Biomimetic engineering of materials based on hagfish slime thread proteins

Fu, Jing

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**NANYANG
TECHNOLOGICAL
UNIVERSITY**

**BIOMIMETIC ENGINEERING OF MATERIALS
BASED ON HAGFISH SLIME THREAD PROTEINS**

FU JING

SCHOOL OF MATERIALS SCIENCE AND ENGINEERING

2016

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A thesis submitted to the Nanyang Technological
University in partial fulfilment of the requirement for the
degree of Doctor of Philosophy

2016

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research and has not been submitted for a higher degree to any other University or Institution.

05/08/2016

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Date

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Fu Jing

Abstract

Biomimetic materials inspired from Nature have shown unique potential to create a new class of materials with a set of properties not found in synthetic materials. Silk, one classical example of biological protein-based materials, exhibits excellent mechanical performances for diverse applications. However, it suffers from drawbacks with regard to bioengineering processing. Another promising –but far less explored– biological fibrous material model is the hagfish slime thread, which in the native state can be as stiff and tough as silks. At the molecular level, hagfish slime threads (from the species *Eptatretus stoutii*, Es) are built from α -helical coiled-coil proteins (**EsTK α** and **EsTK γ**), which self-assemble into hetero-dimeric intermediate filaments (IFs), and these IFs are bundled intra-cellularly with a high degree of axial alignment into macroscopic threads. Upon draw-processing, native hagfish slime threads exhibit an irreversible structural transition from α -helical coiled-coil to aligned β -sheet (**$\alpha \rightarrow \beta$ transition**). This strain-induced phase transformation endows native threads with remarkable mechanical properties that rival silks and approach Kevlar.

This thesis describes the methods to create artificial EsTK-based fibers by using self-assembled coiled-coil nano-filaments produced by recombinant (rec) protein expression as building blocks. (rec)**EsTK α** and **EsTK γ** are expressed in an *E. coli* system, and subsequently purified by microfluidization and size exclusion chromatography (SEC). Using a self-assembly process based on step-wise removal of urea, (rec)EsTK-based filamentous networks are exploited. Both circular dichroism (CD) measurement and transmission electron microscopy (TEM) images confirm the helical coiled-coil filaments formation, which provides the basis for the production of larger scale materials fabrication. Notably, (rec)EsTK-based filamentous networks undergo $\alpha \rightarrow \beta$ transition under oscillatory shearing, which mimics the native hagfish threads mechanical strengthening process.

Using the self-assembled coiled-coil filaments as building blocks, artificial (rec)EsTK-based fibers are then developed. These artificial fibers experience mechanical performance enhancement during draw-processing, which is attributed to

high content of aligned β -sheet structure formation, as evidenced by synchrotron wide-angle X-ray scattering (WAXS) and polarized Raman spectroscopy. The fibers are believed to exhibit a multi-step conformational transition, from coiled-coils to cross β -sheets during fiber picking-up formation, followed by cross β -sheets to elongated parallel β -sheets during draw-processing. Moreover, chemical cross-linking between lysine residues further enhances the stiffness and ultimate tensile strength (UTS), which approaches that of silks.

Microfluidic devices are also utilized to create (rec)EsTK-based fibers. Using pressure as the driving force, the majority of self-assembled nano-filaments could be stretched, elongated, and aligned parallel to the direction of fluid flow. Although the resulting fibers are shorter and more brittle than manually picking up from the solution, microfluidics provide an elegant method for the alignment and bundling of (rec)EsTK-based filaments and will be further explored.

Generally speaking, this thesis provides the foundation to produce protein-based artificial fibers by a ‘bottom-up’ approach from recombinant protein production, using self-assembled coiled-coil filaments as the fundamental building blocks. The tough and tunable mechanical responses of these protein-based fibrous materials could lead to a variety of engineering and biomedical applications and could notably rival artificial silk.

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30 μm , 20 μm , 10 μm). **(d)** Constrained region under optical microscope using final width 50 μm as the example.

Figure 6.2 Schematic illustrations showing the second microfluidic device design with 1-inlet-1-outlet. **(a)** Illustration of microfluidic device used in this study. **(b)** AutoCAD illustration using a final width of 10 μm as an example. **(c)** Constrained region under optical microscope using final width 10 μm as the example.

Figure 6.3 Schematic illustrations showing the microfluidic device design with 3-inlet-1-outlet. **(a)** Illustration of microfluidic device used in this study. **(b)** AutoCAD illustration using the main channel width of 30 μm shown as an example. **(c)** Actual PDMS device tested by injecting a red dye solution through the inlets. **(d)** Connecting region where the main central channel and the two side channels meet, visualized under an optical microscope using a main channel width of 30 μm as an example.

Figure 6.4 Optical microscope and FESEM micrographs of fibers formed in the 1-inlet and 1-outlet design of microfluidic devices. The final channel width was 20 μm and the flow rate was 20 $\mu\text{L/h}$. **(a)** From top to bottom: under light microscopy; under polarized light microscopy; under polarized microscopy with analyzer rotating 45°. **(b)** FESEM micrograph showing the surface nano-structures.

Figure 6.5 Optical microscope and FESEM micrographs of the formed fiber from 1-inlet-1-outlet second design of microfluidic devices. The testing condition was with a final channel width of 15 μm , a channel height of 15 μm , and a flow rate of 20 $\mu\text{L/h}$. **(a)** From top to bottom: under light microscopy; under polarized light microscopy; under polarized microscopy with analyzer rotating 45°. **(b)** FESEM micrograph showing the surface nano-structures.

Figure 6.6 Optical microscope and FESEM micrographs of the fiber formed in 3-inlet-1-outlet design of microfluidic devices. The main flow channel width was 30 μm and the flow rate was 30 $\mu\text{L/h}$ in main channel and 150 $\mu\text{L/h}$ in side channel (main-to-side ratio

1:5). Methanol was used as the side flow. **(a)** From top to bottom: under light microscopy; under polarized light microscopy; under polarized microscopy with analyzer rotating 45°. **(b)** FESEM micrograph showing the surface nano-structures.

Figure 6.7 Optical microscope and FESEM micrographs of the fiber formed in 3-inlet-1-outlet design of microfluidic devices. The main flow channel width was 60 μm and the flow rate was 20 $\mu\text{L/h}$ in main channel and 400 $\mu\text{L/h}$ in side channel (main-to-side ratio 1:20). Methanol was used as the side flow. **(a)** From top to bottom: under light microscopy; under polarized light microscopy; under polarized microscopy with analyzer rotating 45°. **(b)** FESEM micrograph showing the surface nano-structures.

Figure 7.1 The overall process from recombinant protein production to the final macroscopic fiber. **(a)** pJexpress vectors encoding EsTK α and EsTK γ genes (red region) respectively. **(b)** Self-assembly of (rec)EsTK α and γ into α -helical coiled-coil IFs by step-wise dialysis against urea concentration. **(c)** Predicted domain architecture of self-assembled IFs with central coiled-coil region separated by 3 flexible linker domains. **(d)** Fiber formation by picking-up method. **(e)** Illustration of structural transition after draw-processing and chemical cross-linking.

Figure 7.2 Cyto-toxicity testing on artificial (rec)EsTK-based fibers. **(a)** and **(c)** Fluorescence and light images of Human breast cancer cell MDA-MB-231 (nucleus in blue, cytoplasm in red) grown on fibers. **(b)** and **(d)** Fluorescence micrographs of Human breast cancer cell MDA-MB-231 cells (nucleus in blue, cytoplasm in red) growing on fibers.

Figure 7.3 Fluorescent micrographs of neurites (red) growth tracking hagfish thread fibers (green). The arrows point out to neurite outgrowth preferentially following the contour of hagfish thread fibers. (Courtesy of Dr. Paul Guerette)

Figure 7.4 Schematic illustrations of AFM manipulation experiments on single IF in the tapping mode^[14]. The arrows on the left corner

indicate the scan direction. (a) Normal scan. (b) Along one scan line the force applied increases and immediately decreases. (c) The amount of stretching induced by the tip motion can be approximated as $(L_1+L_2)/L_0$. Similar experiments could be conducted on (rec)EsTK filaments.

Figure 7.5 **AFM image of (rec)EsTK-based coiled-coil filaments indicated by arrows.**

Abbreviations

(rec)EsTK α	Recombinant <i>Eptatretus stoutii</i> Thread Keratin α
(rec)EsTK γ	Recombinant <i>Eptatretus stoutii</i> Thread Keratin γ
$\alpha \rightarrow \beta$ transition	Conformational Transition from α -helix into β -sheet
AAA	Amino Acid Analysis
AFM	Atomic Force Microscopy
BSA	Bovine Serum Albumin
CD	Circular Dichroism
DTT	Dithiothreitol
<i>E. coli</i>	Escherichia coli
EDTA	Ethylenediaminetetraacetic Acid
FESEM	Field Emission Scanning Electron Microscope
FPLC	Fast Protein Liquid Chromatography
GA	Glutaraldehyde
GMC	Gland Mucus Cell
GTC	Gland Thread Cell
HK	Human Keratin
IF	Intermediate Filament
IPTG	Isopropyl β -D-1-thiogalactopyranoside
LB	Lysogeny Broth
MALDI-TOF	Matrix Assisted Laser Desorption Ionization Time-Of-Flight Mass Spectrometry
MES	2-(N-morpholino)ethanesulfonic Acid
NF	Neurofilament
PBS	Phosphate Buffer Saline
PDMS	Polydimethylsiloxane
PEG	Polyethylene Glycol
PEO	Polyethylene Oxide
PMSF	Phenylmethane Sulfonyl Fluoride
PVA	Polyvinyl Alcohol
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SEC	Size Exclusion Chromatography

Suckerin-19	<i>D. gigas</i> sucker ring teeth protein 19
TEM	Transmission Electron Microscope
UTS	Ultimate Tensile Strength
WAXS	Wide-Angle X-ray Scattering

Chapter 1

Introduction

Natural protein-based fibers exhibit a very promising potential for diverse applications. However, some limitations have driven researchers to explore alternative protein-based strong fibers other than the classical silk-based ones. Hagfish slime threads represent such promising proteinaceous materials that can be processed into stiff and tough fibers. The threads exhibit a strain-induced $\alpha \rightarrow \beta$ transition during draw processing, which enhances the fibers mechanical properties and can rival silks and even approach the stiffness and strength of Kevlar. This Chapter lays out the hypothesis of the project: strong and tough artificial hagfish slime thread protein based fibers with tunable mechanical properties could be produced by using 'bottom-up' approach of biomimetic engineering techniques. The objectives and scopes are hence designed accordingly.

1.1 Background and Hypothesis

Nature exhibits abundant structural materials, which are used for different functions. Turtles use their shells to protect themselves^[1]; mantis shrimp use their dactyl clubs to hunt for food^[2-3]; insects use their cuticles to support and protect their bodies^[4]. Among these, an important class of biological structural materials is protein-based fibers. They have received lots of research attention due to their remarkable properties. Table 1.1 outlines some examples of natural protein-based fibers and their related locations and functions.

Table 1.1 Some examples of natural protein-based fibers studied over the past decades.

Protein type	Location	Function	Ref
Collagen	Extracellular and exoskeleton	Form connective tissues, skin and bones.	[5]
Elastin	Extracellular	Provide elasticity to tissues.	[6]
Flagellin	Extracellular	Facilitates motility of flagellated bacteria.	[7]
Keratin	Extracellular and exoskeleton	Scaffold in nails, hair and feathers.	[8]
Resilin	Extracellular and exoskeleton	Hinge of insect wings.	[9]
Tubulin	Cytoplasm	Building blocks of microtubules.	[10]
Silks	Exoskeleton	Building materials for cocoons or spider webs.	[11]

Protein-based fibers have a variety of applications. For instance, in the tissue engineering field, protein-based fibers are being explored as membranes and scaffolds for cell growth and tissue function^[12]. In the medical field, the fibers are used as surgical threads and sutures^[13]. Other applications include, but are not limited to, implants^[14], wound healing^[15], pharmaceutical delivery systems^[16], biosensors^[17], personal care products^[18], and even ligament replacement^[19].

Although these protein-based fibers exhibit unique properties and have diverse usage, they exhibit some limitations. For instance, hydrogel is a common material used in tissue engineering and drug delivery system, which is normally formed from natural protein fibers such as collagen and elastin^[20-23]. However, protein fibers reconstituted from denatured collagens or made of elastin cannot sustain high mechanical loads^[24]. Silk, on the other hand, exhibits remarkable mechanical performances with biocompatibility and biodegradability, and has thus been intensively explored^[25]. However, while silk is undeniably receiving large attention in the biomaterials community, it also exhibits some disadvantages with regard to bioengineering process. For example, it has genetic instability to produce full-length fibroins, low solubility of silk proteins in aqueous-based solvents, and usually requires harsh conditions for processing, including the use of hexo-fluoro-isopropanol (HFIP) or 6M Lithium Bromide (LiBr) to facilitate solubilization ^[26-28]. As the result, alternative protein-based strong fibers are being explored all the time.

Recently, hagfish slime threads have emerged as a promising biological material of stiff and tough protein-based fibers^[29-31]. They are built from α -helical coiled-coil proteins (**EsTK α** and **EsTK γ**) that self-assemble into hetero-dimeric intermediate filaments (IFs), and these IFs are bundled intra-cellularly with a high degree of axial alignment into macroscopic threads^[32-34]. Native hagfish slime threads exhibit an irreversible α -helical coiled-coil to aligned β -sheet transition (**$\alpha \rightarrow \beta$ transition**) when draw-processed. This strain-induced phase transformation endows native threads with remarkable mechanical properties that rival silks and approach Kevlar^[35].

Based on the characteristics of native hagfish slime threads mentioned above, we proposed the following main question for this project: are we able to learn from, and later duplicate, hagfish slime threads to make strong and tough recombinant protein-based fibers through biomimetic engineering?

1.2 Objectives and Scope

This project aims to fabricate strong microscopic artificial fibers based on recombinant EsTKs by using a ‘bottom-up’ approach. In order to achieve this goal, several aspects are required. First, efficient recombinant protein production and purification are essential in order to provide sufficient quantities of materials for laboratory-scale experiments. Second, self-assembly of the recombinant proteins into α -helical coiled-coil intermediate filament (IF) is needed as the molecular basis to biomimic hagfish slime threads. Third, IFs will be used as the building blocks to fabricate microscopic artificial EsTK-based materials through alignment and bundling. Last but not least, draw-processing of the artificial EsTK-based fibers will be applied to impose the $\alpha \rightarrow \beta$ conformational transition, which is essential to tailor the mechanical performance. Subsequent chemical cross-linking of the fibers will also be employed to further enhance the mechanical performance of artificial EsTKs-based micro-fibers.

To address the aspects stated above, the project objectives are as follow:

[1] To set up an efficient recombinant EsTKs expression and purification system:

The genes encoding EsTK α and γ will be cloned into pJexpress vectors and then transferred into *E. coli* cells for expression. Protein purification will be achieved by size exclusion chromatography (SEC) or fast protein liquid chromatography (FPLC) system. The purity will be checked by Matrix Assisted Laser Desorption Ionization Time-of-flight (MALDI-TOF) and Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE).

[2] To establish suitable conditions to self-assemble (rec)EsTKs into IFs and to characterize the IFs subsequently:

The self-assembly behavior of other IF systems has been characterized in detail in previous years^[36-41]. The self-assembly of (rec)EsTKs into coiled-coil IFs will be based on these previous studies. Purified (rec)EsTKs will be self-assembled in 1:1

(w/w) ratio by step-wise dialysis against urea concentration under various conditions, including different pH and temperature. The obtained products will be characterized by transmission electron microscopy (TEM) for filamentous structure formation, circular dichroism (CD) to assess their protein secondary structure, and rheology testing to investigate their micro-mechanical response.

[3] To create artificial (rec)EsTK-based fibers by using IFs as building blocks:

The self-assembled (rec)EsTK-based filaments will be aligned and bundled to form artificial fibers. Based on the α -helical coiled-coil filaments acting as building blocks, strong macroscopic fibers could be obtained by a ‘picking-up’ method from the liquid-air interface.

As an alternative, microfluidics will also be applied to make artificial fibers. Different microfluidic devices with various geometries will be designed and tested. The self-assembled filaments are expected to be aligned by flow in aqueous solutions. Elongational flow regimes will be created on the micro-scale to enable the efficient filaments alignment. Subsequent coagulation bath will be used to solidify and bundle the filaments into macroscopic fibers.

[4] To trigger the $\alpha \rightarrow \beta$ transition of the artificial fibers through draw-processing and chemical cross-linking:

The obtained artificial (rec)EsTK-based fibers will then be subjected to draw-processing in order to induce the $\alpha \rightarrow \beta$ structural transition. The mechanical properties of the fiber will be further enhanced by chemical cross-linking. The mechanical performances of all the fibers mentioned above will be measured by tensile testing. Field emission scanning electron microscopy (FESEM) will be used to examine fibers’ topography. The structural transition will be qualitatively analyzed by Congo red staining, and quantitatively by synchrotron wide-angle X-ray diffraction (WAXS) as well as Raman spectroscopy.

We expect that by meeting the above objectives, strong artificial protein-based fibers with tunable mechanical performance could be obtained and hence could provide a new design strategy to produce strong protein-based fibers from coiled-coil IF-like building blocks.

1.3 Dissertation Organization

This thesis describes the biomimetic engineering process to produce artificial (rec)EsTK-based fibers by a ‘bottom-up’ approach.

Chapter 1 gives a brief introduction of protein-based materials, especially protein fibers. Hypothesis, project objectives and scopes are also provided.

Chapter 2 presents the literature background of previous research on bio-inspired and biomimetic fibrous materials, such as cocoon silks and spider silks. The model organism used in this study (pacific hagfish) and the extra-cellular slime from which the protein threads are derived are also presented and lay the foundation of the whole project.

Chapter 3 provides the rationale and the methodologies of the experimental set-ups employed for the overall process, starting from recombinant protein production, followed by self-assembly into coiled-coil filamentous structures, until the final microscopic fibers creation. The draw processing and chemical cross-linking are also introduced in this chapter. Different characterization methods are discussed as well.

Chapter 4 describes (rec)EsTKs expression, purification and self-assembly into coiled-coil filamentous structures, which are amenable to α -helix to β -sheet conformational transition under oscillation shearing.

Chapter 5 describes the approach to produce artificial (rec)EsTK-based fibers with high and tunable stiffness by using the self-assembled coiled-coil filaments obtained in Chapter 4 as building blocks,. The fibers are mechanically, spectroscopically, and chemically characterized.

Chapter 6 presents the microfluidic devices designed to align and bundle self-assembled coiled-coil filaments, and hence induce artificial (rec)EsTK fiber formation.

Chapter 7 provides a brief summary on the main findings of this work, and some proposed future research directions.

1.4 Findings and Outcomes

This research has led to several new outcomes:

[1] Setting up efficient (rec)EsTKs expression and purification systems.

[2] Establishing suitable self-assembly conditions to produce (rec)EsTK-based coiled-coil filaments.

[3] Establishing (rec)EsTK-based artificial fibers fabrication methods using the self-assembled coiled-coil filaments obtained in [2] as building blocks.

[4] Achieving a tunable mechanical performance of these artificial fibers with a high elastic modulus after draw-processing and chemical cross-linking, with mechanical properties approaching that of silk.

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Chapter 2

Literature Review

Biomimetic materials inspired from Nature have been demonstrated to exhibit a large potential as novel materials with combination of interesting properties. Silk, a classical example of biological fibers, exhibits excellent mechanical performances with a wide range of applications reported in the past decade. However, silk production faces significant challenges with regard to bioengineering processing. As an alternative to silks, hagfish slime threads are a type of much-less explored protein-based fibers, which can be processed into strong fibers and even surpass the mechanical performances of silks. Notably, after draw-processing these threads exhibit a strain-induced α -helical coiled-coil to aligned β -sheet transition ($\alpha \rightarrow \beta$ transition), which yields remarkable tensile properties. This Chapter describes the overall scope and sets out the foundation of the project.

2.1 Biomimetic and Bio-Inspired Materials

Nature has inspired human innovation in a wide range of fields for centuries. In the past few decades, a huge number of model biological organisms have been explored to elucidate the unique properties of biological structures. For example, lotus leaves exhibit super-hydrophobicity and self-cleaning surfaces^[1] (Figure 2.1a); Gecko feet have excellent adhesive ability through hydrophobic interactions^[2] (Figure 2.1c); shark skin has remarkable water repellency which minimizes hydrodynamic drag^[3] (Figure 2.1b). Researchers began to ask if it is possible to mimic these natural structures and to create bio-inspired materials with comparable and even better performances.

Nature-inspired biomimetic materials have attracted a large interest in the past decade^[4]. There are three complementary directions in biomimetic materials research, namely: *(i)* revealing structure–function relationships in biological model systems; *(ii)* exploring the fundamental principles behind the relationship all the way down to the molecular level; and *(iii)* developing synthetic strategies and manufacturing techniques to produce new biomimetic materials^[5]. Combining chemistry, physics, biology, materials science and engineering technologies, there is a strong belief that biomimetic principles learned from Nature will expand the field of materials science^[6].

Figure 2.1 Illustrations of some biological structures that have bio-inspired materials. (a) Lotus leaf (top) and SEM micrograph of upper leaf side (bottom)^[1]. (b) Shark skin and surface structures in different locations of the body^[3]. Scale bar: 0.5 mm. (c) Gecko feet and the hierarchical structure organization^[2].

2.2 Cocoon Silks and Spider Silks

Man-made fibers based on petrochemical raw materials have played an important role in the polymer industry since Second World War^[7]. They are found to have comparable or even better properties than many natural derivative fibers^[8-9]. These materials are suitable for diverse field usage and applications such as food packaging and clothing. Nylon, for example, possesses excellent mechanical properties including

high extensibility and stiffness as well as a high resistance to many chemicals, which makes it one of the most commonly used synthetic polymers in daily life^[10-11]. However, most of the petroleum-based materials are manufactured from non-renewable resources and are far from being environmentally friendly^[12]. As the demand for materials with robust and biodegradable properties is growing significantly, researchers are increasingly looking into biology to develop robust materials based on natural polymers.

Silk, defined as externally spun fibrous protein secretions, is produced in many animals^[13]. One traditional example is silkworm cocoon silk (Figure 2.2a). Breeding silkworms has been applied for at least 5,000 years in China in order to get silks for various purposes^[14]. To these days, it still remains one of the most important sources of silk production. Each cocoon is made of a thread of raw silk which is around 900 m long and 10 μm in diameter^[15]. Typical commercial silkworm silk from *Bombyx mori* cocoons has a stiffness (Young modulus E) of ~ 7 GPa, an ultimate tensile strength (UTS) of ~ 500 MPa and a breaking strain of $\sim 20\%$ ^[16] (Figure 2.2b). The unique combination of high strength, long durability, and high extensibility makes cocoon silk a suitable material in the textile industry^[17]. In order to grab one pound of raw silk, more than 2,000 cocoons are required^[18]. Cocoon silk is considered as one of the strongest natural fibers, but one drawback is that its strength in hydrated condition decreases up to 20% of the value in dry condition^[17, 19-20]. Recently, research has focused on using recombinant cocoon silk proteins to make artificial silks with properties comparable to natural silks^[21]. However, researchers have still not been able to reach the mechanical performance of native cocoon silk.

Figure 2.2 Silkworm cocoon silks and mechanical properties. (a) Photograph of a silkworm, its cocoon and raw silks. (b) Tensile behavior of silks of different animal species^[16].

Another type of silk that has gained a lot of attention is spider silk. Vollrath *et al.* have discussed that spider silks from different glands exhibit specific mechanical properties, which are used by spiders for different biological functions^[22]. Among them, dragline silk which is produced in the major ampullate gland, exhibits exceptional mechanical properties including high breaking stress and extreme failure energy (Figure 2.3). Spider dragline silk has a tensile strength of roughly 1.2 GPa, which is slightly lower than the tensile strength listed of steel 1.65 GPa^[23]. However, spider silk is a much less dense material compared to steel. Therefore, spider silk has a higher specific toughness and modulus (property per unit weight) than steel^[24].

Spider silk also has a very good capacity for absorbing strain energy^[9]. Perhaps more critical, spiders spin their recyclable fibers under environmentally benign conditions, namely low temperature, pressure, and using naturally-occurring chemicals. Such specific physicochemical characteristics of spider silks have gathered significant attention by the materials research community, and major companies like DuPont have invested capital to artificially reproduce artificial silks^[25]. Recently, a number of start-up companies, including AMSilk in Germany, Spiber in Japan, or Bolt Threads in the US, have also obtained significant fundings to produce and spin artificial silks by using portion of native silk gene sequence, and the business is undoubtedly gaining traction.

Figure 2.3 Stress-strain curves of spider silks from *Nephila* golden silk spider^[22]. Silks from different glands have specific properties and used by the animal for different biologic functions. The highlighted line in each plot is the gland-specific silk. For Flagelliform silk, D and W represent dry and wet conditions, respectively.

Since feeding spiders is much more difficult than feeding silkworms and since spiders are cannibals, bioengineering production of artificial silks with properties comparable to that of native spider silks has been explored for decades^[26-29]. Nevertheless, in-depth research has revealed several significant obstacles. Firstly,

synthesizing full-length spider silk protein gene which is a large and repetitive molecule (250-320 kDa) is complex. The instability of spider silk protein genes in bacteria or yeast often prohibits the effective recombinant spider silk proteins production^[30]. Secondly, native-size spider silk proteins tend to aggregate in aqueous solutions, and are difficult to solubilize. In fact, the production of artificial spider silks from native-size silk proteins using spinning methods can be only achieved in harsh solvents such as hexa-fluoro-isopropanol (HFIP) or 6 M Lithium Bromide (LiBr). This makes the industrial scale-up of artificial spider silks challenging^[26]. Finally, similar to cocoon silks, in the dry state native dragline silks exhibit impressive strength and toughness. They can shrink and contract up to 45% of their length when wet, leading to a process known as “super-contraction”. The cause of super-contraction is still not fully elucidated, although there are some evidence that amorphous coil domains collapse when hydrated, and breakage of hydrogen bonding has also been reported^[31-33]. The resulting native silks are rubbery with entropic elasticity, which may lead to significant limitations for applications ^[23, 34].

2.3 Hagfish Slime Threads

Another interesting biological protein-based fiber exhibiting silk-like properties, but yet has received much less attention is the hagfish slime thread. Pacific hagfish (*Eptatretus stoutii*, Es) are benthic marine scavengers. When facing attack, within a very short time they can secrete large volume of slime that can clog the breathing gills of its predators^[35-36] (Figure 2.4). The slime production is achieved through the combined interaction of Gland Mucous Cells (GMCs) and Gland Thread Cells (GTCs) with seawater^[37]. These cells are secreted together from the epithelial slime glands which are ventro-laterally located below the skin surface^[35-36]. GTCs are tightly packed ellipsoidal bundles which are ~65 μm in diameter and ~125 μm in length^[36-38] (Figure 2.5a). When GTCs and GMCs interact together with seawater, their cell membranes are rapidly disrupted and the packed threads unwind. A closer examination after uncoiling indicates that GTCs contain a single cytoplasmic proteinaceous thread which is ~15 cm long and ~3 μm in diameter^[38-40] (Figure 2.5b). Later, these unwound threads, together with mucin proteins from GMCs, interact with

seawater and expand dramatically to form an ultra-soft, hydrogel-like slime^[41], which consists of 99.996% seawater, 0.0015% mucin protein and 0.002% slime threads^[40].

Figure 2.4 Photographs of (a) *Eptatretus stoutii* (Es) and (b) its slime secretion^[41].

Native hagfish slime threads exhibit intriguing mechanical performance, which can be modulated during draw-processing. In the hydrated state, they exhibit an initial elastic modulus of ~ 6 MPa and a yield stress of ~ 3 MPa. After 70% extension, the threads experience a strain hardening process, resulting in an UTS of ~ 200 MPa and strain failure up to 220%^[42] (Figure 2.6a). Another notable feature of these threads is that their mechanical response can be tailored by a draw-processing mechanism as follows. The drawn threads are processed by subjecting hydrated native threads to a certain strain under wet conditions, and dried afterwards. The process results in a remarkable increase in both stiffness and UTS, whereas failure strain decreases^[43]. In addition, the mechanical properties of these fibers can be further enhanced by post chemical cross-linking^[44]. In this way, the optimally processed threads exhibit enhanced mechanical properties with high initial elastic modulus (~ 8 GPa), UTS (~ 800 MPa) and strain energy to failure of about 200 MJ/m^3 , which is comparable to that of spider dragline silks and even approach high-performance Kevlar^[43] (Figure 2.6b). The drawn-processed hagfish slime threads can thus potentially provide a broad range of tensile response, which may be used in a variety of applications, such as reinforcing fibers for composites^[45].

Figure 2.5 Micrographs of GTCs and intermediate filaments (IFs). (a) SEM image of packed GTCs^[39]. (b) SEM image of uncoiling GTCs showing single proteinaceous thread^[39]. Scale bar: 20 μm . (c) TEM image of IFs from the thread^[46]. Scale bar: 100 nm.

Figure 2.6 Stress-strain curves of hagfish threads and comparison with dragline silk and Kevlar^[43]. (a) Hydrated hagfish slime threads show low stiffness and strength, but high extensibility. (b) Drawn hagfish slime threads at different levels of draw processing compare with native hagfish slime thread, spider silk, nylon, thermoplastic polymers, and Kevlar. Along with draw-processing degree of hagfish threads increases, their stiffness and strength increases.

At the nano-scale, hagfish slime threads are comprised of bundles of intermediate filaments (IFs), which are roughly 10 nm in diameter and 200 nm in length^[46] (Figure 2.5c). The building blocks of these IFs are self-assembled coiled-coils made of two α -helical proteins winding together^[39, 46]. The microscopic threads are formed by bundling IFs intra-cellularly with a high degree of axial alignment^[47-48]. The molecular basis of the observed property enhancement during draw-processing is due

to the strain induced conformational transition from α -helical coiled-coil to aligned β -sheet rich structures of the constituent proteins (hereafter referred to as the $\alpha \rightarrow \beta$ transition)^[42]. This transition has been confirmed by wide-angle X-ray scattering (WAXS), which indicates that the mechanical response enhancement is associated with the formation of β -sheet reinforced supra-molecular network along with draw-processing^[43] (Figure 2.7).

Figure 2.7 WAXS patterns for hagfish threads of various drawn degrees^[43]. (a) Unstrained threads with the pattern of α -helix. (b) Extended threads to a strain of 60% with a mixed pattern of both α -helix and β -sheet. (c) Extended threads to a strain of 100% with the pattern of β -sheet.

In further details, the $\alpha \rightarrow \beta$ transition occurs as follow: in the native state two α -helical proteins wind into coiled-coils. Under stress, the coiled-coils start to unravel into random coils at specific stress values. When loading increases, the random coils are extended further and begin to form extended β -sheet structures with inter-chain hydrogen bonds. Eventually, the majority of α -helices unravel, and β -sheets extend and align^[49] (Figure 2.8).

Figure 2.8 Schematic illustration of $\alpha \rightarrow \beta$ transition^[49]. Along with draw-processing, β -sheet rich structures are formed.

Draw-processing of the native hagfish threads results in the formation of a β -sheet reinforced supra-molecular network, which is similar to spider silk at the molecular level. The achieved β -sheet rich structures strengthen the threads and thus lead to mechanical properties comparable to that of spider silk, and even competitive with high-performance crystalline polymers^[43]. In addition, studies have shown that drawn-processed hagfish threads exhibit nominal contraction when re-hydrated, which is contrast to cocoon silks and native spider silks which shrink dramatically when wet^[16]. In turn, the drawn fibers largely maintain their tensile response in the hydrated state. All the properties indicated above make hagfish slime threads an extremely attractive model for the biomimetic engineering of high-performance and mechanically tunable biomaterials.

2.4 Hagfish Slime Thread Proteins (EsTK α and EsTK γ)

Detailed studies on the native hagfish slime threads have provided important insights into the molecular design and processing mechanisms of this material. These single cytoplasmic proteinaceous threads are assembled from IFs, which are made of two major coiled-coil α -helical protein constituents, namely *Eptatretus stoutii* Thread Keratin α and γ (hereafter referred to as **EsTK α** and **EsTK γ**)^[47-48, 50]. **EsTK α** has a molecular weight of 66.7 kDa with an isoelectric point ~ 7.5 , while **EsTK γ** has a molecular weight of 62.8 kDa with an isoelectric point ~ 5.3 . Both EsTKs subunits have non-helical N- and C-terminals, which are connected to the central α -helical rod domains (1A, 1B, 2A, 2B) through the linker domains (L1, L12, L2) (Figure 2.9). The central rod domains are made of heptad peptide motifs *abcdefg*, where *a* and *d* are hydrophobic residues, which is the classical repeat motif of α -helical coiled-coil forming IF-proteins, as explained in further details in Section 2.5. Furthermore, the sub-domain 2B of both EsTKs subunits contains a “stutter”, which is a discontinuity found in heptad repeats. The molecular structure is altered in this region and therefore leads to imperfect coiled coil formation. It has shown that such stutter is not stable in α -helical status and thus may play an important role in the unfolding of the coiled-coils under applied force^[51]. These two subunits likely self-assemble in a manner similar to many other coiled-coil hetero-dimeric complexes, where hydrophobic Crick-like knob-in hole packing occurs and the coils are further stabilized by ionic interactions^[42-43, 46, 52]. The resulting coiled coils then self-assemble further intracellularly to form IFs, which are the meso-scale filaments observed in many cells and will form the central building blocks for this project. More details are discussed in Section 2.6.

Figure 2.9 Predicted architecture domain of hagfish thread IFs based on the protein sequence. Both N- (left) and C- (right) terminals are non-helical. Rectangular represents the central α -helical rod domains (from left to right: 1A, 1B, 2A, 2B) which are connected through the linker domains (from left to right: L1, L12, L2).

Native hagfish slime thread proteins have been isolated and purified^[46]. Self-assembly of the proteins was carried out by the stepwise removal of urea experiments. The resulting products were 9-11 nm wide and ~300 nm long IFs under transmission electron microscopy (TEM)^[53]. This work strongly implies that recombinant EsTKs have the potential to self-assemble into IFs under the appropriate conditions. The genes encoding hagfish slime thread proteins are less repetitive compared to that of spider silks, which makes them more stable for expression and potentially easier to produce.

2.5 The α -Helical Coiled-Coil Structures

The α -helix structure, which adopts the spring-like shape, is one of the most important and abundant secondary conformations in protein-based fibers^[54]. It is a right-handed coil and the backbone N-H group (position i) forms the hydrogen bond with the backbone C=O group of four residues ahead (position $i+4$) (Figure 2.10a). On average, the helix has 3.6 residues per turn and the distance between each turn (pitch distance) is 5.4 Å^[55]. The α -helical coiled-coils are formed by two or more α -helix chains winding together, and the resulted left-handed twist is in a more stable form (Figure 2.10b, using two chains as an illustrative example). Coiled coils contain the repeating heptad peptide modules $(abcdefg)_n$. Positions a and d are typically hydrophobic residues facing the inside of the helices and provide stabilization through hydrophobic interactions. Positions e and g are typically charged residues sitting against the hydrophobic core. They provide ionic interactions for further stabilization of the coiled-coil assemblies. Positions b , c and f are facing outside and they facilitate

solubilization^[56]. In protein engineering of coiled-coils, they provide ways to control larger-scale assemblies^[57].

Figure 2.10 Schematic illustrations of α -helix and two chain coiled coils. (a) Top view and side view of α -helix^[58]. (b) Top view and side view of coiled coils in parallel orientation relative to each other^[59].

2.6 Intermediate Filament (IF) and Its Self-Assembly

IFs are comprised of proteins which consist of long, uninterrupted segments of α -helices that usually adopt a rope-like coiled coil structure^[60]. Typical examples include type I and II keratins and type III vimentins and desmins^[61-63] from their cell cytoskeleton. Figure 2.11 shows a coiled-coil IF dimer, which can be either homo- or hetero-dimers. Each IF molecule consists of non-helical N- and C-terminals, with a central α -helical coiled coil rod domain separated by three flexible linker domains^[64] (Figure 2.11).

Figure 2.11 Schematic illustration of an IF dimer molecule^[65]. Green regions are the central α -helical coiled coil rod domains. Purple regions are the flexible linker domains. Red regions are the non-helical N- (head) and C- (tail) terminals.

One key biochemical characteristic of IFs is that they can self-assemble from the building block proteins both *in vivo* and *in vitro*^[66]. Taking type III vimentin as an example, the first step of self-assembly process involves two α -helical individual protein strands associating in parallel to form stable coiled-coil homo- or hetero-dimers, which are 40-50 nm long with diameters of *ca.* 2 nm^[67]. Tetramers are formed by two dimers with either parallel or anti-parallel alignment through cohesive forces between adjacent dimers. Next, eight tetramers associate laterally to form a unit-length filament (ULF) with approximately 60 nm long and 20 nm wide, while these ULFs anneal longitudinally and give rise to less compact filaments. Lastly, the elongated filaments radially compact to yield full IFs which are 100-200 nm long with diameters of 10 nm^[68]. Figure 2.12 illustrates the self-assembly process of recombinant vimentin *in vitro*^[69].

Figure 2.12 Schematic illustration of self-assembly pathway of type III vimentin^[69]. Two dimers align either parallel or anti-parallel to form tetramers. Eight tetramers associate laterally to form ULF, while ULFs anneal longitudinally to form less compact filaments. Lastly, the elongated filaments compact to yield full IFs.

The essential steps of *in vitro* IFs self-assembly process involve fully solubilizing proteins in buffers with 8 M urea and stepwise removal of urea through dialysis^[39], for instance from 8 M to 6 M to 4 M to 2 M and finally to 0 M. However, different structures and morphologies of IFs might be obtained in different self-assembly conditions. For example, keratins can self-assemble in low ionic strength and higher pH (2 mM Tris, pH=9), while vimentins can only self-assemble with high salt concentrations and low pH (5 mM Tris, 20 mM NaCl, pH=7.5)^[70]. Some of the self-assembly conditions are summarized in **Table 2.1**. The later self-assembly protocols for recombinant EsTKs in Chapter 4 were designed based on the above discussions and Table 2.1.

Table 2.1 Various conditions used for self-assembly of different IF protein types.

Protein type	Urea removal steps	Buffer recipe	pH	Temperature
Keratin ^[71-72]	8 M → 6 M → 4 M → 2 M → 0 M	0.7 mM sodium phosphate, 40 mM KCl, 1 mM DTT	7.5	37°C
	8 M → 6 M → 4 M → 2 M → 0 M	2 mM Tris-HCl, 1 mM DTT, finally mixed with 20 mM Tris-HCl	7.0	Room temperature
	8 M → 6 M → 4 M → 2 M → 0 M	10 mM Tris-HCl	7.3	37°C
	8M → 6M → 4M → 2M → 0M	5 mM Tris-HCl, 1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, finally mixed with 100 mM NaCl, 40 mM Tris-HCl	7.5	Room temperature
	8M → 4M → 2M → 0M	25 mM Tris-HCl, 50 mM NaCl	7.5	37°C
Vimentin, Keratin ^[71]				
Vimentin, Desmin, Keratin ^[73]				

2.7 IF-based Fibers

Hagfish slime threads are formed in the way that IFs are bundled intra-cellularly with a high degree of axial alignment^[47]. The threads are matrix-free since they only consist of tightly packed and aligned IFs^[74]. There is no reporting mechanism to date how IFs are aligned and packed in Hagfish glands. It is speculated that water molecules bring IFs close and the subsequent removal should increase the lateral adhesion of IFs which thereby facilitating threads formation.

2.8 Technical Methods Used to Fabricate Artificial Protein-Based Fibers

In Nature, plenty of protein-based fibers with tailorable properties are available, which provides a broad range of applications. Common examples include wool from cotton and silks from silkworm cocoons or spiders. Wool is easier to obtain and cheap, although it still depends on farming and other natural factors^[75]. Cocoon silks are harvested from feeding silkworms (*Bombyx mori*), which are not difficult to obtain but still relatively expensive, and thus has been largely limited to luxury goods^[76]. In the past decade, however, research on *B. Mori* silks have made interesting advances in the biomedical field or for advanced engineering applications^[77-81]. As stated above, different methods have been developed to fabricate artificial protein-based fibers and are briefly presented below.

2.8.1 Electro-Spinning

Electro-spinning involves having a polymeric solution in a syringe designed with a “spinneret” at the extremity, with the syringe at a certain distance whereas a collector is kept at the ground level. Under application of a high voltage, the electrically charged polymer solution is pulled through the spinneret to some distance such that the polymer solution surface tension is overcome. It then bends to follow a spiral path before reaching the collector. The solvent evaporates along the way and the polymer solution forms a non-woven fibrous mat on the surface of the collector^[82-84]. Figure 2.13 shows a schematic illustration of the electro-spinning principle and the resulting fibers’ diameters are usually in the nano- or micro-scale range^[85].

Figure 2.13 Schematic illustration of electro-spinning principles^[85].

One example using electro-spinning to fabricate artificial protein-based fibers is the utilization of regenerated keratins from wool or hair^[86]. Aluigi *et al.* showed that mixing aqueous keratin solutions with PEO powder would create combined keratin/PEO materials^[87-88]. When the mixing ratio was 50:50 with 100 mg/mL polymer concentration, the electro-spun fiber could be obtained. However, further analysis indicated that the self-assembled keratin IFs structure (section 2.5) was lost during the electro-spinning process and the resulted fibers had poor water instability and mechanical responses^[87-88]. On the other hand, Zoccola *et al.* showed that by adding silk fibroins under the same conditions, the fibrous materials of keratins could also be obtained^[89]. Structural analysis indicated that the resulted fibers had smaller diameters and possessed α -helix coiled-coil structure^[89]. Thus, electro-spinning exhibit an interesting potential for the fabricatation of artificial protein-based fibers. There are several parameters which can be controlled during the process, including protein solution properties (concentration, viscosity, conductivity, surface tension) and equipment setups (electric potential, flow rate, distance between the spinneret and the plate)^[90-92].

2.8.2 Wet Spinning

Wet spinning is another method used to produce artificial fibers, which is based on protein solidification process. A fiber is formed by extrusion of prepared protein dope solution into a coagulation bath through a spinneret. Subsequent drawing is able to facilitate molecule backbone alignment. This method is traditionally used in the textile industry to manufacture synthetic fibers, such as Acrylic, Rayon, Aramid, and Spandex^[93-95].

Figure 2.14 Schematic illustration of wet pinning principles^[96].

Recently wet spinning has been explored to create individual protein-based fibers. Similar to electro-spinning, artificial keratin-based fibers have also been fabricated using this method^[86]. Katoh *et al.* showed that the fibers could be formed by applying a viscous mixture of an aqueous keratin solution and PVA into the coagulation bath containing sodium sulfate, and subsequently drawn^[97]. This combination of keratin and PVA is proved to enhance the material properties including mechanical strength and waterproof characteristics. Wet spinning also exhibits a high potential for the fabrication of artificial protein-based fibers. There are several parameters which can be controlled during the process, including protein dope solution properties (concentration, viscosity) and equipment setups (flow rate, coagulation bath contents and concentrations)^[98-100].

2.8.3 ‘Picking Up’ method

This ‘picking up’ method is similar to wet spinning, involving a highly concentrated dope solution and a coagulation bath. Until now, only the recombinant IF protein vimentin and native hagfish proteins have shown the ability to form artificial fibers using this method^[101-102].

Figure 2.15 Schematic illustration of ‘picking up’ method^[101].

In a specific example, a protein dope solution where vimentin or native hagfish proteins were solubilized in formic acid with ultra-high protein concentration (~100 mg/mL) was required. Applying the dope solution onto the coagulation bath containing MgCl_2 would create the solidified protein film. The single fiber would then be formed by ‘picking up’ the protein film using tweezers. There are several parameters which can be controlled during the process, including the protein dope solution properties (concentration, applying volume), coagulation bath properties (content, concentration) and picking up velocity.

2.8.4 Microfluidics

Microfluidics encompasses techniques to manipulate liquid on the micrometer, or even sub-micrometer scale, to offer various lab-on-chip applications^[103-104]. The biggest advantage of this technique is that the flow becomes laminar rather than turbulent in the micro-channel, and this has the advantage to prevent unnecessary mixing and molecules transportation. One aspect of microfluidics engineering is to fabricate artificial fibers under elongational flow. Microfluidic devices involve one or

more inlets, with core flow of sample solution and side flows of sheath solutions. The sample molecules are stretched and aligned in the channel and come out at the end of outlet. In some instances, a coagulate bath outside the outlet is necessary to solidify the aligned sample molecules into consistent fibers^[105]. Similar to larger scale fluidics, applying a pressure difference across the two ends of channel is one of the most common ways to provide driving force and create flow^[106-107]. By this means, the flow rate inside the channels can be precisely controlled.

One example of using microfluidics to fabricate artificial protein-based fibers is that of silks. Microfluidic devices have been designed so that the artificial silk can be produced by using hydrodynamic focusing at the 3-inlet cross-section (Figure 2.16)^[27]. These conditions mimic the elongational flow observed in the cross-section of the lumen of the spinning gland, which aligns the silk fibroin in the flow direction. Other examples using similar 3-inlet design to fabricate artificial fibers include gelatin^[108], alginate/collagen^[109] and alginate/fibroin^[110]. This type of hydrodynamic focusing provided the concept of microfluidics design in this project. There are lots of parameters which can be controlled in microfluidics, such as the core protein solution properties (concentration, viscosity), side solution properties (content, concentration), devices geometries, flow rates and coagulation bath properties (content, concentration).

Figure 2.16 Schematic illustration of the 3-inlet cross-section design used in production of artificial silks^[27].

2.9 Mechanical Aspects of Biological Materials

Different biological materials have a remarkable broad range of structural-mechanical property relationships. In order to do the comparison for material

selection in engineering aspects, Wegst and Ashby summarized and organized these materials into charts and diagrams as convenient tools, which is also called ‘Ashby plots’^[111].

The knowledge of mechanical performances of biological materials is important for diverse applications. Figure 2.17 is the Ashby plot of representative soft biomaterials with strain to failure versus elastic modulus^[112]. Silk, known as the form of stiff and tough fibers, has low extensibility (10-20%). Elastin, on the other hand, is elastically stretchable ($> 100\%$) but with low elastic modulus (~ 1 MPa). Notably, as stated in section 2.3, native hagfish threads has high extensibility ($\sim 220\%$) and low initial elastic modulus (~ 6 MPa), which locate in the range of soft materials. However, after draw-processing, the threads appear to be stiffer which achieve similar properties as silks. This mechanical response tailorability of hagfish threads between soft and stiff fibers could provide a wide range of properties for applications in diverse fields. For instance, when bonding difference tissues with different mechanical responses together, this mechanical tailorable biological material would be useful^[113].

Figure 2.17 Mechanical properties of representative soft biomaterials^[112]. Strain to failure versus elastic modulus, with dotted lines representing the guidelines along which different materials show the same strain energy to failure.

2.10 Scope and Novelty of Project

Native hagfish slime threads have emerged as a promising biological material of stiff and tough protein-based fibers. The irreversible α -helical coiled-coil to aligned β -sheet transition after drawing-processing endows native threads with remarkable mechanical properties that rival silks and approach Kevlar. Most of the work in the field makes the use of native hagfish slime threads, or proteins purified from the threads directly. However, ‘farming’ hagfish to obtain native threads is not a reasonable solution for engineering applications in the future. Especially the content of native threads in the secreted slime is relatively low (Section 2.3). Therefore, recombinant protein production and artificial hagfish protein-based fibers are being explored in this project. Self-assembly of recombinant proteins into IFs is a basis to provide building blocks. The subsequent draw-processing and further chemical cross-linking are applied to enhance and tailor the properties of artificial fibers. The two aspects are identified as the key basis to biomimetic engineering of hagfish protein-based artificial fibers.

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Chapter 3

Experimental Methodologies

In order to fabricate and characterize protein-based biomaterials, a wide range of experimental approaches have been used in this project. This chapter briefly introduces these techniques including their main physical principles. These techniques include expression and purification of recombinant proteins, which provides the foundation for subsequent biomaterial fabrication, as well as the different structural characterization methods employed to characterize the self-assembled filamentous structures, together with the mechanical and biochemical characterization methods of the macroscopic fibers.

3.1 Rational for the Selection of Methods

In order to produce (rec)EsTKs-based biomaterial for biomedical and engineering applications, large scales of (rec)EsTKs are required. One way to achieve this large quantity is to use recombinant protein expression system together with downstream purification techniques. In order to make structural active filamentous structures, self-assembly experiments of (rec)EsTKs mixtures are conducted, and therefore the structural characterization methods are subsequently applied since they can provide a deep understanding of self-assembled filaments which provide the basis for later (rec)EsTKs-based biomaterial fabrication. After obtaining the protein-based materials, mechanical and biochemical characterization methods are used to study the fabricated fibers properties in order to lay the foundation for future engineering application.

3.2 Recombinant Protein Expression System

Recombinant proteins are produced by genetically engineered organisms, and involve cloning the foreign gene which encodes the target protein into an organism genome^[1]. This technology is widely used in the molecular biology field since large quantity of target proteins can be produced, enabling later studies such as molecular structural analysis, pharmaceutical compounds production, as well as biomaterials fabrication^[2]. One important aspect in recombinant protein expression system is to ensure that only target proteins are produced in large scale with a low contamination level from other non-specific proteins. This can be achieved by encoding the specific antibiotics gene into the recombinant plasmid at the same time^[3]. Therefore, in the growth environment with specific antibiotics, only the target genetically-engineered organism is able to survive and perform the desired protein production (Figure 3.1).

Figure 3.1 Schematic illustrations showing the process of protein production by recombinant expression. DNA of targeted protein is cloned into plasmid with specific antibiotics gene. The recombinant plasmid is then transformed into selected organism (here *E. coli* as an example). In the growth environment with the specific antibiotics, only genetically engineered organisms can survive and produce the target protein in large quantity.

The genes of (rec)EsTK α and γ were obtained as expression-ready inserts cloned in the pJ414 expression vectors separately from DNA 2.0 (Menlo Park, California). The plasmids were transformed into *E. coli* TOP 10 cells and stocks were prepared in 20% glycerol and stored at -80 °C accordingly. 1 μ L of TOP 10 cultures was then incubated in 1 mL LB medium (BD Bioscience) overnight and the reproduced plasmids were purified using a Qiaquick DNA purification kit (Qiagen) and stored in MilliQ water. Approximately 10 ng of each plasmid was transformed into BL21 (DE3) cells by heat shock and stocks were prepared in 20% glycerol and stored at -80 °C separately. 10 μ L of each stock was used to inoculate 4 mL of LB containing ampicillin (50 μ g/mL, Sigma) and chloramphenicol (34 μ g/mL, Sigma). Pre-cultures were incubated overnight at 37 °C with a shaking speed of 250 rpm. 2 mL from the pre-culture was then transferred into 600 mL of fresh LB containing the same ampicillin and chloramphenicol concentrations as described above. The cultures were incubated at 37 °C with a shaking speed of 250 rpm until OD₆₀₀ reached 0.6-0.8. 1 mM IPTG (Sigma) was added into the medium for protein induction with further

incubation at 37 °C and a shaking speed of 250 rpm for an additional 3 hours. Cells were harvested by centrifugation at 15,700 g for 10 minutes at 4 °C and the resulted pellets were stored at -80 °C for further purification.

3.3 Recombinant Protein Purification

After (rec)EsTk α and γ are successfully expressed, the next step is to purify them so that they can be used for further self-assembly experiment to make filaments.

3.3.1 Microfluidization

Microfluidization is a cell disruption method, which is used to lyse cells using high shear rate to extract induced proteins from organisms^[4]. This technique has a higher cell disruption efficiency with better protein recovery as compared to tradition cell lysis methods such as ultra-sonication and bead-beating homogenization^[5-7]. Concurrently, the experiment condition is mild to proteins, although tough on cells, which makes the further purification easier^[8].

Here microfluidization was applied using a Microfluidics apparatus (M-110P, Microfluidics, USA). The harvested pellets were re-suspended with lysis buffer (50 mM Tris, 200 mM NaCl, 1 mM PMSF, pH 7.4) and lysed 6 times. Cell lysates were then subjected to centrifugation at 52,000 g for 1 h at 4 °C and the resulting pellets were washed twice with 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). Inclusion bodies were collected by low speed centrifugation (5,000 g for 15 min at 4 °C) between each wash steps. The final pellets were denatured and re-solubilized in running buffer (8 M urea, 0.02 M NaH₂PO₄, 0.5 M NaCl, pH 7.8) for further purification.

3.3.2 Size Exclusion Chromatography (SEC)

SEC is used to separate biological macromolecules in solutions based on their sizes and molecular weights^[9-11]. The stationary phase inside the column is agarose,

which has been cross-linked to form gel-like matrix. There are pores of different sizes inside the matrix and thus provide various separation ranges^[12]. In the stationary phase, smaller molecules are trapped in the pores, while larger molecules simply pass through the column and elute first^[13] (Figure 3.2). In this way, the separation of molecules with different sizes is achieved.

Figure 3.2 Schematic illustrations showing the principle of SEC. Bigger molecules migrate faster in the column while smaller molecules are trapped in porous adsorbent materials and eluted later.

Here, SEC was used to further purify the proteins after micro-fluidization. The experiments were performed using an AKTA-purifier FPLC system (Fast Protein Liquid Chromatography, GE Healthcare, Life Sciences) equipped with an UV detector ($\lambda = 280$ nm) using a Superose 6 10/300 gel filtration column (GE Healthcare, Life Sciences) at a flow rate of 0.4 mL/min with the running buffer (8 M urea, 0.02 M NaH_2PO_4 , 0.5 M NaCl, pH 7.8). Each purification run involved the injection of 2 mL

protein samples from microfluidization. In each case, thirty 1 mL fractions were obtained with an in-line fraction collector module.

3.3.3 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE is used to investigate the molecular weight and purity of protein samples according to their electrophoretic mobility^[14]. After heating with reduced and denatured reagents such as Dithiothreitol (DTT) and SDS, protein strands are linearized and bind with SDS so that to distribute even charges onto protein molecules per unit mass^[15]. In this way the electrophoretic mobility of protein molecules is only relative to molecular size^[16]. Molecules with a larger molecular weight migrate slower in a gel while with smaller molecular weight move faster^[17]. Running together with a standard protein ladder, the rough molecular weight can be examined (Figure 3.3).

Figure 3.3 Schematic illustrations showing the principle of SDS-PAGE. After heating with denatured reagent (here DTT as an example) and SDS, folded protein molecules are linearized and got distribution of negative charges. The molecules are separated based on the difference of molecular weight in electric field.

Here SDS-PAGE was applied to evaluate the molecular weight and purity of proteins after SEC purification. Protein fractions were mixed with Laemmli buffer (Bio-Rad, California, USA) at 1:1 ratio and DTT (Bio-Rad), followed by boiling for 5 min. After loading into SDS-PAGE (4% stacking gel and 12% resolving gel), it was run at 10 mA and 250 V for 50 minutes. The gel was subsequently stained with Coomassie Blue 250 (CBR-250, AppliChem, Germany) to show and evaluate protein bands.

3.3.4 Matrix Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF)

MALDI-TOF is used to accurately determine the molecular weight of proteins^[18]. After mixing protein samples with a matrix, the ionization process protonates or deprotonates the matrix molecules and hence charges the analyte molecules. The charged molecules are accelerated through an electric field generator in a quadrupole mass analyzer and by measuring the flight time in the tube with a fixed distance, the molecular weight-to-charge ratio can be obtained ^[19-20] (Figure 3.4).

Figure 3.4 Schematic illustrations showing the principle of MALDI-TOF. The mixture of matrix and sample is charged by laser and accelerated by electric field generator. Molecules with different molecular weight to charge ratio have different flight time in the tube. By measuring the flight time, molecular weight is able to be examined.

Here MALDI-TOF was applied to verify the molecular weight and purity of (rec)**EsTk α** and γ after SEC for further confirmation. The measurements were done by using an AXIMA-TOF2 (Shimadzu, UK) equipped with an N₂ laser (337 nm, 4 ns pulse width). 2 μ L of the protein sample was mixed with 2 μ L sinapinic acid matrix (10 mg/mL) (Sigma) which was dissolved in a 50/50 MilliQ water/acetonitrile mixture and applied to a MALDI-TOF sample plate. A 20 kV accelerating voltage was used, and the spectra were recorded in a linear mode by averaging at least 100 laser shots at a power setting of 120 system units.

3.4 Folding Testing of Individual (rec)EsTKs Subunits and Self-Assembly Experiments of 1:1 Mixtures

Molecular self-assembly is a process during which molecules re-arrange into specific conformations by themselves without external guidance or action^[21]. There are only non-covalent forces involved in the process, such as hydrogen bonding, hydrophobic forces and π - π interactions^[22]. In this project, protein self-assembly signifies protein molecules rearrange to form secondary, tertiary and/or quaternary structures. Incorrect protein self-assembly may result in insoluble and inactive form^[23-24]. Therefore, proper protein folding is usually required to induce the process^[25-26]. After purification from SEC, (rec)EsTK subunits are in 8M urea buffer which is in denatured linearized form. Self-assembly experiments are required to achieve the proper protein conformation mimicking the native environment. In this case, filaments are formed after self-assembly experiments through a step-wise dialysis against urea concentration from 8 M to final 0 M.

Previously, purified *native* **EsTk α** and γ have been successfully self-assembled into IF-like filaments^[27], although those experiments have not been replicated^[28]. The reason might be due to the presence of post-translational modifications in native EsTKs. Therefore, recombinant EsTKs are used in this project to overcome this potential issue. Moreover, the self-assembly pathways of other similar IF proteins such as keratins and vimentins also provide some clues, which can be used as an additional foundation for (rec)EsTKs self-assembly^[29-31]. Folding testing of individual (rec)EsTKs subunits are conducted first to obtain the solubility window for the

following self-assembly experiments of 1:1 mixtures. A panel of buffers including different pH and temperature was then developed.

Lyophilized purified (rec)**EsTk α** and γ were dissolved in the 1 mM desired buffers with 8 M urea at specific pHs and temperatures to achieve 0.2 mg/mL concentration. The mixture of 1:1 (w/w) (rec)**EsTk α** and γ together with individual EsTKs subunits were then subjected to step-wise dialysis against 4 M, 2 M and finally 0 M urea with the same buffer and pH conditions, where each dialysis step was carried for 24 h. This generalized protocol was used to conduct a panel of experiments with a set of buffers including 2 mM Sodium Citrate, 2 mM PBS, 2 mM Tris and 2 mM MES. A range of pH values (pH 4, 5, 6, 7, 8.4 and 9) and temperatures (4, 22 and 37 °C) were tested in order to identify favorable conditions for filaments formation. In each case, the final dialysis products were evaluated visually to gauge the presence or absence of a pellet following centrifugation at 13,000 g.

3.5 Structural Characterization of Folded Individuals and Self-Assembled Mixtures

In order to analyze the folding of individual (rec)EsTKs subunits and self-assembly of 1:1 mixtures under different conditions, subsequent structural characterization techniques were applied. CD measurements were conducted to determine the protein secondary structure of folded individuals and self-assembled mixtures. Both standard (with stained samples) and cryo-TEM were used to reveal the details of folded individuals and self-assembled mixtures. Rheology was used to study the micro-mechanical response of filamentous structural networks. Combining of all these investigations will provide a deep understanding of (rec)EsTKs-based self-assembled filamentous structure network, which could lay the foundation of subsequent material fabrication.

3.5.1 Circular Dichroism (CD)

CD is used to study the unequal absorption of left-handed and right-handed light when the light passes through optical active chiral molecules^[32-33]. When the

chromophores of the amides of the polypeptide backbone of proteins (absorption below 240 nm, far UV region) are aligned in arrays, their optical transitions are shifted or split into multiple transitions as a result of ‘exciton’ interactions^[34]. In more details, there is a weak but broad $n \rightarrow \pi^*$ transition centered around 220 nm and a more intense $\pi \rightarrow \pi^*$ transition around 190 nm^[35]. Therefore, different protein secondary structures have specific CD spectra, and CD measurements are often used to study biological molecules in solution^[36]. Specifically, proteins with α -helices show two minima at 208 nm and 222 nm with similar intensity and one maximum at 194 nm. Proteins with β -sheet structure show one broad minimum at 216 nm and one maximum at 196 nm. Proteins with β -turn structures exhibit two minima at 225 nm and 190 nm and one maximum at 208 nm. Random coils only give one minimum at 198 nm (Figure 3.5).

Figure 3.5 Representative CD spectra of various protein secondary structures^[36]. Solid line: α -helix; Long dashed line: β -sheet; Dotted line, β -turn; Cross dashed line, extended 3_1 -helix or poly (Pro) II helix; Short dashed line: random coils.

The secondary structures of self-assembled mixtures together with individual subunits were characterized by CD using a Chirascan spectropolarimeter (Model 420, AVIV Biomedical Inc.). The concentrated proteins were diluted to 1 mg/mL prior to each set of CD measurements. Corresponding dialysis buffers were used as background in each case and were subtracted from the final spectra. Measurements were obtained as the average of triplicate across wavelengths ranging from 190 to 260 nm, using a 1 nm step size and 1 nm bandwidth. Spectra were smoothed using the Savitzky-Golay method with a second order polynomial.

3.5.2 Transmission Electron Microscopy (TEM)

TEM is used to image small objects at higher resolution compared with optical microscopy^[37]. Based on Abbe's equation, the resolution of microscopy is directly proportional to the wavelength (λ) of incident source^[38]. Using highly-accelerated electron beam ($\lambda = 0.3$ nm) as the incident source, fine structural details down to nano-meter scale resolution can be obtained under TEM. The incident electron beam, which is focused by electromagnetic lens, interacts with the atoms of an ultra-thin specimen, and parts of the electron beam are transmitted. This transmitted portion of electrons are focused and magnified before projected on a fluorescent screen and finally imaged^[39]. Image contrast comes from the amount of transmitted electrons. Darker areas represent the sample areas where fewer electrons are transmitted, while the lighter areas represent the sample areas where more electrons are transmitted^[39]. For self-assembled mixtures, the nano-scale details can be examined in this way.

3.5.2.1 Heavy Metal Stained TEM

Heavy metal stains are used to stain protein molecules for better contrast since biological samples have lower electron-scattering power^[40-41]. Negative staining and positive staining are the two common methods for contrasting the specimen. Classical examples of negative staining include ammonium molybdate, uranyl acetate (UA) and phosphotungstic acid (PTA). The background is stained with an electron dense heavy metal ions, and the actual specimen is left bright and visible^[42-43]. On contrary, positive staining involves binding of heavy metal atoms to the specimens whose

contrast is thus enhanced compared with the bright background. Classical examples include cadmium iodide and osmium tetroxide^[44]. After staining, the specimen must be dehydrated before imaging in TEM under ultra-high vacuum conditions. In this study, UA was chosen as a negative stain.

Aliquots of 4 μL of folded individuals and self-assembled mixtures were applied to glow-discharged, carbon-coated grids (Ted Pella), adsorbed for 30 seconds, and then fixed with 2% glutaraldehyde (Grade I, Sigma). Excess liquid was removed using filter paper, and the samples were stained with 1% UA (Sigma) subsequently. TEM examination was carried out with an Energy filtered Carl Zeiss LIBRA 120 Plus microscope equipped with an in-column Omega spectrometer. Images were obtained at a magnification of $25,000\times$ and analyzed using ImageJ (NIH).

3.5.2.2 Cryo-TEM

Since the binding of heavy metal atoms with protein molecules and the following dehydration may affect the native structures, cryo-TEM is utilized to study specimens at cryogenic temperatures (lower than $-150\text{ }^{\circ}\text{C}$) to avoid any irreversible conformational change during the staining and dehydration process^[45-46]. In standard TEM, the specimen must be prepared in a dehydrated form since the imaging is under ultra-high vacuum conditions. In cryo-TEM, on the other hand, the specimen is frozen to maintain the hydration status. In short, after applying liquid samples onto the TEM grid, the grid is blotted and vitrified in liquid ethane in a short time (within 1 second) so that water does not have enough time to form crystalline ice. In this way, the specimen is immobilized under vitreous ice and the native structures can be maintained. Cryo-imaging occurs at $-172\text{ }^{\circ}\text{C}$ which could prevent water crystallization and sublimation^[47-48]. As opposed to standard TEM where high electron doses are used to improve the contrast, in cryo-TEM specimens must be imaged with low electron doses to prevent radiation damage, which results in very poor contrast^[49].

Aliquots of 3.5 μL of self-assembled mixtures were applied on to glow-discharged, holey-carbon grids (Ted Pella), adsorbed for 30 seconds, and then double-side blotted for 5 seconds using a Gatan Cryoplunge 3 instrument. The same step was

repeated for same grid in each case. The specimens were vitrified in liquid ethane using the Gatan Cryoplunge 3 and loaded into a Gatan 626 cryo-holder. TEM examination was carried out with an Energy filtered Carl Zeiss LIBRA 120 Plus microscope equipped with an in-column Omega spectrometer. Images were obtained at a magnification of $16,000\times$ and analyzed using ImageJ (NIH). In cryo TEM experiments, sample temperatures were maintained at approximately $-172\text{ }^{\circ}\text{C}$.

3.5.3 Rheology

In the biomaterial science field, rheology is often used to study the micro-mechanical response and dynamic viscoelasticity of biological macromolecules such as proteins^[50]. Using oscillatory rheology by applying dynamic shearing force, the applied stress (σ) is varied sinusoidally with time (t), and the response strain (ε) from the viscoelastic material will also exhibit as sinusoidal curves. The lag phase (δ) comes from the necessary time for molecular motions and relaxations to occur (Figure 3.6). The applied stress is expressed as $\sigma = \sigma_0 e^{i(\omega t + \delta)}$, where i is the imaginary unit and ω is the angular frequency. Similarly, the response strain from the viscoelastic material is expressed as $\varepsilon = \varepsilon_0 e^{i\omega t}$. Hence, the shear modulus from dynamic mechanical test can be deduced based on the definition as $G^* = \frac{\sigma}{\varepsilon} = \frac{\sigma_0 e^{i(\omega t + \delta)}}{\varepsilon_0 e^{i\omega t}} = \frac{\sigma_0}{\varepsilon_0} e^{i\delta} = \frac{\sigma_0}{\varepsilon_0} (\cos\delta + i\sin\delta) = G' + iG''$. Here, G' is the storage modulus, which is associated with elastic energy storage properties and is expressed as $\frac{\sigma_0}{\varepsilon_0} \cos\delta$. G'' is the loss modulus, which is related to viscous energy dissipation and is expressed as $\frac{\sigma_0}{\varepsilon_0} \sin\delta$. Taken together, the storage and loss moduli (G' and G'') provide the molecular structure information^[51]. There are mainly two types of measurements conducted in oscillation testing, namely strain-dependent testing and frequency-dependent testing. In strain-dependent testing, G' is measured as a function of the applied strain (γ) with a constant oscillation frequency (f). The critical strain level at which G' becomes independent of the strain can be measured. When the linear response regime of material is disrupted beyond this critical strain, G' starts to decrease. The other type of measurement commonly done in oscillatory rheology is frequency-dependent tests. Both G' and G'' are measured as a function of the applied oscillation frequency (f) at constant strain (γ), usually below the critical strain defined

above. When G' is higher than G'' where $\delta < \frac{\pi}{2}$, the material exhibit gel-like behavior. With G' is lower than G'' where $\frac{\pi}{2} < \delta < \pi$, the material exhibit liquid-like behavior^[51].

Figure 3.6 Stress and strain versus time (t) in dynamic loading of a viscoelastic material. σ represents the applied stress, ϵ represents the response strain, and δ represents the lag phase.

There are several types of rheometers that mostly differ from each other by the type of geometry used to apply the external shearing force. These include concentric cylinders (used for materials with very low viscosity), cone and plate (used for materials with low to medium viscosity), parallel plates (used for materials with high viscosity to soft solids) and rectangular torsion (used for solid materials)^[52]. Different geometries are selected based on the samples' characteristics. In this project, cone-plate geometry was chosen since the protein sample is in liquid form with a relatively low viscosity (Figure 3.7).

Figure 3.7 Schematic illustration showing the cone-plate geometry rheometer. Liquid sample is applied between the cone and the plate. By turning the cone and keeping the plate static, the mechanical response of protein samples can be measured.

Here rheology was used to characterize the visco-elastic properties of individual subunits and self-assembled mixtures. The experiments were conducted using a Physica MCR 501 (Anton Paar with TruGap™ system) with a cone-plate geometry (diameter 25 mm, cone angle 1°). A solvent trap was used to minimize buffer evaporation. The strain dependence of G' was measured at $\gamma = 0.1$ -100% with $f = 1$ Hz in order to determine the critical strain point. The frequency dependence of G' and G'' were measured at $f = 0.1$ -100 Hz at critical strain $\gamma = 1\%$. Time of recovery for G' experiments were conducted using $\gamma = 1\%$ and $f = 1.6$ Hz.

3.5.4 Divalent Cation Effects

Divalent ions such as Mg^{2+} and Ca^{2+} have been demonstrated to cross-link IFs into stiffened filamentous networks^[53-54]. The cross-linking is believed to take effect through Coulomb interactions between divalent ions and the charged amino acids of the C-terminal^[54].

To test the effect of divalent cation on the (rec)EsTKs-filaments, 1:1 (w/w) mixtures of (rec)EsTK α and γ were dialyzed in a stepwise manner (8 M $\rightarrow$ 4 M $\rightarrow$ 2 M $\rightarrow$ 0 M urea) with 5 mM Tris and 1 mM DTT, pH 8.4 at 4 °C. Following dialysis, the final buffer was exchanged over the course of 2 hours with an equal volume of “cross-linking buffer” (20 mM Tris, 1-3 mM $MgCl_2$ and 1 mM DTT, pH 7.0) at 25 °C. The samples were subsequently concentrated to 1 mg/mL using Vivaspin 2 (100 kDa molecular weight cut off, GE Healthcare) and analyzed by rheometry.

3.6 Mechanical and Biochemical Characterization of Fabricated (rec)EsTK-Based Materials

In order to characterize the biophysical properties of fabricated (rec)EsTKs-based materials, different techniques were used. Nano tensile testing was conducted to measure the mechanical properties of the fibers in tension. Raman and WAXS were employed to reveal the structural transitions, as well as the backbone molecule alignments of the fiber after draw-processing. FESEM was used to obtain the materials' morphology under different conditions. AAA was applied to verify the

chemical cross-linking degree in the materials. All the techniques will help to understand more about the materials and lay the foundation of further engineering applications in various fields.

3.6.1 Nano Tensile Testing

Nano tensile testing is used to characterize the mechanical properties of micro-/nano-materials of small dimensions, which cannot be measured with standard universal mechanical testing equipments^[55-56]. A nano tensile testing equipment is similar to a standard mechanical testing machine, but is equipped with much more sensitive force transducer capable of measuring tensile forces in the micro-Newton range^[57]. In this project, (rec)EsTK-based fibers will be measured to obtain the mechanical response of fibers with micron-scale diameters. In a typical test, the fibers will be pulled vertically at a specified low rate and the resulting force is measured together with the displacement^[58]. After including the specimen dimension parameters (fiber length and diameter), stress-strain curves can be obtained, from which the classical mechanical properties can be extracted, including the elastic modulus (E), the ultimate tensile strength (UTS), and failure strain^[59-60] (Figure 3.8).

Figure 3.8 A typical engineering stress-strain curve from tensile testing. Materials mechanical properties can be calculated, including the Young's modulus (E), the ultimate tensile strength (UTS), and the failure strain.

Tensile testing was performed by using a nano tensile testing system (Nano Bionix® System, MTS, USA). The individual fiber was glued onto a small cardboard frame using double-side tape to secure the fiber for tensile testing (Figure 3.9a). The cardboard frame with individual fiber was mounted on the tester grips (Figure 3.9b). The gauge length of individual fibers ranges from 15 mm to 25 mm. Fiber diameters were measured at 5 different locations on each fiber and the average value was used in the calculation of stress. The side of the cardboard frame was cut open before the uniaxial tensile loads at a strain rate of 10^{-3} s^{-1} until fiber failure.

Figure 3.9 Photographs of (a) individual fiber fixed on the cardboard frame with gauge length 20 mm and average width $80 \mu\text{m}$; and (b) cardboard frame with individual fiber mounted on the tester grips and side cut open right before testing.

3.6.2 Raman Spectroscopy

Raman spectroscopy is a technique used to provide structural information of molecules through the shift in wavelength of the inelastic scattered radiation^[61]. Briefly, a laser source emits photons at a desired frequency, which is absorbed by the specimen and excites its electrons into the virtual energy states. After the electrons return back to the original ground state, photons are re-emitted. Molecules with non-Raman-active effect re-emit photons with the same frequency, which is the type of elastic scattering called Rayleigh scattering^[62]. Molecules with Raman-active effect

re-emit photons with a higher or lower frequency, a type of inelastic scattering called anti-Stoke or Stoke Raman scattering respectively^[62] (Figure 3.10).

Functional groups exhibit similar vibrational signatures. Proteins with the same secondary structures have similar inter and intramolecular characteristics, such as peptide bond angle and distance, and hence similar vibrational frequencies^[63]. In this way, different protein secondary structures can be differentiated by inspecting the frequencies at which amide bonds absorb the radiation. In Raman spectroscopy, the amide I ($1600\text{--}1700\text{ cm}^{-1}$, resulting from C=O stretching) and III ($1200\text{--}1340\text{ cm}^{-1}$, resulting from C-N stretching and N-H bending) bands are mainly used to assign protein secondary structures^[63]. In particular, β -sheets exhibit a shift peaks at $\sim 1670\text{ cm}^{-1}$ in the amide I region and at $\sim 1240\text{ cm}^{-1}$ in amide III region. α -helices exhibits shift peaks at $\sim 1656\text{ cm}^{-1}$ in amide I region and at $\sim 1270\text{ cm}^{-1}$ in amide III region^[63-65]. In this project, Raman microscopy was used to study the structure of the artificial (rec)EsTK-based fibers and the backbone molecules alignment.

Figure 3.10 Schematic illustrations showing the different types of scattering. (a) Elastic Rayleigh scattering. Electrons in molecules with non-Raman-active effect are excited from the ground state to virtual state and finally returned to the original ground state. **(b)** Anti-Stoke scattering. Electrons in molecules with Raman-active effect are excited from the ground state to virtual state and finally returned to a higher ground state. **(c)** Stoke scattering. Electrons in molecules with Raman-active effect are excited from a higher ground state to virtual state and finally returned to a lower ground state.

Raman spectra were collected by focusing a continuous laser beam ($\lambda = 532$ nm) through a 20 $\times$ Nikon objective (NA = 0.4) onto the sample through a confocal Raman microscope (alpha300, WITec) equipped with a piezo-scanner (P-500, Physik Instrumente). The spectra were acquired using a CCD (DV401-BV, Andor) behind a 600 g/cm grating spectrograph (UHTS 300, WITec) with a spectral resolution of ~ 3 cm^{-1} . Data was collected with ScanCtrlSpectroscopyPlus software (version 1.38, WITec) and analyzed with OPUS software (version 7.0, Bruker). Fiber samples were mounted in a custom-built hand-driven tensile tester in the hydrated state. Spectra were collected with an integration time of 2 s and 15 accumulations from different points along two different fibers in both the undrawn (0% strain) and drawn (100% strain) states. Measurements were made in both strain states with the polarizer oriented parallel (Z-polarization) and perpendicular (X-polarization) to the fiber axis and six spectra from each combination of polarization and strain state were baseline corrected, averaged and normalized to the intensity of the Phe peak at 1004 cm^{-1} .

3.6.3 Wide-Angle X-ray Scattering (WAXS)

WAXS is the technique used to study the crystalline structures of polymers^[66]. Briefly, crystalline structures contain arrays of atoms with inter planar distance d . A collimated monochromatic X-ray beam with wavelength (λ) comparable to atomic spacing interacts with the specimen's atoms and hence get elastically scattered. The scattered X-rays by two adjacent inter planes add constructively (constructive interference) and remain in phase. The difference of path lengths between the two waves is equal to an integer (n) multiple of the wavelength (λ), which is also given by $2d\sin\theta$ where θ is the scattering angle^[67]. On the basis of the Bragg's law which is expressed as $n\lambda = 2d\sin\theta$, the intensity of the scattered X-rays is measured as a function of the scattered angle^[68] (Figure 3.11).

The angular dependence of intensity can be measured using a two-dimensional X-ray detector scanned through various angles. In the biomaterial field, WAXS can be used to investigate secondary structures of protein-based materials. Particularly, different protein secondary structures exhibit different two-dimensional scattering signatures. A typical α -helical pattern shows a meridional reflection at 5.2 Å

(corresponding to the distance between each turn, also called α -helical pitch) and an equatorial reflection at 9.6 Å (corresponding to the diameter of the α -helix), while a typical β -sheet pattern shows strong equatorial reflections at 9.7 Å (corresponding to the distance between each β -crystallite plane) and 4.7 Å (corresponding to the distance between each β -strand)^[69] (Figure 3.12).

A synchrotron is a type of cyclic particle accelerator^[70]. Briefly, electrons are produced by the gun in the center and accelerated to almost the speed of light by the linear accelerator. The accelerated electrons are then transferred to the booster ring to increase energy, followed by transferred to the outer storage ring. These electrons move in a circular way around the storage ring with a series of magnets. When the electrons are deflected under the magnetic field, they generate electromagnetic radiation, and hence a beam of synchrotron X-ray is produced. This beamline is used for specimen characterization. The synchrotron X-ray has much higher intensity compared with the lab source X-ray. Therefore, high signal-to-noise ratio can be achieved for better resolution^[71]. In this project, synchrotron WAXS is used to characterize the structural transition of the artificial (rec)EsTK-based fibers, both drawn and non-drawn.

Figure 3.11 Schematic illustration showing the principle of incident X-ray scattering from crystal planes on the basis of Bragg's law. Red dots represent the specimen's scattering centers, d is the distance between each plane, θ is the scattering angle, and λ is the wavelength of the incident X-ray beam.

Figure 3.12 Schematic illustrations showing the principle of WAXS^[69]. The intensity of the scattered X-ray is measured as a function of scattered angle θ on the basis of Bragg's law. **(a)** Typical α -helical pattern consist of meridional reflections at 5.2 Å and of equatorial reflections at 9.6 Å. **(b)** Typical β -sheet pattern consists of equatorial reflections at 9.6 Å and 4.7 Å for β -strands oriented parallel to the fiber axis.

WAXS measurements were carried out at the microfocus beamline at the BESSY II synchrotron radiation facility (Helmholtz Zentrum, Berlin, Germany). WAXS patterns were recorded with a 2D CCD (charge coupled device) detector (MarMosaic 225, Rayonix) with a total area of $3,072 \times 3,072$ pixels and a pixel size of $73 \mu\text{m}$. $2 \times$ software binning resulted in final scattering patterns with $1,536 \times 1,536$ pixels. The energy of the monochromatic incident X-ray beam was 15 keV. The diameter of the beam at the sample position was $\sim 50 \mu\text{m}$ and the sample-to-detector distance was 376 mm. The measurement time for each scattering pattern was 60 seconds. The fibers were glued onto foliar frames and mounted vertically and perpendicular to the incident X-ray beam. The obtained scattering patterns were calibrated (sample-to-detector distance, beam center) and analyzed with the DPDAK software (DESY/MPiKG, v. 1.2.0). All diffraction patterns were background subtracted by acquiring the scattering background prior to the measurements.

3.6.4 Field Emission Scanning Electron Microscopy (FESEM)

FESEM is used to image samples' topography and analyze their chemical composition^[72]. In FESEM, an electrostatic field is used to extract electrons from metal filaments with higher brightness. Electron beam is then accelerated by an electrical anode, which is further confined and focused by electromagnetic lenses^[73]. The focused electron beam interacts with specimen and the detector collects the

scattered electrons emitted from the sample surface^[74]. Different interactions lead to different electron signals. To image the sample topography, inelastically scattered secondary electrons with low energy emitted from 1-50 nm zone on the sample surface are collected and analyzed^[75]. To characterize the sample chemical composition, elastically backscattered electrons emitted from 50-300 nm zone on the sample surface are detected^[76]. In this project imaging the topography of artificial fibers is required and hence secondary electron detector is used (Figure 3.13).

Figure 3.13 Schematic illustration showing different electrons emitted from the sample surface when interacting with incident electron beam^[77]. Here inelastically scattered secondary electrons from the sample surface (1-50 nm) are used in FESEM to image specimens' topography.

Here the FESEM (JEOL 7600F) equipped with an acceleration voltage of 30 keV was applied to study the fibers' topography. To make the specimen electrically conductive while minimizing surface charging, a very thin layer (8-10 nm) of platinum was sputter coated (JFC-1600, JEOL, USA) on the sample surface prior to imaging. For further reduction of surface charging, a low acceleration voltage of 5 kV was used.

3.6.5 Chemical Cross-Linking

Glutaraldehyde is commonly used to cross-link amino acids with free amines such as lysine^[78], through a nucleophilic addition then elimination process which involves the formation of a covalent linkage at the amines leading to imine formation^[79]. As a result, two proteins are irreversibly cross-linked (Figure 3.14). There is around 4 mol% lysine in (rec)EsTKs, which are hence targeted as potential cross-linking sites for final stabilization of the drawn fibers.

Figure 3.14 Schematic illustration showing the interaction between glutaraldehyde and free amines in the protein. The proteins are cross-linked through nucleophilic addition then elimination process and form covalent bonds.

Cross-linking was conducted either before, or after draw-processing using 4% glutaraldehyde (Sigma, Grade I). The process involved immersing fibers into 4% glutaraldehyde for 2 hours, followed by washing with MilliQ water 3 times. Tensile mechanics described above were tested after drying overnight.

3.6.6 Amino Acids Analysis (AAA)

AAA is used to quantitatively obtain the amino acid composition of proteins, by using ion exchange liquid chromatography of a protein hydrolysate^[80]. The protein samples are first hydrolyzed into free amino acids mixtures, following which are injected in an AAA in the liquid state. After eluting from a cation exchange column using a pH gradient, the free amino acids are mixed at high temperature with ninhydrin reagent, resulting in Ruhemann's purple (except proline) which absorbs in the visible range at 582 nm. The absorption intensity is measured which is directly

proportional to the quantity of amino acids present in the protein sample^[81-82] (Figure 3.15). Side-chain cross-link products can also be detected since they also have free amines following hydrolysis that can react with ninhydrin. External standards are required in order to determine the elution time of putative cross-links.

Figure 3.15 Ninhydrin-based AAA working principle^[83]. Amino acids hydrolysates are separated in an ion exchange column first before reaction with ninhydrin. The amount of light absorption by the reacted products is measured and the quantity of amino acids in the sample can be determined.

The fabricated (rec)EsTK-based fibers were cross-linked by glutaraldehyde through free amines of lysine. The cross-linked fibers were hydrolyzed in 200 μL of 6 M HCl with 10 μL phenol (Sigma) at around 110 $^{\circ}\text{C}$ for 24 hours. The hydrolysis product was washed using a ScanVan (Labogen) vacuum concentration with 100 μL MilliQ water 3 times, followed by a 100 μL ethanol final wash. Hydrolysates were dissolved in 300 μL sample dilution buffer (Sykam GmbH) and spun at 13,000 g for 5 minutes to remove any insoluble product before being injected to the AAA. Non cross-linked fibers were prepared in the same way and acted as controls. In order to determine the elution time of cross-linked lysine, pure lysine amino acid was cross-linked by glutaraldehyde under the same conditions. The cross-linked lysine was freeze dried and re-dissolved in the sample dilution buffer, and then mixed with an amino acid standard solution. The AAA chromatogram of the mixture was obtained and used to identify the elution time of the cross-linked lysine product.

3.7 Microfluidics

In order to produce protein-based fibers, microfluidic devices are designed such as to create elongational flow regimes at the micro-scale^[84]. In elongational flow, polymers can be stretched under a velocity gradient along the direction of flow due to the mechanical stress produced by the solution's drag^[85]. This elongational flow can be achieved in several ways. One is by constraining the channels physically and hence creating the mechanical pulling force^[86]. Another involves creating shearing force by introducing another flow with different flow rates^[87]. Both of the designs are discussed in Chapter 6 in more details. Polymer chains alignment can also be achieved along the flow direction. The goal in this project is to enable efficient stretching and alignment of self-assembled (rec)EsTK-based filaments. Subsequently coagulation baths are then used to help solidify and bundle the filaments into macroscopic fibers.

3.7.1 PDMS Device Fabrication

Microfluidics refers to the techniques used to control and manipulate fluids in microscale channels, which offers lab-on-chip applications^[88]. One advantage of this technique is that the flow becomes laminar rather than turbulent, so that to avoid unnecessary mixing and molecule transportation^[89]. Using pressure as a driving force, the majority of nano-scale filaments can be stretched, elongated, and aligned parallel to the direction of fluid flow^[90] (Figure 3.16). The system can be controlled by flow rate, channel width and height^[91]. Microfluidics therefore provides an elegant method for the alignment and bundling of (rec)EsTK-based filaments.

Figure 3.16 Schematic illustration showing the stretching and alignment of polymers under elongational flow.

A silicon wafer was designed with Dr. Andrea Pavesi from Singapore-MIT Alliance for Research and Technology (SMART). Microfluidic structures were cast from the mold with PDMS elastomer (Dow Corning). After curing at 80°C, the polymer was removed from the mold. After cooling down to room temperature, each piece with channels in the middle was cut with a razor blade. The resulting polymer pieces and glass coverslips were then treated with oxygen plasma, forming hydrophilic micro-channels and glass surface in order to bond each other and generate closed channels. More details about the various designs for testing are discussed in Chapter 6.

3.7.2 Fiber Formation Using Microfluidic Devices

100 μ L Hamilton syringes containing different concentrations of (rec)EsTK-based IFs aqueous solution were connected to syringe pump (11 Pico plus Elite, Harvard Apparatus, USA) and connected to fabricated PDMS devices by Teflon tubes (inner diameter 1 mm). In order to solidify the protein solution coming out of the device channels, methanol (Sigma) was used in coagulation bath at the end of outlet. The products inside the coagulation bath were applied onto glass slides and vacuumed dried overnight before characterization by using FESEM and polarized optical microscopy. More details about the various testing conditions are discussed in Chapter 6.

3.8 Overview for Methodology

In this chapter, different techniques used in this project were introduced. These techniques include how to express and purify recombinant proteins which will be used as the foundation of later biomaterial fabrication, as well as different structural characterization methods for self-assembled filamentous structures, together with mechanical and biochemical characterization methods for fabricated protein-based fibers.

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Chapter 4*

Self-Assembly of Recombinant Hagfish Thread Keratins Amenable to α -Helix to β -Sheet Transition

*Native hagfish threads are assembled from protein-based bundles of intermediate filaments (IFs) which undergo α -helical coiled-coil to β -sheet transition ($\alpha \rightarrow \beta$ transition) upon draw-processing. In this chapter, several aspects which lead to biomimetic engineering of recombinant hagfish protein based materials have been established. Two proteins found in hagfish *Eptatretus stoutii* thread, **EsTK α** and **EsTK γ** , were expressed in *E. coli* system and purified. Self-assembly of EsTK-based filaments was obtained using a step-wise urea removal process, leading to the formation of (rec)EsTK-based IF-like filamentous network. A comprehensive characterization of self-assembled products was carried out including circular dichroism (CD), transmission electron microscopy (TEM) and rheology. Notably, (rec)EsTK-based filamentous network underwent an $\alpha \rightarrow \beta$ transition under oscillatory shearing, which mimics the native hagfish threads mechanical strengthening process.*

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4.1 Introduction

As stated in Chapter 2, Hagfish slime threads are built from α -helical coiled-coil proteins self-assembled into intermediate filaments (IFs) and these IFs are further bundled with high degree alignment into macroscopic threads^[1-4]. Mechanical properties of the threads can be enhanced through draw-processing, owing to conformational transition from predominant α -helix into rich aligned β -sheets (**$\alpha \rightarrow \beta$ transition**)^[5]. The genes encoding the two hagfish threads proteins, namely *Eptatretus stoutii* Thread Keratin α and γ (**EsTK α** and **EsTK γ**), have been cloned and sequenced, with molecular weights of 66.7 and 62.8 kDa, respectively, and isoelectric point of 7.5 and 5.3, respectively^[2-3]. Although both of the protein subunits exhibit low sequence homology to most vertebrate keratins, recently they have been reported to maintain a high primary sequence homology with Thread Keratins (TKs) from the lamprey, teleosts and amphibians^[6]. Phylogenetic analysis of the TK-genes intron-exon organizations have shown that **EsTK α** is related to vertebrate Type I keratins while **EsTK γ** is related to vertebrate Type II keratins. Therefore, studying the two protein subunits may also build an important link in the evolution of vertebrate keratins from Type III IF-proteins^[6].

Native hagfish slime threads have been solubilized in 98% formic acid, which denatured the proteins structures. Attempts to form fibers from the protein solution yielded fibers with lower mechanical properties (failure stress 150 MPa) compared to native threads (failure stress 800 MPa)^[7]. This study indicates that accurate assembly into coiled-coil filaments, together with bundling and alignment of the filaments, play an important role for the fabrication of tough fibers.

In order to fabricate EsTK-based biomaterials, large quantities of EsTKs are required, which can be achieved through recombinant protein production and downstream purification. Next, biomimetics of native hagfish threads forming process requires the suitable circumstance where recombinant EsTK α and γ ((rec)**EsTK α** and (rec)**EsTK γ**) can be self-assembled into hetro-dimeric coiled-coil filamentous structures. Fulfilling these two conditions will ensure that EsTK-based filaments can be used as building blocks to fabricate macroscopic scale materials such as films, gels,

tissue scaffolds or fibers. In addition, the final mechanical performance may be tailored through $\alpha \rightarrow \beta$ transition upon draw-processing.

4.2 Experimental Methods

(rec)EsTK α and γ were expressed and purified. Folding of individual (rec)EsTKs subunits and self-assembly of the mixtures were conducted using different conditions and the resulted products were evaluated by CD, TEM as well as rheology. The role of divalent ions on the rheological response of (rec)EsTK-based filamentous networks was also explored.

4.2.1 Expression and Purification of (rec)EsTKs

In order to obtain a sufficient amount of purified (rec)EsTK α and γ for subsequent folding and self-assembly experiments, both proteins were expressed in *E. coli* system respectively. Cell lysis was carried out through 6 rounds of microfluidization with two buffer washing steps. Further purification was conducted by using SEC/FPLC. Protein molecular weight and purity was checked by SDS-PAGE and MALDI-TOF. For more details, please see to section 3.2 and 3.3.

4.2.2 Folding Testing of Individual (rec)EsTKs Subunits and Self-Assembly Experiments of 1:1 Mixtures

After purification, both (rec)EsTK α and γ were in the denatured form. In order to achieve the proper protein structure mimicking the native environment, folding and self-assembly experiments were carried out by a step-wise dialysis against urea at decreasing concentrations, from 8 M to 0 M. A panel of conditions with different buffers was tested, including various pH and temperature, to determine the conditions which favored the solubility of individual (rec)EsTKs subunits, and hence filaments formation. Mixtures of 1:1 (w/w) (rec)EsTK α and γ were used in self-assembly experiments while individual (rec)EsTKs subunits were used as controls to study the solubility and folding behaviour. For more details, please refer to section 3.4.

4.2.3 Structural Characterization of Folded Individuals and Self-Assembled Mixtures

In order to verify the folding of individual (rec)EsTKs subunits and self-assembled mixtures under different conditions, various structural characterization techniques were used to analyze and quantify the self-assembled products as well as individual controls.

4.2.3.1 CD

CD was used to evaluate the protein secondary structures for self-assembled products (1:1 mixture of (rec)EsTK α and γ) as well as for individual (rec)EsTKs subunits that were subjected to the same protocols. The concentrated proteins were diluted to 1 mg/mL prior to each set of CD measurements. Thermal stability analysis was also conducted by measuring the CD spectra from 10 °C to 90 °C in 5 °C steps. Protein samples were allowed to equilibrate for 5 min at each temperature. After melting, protein samples were cooled slowly to 10 °C over 30 min before measuring CD signal again. Further experimental details are provided in section 3.5.1.

4.2.3.2 TEM

TEM was used to reveal the structures of self-assembled filaments as well as folded individual (rec)EsTKs individuals. Each sample was applied to glow-discharged, carbon-coated grids (Ted Pella), fixed with 2% glutaraldehyde (Grade I, Sigma), and followed by staining with 1% UA (Sigma). In order to verify if there was any conformational change due to dehydration and staining, cryo-TEM was conducted. Self-assembled mixture was applied to glow-discharged, holey-carbon grids (Ted Pella), double-side blotted, and followed by vitrification in liquid ethane. For more details, please refer to section 3.5.2.

4.2.3.3 Rheology

Rheology was used to study the visco-elastic properties of self-assembled (rec)EsTK-based filamentous networks as well as folded individual proteins. Each sample was applied on a cone-plate geometry. The strain dependence of storage modulus (G') was measured at strain (γ) in the range 0.1-100% with oscillation frequency (f) 1 Hz in order to determine the critical strain point. The frequency dependence of storage and loss moduli (G' and G'') were measured at f in the range 0.1-100 Hz at critical γ 1%. Time of recovery for G' experiments were conducted using $\gamma = 1\%$ and $f = 1.6$ Hz. More details are provided in section 3.5.3.

4.2.3.4 Role of Divalent Cations

In order to test the role of divalent cations on the (rec)EsTK-based filamentous networks, a buffer containing different concentrations of $MgCl_2$ was used in the final step of self-assembly experiments. The resulted self-assembled product was analysed by rheology as described above. For more details, please refer to section 3.5.4.

4.2.4 Data Analysis

SDS-PAGE and TEM images were analyzed using ImageJ (NIH). Data plotting were done by using OriginPro 8.5. A total number (n) of 100 diameter along the filaments in stained TEM and a total number of 10 diameters of filaments in cryo-TEM were obtained to calculate the mean and STD of diameter values. Sample mean average was calculated based on the formula $\frac{1}{n} \times \sum_{i=1}^n x_i$, where n is the sample size and x_i is each individual item. STD was calculated based on the formula $\sqrt{\frac{\sum (x - \bar{x})^2}{(n-1)}}$, where x is each individual item, $\bar{x}$ is the sample mean average and n is the sample size.

4.3 Results and Discussion

4.3.1 Expression and Purification of (rec)EsTKs

In order to test whether (rec)EsTK α and γ were successfully expressed in *E. coli* cell lines, the expression products before and after IPTG induction were analyzed by SDS-PAGE (10%). The results are shown below (Figure 4.1). SDS-PAGE indicated that the majority of the expressed proteins were in insoluble inclusion body fractions in both cases. While soluble recombinant proteins are often highly desirable for downstream applications such as protein crystallization studies, the formation of inclusion bodies facilitates the enrichment and purification of high yields of target proteins with low contamination level^[8-9]. In addition, as stated in section 3.3.1 microfluidization has been shown as an efficient method to increase inclusion body lysis yields and also provide some basic purification^[10-11]. Following microfluidization, both (rec)EsTK α and γ inclusion bodies were solubilized in buffer with 8 M urea and both showed high expression level (Figure 4.1). The protein expression yield was 40-50 mg per liter culture for both (rec)EsTK α and γ .

Figure 4.1 (a) and (b) are SDS-PAGE of (rec)EsTK α and γ expression respectively. L: Molecular weight ladder; -: Non-induced; +: Induced; S1: Supernatant after microfluidization; P1: Pellet after microfluidization; S2: Supernatant of P1 solubilized in buffer with 8 M urea.

Further purification of expressed (rec)**EsTK α** and γ were carried out in SEC by using an AKTA-purifier FPLC equipment (Figure 4.2a). For (rec)**EsTK α** the chromatogram indicated 3 major peaks. However, only the peak ranging from 13 to 15 ml elution volumes (highlighted in green) contained the protein with target molecular weight, as indicated by SDS-PAGE (Figure 4.2b). The molecular weight and purity of these fractions were further verified by MALDI-TOF. A single intense peak was observed at 66.7 kDa, which corresponded to the calculated molecular weight of (rec)**EsTK α** from the original protein sequence (Figure 4.2c).

Similarly, further purification of expressed (rec)**EsTK γ** was also conducted by using SEC/FPLC (Figure 4.2d). In this case, the chromatogram exhibited 3 peaks, and only fractions ranging between 13 and 15 ml elution volumes (highlighted in green) contained the protein with target molecular weight, which was further verified by SDS-PAGE (Figure 4.2e). MALDI-TOF analysis of these fractions resulted in the peak at around 62.8 kDa, which was consistent with the predicted molecular weight from the original protein sequence (Figure 4.2f).

This whole protein expression and purification approach, involving microfluidization and SEC, resulted in yields of 10-20 mg of protein per liter of culture for both (rec)**EsTK α** and γ . In each case, the protein purity was estimated to be greater than 90%, which was considered sufficient for subsequent solubility testing of individual subunits and self-assembly experiments.

4.3.2 Solubility, Folding and Structural Characterization of Individual (rec)EsTKs Subunits

Spitzer *et al.* previously reported the successful self-assembly of *native* **EsTK α** and γ into IFs which was confirmed by TEM^[4]. However replication of these findings could not be achieved^[12-13]. The reason might be due to the presence of post-translational modifications in native EsTKs. The recombinant proteins do not contain such post-translational modifications. In addition, *in vitro* self-assembly studies were undertaken on the basis of self-assembly studies of other IF proteins such as keratin and vimentin, which has been successfully achieved from their recombinant form^[14-15].

One goal of this chapter was to establish the suitable self-assembly conditions for (rec)EsTK α and γ to generate α -helical coiled-coil heterodimers and further associate into oligomer complexes which mimicked the native hagfish thread IFs. Therefore, the first critical step was set up to explore the solubility and folding behavior of individual (rec)EsTK α and γ subunits, using conditions similar to those reported for the self-assembly of other IF proteins^[15-17]. For more details, please refer to section 2.6.

Figure 4.2 Purification of (rec)EsTK α (left) and (rec)EsTK γ (right). (a) and (d) FPLC/SEC absorption spectra at 280 nm. (b) and (e) SDS-PAGE analysis of SEC purification

fractions. L: Molecular weight ladder; 8-20: SEC fractions. (c) and (f) MALDI-TOF spectra from SEC fractions 13-15.

The results of the step-wise dialysis against urea concentration of individual (rec)EsTK α subunits using various buffers and pH conditions at room temperature (22°C) are summarized in Table 4.1. At pH 4 to 7 the majority of (rec)EsTK α formed aggregates during the final dialysis step. The soluble fractions were analyzed by CD which yielded low intensity indicating random coil structures (Figures 4.3a). These conditions were considered as unsuitable for later self-assembly experiments. On the other hand, in Tris buffer of pH 8.4 and 9 (rec)EsTK α remained soluble. The CD spectra showed a broad peak with one minimum centered at 216 nm, indicating the presence of β -sheet structure (Figure 4.3b). Temperature also played an important role in self-assembly experiments. It is worth reminding that the hagfish live in the deep ocean, and hence native EsTK IFs are assembled within GTCs at low temperatures. Therefore, the influence of temperatures on folding was explored accordingly. Individual (rec)EsTK α subunits were step-wise dialyzed against urea concentration in Tris buffer of pH 8.4 at 4 °C and 37 °C. In both cases the dialysis products remained soluble and their CD spectra exhibited β -sheet signatures with one minimum at around 216 nm. The 4°C dialysis product resulted in the highest CD signal intensity (Figure 4.3c).

(rec)EsTK α dialysis products at pH 8.4 at 4 °C, 22 °C and 37 °C were also evaluated by TEM. The samples appeared predominantly granular for all three temperatures, and one representative micrograph for the self-assembled products conducted at 4 °C is shown in Figure 4.5a. In this case, the folding of individual (rec)EsTK α subunits did not show α -helical structures, nor observed filaments, under any of the conditions tested. Nevertheless, the solubility window for (rec)EsTK α folding has been identified, which will be used for the subsequent self-assembly experiments using (rec)ExTKs mixtures.

Table 4.1 Characterization of individual (rec)EsTK α subunits folded in respective buffers of different pH by removal of urea in step-wise dialysis (8 M $\rightarrow$ 4 M $\rightarrow$ 2 M $\rightarrow$ 0 M) at 22 °C. All are with 1 mM DTT.

Buffer component	pH	Solubility	CD spectra	TEM
Sodium citrate	4	×	Random Coil	-
Sodium citrate	5	×	Random Coil	-
MES	6	×	Random Coil	-
Phosphate	7	×	Random Coil	-
Tris	7	×	Random Coil	-
Tris	8.4	√	β -sheet	Non-Filamentous
Tris	9	√	β -sheet	Non-Filamentous
Tris + 100mM NaCl	8.4	×	Random Coil	-

* √: Product was soluble;

×: Product was not soluble;

–: Non-applicable.

In parallel, the solubility and folding behavior of individual (rec)EsTK γ subunits were explored under the same conditions at room temperature (22 °C) as used for individual (rec)EsTK α subunits (Table 4.2). Here at pH 4 and 5 (rec)EsTK γ was found to precipitate during the final dialysis step, and the soluble fractions showed similar CD spectra as random coil structure. In MES buffer of pH 6 and Tris buffer of pH 7 to 9, (rec)EsTK γ remained soluble. The pH 6 and 7 dialysis products resulted in broad CD spectra with one minimum ranging from ~208 to 222 nm, suggested the presence of a mixture of β -sheets and α -helices (Figures 4.4b-c). On the other hand, in Tris buffer of pH 8.4 and 9, the dialysis product showed clear α -helical signatures in CD, with two minima at 208 and 222 nm (Figure 4.4c). Following that the temperature influence was evaluated by step-wise dialyzing individual (rec)EsTK γ subunits against urea concentration in Tris buffer of pH 8.4 at 4 °C and 37 °C. Each temperature treatment yielded similar α -helical structure in CD measurement, with two minima at 208 and 222 nm and increasing signal intensity associated with decreasing temperature.

Figure 4.3 CD spectra of folding testing of individual (rec)EsTK α subunits. (a) and (b) Folded in respective buffers of different pHs. Sodium citrate pH 4 and 5; MES pH 6; Tris pH 7, 8.4 and 9. **(c)** Folded in Tris buffer of pH 8.4 at different temperatures. These experiments were conducted with 1 mg/mL protein concentration.

Table 4.2 Characterization of individual (rec)EsTK γ subunits folded in respective buffers of different pH by removal of urea in step-wise dialysis (8 M $\rightarrow$ 4 M $\rightarrow$ 2 M $\rightarrow$ 0 M) at 22 °C. All are with 1 mM DTT.

Buffer component	pH	Solubility	CD spectra	TEM
Sodium citrate	4	×	Random Coil	-
Sodium citrate	5	×	Random Coil	-
MES	6	√	Mixture α -helix and β -sheet	-
Phosphate	7	×	Random Coil	-
Tris	7	√	Mixture α -helix and β -sheet	-
Tris	8.4	√	α -helix	Non-filamentous
Tris	9	√	α -helix	Non-filamentous
Tris + 100mM NaCl	8.4	√	Mixture α -helix and β -sheet	-

* √: Product was soluble;

×: Product was not soluble;

—: Non-applicable.

Similarly, the (rec)EsTK γ dialysis products from all three temperatures were evaluated by TEM. Again in each case all the samples appeared granular and one example at 4 °C was shown in Figure 4.5b. Here the folding of individual (rec)EsTK γ subunits showed α -helical structures, but not observed filaments.

Figure 4.4 CD spectra of folding testing of individual (rec)EsTK γ subunits. (a) and (b) Folded in respective buffers of different pHs. Sodium citrate pH 4 and 5; MES pH 6; Tris pH 7, 8.4 and 9. (c) Folded in Tris buffer of pH 8.4 at different temperatures. These experiments were conducted with 1 mg/mL protein concentration.

Figure 4.5 TEM micrographs of individual (rec)EsTKs subunits folded in Tris buffer of pH 8.4 at 4 °C. (a) (rec)EsTK α . (b) (rec)EsTK γ .

Taken together the results of the solubility, folding structure and morphology of individual (rec)EsTK α and γ subunits, the best condition was selected where both proteins remained soluble (in Tris buffer of pH 8.4 at 4 °C). Although individual (rec)EsTK α did not yield α -helical structure and observed filaments whereas individual (rec)EsTK γ yielded α -helical structure but no observed filaments, the formation of α -helices and coiled-coil structures might be facilitated by adding the two subunits together^[18]. Overall this section provided a potential starting point for self-assembly experiments of 1:1 mixtures of (rec)EsTK α and γ .

4.3.3 Self-Assembly and Structural Characterization of (rec)EsTK Dialysis Products

A general mechanism that describes the assembly of IFs has been developed. First, coiled-coils are formed by parallel winding of protein subunits into 40-50 nm long rod-like dimers with diameters of around 2 nm, which are stabilized by internal hydrophobic forces^[19]. Dimers self-assemble further into oligomers, and then into unit length filaments (ULFs) which are approximately 60 nm long and 20 nm wide^[20]. Moreover, ULFs self-assemble from both ends to form filaments which are about 10 nm in diameter and approximately 0.5 to 1 μ m length^[16, 19].

In order to investigate the self-assembly potential of (rec)EsTKs, 1:1 (w/w) mixtures of both subunits were step-wise dialyzed against urea concentration in the buffers selected from the above section where both subunits maintained soluble (Tris buffer of pH 8.4) at different temperatures. These experiments are summarized in Table 4.3. After centrifugation at 13,000 g for 10 min, there were no visible precipitates for all dialysis products. CD spectra of these dialysis products showed two minima at 208 and 222 nm (Figure 4.6a), with highest signal intensity at 4 °C. Notably, the signal intensity was greater than that of individual (rec)EsTK γ subunits, indicating that (rec)EsTK α should participate in α -helical structure formation together with (rec)EsTK γ . At pH 8.4 and 4 °C the 222/208 intensity ratio was 0.77 for individual (rec)EsTK γ subunits, while this ratio was 0.96 for the dialysis products. It has been reported that proteins with a 222/208 nm ratio above 1 are considered to exhibit α -helical coiled-coil structure. This ratio increase suggested that the 1:1 mixture of dialysis products adopted at least partial coiled-coil conformations^[21].

Table 4.3 Characterization of folded individuals and 1:1 self-assembled mixtures of (rec)EsTKs in Tris buffer of pH 8.4 at different temperatures by removal of urea in step-wise dialysis (8 M $\rightarrow$ 4 M $\rightarrow$ 2 M $\rightarrow$ 0 M). All are with 1 mM DTT.

Temperature	Protein Sample	CD	TEM
22 °C	(rec)EsTK α	β -sheet	Non-Filamentous
	(rec)EsTK γ	α -helix	Non-Filamentous
	Self-assembled product	α -helix	IF-like Filaments
37°C	(rec)EsTK α	β -sheet	Non-Filamentous
	(rec)EsTK γ	α -helix	Non-Filamentous
	Self-assembled product	α -helix	IF-like Filaments
4°C	(rec)EsTK α	β -sheet	Non-Filamentous
	(rec)EsTK γ	α -helix	Non-Filamentous
	Self-assembled product	α -helix	IF-like Filaments

Figure 4.6 CD spectra of folding testing of individual (rec)EsTKs subunits and self-assembly experiments of 1:1 mixture. (a) 1:1 self-assembled mixtures at different temperatures. (b) Temperature-dependent CD spectra of individual (rec)EsTK α subunits. (c) Temperature-dependent CD spectra of individual (rec)EsTK γ subunits. (d) Temperature-dependent CD spectra of 1:1 self-assembled mixtures. These experiments were conducted with 1 mg/mL protein concentration.

These dialysis products showed filamentous structures under TEM (Figure 4.7a-c), which approached to the common known IFs. One hundred (rec)EsTK-based filaments self-assembled at pH 8.4 at 4 °C were selected from 15 different TEM images. They exhibited a mean diameter of 15 ± 2 nm and a mean length of 200 ± 40 nm ($n = 100$). In some cases the filaments were smooth, while in other cases the ragged edge morphologies were observed, which may be due to imperfect or incomplete self-assembly.

In order to get rid of heavy metal staining effects, cryo-TEM was carried out on the (rec)EsTK-based filaments self-assembled at pH 8.4 at 4 °C since these filaments showed smoother edges in stained TEM (Figure 4.7d). Ten filaments were measured from two cryo-TEM images. These filaments exhibited lower mean diameters (7 ± 1.5 nm; $n = 10$) than those observed in stained TEM, suggesting that crystallization of TEM staining reagents may affect the observed morphology and dimensions of self-assembled filaments. All data in this section suggested the best condition (in Tris buffer of pH 8.4 at 4 °C) which were suitable for (rec)EsTKs self-assembly into filaments.

Figure 4.7 TEM micrographs of 1:1 self-assembled products of (rec)EsTKs. (a) Stained TEM of self-assembled products at 4 °C. **(b)** Stained TEM of self-assembled products at 22 °C. **(c)** Stained TEM of self-assembled products at 37 °C. **(d)** Cryo-TEM of self-assembled products at 4 °C.

4.3.4 Thermal Stability Analysis of Folded Individual (rec)EsTKs Subunits and Self-Assembled Mixtures

In order to study the thermal stability of (rec)EsTK self-assembled products and individual subunits, the temperature dependence of CD spectra was evaluated. For individual (rec)EsTK α subunits, the CD spectra showed β -sheet structure across the entire temperature range tested between 10 °C and 90 °C, suggesting a high thermal stability for (rec)EsTK α based β -sheets. There was no significant structural change after cooling to 10 °C (Figure 4.6b). Under the same heat ramping conditions, individual (rec)EsTK γ subunits showed α -helical structure between 10 °C and 30 °C, after which melting of the helices was observed and random coils started to form. At 90 °C the majority of the structures were random coils. Interestingly, the α -helical structure was recovered after cooling to 10 °C (Figure 4.6c). On the other hand, self-assembled products showed α -helical coiled-coil signature between 10 °C and 40 °C, after which a conversion to β -sheet structure appeared. Upon cooling to 10 °C, the β -sheet signature was retained (Figure 4.6d). This thermal stability is comparable to those observed in tropomyosin^[22] and leucine zippers^[23], whose melting temperatures are in the range of 40-60 °C. It was speculated that the slightly increase of melting points from individual (rec)EsTK γ subunits to self-assembled products was due to additional coiled-coil stabilization interactions, although the increase is not significant.

4.3.5 Rheological Properties of Folded Individual (rec)EsTKs Subunits and Self-Assembled Mixtures

In order to test the mechanical response of (rec)EsTK self-assembled products and individual subunits, rheology experiments were conducted. In frequency sweeps from 0.1 to 10 Hz with constant shear strain at 1%, both individual (rec)EsTK α and γ subunits (0.5 mg/mL protein concentration) exhibited a liquid like behavior with loss modulus (G'') always higher than storage modulus (G') (Figure 4.8a-b). All self-assembled products with concentrations above 0.2 mg/mL exhibited a gel like behavior with G'' always lower than G' , which provided the concentration threshold above which the network of filaments was formed (Figure 4.8c). This rheological response of the self-assembled filaments was consistent with the presence of

entangled elastic IF networks^[24]. It has been reported that vimentin-based IF networks (1 mg/mL protein concentration) and type I and II Human Keratins HK5/14 based IF networks (1 mg/mL protein concentration) exhibit G' values between 2-4 Pa^[24-25]. Here G' of (rec)EsTK-based self-assembled filament network was higher and achieved around 6 Pa (Figure 4.8c). The plateau modulus (G_0 , storage modulus value at 1 Hz) was found to be dependent of concentration and follow the power law as $G_0 \propto c^{0.71}$ (Figure 4.8d). Notably, the power law coefficient (0.71) was identical to desmin based IF networks and even higher than the values for Human Keratin K8/K18 based IF networks (0.53) and vimentin-based IF networks (0.48) in the similar concentration range^[26-27]. For semi-flexible filaments, the power law coefficient is in the range of 2 to 2.5^[28], and hence the (rec)EsTK-based filament networks exhibited weaker concentration dependence. It was speculated that this weak concentration dependence was due to the more stretched filament strands in the initial states since the similar trend was also found in Human Keratin K8/K18 based IF networks^[26]. This was also confirmed by the large initial storage modulus ($G' > 1$ Pa for 0.2 mg/mL).

Next, the shear strain sweeps from 0.1 to 1000% with constant frequency at 1 Hz on (rec)EsTK-based IF networks (1 mg/mL protein concentration) were conducted. Here G_0 (or G_i' , initial plateau modulus) was around 1.8 Pa between shear strain of 0.4 and 10%, followed by a strong decay to 0.2 Pa due to network rupture (Step 1 in Figure 4.8e). The similar behavior was also observed in uncross-linked Human Keratin HK5/14 based IF networks (1 mg/mL protein concentration)^[25] and K8/K18 based IF networks (1 mg/mL protein concentration)^[26]. After quick recovery to 0% strain (~ 2 sec), the (rec)EsTK-based filament networks were subjected to oscillatory shear-stain test with fixed strain and frequency values ($\gamma = 1\%$, $f = 1.6$ Hz). Interestingly, G_0 (or G_r' , recovery plateau modulus) was found to achieve a higher value around 6 Pa, which was almost 3-times higher than that of obtained in Step 1 (Step 2 in Figure 4.8e). After relaxing for 30 min, the identical set-up experiment for (rec)EsTK-based filament networks were conducted as in Step 1. This time, G_0 was remained the same value as in Step 2 in the range of 6-7 Pa (Step 3 in Figure 4.8e). Following Step 4 was identical to Step 2 and resulted in the similar trend. After relaxing for another 15 min, Step 5 which was identical to Step 3 resulted similar

trend again. For fully recoverable polymer networks, the ratio G_r'/G_i' should approach to 1, which was happened for Human Keratin K8/K18 based IF networks, as well as here in Step 3, 4 and 5. However in Step 1 and 2, (rec)EsTK-based filament networks not only regained the value after the initial straining cycle, but exhibited the ratio G_r'/G_i' even 3-fold higher.

Based on the data shown above, a possible hypothesis was proposed that there might be some structural change before the oscillatory shearing and after, and the change was irreversible. To verify, CD measurement was conducted to identify protein secondary structures of (rec)EsTK-based filament networks before and after the rheology test (Figure 4.8f). CD spectra clearly indicated that the proteins underwent a structural transition from α -helix (two minima at 208 nm and 222 nm) to β -sheet (one minimum at 216 nm). For native hagfish slime threads, the stretch along the axis is able to generate the $\alpha \rightarrow \beta$ transition, which causes the strain hardening of the thread since the transition is irreversible and hence locks the structure^[5]. Here the increase of G_0 from Step 1 to Step 2 indicated that the similar mechanical strengthening mechanism may dominant in (rec)EsTK-based filament networks, and these data provided the first evidence for the strain-induced $\alpha \rightarrow \beta$ transformation of recombinantly engineered (rec)EsTK-based materials.

4.3.6 Effect of Divalent Cations Cross-Linking on (rec)EsTK-based Filament Networks

Assembled IF networks exhibit an increase of storage modulus after cross-linking^[29]. For instance, the stiffness of vimentin-based IF networks increased after introducing $MgCl_2$ or $CaCl_2$ in the final self-assembly buffer. This is likely due to the interaction between the divalent cations and highly charged C-terminal domains^[7]. Based on this, the ability of divalent cations to cross-link (rec)EsTK-based filamentous networks was tested. The rheology experiments showed that with only 0.5 mM Mg^{2+} the network was disrupted and exhibited liquid like behavior ($G' < G''$, Figure 4.9). Similar results were obtained in the presence of Ca^{2+} (data not shown). The reason might be due to the terminal domain of (rec)EsTKs containing less charged residues (**EsTK α** 6 mol%; **EsTK γ** 8 mol%) compared with that of vimentin

(31 mol%). The data suggested that the (rec)EsTK-based filamentous networks either lie hidden or do not present the domains which could interact with divalent ions worked for other IF networks. Other than divalent ions there are other biological cross-linking methods such as intermediate filament associated proteins (IFAPS) and Filaggrin-like proteins^[30], which could be tested in the future study.

Figure 4.8 Frequency dependence of G' and G'' of folded individual (rec)EsTKs subunits and 1:1 self-assembled mixtures. (a) 0.5 mg/mL folded (rec)EsTK α . (b) 0.5 mg/mL folded (rec)EsTK γ . (c) 1:1 self-assembled products with different protein concentrations. (d) Plateau modulus G_0 of self-assembled products versus protein concentration. The power law coefficient (0.71) was identical to the desmin-based IF networks. (e) G' of the 1:1 self-

assembled products (1 mg/mL) versus strain until network rupture (Step 1), followed by G' versus time after 2 sec recovery (Step 2, measured at $\gamma = 1\%$, $f = 1.6$ Hz). In Step 3, G' versus strain until network rupture was re-measured following 30 min relax. Steps 4 and 5 are identical to Steps 2 and 3, respectively. G'_i is the initial plateau storage modulus and G'_r is the recovery plateau storage modulus. (f) CD spectra of 1:1 self-assembled products before the oscillatory shearing and after.

Figure 4.9 Frequency dependence of G' and G'' for 1:1 self-assembled products of (rec)EsTKs with different Mg^{2+} concentrations. (a) 0 mM; (b) 0.5 mM; (c) 1.0 mM; and (d) 1.5 mM. All are with protein concentration of 0.5 mg/mL and measured at $\gamma = 1\%$.

4.4 Conclusion

In this chapter, several aspects were established for the foundation of subsequent EsTK-based material production. First, (rec)EsTK α and γ proteins were expressed in *E. coli* cell lines and further purified by microfluidization and SEC. Second, the solubility and folding behavior of individual (rec)EsTK α and γ subunits were evaluated by a step-wise dialysis against urea in decreasing concentration, from 8 M to 0 M in various buffers, different pHs, and different temperatures. Conditions at which both individual (rec)EsTKs subunits could maintain solubility were identified, and subsequently selected for the self-assembly of 1:1 (wt/wt) mixtures of (rec)EsTK α and γ . CD measurements and TEM observations indicated the formation of coiled-coil (rec)EsTK-based filaments. Rheology tests confirmed that these filaments exhibited entangled networks with gel like behaviour comparable to other IF-based networks. Notably, the $\alpha \rightarrow \beta$ transition was achieved under oscillatory shearing which was observed in CD, and thus mechanical strengthen process was accomplished. However, contrary to other IF-based networks, divalent ions were found to disrupt the (rec)EsTK-based filament networks rather than cross-linking and further stiffening them.

In summary, the studies from this chapter could lead to the production of (rec)EsTK-based filamentous networks, which would be used for larger scale materials fabrication, for example fiber formation through filament bundling and alignment which is the topic of Chapter 5.

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Chapter 5

Artificial Recombinant Hagfish Thread Keratins Based Fibers with Ultra-High and Tunable Stiffness

Hagfish slime threads are attractive protein-based biological materials, which have intriguing mechanical properties that can be tailored by draw-processing. This chapter describes the method to develop artificial (rec)EsTK-based fibers using self-assembled coiled-coil filaments as building blocks. These artificial fibers exhibit mechanical performance enhancement during draw-processing, which is due to the high content of aligned β -sheets formation. The fibers are shown to exhibit a multi-step conformational transformation, from coiled-coils to cross β -sheets during fiber picking-up formation, followed by cross β -sheets to elongated parallel β -sheets during draw-processing. Moreover, chemical cross-linking between lysine residues further enhances the stiffness and ultimate tensile strength, which is higher than most artificial protein-based fibers produced to date.

5.1 Introduction

As stated in Chapter 2, one classic example of biological stiff fibers is silk, which has been studied for years. Both native^[1-2] and artificial^[3-5] forms of silks are used nowadays and have found various applications in many fields. For instance, the unique combination of high strength, long durability, and high extensibility makes silk a good material in the textile industry^[6].

Due to the challenges associated with silk bioprocessing (see section 2.2) alternative biological materials are being explored^[7-9]. In the past decade, hagfish slime threads have received significant interest as a promising alternative to silks shown as bio-based fibers. Hagfish slime threads are made of two α -helical coiled-coil proteins, namely *Eptatretus stoutii* Thread Keratin α and γ (**EsTK α** and **EsTK γ**), which self-assemble into intermediate filaments (IFs) and these IFs are further bundled with high degree alignment into macroscopic threads^[10-13]. The threads exhibit enhancement in mechanical properties via draw-processing, which induced an irreversible conformational transition from α -helices into aligned β -sheets ($\alpha \rightarrow \beta$ transition)^[14].

Attempts to form macroscopic fibers from protein solution of native hagfish threads solubilized in 98% formic acid have been reported^[15], but they generated relatively weak mechanical response (failure stress 150 MPa) compared to the native threads (failure stress 800 MPa)^[14]. To date, there have been no reports of recombinant EsTK-based fibers and a major goal of this thesis was to demonstrate that artificial fibers could be produced from recombinant EsTKs.

5.2 Experimental Methods

Artificial (rec)EsTK-based fibers were produced using the self-assembled (rec)EsTK-based filaments as building blocks, the protocols of which was established in Chapter 4. Both drawn and non-drawn fibers were mechanically and biochemically characterized. The mechanical tests of the resulted fibers were performed by nano tensile testing, while the surface morphology was observed by FESEM. The protein

structure and molecular alignment were analyzed by Congo red staining, Raman spectroscopy as well as WAXS. The effect of chemical cross-linking on the fibers' tensile response was also explored, and the cross-linking chemistry was verified by AAA.

5.2.1 Expression, Purification and Self-Assembly of (rec)EsTKs

Recombinant **EsTK α** and γ were expressed in *E. coli* BL21. Each protein was purified by microfluidization and SEC. The purified 1:1 mixtures of (rec)EsTKs were self-assembled by step-wise dialysis against urea concentration (8 M $\rightarrow$ 4 M $\rightarrow$ 2 M $\rightarrow$ 0M) in Tris buffer of pH 8.4 at 4 °C. Both individual subunits and self-assembled mixtures have been fully characterized by CD, TEM and rheology, and the formation of α -helical coiled coil filamentous structures was confirmed. For more details, please refer to Chapter 4. The self-assembled mixtures were freeze-dried and stored at 4 °C for the subsequent protein dope solution preparation and fiber production.

5.2.2 Protein Dope Solution Preparation and Characterization

Lyophilized self-assembled mixtures containing coiled-coil filaments were re-solubilized in formic acid (98%, Sigma) at a 100 mg/mL concentration. The solution was used to make fibers within 15 minutes. To investigate whether the coiled-coil structure was maintained in the protein dope solution, CD measurements were conducted. The protein solution was stabilized for 1 hour before diluted using MilliQ water to 1 mg/mL, 2 mg/mL, 5 mg/mL and 8 mg/mL, respectively, with 8 mg/ml corresponding to the maximum concentration at which a CD signal could be obtained. For more details, please refer to section 3.5.1.

5.2.3 Fiber Production with Different Drawn Degrees

Two μ l of protein dope solution (100 mg/mL) was applied onto the surface of ice cold electrolyte buffer (20 mM HEPES, 100 mM MgCl₂, pH 7.5). After stabilizing for 30 seconds, fibers were formed by slowly picking up off the film on the surface using tweezers. Overnight drying was allowed before the individual fiber was glued onto a

cardboard to secure the fiber for subsequent tensile testing. The gauge length of individual fibers ranges from 15 mm to 25 mm (Figure 3.9).

Draw-processing was formed by using a nano tensile testing system (Nano Bionix® System, MTS, USA). The individual wet fiber was fixed onto a cardboard frame and mounted on the tester grips. The side of the cardboard frame was cut open before a uniaxial tensile load at a strain rate of 10^{-4} s^{-1} was applied until the desired drawn degree was achieved (0%, 10%, 25%, 50% and 100%). For instance, if the original picked-up fiber is 20 mm long, 100% drawn degree refers to stretching the fiber to 40 mm in length. The other samples were obtained in the same manner. The fibers with different drawn degrees were dried overnight and fixed onto a new cardboard frame individually for subsequent mechanical testing.

5.2.4 Structural and Biochemical Characterization of Fibers

Different structural and biochemical characterization methods were used to analyze and quantify the (rec)EsTK-based fibers. Chemical cross-linking effect on the (rec)EsTK-based fibers was also evaluated subsequently.

5.2.4.1 Mechanical Testing using Nano Tensile Tester

Nano tensile tester was used to characterize the mechanical properties of (rec)EsTK-based fibers before and after draw-processing. For more details, please refer to section 3.6.1.

5.2.4.2 FESEM

The surface features of the fibers were imaged with FESEM. The cross-section was obtained by fracturing the fiber in liquid nitrogen to avoid any deformation. All specimens were sputter-coated with a thin layer of Pt prior to imaging to make the conductive surfaces with minimum charging. For more details, please refer to section 3.6.4.

5.2.4.3 Congo-Red Staining

The fibers were stained by 1% Congo red (Sigma) in 10% ethanol solution for 2 hours, followed by destaining in MilliQ water for 1 hour. After overnight drying, visualization was conducted under an Axio Scope.A1 polarizing microscope (Zeiss, Germany).

5.2.4.4 Raman Spectroscopy

Raman spectroscopy was used to investigate the structures and backbone molecules alignment of fibers before and after draw-processing. For more details, please refer to section 3.6.2.

5.2.4.5 WAXS

WAXS was used to characterize the structural transition of artificial (rec)EsTK-based fibers before and after draw-processing. For more details, please refer to section 3.6.3.

5.2.4.6 Chemical Cross-Linking

Glutaraldehyde (4%, Sigma Grade I) was used to cross-link protein fibers before or after draw-processing. For more details, please refer to section 3.6.5.

5.2.4.7 AAA

Ninhydrin based AAA was used to evaluate the amino acid composition of cross-linked fibers. Non cross-linked fibers were hydrolysed in the same way, acting as negative controls. In parallel, pure lysine amino acid was cross-linked by glutaraldehyde under the same conditions and also analyzed by AAA in order to determine the elution time of the cross-link adduct. For more details, please refer to section 3.6.6.

5.2.5 Data Analysis

FESEM and WAXS images were analyzed by using ImageJ (NIH). Data plotting was done by using OriginPro 8.5. A total number (n) of 7 fibers were tested for each condition in tensile testing study. Sample mean average was calculated based on the formula $\frac{1}{n} \times \sum_{i=1}^n x_i$, where n is the sample size and x_i is each individual item. STD was calculated based on the formula $\sqrt{\frac{\sum(x-\bar{x})^2}{(n-1)}}$, where x is each individual item, $\bar{x}$ is the sample mean average and n is the sample size.

5.3 Results and Discussion

5.3.1 Expression, Purification and Self-Assembly of (rec)EsTKs

Recombinant **EsTK α** and γ were successfully expressed in *E. coli* BL21 and purified by microfluidization and SEC/FPLC. The purity was verified by SDS-PAGE and MALDI-TOF. The favorable conditions (Tris buffer at pH 8.4 at 4 °C) through step-wise dialysis again urea concentration (8 M $\rightarrow$ 4 M $\rightarrow$ 2 M $\rightarrow$ 0M) were selected as both individual (rec)EsTKs subunits could maintain soluble, prior to using self-assembled 1:1 (wt/wt) mixtures of (rec)**EsTK α** and γ . CD measurements and TEM observations indicated the formation of α -helical coiled-coil (rec)EsTK-based filaments. Rheology tests confirmed that these filamentous structures exhibited entangled networks with gel-like property comparable to other IF-based networks. Notably, these filamentous networks showed the ability to transit from α -helix to β -sheet under oscillatory shearing. In general, the resulted filaments would be used as the building blocks for the fiber production described in this chapter. For more details, please refer to Chapter 4.

5.3.2 Preparation and Characterization of (rec)EsTKs Dope Solution

In order to achieve high concentration of protein dope solution, different buffers including Tris, MES, PBS, 5% acetic acid and 98% formic acid were used to resolubilize freeze-dried self-assembled (rec)EsTK-based filaments. A high protein

concentration of 100 mg/mL could only be obtained in formic acid. This high concentration solution proved to be a critical aspect to draw long and strong macro-fibers. After the protein dope solution was stabilized for 1 hour, it was diluted using MilliQ water to a series of concentrations (1, 2, 5 and 8 mg/mL) respectively. CD spectra confirmed the α -helical coiled-coil reservation in diluted protein solution for all concentrations, with clearly two minima at 208 nm and 222 nm (Figure 5.1).

Figure 5.1 CD spectra of diluted self-assembled (rec)EsTK filaments at different concentrations. The samples were obtained by dissolving self-assembled filaments in formic acid, which was then subjected to serial dilutions. The solutions exhibited an α -helical coiled-coil signature at all concentrations.

5.3.3 Production, Draw-Processing and Mechanical Testing of (rec)EsTK-Based Fibers

After applying the protein dope solution (100 mg/mL) onto the surface of ice cold electrolyte buffer containing MgCl_2 , a thin layer of solidified protein film formed at the solution/air interface, which could be picked up using tweezers to form protein fibers (Figure 5.2 and 5.3a), typically 1.5-2.0 cm long and 80-100 μm wide. Different salts including CaCl_2 and NaCl were used in electrolyte buffer and both showed the ability to form protein film at the interface (data not shown). FESEM images revealed a compact structure with small cross-sectional porosity content, and regular ridges on the fibers surface (Figure 5.4a and c). Notably, repeating the same process using self-assembled (rec)EsTKs re-solubilized in 8 M urea, which disrupted the coiled-coil structures, did not lead to film formation at the electrolyte buffer solution/air interface,

and no fibers could be picked up from the solution. Additional control experiments included attempts to form protein films on the electrolyte buffer surface using protein solutions of lysozyme, bovine serum albumin (BSA), collagen, elastin, and silk like suckerin-19^[16] using identical conditions, but none of them resulted in protein film formation. In contrast, only white aggregates were observed (Figure 5.3b, suckerin-19 as an example). All these control experiments suggested that (rec)EsTKs must be initially self-assembled into coiled-coil filaments in order to successfully pick up fibers from the air/solution interface.

Figure 5.2 Schematic illustrations showing artificial fiber formation by a picking-up method. Protein dope solution containing self-assembled coiled coil filaments was applied on the surface of electrolyte buffer. A thin layer of film formed at the solution/air interface, which could be picked up using tweezers to form protein fibers.

Figure 5.3 Photo images of (a) picked-up fiber from the solution/air interface and (b) white aggregates in the case of suckerin-19.

The obtained picked-up fibers were then subjected to draw-processing under wet conditions using the nano tensile tester until desired drawn degrees (0%, 10%, 25%,

50% and 100%). This was to ensure fiber homogeneity along the whole length. FESEM images indicated a smoother surface for 100% drawn fibers compared to the non-drawn fibers (Figure 5.4b).

Figure 5.4 FESEM images of artificial (rec)EsTK-based fibers. (a) Side-view of 0% drawn fiber. **(b)** Side-view of 100% drawn fiber. **(c)** Freeze-fractured cross-section of 0% drawn fiber. **(d)** Side view of tear down part after mechanical testing until failure.

Figure 5.5a displays the representative stress-strain curves of fibers at various draw-processing degrees. Wet fibers exhibited high extensibility ($\sim 100\%$) but relatively low stiffness (0.04 GPa) and UTS (11 MPa), likely due to water molecules trapped inside the fibers acting as plasticizers. The ultra-soft property was consistent with hydrated native hagfish threads^[14]. After overnight drying, all fibers appeared stiffer and stronger. Draw-processing further increased stiffness from 0.5 GPa to 4 GPa and UTS from 25 MPa to 150 MPa, while the strain-to-failure decreased from 85% to 20% (Figure 5.5b-d). It is hypothesized that similar to native Hagfish slime threads, the structural transition during draw-processing might lead to this mechanical property enhancement of artificial fibers.

Figure 5.5 Tensile behaviour and mechanical properties of fibers at different drawn degrees. (a) Representative tensile curves of fibers at different drawn degrees until failure; (b) Stiffness verses fiber drawn degrees; (c) Strength verses fiber drawn degrees; (d) Extensibility verses fiber drawn degrees.

However, compared with native hagfish threads (stiffness 8 GPa and UTS 800 MPa), the stiffness and UTS of artificial (rec)EsTK-based fibers are still inferior. It has been reported that in native hagfish threads, IFs are well aligned and tightly packed, which ensures the high mechanical response^[10]. Here in artificial (rec)EsTK-based fibers, self-assembled filaments were not fully aligned, and the package was not ideal. The cross-section of undrawn fibers exhibited a porous structure under FESEM (Figure 5.4c). These imperfections were likely detrimental to the mechanical performance of artificial (rec)EsTK-based fibers, suggesting that there is still room for improvement.

5.3.4 Structural Transition Characterization

In order to test the hypothesis that the mechanical property enhancement during draw-processing is related to building blocks alignment, the fibers were subjected to

Congo red staining, as well as synchrotron wide-angle X-ray scattering (WAXS) and Raman spectroscopy measurements. Congo red is a diazo dye which emits apple-green birefringence under polarized light upon interacting with protein-based materials made of β -sheet structure^[17-18]. After draw-processing, the fibers stained with Congo red exhibited green birefringence, indicating the presence of aligned β -sheets (Figure 5.6a, second from top).

For more quantitative analysis, WAXS was conducted on the fibers before and after draw-processing. Figure 5.6b shows WAXS patterns of non-drawn fibers, which exhibited reflections consistent with β -sheet structure based on the broad meridional reflection at $D = 0.46$ nm (inter β -strand spacing) and the equatorial reflection at $D = 0.97$ nm (inter-sheet spacing of β -crystallites)^[19]. The azimuthal intensity distribution of the 0.46 nm reflection indicates that β -crystallites were loosely oriented with β -strands primarily perpendicular to the fiber axis^[20]. Since CD spectra indicated a coiled-coil conformation of the protein solution (Figure 5.1), these data suggest that the filaments exhibit a coiled-coil to cross β -sheet conformational transition at the liquid/air interface during fiber picking-up formation.

Radial integration of the WAXS pattern from draw-processed fibers shows a nearly identical profile to unprocessed fibers. However, in contrast to undrawn fibers, the azimuthal intensity distribution of the 0.46 nm reflection was much sharper and oriented along the equatorial direction, indicating a transition of β -strands in draw-processed samples from a perpendicular to parallel orientation relative to the fiber axis (Figure 5.6b). These results can be attributed either to the conversion or to the re-orientation of cross- β sheets into parallel β -sheets during draw-processing, with residual cross- β remaining in the fiber (Figure 5.7). Both scenarios are plausible and would result to the same final structure, namely parallel β -sheets preferentially oriented along the fiber axis. For the first scenario, since cross- β amyloid-like fibers could be draw-processed up to 100% elongation, the implication is that cross- β crystallites would provide “hidden lengths” at the molecular level that unfold when an external load is applied. In the second case draw-processing would lead to the uncoiling of amorphous linkers and in strain-induced β -crystallites re-orientation. The ability of cross β -sheet structures to unfold under mechanical load has been

previously demonstrated in the egg stalk silk of lacewing insects^[21], as well as in mussel byssus attachment fibers^[22], whereas strain-induced β -sheets re-orientation has been proposed during thermo-processing of β -sheet-rich squid sucker ring teeth^[23].

To further investigate conformational transitions during draw processing, fibers were measured with polarized confocal Raman micro-spectroscopy during stretching. Consistent with WAXS measurements, Raman spectra of both 0% and 100% drawn fibers suggested the presence of β -sheet structure based on the amide I band maximum at 1670 cm^{-1} and the higher intensity of the band at 1245 cm^{-1} relative to the peaks around $\sim 1330\text{ cm}^{-1}$ in the amide III region ($1200\text{--}1375\text{ cm}^{-1}$)^[24]. However, the broad shape of the amide I band indicated a significant portion of random coils or β -turns as well^[25]. Polarization-dependent Raman measurements on 0% drawn fibers exhibited a slightly higher amide I band intensity when the laser was polarized parallel (Z polarization) vs. perpendicular (X polarization) to the fiber axis. This implies that the β -strands are preferentially oriented perpendicular to the fiber axis^[26]. However, in agreement with WAXS measurements, the degree of alignment of the β -crystallites was moderate. Comparison between the relative intensities of the amide I band under X and Z polarization between undrawn and drawn fibers revealed a switch in polarities, indicating a preferential orientation of β -strands along the fiber axis in drawn fibers. These data are consistent with the partial re-orientation of cross β -sheets to parallel β -sheets during draw processing. The low magnitude of the Raman polarization dependence indicated a significant fraction of disordered or unaligned domains in drawn fibers, suggesting even higher fiber stiffness and strength may be attainable with optimized alignment during draw-processing. Figure 5.7 shows a schematic illustration of the structural transition from cross β -sheet to aligned β -sheet during draw-processing.

Figure 5.6 Structural transition characterization of artificial (rec)EsTK-based fibers during draw-processing. (a) Congo red staining (from top to bottom: no stain, 100% drawn,

cross-linking after 100% drawn, cross-linking before drawing). **(b)** WAXS patterns from undrawn (top left) and drawn (top right) fibers with β -sheet reflections indicated. The complete integrated 1D radial intensity profiles of both patterns are displayed on the bottom left and the 1D azimuthal intensity profile of the radially integrated peak at $d = 0.46$ nm (indicated by the grey shaded area in the radial intensity profile) is displayed on the bottom right. Values are normalized to the maximum intensity. **(c)** Polarization dependent Raman spectra from undrawn and drawn fibers normalized to the Phe peak at 1004 cm^{-1} .

Figure 5.7 Schematic illustrations showing the structural transition and/or re-alignment of cross β -sheet into aligned β -sheet during draw-processing.

5.3.5 Effects of Chemical Cross-Linking on (rec)EsTK-Based Fibers

In many biological fibers (*e.g.* tendon collagen) post-processing cross-linking is necessary to maximize the tensile performance. There are approximate 4 mol% lysine residues in individual (rec)EsTK α and γ proteins, most of which locate in the central rod domain of coiled-coil regions facing outside^[11-12]. Protein structures of individual (rec)EsTK α and γ subunits were predicted by using each protein sequence on I-TASSER (Iterative Threading ASSEmbly Refinement) approach. Figure 5.8 was obtained by using PyMOL visualization software to illustrate the predicted protein structures with lysine residues highlighted. Glutaraldehyde is an effective chemical cross-linker which could interact with primary amines and form covalent bonds

between them^[27-28]. Therefore, these lysine content present in the (rec)EsTK proteins was harnessed to obtain intermolecular covalent cross-linking. The fibers were cross-linked either before or after draw-processing, which involved incubating the respective fibers in 4% glutaraldehyde (GA) with the objective to induce nucleophilic addition then elimination between free amines of lysine side chains.

Figure 5.8 Predicted structure domain of hagfish thread IFs obtained in PyMOL visualization software based on the I-TASSER prediction model, with the distribution of lysine resides highlighted (yellow sticks). (a) (rec)EsTK α , (b) (rec)EsTK γ .

Representative stress-strain curves of cross-linked fibers before draw-processing at various drawn degrees are shown in Figure 5.9a. Compared with non-cross-linked fibers without drawn, these fibers exhibited a much higher stiffness (3 GPa) and UTS (130 MPa) and a lower extensibility (4%). However, at higher draw-processing degree, the stiffness decreased from 3 GPa to 1.5 GPa, while UTS decreased from 130 MPa to 60 MPa (Figure 5.9b-c). This decrease was attributed to the formed covalent

bonds between protein strands, which hindered the conformational transition or re-orientation of β -sheets during the subsequent draw-processing. Congo-red staining showed that fibers cross-linked before draw-processing exhibited red birefringence, indicating the absence of aligned β -sheet conformation (Figure 5.6a, bottom-most image).

Figure 5.9 Tensile behaviour and mechanical properties of cross-linked fibers before draw-processing at different drawn degrees. (a) Representative tensile curves of cross-linked fibers before draw-processing at various drawn degrees until failure; (b) Stiffness verses fiber drawn degrees; (c) Strength verses fiber drawn degrees; (d) Extensibility verses fiber drawn degrees.

Representative stress-strain curves of cross-linked fibers after draw-processing at various drawn degrees are shown in Figure 5.10a. The stiffness increased from 3 GPa to 17 GPa with higher drawn degrees (Figure 5.10b), which matches that of the stiffest artificial silk spiders for which values in the range 17 to 25 GPa have been reported^[29]. At the same time, UTS also increased from 130 MPa to 250 MPa (Figure 5.10c). As a comparison, short amyloid fibers typically exhibit elastic moduli ranging

from 4 to 10 GPa^[30], whereas that of cyclic peptide nanotubes is 11 GPa^[31]; however these materials have to the best of our knowledge not been engineered into macroscopic fibers. In another example, films made of recombinant silk-like suckerin-19 exhibit a modulus between 8 and 9 GPa after photo covalent cross-linking^[32]. The mechanical properties enhancement is believed to be due to the formation of covalent bonds between adjacent β -sheet protein strands previously formed during draw-processing.

Figure 5.10 Tensile behaviour and mechanical properties of cross-linked fibers after draw-processing at various drawn degrees. (a) Representative tensile curves of cross-linked fibers after draw-processing at various drawn degrees until failure; **(b)** Stiffness verses fiber drawn degrees; **(c)** Strength verses fiber drawn degrees; **(d)** Extensibility verses fiber drawn degrees.

In order to verify the chemistry and degree of cross-linking, Ninhydrin based AAAwas conducted, with the elution time of the putative lysine-lysine cross-link determined by cross-linking free lysines using identical conditions. Figure 5.11 shows the AAA chromatogram of hydrolysed artificial fibers cross-linked after draw-

processing, together with that of the cross-linked lysine-lysine and the non-cross-linked fiber. Lysine content significantly decreased from 3.9 mol% for the non-cross-linked fiber to 0.4 mol% after cross-linking, and a new peak with an elution time corresponding to cross-linked lysine-lysine concomitantly appeared. On the other hand, the relative content of other amino acids did not change significantly. These data confirmed that most lysine residues were involved in lysine-lysine covalent bonding during the cross-linking treatment, whereas other amino acids did not participate in cross-linking.

Figure 5.11 AAA spectra of cross-linked fibers with two controls. The amount of free lysine decreased from 3.9 mol% (non-cross-linking) to 0.4 mol% (cross-linking), which indicated that most of the Lysine residues have been cross-linked.

5.3.6 Comparison with Other Protein-Based Materials

In order to compare the properties of the artificial (rec)EsTK-based fibers with other materials, an Ashby-type selection chart was plotted^[33], with the Young's Modulus (GPa) in x-axis and the Tensile Strength (MPa) in the y-axis. The chart indicates the position of artificial (rec)EsTK-based fibers in the “Modulus/UTS properties” space among various biological and non-biological materials^[30] (Figure 5.12). Non-drawn fibers sit in the range of keratins while 100% drawn fibers, in contrast, exhibit properties similar to functional amyloids, which is in agreement with the fact that both drawn fibers and amyloids are made of similar oriented β -sheets, as shown in synchrotron X-ray data^[34]. The cross-linked drawn fibers exhibit a higher

stiffness and strength, although still in the range of amyloids, but very close to that of native spider dragline silks, which to the best of our knowledge has not been achieved with other type of protein-based fibers. Another interesting feature is the fact that our fibers exhibit a wide range of properties, which can be tailored by draw-processing and cross-linking.

Figure 5.12 Mechanical properties of artificial (rec)EsTK-based fibers in comparison to biological and non-biological materials^[30]. Young's modulus versus tensile strength for a range of different materials. Red circles represent 0% drawn and 100% drawn of non-cross-linked fibers. Cyan circle represents fibers that were cross-linked after 100% draw-processing.

5.4 Conclusion

This Chapter has described the successful development of methods to fabricate artificial (rec)EsTK-based fibers. The high concentration of protein dope solution with retained α -helical coiled-coil filamentous structures provided the basis to process strong macroscopic fibers. After draw-processing, these artificial fibers exhibited remarkable mechanical performances with increased stiffness and UTS. Congo red staining, as well as WAXS and Raman measurement confirmed that properties

enhancement is due to the presence of aligned β -sheet structure formation. The fibers were shown to exhibit a multi-step conformational transformation, from coiled-coils to cross β -sheets during fiber picking-up formation from the solution interface, followed by cross β -sheets to elongated parallel β -sheets during draw-processing. Notably in the final step, covalent cross-linking of free amine residues further enhances the stiffness and UTS. The data from this Chapter suggests that process may be amenable to other coiled-coil based filaments, including *de novo* designed self-assembled constructs, which may lead to a wide range of molecular opportunities to create tough bio-fibers with a performance that may surpass the fibers described here and even native silk.

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Chapter 6

Microfluidic Devices Design and Application for (rec)EsTK-Based Filaments Bundling and Alignment

Microfluidic devices, which are utilized on the micrometer or even sub-micrometer scale, offer lab-on-chip applications in diverse fields such as blood testing, cell sorting and controllable chemical reactions. In this Chapter, microfluidic devices with 1-inlet-1-outlet and 3-inlet-1-outlet were designed with the goal to produce macroscopic artificial fibers using (rec)EsTK-based filaments. Using pressure as the driving force, the majority of nano-scale filaments could be elongated and flow-aligned parallel to the direction of fluid flow. The formed fibers under various flow rates and channel geometries were characterized by field emission scanning electron microscopy (FESEM) and polarized optical microscopy. Although the resulted fibers were short and rather brittle, microfluidics provided a proof-of-concept method to align and bundle (rec)EsTK-based filaments.

6.1 Introduction

Microfluidics encompasses techniques to manipulate liquid on the micrometer, or even sub-micrometer scale, to offer various lab-on-chip applications^[1-2]. At larger scales, turbulent flow dominates inside the tube, which results in irregularity, stochastics and fluctuations of the flow^[3-4]. However, at the micro- and nano-scales, the flow inside microfluidic devices are regular, and generally laminar, which leads to better control of objects prepared in this way^[5-6]. Another advantage of microfluidics is that, due to the large surface area to volume ratio, surface tension forces are predominant and thus thermal dissipation is fast, and inertial effects are negligible^[7-8]. More importantly, only very small volume of reagents are needed, which provides the possibility of large number of experiments to be performed rapidly and in parallel, and hence enables efficiency which leads to high throughput screening assays^[9-10]. Similar to larger scale fluidics, applying a pressure difference across the two ends of channel is one of the most common ways to provide driving force and create flow^[11-12]. By this means, the flow rate inside the channels can be precisely controlled.

One successful example of using microfluidics in biomimetic engineering area is to study the assembly of silk fibers^[13-14]. Natural silk fiber formation is achieved by transition of a globular protein to highly ordered β -sheets arranged between globular regions^[15]. This highly ordered arrangement is believed to be induced by the elongational flow created in the cross-section of lumen of spinning gland, which aligns the silk fibroin in the direction of flow^[16-17]. In order to mimic the natural silk spinning process, microfluidic devices are designed so that the artificial silk can be produced by using hydrodynamic focusing at the 3-inlet cross-section^[13]. This type of hydrodynamic focusing provided the concept of microfluidics design in this project.

Poly-dimethylsiloxane (PDMS) is the most popular soft material used to fabricate inexpensive microfluidic devices^[18-19]. It has useful physical and chemical properties, such as elasticity, flexible surface chemistry, low permeability to water, and low electrical conductivity^[20-22]. The optical transparency allows the investigation of processing inside the channels^[2]. PDMS is non-toxic to cells and proteins, and thus it is suitable for biological assays^[23-24]. All microfluidic devices used in this project were fabricated using PDMS.

In this Chapter, several experiments have been conducted to form artificial (rec)EsTK-based fibers using microfluidic devices. First, microfluidic devices with 1-inlet-1-outlet and 3-inlet-1-outlet were designed and fabricated. All the devices were tested using different parameters, including various flow rate and channel geometries. The majority of (rec)EsTK-based filaments were elongated and aligned parallel to the direction of fluid flow. The produced fibers were examined by FESEM and polarized optical microscopy. Although the fibers did not yield the expected length and strength, the data showed the potential to use microfluidics to bundle and align (rec)EsTK-based filaments.

6.2 Experimental Methods

6.2.1 PDMS Devices Fabrication

All silicon wafers were designed in collaboration with Dr. Andrea Pavesi from the Singapore-MIT Alliance for Research and Technology (SMART). Microfluidic structures were cast from the mold with PDMS elastomer (Dow Corning). For more details, please refer to section 3.7.1.

6.2.2 Fiber Formation and Characterization

The 100 μ L Hamilton syringe containing (rec)EsTK-based filaments aqueous solution obtained from Chapter 4 was equipped with syringe pump (11 Pico plus Elite, Harvard Apparatus, USA) and connected to fabricated PDMS devices by Teflon tubes (inner diameter 1 mm). In order to solidify the protein solution coming out of the device channels, methanol (Sigma) was used in the coagulation bath at the end of outlet. The fibrillar structures inside the coagulation bath were placed onto glass slides and vacuum-dried overnight before characterization using FESEM and polarized optical microscopy. For more details, please refer to section 3.6.5.

6.2.3 Data Analysis

Both optical microscope and FESEM images were analyzed by using ImageJ (NIH). Each condition used in the devices has been repeated for at least 3 times.

6.3 Results and Discussion

6.3.1 Microfluidic Devices Design

During alignment, minimal overlapping of small filaments is predicted to occur and the system can be controlled by different parameters including various flow rates and channel heights/widths. The width of native hagfish threads is around 3 μm (Section 2.3). Therefore, the devices were designed accordingly so as to mimic and produce artificial fibers of similar size. Due to the limit accuracy of PDMS, the size of channel must be beyond 10 μm . Hence, all the designs have channel geometries in the range of 10-50 μm . In general, two different microfluidic devices were designed in this project. One is with 1-inlet and 1-outlet and the other is with 3-inlet and 1-outlet.

6.3.1.1 1-Inlet-1-Outlet Design

Figure 6.1a shows the schematic illustrations of microfluidic device design with 1-inlet and 1-outlet. The aqueous solution of coiled-coil filaments would flow through the inlet. The elongation and alignment of the filaments was achieved by geometrically constraining the region near the inlet using a smaller diameter. The coagulation bath at the end of outlet was then used to help solidifying the protein solution coming out from the channel to form fibers. For the first design, the channels were designed with 1 cm in length, a starting width of the constrained region of 100 μm , and various final widths (50 μm , 40 μm , 30 μm , 20 μm , 10 μm). The channel height was fixed at 15 μm (Figure 6.1b). The initial test of these PDMS microfluidic devices was done by using a red dye dissolved in MilliQ, which was manually injected with 1 mL syringe and Teflon tubing into the channel from the inlet (Figure 6.1c). All tests were carried out horizontally to minimize gravity effects.

Figure 6.1 Schematic illustrations showing the first microfluidic device design with 1-inlet-1-outlet. **(a)** Illustration of microfluidic device used in this study. **(b)** AutoCAD illustration. From left to right the final width varies (50 μm , 40 μm , 30 μm , 20 μm , 10 μm). **(c)** Actual PDMS device tested by injecting red dye solutions into the channels. From left to right the final width varies (50 μm , 40 μm , 30 μm , 20 μm , 10 μm). **(d)** Constrained region under optical microscope using final width 50 μm as the example.

For the second design with 1-inlet and 1-outlet, the inside channels were designed with sets 2.5 cm in length, 100 μm width at the outlet and a regular decrease down to various final widths (20 μm , 15 μm , 10 μm), as well as various channel height (15 μm and 17 μm). The longer channels were designed in order to provide sufficient time for filamentous structures to be elongated and aligned. (Figure 6.2)

Figure 6.2 Schematic illustrations showing the second microfluidic device design with 1-inlet-1-outlet. (a) Illustration of microfluidic device used in this study. (b) AutoCAD illustration using a final width of 10 μm as an example. (c) Constrained region under optical microscope using final width 10 μm as the example.

6.3.1.2 3-Inlet and 1-Outlet Design

Figure 6.3a shows the schematic illustrations of microfluidic device designed with 3-inlet and 1-outlet. In this arrangement, the aqueous solution of coiled-coil filaments passed through the main inlet flow channel. At the same time, the elongation and alignment was achieved from both side channels at different flow rates with various organic (methanol, IPA, ACN) or non-organic solvents (Electrolyte buffer containing MgCl_2 , PBS). The main flow channels were designed with various widths (30 μm , 60 μm) while the channels connecting to the outlet were fixed at 3 cm in length, 100 μm in width, and 50 μm in height. Initial testing of these PDMS microfluidic devices was done by using a red dye dissolved in MilliQ water, which was manually injected with 1 mL syringe and Teflon tubing into the channel from the 3 inlets (Figure 6.3c). All tests were carried out horizontally to minimize the effect of gravity.

Figure 6.3 Schematic illustrations showing the microfluidic device design with 3-inlet-1-outlet. **(a)** Illustration of microfluidic device used in this study. **(b)** AutoCAD illustration using the main channel width of 30 μm shown as an example. **(c)** Actual PDMS device tested by injecting a red dye solution through the inlets using the main channel width of 30 μm shown as an example. **(d)** Connecting region where the main central channel and the two side channels meet, visualized under an optical microscope using a main channel width of 30 μm as an example.

6.3.2 Fiber Formation and Characterization

6.3.2.1 1-Inlet-1-Outlet Design

(rec)EsTK-based coiled-coil filaments were elongated and aligned through geometrically constrained region with starting width of 100 μm and various final widths. The solidified fibers were formed using methanol as the coagulation bath. Only two devices with the specific geometry parameters and flow rates showed the ability to form fibers, which are summarized in Table 6.1. The fibers seemed to be obtained only when the final channel cross-section geometry was square. A broad

range of flow rates was tested from 100 $\mu\text{L/h}$ to 10 $\mu\text{L/h}$. It should be noted that the filaments may not have sufficient time to bundle together if the flow rate is too fast, whereas the liquid may not overcome the surface tension and come out from the channel if the flow rate is too slow. These tests provided the preliminary information for the subsequent design of microfluidic devices.

Table 6.1 Testing conditions of microfluidic devices with the first 1-inlet-1-outlet design. The starting width of the constrained region was 100 μm , with a fixed channel height of 15 μm , and various final widths. The filaments solution concentration was 3 mg/mL. Methanol was used as the coagulation bath. All conditions have been repeated at least 3 times.

Flow rate ($\mu\text{L/h}$)	Final channel width (μm)				
	50	40	30	20	10
100	×	×	×	×	×
50	×	×	×	×	×
40	×	×	×	×	×
30	×	×	×	×	√
20	×	×	×	√	×
10	×	×	×	×	×

* √: Fiber formed;

×: Fiber did not form.

All the formed fibers appeared to be short (several millimeters long) and brittle. They were too fragile to be picked up by using tweezers. Therefore, the coagulation bath solution containing these small fibers was applied onto glass slides and was vacuum dried before characterization using FESEM and polarized optical microscopy. All the fibers experienced similar morphology and birefringence, and one example (testing condition: final width 20 μm , flow rate 20 $\mu\text{L/h}$) is shown in Figure 6.4. Under FESEM, the fiber appeared to have fibril nano-structures, which indicated that the bundling of filaments occurred in the flow. Under polarized optical microscopy, the fiber showed birefringence with every 45° rotation of the analyzer, suggesting that alignment of filaments was achieved in the flow.

Figure 6.4 Optical microscope and FESEM micrographs of fibers formed in the 1-inlet and 1-outlet design of microfluidic devices. The final channel width was 20 μm and the flow rate was 20 μL/h. **(a)** From top to bottom: under light microscopy; under polarized light microscopy; under polarized microscopy with analyzer rotating 45°. **(b)** FESEM micrograph showing the surface nano-structures.

Since the central channel of the first design was only 1 cm in length, it was speculated that longer channel might provide more suitable conditions for filaments to elongate and align^[1]. In addition, only specific geometries with channel cross-sections approaching a square geometry were able to form fibers in the first design (Table 6.1). Therefore, a second batch of microfluidic devices was designed accordingly. (rec)EsTK-based coiled-coil filaments were elongated and aligned through geometrically constrained region with starting width of 100 μm, various channel heights and final widths. The solidified fibers were formed using methanol as the coagulation bath. Again only several devices with the specific geometry parameters and flow rates demonstrated the ability to form fibers, which are summarized in Table 6.2. Similarly, the fibers seemed to be obtained only when the final channel cross-section approached a square geometry. A range of flow rates was tested and similar to the first design only between 20-40 μL/h the fibers could be acquired.

Table 6.2 Testing conditions of microfluidic devices with the second 1-inlet-1-outlet design. The starting width of the constrained region was 100 μm , with various channel heights and final widths. The filaments solution concentration was 3 mg/mL. Methanol was used as the coagulation bath. All conditions have been repeated at least 3 times.

Flow rate ($\mu\text{L/h}$)	Final channel width/height (μm)					
	20/17	20/15	15/17	15/15	10/17	10/15
500	×	×	×	×	×	×
100	×	×	×	×	×	×
50	×	×	×	×	×	×
40	×	×	×	√	×	√
30	×	×	×	√	×	√
20	×	×	×	√	×	×
10	×	×	×	×	×	×

* √: Fiber formed;

×: Fiber did not form.

The resulted fibers appeared to be longer than the ones from first design, which indicated that longer channel might provide more suitable conditions to create fibers. Both designs with square channel cross-section showed the ability to produce artificial fibers. However, the fibers were still in the mm scale range and not strong enough to be manipulated and picked up from the solution. The solution from the coagulate bath was applied onto glass slides and was vacuum dried before characterization. All the fibers experienced comparable morphology and birefringence, and one representative example (Testing condition: final width 15 μm , channel height 15 μm , flow rate 20 $\mu\text{L/h}$) is shown in Figure 6.5. Under FESEM, the fiber exhibited comparable morphology as with the one from the first design. Under polarized optical microscopy, the fibers exhibited birefringence again, indicating that bundling and alignment of filaments was achieved in these fibers.

Figure 6.5 Optical microscope and FESEM micrographs of the formed fiber from 1-inlet-1-outlet second design of microfluidic devices. The testing condition was with a final channel width of 15 μm , a channel height of 15 μm , and a flow rate of 20 $\mu\text{L/h}$. **(a)** From top to bottom: under light microscopy; under polarized light microscopy; under polarized microscopy with analyzer rotating 45°. **(b)** FESEM micrograph showing the surface nanostructures.

All the data shown above indicated that the bundling as well as the alignment of (rec)EsTK-based filaments can be achieved using microfluidic channels to some degrees. However, the relatively weak mechanical performance of these artificial fibers hindered further tensile testing for characterization and potential application. It was hence speculated that the constrained region alone was not able to provide enough elongational flow for the bundling and alignment of filaments. Therefore, microfluidics devices with 3-inlet-1-outlet were designed subsequently.

6.3.2.2 3-Inlet-1-Outlet Design

For this 3-inlet-1-outlet design, (rec)EsTK-based coiled-coil filaments were elongated and aligned by the two side channels at different flow rates. This kind of design has successfully used to form artificial silks^[13]. Various organic (methanol, IPA, ACN) and non-organic (Electrolyte buffer containing MgCl_2 , PBS) solutions,

which had been tested for the ability to solidify proteins (data not shown), were chosen to be used in the side channels, and hence the additional coagulation bath was avoided. The channels were straight with fixed height 50 μm and width 100 μm . The main flow channels were designed with various widths.

For the first batch of testing, methanol was used as the side channel solution, and different main-channel-to-side-channel flow ratios were tested and summarized in Table 6.3. For the main channel with 30 μm width, the fibers were only formed when the main flow rates were maintained between 20-30 $\mu\text{L/h}$, while the side flow rates varied. It should be noted that the main flow stream would be eliminated if the main flow rate is too slow, whereas the main flow stream would diffuse if the main flow rate is too fast. The longest fiber was obtained with a main flow rate of 30 $\mu\text{L/h}$ main and a side flow rate of 150 $\mu\text{L/h}$ (main-to-side ratio 1:5), and was of the mm length scale, but brittle. Under FESEM and polarized microscopy the vacuum dried fiber appeared smoother compared with the fiber formed with the 1-inlet-1-outlet devices, but yielded similar birefringence (Figure 6.6). For the main channel with a 60 μm wide, the same trend was observed. The fibers only formed when the main flow rate was maintained between 20-30 $\mu\text{L/h}$, while the side flow rates varied. The longest fiber was obtained with a main flow rate of 20 $\mu\text{L/h}$ and side flow rates of 400 $\mu\text{L/h}$ (main-to-side ratio 1:20), with a length in the mm range. Under FESEM and polarized microscopy, the fiber appeared to be similar compared with the ones in Figure 6.6 (Figure 6.7).

Table 6.3 Testing conditions of microfluidic devices with 3-inlet-1-outlet design. The channels were straight, with fixed a height of 50 μm and a width of 100 μm , while the main channel width varied. The filaments solution concentration was 3 mg/mL. Methanol was used in side flows. All conditions have been repeated at least 3 times.

Flow ratio (main : side)	Actual Flow rate ($\mu\text{L/h}$)	Main channel width (μm)	
		30	60
1:5	20 – 100	–	√
	30 – 150	√	–
	40 – 200	×	×
	50 – 250	×	×
	102 – 510	×	–
1:10	10 – 100	×	×
	20 – 200	×	–
	30 – 300	–	–
	40 – 400	–	–
	51 – 510	×	–
1:20	10 – 200	×	×
	20 – 400	×	√
	25.5 – 510	√	–
	30 – 600	–	–
1:30	10 – 300	×	×
	20 – 600	√	–
1:40	10 – 400	×	×

* √: Fiber formed;

×: Fiber did not form;

–: Experiments have not been performed.

Figure 6.6 Optical microscope and FESEM micrographs of the fiber formed in 3-inlet-1-outlet design of microfluidic devices. The main flow channel width was 30 μm and the flow rate was 30 $\mu\text{L/h}$ in main channel and 150 $\mu\text{L/h}$ in side channel (main-to-side ratio 1:5). Methanol was used as the side flow. **(a)** From top to bottom: under light microscopy; under polarized light microscopy; under polarized microscopy with analyzer rotating 45°. **(b)** FESEM micrograph showing the surface nano-structures.

Figure 6.7 Optical microscope and FESEM micrographs of the fiber formed in 3-inlet-1-outlet design of microfluidic devices. The main flow channel width was 60 μm and the flow rate was 20 $\mu\text{L/h}$ in main channel and 400 $\mu\text{L/h}$ in side channel (main-to-side ratio 1:20).

Methanol was used as the side flow. **(a)** From top to bottom: under light microscopy; under polarized light microscopy; under polarized microscopy with analyzer rotating 45°. **(b)** FESEM micrograph showing the surface nano-structures.

Using methanol as the solution of in the side channels, the filaments could be bundled and aligned to create microscopic fibers, which were verified by FESEM and polarized optical microscope. However, the achieved fibers were still not very long and not strong enough for further characterization and potential applications. Therefore, other solutions were also tested for the side channels. Both isopropanol (IPA) and acetonitrile (ACN) showed the ability to solidify proteins. However, only short fibers (less than 1 mm) with loose structures (data not shown) were obtained in the vacuum dried coagulation bath. Non-organic solutions with high ionic strength (Electrolyte containing MgCl_2 , PBS) also showed the ability to solidify proteins. Nevertheless, only granular aggregates (data not shown) were observed after vacuum dried.

It has been reported that solutions with a high viscosity (such as PEO) can be used as the side flow solution to create artificial silks by microfluidics^[13]. Therefore, polymer solutions with a high viscosity could be tested in future studies. The influence of these solutions on the solidification behavior will however need to be studied prior.

The protein solution concentration used in the main flow was only 3 mg/mL. This concentration may not be sufficient for filaments to align and bundle together to form strong fibers. For instance, microfluidic devices were used to create silk fibers and the silk solution had a high concentration of 80 mg/mL^[13]. Therefore, protein solution with a higher concentration within the solubility screening window should be tested in the future.

Lastly, geometrical parameters must be considered as well. For instance, the cross-shaped channels were used in microfluidics to fabricate silks. The tiered design ensured all-direction hydrodynamic focusing of proteins, which mimicked the silkworm silk gland^[13]. Although there is no report on how Hagfish produces the

threads from epithelial slime glands, it is speculated that similar production process might occur. Therefore, the same microfluidics design could be applied and tested.

6.4 Conclusion

This Chapter has described the development of methods to produce (rec)EsTK-based fibers using microfluidic devices. In contrast to the way artificial fibers were formed in Chapter 5 where manually “picking-up” method was applied, lab-on-chip technology was introduced in this Chapter, similar to what has been used to form microscopic artificial silk fibers^[13]. Both 1-inlet-1-outlet and 3-inlet-1-outlet microfluidic devices were designed accordingly. Among them, the 3-inlet-1-outlet microfluidic devices resulted in longer fibers. The whole process involves the alignment and bundling of self-assembled (rec)EsTK-based filaments under elongational flows, and the fabricated fibers were observed by FESEM and polarized optical microscopy to verify the alignment. Although the length of all artificial fibers was within the millimeter range and too brittle to be mechanically tested, we believe that these results provide the foundation for further development of microfluidic device designs. Moreover, using transparent PDMS as the device material allows to monitor the fluid behaviour within the channel, for instance allowing to simultaneously detect the alignments of molecules using polarized light microscopy^[2].

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Chapter 7

Discussion and Proposed Future Work

This Chapter provides the summary of the whole findings presented in Chapters 4, 5 and 6. In this chapter, possible applications for the artificial (rec)EsTK-based fibers are briefly discussed as future research areas, which are mostly focused on the biomedical fields such as tissue engineering. Potential future work is also proposed, such as nano-manipulation of filaments, further microfluidic devices designs and applications, electro-spinning and wet spinning methods to create artificial (rec)EsTK-based fibers, as well as truncated and mutagenesis of (rec)EsTKs to study in more detail the self-assembly mechanisms.

7.1 General Discussion and Conclusion

Hagfish slime threads have emerged as a promising biological material of stiff and tough protein-based fibers. They are built from α -helical coiled-coil proteins (**EsTK α** and **EsTK γ**) that self-assemble into hetero-dimeric intermediate filaments (IFs), and these IFs are bundled intra-cellularly with a high degree of axial alignment into macroscopic threads^[1-4]. Native hagfish slime threads exhibit an irreversible α -helical coiled-coil to β -sheet transition (**$\alpha \rightarrow \beta$ transition**) when draw-processing^[5]. This strain-induced phase transformation endows native threads with remarkable mechanical properties that rival silks and approach Kevlar^[6].

This thesis has provided an approach to create artificial EsTK-based fibers using self-assembled coiled-coil IF-like filaments as building blocks from recombinant protein production (Figure 7.1). The high content of aligned β -sheet structure formation during picking-up formation and further chemical cross-linking provide the artificial fibers ultra-high and tunable mechanical properties. We believe that these mechanical response tunable materials could lead to various application opportunities in the future.

Figure 7.1 The overall process from recombinant protein production to the final macroscopic fiber. (a) pJexpress vectors encoding **EsTK α** and **EsTK γ** genes (red region) respectively. (b) Self-assembly of (rec)**EsTK α** and γ into α -helical coiled-coil IFs by step-wise dialysis against urea concentration. (c) Predicted domain architecture of self-assembled IFs with central coiled-coil region separated by 3 flexible linker domains. (d) Fiber formation

by picking-up method. (e) Illustration of structural transition after draw-processing and chemical cross-linking.

In summary, all four objectives proposed in Chapter 2 have been achieved, and new findings are derived as follow:

[1] An efficient recombinant EsTKs expression and purification system has been set up:

Chapter 4 described the successful development of methods to express and purify (rec)EsTK α and γ proteins. The purity of each protein subunit was checked by MALDI-TOF and SDS-PAGE. The availability of purified recombinant proteins enabled subsequent self-assembly experiments, as well as artificial fiber formation. Besides, it also enables further research on EsTKs, such as protein crystallization studies to understand in more detaile EsTKs structures, or genetic engineering of truncated (rec)EsTKs to reveal detailed self-assembly mechanisms.

[2] Suitable conditions have been established to self-assemble (rec)EsTKs into coiled coil filaments:

In Chapter 4, suitable conditions were explored for (rec)EsTK α and γ self-assemble into coiled-coil filaments. Both individual subunits and self-assembled mixtures were characterized by CD measurements and TEM observations, which confirmed the (rec)EsTK-based IF-like filaments formation. Rheology tests indicated the formation of entangled filamentous networks, and more importantly the $\alpha \rightarrow \beta$ transition under oscillatory shearing. Taken together we believe it could lead to the production of (rec)EsTK-based filaments which would be used for larger scale materials fabrication, for example fiber formation through filament bundling and alignment, which were then described in more details in Chapter 5 and 6.

[3] Artificial (rec)EsTK-based fibers have been produced:

Chapter 5 described the successful development of methods to fabricate artificial (rec)EsTK-based fibers. The high concentration of protein dope solution with retained

α -helical coiled-coil filamentous structures provided the basis to biomimic hagfish threads. Artificial (rec)EsTK-based fibers were formed using a ‘picking-up’ method. The hydrated fibers exhibited rubber-like properties with relatively low stiffness and strength, as well as large extensibility, which is consistent with that of native hagfish threads. After dehydration all fibers appear stiffer and stronger.

Chapter 6 described the development of methods to produce (rec)EsTK-based fibers by using microfluidic devices where lab-on-chip technology was introduced. Both 1-inlet-1-outlet and 3-inlet-1-outlet microfluidic devices were designed. Self-assembled coiled-coil filaments as building blocks were used and the whole process led to the alignment and bundling of filaments into macroscopic fibers. The resulted artificial fibers were observed by FESEM and polarized optical microscopy. The 3-inlet-1-outlet microfluidic devices result in the longest fibers among all the tests, however they were still within the mm range. In addition, the artificial fibers were too brittle to conduct tensile testing. Nevertheless, we still believe that this Chapter provides the foundation for further development in using microfluidic devices to produce homogenous and mechanically performant fibers.

[4] The artificial fibers have been engineered through draw-processing and chemical cross-linking:

In Chapter 5, the artificial (rec)EsTK-based fibers exhibited enhancement of their mechanical performance after draw-processing, which was due to the high content of aligned β -sheets formation. Based on Congo-red staining, as well as Raman and WAXS measurements, the fibers were shown to exhibit a multi-step conformational transformation first from coiled-coils to cross β -sheets during fiber picking-up formation, and second by cross β -sheets to elongated parallel β -sheets during-draw processing. Moreover, chemical cross-linking between lysine residues further enhanced the stiffness and ultimate tensile strength, which approached that of silks. The cross-linking degree was verified by AAA, which confirmed that the majority of lysine residues in (rec)EsTKs were cross-linked.

7.2 Potential Applications in the Biomedical Field

As stated in Chapter 2, protein-based fibers have been used in a variety of applications in the biomedical field. Artificial (rec)EsTK-based fibers share many similarities with these protein-based fibers, both their structures and in performance. For example, draw-processed (rec)EsTK-based fibers that were covalently cross-linked exhibit mechanical properties which are comparable to silks. Therefore, the applications demonstrated for silks may also be achieved with (rec)EsTK fibers. Notably, in preliminary studies, (rec)EsTK-based fibers have been shown to be non cyto-toxic against Human breast cancer cell MDA-MB-231. Figure 7.2 shows that MDA-MB-231 cells were able to proliferate along, and on the top of the fibers, with very little cell death observed after 72 hours.

Figure 7.2 Cyto-toxicity testing on artificial (rec)EsTK-based fibers. (a) and (c) Fluorescence and light images of Human breast cancer cell MDA-MB-231 (nucleus in blue, cytoplasm in red) grown on fibers. (b) and (d) Fluorescence micrographs of Human breast cancer cell MDA-MB-231 cells (nucleus in blue, cytoplasm in red) growing on fibers.

7.2.1 Promotion of Peripheral Nerves Regeneration

Neurofilaments (NFs), a family member of intermediate filament (IF), are one of the most abundant cytoskeleton components in the central and peripheral nervous system^[7]. Native human NFs have complex organizations, which are co-assembled into coiled-coils by several protein subunits including NFL (low-molecular-weight subunit), NFM (medium-molecular-weight subunit), NFH (high-molecular-weight subunit), α -internexin and peripherin. The ratios of these subunits vary in different nervous systems^[8]. Research has shown that the correct expression and formation of NFs is important and crucial to enhance axon stability and caliber, especially in axonal regeneration^[9]. As another IF family member, artificial (rec)EsTK-based fibers might share similar working functions as NFs.

A potential material for peripheral nerve repair should provide suitable surface binding positions, appropriate mechanical stability and biocompatibility^[10]. Traditional materials used in this field include chemically synthetic polymers and naturally biopolymers^[11]. While synthetic polymers such as poly-lactic acid possess high versatility in fabrication process, they usually lack of biologically active sites and hence cannot mimic the *in vivo* environment very well^[12]. On the other hand, naturally biopolymers such as pure collagen provide high levels of biocompatibility, degradability and low levels of toxicity. However, they do not have sustainable mechanical properties working as scaffolds for peripheral nerves regeneration unless cross-linked^[13]. Recently, silk fibroins are attracting researchers' attention due to their remarkable mechanical properties and biocompatibility^[14]. Nevertheless, as stated in Chapter 2, in-depth research has revealed significant obstacles in bioengineering production of silk proteins. Artificial (rec)EsTK-based fibers have shown non cytotoxicity and the ability to tailor the mechanical properties by physical means which rival that of silks. Therefore, it might be considered as the potential method for peripheral nerves regeneration.

Previous research has revealed that fibers electro-spun from native hagfish slime thread proteins have the ability to induce the growth of chick dorsal root ganglion (DRG) (Figure 7.3). The arrows clearly point out that neurite outgrowth preferentially

follows that contour of the electro-spun hagfish thread fibers. At the molecular scale, these hagfish thread fibers have similar coiled-coil filamentous structures as NFs with preferred alignment, which could help the orientation control over axonal outgrowth. Furthermore, the biocompatibility and flexibility strongly support the view that fibers from native hagfish slime thread proteins are potential viable neural tissue scaffolds.

These preliminary results (unpublished) from native hagfish fibers demonstrates that the artificial fibers from recombinant hagfish slime thread proteins may work in a similar way, and hence represent a good model system for scaffolds in neural engineering. For further studies, dissociated rat dorsal root ganglion neurons could be used for the test since they are simple to culture and also represent the origin for the ascending sensory axons. The neurons could be dissociated and seeded onto the fabricated artificial hagfish fibers tethered to the base of cell culture wells. Neurite outgrowth could be digitally measured on video-captured images.

Figure 7.3 Fluorescent micrographs of neurites (red) growth tracking hagfish thread fibers (green). The arrows point out to neurite outgrowth preferentially following the contour of hagfish thread fibers. (Courtesy of Dr. Paul Guerette)

7.2.2 Utility in Biomedical Applications

Silk, which has been discussed in Chapter 2, shows the ability to be used as sutures for wound ligation^[15]. The tensile strength and elasticity of silk matches, or is even better, than many biomaterials currently used in restorative surgery. Further

coating treatment of silk with waxes or silicon to enhance their mechanical properties and reduce fraying makes silks useful for commercial developments as sutures (e.g., Perma-Hand™)^[16]. However, in-depth research has revealed significant obstacles in bioengineering production of silk proteins, including the instability of spider silk protein genes in bacteria or yeast, and harsh solvent such as hexa-fluoro-isopropanol (HFIP) or 6 M Lithium Bromide (LiBr) used in the production process. These obstacles make the industrial scale-up of artificial spider silks challenging. Therefore, other potential biomaterials with similar properties are explored in biomedical applications.

Artificial (rec)EsTK-based fibers, which exhibit properties comparable to silks after draw-processing and chemical cross-linking, might be applicable in similar application area of spider silks^[17]. Additional biological research on their biodegradability –the fibers must be metabolized by the host once their repair function has been completed– and on tissue reactivity are required^[18]. Further improvement of the fiber's mechanical performance must also be considered.

7.3 Proposed Future Work

7.3.1 Nano-Manipulation of Single Self-Assembled Filament

Atomic force microscope (AFM) based protocols have previously been used to directly probe the mechanical properties of single cytoplasmic IFs adsorbed onto a solid support in physiological buffer environment^[19]. In the tapping mode, single IF can be manipulated and stretched. Therefore, the micro-mechanical performances of single IF can be examined. The forces applied to the nano-scale filaments are less than 1 nN in the tapping mode. However, by reducing the amplitude of the cantilever oscillation, forces in the range of 30-40 nN can be applied to the solid support surface. In this way, the mechanical properties of single IF could be probed.

Figure 7.4 Schematic illustrations of AFM manipulation experiments on single IF in the tapping mode^[19]. The arrows on the left corner indicate the scan direction. (a) Normal scan. (b) Along one scan line the force applied increases and immediately decreases. (c) The amount of stretching induced by the tip motion can be approximated as $(L_1+L_2)/L_0$. Similar experiments could be conducted on (rec)EsTK filaments.

As another IF family member, the mechanical behavior of (rec)EsTK-based coiled-coil filament might be measured in a similar way. The persistent length –a mechanical property quantifying the stiffness and flexibility of a polymer– of single filament could be obtained^[20]. After comparison with rheology and tensile testing data, it would provide a more direct insight into the mechanical behavior of (rec)EsTK-based coiled coil filaments and artificial fibers. Single (rec)EsTK-based coiled-coil filament has been monitored in AFM in the tapping mode (Figure 7.5). These observations suggest that it may be possible to conduct similar nano-manipulation experiments described above.

Figure 7.5 AFM image of (rec)EsTK-based coiled-coil filaments indicated by arrows.

7.3.2 Further Microfluidic Devices Design and Application

In Chapter 6 two kinds of microfluidic devices with 1-inlet-1-outlet and 3-inlet-1-outlet were designed and tested. Although macroscopic long fibers were not obtained, the experiments provided the basic proof-of-concept that microfluidics technology could be applied to process (rec)EsTK-based nano-filaments into macroscopic fibers using elongational flow. This Chapter opened the door to such research but a wide range of improvements could be implemented.

First, the testing protein solution containing self-assembled filaments had only a concentration of 3 mg/mL. However, this concentration may not be sufficient for filaments to bundle together and form thick fibers. For instance, it has been reported that microfluidic devices were used to create silk fibers and the silk solution had a high concentration of 80 mg/mL^[21]. Therefore in the future, IF solution with a higher protein concentration should be tested whereas the solubility screening window should be considered. Second, for the 3-inlet-1-outlet design, both organic and inorganic solutions with low viscosity were used in the side channels to elongate and align the filaments coming from the main flow, but only yielded short and brittle fibers. On the other hand, solutions with high viscosity could be tested in this circumstance since they might provide a larger shearing force to elongate and align IF molecules. For instance, Kinahan et al. has shown that artificial silks could be obtained in microfluidic devices by using PEO as the side flow solution to avoid pearling instabilities^[21]. Therefore in the future, various solutions with high viscosity such as PEO and PEG could be examined. However, the protein solution coagulation process as well as PEO or PEG removal at the final step should also be considered. Third, the geometrical parameters could be altered as well. For the 1-inlet-1-outlet design, artificial fibers were obtained only when the channel cross-section approaching to square. This specific geometry could be applied to 3-inlet-1-outlet design, with square cross-section channels connecting to the outlet. Compared with 1-inlet-1-outlet design, 3-inlet-1-outlet design had larger channel dimensions, which did not match the size of native hagfish threads. Therefore, 3-inlet-1-outlet devices with smaller channel geometry parameters could be designed and tested in the future. Lastly, although a broad range of flow rates have been tested for each design, more precise flowing conditions could be examined. For instance, for the 1-inlet-1-outlet

design, only in 20, 30 and 40 $\mu\text{L}/\text{h}$ the fibers could be acquired. However, more refined flow rates such as 25 $\mu\text{L}/\text{h}$ could be tested since microfluidic devices are utilized on the micrometer scale and small changes may cause huge effects.

7.3.3 Other Methods to Fabricate Artificial (rec)EsTK-Based Fibers

As stated in Chapter 2, electro-spinning and wet spinning have been used to produce nano-sized artificial non-woven fibrous mat. Some successful examples include using keratin/PEO mixture, native cocoon silk and native spider silk^[22-24]. However, there are several challenges that need to be considered before using similar approaches for (rec)EsTKs.

Sufficient protein solutions with relatively high concentration and reasonable viscosity are required^[25]. At this stage, the production yield of (rec)EsTKs (10-20 mg/L) remains too low to apply these techniques. Therefore, increasing the production yield is the first challenge to be addressed. Possible methods include varying *E. coli* growth environments (for example changing fresh medium or add more induction chemicals) or using other organisms (for example yeast) for recombinant protein production^[26-27]. At the moment, the highest filament solution concentration is up to 200 mg/mL, which involves dissolving lyophilized coiled-coil filaments into 98% formic acid. However, higher concentration may be needed for electro-spinning and wet spinning testing. What's more, other than microfluidics which only needs small volume of testing sample, electro-spinning and wet spinning require larger sample volumes. For instance, when using electro-spinning to fabricate collagen nano-fibers, a high concentration collagen solution of 83 mg/mL is needed, together with 1.0 mL syringe and several cm long tubes^[28]. Therefore, protein solution containing (rec)EsTKs-based filaments must have high concentration as well as sufficient sample volume for electro-spinning and wet spinning testing. Furthermore, in order to achieve reasonable viscosity, filaments solutions may have to be mixed with polymer powders such as PEO or poly-lactic acid (PLA). Hence, the mixing conditions should be investigated as well.

7.3.4 Truncated and Mutagenesis of (rec)EsTKs to Study More Detailed Self-Assembly Mechanisms

In order to study the self-assembly mechanisms of hagfish slime thread proteins into filaments, the functional role of non-helical head and tail domains, as well as the central helical rod, may be examined by using truncated versions of (rec)EsTKs. For example, headless, tailless, headless/tailless of truncated (rec)EsTKs can be obtained through genetic engineering. Attempts to form self-assembled coiled-coil filaments for each case would then allow understanding the self-assembly process in greater details. Mutagenesis studies on the central rod domains might shed light on the key residues generating interactions in the coiled-coil regions, and it may also be possible to further improve the mechanical performance of the fibers by optimizing (rec)EsTKs at the molecular level.

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List of Publications

1. **Fu, J.**, Guerette, P.A. and Miserez, A., 2015. Self-Assembly of Recombinant Hagfish Thread Keratins Amenable to a Strain-Induced α -Helix to β -Sheet Transition. *Biomacromolecules*, 16(8), pp.2327-2339.
2. **Fu, J.**, Guerette, P.A., Pavesi, A., Horbelt, N., Lim, C.T., Harrington, M.J., and Miserez, A., 2016. Artificial Hagfish Protein Fibers with Ultra-High and Tunable Stiffness. (*Advanced Materials*. Submitted)
3. Ding, D., Guerette, P.A., **Fu, J.**, Zhang, L., Irvine, S.A. and Miserez, A., 2015. From Soft Self-Healing Gels to Stiff Films in Suckerin-Based Materials through Modulation of Crosslink Density and β -Sheet Content. *Adv Mater*, 27(26), pp.3953-3961.

Appendix

Illustration of EsTK α construction map

Protein sequence of (rec)EsTK α

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MSISQTVSKSYTKSVSRGGQGVSYSQSSSHKVGGGSVRYGTTYSSGGISRVLGFQGGAGGAA
SAGFGGSVGGSGLSRVLGGSVMVSGYRSGMGVGGLSLSTAGLPVSLRGVGAGKALHAITSAF
RTRVGGPGT SVGGYGVNYSFLPSTAGPSFGGPFGGPFGGPLGPGYIDPATLPSPDTVQ
HTRI REKQDLQTLN TKFANLVDQVRTLEQHNA ILKAQISMITSPSDTPEGPVNTAVVASTVT
ATYNAQIEDLRTNTALHSEIDHLTTIINDITTKYEEQVEVTRTLETDWNTNKNIDNTYLT
IVDLQTKVQGLDEQINTTKQIYNARVREVQAAVTGGPTAAYSIRVDNTHQAIDLTTSLQEMK
THYEVLATKSREEAFTQVQPRIQEMAVTVQAGPQAI IQAKEQIHVFKLQIDSVHREIDRLHR
KNTDVEREITVIETNIHTQSDEWTNNINSLKVDLEVIKKQITQYARDYQDLLATKMSLDVEI
AAYKKLLDSEETRISHGGGITITTNAGTFPGGLS AAPGGGASYAMVPAGVGGVGLAGVGGYG
FRSMGGGGGVGYGAGGGGVGYGVGGGFGGGMGMSMRSMGA AVGGGSYSGSGYSGGFGLS
SSRAGYSASRKSYS SARSSSRIY

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Illustration of EsTKy construction map

Protein sequence of (rec)EsTKy

MASHSSVSYSVRTGGTSAMIGSSGYGGSSSSSRAMGLGMGAAGLSMGGGSFRVGSAGIGGMG
 ISSGIGGMGISSRAGGMSAYGGAASGGAGGFVSGGVPMLGYGGGAGGFIGGVSPGIMASPAF
 TAGRAITSAGMSGVVGTLGPAGGMVPSLVSDEVKNILGTNLNQLASYVDKVRQLTIENETM
 EEELKNLTGGVPMSPDSTVNLENVETQVTEMLTEVSNLTLERVLEIDVDHLRATADEIKSK
 YEFELGVRMQLETDIANMKRDLEAANDMRVDLDSKFNFLEELTFQRKTQMEELNTLKQQFG
 RLGPVQTSVIELDNVKS VNLT DALNVMREEYQQVVTKNVQEAETICKMQIDQIQGISTQTTE
 QISILDKEINTLEKELQPLNVEYQRLTTYQTLGDRLTDLQNRESIDL VQFQNTYTRYEQEI
 EGNQVDLQRQLVITYQQLLDVKTALDAEIAITYKKLLEGQELMVRTAMADDFAHATVVRSGTLG
 GASSSSVGYGASSTTLGAISGGYSTGGGASYSAGAGGASYSAGAGGASYGVGGGYSGSSAM
 MEGSSSGHSMYSSSSMKRSSSKSASASAGGYGTS GHDSTIILQQ