Preparation and characterization of biomimetic materials inspired by jumbo squid sucker ring teeth

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NANYANG
TECHNOLOGICAL
UNIVERSITY

**Preparation and Characterization of
Biomimetic Materials Inspired by Jumbo Squid
Sucker Ring Teeth**

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School of Materials Science and Engineering

2016

Preparation and Characterization of Biomimetic Materials Inspired by Jumbo Squid Sucker Ring Teeth

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List of Publications and Conference Presentations

Publications

1. Victoria Latza, Paul Guerette, **Dawei Ding** *et al.*, Multi-Scale Thermal Stability of a Hard Thermoplastic Protein-Based Material. *Nature Communications* (2015). DOI:10.1038/ncomms9313.
2. Bram Cantaert, **Dawei Ding** *et al.*, Stable Formation of Gold Nanoparticles onto Redox-Active Solid Bio-Substrates Made of Squid Suckerin Proteins. *Macromolecular Rapid Communications* 36 (2015) 1877-1883.
3. **Dawei Ding** *et al.*, From Soft Self-Healing Gels to Stiff Films in Suckerin-Based Materials Through Modulation of Crosslink Density and β -Sheet Content. *Advanced Materials* 27 (2015) 3953-3961.
4. **Dawei Ding** *et al.*, Biomimetic Production of Silk-Like Recombinant Squid Sucker Ring Teeth Proteins. *Biomacromolecules* 15 (2014) 3278–3289.
5. Paul Guerette, Shawn Hoon, **Dawei Ding** *et al.*, Nanoconfined β -Sheets Mechanically Reinforce the Supra-Biomolecular Network of Robust Squid Sucker Ring Teeth. *ACS Nano* 8 (2014) 7170-7179.
6. Paul Guerette *et al.*, **Dawei Ding**, Ali Miserez, Accelerating the design of biomimetic materials by integrating RNA-seq with proteomics and materials science. *Nature Biotechnology* 31 (2013) 908-918.

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Abbreviations

3D	Three dimensional
5-FU	Fluorouracil
AAA	Amino acid analysis
ABS	Acrylonitrile butadiene styrene
AFM	Atomic force microscopy
Ala	Alanine
APS	Ammonium persulfate
ATR	Attenuated total reflection
AUC	Area under curve
<i>B. mori</i>	<i>Bombyx mori</i>
CBR	Coomassie blue R
CD	Circular dichroism
<i>D. gigas</i>	<i>Dosidicus gigas</i>
DLS	Dynamic light scattering
DMEM	Dulbecco's modified eagle medium
DSC	Differential scanning calorimetry
<i>E</i>	Young's modulus or elastic modulus
ECM	Extracellular matrix
<i>E. coli</i>	<i>Escherichia coli</i>
EDX	Energy-dispersive X-ray spectroscopy
EthD-1	Ethidium homodimer-1
FESEM	Field emission scanning electron microscopy
FPLC	Fast protein liquid chromatography
FTIR	Fourier transform infrared spectroscopy
Gly	Glycine
<i>H</i>	Hardness
HCDC	High cell density cultivation
HDF	Human dermal fibroblasts
HEK 293 cells	Human embryonic kidney 293 cells
His	Histidine
hMSCs	Human mesenchymal stem cells

HPLC	High performance liquid chromatography
Hyp	Hydroxyproline
IEP	Isoelectric point
IFs	Intermediate filaments
IPTG	Isopropyl β -D-1-thiogalactopyranoside
IMAC	Immobilized-metal affinity chromatography
LCST	Lower critical solution temperature
MALDI-TOF	Matrix-assisted laser desorption ionization time-of flight
MES	2-[N-Morpholino]ethanesulfonic acid
MW	Molecular weight
Ni-NTA	Nickle-Nitrilotriacetic acid
PBS	Phosphate buffer saline
PCL	Polycaprolactone
PDMS	Poly(dimethylsiloxane)
PEEK	Polyether ether ketone
PEO	Polyethylene glycol
PMMA	Poly(methyl methacrylate)
PMSF	Phenylmethanesulfonyl fluoride
Pro	Proline
s.d.	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
s.e.	Standard error
<i>S. esculenta</i>	<i>Sepia esculenta</i>
<i>S. lessoniana</i>	<i>Sepioteuthis lessoniana</i>
SRT	Sucker ring teeth
SUMO	Small ubiquitin-related modifier
TCP	Tissue culture plastic
TEV	Tobacco etch virus
TGA	Thermal gravimetric analysis
Thr	Threonine
Tyr	Tyrosine
Val	Valine
WAXS	Wide-angle X-ray scattering

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Abstract

Dosidicus gigas (*D. gigas*, or Jumbo squid) sucker ring teeth (SRT) are newly discovered biological materials with intriguing combination of mechanical and physico-chemical properties. Notably, recent studies have shown that SRT are devoid of minerals, chitin and inter-chain covalent cross-links, and are entirely constituted of proteins called “suckerins” which form a polymer network reinforced by self-assembled nano-scale β -sheet structures. At the molecular (primary sequence) scale, suckerins display a block co-polymer sequence consisting of two tandem alternating modules. One is rich in alanine (Ala) and resembles the poly-Ala or poly(glycine (Gly)-Ala) domains of silk proteins and form β -sheets, and the other is dominated by Gly accompanied with high contents of tyrosine (Tyr) and leucine (Leu) residues. These characteristics make SRT a promising model for biomimetic materials research. In this project, therefore, we hypothesize that functional materials with β -sheet structures could be engineered with SRT proteins and these materials could be utilized in a broad range of biomedical and engineering applications that have been demonstrated for silks. Furthermore, the goal of the project is to further deepen the structure/property relationships of SRT, notably to link their thermo-mechanical response with the protein structure of suckerins, which is unknown prior to this work. We hypothesize such structure/property relationships would provide molecular scale biomimetic principles to develop a new range of protein- and peptide-based materials.

Characterization of SRT by nanoindentation was conducted in different solvents that could disrupt their putative secondary structures. We found that β -sheets disruption in SRT was correlated to a concomitant decay of Young's modulus (E), which provided the first direct evidence of hydrogen bonding localized in β -sheets as an essential contributor to SRT mechanics. This also explained the fundamental

structure-property relationship of SRT. SRT and suckerins also displayed thermoplastic properties, where the crystalline β -sheets and amorphous domains exhibited different thermal stabilities and thermo-mechanical responses. These intriguing characteristics motivated us to fabricate materials with native SRT proteins, including melt-spun fibers and electro-spun nanofibers. The melt-spun fibers displayed polymer chain alignment and comparable mechanical strength to regenerated silk fibers. Electro-spun nanofibers were made by using an additive and a systematic optimization of electro-spinning parameters that led to smooth nanofibers.

In order to sustainably and efficiently supply SRT proteins for materials engineering, a recombinant expression system for suckerin-19 was established. The production of two suckerin-19 variants was achieved and a facile chromatography-free purification method was developed, with promising protein yields. In order to demonstrate the hypothesis that recombinant SRT proteins were also able to produce materials, suckerin-19 was fabricated into tunable materials by a Ru^{II} mediated photo-cross-linking method. This method allowed the formation of hydrogels and films whose surface roughness, cross-link density and β -sheet content could be tailored. Importantly, the stiffness of these gels and films could be modulated over 7 orders of magnitude by varying the cross-linking conditions or by the addition of a common plasticizer. This tunability of E enables them to match the elasticity of a wide range of human tissues from soft liver to stiff bone, opening promising avenues in tissue engineering and restorative applications. Overall, this research demonstrates that suckerins' characteristics, namely β -sheet self-assembly, modular sequence design, impressive mechanical properties and biocompatibility, provide a novel toolbox of protein-based materials to biomaterial scientists, with a broad spectrum of potential biomedical and engineering applications.

Chapter 1 Introduction

Material science is a vibrant field which is continuously playing a critical role in driving human being's life and society. A century ago, only around 10 types of materials were widely used by human beings: wood, cotton, brick, several types of metals, and a few plastics.^[1] However, thousands of new materials are emerging in modern society and filling up the needs of a vast number of applications, from solar cells to environment-friendly paints, or from high performance alloys to multi-functional carbon nanotubes. While materials science initially focused on integrating rigorous scientific disciplines such as solid-state physics and chemistry to create new complex materials which display properties beyond those of pure and simple solids, it has increasingly looked into the biological world in recent decades.^[2] The rationale behind this pursuit is that Nature has developed an enormous range of biological materials over millions of years of Evolution –from those present in plants to those synthesized by living organisms– with useful material properties and multi-functionality.^[3]

1.1. Biological and biomimetic materials

Biological materials are chemical substances or mixtures of substances present or produced by a living organism. These materials allow organs to function, plants to stand upright and animals to fly or move. Importantly, there exist a few interrelated characteristics which distinguish them from their synthetic counterparts: **(i)** The choice of elements. Nature usually uses only a few elements in the synthesis of biological minerals and biopolymers including proteins and polysaccharides: C, H, O, N, P, S, Ca, Fe etc.^[4] On the other side, man-made materials employ many more elements for materials fabrication.^[3] **(ii)** Biological materials are produced by

precisely controlled self-assembly mechanisms. In contrast to many synthetic materials, biological materials are usually assembled by “bottom up” strategies rather than “top down” ones.^[2] For example, in order to form chitin nanofibrils which are the major structural component of cell walls in fungi and yeast, the exoskeleton of arthropods, and mollusk shells,^[5] 19 to 25 chitin chains self-organize in an anti-parallel way into long crystalline α -chitin nanofibrils (Figure 1.1).^[6] **(iii) Hierarchy.** Biological materials are virtually all multi-scale structures from the nano- to the macroscopic scale.^[2] The cuticle of lobster is a representative model, whose structure can be divided into various length scales, starting from single chitin molecules to chitin nanofibrils, further to chitin-protein fibers embedded in a mineral-protein matrix, and finally to honeycomb-like chitin-protein planes which gradually rotate around the normal direction of the cuticle, resulting in a twisted “plywood”. These plywood structures form three macroscopic layers with different contents of minerals that build the final cuticle (Figure 1.1).^[6] **(iv) The physico-chemical conditions at which the materials are made.** Most biological materials are produced at room temperature, atmospheric pressure, and in an aqueous environment.^[2, 7] **(v) Multi-functionality.** Many biological materials serve more than one purpose.^[4] For instance, bones are able to provide structural support, while at the same time facilitating the production of red blood cells. Because all of these reasons, biological materials serve as intellectually-stimulating sources of inspiration to solve a number of challenges in materials science, which has led to the development of “biomimetic materials science”. Biomimetic materials science aims to elucidate structure-function relationships of biological models and later to apply and translate these underlying design principles into the fabrication of new materials which mimic natural ones.^[8]

Figure 1.1 Self-assembly and hierarchical structure of the lobster cuticle: (I) *N*-acetyl-glucosamine molecules, (II) antiparallel chains of α -chitin, (III) chitin-protein nanofibrils, (IV) chitin-protein fibers in a mineral-protein matrix (not shown), (V) cuticle with pore canal system (in-plane cross-section), (VI) twisted plywood structure, and (VII) three-layered cuticle. The figure is adapted from Ref.^[6]

1.2. Jumbo Squid Sucker Ring Teeth

Within the numerous biomimetic animal model systems, cephalopods (especially squids) have attracted increasing interest in various areas of biomimetic engineering due to a number of physiological characteristics, including their highly evolved sensory systems,^[9] remarkable camouflage abilities,^[10] fast and strong tentacles and arms,^[11] as well as strong ring-shape teeth within their suckers.^[12]

In this project, we are interested in the jumbo squid (Humboldt squid), *Dosidicus gigas*, a large, aggressive, and predatory species commonly encountered throughout the Eastern Pacific (Figure 1.2a).^[13] These squids possess two hard tissues used in their predation strategies, which have triggered broad interest from the biomimetic materials research community. The first one is the tough, wear-resistant beak (Figure

1.2a) used to lacerate tissues and subdue preys, and whose tip is one of the hardest and stiffest materials entirely composed of organic building blocks.^[14] It is composed of a biomolecular composite made of hydrated chitin and Gly- and Histidine (His)-rich proteins.^[15] These building blocks exhibit an opposing compositional gradient, resulting in a mechanically graded material which is tailored through inter-protein and protein-chitin covalent cross-linking.^[16]

Besides the beak, jumbo squids possess another load-bearing material of interest: the sucker ring teeth (SRT) located inside the suckers that are lining the squid's arms and tentacles (Figure 1.2a). Each SRT contains a basal ring and a series of formidable triangular teeth (Figure 1.2b),^[12] which enhances the predatory efficiency of jumbo squid.^[17] These tentacles help the animal grabbing its prey by suction and movement restriction, while the inward bent sucker rings penetrate into the flesh or skin of the prey, allowing them to firmly hold onto it.^[4]

Figure 1.2 Overview of *D. gigas* and its sucker ring teeth. **(a)** A schematic for morphology of jumbo squid and its two hard tissues: beak and sucker ring teeth (Adapted from Ref.^[18]). **(b)** An image of jumbo squid SRT (Adapted from Ref.^[19]).

1.2.1. Microstructure of SRT

Studies have shown that the SRT's microstructure is composed of parallel, nano-scale tubular elements (Figure 1.3). The nano-sized channels are oriented parallel to

the tooth axis and spread out through the base (Figure 1.3a-c). The pore fraction across a given tooth is not uniform: there is a gradual decrease in pore fraction from the center to the periphery of the tooth in the cross-section (Figure 1.3d). This is achieved by both a decrease in channel diameter and an increase in channel spacing. The channel diameter increases from 100 nm (close to the tooth surface) to 260 nm (going inside about 20% of the tooth diameter from the tooth surface), while the pore fraction increases from <2% (near the periphery) to approximately 20% (tooth core).^[12]

Figure 1.3 Microstructure of *D. gigas* SRT. (a) A scheme of SRT cross-section revealing the channel organization from a tooth tip to the basal ring. The SEM images shown in (b) and (c) reveal the highly parallel nature of the channels. (d) Pore fraction as a function of distance from the tooth periphery to the core, and including two representative SEM images of tooth longitudinal sections, illustrating the changes in both channel diameter and spacing as a function of location. The Figure is adapted from Ref. ^[12]

1.2.2. Mechanical properties of SRT

The nano-sized parallel channel microstructure of SRT directly influence their mechanical properties (Figure 1.4).^[12] In the dehydrated state, the elastic modulus (E) and hardness (H) reach values as high as 8-10 and 0.7 GPa respectively (near the tooth periphery), which match the values of strong synthetic polymers such as PMMA, PEEK, or polyamides.^[20] Interestingly, there is a gradual decrease in both E and H from the periphery to tooth core on the cross-section: they reach a minimum of ~4.5

and 0.4 GPa respectively in the tooth core (Figure 1.4b). The teeth in hydrated conditions exhibit similar gradients, with the modulus varying from 2.75 GPa (periphery) to 1.75 GPa (core) and the hardness from 0.25 to 0.15 GPa.^[12]

Figure 1.4 Micromechanical properties measured on cross-sections of *D. gigas* SRT. (a) Illustration of the microtome cutting plane. (b) Maps of modulus and hardness in dehydrated and hydrated conditions measured by nanoindentation. The Figure is adapted from Ref.^[12]

1.2.3. Chemical composition and molecular structure of SRT

In SRT, C, N, O, are uniformly distributed, and trace amounts of Cl and S have also been detected, but no metals or minerals, the common phases used by Nature to make hard tissues, are found (Figure 1.5a).^[12] Chitin, a common biological structural polysaccharide, is also absent in SRT (Figure 1.5b),^[14] which is a surprising fact since chitin comprises about 20 wt% of the *D. gigas* beak tip and is majorly responsible for the beak's mechanical properties.^[15, 21] In previous literature, the teeth are often wrongly described as being “chitinous”. On the other hand, SRT are entirely constituted by proteins called “suckerins” which are overall rich in glycine (Gly, 37 mol. %), tyrosine (Tyr, 14 mol. %), and histidine (His, 13 mol. %) (Figure 1.5c&d).^[12, 17, 19]

Natural and synthetic bulk polymers are mechanically stabilized or reinforced by a few different physico-chemical strategies. The first is chain entanglement as found

in classic amorphous thermoplastic polymers.^[17] The second strategy is dense covalent inter-chain cross-linking, which are commonly found in thermoset resins for synthetic materials, and insect exoskeletons^[22] and squid beaks for natural biopolymers composites.^[15] In addition, many materials are strengthened by the addition of a reinforcement phase, such as fine mineral particles in bone and other biominerals^[23] or in engineered nanocomposites.^[24] Strikingly, there are no inter-chain covalent cross-links present between proteins in SRT. Instead, suckerin proteins assemble into nano-confined β -sheets to form a supramolecular network, thus reinforcing SRT as described below.^[17, 19]

Figure 1.5 Chemical composition of *D. gigas* SRT. (a) EDX spectrum of SRT. (b) Amino acid analysis spectra (blue curve) showing the region where the acid hydrolysis product of chitin, glucosamine, would be expected, compared to a glucosamine standard (pink curve); no chitin is detected. (c) SDS-PAGE analysis of sucker ring teeth proteins. (d) Primary sequence of representative suckerins illustrating their modular architecture with alternating Ala- and His-rich (red) and Gly- and Tyr-rich domains (blue). These domains are composed of smaller

repeats with homology to dragline silks. (e) Color-coded detailed primary sequence of suckerin-19. The panels are adapted from Refs. ^[12, 17, 19]

At the molecular level, suckerins (the most abundant protein being suckerin-19 based on SDS-PAGE) are block co-polymer like biomolecules displaying a regular modular sequence design^[25] which contains two major alternating modules (Figure 1.5d&e). The first module is dominated by alanine (Ala) with significant His residues and is reminiscent of poly-Ala domains found in spider silk proteins (spidroins),^[26] which form β -sheet structures to reinforce the material.^[27] The second module is featured by Gly-rich domains with a large amount of Tyr and leucine (Leu) residues appearing as tri- and tetra-peptides, for example GGY, GGL, or GGLY. Micro-Raman spectroscopy (Figure 1.6a) on ultra-microtomed SRT cross-sections indeed indicate a clear β -sheet band across the entire ring in the amide I band matching that of *Bombyx mori* silk.^[28] In contrast to spider dragline and silkworm silks, polarized Raman spectra suggest that the β -sheet domains are randomly oriented inside SRT (Figure 1.6b). Further detailed examinations of *D. gigas* SRT by synchrotron Wide Angle X-ray Scattering (WAXS) have indicated a circular scattering pattern, with equal intensity at all azimuths, confirming the random orientation of β -sheet domains (Figure 1.6c). Integration across all azimuthal angles revealed peaks attributed to reflection coming from [120], [200], [002] and [100] planes (Figure 1.6d), which are in agreement with silkworm^[29] and spider silks.^[30] β -sheet dimensions are estimated to be 2.4-2.6 nm in the hydrogen bond direction and 3-3.5 nm in the peptide backbone direction by peak broadening analysis of the deconvoluted pattern using the Scherrer equation (Figure 1.6d), corresponded to 5 strands in width and ~8-10 amino acids in length, respectively.^[17] Importantly, proline (Pro) is highly conserved within the sequences (Figure 1.5e). Pro is a well-known β -sheet disruptor and is found to flank

the Ala- and His-rich modules, thereby constraining β -sheet size to only 10-11 residues within this module (corresponding to β -strands ~ 3.1 - 3.5 nm in length, in a good agreement with WAXS data). In addition, amorphous domains are also detected in SRT (Figure 1.6d), leading to an overall semi-crystalline structure made of amorphous domains reinforced by randomly oriented nanoconfined β -sheets (Figure 1.6e).^[17] The isotropic distribution of β -sheet domains is consistent with the predatory function of jumbo squid SRT, which must resist complex compressive, shear and torsional load regimes, with β -sheet domains providing the load-bearing function.^[25]

Figure 1.6. Protein structures of *D. gigas* SRT probed by Raman spectroscopy and WAXS. **(a)** Raman spectrum of a microtomed SRT section compared against a *B. mori* silk standard, showing matching of the amide I band centered at $1,666\text{ cm}^{-1}$, which is attributed to β -sheet conformation. **(b)** Raman spectra in cross-polarized modes suggest that the β -sheet domains in SRT are randomly oriented. **(c)** WAXS pattern of SRT (tip region) and **(d)** integrated spectrum (all azimuthal angles), indicating reflection positions consistent with β -sheets (inset: estimation of β -sheet size using the Scherrer analysis). Deconvolution indicates a broad amorphous halo. **(e)** Semicrystalline structure of SRT, consisting of randomly oriented β -sheets within an amorphous matrix. The panels are adapted from Refs.^[17, 25]

1.3. Scope of the Project

1.3.1. Hypothesis of the project

Given the outstanding mechanical properties of jumbo squid SRT, their sole protein composition with a large β -sheet content reminiscent to silks, the modular “block co-polymer” like primary sequences of SRT proteins and their homology to silk fibroin sequences, we hypothesize that diverse forms of functional materials could be prepared with SRT proteins, similarly to silk proteins. Like native SRT, recombinant suckerins self-assembly will be driven by β -sheet formation and these materials could be used in a wide range of engineering and biomedical applications. Considering the broad biomedical and engineering applications that have been demonstrated for silk materials, including in tissue engineering,^[31] drug delivery,^[32] and photonics,^[33] we hypothesize that similar applications could be demonstrated with suckerins. In addition, although the mechanical properties, chemical composition, protein sequencing and primary sequences have been investigated for jumbo squid SRT, we hypothesize that in-depth structure/property relationships will result in molecular-scale design principles that could be used for *de novo* development of protein and peptide-based materials.

1.3.2. Objectives and scope of the project

Considering the hypotheses stated above, four detailed aims are devised in this project, which are corresponded to the following 4 chapters of the thesis.

Aim 1: To further characterize the physico-chemical and thermo-mechanical properties of SRT (Chapter 3).

Although β -sheets have been identified in jumbo squid SRT, the direct evidence of β -sheets' contribution to the impressive mechanical properties still needs to be

elucidated. We will measure the elastic modulus by Depth-Sensing Nanoindentation (DSI) of SRT in different solvents, including water, ethanol and a serial of urea solutions at different concentrations in order to dissect out the role of different chemical and physical bonds in SRT intrinsic mechanical properties, including hydrogen bonding and hydrophobic interactions. In addition, thermo-mechanical response of *D. gigas* SRT will be analyzed by a combination of techniques for the purpose of understanding the thermoplastic features of SRT, which could inspire the thermo-processing of materials with SRT proteins.

Aim 2: To fabricate materials from native SRT proteins (Chapter 4).

The sole presence of proteins in SRT, together with the absence of covalent cross-links, the thermoplastic features of SRT and the high solubility of SRT proteins in mild acidic solutions (*e.g.* 5% or higher concentration of acetic acid) enable the straightforward extraction and solubility of SRT proteins. Inspired by the above characteristics, extracted SRT proteins are fabricated into fibers by melt-spinning or nanofibers by electro-spinning, and they will be characterized by scanning electron microscopy (SEM), Fourier Transform Infrared Spectroscopy (FTIR), differential scanning calorimetry (DSC), as well as nano-tensile testing.

Aim 3: To setup a recombinant expression system to produce suckerins artificially (Chapter 5).

In order to obtain a convenient and sustainable source of SRT proteins for materials engineering, we employ a genetic engineering approach to produce recombinant suckerins. We will synthesize and clone the gene of suckerin-19, transform the gene into *Escherichia coli* (*E. coli*) and express the encoded protein in relatively high yields. The protein will be subsequently purified by chromatographic

and non-chromatographic techniques. The key finding of this chapter is that *E. coli* transformed with suckerin-19 gene is able to express the protein, which is purified by facile non-chromatographic methods and displays promising in-solution features for materials processing.

Aim 4: To engineer biomimetic materials with recombinant suckerins (Chapter 6).

In order to demonstrate the potential of suckerins in novel protein-based materials engineering, recombinant suckerin-19 will be used for materials fabrication. Hydrogels and films are prepared by a photo-cross-linking method and their structural and mechanical features will be examined. Furthermore, their cytotoxicity will also be assessed in order to verify their potential for biomedical applications. The key finding is that the mechanical properties of suckerin-19 materials could be tuned by several orders of magnitude by adjusting the cross-linking density, which in turn controls the β -sheet content. In addition, suckerin-19 materials display negligible cytotoxicity in *in vitro* cell culture, suggesting a promising potential for suckerins in biomedical applications.

Chapter 2 Literature Review

Biological materials contain relatively few elements. Only around 10% of stable elements of the periodic table are found as the building blocks of biological tissues.^[4] Biological materials can broadly be divided into 2 broad categories, albeit this differentiation is sometimes blurry:^[2] **(i)** non-mineralized (“soft”) materials which are comprised of biopolymers (*e.g.* collage, keratin, elastin, silk fibroin, chitin and so on) and display a wide range of mechanical properties depending on their functions, and **(ii)** mineralized (“hard”) materials, which are usually hierarchically assembled composites of minerals (*e.g.* hydroxyapatite, calcium carbonate and calcium sulfate) and fibrous biopolymers, mainly collagen and chitin. The organic constituents of biological materials are either structural proteins (primarily collagen, keratin, elastin, resilin, and silk fibroin) or polysaccharides (mainly chitin and cellulose),^[4] although in some cases they are complexes of these main types of building blocks, for instance glycosylated proteins such as mucins found in joint articulations.^[34]

2.1. Structural Proteins

Proteins are long-chain of amino acids with specific sequence, whose side chains are different in size, charge, and hydrophobic/hydrophilic characteristics. Crystalline protein molecules have a few well-defined secondary structures such as α -helix, β -sheet, and β -turn. Structural proteins that constitute biological materials can be further divided into two classes:^[4] **(i)** proteins dominated by stiff secondary structure regions (such as triple-helix in collagen and β -sheets in silk fibroins), and **(ii)** those that are majorly composed of amorphous random coil domains (*e.g.* elastin^[35] and resilin^[36]). The presence of crystalline structures in structural proteins plays a crucial role in the mechanical properties of protein based biological materials: The stiffness

of these crystalline biopolymers is usually several orders of magnitude higher than the amorphous ones, but their failure strains are a few orders of magnitude lower than the later.^[4] Collagen,^[37] keratin,^[38] elastin,^[35] resilin^[39] and silk fibroins^[40] are well-known examples of structural proteins that have caught the attention of biomimetic material scientists (Table 2.1).^[41] They will be discussed in the following.

Table 2.1 Basic features of major structural proteins and their potential applications in the biomedical field.

Protein	Basic structure	Main function in biology	Relevant properties	Biomedical applications	Ref.
Collagen	Three parallel polypeptide chains formed by GXY (G-Gly, X- usually Pro, Y-usually Hyp) repeats and arranged in triple helix	Structural protein in tissues such as connective tissue, tendon, skin, bone and cartilage.	High abundance, biodegradability, low antigenecity and biocompatibility	i) Drug delivery (from small molecules to protein drugs); ii) Vascular and dermal tissue engineering iii) Cartilage and bone repair.	[42, 43] [44, 45] [46, 47]
Keratin	Formed by α -helical coiled-coil dimers assembled into 10 nm wide filaments	Structural protein in the cytoskeletons of vertebrate epithelial cells and epidermis appendages such as hair, nails and wool	Biocompatibility, good cell attachment and growth	i) Nerve injury repair; ii) Liver repair; iii) Bone regeneration; iv) Vascular tissue engineering.	[48] [49] [50] [51]
Silk fibroin	Highly repetitive core domain of alternating hydrophobic poly-A and G rich hydrophilic motifs	Building element of many arthropod nests, cocoons and prey traps.	Self-assembly and remarkable mechanical properties	i) Drug delivery (from small molecules to protein drugs); ii) Tissue repair and engineering (tendon, skin, vascular grafts, bone and corneal regeneration, cartilage repair, cardiac engineering)	[32] [41, 52-54]
Elastin	Cross-linked units of tropoelastin formed by hydrophobic and hydrophilic domains	Structural protein found predominantly in connective tissue of arteries, ligaments, skin and lung	Temperature dependent self-assembly and phase separation behavior	i) Drug delivery; ii) Tissue repair and regeneration (skin repair, vascular tissue engineering and bone repair).	[55-57] [35, 58-62]

2.1.1. Helix based structural proteins

Collagen is the most abundant protein in mammalian bodies, accounting for 20–30% of the total protein content.^[41, 63] It is a stiff (load-bearing) protein majorly produced by fibroblast cells and is present in different organs and tissues such as bones, teeth, cartilage, and blood vessels.^[4, 64, 65] The primary functions of collagen in mammals are to mechanically support the body^[66] and to control cell adhesion, cell migration as well as tissue repair.^[67] There are 29 types of collagens whose amino acid sequence have been identified and this number is continuously increasing. However, 90% of the collagen in the human body comes from five types: **(i)** type I collagen that is majorly found in the skin and bone, **(ii)** type II collagen, the chief component of cartilage, **(iii)** type III collagen that usually comes along with type I, **(iv)** type IV collagen which is found in the cell basement membrane and **(v)** type V collagen, the chief component of hair and the placenta.^[4]

A key feature of collagen is the frequent occurrence of the amino acid sequence Gly-Pro-X or Gly-X-Hyp (Gly: glycine; Pro: proline; Hyp: hydroxyproline; X: other amino acids) (Figure 2.1a). All collagens share the same triple-helical structure at the molecular scale, where three parallel polypeptides (α -chain) coil around each other to form a right handed triple helix chain called tropo-collagen (Figure 2.1b).^[41] The high content of Gly, the smallest amino acid with only a single hydrogen atom on the side chain, allows hydrogen bonding between chains within the helix and stabilizes the triple helical structure of collagen.^[68] The tropo-collagens undergo a hierarchical organization into a staggered array to form collagen fibrils that are $\sim 1\ \mu\text{m}$ in diameter. The staggering of tropo-collagen gives rise to two important dimensions, the gap and the overlap regions that work together to form the characteristic banded structure of

67 nm spacing (Figure 2.1b).^[4, 63] At the next level of hierarchy, multiple fibrils make up the collagen fibers (~10 μm in diameter) which are found in different tissues.^[65]

Figure 2.1 Structure of collagen. (a) major amino acid residues; (b) tropo-collagen assemble into a triple helix of collagen strands; tropo-collagen assembles to form collagen fibrils, a staggered arrangement of 300 nm long molecules form gap and overlap regions in the fibril (~1 μm diameter). The figure is adapted from Refs.^[4, 65]

Keratin is another structural protein dominated by helical structures, which is commonly found in hair, nails, hooves, horns and the epidermal layer of skins in mammals.^[69] Keratin molecules are also hierarchically organized to form keratin fibers in diverse tissues: two single α -helix chains of keratin bundle together to form a superhelix dimer (coiled-coil) that is usually stabilized with disulfide cross-links. The coiled coils assemble into proto-filaments and then dimerize to form intermediate filaments (IFs), which are embedded in a sulfur-rich amorphous keratin matrix,^[70] resulting in a polymer/polymer composite reinforced by crystalline IFs.^[4] There are a few key properties of keratins that open a wide avenue for the development of keratin-based biomaterials for biomedical applications: (i) the intrinsic ability to self-assemble and polymerize into porous, fibrous scaffolds;^[71] (ii) the presence of cell binding domains in the protein sequence, including leucine-aspartic acid-valine (LDV) and glutamic acid-aspartic acid-serine (EDS) binding motifs;^[72] (iii) the ability to

mimic the extracellular matrix (ECM).^[51] Extracted keratins can be processed into films, scaffolds, hydrogels, and electrospun fibers for the engineering/regeneration of a variety of tissues, such as the nerve injury repair,^[48] liver repair,^[49] bone regeneration^[50] and vascular tissue engineering.^[51]

2.1.2. β -sheet based structural proteins

Structural proteins dominated by β -sheet domains are another class of load-bearing proteins in biological materials. Silk fibers are typical materials made of such proteins. Silk is a material system characterized by its outstanding mechanical properties that can out-compete high performance manmade fibers such as Kevlar, nylon and high-tensile steel (at room temperature).^[41, 73] They are produced by spiders^[73] and insects^[74] for different uses such as producing cocoons and nests. Silk fibers are primarily composed of proteins (silk fibroin) and some other macromolecules like lipids.^[75] For example, the silkworm silk produced by *Bombyx mori* (*B. mori*) is formed by two microfilaments embedded in a family of hydrophilic sericins which glue the fibroins together. Each microfilament results from the assembly of a hydrophobic ~350 kDa heavy-chain fibroin and a relatively hydrophilic ~25 kDa light-chain fibroin.^[76] Spider silk is in a slightly different structure where two spidroins, major ampullate spidroin protein 1 (MaSp1) and 2 (MaSp2) form an inhomogeneously distributed core filament that is coated by glycoproteins and lipids.^[41, 77]

Studies have revealed that the impressive mechanical properties of silkworm and spider silk fibers are due to the antiparallel β -sheets crystals at the nanoscale, consisting of highly conserved poly-(Gly-Ala) and poly-Ala repeats.^[27, 78] For example, *Bombyx mori* silkworm fibroin has a hydrophobic sequence

GAGAGSGAAG[SG(AG)₂]₈Y that are known to form β -sheets,^[79] whereas spidroins are β -sheet-rich owing to repetitive stretches of poly-Ala.^[27, 78] Importantly, large-scale molecular dynamics simulations have revealed that β -sheet nanocrystals confined to a few nanometres are correlated to higher stiffness, strength and mechanical toughness than larger nanocrystals.^[27] β -sheet nanocrystals provide stiff orderly cross-linking domains embedded in a semi-amorphous matrix that consists of less orderly structures like β -turns^[80] (Figure 2.2). They constitute about 10-15% of the silk volume, and can go to 50% with less orderly extended structures.^[28, 81]

Figure 2.2 Schematic of the hierarchical spider silk structure that ranges from nano to macro. It displays key structural features of silk, including the electron density at the Angstrom scale, hydrogen bonded β -strands, β -sheet nanocrystals, a hetero-nanocomposite of stiff nanocrystals embedded in a softer semi-amorphous phase and silk fibrils, which assembles into macroscopic silk fibers. The figure is adapted from Ref.^[27]

2.1.3. Elastic structural proteins

Elastic proteins are also an important class of structural proteins, which can quickly and efficiently release energies in biological materials. They are flexible under relatively low stress and have some extent of covalent/non-covalent cross-links to form a network. They provide extensive elasticity and resilience in tissues that undergo reversible and repetitive deformation.^[4, 82] At the molecular scale, elastic proteins all contain at least one domain that is made up of elastic repeat sequences, and they all contain cross-links between residues in either the elastic or non-elastic domains.^[36, 83] Elastin, resilin and abductin are typical members in the family of

elastic proteins. Whereas elastin is found in vertebrates, resilin is produced in insect flight systems, and abductin usually appears in the hinges of bivalve mollusks.^[4, 84]

Elastin (together with collagen) is the major protein component of ECM in many tissues such as skin, lung, artery, ligament and cartilage.^[85] Elastin appears in different morphologies among various tissues: **(i)** rope-like networks in the skin and lungs; **(ii)** thin sheets in blood vessels; and **(iii)** three-dimensional cellular structures in cartilage.^[86] Elastin is typically considered as a polymer network of tropoelastin monomeric precursor with cross-linked, randomly coiled peptide chains.^[41] Therefore native elastin is composed of two types of domains in amino acid sequence, the elastomeric domains and cross-linking domains. The elastomeric domains are rich in hydrophobic amino acids that often appear in repetitive tetra-, penta-, and hexapeptides motifs, including VPGG, VPGVG, and APGVGV respectively. The cross-linking domains are rich in Ala and lysines (Lys) that are directly involved in cross-linking.^[85] Hydrophobic domains present in the elastin sequence are contributing to the elastic properties.^[87] These domains result in a high mobility of protein backbone. The configurational entropy is reduced on stretching, which provides the restoring force upon unloading^[88] (Figure 2.3a). Therefore, elastin can withstand more than 200% strain before the protein strands are completely straightened out.^[89]

Figure 2.3 (a) The scheme of elasticity for elastin. The low stiffness of elastin is due to the tangled nature of the protein strands in elastin that are embedded in a gel-like matrix. Applying a force to elastin results in the tangled chains straightening out but remaining cross-linked which returns to the tangled state when the force is released. The panel is adapted from Ref.^[85] (b) Structure of di-Tyr in cross-linked resilin. The panel is adapted from Ref.^[36]

Resilin is another member of the elastic protein family. It is a “super elastic rubber” commonly found in the specialized cuticle of a wide range of insects where extension and retraction are needed millions of times over the lifetime of the animal.^[90, 91] Naturally cross-linked resilin exhibits low stiffness, high strain and resilience (recovery after deformation), very high fatigue lifetime and efficient energy storage^[82, 92] to these tissues, thus playing an important role in insect flight, the remarkable jumping ability of fleas and spittle bugs.^[36] The origin of elasticity for resilin has not been fully understood. However, it is generally accepted that the randomly coiled polypeptide chains of resilin undergo a decrease in entropy upon straining,^[93] while the di- and tri-tyrosine^[92] (Figure 2.3b) based chemical cross-links retains the integrity of the network. Upon unloading, the system goes back to a state of maximum entropy. It has also recently been reported that a reversible β -turn transition is key to the elasticity mechanism of native full-length resilin,^[90] The hydrophilic-hydrophobic-hydrophilic polymer-like proteins self-assembly into micelles and form nanoporous patterns with island-like structures upon input of energy.^[90]

2.2. Biomimetic Materials Inspired by Structural Proteins and Their Biomedical Applications

The unique properties of different structural proteins (elastic or stiff) have recently triggered intense interest from the biomaterials community, which has led to a wide range of applications. In addition, due to their non-antigenicity, biodegradability, and biocompatibility, natural proteins and protein-based materials are particularly utilized in biomedical research, including for tissue repair and engineering, and drug delivery.^[41, 94] Tissue engineering is an interdisciplinary field which aims to develop biological substitutes that can restore, maintain or improve tissue function.^[95, 96] Typically, a highly porous scaffold is crucial to control the tissue formation in three dimensions. This scaffold mimics the *in vivo* ECM, which provides the microenvironment for cell attachment, migration, proliferation, as well as differentiation and *de novo* tissue generation.^[53, 97] The scaffolds' biocompatibility, biodegradability, ease of processing into porous structures, as well as mechanical properties are required for the efficient regeneration of different tissues.^[40, 95] In addition, biomaterials for controlled drug delivery systems need to fulfill several requirements, including protection of the drug from degradation, control of drug release, limited toxicity, low cost and easy processability into different morphologies.^[32, 98]

2.2.1. Collagen-based materials

Collagen is a promising biopolymer for applications in the medical and pharmaceutical fields due to its ease to process and modify, its abundance, low antigenicity, biodegradability, biocompatibility and mechanical properties (plasticity).^[41, 99] Most types of collagens are soluble in acidic aqueous solutions and can be processed into different forms such as sheets, tubes, foams, nanofibrous

matrices, particles and aqueous injections for biomedical applications.^[42] Moreover, thanks to their high reactivity, collagens can be cross-linked into gels by cross-linking agents (*e.g.* glutaraldehyde), thermal treatments or high-energy irradiation.^[99] Recent years have seen extensive investigations of collagen-based materials for the localized delivery of low molecular weight antibiotics, protein drugs (*e.g.* growth factors, insulin and monoclonal antibodies), and small molecule anti-cancer drugs (*e.g.* cisplatin and 5-FU).^[42, 43] For example, several collagen-based gentamicin delivery vehicles that have been developed and are commercially available (Sulmycins-Implant, Collatamp®-G and Septocolls) show a sustained local delivery of drugs with very low systemic exposure.^[100] In addition, the physical properties of the gel, including porosity, density and degradation rate, are able to control the drug release from collagen matrices.^[99]

Being the major component of ECM and serving as a natural substrate for cell attachment, proliferation and differentiation, collagens are also catching the interest of researches as a matrix material for tissue engineering and wound dressing applications.^[99] Collagen scaffolds have been used for the repair of cartilage,^[47] bone,^[46] as well as the engineering of vascular grafts^[45] and dermal tissues^[44] (Table 2.1). In particular, some of these materials have been clinically used. For example, Promogran®, a novel sponge made of collagen matrix has been clinically used in US and Europe to treat diabetic and ulcer wounds.^[101] Integra® Dermal Regeneration Template, a bilayer acellular skin substitute that has a dermal layer comprised of cross-linked bovine collagen and glycosaminoglycan and an epidermal layer composed of polysiloxane, is currently in the market for the treatment of thermal injury.^[102] In addition, several bioengineered skin equivalents have also been commercially developed by the encapsulation of human keratinocytes in collagen-coated polymer

substrates (TransCyte®) and incorporation of human keratinocytes and fibroblasts in collagen-based bilayer dressings (Orcel® and Apligraf®).^[99]

2.2.2. Silk fibroin-based materials

Since its initial development in China, silk has been used as luxury fabrics.^[27] It is also an attractive and versatile candidate material used in lightweight insulation, surgical sutures and, more recently, drug delivery vehicles and tissue engineering scaffolds in biomedicine.^[27, 40, 73, 95, 103]

Besides their remarkable mechanical properties, silk proteins from silkworms and spiders are biocompatible, biodegradable, and easy to fabricate into diverse morphologies (Figure 2.4),^[40, 104, 105] all of which make them promising candidates to fabricate scaffolds for tissue regeneration. In particular, silk sutures have been used for tendon tissue engineering.^[52] Silkworm *B. mori* silk is being studied as a potential scaffold in skin and corneal regeneration, cartilage repair, vascular grafts and bone regeneration as well as cardiac tissue engineering.^[41, 53, 54] In addition, the silk protein from the Indian tropical tasar *Antheraea mylitta* has been fabricated into scaffolds for the engineering of an *in vitro* cardiac patch.^[53] The remarkable mechanical properties of silk proteins meet the requirements of mechanical strength in the scaffolds of tissues like bone, cartilage and heart in tissue regeneration. Moreover, with the development of biotechnology tools, it has been possible to engineer silk genes to produce recombinant silk-like proteins for tissue engineering.^[106]

Figure 2.4 Schematic of material forms fabricated from silk fibroins using different procedures. The figure is adapted from Ref.^[105]

Silk proteins have also been intensively used in controlled drug delivery systems as they meet the above-mentioned requirements. A wide range of morphologies for silk fibroins, such as hydrogels, films, microparticles, nanoparticles, and scaffolds, have been developed (Figure 2.4). They are fabricated with various techniques, including film casting^[107] or combined with subsequent sonication,^[108] gelation,^[109] lipid-templates,^[110] vibrational splitting of laminar jet^[111] as well as self-assembly.^[112] Diverse drug molecules are incorporated therein ranging from low molecular weight compounds such as propranolol hydrochloride and salicylic acid, to protein drugs like horseradish peroxidase and lysozyme, and various growth factors.^[32] At the same time, recombinant spider silk proteins are also used in controlled drug delivery and they can integrate new features compared to native counterparts.^[113]

2.2.3. Elastin-based materials

Native elastin is an extremely insoluble protein which forms fiber networks due to the cross-linking between tropoelastin monomers and the interactions between hydrophobic domains, which limits the use of elastin from animal origin.^[35, 41] However, genetic engineering has made it possible to produce recombinant elastin-like polypeptides (ELPs) which contain the key amino acid repeats of elastin, for example (Val-Pro-X-Y-Gly)_n (X stands for Gly or Ala and Y represents any amino acid except proline) to mimic the pentapeptide (Val-Pro-Gly-Val-Gly) in the elastomeric domains.^[85, 114, 115] A key feature of these elastin-like proteins is that they can perform a reversible, temperature-dependent self-assembly from aqueous solutions, similar to the behavior of native elastins.^[116, 117] The self-assembly gives rise to a spontaneous phase separation above a lower critical solution temperature (LCST), which is accompanied with a conformational change of protein secondary structure from random coils to β -turns as the temperature is getting close to the transition temperature (T_t).^[117, 118] In addition, the substitution of certain amino acids in the protein sequence results in tunable properties of the proteins and materials thereof.^[85, 119] For example, the replacement of Ala by Gly at the third amino acid of the pentapeptide leads to a change of the mechanical properties of the materials from elastic to plastic, while the change of the fourth amino acid tailors the temperature dependent phase behavior of the materials.^[120] Moreover, another strategy to tune the properties of elastin-like protein materials by genetic engineering is to design block co-polymers.^[85] For instance, a triblock copolymer design composed of two identical hydrophobic elastin domains in both ends and a hydrophilic block in the center is able to tune the size and chemical properties of individual blocks, resulting in many opportunities to adjust the properties of the material made of these protein co-

polymers.^[85] The self-assembly process and tunable properties of elastin-like proteins result in the generation of recombinant elastin-mimetic protein materials, including micelles, injectable gels, films, hydrogels and electro-spun fiber scaffolds,^[35, 85] that are finding applications in tissue engineering^[114] and drug delivery.^[121]

Elastin is a major component of skin dermis, therefore it has been used in the repair of skin for cases of severe burns and chronic wounds, in the format of hybrid materials with collagen.^[58] Besides, elastin-based materials have also been used as surface coatings on vascular grafts for the purpose of improving the long-term patency and function, as elastin is one of the essential structural components in the vascular walls.^[59] These elastin based materials are able to provide mechanical strength and the regulation of cell behaviors to obtain a desired clinical response.^[35] Elastin-like proteins can also be electrospun into hybrid fiber scaffolds with other proteins (e.g. collagen) and synthetic polymers (e.g. polycaprolactone (PCL)) or both for the engineering of vasculature,^[62] cardiac tissues^[60] and bone repair.^[61]

The temperature-dependent self-assembly of elastin-mimetic block copolymers allows the formation of hydrophobic aggregates ranging in dimensions at the nano- and micron scales. This process facilitates the formation of micellar nanoparticles^[57] and injectable gels^[56] with elastin-like proteins for drug delivery. The size, composition and sequence of the blocks can be varied to precisely control molecular self-assembly in different conditions, including various temperature, pH, and ionic strength of the aqueous solution.^[85] For example, a well-designed di-block copolymer of elastin like polypeptides allows the self-assembly into temperature-responsive nanoparticles within the clinically relevant temperature range (37-42 °C) for the targeted drug delivery to cancer cells.^[122] Such systems have been designed for the

delivery of a range of drugs and molecules, including small molecule hydrophobic drug (*e.g.* doxorubicin),^[55] hydrophilic drugs (*e.g.* vitamin B13) and proteins (*e.g.* ovalbumin).^[56]

2.3. Bioengineering of proteins at the molecular level

Protein synthesis in living cells is accomplished on ribosomes according to the sequence of mRNA encoded by specific genes, and then these polymer chains self-assemble or fold into large and complex molecules with diverse specific functions. This process inspires bioengineer scientists to control the biosynthesis of proteins at the molecular level. In the past decade, the use of molecular biology and protein engineering methods, especially recombinant DNA technologies has allowed us to produce protein-based biopolymers.^[123] These techniques provide well established protocols for cloning, mutation and gene fusion in different host cells for the expression of proteins with a broad range of size^[124, 125] and an exquisite control over the molecular mass, polydispersity, composition, as well as sequence.^[123] Bacteria, especially *Escherichia coli* (*E. coli*) and yeast have been the most widely used hosts for expression of recombinant proteins because they are well characterized in terms of their molecular genetics and cell physiology. Their genomes are easy to manipulate, and their cultivation is simple and low-cost.^[126, 127] Mammalian, insect, microalgae or plant cell bioreactors are also used due to their ability to introduce proper post-translational modifications onto proteins.^[127, 128]

Genetically engineered proteins with the use of 20 natural amino acids provide a variety of advantages. First, recombinant DNA technologies allow the introduction of tailored synthetic genes into the expression vector that produces recombinant proteins.^[129] Second, the degree of control and the complexity obtained in genetic

engineering is higher than that in conventional chemical synthesis. These two are the key drawbacks of conventional chemical synthesis, where any increase in the complexity of the final product usually requires a nearly exponential increase in the time and cost of synthesis. Third, the production cost of recombinant proteins is not related to their complexity. Although the gene construction is moderately time and money consuming, the fast growth and low requirements in culture and expression conditions of genetically engineered microorganisms will compromise the cost in gene construction.^[123] Finally, besides engineering biological components that are already present in nature, recombinant DNA technology makes it possible to design new peptide and protein sequences that are not found in biology. For example, new artificial self-assembling peptides and fusion proteins with different bioactive domains or protein motifs that are not otherwise found together in nature can be produced.^[124] Recombinant protein production is an enormous and quickly expanding field with a wide variety of applications. For example, in the pharmaceutical field, progresses in recombinant DNA technology in the past decades have brought many therapeutic proteins into clinical trials, with hundreds of them already used into clinical applications.^[130]

2.3.1. Recombinant expression of silk proteins

Given the broad applications of silk proteins in engineering and biomedical fields and that the collection of spider silk proteins from webs is a time-consuming job,^[131, 132] there have been significant efforts to achieve high yield recombinant expression of silk proteins, especially spidroins over the past decade (Table 2.2). On the other hand, the long and repetitive sequence and the guanine/cytosine (G/C) rich nature of fibroin genes make the sequencing, cloning and expression of spidroins complicated.^[133] Thus, it was only recently that the first full-length silk fibroins were

recombinantly sequenced^[134, 135] and the recombinant production of spidroins were initially focusing on the expression of partial spidroin (“silk-like”) proteins which contained typical repetitive domains of spidroins.

Table 2.2 Summary of recent advances in the recombinant expression of silk proteins.

Typical examples of silk protein expression in each type of host are shown.

Protein	Origin	Host	MW (kDa)	Purification strategy	Yield (mg/L)
MaSp-1 ^[136]	<i>Nephila clavipes</i>	<i>E.coli</i>	65-163	IMAC	300
MaSp-1 ^[137]	<i>Nephila clavipes</i>	<i>E.coli</i>	100-285	Ammonium sulphate	1200 ^a
MaSp-1 ^[138]	<i>Nephila clavipes</i>	<i>P. pastoris</i>	65	Ammonium sulphate	663
MaSp-1 ^[139]	<i>Nephila clavipes</i>	<i>B. mori</i>	70	IMAC	6 mg/larva
MaSp-1 ^[140]	<i>Nephila clavipes</i>	<i>N. tobaccum</i>	13-100	Ammonium sulphate	0.5-2.0% total protein
ADF-3 ^[141]	<i>Araneus diadematus</i>	BHK cells	60-140	Ammonium sulphate	25-50

a: High cell density cultivation (HCDC) of the engineered cells was performed to achieve high yield of proteins.

Because of the merits mentioned above, *E. coli* is the most commonly used host for recombinant silk protein production,^[133, 142] which also offers excellent flexibility for industrial-scale production.^[143] However, the highly repetitive, GC-rich silk gene sequences are often unstable in *E. coli*. Codon biases, tRNA pool depletion, homologous recombination, segmental deletions and expansions, transcription errors, unusual mRNA structure, truncated synthesis, translational pausing, and protein aggregation (low solubility) may all limit the efficiency of silk production in *E. coli*.^[132, 136, 142-144] To this end, a wide range of systems including yeast, plants, mammalian cell lines and transgenic organisms have been evaluated for the generation of recombinant silk proteins, each of which has met with varying degrees

of success as reviewed elsewhere (Table 2.2).^[132, 143, 145, 146] The methylotrophic yeast *P. pastoris* has been utilized for the purpose of minimize truncations due to translation stops^[138] and allowing the extracellular secretion of expressed products.^[147] Various plants including tobacco and potato have been used as a cost-efficient approach for scale-up of protein expression.^[133, 140] Moreover, inspired by the production of silks by silkworms, the expression of spidroins in larvae of *B. mori* has been tried, but protein solubility remains a major problem.^[139] Mammalian cells, including baby hamster kidney (BHK) cells and bovine mammary epithelial alveolar cells, have also been utilized to produce high-molecular-weight spidroins.^[141] In addition, transgenic production of spidroins in mammary glands and secretion into milk has been employed in mice^[148] and goats,^[149] but fairly low yields remains a limitation.

Native silk fibers have evolved to sustain high mechanical loads and the high molecular weight of their constitutive silk proteins (~250-350 KDa) is key to their impressive mechanical properties.^[150] While recombinant proteins that directly mimic the full size of the native proteins are desirable from a biomimetics perspective, the cloning and expression of full-length native spidroin sequences thus remains a major challenge. Therefore, efforts to generate recombinant silk proteins have focussed on the development of methods to assemble modular constructs that approach or mimic the size of the native genes.^[137] Among the most significant advances, Xia et al^[137] have reported high yield (1.2g/L from high density cell cultures) with a 285 kDa dragline silk protein mimetics produced in metabolically engineered *E. coli* cells which were designed to accommodate the heavy codon bias by increasing glycyl-tRNA pools (Table 2.2). In this case, the recombinant proteins obtained were subsequently spun into fibers whose mechanical properties approached those of the native materials.

Chapter 3 Characterization of *D. gigas* Suckerin Ring Teeth

3.1. Introduction

The mechanical properties, microstructure, chemical composition and protein sequences of *D. gigas* SRT have recently been investigated (refer to section 1.2, Chapter 1).^[12, 19, 25] The protein-only composition (suckerins), the β -sheet containing structures, and the sequence homology to silk proteins strongly suggest that jumbo squid SRT are a good model for biomimetic materials research. However, further information is needed to better understand SRT and to elucidate the structure-property-function relationship of the native material. For example, both intriguing mechanical properties and β -sheet structures of SRT have been identified, but the clear evidence that β -sheets play a key role in contributing to SRT mechanics is still lacking. In addition, how the mechanical properties of jumbo squid SRT are reminiscent of that of other species in cephalopods superfamily (*e.g.* cuttlefish) are still unknown. Last but not least, straightforward melting strategy has enabled the fabrication of materials based on suckerins including nanopillars,^[25] but the detailed thermoplastic features of SRT are still not elucidated. Because of these unknown characteristics, jumbo squid SRT were further characterized using a combination of techniques, with an emphasis on their mechanical properties under different conditions and their thermo-mechanical responses at elevated temperatures. The information obtained from these characterizations will offer directions for the engineering of biomimetic materials with suckerins.

3.2. Materials and methods

3.2.1. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) of SRT from squids and cuttlefish

The jumbo squid (*D. gigas*) were previously caught along the east coast of Mexico. The bigfin reef squid *Sepioteuthis lessoniana* (*S. lessoniana*) and the golden cuttlefish *Sepia esculenta* (*S. esculenta*) were commercially obtained in Singapore. The animals were euthanized, and their SRT were collected immediately by dissection. The harvested SRT were cleaned by sonication in water and stored at -20 °C for further use.

D. gigas SRT protein powders were achieved by grinding sucker rings under liquid nitrogen and dried overnight in a chemical hood. The protein powders were dissolved in 8 M urea and 5% acetic acid at a concentration of around 1-2 mg/ml before being subjected to SDS-PAGE: 6-10 µL sample was mixed with equal volume of loading buffer (Bio-Rad) and boiled at 100°C for 3-5 min before loading to the 10% SDS-PAGE gel. The protein loaded gels were run at constant current (0.01 A/gel), followed by 2 h staining with Coomassie blue R (CBR) (in 10% (v/v) acetic acid and 50% (v/v) methanol), and an overnight de-staining with 7% (v/v) acetic acid and 5% (v/v) methanol.

3.2.2. Nanoindentation of SRT from squids and cuttlefish

The mechanical properties of SRT from the three species were investigated by nanoindentation with a TriboScan 950 (Hysitron) equipped with a standard force transducer (maximum load: 12 mN) and a fluid cell cube-corner tip following previously described procedures^[25] to measure the elastic modulus (*E*). Single SRT were embedded in Epofix resin and cut by ultra-microtomy to obtain flat and smooth

cross-section surfaces. Microtome-treated samples were placed into a custom-made glass Petri dish designed for measurement under both dry and hydrated conditions. In dry conditions, cross-sections of SRT were probed with a maximum load of 1000 μN with 200 $\mu\text{N/s}$ loading and unloading rates and a holding time of 2s at peak load. Indents were placed according to grids that completely covered the entire tooth cross-sections. In wet conditions, the petri dish was filled with water until the sample was fully submerged and the system was stabilized for at least 1 h for complete hydration. The hydrated teeth surface was probed using the same experimental conditions. E was obtained from the load–displacement curves according to the classical Oliver and Pharr method.^[151] The cuttlefish SRT were measured at the ring basal since the tips were too small to be probed.

3.2.3. Nanoindentation of jumbo squid SRT in different solutions

Jumbo squid (*D. gigas*) SRT were embedded in glass ionomer cement (Riva Luting, SDI) and polished with diamond paste down to a particle size of 0.25 μm for the purpose of obtaining flat cross-sections (Figure 3.1). The polished SRT were then placed in a glass Petri dish. The Petri dish was covered by a custom-made lid with a small hole in the center, which was designed to (i) enable the immersion of SRT in solutions, (ii) prevent solvent evaporation, and (iii) probe sample surfaces in the longitudinal direction (Figure 3.1). A narrow but long grid of indents were performed (20 indents/line, with an indent spacing of 15 μm) using TriboScan 950 (Hysitron) and a fluid cell cube-corner tip, in a relatively small region of SRT cross-sections for each condition. The location of the measurements on the SRT cross-sections was chosen such that it corresponded to regions where the mechanical properties were previously demonstrated to show little variations in modulus (there is an intrinsic gradient in E from the periphery to the center of tip on cross-sections, which needs to

be minimized, thus all measurements were conducted on the periphery). The loading conditions were as follow: samples were probed with a loading time of 5 s, an unloading time of 5 s and a holding time of 2 s at peak load before unloading. The peak load was adjusted for each solution. For example, the peak load was 200 μN for the measurements in 0.2 M urea, but was 1000 μN for those in ethanol. For each set of measurements, the Petri dish was carefully filled with the solution before starting the indentations process in order to fully immerse the SRT, and the solutions were re-introduced if necessary to ensure total immersion of SRT sections during the measurements.

Figure 3.1 Schematic showing the experimental design for nanoindentation of *D. gigas* SRT in various solutions.

3.2.4. Raman spectroscopy of jumbo squid SRT in urea solution

Polished *D. gigas* SRT cross-sections were examined with a confocal Raman microscope (LabRAM HR800, Horiba Jobin Yvon) equipped with a diode-pumped 785 nm near infrared laser (PilotPC 500, Sacher Lasertechnik) and a long working distance 50 $\times$ objective (Olympus, numerical aperture = 0.5), under the control of Labspec 5 software (Horiba Jobin Yvon). Spectra with a 60 s integration time were obtained at different time intervals from 0 to 90 min after immersing the SRT in 0.2 M urea and the spectra in protein Amide III (1190-1350 cm^{-1}) were integrated.

3.2.5. Differential scanning calorimetry (DSC) of *D. gigas* SRT proteins

SRT protein powders were obtained as above. The thermal behaviors of SRT proteins were investigated by differential scanning calorimetry (Q10, TA Instruments Co.). Around 5 mg of proteins were subjected to 4 cycles of heating and cooling at a ramping/decreasing rate of 10 °C/min under nitrogen atmosphere: (i) the 1st cycle ramping from room temperature to 125 °C and going back to 20 °C; (ii) the 2nd to 4th cycles ramping from 20 °C to 125, 175 and 225 °C respectively and returning to 20 °C before the next cycle. Three minutes isothermal incubations were introduced between each ramp. In another trial, SRT proteins were heated from 20 °C to 300 °C at a ramping rate of 10 °C/min before a pre-heating cycle from room temperature to 150 °C to remove all bound water.

The glass transition temperature (T_g) of SRT proteins in water was determined by differential scanning calorimetry (DSC Q10, TA Instruments Co.). Fifty mg of SRT with distilled water (1:2, w:w) were mixed and sealed in a stainless steel pan. The temperature scanning range was 10 to 90 °C at a heating rate of 5 °C/min under nitrogen atmosphere.

3.2.6. Thermal gravimetric analysis (TGA) of jumbo squid SRT proteins

The decomposition profile of SRT proteins was characterized by thermal gravimetric analysis (Q500, TA Instruments Co.). Around 20 mg SRT proteins were heated from room T to 900 °C at a ramping rate of 10 °C/min under air atmosphere.

3.2.7. Nanoindentation of SRT at high temperature

The mechanical properties of SRT in dry and wet conditions under elevated temperatures were investigated by high temperature nanoindentation. The measurements were done with a TriboScan 950 (Hysitron) equipped with a heating

control system which consists primarily of: (i) a heating stage with a thermocouple positioned below the specimen, (ii) a cooling system that is used to remove heat from the surrounding stage and that also goes through (iii) an aluminum heat shield located between the stage and the standard transducer to protect the latter, and (iv) a temperature controller (Figure 3.2a). *D. gigas* SRT were embedded in heat-conductive high temperature chemical set cements (OMEGABOND OB-600) and polished with #4000 sand paper and polishing pastes with particle size of 3 and 0.04 μm respectively to achieve a flat and smooth cross section surface of SRT. The sample thickness was around 1 mm to reduce the temperature gradient upon heating (Figure 3.2b top). In dry conditions, cross sections of SRT were probed with a Berkovich-high temperature tip with a maximum load at 120 μN , 24 $\mu\text{N/s}$ loading and unloading rates and a holding time at peak load of 2s in a piezo automation method. The temperatures were set at 20, 60, 100, 120, 140, 160, 180, 200 and 220 $^{\circ}\text{C}$ respectively. Before the measurements at each elevated temperature, the samples were heated and kept for 30 min to ensure the temperature was homogeneous within the sample. In wet conditions, the sample was fixed in a custom-designed aluminum dish (Figure 3.2c), hydrated in water for 1 h and cross-sections of SRT were probed by a cube-corner fluid cell tip (with the standard transducer without any heat shield) through the hole of dish lid with a maximum load at 80 μN , 3s loading and unloading and a holding time at peak load of 2s in a piezo automation method. The sample was first incubated in water for 1 h at 20 $^{\circ}\text{C}$ to ensure full hydration and measured before the temperature was increased to 50 $^{\circ}\text{C}$ with an increase of 5 $^{\circ}\text{C}/\text{step}$. Between each set of measurements at high temperature, the sample was stabilized similarly as in dry conditions. In both conditions, the sample was probed with a single line of indents around the boundary

region in order to minimize the possible influence of the micro-scale elastic modulus gradient of jumbo squid SRT discussed before (Figure 3.2b bottom).

Figure 3.2 Experimental design of SRT nanoindentation at elevated temperatures. (a) The set-up employed for high temperature nanoindentation. (b) SRT embedding and the position of indents on a single tooth. (c) A custom-made aluminium dish for high temperature nanoindentation of SRT in wet conditions.

3.3. Results and discussions

3.3.1. Protein composition and mechanical properties of SRT from squids and cuttlefish

Predation success and survival of squids and cuttlefish is intimately correlated to the presence of SRT that line their arms and tentacles and play a crucial role in prey grabbing during predation. Therefore, to elucidate and compare the composition and mechanical properties of SRT from the three species, it is important to reveal the interplay between the physico-chemistry and the mechanical performance. SRT from different species vary in size in a way that is proportional to their body sizes (Figure

3.3a). Similarly to *D. gigas* SRT, SDS-PAGE revealed that the SRT of *S. lessoniana* and *S. esculenta* are composed of a protein mixture with molecular weights below 50 kDa (Figure 3.3b). We also notice that *D. gigas* SRT contain a larger population of proteins than those of the other two other species, potentially playing a role on their mechanical properties, as discussed below.

Figure 3.3 Characterization of *D. gigas* SRT. (a) Photographs of SRT from *D. gigas*, *S. lessoniana* and *S. esculenta* respectively. (b) SDS-PAGE of SRT proteins from *D. gigas* (lane 3), *S. lessoniana* (lane 4), and *S. esculenta* (lane 5). MW: molecular weight. (c) Low-magnification optical micrograph of *D. gigas* SRT tip cross-sectioned by ultra-microtomy and subsequently used for nanoindentation experiments. The red box highlights the half tip probed by nanoindentation. (d) *E* map of cross-section from *D. gigas* SRT shown in (c). (e) *E* map of a single spike acquired on a *S. lessoniana* SRT cross-section. (f) Average *E* of SRT from the three species in both dry and wet conditions. (n=35-60. *** $P < 0.001$, one-way ANOVA analysis).

The SRT from three species showed comparable mechanical properties.^[12] The decrease of modulus from the periphery to the center on cross-sections of SRT previously reported for *D. gigas* was first confirmed for *D. gigas*, and was also observed for *S. lessoniana* SRT (Figure 3.3c-e). But the variation was less marked, with the elastic modulus of *S. lessoniana* SRT varying between 5 and 6.5 GPa (versus

10 to 5 GPa for *D. gigas* SRT). This suggests a possible difference in microstructure. Since the pore fraction gradient due to nano-channels in *D. gigas* SRT directly affects the elastic modulus gradient, these results suggest a narrower pore fraction distribution for *S. lessoniana* SRT, although further investigation is needed to confirm this. Since *S. esculenta* SRT spikes were too small to be probed by nanoindentation, only the basal location of these SRT were probed for comparison with the other samples shown in Figure 3.3f. All samples displayed an average E of ~6-7 GPa in dry conditions, which was comparable to that of many synthetic polymers including polystyrene, PMMA, and ABS.^[20] In wet conditions, the average E value for all SRT significantly dropped, but that of *D. gigas* (2.4 ± 0.6 GPa) was significantly higher than the other SRT (1.6 ± 0.6 GPa and 1.4 ± 0.4 GPa for *S. lessoniana* and *S. esculenta* respectively) ($P < 0.001$). This is consistent with the predatory functions of SRT for the three animals. Jumbo squid is a bigger animal, whose stronger SRT may allow the animal to capture bigger preys, since it has been accepted that the size of predators are positively correlated to the size of preys that are able to successfully coexist with them.^[152] The difference in E for three SRT under wet conditions could perhaps be attributed to the distribution of protein molecular weights for the 3 species. High molecular weight proteins are more abundant in *D. gigas* SRT. Given the correlation between high MW and better mechanical properties in synthetic polymers^[153] (longer polymer chains and thus more potential inter-chain interactions), a similar mechanism may be happening here, but this remains to be fully established. Furthermore, together with the similarity in protein modularity^[17] between the SRT from these species, their similarity in mechanical response suggest that the SRT structure appeared very early on in the evolutionary history of cephalopods.

3.3.2. Contribution of hydrogen bonds and β -Sheets to SRT mechanics

In order to further dissect the relationship between suckerin protein structure and SRT mechanics, complementary nanoindentation and micro-Raman spectroscopy investigations were carried out on *D. gigas* SRT at conditions designed to disrupt hydrogen bonds and hydrophobic interactions present within the SRT. In the dry, hydrated, and ethanol treated samples, the E did not change significantly over time (Figure 3.4a). Considering that there is a relatively high amount of hydrophobic residues in suckerin present in both Ala, His-rich and Gly, Tyr-rich domains^[17], the fact that SRT subjected to ethanol exhibited similar properties to dry samples indicated only minor contributions of hydrophobic interactions for SRT mechanics. However, we observed quick and dramatic decrease of the elastic modulus of SRT when the samples were incubated with urea, which is a strong hydrogen bond disruptor. In these samples, the final plateau modulus after the initial decay strongly depended on the urea concentration. At 2 M urea, the final plateau modulus was ~20 MPa which is much lower than the value in water (~2-3 GPa). With 0.2 M urea, the plateau modulus was between 100 to 200 MPa, whereas with 0.02 M it was 300-400 MPa (Figure 3.4a). These data provide the first direct evidence that hydrogen bonds play a crucial role in contributing to SRT mechanical properties. Furthermore, Raman spectroscopy measurements were performed on SRT in parallel to investigate the changes in protein secondary structures under urea treatments (Figure 3.4b). When SRT sections were subjected to 0.2 M urea, a clear decrease of the Raman shift peaks for the amide III band at 1236 and 1339 cm^{-1} were observed, which can be attributed to a loss of β -sheet structure.^[154] Therefore, we suggest that there is a direct correlation between the modulus decrease and the disruption of β -sheets in SRT, as illustrated in Figure 3.4c where urea disrupted the hydrogen bonding, causing the

dissolution of β -sheets and a decay of E in a concentration dependent manner. Thus we conclude that hydrogen bonds localized in β -sheet structures play a major role in stabilizing and reinforcing SRT. From a biomimetic material perspective, these structure-property relationships also suggest opportunities to engineering biomimetic materials with suckerin proteins: incorporation of β -sheet structures in a controllable manner should potentially allow to modulate the stiffness of suckerin-based materials, as demonstrated in Chapter 6.

Figure 3.4 Mechanical properties and secondary structure of *D. gigas* SRT sections. **(a)** E as a function of time measured by nanoindentation in air, water, 100% ethanol, and various concentrations of urea. **(b)** Micro-Raman spectroscopy of *D. gigas* SRT incubated in 0.2 M urea. Dotted lines correspond to β -sheet structure and its disruption with exposure to urea (note the recovery of β -sheet structure upon removal of urea and drying). **(c)** Schematic of hydrogen bond disruption accompanied by β -sheet dissolution upon contact of SRT surface with urea.

3.3.3. Thermoplastic properties of *D. gigas* SRT

Thermoplastic polymeric materials are commonly used in a multitude of applications due to their ease of processing. The ability to melt thermoplastics into any complex shape makes them suitable for processing methods such as extrusion, injection molding, or drawing.^[155] Natural biopolymers, such as protein-based materials, typically do not exhibit thermoplastic characteristics^[156] due to the irreversible degradation of their macroscopic physical properties during processing and due to their low solubility. However, SRT represent an intriguing exception of natural biopolymer featuring thermoplastic behaviour. This is mainly because inter-chain covalent cross-links are not found in SRT and the structure is instead stabilized primarily through hydrogen bonds localized in β -sheets. Therefore, SRT proteins can be melted by simple heating in water and reshaped into a variety of materials with diverse morphologies,^[25] just like standard thermoplastics such as polyethylene. This provides intriguing lessons for eco-friendly processing and demonstrates the potential to use SRT-based materials as thermoplastic biopolymers.

Reliable processing of SRT proteins as thermoplastics requires a detailed understanding of their thermal behavior. Therefore, SRT were further investigated by a combination of relevant techniques, including thermal gravimetric analysis (TGA), differential scanning calorimetry (DSC), and nanoindentation at elevated temperatures. TGA showed that SRT contained ~13 wt.% water as illustrated by the cumulative weight loss up to 100 °C (Figure 3.5a). Since the sample was thoroughly dried before subjected to TGA, these water molecules are believed to be molecularly bound to protein chains as apposed to free water. This low content of water, however, plays a key role as a plasticizing agent which is critical for processability, as shown by the thermo-mechanical measurements in the 20–120 °C range detailed below. No

further significant weight loss was observed until ~ 250 °C, indicating a wide range of thermal stability for SRT. Moreover, two degradation onset temperatures at ~ 250 °C and 500 °C respectively were detected, indicating a bi-phasic, semi-crystalline biopolymer network in SRT, named β -sheet-dominated crystalline domains embedded in random coil based amorphous phase which displayed different thermal stabilities, in agreement with recent work.^[19, 25] In addition, there was only a 0.5 wt.% residual at 900 °C, which further confirmed the absence of minerals in SRT.

Next, our goal was to link the mechanical properties of SRT with their thermally-induced phase transitions by combining DSC with temperature dependent nanoindentation under both hydrated and dry conditions. Under hydrated conditions, there existed a clear slope change in the DSC curve at *ca.* 36 °C (Figure 3.5b). Concomitantly, an obvious drop in elastic modulus appeared from ~ 3 GPa to 100-200 MPa (Figure 3.5c, left inset) as the temperature ramped from room temperature to 50 °C (which represents the limit of the Nanoindenter equipment for measurements under hydrated conditions). Since β -sheets are thermally stable up to beyond 200 °C (as shown below), we speculate that the modulus decrease could be attributed to increased chain mobility of the amorphous domains in the presence of water.

Figure 3.5 Thermo-mechanical properties of SRT. **(a)** TGA analysis from room temperature up to 900 °C. Following water evaporation, SRT remain stable until *ca.* 250 °C. **(b)** DSC curves for hydrated (blue) and dry (red) samples. Under hydrated conditions, the heat flow was measured from 20 °C to 60 °C. Under dry conditions, a first cycle (not shown) was ramped up to 120 °C in order to remove residual water and subsequent cycles were ramped up to 120 °C (cycle 2), 170 °C (cycle 3), and 250 °C (cycle 4). Exothermic peaks at 137 °C and 150 °C were reproducible from cycle to cycle. The exothermic peak at 220 °C is attributed to β -sheet melting. **(c)** Reduced elastic modulus (E_r) measured by high temperature indentation using a temperature-ramping stage under both hydrated (blue) and dry (red) conditions.

The thermo-mechanical response of SRT was very different under dry conditions. We first observed a modulus increase from 6 GPa to ~12 GPa when the temperature increased from room temperature to 100-110 °C. Values above 10 GPa are extremely high for a non-cross-linked thermoplastic polymer.^[20] Since no phase transitions were detected by DSC up to 120°C (Figure 3.5b), this behavior could be attributed to loss of residual water detected by TGA, where stiffening happened due to loss of chain mobility as water escaped from the polymer network. At higher temperature, DSC measurements indicated reversible small exothermic peaks between 137°C and 150°C, as illustrated by thermal cycling experiments (cycles 3 and 4, Figure 3.5b), as well as an additional peak at 190°C (Figure 3.5b). In the same temperature range, there was only a mild decay in modulus which remained at 6 GPa up to 200°C (Figure 3.5c), which indicated limited thermal damage or structure change of SRT. At 220°C, however, a significant exothermal peak was detected by DSC (Figure 3.5b), which was accompanied by a dramatic decrease in elastic modulus from 6 GPa down to ~100 MPa (Fig 3.5c, right inset). This could result from the melting of β -sheet structures, consistent with the above results where chemical disruption (urea incubation) of β -sheets also gave rise to a drop in modulus by an order of magnitude.^[17] At temperatures beyond 220 °C, the SRT biopolymers no longer displayed structural integrity before the degradation at ~250 °C (Figure 3.5b inset), which was in agreement with TGA data (Figure 3.5a).

Interestingly, a class of supramolecular block copolymers resembling the molecular design of suckerins, namely composed of a stiff crystalline phase embedded in a soft matrix, have recently been shown to exhibit excellent high-temperature processability.^[157] Therefore, the present data suggest that SRT molecular structure serves as a design paradigm to create bio-inspired thermoplastic materials

not only from recombinant suckerins,^[19, 25] but also more generally to design *de novo* modular protein-based materials or block co-polymers with thermoplastic properties. The data also suggest that suckerins represent promising eco-friendly materials for additives manufacturing, which could be employed in printed wearable electronics or in other multi-purpose biomedical devices.

3.4. Conclusion

Jumbo squid SRT are a new class of load-bearing biological materials with mechanical properties that rival those from many engineered synthetic polymers and thus represent an intriguing model of biomimetic materials research. They are only made of proteins, which assemble into β -sheet structures embedded in an amorphous matrix. In this Chapter, the mechanical properties of *D. gigas* SRT were found to be comparable to that of another squid species (*S. lessoniana*) and cuttlefish (*S. esculenta*). Further studies will be needed to elucidate whether all species share the similar nanostructure design of β -sheet embedded within an amorphous matrix. The primary structure of SRT from these other species has however been elucidated and also found to consist of a block copolymer-like structure made of alternating β -sheet and amorphous domains.^[17] Hydrogen bonds within the β -sheets are playing a central role in providing the load-bearing properties of *D. gigas* SRT, as revealed by nanoindentation measurements in a range of solutions in combination with micro-Raman spectroscopy. These data serve as the first direct evidence that β -sheets are directly responsible for providing load-bearing to SRT and provide useful structure-property relationships to engineer structural biopolymers with artificial suckerins.

Furthermore, the presence of cooperative weak interactions (hydrogen bonding) and the absence of covalent cross-links make SRT an intriguing model system of a thermoplastic biopolymer. Characterizations by TGA, DSC and high temperature

nanoindentation revealed further insights into the thermoplastic properties of SRT. The crystalline β -sheet structures and amorphous random coils of the bi-phasic, semi-crystalline biopolymer exhibited different thermal stabilities in dry and hydrated conditions. These data offer important information for the molecular design of biomimetic proteins that could be fabricated into thermoplastic biopolymers with potential biomedical and 3D printing applications.

Chapter 4 Fabrication and Characterization of Materials with Native SRT Proteins

4.1. Introduction

In order to fabricate materials with silk fibroins, one must use a complex and time-consuming protein extraction procedure from the native fibers or raw cocoons (Figure 4.1).^[105] The cocoon pieces need to be boiled in 0.02M sodium carbonate solution first for de-gumming and rinsed with water for multiple times. The molecular weight of the resulting proteins depends on the degumming time, while 30 min degumming of cocoons results in a molecular weight of silk fibroins centered at around 100 kDa.^[105, 158] The boiled cocoon pieces are then dried overnight and dissolved in 9.3 M lithium bromide (LiBr) for 4 h at 60 °C. In the following steps, the dissolved proteins are subjected to dialysis against deionized water for 48 h and centrifuged to remove insoluble impurities before an aqueous solution of silk fibroin is achieved. The whole processing from cocoons to the final aqueous solution takes 4 days, which is even longer if lyophilized silk proteins are required. Therefore, it would be interesting to find a similar material system but that requires a simpler and shorter protein extraction procedure.

Figure 4.1 Schematic of the silk fibroin extraction procedure. Going from the raw material (cocoons) to the final aqueous-based solution takes 4 days. The figure is adapted from Ref.^[105]

On the other hand, it is very straightforward to extract suckerins from SRT due to the absence of non-suckerin components in SRT and the high solubility of suckerins in the extraction solution (see below). Furthermore, native suckerins exhibit synthetic polymer-like thermoplastic characteristics, which endow the proteins with excellent high temperature processability (Chapter 3). These two characteristics, combined with the modular primary sequence design of suckerins that includes β -sheet forming domains, suggest an intriguing potential to use native suckerin mixtures as building blocks to engineer protein-based materials that could rival those made from silks. In this Chapter, diverse materials including melt-spun fibers and electro-spun nanofibers are prepared from native suckerin mixtures and assessed by a range

of techniques to characterize their protein secondary structure, thermal, and mechanical properties.

4.2. Materials and methods

4.2.1. Preparation of fibers from native suckerins

Fibers made of native suckerins were prepared by melt-spinning. The *D. gigas* SRT powder (protein mixture) was achieved in the same way as in the previous Chapter (section 3.2.1). Ten mg of the dry powder was re-suspended in 6 mL deionized water inside a glass Petri dish. The dish was heated in a microwave (900 W) for 25 seconds, leading to a viscous melt from which fibers could be spun using a sharp spatula that was immersed into the solution surface and then drawn out. The protein mixture was put back into the microwave for 15 seconds to spin more fibers once it had cooled down. The mixture volume was kept relatively constant by refilling deionized water during the spinning cycles. Fibers were let dry on glass slides, from which they were collected for further analyses.

4.2.2. Diameter, chain alignment and birefringence of suckerin fibers

Polarized light microscopy is a technique used to detect birefringence in materials, which can arise from structural anisotropy in the materials such as polymer chain alignment.^[159] We used polarized light microscopy to assess chain alignment of drawn suckerin fibers. Fibers mounted on glass slides were observed under an Axio Scope A1 polarized light microscope (Carl Zeiss). The diameter of each fiber was obtained as the average of at least 3 fragments along the fiber, with at least 10 readings taken for each fragment. Representative fibers were also stepwise rotated by 45° to observe the birefringence.

4.2.3. Morphology of suckerin fibers

The morphology and microstructures in both the longitudinal and cross-section of suckerin fibers was examined by scanning electron microscopy (SEM) (JSM-6340F, JEOL Co.) with 5 kV of acceleration voltage. Before placing the samples in the SEM chamber, the samples were mounted onto a copper stud and sputter-coated with platinum for 45 s (JFC-1600, JEOL Co.) to prevent charging.

4.2.4. Protein secondary structure of suckerin fibers

The protein secondary structure of suckerin fiber was analyzed by Fourier transform infrared spectroscopy (FTIR) in the Attenuated Total Reflection (ATR) mode. The fiber was measured using a Vertex 70 infrared spectrometer (Bruker) with 250 scans in the region of $4000\text{--}400\text{cm}^{-1}$ at room temperature. The Amide I region was analyzed by standard curve-fitting based on 2nd derivatives to calculate the percentage of different secondary structures.^[160]

4.2.5. Thermal property of suckerin fibers

The thermal responses and stability of the melt-spun fibers were investigated using DSC (DSC Q10, TA Instruments Co.) under nitrogen atmosphere at a flow rate of 50 mL/min. At least 40 suckerin fibers were mounted into the DSC pan and then were heated from 25 to 300 °C at a heating rate of 10 °C/min.

4.2.6. Mechanical properties of suckerin fibers

The mechanical properties of the suckerin fibers were investigated using an Agilent UTM T150 testing instrument (Agilent) at room temperature. Tensile tests were performed at an extension rate of 0.01 l^{-1} , with a gauge length of 20 mm. The diameter of the samples was measured under the polarized light microscope and used to calculate the Young's modulus and ultimate strength.

4.2.7. Preparation of electro-spun suckerin nanofibers

The suckerins powder was made as above and dissolved in 50% acetic acid (v/v) at 100 mg/ml. A sonication of 5 min was used to facilitate dispersion of proteins, which were centrifuged at 16,000 g for 5 min to eliminate minor insoluble aggregates. The resultant proteins were used for electrospinning of fibers with the equipment illustrated in Figure 4.2. The accelerating voltage was varied between 9 to 20 kv, while the flow rate of proteins solution was set between 0.1 to 1.0 ml/h. The distance between the spinneret and the aluminum foil for fiber collection was set constant at 15 cm. The protein concentration was also adjusted for optimization. In order to improve the spinnability of proteins, polyethylene glycol (PEO, MW: 900 kDa) was added to the protein solution at different ratios.

Figure 4.2 A scheme showing the common setup and working principle of electrospinning used in this study. The figure is adapted from Ref.^[161]

4.2.8. Scanning electron microscopy of electro-spun suckerin nanofibers

The electrospinning products of suckerin proteins were investigated by SEM (JSM-6340F, JEOL Co.) with an acceleration voltage at 5 kV. Before placing the samples in the SEM chamber, the samples were sputter-coated with platinum for 45 s (JFC-1600, JEOL Co.) to prevent charging.

4.3. Results and discussions

4.3.1. Melt-spun suckerin fiber

Fibers made of extracted suckerin mixture (suckerin fibers) can be easily spun by drawing from the protein melt. The longest fiber that could be spun was more than 1.0 m in length (data not shown). The mean diameter measured by the polarized light microscope was $20.6 \pm 5.2 \mu\text{m}$ (Table 4.1), which is quite close to native silk fiber (varying between 15 to 19 μm including the sericin coating).^[162] This spinning technology and the fibers possess a few intrinsic advantages compared to those spun from regenerated silk fibroins: (i) The melt-spinning is a very simple and straightforward processing, which does not need the complicated degumming, solubilization, and dialysis of silk fibroins used for the fabrication of regenerated silk fibers.^[163, 164] (ii) This strategy is environment-friendly. It avoids the use of harsh chemicals treatment such as boiling in Na_2CO_3 and using highly concentrated LiBr .^[163-166] (iii) This processing is completely water-based, which provides a much more versatile strategy than the harsh treatments used in the fabrication of many other types of silk protein-based materials, including tissue engineering scaffolds, films, or microspheres.^[105, 167, 168]

4.3.2. Chain alignment and birefringence of suckerin fibers

The protein chain alignment of suckerin fibers was observed under by polarized light microscopy (Figure 4.3). Birefringence was observed by rotating the fibers to different angles under the polarized microscope. Birefringence is positively correlated with molecular orientation, which indicates the presence of crystalline structures, possibly β -sheets in the fibers.^[141, 169] The melt-spinning is associated with rapid solvent evaporation and fiber solidification, since it took a few seconds for the formation of fibers. This short time scale gave rise to a significant elongation of the

polymer chains. Therefore, melt-spun fibers exhibited orientational order along the main fiber axis. The chain alignment might facilitate inter-molecular interactions between polymer chains (*e.g.* hydrogen bonds between chains), which can mechanically reinforce the material.

Figure 4.3 Chain alignment and birefringence of suckerin fibers investigated by polarized light microscopy. The fiber was rotated by 45° (b) and 90° (c) from the original position (a). Bright and extinct contrast is related to birefringence and fiber elongation.

4.3.3. Morphology of suckerins fibers

The morphology and microstructure of suckerin fibers was investigated by SEM. The diameter of the fibers was found to be around 20 μm (Figure 4.4). The fibers were not uniform and smooth, but composed of thinner sub-layers. The layers were bundled together and continuously aligned along the direction of the fiber axis (Figure 4.4a). The image at the cross-section of a fiber confirmed the sub-layer microstructure of suckerin fibers, which showed different layers combined together with a thickness varying from about 200 nm to 500 nm (Figure 4.4b). It is worth noting that this microstructure is different from native and regenerated silk fibers, which are more uniform, compact and smooth.^[165, 170] Chanel-like gaps were observed between the thin layers with a diameter from a few hundred nanometers to beyond 1 micron. This type of microstructure makes the real diameter of fibers smaller than that observed from the longitudinal direction in SEM and polarized light microscopy, which might play a role in influencing the mechanics of suckerins fibers.

Figure 4.4 Morphology and microstructure of a drawn suckerin fiber. The longitudinal view is shown in (a). The microstructure of the cross-section is shown in (b).

4.3.4. Protein secondary structures of suckerin fibers

FTIR spectroscopy is a useful technique used to study protein secondary structure, and reveals absorption bands sensitive to molecular confirmation of proteins.^[170, 171] The FTIR spectrum of the melt-spun suckerin fiber (Figure 4.5a) was characterized by strong absorption bands at 1668 cm^{-1} and 1640 cm^{-1} (amide I) attributed to β -turn and random coil structures respectively, as well as peaks at 1540 cm^{-1} and 1516 cm^{-1} (amide II) assigned to random coil and β -sheet conformation respectively.^[169-175] In order to clarify the secondary structure of drawn suckerin fibers in details, the amide I profiles were de-convoluted^[160] using the standard 2nd derivative method as shown in Figure 4.5b. The de-convoluted curves indicated that suckerins fibers were highly rich in random coil and β -turn characteristics at 37.8% and 34.0% respectively. However, suckerin fibers also exhibited a high level of β -sheets at 24.5% (1623 , 1633 and 1696 cm^{-1} respectively).^[171, 172, 176] It is also noted that the ordered structure (β -sheets and β -turns) can go up to beyond 50%,^[27, 28] implying a promising potential to tune the mechanical properties of the fibers by adjusting the content of these structures.

Figure 4.5 (a) ATR-FTIR spectra of suckerin fiber obtained by melt-spinning; (b) Deconvoluted ATR-FTIR profiles of amide I of suckerin fiber obtained by melt-spinning.

4.3.5. Thermal behavior of suckerin fibers

The thermal behavior of suckerin fibers was examined by DSC. The fibers showed two broad and eminent endothermal peaks, one at low temperature starting at ~ 80 °C up to ~ 120 °C which was associated to water evaporation, and another at high temperature (endothermal peak at 260 °C), attributed to thermal melting of β -sheets in the structure (Figure 4.6a).^[171] This indicated a high thermal stability of suckerin fibers. Between the water evaporation peak and melting peak, no additional peak was noticed, denoting that no further thermal-induced crystallization occurred in the fibers.^[174]

Figure 4.6 (a) DSC curves of melt-spun suckerin fibers. The fibers were heated from 25 °C to 300 °C with a temperature increase rate of 10 °C/min. (b) Strain-stress curves for melt-spun suckerin fibers obtained by nanotensile testing.

4.3.6. Mechanical properties of suckerin fiber

In order to characterize the mechanical performance of suckerin fibers, nanotensile tests were performed under dry conditions and the results are shown in Figure 4.6b and Table 4.1. Stress-strain curves of melt-spun fibers (Figure 4.6b) had three distinct regions that began with a stiff region and yield from a strain about 1%. The minor drop in the stress at the yield point led to a shallow plateau region. Most fibers failed in the plateau region, while with the exception of one fiber, the plateau region led to a strain-stiffening region that preceded failure. The average Young's modulus of the fibers was 1.75 ± 0.65 GPa, and the average tensile strength of these fibers was 18.5 ± 12.5 MPa, with an average breaking strength at 15.9 ± 11.1 MPa. The large deviation in ultimate strength arises from one outlier fiber with higher Young's modulus, tensile strength and breaking strength compared to the others (Figure 4.6b). Although more work is needed to optimize the process, the results show that in terms of mechanical performance, the melt-spun fibers can potentially rival those from other regenerated silk and protein fibers without post-spinning processing. [103, 164, 166, 177] At the same time they are formed by a water-based eco-friendly processing. The low average mechanical strength is attributed partially due to

extensive porosity found between different sub-layers inside the fibers, leading to localized weak regions, stress concentrators that lead to premature failure. The fundamental step in natural silk fiber formation is the transformation of the protein solution into the β -sheet rich secondary structures responsible for the outstanding mechanical properties.^[103] Therefore, future effort will be focused on optimizing the spinning conditions to improve the β -sheet contents in suckerin fibers. It has been reported that the spinning speed, the temperature, humidity, and species type (protein composition) may influence the mechanical strength of silkworm and spider silks.^[173, 178, 179] These factors will affect the chain alignment of proteins in the fibers, subsequently influencing the formation of β -sheets and mechanical properties of the fibers. Therefore, an automatic spinning system will be constructed to optimize the spinning of suckerin fibers. The device consists of a motor, a speed controller, and a bobbin. The speed controller of the motor will precisely control the spinning speed, while the dish can be heated to different temperatures in order to vary processing conditions. Therefore, important parameters including the spinning speed and the temperature of melted proteins, can be optimized with this setup. It has also been shown that post-spin drawing can alter the polymer chain alignment and affect mechanical properties of silk fibers.^[103] Therefore, different ratios of post-spin drawing right after the formation of fibers, including 10%, 20%, 50% and 100%, will be used to improve the mechanical properties of suckerin fibers. Besides the improvement of β -sheet content, covalent cross-links can be introduced to enhance the mechanical properties of suckerin fibers, given that SRT protein are highly rich in Tyr (7-16 mol.%). A similar way used in cross-linked suckerin-19 films will be employed for this purpose.

Table 4.1 Summary of mechanical properties of melt-spun suckerin fibers.

Fiber	Specimen Diameter (μm)	Modulus (GPa)	Breaking Strength (MPa)	Tensile Strength (MPa)
Mean	20.55	1.75	15.86	18.51
Std. Dev.	5.20	0.65	11.08	12.49

4.3.7. Electro-spun suckerins nanofiber

Electro-spinning is a widely used technology for the preparation of micro-scale fibers with high aspect ratios. It employs an electrical potential to produce fibers with diameters ranging from a few nanometers to tens of microns from the solutions of a wide spectrum of native and synthetic polymers.^[180, 181] In recent years, there have been increasing interests in research and commercial applications of fibers produced by electro-spinning.^[180, 182] This is not only due to the versatility of the technique in spinning a great variety of polymer solutions, but also due to the ability of electro-spinning to make polymeric fibers in the nanometer range, which is difficult to reach by other standard fiber-spinning techniques.^[183] Electro-spun fibers are also advantageous due to (i) an extraordinary high surface-to-volume ratio, (ii) tunability in sizes and shapes, and (iii) the ability to control the composition of nanofibers to obtain desired properties and functionalities.^[161] To this end, electro-spun fibers have been widely used in a range of fields, including filtration, pharmaceutical, tissue engineering, catalysis, protective clothing and sensors.^[184]

Due to their good biocompatibility, biodegradability, minor antigenicity and excellent mechanical properties, silk fibroins are promising natural polymers which can be utilized in the fabrication of nanofibers by electro-spinning.^[161] Given the advantages of fibers in nanometer size and those of silk fibroins themselves, electro-spun silk nanofibers are widely used as tissue engineering scaffolds, wound dressings and drug delivery systems. However, a strong solvent (hexafluoro-2-propanol, HFIP)

and polymer polyethylene glycol (PEO) are often used to successfully electro-spin silk fibroins.^[105, 185]

The high solubility of suckerins in concentrated acetic acid prompted us to attempt their electrospinning in this solvent. We found that varying the electrospinning parameters, including the flow rate of protein solution from 0.1 to 0.4 ml/h, did not lead to suckerin nanofibers (Figure 4.7a-c). Instead, inhomogeneous particles with dimensions from ~100 nm to >1 μm were observed. The small nanoparticles were consistent with the original nanoparticles observed in the suckerins solution before electro-spinning (Figure 4.7d) and those big particles might be due to the aggregation of small nanoparticles during electrospinning. PEO is a useful additive to increase the viscosity of polymer solutions because it improves their spinability, including for nanofibers made of silk fibroins. Therefore, 10 mg/ml PEO (MW: 900 kDa) was added to suckerin solutions at 1:7 ratio (PEO/suckerins, w/w). Clear improvements were obtained for the electro-spun products, as shown by the connecting fibers between big suckerin particles (Figure 4.8). However, there existed no long and continuous nanofibers at various flow rates (0.4 and 0.6 ml/h) and various accelerating voltages (12 kv and 15 kv). It is also noticed that higher accelerating voltage (15 kv) resulted in stretched spindle-like suckerin particles, which implied an improved spinnability (Figure 4.8c), but such conditions were still not able to produce mature nanofibers. Afterwards, PEO concentration was increased to 30 mg/ml in the protein solution (1:2, w/w, PEO/protein). Significant improvement was obtained, as indicated by the presence of continuous nanofibers (Figure 4.9), either at different accelerating voltages (9 kv and 13 kv) or at different flow rates (0.4, 0.7 and 1.0 ml/h). There was no significant difference in fiber diameter at different electrospinning conditions. However, the fiber morphology was not uniform due the presence of

micron-size protein beads, showing that further optimization will be necessary in the future.

Figure 4.7 Electrospraying of 100 mg/ml suckerins at 0.1 ml/h (a), 0.2 ml/h (b) and 0.4 (c) ml/h in 50% acetic acid. The particle size distribution of suckerins before electrospraying at this condition is shown in (d).

Figure 4.8 Electrospraying of suckerins with 10 mg/ml PEO at different conditions: (a) 0.4 ml/h, 12 kv; (b) 0.6 ml/h, 12 kv; (c) 0.6 ml/h, 15 kv.

Figure 4.9 Electrospinning of suckerins with 30 mg/ml PEO at different conditions: (a) 0.4 ml/h, 9 kv; (b) 0.7 ml/h, 9 kv; (c) 1.0 ml/h, 9 kv; (d) 0.4 ml/h, 13 kv; (e) 0.7 ml/h, 13 kv; (f) 1.0 ml/h, 13 kv.

Next, PEO content in the protein solution was further increased to 1:1 (PEO/protein, w/w). A low protein concentration at 15 mg/ml was first tried for the electrospinning, where relatively smooth and uniform nanofibers with diameters around 150-200 nm were found under flow rates ranging from 0.2 to 0.4 ml/h (Figure 4.10a-c), while 0.5 ml/h flow rate gave rise to beaded structure (Figure 4.10d). These data are suggesting that flow rate is playing an important role in tuning the fiber morphology. However, a closer examination of these nanofibers revealed that suckerin nanofibers were not internally homogeneous, as many chunk-like particles were observed inside the nanofibers, which we speculate were suckerin protein nanoparticles (Figures 4.10e and f). At high protein concentration (30 mg/ml) with the same PEO weight ratio, smoother, more uniform and thicker fibers with diameters ~200 to 300 nm were produced at relatively low flow rates (0.2 and 0.3 ml/h) and they were independent of the accelerating voltage (Figure 4.11a-c). Similar to nanofibers prepared at low concentration, these nanofibers were also internally composed of protein particles (Figure 4.11d-f). It is worth noting that these particles are consistent

with those observed data before and after electrospinning (Figures 4.7 and 4.8) and indicated that a significant amount of suckerin particles may be insoluble during the electro-spinning process. Subsequently, suckerins will not be able to interact with each other to form stable continuous nanofibers while water is employed to remove PEO.^[105] Therefore, a stronger solvent (*e.g.* formic acid) could be used in the future to disrupt interactions between protein molecules and prevent the formation of protein nanoparticles. This could facilitate homogeneous distribution of suckerins inside the nanofibers and could result in uniform and continuous fibers after the removal of PEO.

Figure 4.10 Electrospinning of suckerins/PEO (15 mg/ml for each) at different flow rates from 0.2 to 0.5 ml/h (**a-d**). The high magnification images of nanofibers electrospun at 0.2 and 0.4 ml/h are shown in (**e**) and (**f**) respectively.

Figure 4.11 Electrospinning of suckerins/PEO (30 mg/ml for each) at different conditions. (a) 0.3 ml/h, 13 kv; (b) 0.2 ml/h, 15 kv and (c) 0.2 ml/h, 20 kv. The high magnification images of nanofibers are shown in (d), (e) and (f) respectively.

4.4. Conclusion

The mechanical properties of *D. gigas* SRT, their protein-only composition, ease of protein extraction, and high solubility, as well as their thermoplastic characteristics allow for the straightforward preparation of materials with native suckerins. In this Chapter, suckerins mixtures were first processed into macroscopic fibers by a melt-spinning method. This is an environment-friendly method to make protein fibers, which overcomes some of the issues associated with fabrication of regenerated silk fibers, such as the usage of harsh chemicals. Fiber characterization revealed a diameter comparable to native silk fibers and protein chain alignment. FTIR and DSC investigations confirmed the significant presence of β -sheet configurations in the fibers, which is likely responsible for their mechanical strength that can rival regenerated silk fibers without post-spinning treatments. However, the mechanical strength is still much lower than native silk fibers and post-processed regenerated silk

fibers, making it necessary to optimize the spinning conditions for improvement of SRT based fibers, with an emphasize on increasing the β -sheet content in the future.

Extracted suckerins mixture was also utilized for the fabrication of nanofibers by electro-spinning. Suckerins alone displayed a low spinnability even at a very high concentration. But the addition of PEO significantly improved the spinnability of suckerin solutions. In particular, an equal amount of PEO and suckerins at high concentration (30 mg/ml) led to smooth and uniform nanofibers with diameters ranging from 200 to 300 nm. However, an inhomogeneous internal structure of nanofibers was observed, with the formation of locally aggregated suckerin nanoparticles dispersed in PEO matrix. Therefore, a more efficient solvent would be recommended in future studies to prevent the aggregation of suckerins and facilitate the homogeneous distribution of proteins inside nanofibers, which will guarantee the stability of suckerin nanofibers once PEO is removed.

Chapter 5 Recombinant Expression, Purification and Characterization of Suckerin-19^a

5.1. Introduction

Chapter 1 has revealed that *D. gigas* suckerins –the most abundant being suckerin-19– display a regular modular sequence design^[25] comprising of two main types of alternating modules: (i) Ala-rich modules that also contain significant of His residues which are reminiscent of poly-Ala β -sheets^[26] that mechanically reinforce spider dragline silks,^[27] and (ii) the Gly- Tyr- and Leu-rich modules containing tri- and tetra-peptides such as GGY, GGL and GGLY. It is key to keep in mind that the homology of suckerins to silk proteins is appealing given the wide range of biomedical and engineering applications demonstrated for silks, for example in tissue engineering,^[31] drug delivery vehicles^[32] and photonic devices^[33] to name just a few.

Over the past decade there have been significant efforts to achieve high yield recombinant expression of silk proteins and to establish streamlined green processing protocols that maintain silk protein solubility with minimal usage of harsh and toxic solvents. These two goals have met several major challenges. Recombinant spidroins have been expression in *E. coli*, yeast, plants, silkworms (*B. mori*), plants, mammalian cells and glands, as reviewed in Chapter 2 (Section 2.3.1). Another major goal in silk biotechnology is to develop efficient methods for the solubilization and processing of native and recombinant silk proteins. These proteins have a well-known tendency to prematurely adopt β -sheet structures and aggregate under aqueous-based conditions. While an impressive range of structures and functional materials have been generated

^a This Chapter mostly comes from my first-author paper entitled “Biomimetic Production of Silk-Like Recombinant Squid Sucker Ring Teeth Proteins” (*Biomacromolecules* 2014, 15, 3278–3289).

using both native silk fibroins and recombinant silk-like proteins,^[73, 105] many published processing strategies involve the use of harsh conditions, including 9 M lithium bromide (LiBr) and HFIP to solubilize and process these proteins. On the other hand, recent studies have also focused on processing fibroins from the aqueous phase under more environmentally-friendly conditions, with promising results.^[105, 186]

Gram quantities of native SRT can be collected for laboratory-scale experiments. However, large-scale production of suckerin for biomedical and engineering applications will require a reliable source of recombinant proteins. Moreover, suckerins in SRT are too similar to each other in modular sequence and thus the biophysical features, making it tough to purify single protein from the native mixture through chromatographic methods. Therefore, in this Chapter, we first aim to test and establish streamlined recombinant expression and purification strategies for suckerins, which will eventually be suitable for large-scale production. Second, we aim to establish the conditions which maintain solubility of recombinant suckerins. Two versions of full-length suckerin-19 were expressed in *E. coli*: (i) a codon optimized, poly-His tagged suckerin-19, and (ii) a non-tagged native coding sequence of suckerin-19. Our data show that full-length recombinant suckerin proteins were readily expressed in inclusion bodies and easily purified by chromatographic and non-chromatographic methods. Recombinant suckerin-19 was found to exhibit high solubility in mild acidic environments and water alone. Solubilized suckerins were able to assemble into colloids of discrete dimensions, which are suggested to offer a route toward the facile processing of these proteins into hierarchical structures. These results establish relevant parameters for the processing of suckerin-based materials from aqueous solutions and for the green processing of high-performance “bioplastics”.

5.2. Materials and methods

5.2.1. Cloning of His-suckerin-19 and suckerin-19

The full-length open reading frame (ORF) of suckerin-19 was originally obtained from the transcriptome assembly of jumbo squid sucker tissue^[25] and confirmed by RACE-PCR. The codon optimized gene encoding suckerin-19 was purchased from DNA 2.0 (Menlo Park, California). A PCR product containing this gene was then generated using forward primers that included LIC cloning sequences: His-suckerin-19-FWD primer: 5'-TGTGAGCGGATAACAATTCC-3'; His-suckerin-19-REV primer: 5'-AGCAGCCAACTCAGCTTCC-3'. The PCR product was isolated using a QIAquick Gel Extraction Kit (Qiagen) and cloned into the LIC sites of the pNIC-28-BsaIV plasmid vector (Figure 5.1a) that included sequences encoding a 5' poly-His tag and a linker peptide containing a TEV protease cleavage site. The plasmid constructs were then transformed into *E. coli* Rosetta Cells.

For non-tagged suckerin-19, the full-length ORF omitting the signal peptide sequence was amplified by RT-PCR using KOD polymerase and the following primers: suckerin-19-FWD primer: 5'-AAAAAAGCTAGCATTTTGCCAGCGGCAACATCTG-3'; suckerin-19-REV primer: 5'-AAAAAACTCGAGTTAGTGGAGGAGACCATATCCAC-3'. The PCR product was then purified using a QIAquick Gel Extraction Kit (Qiagen) and cloned into the into the NheI/XhoI site of a pET23a vector (Novagen) (Figure 5.1b), which was transformed into *E. coli* BL21 (DE3) cells. Cell stocks were made in 20% glycerol. The sequences of both His-tagged and non-tagged suckerin constructs were verified by Sanger sequencing.

Figure 5.1 Plasmid vector configurations for the recombinant expression of His-suckerin-19 (a), suckerin-19 (b) and TEV protease (c). The detailed sequence of His-tag and linker peptide of His-suckerin-19 is shown in (a).

5.2.2. Expression of His-suckerin-19 and suckerin-19

10 μ l of cells from both glycerol stocks were streaked onto LB agar plates containing kanamycin (30 μ g/mL) and ampicillin (100 μ g/mL) respectively, and grown overnight at 37 °C. Single colonies were inoculated into selective LB media (30 μ g/mL kanamycin and 100 μ g/mL ampicillin for His-suckerin-19 and suckerin-19 cells, respectively) for overnight shaking cultures at 37 °C. The next day, cell cultures were diluted 100-fold into fresh LB media (with the same concentrations of antibiotics) and grown under the same conditions until an OD₆₀₀ of ~0.6-1 was achieved. Protein expression was induced by adding 0.5 mM IPTG and cultures were further incubated with shaking for 3-4 hours before harvesting by centrifugation at 14,000 g for 10 min and frozen at -20 °C.

5.2.3. Purification of His-suckerin-19 by Immobilized Metal Affinity Chromatography (IMAC)

Cell pellets were re-suspended in lysis buffer (20 mM Tris-HCl, pH 7.4), lysed by sonication (40% amplitude, 1 second pulse + 1 second pause, 7.5 min sonication) and clarified by centrifugation at 23,000 g for 10 min at 4 °C. The pellet was re-

suspended in 8 M urea and 5% acetic acid at 4 ml per gram wet weight of pellet. Small aliquots (10 μ l) of both supernatants and urea-solubilized pellets were loaded into SDS-PAGE gels to verify the protein expression for each cell line. His-suckerin-19 was purified from inclusion bodies under denaturing conditions by IMAC. His-suckerin-19 inclusion bodies were dissolved in a solution of 5% acetic acid with 8 M urea, and 0.5 ml of this solution was mixed with 7.5 ml of binding buffer (8 M urea, 20 mM NaH_2PO_4 , 0.5 M NaCl, 10 mM imidazole, pH 7.8). The sample was then incubated with 1 ml Ni-NTA resin (Qiagen), allowing the binding of proteins to the resin. Two parameters were evaluated, namely the binding temperature (4 °C and room temperature) and binding time (1h and 15 h). After binding, the flow-through from the column was collected and the resin was rinsed twice with 5 ml wash buffer (8 M urea, 20 mM NaH_2PO_4 , 0.5 M NaCl, 30 mM imidazole, pH 7.8). Finally, the protein was eluted from the resin with 10 ml elution buffer (8 M urea, 20 mM NaH_2PO_4 , 0.5 M NaCl, 500 mM imidazole, pH 7.8). The samples were then dialyzed against 5% acetic acid, freeze-dried, solubilized in 0.5 ml 5% acetic acid, and subjected to SDS-PAGE analysis. Their purity and molecular weights were further confirmed by Matrix-Assisted Laser Desorption Ionization Time-of-Flight (MALDI-TOF) mass spectrometry (AXIMA-TOF2, Shimazu) with Sinapinic acid (Sigma) dissolved in a mixture of 50/50 Q-water/ACN with 0.1% TFA as the matrix.

5.2.4. Purification of His-suckerin-19 and suckerin-19 by salting-out

Cell pellets were lysed as described above and His-suckerin-19 was purified by salting-out using a buffer with a pH close to its calculated isoelectric point (IEP) (8 M urea, 20 mM NaH_2PO_4 , 0.5 M NaCl, 10 mM imidazole, pH 7.8). Inclusion bodies were solubilized in 8 M urea and 5% acetic acid at 4 ml solution/gram of inclusion bodies and dialyzed twice against the salting out buffer at a volume ratio of 1:1000.

The precipitated proteins were collected by centrifugation at 16,000 g for 10 min and the pellet was re-solubilized in 5% acetic acid. Suckerin-19 was purified with a similar salting-out method, where the pH of the binding buffer was adjusted to 8.4 to account for the higher IEP of non His-tagged suckerin.

5.2.5. Purification of suckerin-19 by microfluidization followed by inclusion body purification

Harvested cell pellets were washed twice with lysis buffer (20mM Tris, pH8). Each pellet from 1L culture was resuspended in 50 ml of lysis buffer (50mM Tris pH7.4, 200mM NaCl, 1mM PMSF) and lysed by 6 passes of microfluidization (Microfluidics M-110P). The lysate was clarified by centrifugation at 52,000 g for 1 h at 4°C. The pellet was washed twice with 20 ml wash buffer 1 (100 mM Tris, 5 mM EDTA, 2 M urea, 2% (v/v) Triton X-100, 5 mM DTT, pH 7.4) followed by two washes of wash buffer 2 (100 mM Tris, 5 mM EDTA, 5 mM DTT, pH 7.4). The inclusion bodies were collected by low speed centrifugation (5,000 g for 15 min at 4°C) between wash steps. Finally, the pellet was re-solubilized in 5% acetic acid.

5.2.6. Protein expression of TEV protease

A solubility improved variant of TEV protease was achieved by directed evolution.^[187] The gene giving rise to the highest solubility of protein was amplified with primers adding NheI sites in both ends and transferred to a pTH24 vector (Novagen) (Figure 5.1c) providing a C-terminal hexahistidine (His₆) tag for up-scaled expression using the Gateway recombination technology. Pre-culture in 10 ml LB with ampicillin (50 µg/ml) and chloramphenicol (34 µg/ml) were grown overnight at 37 °C and used to inoculate 500 ml cultures the next day. The cultures were incubated at 37 °C until OD₆₀₀ reached 0.6-1. Protein expression was induced by addition of

IPTG to a final concentration of 1 mM, and the temperature was lowered to 20 °C. The expression was continued for 20 h.

5.2.7. Protein purification and activity test of TEV protease

After expression, the cells were centrifuged at 12,000 g for 10 min at 4 °C. The pellet was re-suspended in the lysis/binding buffer (20 mM HEPES, 500 mM NaCl, 10 mM imidazole, 0.5 mM TCEP, pH 7.5) and lysed by sonication. Then, the lysate was subsequently centrifuged by 16,000 g for 10 min at 4 °C. The pellet was re-suspended in 8 M urea and 5% acetic acid. Both the supernatant and pellet were loaded into the SDS-PAGE gel to verify the expression.

TEV protease purification was conducted by IMAC under native conditions. 1 ml Ni-NTA resin was washed with deionized water and equilibrated with the binding buffer. 10 ml supernatant from the cell lysate was mixed with the equilibrated resin to allow an overnight binding at 4 °C. Afterwards, the flow-through from the purification column was collected and the resin was washed with 4 ml wash buffer 1 (20 mM HEPES, 500 mM NaCl, 10 mM imidazole, 0.5 mM TCEP, 10% glycerol, pH 7.5) and wash buffer 2 (20 mM HEPES, 500 mM NaCl, 25 mM imidazole, 0.5 mM TCEP, 10% glycerol, pH 7.5), respectively. The protein was eluted from the resin with 10 ml elution buffer (20 mM HEPES, 500 mM NaCl, 500 mM imidazole, 0.5 mM TCEP, 10% glycerol, pH 7.5). The elution fractions were further subjected to a desalting column (PD-10, GE Healthcare) and eluted with the gel filtration buffer (20 mM HEPES, 300 mM NaCl, 5 mM EDTA, 0.5 mM TCEP, 10% glycerol, pH 7.4). The flow-through, washing, and elution fractions (both from the IMAC and desalting) were analyzed with SDS-PAGE.

The activity of TEV protease was investigated on the model substrate, the small ubiquitin-related modifier (SUMO) fusion protein which contained an engineered TEV protease cleavage site.^[188] 50 μ L of the substrate (1.6 mg/ml) was mixed with 18 μ L TEV protease (0.24 mg/ml), with a substrate-to-enzyme molar ratio of 10:1. The reaction was conducted at 4 °C and 8 μ L of sample was taken from the reaction for SDS-PAGE analysis at different time intervals of 2 h, 4 h, 8 h, 24 h, 48 h and 96 h.

5.2.8. Screening of buffers to cleave the His-tag from His-suckerin-19 using TEV protease

In order to cleave the His-tag from the recombinant His-suckerin-19, various buffer conditions were screened, with the goal of keeping His-suckerin-19 soluble while maintaining TEV protease activity. Buffers containing 0.5 mM EDTA, 1 mM DTT, and different concentrations of urea from pH 4.0 to 9.0 were screened. Eluted fractions of His-suckerin-19 with concentrations ranging from 0.1-0.2 mg/ml were dialyzed against these buffers overnight and centrifuged at 16,000 g for 5 min to spin down possible precipitates. Supernatants and pellets (dissolved in 5% acetic acid) were analyzed by SDS-PAGE. In addition, the enzymatic activity of TEV protease in each buffer was evaluated using a SUMO fusion protein^[188] with a similar procedure as above. Buffers were considered not useful if His-suckerin-19 precipitation occurred or if TEV protease did not cleave the SUMO fusion protein in test digestion experiments, which involved verifying the presence of two bands by SDS-PAGE in the case of successful cleavage.

5.2.9 Evaluation of solubility of suckerin-19 in different solutions

Freeze-dried suckerin-19 was dissolved in different solutions including milli-Q water, 5% acetic acid (v/v), and 8 M urea containing 5% acetic acid (v/v). In order to

measure the maximum solubility of suckerin-19 in each solution, small volumes of solution were sequentially added to the suckerin-19 powder until the majority of the proteins were completely dissolved. Five minutes of sonication was used to facilitate the dispersion. The suspension was then centrifuged for 5 min at 16,000 g to remove the minor fraction of insoluble products and the protein concentration of each supernatant was analyzed with Bradford protein assay kit (Thermo Scientific). Each sample was diluted to below 1 mg/ml with respective solvent before the measurement. The maximum protein solubility was measured 3 to 4 times for each solvent tested and calculated using a standard curve.

We also evaluated the solubility and refolding/assembly behavior of suckerin-19 in several buffered saline solutions. For these experiments 0.1 mg/ml of 5% acetic acid solubilized protein was dialyzed against each buffer. The dialysate was then centrifuged at 16,000 g for 15 min. The supernatant was concentrated to a volume of ~100 μ L with a Vivaspin Sample Concentrator (GE Healthcare) and the pellet was dissolved in an equivalent volume of 5% acetic acid as that in dialysis. Both fractions were then evaluated by SDS-PAGE to infer solubility/insolubility (data not shown) and suckerin concentrations in the supernatant fractions were quantified using Bradford assay as described above.

5.2.10 Biophysical characterization of suckerin-19

Particle dimensions of suckerin-19 were determined by dynamic light scattering (DLS). Suckerin-19 was re-suspended in 5% acetic acid and water at 1 and 6 mg/ml or in 0.05 M MES, pH 5.5 at 0.64 mg/ml, and the samples were analyzed with a 90Plus particle size analyzer (Brookhaven Instruments) equipped with a 658.0 nm monochromatic laser. To minimize the reflection effect, all measurements were taken

at a scattering angle of 90° . The number-weighted histogram profiles of the proteins in solution are reported. The mean particle size of suckerin-19 colloids in water was also investigated by the Atomic Force Microscopy (AFM) (MFP-3D-BIO, Asylum Research). The protein was diluted to 10 $\mu\text{g/ml}$ with water and single drops of the solution were placed on mica substrates, and allowed to equilibrate for 20 min followed by rinsing with water. Finally, the sample was air-dried and subjected to AFM imaging. The mean size of particles from three images was measured by Image JTM. The zeta-potentials of these samples were measured in triplicate at 25 $^\circ\text{C}$ with 15 scans for each measurement using a Nano-ZS Zeta sizer (Malvern).

The secondary structure of suckerin-19 was characterized by circular dichroism (CD) using a Chirascan spectropolarimeter (Model 420, AVIV Biomedical Inc.). 1 mg/ml and 6 mg/ml of suckerin-19 in water and in 5% acetic acid, as well as 0.64 mg/ml suckerin-19 in 0.05 M MES at pH 5.5 were used for the measurements, which were conducted in triplicate at wavelengths ranging from 190 to 260 nm, with 1 nm step size and 1 nm bandwidth. The spectra were smoothed by the Savitzky-Golay method with a polynomial order of 2. The secondary structure of suckerin-19 in different solutions was also measured by FTIR spectroscopy. A Vertex 70 instrument (Bruker) was employed in the Attenuated Total Reflection (ATR) mode, with 4 min scanning and a resolution of 4 cm^{-1} in the $4000\text{--}400\text{ cm}^{-1}$ region. The respective solvents were used as the controls. The protein concentration was 6 mg/ml for suckerin-19 in 5% acetic acid and water, and 0.64 mg/ml for that in 0.05 M MES (pH 5.5). In order to estimate the fraction of different secondary structures, the Amide I band was deconvoluted using OPUS 6.5 software (Bruker). The positions of the fitting peaks were fixed based on peaks identified in the second derivative of Amide I. In this case, the widths of the fitted peaks were approximately the same.

5.3. Results and discussions

5.3.1. Expression of His-suckerin-19 and suckerin-19

Two types of *E.coli* cells, Rosetta cells and BL21 (DE3) cells were cultured for suckerin expression. Both His-suckerin-19 and suckerin-19 were almost exclusively found in the pellet after cell lysis (Figure 5.2a), demonstrating that these proteins were predominantly assembled into inclusion bodies. The appearance of inclusion bodies is usually explained by protein overexpression that can result in protein misfolding and formation of insoluble aggregates. While misfolding and the formation of inclusion bodies are usually problematic for downstream applications, it is advantageous in the context of protein-based biomaterials because it provides an efficient pre-separation of relatively pure target proteins from the bacterial cytoplasmic proteins. As seen in Figure 5.1a, the inclusion bodies contained a relatively low number of proteins.

Figure 5.2 Expression and purification of suckerin-19 variants. **(a)** SDS-PAGE of expression products of His-suckerin-19 and suckerin-19. MW: molecular weight; M: Molecular weight markers; S1 and P1: supernatant and pellet from cells expressing His-suckerin-19 respectively; S2 and P2: supernatant and pellet from cells expressing suckerin-19 respectively; **(b)** Re-concentrated elution fractions of His-suckerin-19 purified by IMAC with 1h binding at room temperature (E1), 1h binding at 4 °C (E2) and 15h binding at 4 °C (E3); **(c)** MALDI-TOF spectra of His-suckerin-19 purified by IMAC at different conditions (E1, E2 and E3 fractions shown in (b)).

5.3.2. Purification of His-suckerin-19 by IMAC

Urea-solubilized His-suckerin-19 was bound to Ni-NTA resin under varying conditions (1 h at room temperature, 1 h at 4 °C and 15 h at 4 °C) and eluted under denaturing conditions. The elution fractions were subsequently dialyzed against 5% acetic acid, freeze-dried, and reconstituted in 5% acetic acid. All three binding conditions resulted in pure His-suckerin-19 preparations as shown by SDS-PAGE analysis (Figure 5.2b). 15 h of binding at 4 °C was identified as optimal for His-suckerin-19 IMAC binding. The purity and molecular weight of His-suckerin-19 were then evaluated by MALDI-TOF. The elution fractions exhibited a major peak at around 42 kDa (Figure 5.2c), corresponding to the expected MW of suckerin-19 (39.4 kDa) with His-tag and linker peptide (2.7 kDa). Doubly and triply-charged species were also detected. A few low molecular weight impurities were observed, but their intensity was extremely low. These data demonstrate that His-suckerin-19 purification by IMAC is straightforward. However, the presence of the His-tag may be problematic for some applications and for subsequent self-assembly.

5.3.3. Expression, purification and enzymatic activity of TEV protease

The catalytic domain of TEV protease (27 kDa) recognizes a linear epitope of the general form E-Xaa-Xaa-Y-Xaa-Q-(G/S) and efficiently cleaves between Q and G/S.^[189] TEV protease tended to form insoluble inclusion bodies when expressed in 37 °C (data not shown), as previously reported.^[187] Insoluble TEV protease has no enzymatic activity. Thus expression conditions were optimized by inducing protein expression at lower temperatures to obtain soluble TEV protease. After 20 h expression at 20 °C, SDS-PAGE analysis revealed that the enzyme was exclusively present in the supernatant (Figure 5.3a). After an overnight binding at 4 °C to the IMAC resin, TEV protease was obtained at high purity with faint bands at higher

molecular weights (Figure 5.3b) attributed to TEV oligomers. TEV protease was subjected to a final desalting step where the protein buffer was changed to remove the high concentration of imidazole (Figure 5.3c). Recombinant TEV activity was evaluate using the established model SUMO fusion protein, which is a fusion protein composed of two SUMO subunits linked by a flexible polypeptide containing a TEV protease cleavage site ((GGGS)2-ENLYFQ-(GGGS)2).^[188] After incubation of the SUMO protein with TEV protease, two bands at ~ 20 kDa and 25 kDa appeared after 2 hours and the cleavage was completed within 4 hours (Figure 5.3d), indicating that TEV protease was active and suitable for cleavage of the His-suckerin-19 tag.

Figure 5.3 Expression, purification, and activity evaluation of TEV protease. (a) Recombinant expression of TEV protease. MW: molecular weight; M: Molecular weight markers; S1 and P1: supernatant and pellet of cell lysates induced by IPTG; S2 and P2:

supernatant and pellet of non-induced cell lysates; **(b)** Purification of TEV protease by Ni-NTA resin. FL: flow-through of TEV protease after binding; W1 and W2: washing fractions 1 and 2 with wash buffer; E: elution fraction; **(c)** Removal of small molecules by a de-salting column; **(d)** Cleavage of Sumo fusion protein by TEV protease. Ctrl: control sample of Sumo fusion protein; TEV: TEV protease.

5.3.4. Cleavage of His-tag from His-suckerin-19 with TEV protease

While affinity tags can provide several benefits to recombinant proteins, such as facilitating purification, increasing yield and solubility, and even promoting the folding of target proteins,^[190] they have also been reported to negatively affect the target proteins. For instance they can affect protein conformation, alter the biological activity, lead to undesired flexibility in structural studies, and induce toxicity.^[191] Therefore, we established protocols to remove the tag from His-suckerin-19.

In order to cleave the His-tag, we screened a series of buffers to establish conditions where His-suckerin-19 was soluble and TEV protease was active. Any conditions that failed to fulfill these two requirements were eliminated and the results are summarized in Table 5.1. The standard working buffer of TEV protease is 50 mM Tris-HCl containing 0.5 mM EDTA and 1 mM DTT (pH 8.0) and its activity is not decreased at pH between 4 and 9.^[192] Therefore, we first investigated the solubility of His-suckerin-19 in buffers ranging from pH 4.0 to pH 9.0 containing 50 mM sodium acetate, MES or Tris-HCl according to the enzyme's compatibility.^[192] His-suckerin-19 was not soluble in any of these buffers and we found that a minimum of 2M urea was required to maintain solubility in these buffers. Under such denaturing conditions, His-suckerin-19 precipitated at pH 6.5 and above. It has been reported that TEV protease can tolerate high concentrations of denaturants, such as urea, up to 4M.^[193] We verified TEV protease activity with 2M urea and found that it remained active at pH >5. In summary, this screening study established that suitable conditions for His-

suckerin-19 solubility and TEV protease activity were MES buffer with 2M urea at pH 5.5 (fully soluble) and pH 6 (partially soluble), as illustrated in Figure 5.4a and 5.4b. These conditions were then used to cleave the His-tag of His-suckerin-19. The results revealed that cleavage was time-dependent. Approximately half of the His-tags were removed within 2 h and the cleavage continued until the His-tags were totally removed by TEV protease after 24 h at room temperature (Figure 5.4c), giving rise to de-tagged His-suckerin-19. The cleaved His-Suckerin-19 was separated from TEV protease by ion exchange FPLC, where two distinct peaks were eluted from the column at around 37 ml and 43 ml (Figure 5.4d). SDS-PAGE analysis revealed that the first peak corresponded to pure de-tagged His-suckerin-19 (Fig. 5.4d inset), whereas the second peak was TEV protease (SDS-PAGE data not shown). We found it was challenging to purify de-tagged His-suckerin-19 from TEV protease by IMAC although the later had a poly-Histidine tag. This may be due to the high His content of suckerin-19,^[25] making it prone to bind to Ni-NTA resin and to be eluted together with TEV protease. The molecular weight of de-tagged suckerin-19 was confirmed by MALDI-TOF (Figure 5.4e), with a peak at 39.4 KDa matching the expected MW.

Table 5.1 Screening of buffers to solubilize His-suckerin-19 and maintain TEV protease's activity for His-tag removal.

Urea concentration	pH	Solubility of His-suckerin-19	Activity of TEV protease
0	4.0	×	-
	5.5	×	-
	6.5	×	-
	7.5	×	-
	8.5	×	-
	9.0	×	-
1 mol/L	4.0	-	×
	5.0	-	×
	5.5	×	-
	6.0	×	-
	6.5	×	-
	8.0	×	-
2 mol/L	4.0	✓	×
	5.0	✓	×
	5.5	✓	✓
	6.0	✓	✓
	6.5	×	-
	8.0	×	-

×: His-suckerin-19 was not soluble or TEV protease was not active;

✓ w His-suckerin-19 was soluble or TEV protease was active;

-: Non-applicable.

Figure 5.4 Cleavage of His-tag from His-suckerin-19. **(a)** Products of His-suckerin-19 in IMAC elution buffer dialyzed against buffers with different pH (S: supernatant, P: pellet); **(b)** Cleavage of Sumo fusion protein by TEV protease in the buffers used in (a); **(c)** Time-dependent His-tag cleavage from His-suckerin-19 from 2 h to 24 h; **(d)** Ion exchange-FPLC profile of de-tagged His-suckerin-19. The inset shows the SDS-PAGE analysis of the de-tagged His-suckerin-19 peak: Fractions 35-40; **(e)** MALDI-TOF spectrum of de-tagged His-suckerin-19 after ion exchange FPLC purification.

5.3.5. Purification of suckerin-19 by salting-out

In addition to IMAC purification, simpler non-chromatographic methods for His-suckerin-19 and suckerin-19 purification were also established. We noticed that His-suckerin-19 tended to precipitate when their inclusion body solubilized in 5% acetic acid/8 M urea were dialyzed against the IMAC binding buffer at pH 7.8. Following centrifugation, we observed that the pellet contained His-suckerin-19 at relatively

high purity (Figure 5.5a left). This result is not surprising given that the pH of the binding buffer was close to the theoretical isoelectric point of His-Suckerin-19 (Calculated IEP = 7.6, based on the full sequence including the poly-His tag and linking peptide), leading to a protein net charge close to zero, thus facilitating its isoelectric precipitation. Notably, His-suckerin-19 aggregated despite the presence of 8M urea. The high purity was confirmed by MALDI-TOF analyses which indicated peaks at around 14, 21, and 42 KDa corresponding to the triply, doubly, and singly charged His-suckerin-19, respectively (Figure 5.5a right). From these observations, this salting-out method was evaluated with non-tagged suckerin-19. Based on its higher IEP of 8.4 (compared to 7.6 for the His-tagged version), the same buffer adjusted to pH 8.4 was used for the dialysis. As shown by SDS-PAGE and MALDI-TOF analysis (Figure 5.5b), high purity suckerin-19 was obtained after dialysis. In contrast to IMAC purification, this method was more efficient, eliminating the need for His-tag removal and requiring fewer steps and reagents.

Figure 5.5 Purification of His-suckerin-19 and suckerin-19. **(a)** Salting-out products of His-suckerin-19 in IMAC binding buffer at pH 7.8 (left) and corresponding MALDI-TOF spectrum of the pellet (right). S and P: supernatant and pellet after dialysis respectively; **(b)** Salting-out products of suckerin-19 in IMAC binding buffer at pH 8.4 (left) and corresponding MALDI-TOF spectrum of the pellet (right); **(c)** Purification of suckerin-19 by microfluidization and corresponding MALDI-TOF spectrum of the pellet (right). S: supernatant after cells lysis with a microfluidizer; W1.1, W1.2: supernatant washing fractions from wash buffer 1; W2.1, W2.2: supernatant washing fractions from wash buffer 2; 5% AA: Suckerin-19 solubilized by 5% acetic acid after washing steps.

5.3.6. Purification of suckerin-19 by microfluidization and inclusion body purification

During the purification of recombinant proteins from inclusion bodies, it is important that cells are lysed completely to reduce the amount of contaminating proteins. Additional methods for suckerin-19 inclusion body purification were therefore developed. We found that cell lysis by microfluidization led to high throughput and was effective to reach this goal. Cells collected after culture were re-suspended in lysis buffer and passed through a microfluidizer 6 times in order to completely disrupt the cells and release the inclusion bodies (see Section 5.2 for experimental details). After microfluidization, suckerin-19 remained mostly in the insoluble pellet. The pellet was washed twice with buffers containing 2M urea and 2% Triton X-100 to remove cellular membranes and other contaminating proteins (Figure 5.5c, lanes W1.1 and W1.2). This was followed by two detergent-free washes to remove the Triton X-100 and low-molecular weight contaminants (Figure 5.5c left, lanes W2.1 and W2.2). Very little suckerin-19 loss was observed. Suckerin-19 was obtained by re-solubilizing the pellet in 5% acetic acid, which resulted in sample purity comparable to IMAC purification, where MALDI-TOF indicated the presence of a small quantity of low molecular weight contaminants (Figure 5.5c right).

The yields of suckerin-19 for each purification method are listed in Table 5.2. Around 4.3 mg of protein per liter of culture was obtained by the IMAC method for His-tagged suckerin-19, while the yield of non-tagged suckerin-19 by salting-out was 5.9 mg per liter of culture. By microfluidization, the yield was 20.2 mg/ L of cell culture. Of the three methods tested, microfluidization proved to significantly improve yield and efficiency. While our salting-out method required excessive amounts of urea that would not be suitable for scale-up, the current microfluidization purification

methodology required only 40 ml of 2M Urea per liter of culture, which we consider a reasonable step forward.

Table 5.2 Summary of recombinantly expressed His-suckerin-19 and suckerin-19 in different hosts and purified by different methods.

Protein	Origin	Host	MW (kDa)	Purification strategy	Yield (mg/L)
His-suckerin-19	<i>Dosidicus gigas</i>	<i>E.coli</i>	42	IMAC	4.3 ^a
Suckerin-19	<i>Dosidicus gigas</i>	<i>E.coli</i>	39	Salting-out	5.9
Suckerin-19	<i>Dosidicus gigas</i>	<i>E.coli</i>	39	Microfluidization	20.2

a: Yield before TEV protease cleavage.

The apparent ease of production of recombinant suckerin-19 in *E. coli* likely relates to its manageable size and lower degree of highly repetitive sequences compared to silk proteins. No attempts were made to optimize the expression conditions, such that significant opportunities remain to further increase yield. Future studies in our lab will involve a direct evaluation of the performance of Rosetta cells compared to BL21 (DE3) cells and the quantitative evaluation of yields from codon optimized and native suckerin genes. We will also conduct a systematic optimization of expression yields as has been done for the efficient expression of silk-elastin like peptides (SELPs) in *E. coli*.^[194] SELPs, which can have similar molecular weights to suckerin-19, exhibit excellent aqueous-based solubility and have gained strong traction in the biomimetic engineering of robust functional materials.^[194, 195] We anticipate that expression yields as high as those obtained for silk and SELP constructs should be attainable for suckerins.

5.3.7. Solubility of suckerin-19

The processing of proteins at high concentrations is desirable for several reasons. Natural secretions are often processed at high concentrations, and molecular crowding may facilitate their hierarchical assembly into bulk materials. In addition, processing from dilute preparations can require excessive solvents and thus impact scalability. Native and recombinant silk fibroin proteins generally require harsh solvents to maintain solubility at elevated concentrations where optimal working concentrations for recombinant silk fibroin preparations are usually in the 10 mg/ml to 80 mg/ml range. In order to explore the potential for fabrication of suckerin-19-based materials from concentrated solutions, particularly using mild processing strategies, we investigated the solubility limit of suckerin-19 in different aqueous solutions, including water, 5% acetic acid, 5% acetic acid/8M urea and various buffered saline solutions. Suckerin-19 exhibited a maximum solubility of 7.2 mg/ml in water (Figure 5.6a). In contrast, suckerin-19 exhibited a much higher maximum solubility in 5% acetic acid/8M urea (85.1 mg/ml). Interestingly, the solubility limit of suckerin-19 in 5% acetic acid (70.9 mg/ml) was close to that in 5% acetic acid/8 M urea. Suckerin-19 precipitated in all buffered saline solutions tested (Table 5.3); however it remained soluble up to 0.64 mg/ml in 0.05 M MES at pH 5.5. Remarkably, the solubility of suckerin-19 was about 10-fold greater in 5% acetic acid compared to water (Figure 5.6a). Given that high protein concentration is often a requirement to produce protein-based biomaterials,^[36, 105] the high solubility of suckerin-19 in acetic acid will likely be critical for the fabrication of bulk materials. Furthermore, 5% acetic acid has been shown to be a promising solution for film fabrication of suckerin-19 from initial protein concentrations of ~20mg/ml.^[25] Hence, the higher solubility limit under mild conditions provides an additional degree of versatility that may enable tuning of the

properties of suckerin-19 based biomaterials by adjusting the initial protein concentration. While the underlying reason for the high solubility of suckerin-19 in mild acidic conditions has not been explored, the high His content (9.8 mole%) and absence of other charged residues in suckerin-19 are likely to play a critical role. Below the pKa of His (6.5), suckerin-19 bears a net positive charge due to protonation of the imidazole moieties, resulting in electrostatic repulsion of individual chains and increased protein solubility, as also confirmed by zeta potential measurements discussed in the next Section.

Figure 5.6 (a) Solubility limit of suckerin-19 in different solutions. The inset shows suckerin-19 concentration in log scale (AA: acetic acid); (b) Particle size distribution of suckerin-19 in 5% acetic acid and water at 1 mg/ml and in 0.05 M MES, pH 5.5, at 0.64 mg/ml ; (c) Particle size distribution of suckerin-19 colloids solubilized in water determined by AFM in the dry

state. The inset shows a representative AFM image of suckerin-19 colloidal particles assembled in water and viewed in the dry state (deposited on mica substrate). **(d)** DLS particle size distribution of suckerin-19 colloids in 5% acetic acid and water after two weeks at 4 °C.

Table 5.3 Solubility testing of suckerin-19 in buffered saline solutions.

MES: 2-[N-Morpholino]ethanesulfonic acid; Tris: 2-Amino-2-hydroxymethyl-propane-1,3-diol; HEPES: N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid]; PBS: Phosphate Buffer Saline; CAPS: 3-[Cyclohexylamino]-1-propanesulfonic acid.

Buffer component	pH	Additives	Solubility
0.05 M NaH ₂ PO ₄	4.0	0 M NaCl	×
		0.1 M NaCl	×
		0.15 M NaCl	×
		0.2 M NaCl	×
0.05 M CH ₃ COONa	4.0, 4.5 and 5.0	0 M NaCl	✓
		0.1 M NaCl	×
		0.15 M NaCl	×
		0.2 M NaCl	×
0.05 M MES	5.5, 6.0 and 6.5	0 M NaCl	✓
		0.1 M NaCl	×
		0.15 M NaCl	×
		0.2 M NaCl	×
0.05 M Tris	7.0	0 M NaCl	×
		0.1 M NaCl	×
		0.15 M NaCl	×
		0.2 M NaCl	×
0.05 M HEPES	7.0	0 M NaCl	×
		0.1 M NaCl	×
		0.15 M NaCl	×
		0.2 M NaCl	×
0.1 M PBS	7.4	0 M NaCl	×
		0.1 M NaCl	-
		0.15 M NaCl	-
		0.2 M NaCl	-
0.05 M CAPS	10.0	0 M NaCl	×
		0.1 M NaCl	×
		0.15 M NaCl	×
		0.2 M NaCl	×

×: Suckerin-19 was not soluble;

✓ waSuckerin-19 was soluble;

-: Non-applicable.

5.3.8. Biophysical characterization of suckerin-19

Biophysical characterizations were performed on purified suckerin-19 in order to investigate the influence of aqueous-based environments on assembly and secondary structure. In acidic conditions (5% acetic acid) and at low protein concentration (1mg/ml), DLS measurements indicated that suckerin-19 existed in a monomeric state, with a mean hydrodynamic diameter D_H of about 5-7 nm (Figure 5.6b and Table 5.4). At an increased concentration of 6 mg/ml, D_H increased by 10-fold to ~ 60nm (+/- 22 nm), suggesting the formation of suckerin-19 oligomers. When solubilized in water (~ pH 6.6), the hydrodynamic diameter distribution ranged from 60 to 90 nm at both 1 mg/ml and 6 mg/ml (Figure 5.6b and Table 5.4) with an average at around 70 and 80 nm respectively, suggesting that suckerin-19 oligomers are stable in water up to their solubility limit. In 0.05 M MES at pH 5.5, 0.64 mg/ml suckerin-19 exhibited a D_H distribution around 50 nm (Figure 5.6b and Table 5.4). The polydispersity indices of suckerin-19 in 5% acetic acid, water and 0.05 M MES were similar (around 0.30) at all concentrations tested (Table 5.4).

Table 5.4 Particle diameter and zeta-potential of suckerin-19 colloids.

Solvent	Concentration (mg/ml)	Particle diameter (nm)	Polydispersity indices	Zeta-potential (mV)
5% acetic acid	1.0	6±2	0.31±0.02	43.2±1.8
	6.0	63±22	0.30±0.01	30.3±0.6
	40.0	94±10	0.30±0.02	N.A.
H ₂ O	1.0	69±7	0.31±0.01	44.2±0.2
	6.0	80±7	0.30±0.01	33.6±0.6
0.05 M MES	0.64	52±2	0.24±0.02	19.7±0.4

The particle size of suckerin-19 colloids assembled in water was further investigated by AFM, where globular particles ranging from 15-80 nm in diameter were observed on the substrate (Figure 5.6c inset). In this case the particles exhibited a bimodal size distribution, with one group exhibiting a mean diameter of ~20-30 nm

and the other a mean diameter of ~50-60 nm (Figure 5.6c). Overall, the mean diameter is around 40 nm. We do not consider the mean particle diameters measured by AFM imaging to be a direct reflection of the colloidal particles observed in solution since the sample had to be diluted for imaging and given that the substrate may also influence assembly, potentially affecting the oligomerisation state of the particles. Therefore the DLS and AFM data should not be directly compared.

Circular dichroism (CD) was then used to evaluate the secondary structures of suckerin-19 in 5% acetic acid, water, and 0.05 M MES, pH 5.5 (Figure 5.7). In 5% acetic acid and milli-Q water, a conformational switch was observed with increased concentration. At 1 mg/ml, the CD signature corresponded to predominant random-coil structure with a minimum at around 195-200 nm.^[196] On the other hand, at higher concentration (6 mg/ml), suckerin-19 exhibited a minimum at 215 nm and a maximum at 198 nm, which is characteristic of β -sheet structure.^[196] Thus, there existed a clear transition of the peak minimum, in the form of a red shift, with the increase of protein concentration in both solutions. A β -sheet signature was also observed for 0.05 M MES solubilized suckerin-19 with a minimum at 218 nm and a maximum at 202 nm (Figure 5.7c). (Standard CD curve fitting software did not provide reasonable fits for % structure estimates of the data presented in Figure 5.7 and we restrict our interpretation qualitative observations in this case). Taken together, the data suggest that suckerin-19 can be refolded into β -sheet enriched structures in aqueous-based solutions, with a secondary structure bearing similarity with the native structure of SRT.^[25]

Figure 5.7 Secondary structures of suckerin-19 in solution. **(a)** CD spectra of suckerin-19 at different concentrations in 5% acetic acid; **(b)** CD spectra of suckerin-19 at different concentrations in water; **(c)** CD spectrum of 0.64 mg/ml suckerin-19 in 0.05 M MES, pH 5.5.

The occurrence of β -sheets within 5% acetic acid, water and 0.05 M MES (pH 5.5) solubilized suckerin-19 preparations was supported by FTIR (Figure 5.8). The spectra were characterized by strong absorbance at $\sim 1623\text{--}1641\text{ cm}^{-1}$ assigned to β -sheet structures of the amide I region, as well as by peaks at $\sim 1520\text{ cm}^{-1}$ corresponding to β -sheet conformation in the amide II region.^[174] In addition, peaks at $\sim 1242\text{ cm}^{-1}$ attributed to β -sheet structure were also observed in the amide III region (Figure 5.8a).^[197] Semi-quantitative analysis of the secondary structures of suckerin-19 were obtained by standard peak deconvolution^[160] of the Amide I band. As shown in Figures 5.8b-d, suckerin-19 exhibited a relatively high content of β -sheet conformations in 5% acetic acid (26.2%), water (34.7%) and 0.05 M MES at pH 5.5 (42.7 %). Other conformations were generally less abundant, including helix, random coil and β -turns.^[160, 198] The formation of oligomers coupled with the detection of β -sheets, schematically represented in Figure 5.9 for the various conditions tested, suggests that oligomerization is enabled by the formation of inter-chain β -sheets at higher protein concentrations.

Figure 5.8 ATR-FTIR analysis of recombinant suckerin-19. **(a)** Full FTIR spectra of suckerin-19 in 5 % acetic acid (6mg/ml), water (6mg/ml) and 0.05 M MES, pH 5.5 (0.64 mg/ml); **(b-d)** Deconvoluted amide I spectra suckerin-19 FTIR spectrum for the samples respectively described in (a). The positions of fitting peaks were defined by the 2nd derivative of the Amide I spectra and the width of each peak was fixed.

Figure 5.9 Schematic of oligomerization state and structure of suckerin-19 in 5% acetic acid, water and 0.05 M MES, pH 5.5 at different concentrations.

The zeta-potential of suckerin-19 in different solutions was also measured. As shown in Table 5.4, the zeta potential of suckerin-39 in 5% acetic acid ($\sim$ pH 2.0) was high (≥ 30 mV) at both 1 and 6 mg/mL. These values are consistent with the high abundance of His residues (and the absence of other charged residues) in suckerin-39, which are protonated under acidic conditions. We also observed that the zeta-potential of suckerin-19 in water was similar to that in 5% acetic acid at different concentrations (Table 5.4). The high zeta potential suggests a high colloidal stability of suckerin-19 particles, which was supported by the observation that the particle size distribution remained nearly constant for 2 weeks at 4 °C in both low and high concentrations in both solvents (Figure 5.6d). In addition, the zeta-potential of suckerin-19 in 0.05 M MES was 19.7 ± 0.4 mV at 0.64 mg/mL, indicating relatively stable colloidal particles of suckerin-19 as well.

In summary, our biophysical investigations indicated that suckerin-19 formed soluble oligomers in 5% acetic acid, water and MES, which are soluble, β -sheet-

enriched colloids at higher concentration. While much remains to be discovered regarding the self-assembly of native SRT, we suggest that the tendency of full-length recombinant suckerins to assemble into distinct oligomeric complexes may reflect the native self-assembly behavior of these proteins, where they are likely concentrated within the sucker's specialized epithelial cells and exported into the confined extracellular interface at the base of the SRT during its growth and development. We also suggest that soluble, β -sheet enriched nano-scale suckerin-based oligomers/colloids represent useful precursors that limit unwanted aggregation during processing, which will facilitate the assembly and engineering of solid-state macroscopic materials that mimic the β -sheet reinforced native SRT.

While synthetic silk proteins have been researched and developed over the past two decades, suckerins are newly discovered proteins. However, the suckerins exhibit several parallels with silk proteins, suggesting that they may be suitable for a range of applications. Notably, suckerin-19 exhibits a clear ease of expression of full-length proteins in *E. coli* and favorable solubility in aqueous solvents. Given that suckerins exhibit biocompatibility in cell culture and that they can be readily processed into a variety of structures at a range of length scales,^[25] we suggest that they represent a new class of proteins with potential for the efficient engineering of robust β -sheet reinforced materials.

5.4. Conclusion

Proteins constituting the building blocks of squid SRT display a unique combination of biophysical and mechanical properties, and their molecular structure is reminiscent to that of silks. In the study described in this Chapter, two forms of the most abundant protein of SRT (suckerin-19) were successfully expressed in *E. coli*

and purified by various methods. Critically, full-length suckerins were readily expressed. His-tagged suckerin-19 (His-suckerin-19) was purified from inclusion bodies by IMAC. This version of suckerin-19 may be suitable for a variety of applications. For instance the His-tag may be used for surface functionalization or metal co-ordination. However, in other applications the His-tag must be removed, which required expression of TEV protease followed by cleavage and final separation of TEV protease from de-tagged His-suckerin-19. Our data indicated that non-tagged suckerin-19 was best purified using facile non-chromatographic methods including inclusion body purification by microfluidization. The achieved yields were promising, although they do not yet match those recently obtained with some silk mimics. However, we suggest that yields could be significantly optimized by varying the bioprocessing conditions and by exploring alternative codon-optimized host expression systems. After purification, suckerin-19 exhibited in-solution characteristics that were of direct use for the aqueous-based, eco-friendly processing of suckerin-based biomaterials, including a high solubility in mild acidic conditions, which could be correlated to nanometer-scale colloids stabilized by inter-chain β -sheet interactions upon an increase in concentration. These data thus suggest that recombinant suckerin proteins maintain potential for the industrial fabrication of functional sub-micron structures similar to the micro-spheres and colloids recently developed for silks,^[172] and for the creation of β -sheet reinforced materials that may find use in a range of biomedical and engineering applications.

Chapter 6 Mechanically and Structurally Tunable Materials Fabricated with Recombinant Suckerin-19^b

6.1. Introduction

The previous Chapter has shown that recombinant suckerin-19 can be easily expressed and purified. Given the unique features of this block co-polymer-like protein, we were interested in further exploring the potential of suckerin-19 as a novel biomacromolecular building block.

As previously discussed, suckerins exhibit a block co-polymer structure at the molecular level made of nano-scale β -sheet domains intervened by longer amorphous blocks. This design and assembly makes suckerins promising biomacromolecules for a wide range of load-bearing applications.^[25] A chemically useful characteristic of suckerins resides in their large Tyr content (ranging from 7 to 16 mole%),^[17] which is exclusively found in their amorphous domains. Tyr can readily form di-Tyr cross-links, a chemistry that has been exploited in the formulation of biopolymers, including resilin mimics,^[36] fibrin,^[199] and silk-based hydrogels.^[200] In this Chapter, we exploited this biochemical feature to cross-link suckerin-19 in a controllable fashion. In order to explore the potential of modulating the mechanical properties of suckerin-19 based materials, one target application was to tackle an important challenge encountered in restorative medicine, namely duplicating the mechanical gradients of connective tissues. Indeed, mechanical gradients are prevalent at virtually all attachment sites between tissues with dissimilar mechanical properties.^[201] These so-

^b This Chapter mostly comes from my first-author paper entitled “From Soft Self-Healing Gels to Stiff Films in Suckerin-Based Materials Through Modulation of Crosslink Density and β -Sheet Content” (*Adv. Mater.* 2015, 27, 3953–3961).

called “entheses” –for instance the classical tendon-to-bone interface– play major biomechanical roles by mitigating stress concentrations, thereby lowering the risk of micro- and macroscopic failure. A major challenge in restorative medicine is thus to gain precise control over the mechanical properties of biocompatible materials and to mimic natural gradients to achieve robust mechanical attachments similar to that of the native junction.^[202] This task is exacerbated by the large stiffness disparity between soft and hard tissues. For instance the Young’s modulus (E) of muscle is 0.006-0.01 MPa,^[203] around 10-600 MPa for ligaments,^[204] and 8-20 GPa in compact bone.^[23, 205] In order to fabricate mechanically-graded materials that can mimic native entheses properties, it would be advantageous to formulate biomaterials that are able to intrinsically exhibit a wide range of elastic properties through simple chemical manipulations of their building blocks. Biological materials from diverse sets of organisms constitute a valuable source of inspiration to tackle this question, with notable examples such as the graded protein/chitin biocomposite of squid beaks,^[15, 16] or the collagenous-based threads of mussels in which the gradient is modulated by adjusting the relative content of elastin-like and silk-like domains along the threads.^[206] Thus, protein-based materials are attractive candidates for the production of artificial materials with tunable properties,^[207] owing to their versatile side-chain chemistry that can be exploited to induce cross-linking in a controlled fashion.

In this Chapter, a ruthenium (Ru)-mediated photo-cross-linking method^[199] is described, which allowed to modulate the elastic properties of suckerin-19 based materials. Seven decades of elasticity were achieved, from soft hydrogels with storage moduli ranging from 40 to 500 Pa, to stiff cross-linked films with Young’s moduli in the GPa range. This broad range of moduli was obtained by facile adjustment of the initiator and suckerin-19 concentrations, leading to materials with tunable cross-link

density and β -sheet content, which in turn controlled the final mechanical response. Cell culture studies with various cell lines demonstrated that the cross-linked suckerin films exhibited negligible cytotoxicity. The work demonstrates the broad potential of recombinant suckerins for tissue engineering and a wide range of biomedical applications.

6.2. Materials and methods

6.2.1. Gene cloning, recombinant protein expression and purification

Gene cloning, recombinant protein expression, and purification of suckerin-19 by inclusion bodies washing followed previously established methods.^[19, 25] Please refer to Chapter 5 for further details.

6.2.2. Characterization of suckerin-19 solution

The hydrodynamic diameter of suckerin-19 at 40 mg/ml in 5% acetic acid was measured by DLS (90Plus particle size analyzer, Brookhaven Instruments). The number-weighted histogram profiles of the proteins in solution were used for data analysis. The mean particle size of suckerin-19 colloids in 5% acetic acid was also investigated by AFM (MFP-3D BIO, Asylum Research). Suckerin-19 (40 mg/ml in 5% acetic acid) was diluted to 100 μ g/ml with water in order to keep the protein colloid intact, as previous studies showed that low concentrations of suckerin-19 in 5% acetic acid resulted in perturbation of the protein colloids.^[19] Single drops of the sample were placed on mica substrates, and allowed to equilibrate for 20 min followed by rinsing with water. The sample was then air-dried and subjected to AFM imaging. The mean size of particles from the images was measured by ImageJ.

6.2.3. Preparation of suckerin-19 hydrogels, films and suckerin-19-glycerol films

Suckerin-19 was dissolved in 5% acetic acid at 40 mg/ml for photo-cross-linking. The protein solutions were sonicated for 5 minutes to facilitate the dispersion, mixed with ammonium persulfate (APS) and centrifuged, followed by the addition of Tris(bipyridine)ruthenium(II) chloride (Ru^{II}) at a volume ratio of 18:1:1(suckerin-19:APS: Ru^{II}). The final Ru^{II} concentration was 0.2 mM, while APS was adjusted from 0.5 to 2.5 mM. To fabricate the gels, the “pre-polymer” solutions were transferred to glass vials and exposed for twice to the light from a 500 W halogen lamp for a duration of 3 min, with a 15 min waiting time between each step. For film fabrication, 10 μl of “pre-polymer” solution with different APS concentrations were transferred to glass slides, left in the dark for 2 hours, and illuminated for 3 min for twice as described above. The samples were then slowly dried at room temperature. Softer suckerin-19-glycerol films were prepared with glycerol as a plasticizer. 50 μl of suckerin-10 in 5% acetic acid (40 mg/ml) was mixed with glycerol at ratios of 1:3, 1:5, and 1:7 (suckerin: glycerol; w/w), pipetted onto glass slides and dried at room temperature.

6.2.4. Quantification of Di-Tyr content

Suckerin-19 hydrogels cross-linked with 2 and 2.5mM APS (40 mg/ml) were rinsed in water several times to remove Ru^{II} and other components used in the cross-linking process, and exposed to a 302 nm UV lamp for qualitative detection of fluorescence from di-Tyr cross-links. The cross-linking density of suckerin-19 gels and films was quantified by Ninhydrin-based amino acid analysis (AAA) on a Sykam 433 equipment (Sykam GmbH, Eresing, Germany). Briefly, non cross-linked and cross-linked films made from 20 μl cross-linked/non cross-linked samples were hydrolyzed in 0.2 ml of 6 M HCl with 10 μl phenol (Sigma) at 110 °C under vacuum

for 24 h. Hydrolysis products were washed using a ScanVan (Labogen) vacuum concentrator with 0.1 ml deionized water for 3 times, followed by a final washing with 0.1 ml methanol. Hydrolysates were then dissolved in 0.25 ml sample dilution buffer (Sykam GmbH) and spun at 16,000 g for 5 min before being subjected to AAA. In order to determine the elution time of di-Tyr, pure Tyr amino acid was cross-linked with the same method used for the preparation of 2.5 mM APS cross-linked suckerin-19 films, with Tyr initially dissolved in 20 % acetic acid at 0.65 mg/ml. The solvent was removed by freeze-drying and the sample was dissolved in the AAA sample dilution buffer. The cross-linked product was then mixed with an amino acid standard solution and run on the analyzer. One additional peak –which was not observed on the external AAA standard– was detected at ~35.5 min elution time (right before the elution peak of histidine) and could thus be unambiguously attributed to di-Tyr (Figure 6.4a). Quantification was obtained using the Leu peak as an internal standard, chosen because of its relatively high abundance in suckerin-19 and its distinct elution time. The areas under the di-Tyr and Leu peaks were measured, and the di-Tyr/Leu area ratio was used to calculate the relative mol. content of di-Tyr, assuming an extinction coefficient for di-Tyr equal to that of Tyr.

6.2.5. Rheology of suckerin-19 hydrogels

Rheological measurements were conducted on cross-linked suckerin-19 solutions and gels to determine their viscoelastic properties with an Anton Paar MCR 501 rheometer (Anton Paar) using a 60 mm stainless steel conical plate (angle: 1°) and a 10 mm parallel plate, and the temperature was set to 20 °C. An amplitude sweep was first conducted to determine the viscoelastic regime of suckerin-19. Frequency sweeps from 0.1 to 10 Hz were then carried out to ensure that measurements occurred within the viscoelastic regime. The storage moduli of cross-linked suckerin-19 gels

were also evaluated at protein concentrations ranging from 1.25 to 40 mg/ml with a fixed molar ratio of suckerin-19 to APS. In another amplitude sweep test, suckerin-19 hydrogels were subjected to 300 pct. strain at 1 Hz, after which recovery studies were conducted by measuring the storage modulus as a function of time, at fixed strain (2%) and frequency (1 Hz) values.

6.2.6. Field Emission Scanning Electron Microscopy (FESEM)

The thickness of suckerin-19 films and morphology of their cross-sections were characterized by FESEM (JSM-6340F, JEOL) at an accelerating voltage of 5 kV. Each sample was gold-coated with a sputter coater (JEOL JFC - 1600, Japan) for 75 seconds before imaging.

6.2.7. Atomic Force Microscopy (AFM)

The surface topology and roughness of suckerin-19 films were probed by AFM in the tapping mode (MFP-3D BIO, Asylum Research). The AFM was equipped with rotated tapping-mode etched silicon probes (Ted Pella). Image scans of $20 \times 20 \mu\text{m}$ were performed in air for each sample. The roughness value R_a , representing the average of amplitudes in the height profiles, was calculated from the AFM images using Asylum Research software (Asylum Research).^[208]

6.2.8. Fourier Transform Infrared Spectroscopy (FTIR)

The protein secondary structures of suckerin-19 films and suckerin-19-glycerol films were measured by FTIR with a Bruker Vertex 70 infrared spectrometer (Bruker) in the Attenuated Total Reflection (ATR) mode. Each sample was scanned for 4 min with a resolution of 4 cm^{-1} and with a wavenumber from $4000\text{--}400 \text{ cm}^{-1}$ at room temperature. In order to estimate the fractions of different secondary structures, the amide I bands were deconvoluted using OPUS 6.5 software (Bruker), where the

positions of each fitting peak were fixed at the positions of peaks identified in the second derivative of each amide I band and the widths of the fitted peaks were approximately the same.

6.2.9. Nanoindentation of suckerin and suckerin-glycerol films

The mechanical properties of suckerin-19 films were investigated by nanoindentation with a TriboScan 950 (Hysitron) following previously described procedures^[25] and the Oliver-Pharr method to extract the Elastic modulus E . The films were probed in various locations using a sharp cube corner fluid cell tip at maximum loads of 25, 50 and 100 μN respectively, with the various peak loads used to ensure that substrate stiffness effects did not affect the measurements. 50 indents with a maximum load of 50 μN were carried out in dry conditions at a loading/unloading rate of 10 $\mu\text{N/s}$ with 2 s holding times before unloading. Water droplets were then introduced onto the films and the film was hydrated for 5-10 min before indentation. The films were tested in three different locations at a maximum load of 10 μN for a total of 30 indents. Water drops were re-introduced between each set of indents to ensure that the films remained fully hydrated.

Because suckerin-glycerol films were too soft to be probed by sharp tip indentation, their elastic modulus was measured by Hertzian contact,^[209] using an elongated (fluid cell) cono-spherical tip (nominal radius of 10 μm) and a large extended displacement stage (xZ 500, Hysitron) capable of applying maximum displacement up to 500 μm . For the “1:3” films in dry conditions, 20 indents with a maximum load of 10 μN were carried out at a loading/unloading rate of 2 $\mu\text{N/s}$ with a 2 s holding time before unloading. For the “1:5” and “1:7” films in dry conditions, they were probed using the “air indent” method (25 s loading, 10 s unloading) under the “Image” mode. The indents were performed in the displacement control mode by

imposing 5 to 7 μm displacement into the samples. The elastic modulus of the films was calculated by fitting the initial loading portion of the indentation curves using the Hertz elastic solution for a blunt contact with the equation below:^[210]

$$P = \frac{4}{3} * E * \sqrt{R} * \sqrt{\delta^3} \quad (1)$$

where δ is the longitudinal displacement (in m), P is the load (in N), E is the elastic modulus and R is the radius of tip (10^{-5} m). For all measurements in wet conditions, water droplets were introduced to the films, which were let hydrated for 20 min before indentation. The same protocol used for the “1:5” and “1:7” films in dry conditions was employed. Water drops were added between every several indents to ensure that the films remained fully hydrated during the measurements.

6.2.10. Cell culture on suckerin-19 films

Three cell lines were used for cell culture on suckerin films, namely human embryonic kidney (HEK) 293 cells, human dermal fibroblasts (HDF) and human mesenchymal stem cells (hMSCs). HEK 293 cells were obtained from Cell Labs, Inc. HDF cells and Human MSCs were obtained from Lonza. HDF and HEK 293 cells were cultured in Dulbecco’s modified eagle medium (DMEM) supplemented with 10% fetal bovine serum and 1% antibiotic/antimycotic (Life Technologies). Human MSCs were cultured in the same medium supplied with β -fibroblast growth factor (FGF) (5 ng/ml). For surface seeding, non cross-linked and highly cross-linked suckerin-19 films were prepared on 1 cm^2 glass slides with the same procedure of film preparation as above. Briefly, 50 μl of pre cross-linking solution of suckerin-19, APS (2.5mM) and Ru^{II} were spread on the glass slide to cover the entire surface, and cross-linked as described above. Non cross-linked films were prepared in the same way as before. The resultant suckerin-19 films were incubated in 70% ethanol for 2h for sterilization,

washed with PBS twice and transferred to a low-binding 12-well plate (Nunc brand, ThermoScientific). The samples were washed with PBS twice again to completely remove ethanol as well as the remaining Ru^{II} and sulfate salts. HDF cells and hMSCs were seeded at $\sim 230 \text{ cells/mm}^2$ and allowed to adhere for 12h, while HEK 293 cells were seeded at 140 cells/mm^2 . All films were transferred to fresh wells after cell attachment and refilled with 1 ml fresh culture medium to ensure removal of well surface attached cells and to continue the culture. Cells seeded in 12-well plates of cell attaching tissue culture plastic (TCP) (Nunc brand, ThermoScientific) with the same density respectively were utilized as positive controls for cell attachment. All cell cultures were performed in an incubator maintained at 37°C and $5\% \text{ CO}_2$.

6.2.11. Cell proliferation and cytotoxicity assay of suckerin films

The cellular viability of HDF cells assessed by relative metabolic activity was determined by the cellular activation of resazurin fluorescence using the AlamarBlue reagent (Life Technologies) according to the manufacturer's directions. Medium on each film was gently removed and cells were incubated in DMEM medium with 10% AlamarBlue reagent for 3 h at 37°C with $5\% \text{ CO}_2$. Next, 100 μl aliquots from each sample were transferred to 96 well clear bottom/black wall plates and the fluorescence was quantified using a plate reader (TECAN Infinite M200; (Excitation: 560 nm; Emission: 590 nm). Cell numbers on suckerin-19 films and TCP were calculated against a standard curve, obtained by conducting the AlamarBlue assay of a series of cell cultures across a serial dilution in a 12-well plate assayed using identical reaction protocols as the study. The AlamarBlue reagent was removed after the assay and replaced with 1ml of fresh medium to continue the cell culture. The cell number on each sample at Day 1 post-seeding was compared for cell attachment. From Day 3 onwards, the cell number on each sample was compared for proliferation with time.

Cytotoxicity of suckerin films was performed on HDF cells and hMSCs with a Live/Dead Viability/Cytotoxicity kit (Life Technologies) according to the manufacturer's directions. Briefly, cells were incubated with calcein AM and ethidium homodimer-1 (EthD-1) at 37 °C for 15-20 min to stain live (green) and dead (red) cells respectively. When assessing the live/dead status of the GFP-HEK293 cells only the EthD-1 reagent was required since the HEK293s sustained over expression of green fluorescent protein transgene which is also an indicator of their continued viability.^[211] Cells were imaged using a fluorescence microscope (1X71, Olympus) with excitation at 488 nm and emission at 520 nm for live cells, and excitation at 528 nm and emission at 617 nm for dead cells. Viable/living cells displayed strong green fluorescence whereas dead/non viable cells displayed strong red fluorescence.

6.3. Results and discussions

6.3.1. Cross-linking of suckerin-19 at low concentration

We first established conditions for Ru-mediated cross-linking of recombinant suckerin-19 using polyacrylamide gel electrophoresis (PAGE) and MALDI-TOF mass spectroscopy. Recombinant suckerin-19 was solubilized in 5% acetic acid and di-Tyrosine cross-linking was triggered using a Ruthenium complex (Ru^{II}), with ammonium persulfate (APS) as the initiator. At low suckerin-19 concentration of 1 mg/ml, oligomers started to appear with 0.1 mM of APS (Figure 6.1a). As the APS concentration increased to 0.5 mM, oligomers became more abundant and were of higher molecular weight (MW). At concentration above 2.5 mM, no proteins migrated into the gel, suggesting a concentration dependence of cross-linking. MALDI-TOF spectra confirmed these observations: as the APS concentration increased higher MW oligomers were detected, with higher relative intensity (Figure 6.1b). At 2.5 mM, no peaks were detected, indicating the formation of a fully cross-linked network. We also

tested the impact of light exposure time, protein concentration, and solvent concentration on cross-linking. Exposure times as low as 5 sec. were sufficient to totally cross-link 1mg/ml of suckerin-19 solution, whereas protein concentration as low as 0.25 mg/ml could be cross-linked with 2.5 mM APS (Figure 6.2a). Furthermore, extensive cross-linking could be achieved at solvent concentrations as high as 50% acetic acid (Figure 6.2b). These data thus indicate that a wide range of parameters can be adjusted to tailor suckerin-19 cross-linking by Ru^{II} -mediated photo-cross-linking, including APS, protein, and solvent concentrations, as well as light exposure time.

Figure 6.1 (a) SDS-PAGE analysis of 1 mg/ml suckerin-19 cross-linked with different concentrations of APS in 5% acetic acid. (b) MALDI-TOF spectra of 1 mg/ml suckerin-19 cross-linked with different concentrations of APS.

Figure 6.2 (a) SDS-PAGE analysis of suckerin-19 at different concentrations cross-linked with 2.5 mM APS, and examination of the effect of light exposure time on the cross-linking of suckerin-19 in 5% acetic acid. (b) SDS-PAGE analysis of 0.4 mg/ml suckerin-19 cross-linked with 2.5 mM APS in different concentrations of acetic acid.

6.3.2. Cross-linked suckerin-19 hydrogels

Protein concentration of suckerin-19 was increased for the preparation of solid materials and suckerin-19 gels were initially prepared from 40 mg/ml protein solution. As recently demonstrated,^[19] suckerin-19 at this concentration in 5% acetic acid exists in an oligomeric state, with a mean particle hydrodynamic diameter of ~ 70 nm (Figure 6.3a). The particle size of suckerin-19 colloids was also assessed by AFM, which indicated a smaller average particle size for suckerin-19 colloids of ~ 25 nm that can be attributed to particle dehydration-induced shrinking (Figure 6.3a inset). Upon illumination with Ru^{II} salt in the presence of APS, the colloidal suspension was converted into hydrogels (Figure 6.3b). As the APS concentration increased, the solution became more viscous and gels started to form at 2 mM APS (Figure 6.3b). These gels displayed a strong deep blue fluorescence upon exposure to 302 nm UV

light (Figure 6.3c), which we attribute to di-Tyr cross-links similar to those detected in cross-linked recombinant resilin.^[36] To further verify and quantify the di-Tyr cross-link density, cross-linked samples were analyzed by Ninhydrin-based quantitative AAA. The presence of di-Tyr was confirmed by comparing the elution profiles against a standard AAA solution to which di-Tyr was added (Figure 6.4a), where a clear di-Tyr peak was observed before His in the elution profiles of cross-linked suckerin-19 films with increasing APS concentration. Compared to Leu, the area under curve (AUC) of di-Tyr increased from 1.1% to 3.8 %, which corresponded to a di-Tyr content ranged from 0.1 to 0.4 mole% as the APS concentration increased from 0.5 to 2.5 mM (Figure 6.3d). At the same time, the Tyr relative content in cross-linked samples decreased by 2.7% (to 8.6%) depending on the APS concentration in comparison to uncross-linked films (Figure 6.4b). These results suggest that di-Tyr cross-linking in suckerin-19 materials is controllable by dosing the APS concentration during the cross-linking process.

Figure 6.3 Cross-linking of suckerin-19 into hydrogels. (a) Particle size distribution of suckerin-19 at 40 mg/ml in 5% acetic acid measured by Dynamic Light Scattering (DLS). The

inset shows an AFM image of suckerin-19 particles in 5% acetic acid. **(b)** Suckerin-19 cross-linked with different APS concentrations. **(c)** Photographs of normal light and UV light illuminated suckerin-19 gels cross-linked with 2.0 and 2.5 mM APS, illustrating the fluorescence due to Di-Tyrosine cross-linking. **(d)** Cross-linking density of suckerin-19 hydrogels/films as quantified by Ninydrin-based amino acid analysis ($N = 3$, values presented are mean $\pm$ s.d.).

Figure 6.4 Amino acid analysis (AAA) of cross-linked suckerin-19 gels and films. **(a)** Elution profiles of suckerin-19 films, showing the peak elution times of different amino acids, including di-Tyr, which elutes right before histidine. For quantification, the area under the di-Tyr peak was measured and compared to that of Leu, which was used as an internal standard. **(b)** Relative decrease of Tyr suckerin-19 films for various cross-linking conditions. Values were normalized to the Tyr content of the non cross-linked film ($N = 3$, values shown are mean $\pm$ standard deviation (s.d.)).

Frequency-sweep rheological measurements in the viscoelastic regime revealed that the storage modulus (G') of the gels increased from 5×10^{-3} Pa to 480 Pa as the APS concentration increased from 0 to 2.5 mM (Figure 6.5a). No frequency dependence was observed at high APS concentrations above 0.1 Hz, suggesting that the gels were predominantly elastic in this frequency range.^[212] The loss tangent (G''/G') also decreased with increasing the APS concentration, with the critical cross-

over point corresponding to the transition from a fluid-like to a solid-like behavior ($G'' = G'$) located at $\sim 1\text{mM}$ APS (Figure 6.5b). Strain amplitude sweep measurements were also performed on cross-linked suckerin-19 hydrogels, which indicated a viscoelastic regime in the range 0.5 to 30% strain (Figure 6.5c, left panel). As the strain increased beyond 100%, the G' values of both gels decreased significantly. At 300% strain G' dropped abruptly, indicating failure of the gels. However, within 30 sec. of recovery both hydrogels recovered their storage moduli to their original values (Figure 6.5c, right panel), suggesting that suckerin-19 hydrogels are able to self-heal following failure. Self-healing capacity of hydrogels has previously been established in networks cross-linked by weak ionic or hydrogen bonds,^[213, 214] or by metal ligand complexation.^[213] In these systems self-healing is provided by reversible physical bonds, whereas gels that are covalently cross-linked lose their structural integrity permanently. Given that both types of bonds are present in suckerin-19 gels (hydrogen bonds within β -sheets for the physical cross-links and di-Tyrosine for the covalent cross-links), our data suggest that gel failure and self-healing may occur by β -sheet breakage and re-formation, respectively.

To further understand the polymer network properties of suckerin-19 gels, we explored their scaling relationships by measuring the influence of protein concentration ($C_{\text{suckerin-19}}$) on G' . As shown in Figure 6.5d, G' scales with the concentration as $G' \sim C_{\text{suckerin-19}}^{2.3}$, which is fully consistent with the scaling-law derived for semi-flexible networks,^[215] where a $G' \sim C^{11/5}$ scaling has been predicted and verified experimentally for a range of biopolymeric gels including cross-linked actin^[216] and fibrin.^[217] This agreement with a semi-flexible network suggests that β -sheets are the rigid elements of the gels, whereas the entropic elastic springs of the network are provided by the random-coil domains. We also measured the scaling

relationship as function of cross-linking density and found a $G' \sim C_{\text{APS}}^{6.1}$ dependence (Figure 6.5e). The scaling exponent is remarkably higher than most cross-linked semi-flexible biopolymers. For instance in intermediate filaments networks electrostatically cross-linked with divalent Mg^{2+} ions,^[218] G' scales as $C_{\text{Mg}}^{0.6}$, whereas in cross-linked actin networks, the scaling relationship is $G' \sim C_{(\text{cross-link})}^2$.^[216] The marked cross-linked dependence of suckerin-19 gels hence implies that Ru^{II} -based cross-linking is a very effective method to tune their visco-elastic properties, a behavior that can be rationalized based on the block co-polymer molecular structure of suckerin-19. Tyr residues are segregated in the amorphous blocks where the inter-Tyr separation is typically 3 to 5 amino acids (Figure 6.11a). Therefore, a large number of potential cross-linking sites per unit length exist in the amorphous domains.

Figure 6.5 Rheological measurements of suckerin-19 hydrogels. **(a)** Plots of G' and G'' vs. oscillation frequency for suckerin-19 cross-linked with different APS concentrations. **(b)** Loss tangent (G''/G') of cross-linked suckerin-19 vs. APS concentration, measured at 1 Hz. The cross-over point ($G''/G' = 1$) is at ~ 1 mM APS. **(c)** Amplitude strain sweep of cross-linked suckerin-19 gels at 2 and 2.5 mM APS and their recovery. G' during recovery as a function of time was measured at 2% strain and 1 Hz. **(d)** G' vs. protein concentration (fixed molar ratio of APS to suckerin-19) in log scale. The scaling of $G' \sim C_{\text{suckerin-19}}^{2.3}$ is fully consistent with a cross-linked semi-flexible network. **(e)** G' vs. APS concentration in log scale, with the red line illustrating a scaling of $G' \sim C_{\text{APS}}^{6.1}$.

6.3.3. Microstructure and surface features of suckerin-19 films

In the following, we prepared suckerin-19 films using a simple solvent evaporation method following photo-cross-linking of self-assembled suckerin-19

colloidal particles. Solvent drying is an efficient method to prepare protein-based materials when proteins self-assemble into specific secondary structures during solvent evaporation, including in silk fibroins.^[174, 175, 219] After Ru^{II} -mediated cross-linking, the suspensions were dried into homogenous films 5-10 μm thick (Figure 6.6, insets). High-resolution FESEM showed that all films, whether fabricated from cross-linked or non cross-linked colloids, were composed of nanoparticles with dimensions from 30 to 50 nm (Figure 6.6), indicating that cross-linking had little influence on the film microstructures. The size of the nanoparticles observed on the film cross-section was consistent with that measured by DLS and with AFM imaging prior to solvent evaporation (Figure 6.3a). This size similarity suggests that the colloidal particles assemble into a closely packed film, with individual particles remaining mostly intact during solvent evaporation and compaction. It is worth noting that the film microstructures are distinct from those observed on other β -sheet-rich protein-based films and scaffolds, which usually exhibit fibrous^[167] or porous and lamellar microstructures.^[175, 219]

Figure 6.6 SEM images of suckerin-19 films cross-linked with different APS concentrations. (a) Non cross-linked film. (b-f): suckerin films cross-linked with 0.5 to 2.5 mM APS respectively.

Given the recognized role of surface roughness of materials on cell attachment, migration, proliferation and stem cell differentiation,^[208, 220, 221] we also explored the effect of cross-linking conditions on the films' surface topography using AFM (Figure 6.7). For non cross-linked films, the surfaces exhibited surface roughness in the range of 64.6 nm. As the APS concentration increased, the surface roughness gradually decreased to ~ 28.9 and 15 nm at 1 and 1.5 mM APS respectively, and down to less than 5 nm for 2 and 2.5 mM APS. Therefore the surface roughness exhibits a clear decay at higher APS concentrations (Figure 6.7f). Surface roughness has been reported to play an important role in the proliferation and differentiation of human myogenic and bone marrow stem cells,^[208] as well as for embryonic^[221] and mesenchymal^[222] stem cells. Considering the range of surface roughness values that can be attained with suckerin-19 films (< 5 nm to ~ 65 nm), it may be possible to regulate stem cell differentiation via simple manipulation of cross-linking conditions, a characteristic that will be tested in future studies.

Figure 6.7 AFM surface topography observations of suckerin-19 films cross-linked with different APS concentrations. **(a)** Non cross-linked film. **(b-e)** Suckerin-19 films cross-linked with 1.0 to 2.5 mM APS respectively. **(f)** Surface roughness parameter R_a for the various

suckerin-19 films measured on AFM images. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; one-way ANOVA analysis.

6.3.4. Mechanical properties and protein secondary structures of suckerin-19 films

In order to examine the influence of cross-linking conditions on the films' mechanical properties, we measured their Young's moduli (E) and Hardness properties (H) using Depth-Sensing Nanoindentation in both dry and wet conditions. In the dry state, cross-linking had a minor influence on the modulus, with E values ranging between 8 and 9 GPa (Figure 6.8a) for all films. Interestingly the values are larger than the average E value of native SRT (4-5 GPa) and match or exceed structural synthetic polymers such as PMMA, PEEK, or polyamides.^[20] Figure 6.8b showed representative load-displacement curves of suckerin-19 films measured with different loads in dry state, whereas a higher load (100 μ N) was employed to eliminate the effect of substrates. Under hydrated conditions, on the other hand, the films exhibited wide differences in E as a function of cross-linking conditions (Figure 6.8a), with E decreasing exponentially as the APS concentration increased. With 0.5 mM APS, E values were in the range of 2 GPa, dropped to 9.4×10^{-1} GPa with 1 mM APS, and to 3.8×10^{-2} GPa with 2.5 mM APS. For the non cross-linked film, E was 8 MPa. Figure 6.8c showed representative load-displacement curves on different samples with the same load, where the tip was able to penetrate for different depths into diverse films, thus confirming that these films differed in mechanical strength. In other words, we were able to tailor the Young's modulus of suckerin-19 films over 3-orders of magnitude by simply changing the initiator concentration.

Figure 6.8 Nanoindentation of suckerin-19 and suckerin-19-glycerol films. **(a)** Elastic modulus (E) of suckerin-19 films cross-linked at various APS concentrations and plasticized with different amounts of glycerol in both wet and dry conditions. **(b)** Representative load-displacement curves of suckerin-19 film (2.5 mM APS) at different peak loads. The film was probed at high load (100 μ N to eliminate the effects of substrate). **(c)** Representative load-displacement curves in wet conditions of suckerin-19 film cross-linked with different concentrations of APS. **(d)** Representative load-displacement curve of suckerin-19-glycerol films and the curve fitting based on the Hertzian solution for E calculation.

It is counterintuitive that the modulus displays an inverse dependence on the cross-linking density. Indeed, higher covalent cross-link density is well established to increase the modulus of bulk polymers,^[153] a behavior that has also been established for photo-cross-linked functionalized cellulose-based polymers.^[223] Because E of β -sheet rich protein-based polymers has been shown to strongly depend on the β -sheet content, including in silk^[27, 175] and native SRT,^[17] we hypothesized that the broad variation in Young's modulus was also correlated to changes in β -sheet content, which may vary as a function of cross-linking conditions. To test this hypothesis, we

assessed the films FTIR in the ATR mode. All films exhibited the characteristic 1625 cm^{-1} shoulder peak of β -sheets (Figure 6.9a) in the Amide I region, as well as the signature of β -sheets in the Amide II region at 1520 cm^{-1} .^[174, 198] β -sheet peaks in the Amide III region ($1232\text{--}1235\text{ cm}^{-1}$)^[197] were also observed (data not shown). Comparisons between all films indicated a transition away from β -sheets towards random coil structures as the APS concentration increased. A semi-quantitative analysis by standard peak deconvolution of Amide I^[160] revealed that the β -sheet content significantly decreased as the APS concentration increased, from $\sim 35\%$ for 0.5 mM APS cross-linked film to 25% at the highest concentration of APS (Figure 6.10). Taken together, the data suggest a direct correlation between the Young's modulus and the β -sheet content in cross-linked suckerin-19 films (Figure 6.9b), consistent with the load-bearing capability of nano-scale β -sheets in protein-based biopolymers.^[27]

Figure 6.9 (a) Zoom-in on the Amide I and II bands of suckerin-19 films. (b) E vs. β -sheet content (wet conditions) of cross-linked suckerin-19, illustrating the correlation between the modulus and the β -sheet content.

Figure 6.10 Deconvolution of Amide I spectra for suckerin-19 films with different APS concentrations. (a) 0 mM APS. (b) 0.5 mM APS. (c) 1 mM APS. (d) 1.5 mM APS. (e) 2 mM APS. (f) 2.5 mM APS.

Therefore, the counterintuitive observation that higher cross-link densities lead to softer suckerin-19 films is explained by the counter-balancing effect of increased β -sheet content at lower cross-link densities. We suggest that this unusual behavior is directly related to the molecular structure of suckerin-19, which is essentially a di-block co-polymer consisting of β -sheet forming domains about 12 amino acids long intervened by longer amorphous domains.^[17] While the β -sheet domains are enriched in Ala, Thr, and His residues, Tyr is completely absent from these domains and is instead segregated within the amorphous Gly-rich domains, where it appears in the tri-peptides GGY and GYG, or in the tetra-peptide GGLY (Figure 6.11a). It is reasonable to assume that a certain level of chain flexibility in the amorphous regions facilitates the spatial presentation of adjacent β -sheet strands, therefore enabling β -

sheet “locking” (Figure 6.11b). On the other hand, extensive di-Tyr cross-linking within the amorphous regions limits chain backbone flexibility, thus frustrating β -sheet formation (Figure 6.11c) and providing a mechanism to finely tune the final β -sheet content. Since hydration is playing a softening role in the un-cross-linked films, most likely by increasing chain mobility of the amorphous domains, we note that a minimum amount of cross-links is required in order to prevent extensive water ingress within the amorphous domains and to ensure minimal loss of Young’s modulus. Modulation of the mechanical response of protein-based materials via di-Tyr cross-linking has previously been achieved in fibrinogen,^[199] resilin,^[36] and silk fibroins.^[200] For materials made from recombinant suckerin-19, our data suggest that this chemical functionality is exploited further by taking advantage of two specific features of their primary structure. First, the Tyr content of suckerin-19, at 15.8 mole%, is notably higher than other protein based materials, allowing to potentially achieve a wider range of cross-link densities. Second, the di-block co-polymer modular architecture of suckerins brings an additional level of versatility because the localization of Tyr within the amorphous domains prevents direct disruption of β -sheets.

Figure 6.11 Representative modular peptide domains of suckerin-19 and cross-linking schematic for suckerin-19 films. (a) Typical Ala- and His-rich β -sheet forming peptides (left), and Gly- and Tyr-rich amorphous peptides of suckerin-19 (right). (b) Spatial presentation of β -sheet strands. “ β -sheet locking” occurs with a moderate di-Tyr cross-link density of the amorphous domains. (c) At high di-Tyr cross-link density, β -sheet formation is frustrated by the constrained flexibility of the amorphous domains.

In order to further expand the range of elastic moduli towards the sub-MPa range, we prepared films by mixing suckerin-19 solution with glycerol, a common plasticizer used to tune the mechanical response of protein-based materials.^[224] By varying the suckerin-19 to glycerol ratio, E of dried films could be decreased in the range 10 to 0.30 MPa (without glycerol, ~ 8 GPa), whereas under hydrated conditions, E ranged between 1.5 MPa and 0.15 MPa (Figure 6.8a). Therefore, the addition of a plasticizer allows the further tuning of the elastic modulus of suckerin-19 films by another two decades. Suckerin-19-glycerol films were also analyzed by FTIR. The β -sheet content

decreased with increasing glycerol dosage (Figure 6.12), which was also correlated to a decrease of E . Comparing the elastic modulus of biological tissues with those obtained for suckerin-19 (Figure 6.13), we note that the former can be made to match a wide range of tissues, from soft liver to ligament and tendons, with the stiffest films approaching the Young's modulus of bone. We suggest this makes recombinant suckerins promising candidates in biomedical applications, notably in restorative connective tissues.

Figure 6.12 FTIR spectra of soft suckerin-19-glycerol films. **(a)** Amide I band of suckerin-19 films with different glycerol content. The intensities were normalized to [0, 1] in amide I region. **(b-d)** Deconvolution of Amide I spectra for suckerin-19/glycerol films with different glycerol contents (wt/wt). **(b)** 3x glycerol. **(c)** 5x glycerol. **(d)** 7x glycerol. The relative amount of β -sheet decreases as the glycerol content increases.

Figure 6.13 Tissue elasticity of a range of biological tissues (inspired by and redrawn from Ref.^[207]) and comparison with elastic moduli achieved in this work. Suckerin-19 can be tailored to match soft tissues, stiffer ligament and muscle tissues, and can even approach the modulus of bone in the moderately cross-linked state.

6.3.5. Cell culture studies on suckerin-19 films

In order to verify that suckerin-19 materials are viable candidates for biomedical applications, we cultured a variety of cell lines, including primary cells (human dermal fibroblast (HDF)), stem cells (human mesenchymal stem cells (hMSCs)) and human embryonic kidney (HEK) 293 cells on non cross-linked and cross-linked films (2.5 mM APS). In particular, the cell number of HDF for attachment and proliferation was quantified against a standard curve by alamarblue method (Figure 6.14). HDF cells seeded on suckerin-19 films displayed a similar cell attachment as the control TCP surface (Figure 6.15a), with suckerin-19 films able to support cell proliferation for more than 2 weeks until a plateau was reached (Figure 6.15b). It is noteworthy that suckerin-19 films supported a faster cell proliferation than TCP. For instance at Day 12, a 5-to 6-fold increase in cell density was observed for the suckerin-19 films versus a 3.5-fold increase for TCP, a performance that exceeds that of other di-Tyr cross-linked protein based materials.^[200, 225] Live/dead staining indicated that cells were homogeneously attached and started to spread widely on both films from Day 1 post seeding. The cells continued to spread and proliferate until they were almost confluent on Day 7. Furthermore almost no dead HDF cells were observed on suckerin-19 films over time (Figure 6.15c). Live/dead staining was also performed for hMSCs and a

similar trend of cell attachment, spreading, and proliferation was observed, with very few dead cells on each type of suckerin films (Figure 6.15d). In comparison to TCP surfaces, cells exhibited more spreading on suckerin films (Figure 6.16). The quantitative analysis of live and dead cells in the images revealed a high cell viability constant at ~95% along the time on both films (Figure 6.17a), and more interestingly, there existed a fast increase in live cell number by more than 2 folds within the 11 days post seeding and cells proliferated a bit faster on non cross-linked films compared to cross-linked films (Figure 6.17b). HEK 293 cells also displayed a high attachment and rapidly proliferated until over-growth was observed (1 week after seeding), with very few dead cells noticed until Day 7 (Figure 6.18). Overall, the high attachment, better spreading, fast proliferation, and long-term survival of diverse cells on suckerin-19 films suggest that these materials are non-cytotoxic and biocompatible. These data also confirm the low cytotoxicity of the Ru^{II} -mediated cross-linking method.^[199, 225]

Figure 6.14 Standard curve corresponding the cell number of HDF to fluorescence intensity in Alamar blue assay for the calculation of cell density on suckerin-19 films.

Figure 6.15 Cell culture studies on non cross-linked and cross-linked suckerin-19 films. **(a)** Cell attachment of HDF cells on suckerin films on Day 1 post seeding. **(b)** Cell proliferation on suckerin films and TCP (control) over a 16-day period as determined by Alamar blue assay. Cell proliferation rate was greater for suckerin films than TCP. **(c)** Live (green) and dead (red) staining of HDF cells on suckerin films over a 7-day period. **(d)** Live and dead staining of human mesenchymal stem cells (hMSCs) on suckerin films over an 11-day period. All scale bars represent 100 μ m.

Figure 6.16 Live/dead staining of hMSCs on TCP.

Figure 6.17 (a) Cell viability of hMSCs on suckerin films calculated from live and dead cells in live/dead staining. (n=3, mean $\pm$ s.d.). (b) Increase of hMSC cell number on suckerin films as a function of time (n=3-4, mean $\pm$ standard error (s.e.)).

Figure 6.18 (a) Green Fluorescent Protein expressing HEK 293 cell proliferation on suckerin-19 films. (b) Live/dead staining of HEK 293 cells on suckerin-19 films on Day 7 post seeding.

6.3.6. Comparison between *D. gigas* SRT and silks

Given the sequence homology of *D. gigas* suckerins to silk proteins and a wide range of materials prepared with suckerins and silks as demonstrated in the present project and literatures, we are able to provide useful comparisons between *D. gigas* SRT and silks, especially silkworm silks and spider dragline silks. There exist a wide range of similarities and differences between the two, as illustrated in Table 6.1. Both are mechanically robust materials with high E arising from the reinforcement of the polymer network with β -sheets. Both are composed of proteins consisting of a modular structure at the primary sequence level, with suckerins sharing some

homology to silk proteins in the sub-modules. For example, both proteins are dominated by Ala-rich and Gly-rich domains in the primary sequence. But in contrast to silks, SRT is a bulk material which must withstand complex load regimes combining, tensile, compression and shear stresses. Thus, the β -sheet reinforcing domains in SRT are randomly oriented to provide strengthening in a multi-directional fashion.^[25] Silks, on the other hand, are fibrous structures that are primarily loaded in tension.^[226] Moreover, the molecular weights of proteins from the two materials are different. Silk contains much higher protein molecular weights and a lower number of proteins. To this end, the recombinant expression of silk proteins is not as easy as that of suckerins, as evidenced by the decades of development that were required to produce recombinant silks in good yield. In contrast, SRT recombinant production was established in this work in just a few years.^[25] Therefore, suckerins are representing an alternative to silk proteins for the fabrication of biomaterials, especially in their recombinant expression form. These functional materials include fibers, nanofibers, films, nanostructures, nanospheres, hydrogels, and tissue scaffolds. Fibers, films, nanofibers, microspheres, sponges, hydrogels, and tissue scaffolds have all been engineered with silk proteins and we believe that all of these applications could also be obtained with suckerins.

Table 6.1 Comparison between SRT and silkworm or spider dragline silks.

	SRT	Silkworm and spider dragline silks
Native morphology	Bulk	Fiber
Native mechanical loading regime	Tensile, compression, shear ^[25]	Tensile ^[226]
Mechanical properties	High E (4.5-8 GPa) ^[12, 25]	High E (~10 GPa) ^[226-228]
Structure	β -sheet reinforced network ^[25]	β -sheet reinforced network ^[27, 226]
Protein Composition	Suckerins ^[25]	Fibroins or spidroins ^[105, 226]
Molecular weight	8-50 kDa ^[12, 25]	Heavy chain silk fibroin: 350 kDa; Light chain silk fibroin: 25 kDa; Spidroins: 250-320 kDa ^[134, 229]
Primary amino acid sequence	Ala, His -rich and Gly, Tyr, Leu-rich modules ^[25]	Poly(Gly-Ala)/poly-Ala and Gly-rich Modules ^[27, 226]
Ease of full-length recombinant protein expression	Yes ^[25]	No ^[143, 145]
Engineered materials	Fibers, nanofibers, films, nanostructures, nanospheres, hydrogels, tissue scaffolds ^[25]	Fibers, films, nanofibers, microspheres, sponges, hydrogels, tissue scaffolds ^[40, 105]

6.4. Conclusion

In summary, we have used the recently discovered suckerin-19 protein to fabricate mechanically tunable hydrogels and films. The mechanical response could be modulated by simple variation of the cross-linking conditions or by the addition of a common plasticizer. When suckerin-19 was processed into hydrogels, the rheological response was consistent with a semi-flexible polymer network whose elasticity could be finely tuned by di-Tyr cross-linking. Another key finding is that the Young's modulus of suckerin-19 films could be controlled by manipulating the β -sheet content, which was itself governed by modulating the di-Tyr cross-link density.

Importantly, suckerin-19 films did not exhibit cytotoxicity against any of the cell lines tested. More importantly, the ability to tune the Young's modulus of suckerin-19 materials by several orders of magnitude to match the elasticity of a wide range of human tissues, from soft liver to stiff bone, opens promising avenues in tissue engineering and restorative applications, especially considering the biocompatibility of the photo-cross-linking method employed in the present study.

Chapter 7 Conclusions and Outlooks

7.1 Conclusions

Nature is providing many examples for bio-inspired engineering and increasingly does so at the molecular level. A broad spectrum of sophisticated biological materials are being investigated for the development of environmentally benign routes to synthesize novel materials. In this work, the *D. gigas* SRT, which perform key grappling functions during predation, provides bio-inspired lessons to create load-bearing biomacromolecular materials that can rival a variety of synthetic polymers. In contrast to many other load-bearing biological materials, SRT contain neither chitin, minerals, nor covalent inter-chain cross-links and are instead entirely assembled from proteins called “suckerins”, which form a supramolecular network reinforced by nanoconfined β -sheet structures.

In this project, a comparative study of SRT from different species revealed that their mechanical properties were similar to that of other squid (*S. lessoniana*) and cuttlefish (*S. esculenta*). Nanoindentation measurements of *D. gigas* SRT in different solutions combined with micro-Raman spectroscopy under identical conditions provided the first direct evidence that hydrogen bonding localized in the β -sheets was an essential factor contributing to the mechanical properties of the material. Thermo-mechanical measurements provided deeper insights into SRT and suckerins with thermoplastic features. We found that the crystalline β -sheets and amorphous random coils of the bi-phasic, semi-crystalline biopolymer exhibited different thermal stabilities and mechanical response in dry and hydrated conditions. β -sheets were stable up to 220 °C, at which structural integrity was lost, revealing a relatively high temperature window available for thermal processing of these proteins. These data

offer important suggestions for the molecular design of biomimetic proteins that could be fabricated into thermoplastic biopolymers with potential biomedical and 3D printing applications.

These features suggest that SRT proteins could be used for the fabrication of novel biomaterials. Here, a proof-of-principle was established by re-processing SRT into diverse morphologies (*e.g.* fibers and nanofibers). The melt-spun suckerins fibers displayed protein chain alignment, β -sheets structures and comparable mechanical properties as regenerated silk fibers. Importantly, this water-based spinning strategy is an environment-friendly process which eliminates the complex processing and the usage of harsh chemicals used in the fabrication of regenerated silk fibers. Native suckerin mixtures were also utilized for the fabrication of nanofibers by electro-spinning. PEO was found to greatly improve the spinnability of proteins and gave rise to smooth nanofibers with diameters from 100-300 nm, despite an inhomogeneous distribution of proteins particles inside the fibers. Protein concentration, flow rate and accelerating voltage played important roles in controlling the spinnability and fiber features.

In order to make suckerins an efficient and sustainable alternative to current artificial protein-based materials, a recombinant expression system of suckerin-19 was established with two forms of protein (with or without poly-His tag, respectively) in *E. coli*. Both His-suckerin-19 and suckerin-19 were expressed in inclusion bodies and purified by a combination of chromatographic and non-chromatographic (*e.g.* isoelectric precipitation) methods and His-tag was removed by recombinantly expressed TEV protease. The protein yields were promising although they do not yet match those recently obtained from some silk mimics. Purified suckerin-19 exhibited

in-solution characteristics that were of direct use for the aqueous-based, eco-friendly processing of suckerin-based biomaterials, including a concentration-dependent structure transition from random coils to β -sheets and a high solubility around 70 mg/ml in 5% acetic acid.

In order to investigate its potential for the preparation of materials that may find usage in biomedical and engineering applications, suckerin-19 was fabricated into mechanically tunable materials by a Ru^{II} -mediated photo-cross-linking method. A key finding is that E of these materials could be modulated for 7 orders of magnitude by simple variation of the cross-linking conditions or by the addition of a common plasticizer. For cross-linked suckerin-19 hydrogels, the rheological response was consistent with a semi-flexible polymer network whose elasticity could be finely tuned by di-Tyr cross-linking. For suckerin-19 films, the Young's modulus was controlled by manipulating the β -sheet content, which was itself governed by modulating the di-Tyr cross-link density. The microstructure of suckerin-19 films were all composed of nanoparticles, but their surface roughness can be tailored by APS concentration in the cross-linking. Importantly, suckerin-19 films exhibited negligible cytotoxicity against any of the cell lines tested. The ability to tune the Young's modulus of suckerin-19 materials by several orders of magnitude to match the elasticity of a wide range of human tissues from soft liver to stiff bone, opens promising avenues in tissue engineering and restorative applications, especially considering the biocompatibility of the photo-cross-linking method and suckerin based materials. The di-Tyr based cross-linking strategy also sheds light on other biomedical applications of protein-based materials, for example in cross-linking controlled drug release of hydrogels and nano/micro-particles.

In summary, all of four aims previously defined have been achieved in this thesis and four key findings can be derived from the study of this project:

(1) The impressive mechanical properties of native SRT are directly related to hydrogen bonds located in the nano-confined β -sheet structures that reinforce the material. SRT are a type of thermoplastic semi-crystalline biopolymer whose crystalline β -sheets and amorphous domains exhibit different thermal stabilities and mechanical responses upon heating.

(2) Suckerin proteins can readily be prepared by recombinant expression and the purification can be streamlined without the need to use time-consuming chromatographic methods. These characteristics are important towards a sustainable supply of suckerin proteins for materials engineering and biomedical applications.

(3) The modular structure of SRT proteins' primary sequence, their tendency to self-assemble into β -sheet enriched structures, their homology to silk proteins, and their thermoplastic properties make suckerin proteins a promising biopolymer to engineer of a wide range of functional materials, including fibers, nanofibers, nanoparticles, hydrogels, and films that have been demonstrated in this study.

(4) Suckerin-19 materials display tunable mechanical properties with a Young's modulus that can be varied over 7 orders of magnitude, as well as negligible cytotoxicity. This makes recombinant suckerin a promising material in restorative and regenerative applications of a wide range of human tissues.

7.2. Outlooks and future studies

Besides the above-mentioned achievements, there exists ample room to further explore *D. gigas* SRT, especially for the fabrication of materials with native and recombinant suckerins.

Thermo-mechanical features of SRT have been characterized. These efforts may facilitate the preparation of suckerin materials based on their unique thermoplastic characteristics. For example, micro-fibers have been melt-spun from a melt suckerin mixture. Careful optimization will facilitate the production of high performance fibers made of suckerins mixture whose mechanical properties could be largely improved to approach that native silk fibers.^[227] It has been reported that spinning speed, temperature, humidity, and species type influence the mechanical strength of silkworm and spider silks.^[173, 179] These factors will affect the chain alignment of proteins in the fibers, subsequently influencing the mechanical properties of the fibers. Parameters including the spinning speed, temperature and post-spinning drawing ratio, can be optimized by an automatic spinning system (refer to section 4.3.6) for this purpose. Besides the melt-spun fibers, other materials taking advantage of SRT thermoplastic characteristics could be prepared with suckerins. For instance, suckerins could be mixed with certain plasticizers and heated for thermal processing as a 3D printed material for biomedical and engineering applications.

Nanofibers made of suckerins have also been prepared by electro-spinning. However their homogeneity still needs improvement, as shown by the presence of aggregated suckerin nanoparticles inside the nanofibers. Therefore, a different solvent is recommended in future studies to prevent the aggregation of suckerins and facilitate the homogeneous distribution of proteins within nanofibers, which will guarantee the

stability of suckerin nanofibers once PEO is removed after electrospinning. These nanofibers could be characterized for their protein secondary structures, mechanical properties and biocompatibility by cell culture studies.

Recombinant suckerin-19 has been processed into tunable biomaterials including hydrogels and films whose mechanical properties could be tailored by the cross-linking conditions. We envision that it will also be applicable to produce graded materials that are able to display a continuous gradient in mechanical properties. For instance, one option developed by Claussen *et al.*^[230] consists in storing the protein solution, plasticizer, and the initiator and catalyst into separate high precision syringe pumps that mix the components in a graded fashion by adjusting the flow rates of the individual syringes. Alternatively, the photo-cross-linking conditions could be varied in order to spatially adjust light dosage, using for instance moving light-filters^[223, 231] or photolithographic masks with various transparencies,^[232] thereby achieving graded cross-linking densities and elastic gradients. In addition, similar to native suckerins, recombinant suckerin-19 could also be fabricated into fibers in the future. For example, a microfluidic device utilized in the spinning of silk fibers^[103] could be employed for suckerin-19 fiber spinning. Soft lithography techniques enable the fabrication of a poly(dimethylsiloxane) (PDMS) mold with three inlets and one outlet, where aqueous solution of suckerin-19 flows through the main (central) inlet and reaches the cross intersection where it interacts with two outer streams of the hydrodynamic focusing solution.^[103] The interaction between two solutions at the inter-section causes elongational flow. The suckerin-19 stream is hydrodynamically focused by the outer streams, which could lead to the alignment of the protein chains and possibly leads to fibers rich in β -sheets. The fibers could be examined by a

combination of techniques including polarized light microscopy, FTIR, SEM and nanotensile testing to characterize their structural and mechanical properties.

Finally, a critical step towards the fabrication of materials using recombinant suckerins lies on the production of these proteins in a high throughput way. In this study, 20 mg suckerin-19 could be obtained from 1 liter *E. coli* culture. But for the purpose of commercial application of suckerin based materials, there exists large room to further improve the yield. A possible way to increase the yield is the metabolic engineering of bacterial or optimization of fermentation conditions. For example, the promoter, codon usage, translational enhancers and mRNA stabilizers of suckerin-19 could be used or optimized to enhance protein expression in *E. coli*.^[233] In addition, the high cell density cultivation (HCDC) of bacterial in fermentors could be performed to produce more proteins, where a range of parameters including medium components, dissolved oxygen concentration and pH could be modified.^[137] Considering the facile non-chromatographic methods for protein purification employed in this study, protein purification could easily be scaled-up at the same time, resulting in a high yield of purified protein. In addition, similarly to recombinant silk proteins, the suckerin-19 gene could also be transfected into other hosts including yeast,^[138] plant cells,^[140] or even mammalian cells^[141] to further increase the expression yields.

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