimeric material RZ10 fused with the RGD sequence (derived from fibronectin) has shown the faster cell spreading with well-organized actin structures of human mesenchymal stem cells (hMSCs), suggesting that RGD sequence can be recognized specifically and used for supporting of cell adhesion and spreading within resilin-based materials (Renner et al. 2012a, Renner et al. 2012b, Su et al. 2014). In addition, Resilin RLP12 hydrogels exhibited the comparable mechanical strength and extensibility similar to native vocal fold tissues at high frequencies that corresponded to the human voice (Li et al. 2013). Moreover, resilin RLP24 supported viability and spreading of encapsulated human aortic adventitial fibroblasts useful for cardiovascular applications (Li and Kiick 2013b, Li et al. 2011, Li et al. 2013).

A successful biomedical scaffold in tissue regeneration must allow homogeneous cell distribution within the whole cell culture matrix for inducing cellular activities such as cell attachment, proliferation and even differentiation, and then regenerate complex architectures of various tissues. Three-dimensional (3D) bioprinting technology is coupled with an accurate positioning system based on a deposition/encapsulation system. Recently it has been introduced in tissue engineering because of their efficiency and versatility in cell distribution within the 3D structures, enabling 3D printing of biocompatible advanced materials, cells and supporting components into complex 3D functional living tissues (Kolesky et al. 2014, Murphy and Atala 2014, Wang et al. 2005). With this particularly complex 3D encapsulating printer used, the protein-based biomaterials might be possible to translate directly into cell-laden scaffolds, and the sol-gel transition might also be designed and controlled by quickly heating (not too long to cause thermal shock to cells) and cooling cycle to obtain the advanced hydrogel from protein solutions (Gasperini et al. 2014). It has been believed that the combination of protein-based advanced biomaterials and directed 3D bioprinting technology will offer a promising strategy for future design and manufacture of soft and hard tissue regenerative substitutes and address the need for tissue and organs suitable for transplantation in regenerative medicine (Skardal and Atala 2014).

3.4 Wounds and Burns Dressing

Natural biopolymers including polysaccharides and fibrous proteins can be used widely for wounds and burns dressing materials that speed up the wound healing process because of their biocompatibility, biodegradability and similarity to the extracellular matrix (ECM), providing an optimal microenvironment for cell proliferation, migration and differentiation. Due to their three dimensional cross-linked polymeric networks, wounds and

burns dressing materials made from natural biopolymers can maintain a suitable moisture and oxygen at the wound level, prevent and keep the wound mainly against microorganisms, and improve the wound healing process, which is useful for the regeneration and repairing of dermal and epidermal tissues (Mogosanu and Grumezescu 2014). Recent studies of several promising biopolymers will led to a substantial development of advanced wound dressings for regenerative medicine, such as silk fibroin and collagen (Ghezzi et al. 2011, Wlodarczyk-Biegun et al. 2014, Zhu et al. 2015).

As the natural biopolymer sutures for wound ligation with a long history of applications in the human body, silk fibers fulfill complex surgical needs for advanced wound dressing including good biocompatibility, slow and controllable biodegradability, flexibility (i.e., elasticity), and minimal inflammatory reaction (Altman et al. 2003, Fu et al. 2009, Heim et al. 2009, Lewis 2006, Wharram et al. 2010). Silk sutures have been applied for the treatments of skin wounds, lips, eyes and oral surgeries. Therefore, various systems with silk fibroins (SF) used have been explored for advanced wound dressing or healing, such as silk porous films (Gil et al. 2013), electrospun silk nanofibers with multiwalled carbon nanotubes (Jeong et al. 2014), silk-alginate-blended sponges/membranes (Mehta et al. 2015).

Compared to silk materials, other natural biomaterials including hyaluronic acid, gelatin and alginate may not provide sufficient mechanical strength, with the weakness of accelerated degradation (Altman et al. 2003, Wang et al. 2007, Wong Po Foo and Kaplan 2002). Collagen is the most abundant protein in the human body and the skin and is commonly used for wound dressings with minimal to moderate exudates (An et al. 2013, Muralidharan et al. 2013, Ruszczak 2003). Numerous studies of different collagen dressings have been reported for wounds and burns with various formulations, such as collagen sponges for deep skin wounds, collagen resorbable membranes for oral wounds, collagen electrospun nanofibrous scaffolds for wound repair, and collagen hydrogels for wound infections (Chang et al. 2010, Jorgensen 2003, Kim et al. 2015, Oryan 1995, Rudnick 2006). Collagen can also cross-link with other natural polymers to generate advanced composite wound dressing materials, such as collagen – alginic acid cross-linked biopolymers with thermostability and biodegradability (An et al. 2013, Sarithakumari and Kurup 2013, Sell et al. 2009).

4. Design and Exploration of Artificial Advanced Biomaterials

The fundamental understanding of the sequence – structure – function properties of naturally occurring structural proteins plays a key role in

design and synthesis of novel advanced materials with the ability to self-assemble, respond to stimuli, and/or promote cell interactions. However, the design for advanced biomaterials from natural sources was limited by restricted material amounts, the heterogeneity of post-translational modifications, the inability to readily introduce point mutations into the sequences and other changes tailored for precise control over spatial and temporal release. Unlike the majority of synthetic chemical strategies used, the developments in genetic engineering and DNA manipulation techniques enable the optimization of structure and *de novo* design of protein-based advanced materials, allowing for the production of monodisperse polymers with interesting mechanical and biological properties. Such properties can be attributed to specifically defined structural modules, originating from a modular domain of natural proteins such as silk, elastin, collagen and resilin and unique secondary structures combined with great flexibility, as well as functional elements identified from other proteins such as cell binding sites or enzymatic domains (Grove and Regan 2012).

Recent advances in genetic engineering have provided a promising approach to design and synthesize artificial protein-based advanced biomaterials with similar behaviors to their native counterpart, enabling the self-assembly into fibrous structures with a regularly repeating and well-defined secondary structure. Various protein expression systems including bacteria, yeast, plant and mammalian cells have been investigated for cloning and expression of native and synthetic protein biopolymers to mimic the modular primary structure of proteins with unique physical and biological properties (Fahnestock et al. 2000, Tokareva et al. 2013, Winkler and Kaplan 2000). Among of them, *Escherichia coli* is widely used to express protein biopolymers such as silk, elastin, resilin and other biomimetic protein polymers, because of the easy genetic manipulation of the target genes and simple purification procedures afterwards, even if *E. coli* lacks post-translational modification for eukaryotic coding sequences. Moreover, besides making the large synthetic genes encoding the repetitive amino acid sequences and producing the repetitive protein biopolymers in various host cells, recombinant DNA techniques are ideally suited for the introduction of additional functionalities by re-engineering biologically functional peptide motifs such as cell-binding domains to the repetitive gene, showing their good biocompatibility and biodegradation when implanted and extending their biomedical applications as new advanced biomaterials including drug delivery and tissue engineering (Kim 2013).

Silk as a natural protein fiber plays crucial roles in the survival and reproduction of many silk-spinning insects (Sutherland et al. 2010), exhibiting different compositions, structures and properties based on special sources. Spider silks have not been commercialized for biomaterials production because of the predatory nature of spiders and the relatively

low levels of silk production in an orb web. Recently the strategy of genetic engineering has provided new opportunities to overcome these limitations by cloning and expression of recombinant spider silk genes in bacteria, and multimeric and chimeric silk-like proteins (SLPs) fused in a single protein with multiple properties will be extremely valuable for advanced biomaterial investigations in various applications (Fig. 6). Genetically engineered SLPs may therefore be constructed using synthetic oligonucleotide version of consensus repeats based on highly repetitive amino acid sequences of silks (Altman et al. 2003, Tokareva et al. 2013, Wong Po Foo and Kaplan 2002). A variety of hosts were used for longer SLPs expression, including bacteria (*E. coli*), transgenic silkworms, transgenic plants and mammalian cells. Specially, various types of recombinant spider silks can be expressed in *E. coli* for structure characterization and assembly regulation through genetic manipulation (Partlow et al. 2014, Tokareva et al. 2013, Wong Po Foo and Kaplan 2002), allowing the precise control and efficient packing of silk proteins for the mechanical strength and stability of silk fibers, as well as the control of cell interactions and the rate of degradation (Altman et al. 2003, Horan et al. 2005, Omenetto and Kaplan 2010, Tokareva et al. 2013, Valluzzi et al. 2002). However, recombinant silk materials with limited SLP length (a critical factor defining silk mechanical properties) might not recapitulate the full mechanical potential of native silk fibers and the large-scale yield of spider silks with longer proteins remains challenging

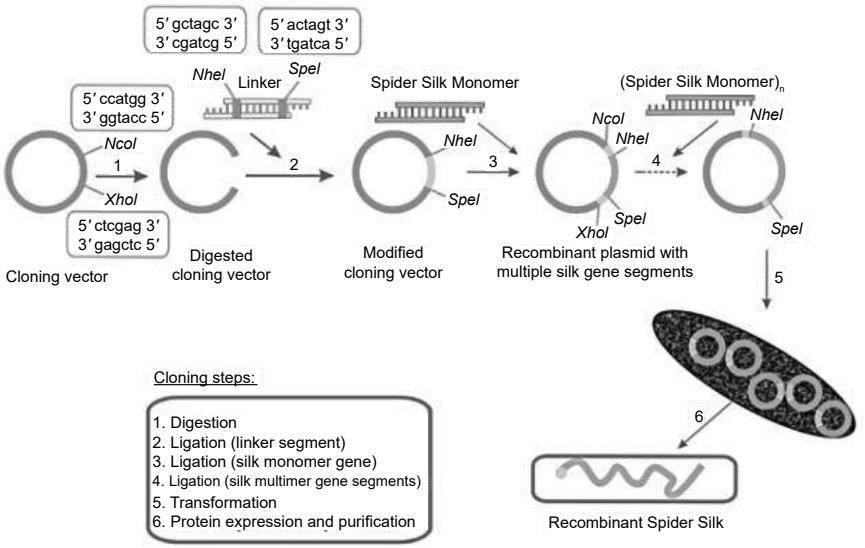

Figure 6. Recombinant DNA approach used to prepare silk-like proteins. Reprinted with permission from Ref. (Tokareva et al. 2013). An open access article under the terms of the Creative Commons Attribution License.

to express in bacteria, because of the insoluble expressed products and high glycine content within highly repetitive sequences. Previously, a SLP composed of spider silk MaSp1-derived sequence was successfully expressed in *E. coli* with a molecular weight of ~ 285 kDa, showing a comparable mechanical properties to native spider silks (Xia et al. 2010).

Elastins are extremely valuable for stimuli-responsive applications because of their high extensibility and stimuli-triggered self-assembly and molecule delivery. Natural elastin can be extracted from tissues by harsh alkaline treatments but with a poor yield (Gasperini et al. 2014). However, recombinant elastin-like polypeptides (ELPs) can recapture the original elasticity and thermo-responsiveness of elastins, which commonly use the hydrophobic domain-derived pentapeptide repeats 'VPGVG' (the most abundant sequence in natural human elastin) or more generally 'VPGXG' ('X' can be any amino acid except proline). Precise control over the ELP sequence can also be used to create the sequence architecture that enables ELP self-assembly. ELP diblock copolymers can be designed for self-assembled materials, consisting of a hydrophobic ELP segment fused to a hydrophilic ELP segment. An example from an elastin-derived material ELP_{BC} composed of a hydrophilic $[VPGEG(IPGAG)_4]_{14}$ block and hydrophobic $[VPGFG(IPGVG)_4]_{16}$ block connected via a VPGEG linker results in a self-assembly behavior of aggregation of the micelles into micron-scale particles (Le et al. 2013, Rodriguez-Cabello 2004, Wright and Conticello 2002). Recombinant ELP proteins are mainly expressed in *E. coli* and purified through relatively easy procedures due to the thermo-responsiveness of elastin with a reversible sol-gel transition upon heating. Recent studies on thioredoxin, tendamistat and virus capsid proteins have also demonstrated the potential of thermo-responsive ELP sequences as fusion tags for purification of other recombinant proteins (Meyer and Chilkoti 2004, Meyer et al. 2001, Rodriguez-Cabello 2004).

Recently, the strategies combining two or more structural proteins offer opportunities to create *de novo* non-natural chimeric advanced biomaterials tailored for specific applications. For example, the silk-elastin chimeric proteins have been generated to make the advanced silk-elastin-like polymers (SELPs) combining the high tensile strength of silk and high resilience of elastin in a single structure, where the silk blocks mimic the natural silkworm fibroin and tend to assemble into β-sheets while the elastin blocks are highly hydrated and disordered and provide thermo-response self-assembly. Different SELPs have been produced by varying the ratio of silk to elastin blocks, exhibiting large differences in solubility and different phase separation and self-assembly below and above the elastin transition temperature (Xia et al. 2011a). When engineered elastin blocks into chimeric fusion proteins, the overall secondary structure, stability and

thermo-responsive self-assembly properties depend on the orientation and number of elastin blocks. Previous studies of chimeric system containing elastin blocks and coiled-coil matrix proteins have shown the decreased thermo transition temperatures from 27.8°C to 59.8°C if increasing elastin block number from one to four, as well as the importance of protein block directionality in fusion system for phase transition behavior, providing the additional tunable features for future advanced biomaterial design (Dai et al. 2011). The other chimeric protein based on resilin-elastin-collagen polypeptides (REC) was also designed and demonstrated the remarkable elasticity and self-assembly into fibrous structures with a Young's modulus between 0.1 and 3 MPa, much softer than collagen-like bundles (Bracalello et al. 2011). Specially the fully biosynthetic analogues to protein-polymer conjugates, mCherry-ELPs fusion proteins containing a thermo-responsive coil-like protein, ELP, and a globular protein, mCherry, have been developed, showing the self-assembly of biofunctional nanostructures such as hexagonal and lamellar phases in concentrated solutions. This new system provides a rich landscape to explore the capabilities of fusion architecture to control supramolecular assemblies for advanced heterogeneous biocatalysts (Qin et al. 2015).

5. Summary

Natural protein-based advanced biomaterials are reviewed in this chapter, looking at the recent advances in a broad range of natural polymeric biomaterials such as silk, elastin, resilin and others. Their protein composition and molecular structure, mechanical properties, and biomedical engineering applications were discussed, as well as the design, bioengineering, and processing of advanced biomaterials for biomedical engineering applications. Naturally occurring polypeptide-based biomaterials show incredibly outstanding properties when compared to synthetic polymer materials. Therefore, the likely roles for the compelling class of advanced biomaterials are likely to increase significantly, allowing mechanically robust, slowly degrading and versatile biomaterial designs with low inflammation and low immune response. Controllable processability and surface modifications also expand their utility in drug delivery and functional tissue engineering. To create novel and enough protein materials, the alternative way instead of growing natural sources including animals and insects, recombinant DNA technique has been introduced to generate these advanced biomaterials on a large scale. It is possible that the material structure and properties may be fine tuned and defined for protein-based natural biopolymers with the advances in protein engineering. A future challenge will be to scale-up the protein production

and purification of recombinant protein biopolymers with all necessary modifications, and finally extend the application of advanced biomaterials in biomedical engineering.

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