CHARACTERIZATION AND BIOMEDICAL APPLICATIONS OF RECOMBINANT SILK-ELASTINLIKE PROTEIN POLYMERS

by

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DEDICATION

To my dad, mom, and my wife
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ABSTRACT

Biomaterials requirements nowadays are becoming more and more specialized to meet increasingly demanding needs for biomedical applications such as matrices for tissue scaffolds. Among various useful classes of biomaterials, protein-based materials have been extensively pursued as they can offer a wide range of material properties to accommodate a broader spectrum of functional and performance requirements. The advent of genetic engineering and recombinant DNA technology has enabled the production of new protein-based biopolymers with precisely controlled amino acid sequence. As an example, silk-elastinlike protein (SELP) polymers consisting of polypeptide sequences from native silk of remarkable mechanical strength and polypeptide sequences from native elastin that is extremely durable and resilient have been produced. In this dissertation, a particular silk-elastinlike protein copolymer, SELP-47K, was cast into film form, and fully characterized for its material properties, including the mechanical property, secondary structure transition, optical transparency, surface, and other physical, chemical properties. The relationship between mechanical property and protein secondary structure was investigated as well. In addition, the material property tunability which can be induced by physical, mechanical, and chemical treatments has been explored. It is worth noting that the physically crosslinked SELP-47K films displayed mechanical properties comparable to those of native elastin obtained from bovine ligament. Secondary structure study through Raman and FTIR spectra showed that methanol treatment is capable of inducing the \( \beta \)-sheet crystallization of silklike blocks, which act as physical crosslinks in the protein polymer chain network, thus
stabilizing the protein structure and conferring the improved material integrity. The SELP-47K protein polymer thin films displayed excellent optical transparency. In particular, its excellent optical transmittance (over 90%) in visible light range may indicate SELPs can be a family of promising biomaterial candidate for ocular applications. Besides material property characterization, SELP-47K protein polymer has been fabricated into a variety of drug delivery devices to sustainably release a common ocular antibiotic, ciprofloxacin over a period of up to 220 h, with near-first order kinetics.
CHAPTER 1: GENERAL INTRODUCTION

1.1. Protein-Based Biomaterials

Medical technologies nowadays have encompassed a range of drug and gene delivery systems [1-3], tissue engineering [4-6] and cell therapies [4, 7], organ printing [8-10] and cell patterning [11], nanotechnology based imaging and diagnostic systems [12-13] and microelectronic devices [14]. Biomaterials, including metals, ceramics, polymers, and biopolymers, etc. are an indispensable part for these medical technologies. The concept and meaning of biomaterials have evolved over the years, and biomaterials have now been defined as “a material intended to interface with biological systems to evaluate, treat, augment or replace any tissue, organ or function of the body” [15]. In current healthcare market, biomaterials requirements are becoming more and more specialized to meet increasingly demanding needs for biomedical applications such as matrices for tissue scaffolds. As the properties of materials depend on the nature of the molecules, the environments, and the interactions between them, one possible approach to meet these performance criteria is to achieve better control over the tailoring of the components and their interactions that govern the material properties.

Among various useful classes of biomaterials, protein-based materials have been extensively pursued as they can offer a wide range of material properties to accommodate a broader spectrum of functional and performance requirements [16-19]. Furthermore, protein polymers could provide a new model system for understanding the relationship between materials properties, functions, and molecular structures. Whether used directly
as new materials [17], or used in composite materials [18], polymer properties can be controlled through fine modification of chain microstructure. The biocompatibility, biodegradability of these protein polymers, the production from renewable resources will make them increasingly attractive in the future.

Natural structural proteins confer critical structural and bioactive properties, such as stiffness, rigidity, and mobility to otherwise fluid biological components. However, one particular protein, which may have useful functions such as mechanical strength, may be found limiting on other critical properties such as elasticity. Nature solves this problem by mixing multiple materials together to form multifunctional composite materials \textit{in vivo}. As an alternative to blending, genetic engineering has enabled generation of combinations or hybrids of recombinant structural proteins to achieve control of functional features.

1.2. Natural Protein Biopolymers

Many natural protein polymers have been studied for their remarkable mechanical, chemical, electrical, optical, and biological properties. Silk [20-24], collagen [25-28], elastin [29-31], keratin [32-34], and resilin [35-37] are some of the most commonly investigated natural protein-based biomaterials. Although the primary amino acid sequences of each class of protein polymers may vary, they have their unique highly repetitive amino acids blocks within the protein chain. The repeating building blocks within the proteins will form long range highly ordered molecular secondary structures which could confer the proteins unique structure-related material properties.
Silk proteins are fibrous proteins synthesized by spiders and silkworms. The primary repeating unit for silk fibroin consists of \([\text{GAGAGS}]_n\) \([24, 38]\). Through a spinning process by silkworms or spider, silk fibroin undergoes a very rapid transition from aqueous state to an insoluble form characterized by antiparallel \(\beta\)-sheet crystals. The antiparallel \(\beta\)-sheet crystals formed by the repeat sequence \([\text{GAGAGS}]_n\) through hydrophobic interactions serve as physical crosslinkers in the materials polymer chain network. Thus, silk fibroins have high tensile strength and toughness.

Collagen (Type I ~ XXVIII) is a group of naturally occurring proteins found in animals, especially in the flesh and connective tissue of mammals. It is the main component of connective tissue such as tendon, ligament, cartilage, and skin, with up to 25% – 35% of whole-body protein content consisting of different types of collagens. Collagen is composed of a right-handed triple helix, formed by three intertwined left-handed helices, and generally consists of different chain compositions depending on the specific type of collagen \([28]\). The most common motif in the amino acid sequence of collagen is \((\text{GPX})_n\), where X is any amino acid other than glycine, proline or hydroxyproline. If collagen is irreversibly hydrolyzed through heat or chemicals, it becomes gelatin, which is also frequently used as a biomaterial. Collagen is one of the long, fibrous structural proteins, and it provides great strength to associated tissues, including skin, and blood vessels.

Elastin is a critical protein in forming elastic fibers in connective tissue such as blood vessel that allow many tissues in the body to resume their shape after stretching or contracting \([30-31]\). The hydrophobic domains of elastin are rich in non-polar amino
acids, a common repeating motif of \([\text{GVGVP}]_n\), while the hydrophilic domains contain a significant amount of lysine that can be crosslinked and help to stabilize elastin structure. Elastin can be isolated from animal tissues such as skin, or it can be made by linking the soluble precursor of elastin, tropoelastin protein molecules, to form a massive insoluble, durable elastin crosslinked network. Tropoelastin is a specialized protein with a molecular weight of 64 to 66 kDa, and an irregular or random coil conformation made up of 830 amino acids.

Keratin is a family of fibrous structural proteins [33-34] that is a key component making up the epidermal appendage structures, such as hair, skins, nails, etc. Keratin monomers assemble into bundles to form intermediate filaments in cytoplasmic epithelia, which are tough and insoluble and form strong unmineralized tissues. Both \(\alpha\)-keratin and hard \(\beta\)-keratin are composed of type I and II protein polymer chains with different composition and molecular weight. The keratin supermolecular aggregation depends on the properties of the individual polypeptide sequence. The \(\alpha\)-helix, \(\beta\)-sheet, and disulfide bridges, which are formed through cysteine-cysteine interactions, are crucial to the tough and durable keratin structures [33].

Resilin is a super elastomeric protein found in many insects which enables the species to jump or pivot their wings efficiently for millions of times without fatigue over their lifetime [36-37]. Resilin is currently the most efficient elastic protein known [37]. Resilin usually does not have any regular secondary structures, but its random coiled chains are crosslinked by tyrosine links which confer the elasticity needed to propel. Crosslinked resilin exhibits high resilience up to 95% under high-frequency motion, and
has deformability over 300%, providing an option for biomaterial application during high-frequency elastic motion.

1.3. Synthetic Protein Biopolymers

It is now recognized that the proteins found in all living species are linear polyamides, formed by the end-to-end linkage of a set of 20 α-amino acids [39]. Smaller linear polyamide sequences are usually called peptides. Each kind of protein has a unique amino acid sequence which in turn determines its specific three-dimensional conformation. The hydrolysis of proteins to smaller molecules generates small chains of amino acids (peptides) and ultimately the amino acids.

The development of synthetic polymer systems in the past few decades has led to the production of protein-based biomaterials with a broad range of applications in human healthcare [40-41]. Currently, there are two major strategies to synthesize protein-based polymers, one is traditional chemical polymerization method; and the other one is biological synthesis, which is mediated by transcription and translation process in living cells. Advances in chemistry, materials science, and cell biology are enabling the development of new protein materials for novel applications.

1.3.1. Chemically Synthesized Biopolymers

Polymerization is a process of combining many small molecules known as monomers into a covalently bonded chain or network. By conventional methods of organic synthesis, the preparation of peptides containing more than just a few amino
acids is a difficult task. Therefore, conventional chemical synthesis is usually utilized to make short peptides. The order in which peptide bonds are formed and the synthesis is carried out determines the strategy of a peptide synthesis. There are two major strategies, stepwise synthesis and segment condensation. In stepwise synthesis, amino acids are added one at a time, from the N-terminal to C-terminal amino acid, to a growing peptide chain. For segment condensation, short segments are constructed and then coupled. Both methods require group protection, activation, coupling, and removal of protecting groups during the synthesis process. Due to polydispersity, the chemically synthesized peptides usually need to go through several purification steps to be at high purity. With genetic engineering and recombinant DNA technology, Polydispersity is no longer a problem for biologically synthesized protein polymers, and functional groups can be placed at precise locations along the chain.

1.3.2. Biologically Synthesized Biopolymers

Genetic engineering of protein-based materials provides material scientist with highest levels of control in material microstructures, properties, and functions [4]. For example, multi-functional block protein copolymers in which individual building block may possess distinct mechanical, chemical, and/or biological properties have been biosynthesized [31, 37, 42-51]. Polypeptide sequences derived from well-studied structural proteins (e.g., collagen [43-44, 47], silk [43-44, 47, 50], elastin [31, 46, 48-49, 51], resilin [37]) are often used as motifs in the design and biosynthesis of new recombinant protein-based materials, in which new functional groups may be
incorporated. The resulting protein polymers often inherit some structure and property characteristics from their parent proteins. For instance, silklike protein polymers form β-sheet crystals that are responsible for the high tensile strength and fracture toughness of native silk [43-44], and elastinlike proteins display the excellent elasticity characteristics of native elastin [48, 51].

### 1.4. Recombinant Silk-elastinlike Protein Polymers

Following a similar strategy, a series of silk-elastinlike proteins (SELPs) consisting of repeating polypeptides sequences derived from silk of superior mechanical strength and elastin that is extremely durable and resilient [47, 50] has been produced. SELPs have been fabricated into various structures, such as micro-diameter fibers and nanofibrous scaffolds, displaying unique mechanical properties that combine high deformability, tensile strength, and resilience [52-54]. The potential of SELP polymers for applications in drug delivery [55-56] and tissue engineering [57-58] is being extensively investigated.

In this dissertation, a particular silk-elastinlike protein polymer SELP-47K was used throughout the study. SELP-47K copolymer has a monomer structure of \((S)_4(E)_4(EK)(E)_3\), in which S is the silklike sequence GAGAGS, E is the elastinlike block GVGVP, and EK is the lysine modified elastinlik block with the penta-peptide sequence GKGVP. The monomer is repeated 13 times in each polymer chain. This design provides a repeating interdispersion of “hard” blocks and “soft” blocks in the same polymer chain enabling each chain to form multiple physical crosslinks with neighboring chains.
Notably, the silklike blocks of SELP-47K are capable of crystallizing into $\beta$-sheets, thereby forming mechanically robust physical crosslinks between elastinlike sequences, which, in turn, lower the crystallinity of the silklike blocks and thus enhance the solubility and processability of SELP-47K.

1.5. Scope of the Work

There is a great need of mechanically robust and biologically functional tissue scaffolds to support cell adhesion, proliferation, and other cellular activities in biomedical sciences. Silk-elastinlike protein (SELPs) polymers, as a novel family of protein-based materials, are being processed into various forms, such as hydrogel, fibers, film, and are being extensively studied for their properties and applications in drug delivery, cell therapy, and tissue engineering. By using SELP-47K protein polymer as a model polymer, the overall objectives of this dissertation are to:

1. Characterize the mechanical properties of SELP-47K, and compare them with the mechanical behavior of their parent proteins;

2. Investigate the protein polymer secondary structures, and how the molecular structure changes is related to its mechanical behavior;

3. Study the effect of mechanical, physical, and chemical treatments on protein polymer properties, such as mechanical property, secondary structures, etc.

4. Characterize the optical transmittance of SELP-47K protein polymer films in both dry and hydrated states; other optical, physical, and surface properties are examined and compared as well;
5. Fabricate various drug eluting devices from SELP-47K polymer, and study its potential drug release capability for ophthalmic applications.
CHAPTER 2: MECHANICAL CHARACTERIZATION AND MORPHOLOGY OF CASTED PROTIEN POLYMER FILMS

2.1. Introduction

Elastin is an important load-bearing and abundant protein in connective tissue that is elastic and allows many tissues, like the blood vessel, skin, lung etc. in the body to resume their shape after stretching or contracting [59-60]. The formation of elastin in vivo involves a complex process from its soluble precursor, tropoelastin. Because elastin is extremely insoluble, analysis of elastin has largely been limited to soluble tropoelastin and soluble segments of elastin [61-62]. Furthermore, this insolubility has precluded the use of native elastin as a useful material in bioengineering and regenerative medicine. Thus, there a great need to engineer materials with excellent elasticity of elastin for various biomedical applications.

Genetic engineering has enabled the synthesis of elastin analogs with high levels of control over their primary structure. For instance, elastin-like protein (ELP) polymers composed of repeated pentapeptide sequences displayed interesting physical behavior such as high deformability and inverse temperature transition (ITT) [63-66]. For materials applications, ELPs are often physically or chemically crosslinked in order to attain sufficient stability and mechanical strength. However, the mechanical resilience of most crosslinked ELPs was far inferior to that of native elastin.

The focus of this study was to analyze the mechanical properties of SELP-47K films, including their resilience and time-dependent viscoelastic deformation. The
capacity of the crystallized silklike blocks to stabilize the SELP-47K structure and to function as physical crosslinks was evaluated. In addition, lysine residues present in SELP-47K chain also allow chemical crosslinking of the elastinlike blocks and possible enhancement of the mechanical properties of SELP-47K. Accordingly, the effects on the mechanical properties of SELP-47K films after glutaraldehyde crosslinking were examined.

2.2. Materials and Methods

2.2.1. Solvent Casting for Sample Preparation

Frozen SELP-47K aqueous solutions at a concentration of 13% (w/w) were generously provided by Protein Polymer Technologies, Inc. (PPTI, San Diego, CA). The protein solution was lyophilized and dissolved in deionized water at a concentration of 20% (w/w) at room temperature. The prepared protein solution was mixed thoroughly by Vortex, and trapped air bubbles were removed by centrifugation prior to use. The protein solution was then poured into polypropylene casting molds and solvent evaporation was performed at room temperature for 24 hours under ambient conditions. After complete solvent evaporation, SELP-47K films, which were denoted as non-treated films, were gently peeled off the casting molds for analysis or further chemical treatments.

2.2.2. Chemical Treatments

SELP-47K cast films were treated with 99.9% methanol (MeOH, Fisher Scientific) for 4 hours, and air-dried before further processing or mechanical analysis. After air-
drying, some MeOH-treated films were cross-linked with 10 ml of glutaraldehyde (GTA, Mallinckrodt Baker) solutions at a concentration of 1% (w/v) in phosphate buffer at pH 7.4 for 24 hours at room temperature, following a procedure used by Bigi et al. [67]. The methanol-treated and then glutaraldehyde-crosslinked films were denoted as crosslinked films. Crosslinked films were extensively rinsed using deionized water prior to mechanical and structural analysis.

2.2.3. Mechanical Characterization

Methanol-treated non-crosslinked and crosslinked SELP-47K films were cut into rectangular samples with dimensions of 30 mm × 5 mm or 15 mm × 2 mm for mechanical analyses. The gauge length (the sample length between two clamps) for the large samples was approximately 10 mm while that of the small samples was between 5.5 and 6.0 mm. Depending on the sample gauge length, the maximum travel distance of the drive shaft of the dynamic mechanical analyzer (DMA) was 20 to 24 mm. Small samples that could be broken using the current set-up were used for measurement of the ultimate tensile strength and deformability of SELP-47K films. Large samples were used to more accurately analyze the mechanical behavior of SELP-47K films at small and medium deformation up to 50% strain. Samples were hydrated in 1X phosphate buffered saline (PBS) at 37 °C, which contained 0.2 mg/ml NaN₃ to prevent biological contamination. Hydrated film thickness was typically 0.2 mm, as measured by optical microscopy.

Mechanical characterization of SELP-47K samples was performed using a PerkinElmer diamond DMA, and samples were immersed in a jacketed beaker filled with
1X PBS at 37 °C. Both methanol-treated non-crosslinked and crosslinked films were evaluated by several mechanical testing protocols including: (i) Uniaxial tensile failure. Three to five replicate small samples of each type of film were monotonically extended to failure so the deformability and ultimate tensile strength were obtained. Displacement was applied at a fixed rate of 0.25 mm/min. The maximum displacement within each loading step was 5 mm due to a limitation of the instrument, so multi-step tension experiments were programmed for the failure study. Loading steps were successively initiated with a time lapse between steps of only a few seconds, inducing negligible stress relaxation in the tensile stress-strain analyses of SELP-47K films (see the small stress drops marked by arrows in Figure 2.1.). The Young’s modulus of a sample was determined as the slope of the stress-strain curve in the first 20% deformation region. (ii) Mechanical preconditioning. Ten to twelve samples of each type of film were cyclically stretched to approximately 50% strain (i.e., 5 mm displacement with a gauge length of approximately 10 mm) for 10 cycles with an off-loading period of 5 minutes between cycles. Displacement of the cross-head was applied during loading and unloading at a fixed rate of 1 mm/min. Resilience was calculated from the loading and unloading curves at a fixed cycle and then averaged across replicate samples.

\[
\text{\% resilience} = 100 \times \frac{\text{area under unloading curve}}{\text{area under loading curve}} \quad (1)
\]

(iii) Cyclic loading behavior. The loading and unloading behaviors of preconditioned samples were examined under different cyclical deformations (e.g., 10%, 20%, 30%, and 40% strains). Displacement was applied during loading and unloading at a constant speed of 0.5 mm/min. (iv) Creep and stress relaxation. Three to five replicate samples were
prepared for creep analysis. Constant stresses were applied for time periods of up to 15 hours. Three to five replicate samples were stretched to 20% strain at a constant rate of 0.5 mm/min, and the stress over time was recorded for 15 hours.

2.2.4. Scanning Electron Microscopy (SEM) Imaging

The surface morphologies of three types of SELP-47K films, including non-treated, methanol-treated, and glutaraldehyde-crosslinked films were examined using a Hitachi-S4800 scanning electron microscope (SEM). Typically, films samples were vacuum-dried in a dessicator overnight and then coated with a thin layer of platinum for 10-30 seconds using a sputter coater. Films which were preconditioned, and stretched to failure were also checked under SEM to evaluate their changes in microstructure. The typical accelerating voltages used in the experiments to record SEM images were 5-15 KV.

2.3. Results and Discussions

2.3.1. SELP-47K films displayed high deformability.

Methanol-treated non-crosslinked SELP-47K films displayed enhanced mechanical strength as compared to nontreated films, which were can readily dissolved back in 1× PBS. Fully hydrated, methanol-treated non-crosslinked SELP-47K films possessed strain at failure of 190 ± 60% and ultimate tensile strength of 2.5 ± 0.4 MPa (Figure 2.1.). They displayed nearly linear deformation response to external applied forces, with an estimated average Young’s modulus of 1.3 MPa. After GTA crosslinking,
tensile stress-strain analysis revealed dramatic increases in deformability (245 ± 58%) and ultimate tensile strength (5.4 ± 1.1 MPa) of crosslinked films (Figure 2.1.). It is worthwhile to compare the mechanical behavior of methanol-treated non-crosslinked SELP-47K films to those of native silks and elastins. When analyzed in dry state, Gosline et al. reported that both the major ampullate (MA) silk of spiders and silkworm silk showed Young’s modulus of 7-10 GPa and maximum deformability of 18-27% [68-69]. Although hydration reduced the Young’s modulus of MA silk by 100-fold, Shao and Vollrath demonstrated that its ultimate tensile strength remained around 600 MPa [70]. In contrast, the mechanical properties of methanol-treated non-crosslinked SELP-47K films are very comparable to those of native elastin from bovine ligament, which displayed a Young’s modulus of 1.1 MPa, ultimate tensile strength of 2 MPa, and deformability of 150% [69, 71].
Figure 2.1. Representative stress-strain analysis of methanol-treated non-crosslinked and crosslinked SELP-47K films. Small stress drops in crosslinked SELP-47K films (noted by arrows) were due to the minimal time lapse between successive steps.

In our model, the methanol-treated non-crosslinked SELP-47K films form a three-phase structure: a semirigid phased formed by the crystallized silklike blocks, a flexible phase comprised of the hydrated elastinlike blocks, and a mixed phased formed by the interpenetrating crystallized silklike and hydrated elastinlike bocks. When an external mechanical force is applied, the flexible elastinlike blocks deform first. A further increase in mechanical force will lead to deformation and disruption of the mixed phase [72-73]. Consequently, the irreversible deformation and subsequently poor resilience of SELP-47K films may be observed. However, the disruption of the mixed phase also facilitates separation of the crystallized silklike and hydrated elastinlike phases. As a result, a sample may appear to be more resilient, if subsequent deformation does not exceed the previous deformation. Thus, it is anticipated that mechanical preconditioning, in which a sample experiences repetitive cyclic strain, will stabilize the microstructures of SELP-47K films and improve their resilience under subsequent deformation that is lower than the preconditioning strain.

2.3.2. Mechanical preconditioning improved the resilience of SELP-47K films

The influence of mechanical preconditioning on the material behavior of methanol-treated non-crosslinked films and crosslinked films was examined by
subjecting hydrated samples to repetitive cyclic displacements of 5 mm, which corresponded to about 45% strain. We observed the accumulation of a small residual deformation and a slight decline in peak stress that stabilized after several loading cycles in both methanol-treated non-crosslinked (Figure 2.2.A) and crosslinked films (Figure 2.2.B). The small residual strains (around 5%) were largely due to deformation-induced structural changes, although re-alignment of an imperfectly loaded sample was responsible for small lags observed in the first loading curve. Without preconditioning, crosslinked films possessed an enhanced resilience (77 ± 2%, Figure 2.2.B) over their methanol-treated non-crosslinked counterparts (resilience of 66 ± 4%, Figure 2.2.A). The difference in resilience suggests that glutaraldehyde crosslinking likely limited the chain rearrangement of hydrated elastinlike blocks, decreasing the hysteresis of SELP-47K films. Nevertheless over 10 loading cycles, the resilience of the methanol-treated non-crosslinked films increased from 66 ± 4% to 86 ± 4% (Figure 2.3. and Table 1, n = 6). Likewise, the resilience of crosslinked films increased from 77 ± 2% to 88 ± 1% after 10 cycles of preconditioning (Figure 2.3. and Table 1, n = 6), a more modest increase in resilience than their methanol-treated non-crosslinked counterparts. The greatest increase in resilience largely occurred after the first cycle, presumably due to stabilization of deformation-induced changes in microstructure. It bears significance that the obtained resilience of 86 ± 4% for preconditioned methanol-treated non-crosslinked films and of 88 ± 1% for preconditioned crosslinked films closely matches that of native elastin, which is 90% [69, 71]. This similarity suggests that under small to medium strain the
elastinlike blocks of SELP-47K in mechanically preconditioned films may be solely responsible for deformation while the crystallized silklike blocks remain intact.

Figure 2.2. Representative mechanical preconditioning of methanol-treated non-crosslinked (A) and crosslinked SELP-47K films (B). Both samples were cyclically
stretched to a maximum displacement of 5 mm for the first 10 cycles. Repeatable stress-strain curves were obtained in both samples after 5 to 6 cycles of preconditioning, suggesting their microstructures were stabilized.

Figure 2.3. Resilience of methanol-treated non-crosslinked (♦) and crosslinked (●) SELP-47K films (n = 6). The resilience of non-crosslinked films increased from 66 ± 4% to 86 ± 4%, and that of crosslinked films increased from 77 ± 2% to 88 ± 1% upon 10 cycles of preconditioning. For clarity, only the negative error bars of the resilience of non-crosslinked films are plotted. The apparent small drop in resilience at cycle 3 was due to the manual repositioning of the sample to remove the buckling that occurred after two cycles of loading and unloading.

Table 2.1. Comparison of Young’s Modulus (E), resilience (R), and tensile strength at 40% strain (σ_{40}) of methanol-treated non-crosslinked and crosslinked SELP-47K films
<table>
<thead>
<tr>
<th></th>
<th>E at 1&lt;sup&gt;st&lt;/sup&gt; cycle (MPa)</th>
<th>E at 10&lt;sup&gt;th&lt;/sup&gt; cycle (MPa)</th>
<th>R at 1&lt;sup&gt;st&lt;/sup&gt; cycle (%)</th>
<th>R at 10&lt;sup&gt;th&lt;/sup&gt; cycle (%)</th>
<th>σ&lt;sub&gt;40&lt;/sub&gt; (MPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-crosslinked</td>
<td>1.57 ± 0.3</td>
<td>1.66 ± 0.37</td>
<td>66 ± 4</td>
<td>86 ± 4</td>
<td>0.63 ± 0.14</td>
</tr>
<tr>
<td>Crosslinked</td>
<td>3.17 ± 0.23</td>
<td>3.34 ± 0.26</td>
<td>77 ± 2</td>
<td>88 ± 1</td>
<td>1.36 ± 0.11</td>
</tr>
</tbody>
</table>

2.3.3. Mechanically preconditioned films demonstrated highly linear elasticity

Both methanol-treated non-crosslinked and crosslinked films displayed linear viscoelastic behavior up to 40% strain (Figure 2.4.). The stress-strain analysis further revealed a Young’s modulus of 1.67 ± 0.37 and 3.34 ± 0.26 MPa for preconditioned methanol-treated non-crosslinked and preconditioned crosslinked films, respectively (Table 1). Therefore, GTA-crosslinking doubled the Young’s modulus of SELP-47K films. Since the lysines capable of GTA-crosslinking are only within the elastinlike blocks of SELP-47K, this reinforced our reasoning that under small to medium strain the elastinlike blocks of SELP-47K films are primarily, if not solely, responsible for deformation.
Figure 2.4. Representative stress-strain curves of mechanically preconditioned films. After 10 cycles of preconditioning at ~45% strain (Figures 2.2.A, 2.2.B), both methanol-treated non-crosslinked and crosslinked SELP-47K films demonstrated highly linear elasticity.

2.3.4. Stress relaxation and creep analysis confirmed the excellent elasticity of preconditioned films

When deformation was held at 20% strain, the initial stress was 683 KPa in mechanically preconditioned crosslinked films and 313 KPa in preconditioned non-crosslinked films (Figure 2.5.). Stress in preconditioned crosslinked films dropped to 645 KPa in the first 5 minutes and stabilized thereafter at 620 KPa. Compared to a 9% decrease in stress of crosslinked films prior to equilibrium, non-crosslinked SELP-47K films exhibited a 16% decrease in stress from 313 to 260 KPa in 15 hours.
Figure 2.5. Representative stress relaxation of preconditioned non-crosslinked and crosslinked SELP-47K films at 20% constant strain.

Creep analysis was conducted by placing films under constant stress that would produce an initial approximate strain of 20-30%. Under a sustained stress of 900 KPa, preconditioned crosslinked SELP-47K films exhibited a creep strain of 4%, from an instantaneous deformation of 25.3% to a final deformation of 29.2% after 15 hours (Figure 2.6.). Likewise, creep for preconditioned non-crosslinked films under 400 KPa stress for 15 hours approached 5%. These modest creep and stress relaxation results suggested that medium deformations (e.g., 20%) and mechanical stresses (e.g., 900 KPa) induced minimal damage to preconditioned SELP-47K films, and that their deformation response was elastic in nature.
Figure 2.6. Representative creep of preconditioned crosslinked (solid line) and preconditioned non-crosslinked (dashed line) SELP-47K films at 900 and 400 KPa constant stress, respectively. The stress-strain analysis of preconditioned methanol-treated non-crosslinked and crosslinked SELP-47K films revealed a linear viscoelastic region up to 40% for both types of films. Different stress levels were chosen for creep tests of preconditioned methanol-treated non-crosslinked and crosslinked SELP-47K films to ensure that the total deformation was within 40% strain, in particular, around 30% strain.

2.3.5. SEM analysis revealed changes in the microstructures of SELP-47K films

Along with the conformational conversion revealed by Raman spectroscopy, methanol treatment also induced changes in the surface morphology and microstructure
SEM analysis revealed a micro-fibrilar structure of non-treated SELP-47K films as prepared from aqueous solution (Figure 2.7.A). A close examination of the SEM images suggested that these randomly oriented micro-fibrils were formed by many protein globules of micrometers in size. This observation is consistent with the hierarchical self-assembling model proposed by Jin and Kaplan for silk [38]. According to their model, silk proteins in aqueous solutions form micelles of 100 to 200 nm in size and these micelles further aggregate into larger globules due to hydrophobic folding and hydrophilic interaction. In SELP-47K aqueous solution, the elastinlike blocks GVGVP may form a relatively loose and less-hydrated core, while the more hydrophilic silklike blocks GAGAGS, the pentapeptide sequence GKGVP with a charged lysine residue, and the two non-repetitive amino acid sequences at the N- and C-termini of the polymer containing more charged residues may form a corona. The core-corona micelles form large globules due to weak hydrophilic interaction. Consequently, non-treated SELP-47K films were unstable under mechanical forces when fully hydrated. Upon methanol treatment, the silklike blocks of SELP-47K molecules coagulated into insoluble silk II crystals, stabilized by strong intermolecular hydrogen bonding. The insoluble β-sheet crystals likely formed an interconnected network, linked by the flexible elastinlike blocks. As a result, SEM analysis of SELP-47K films revealed that the aggregates of core-corona micelles disappeared after methanol treatment (Figure 2.8.B). Instead, the methanol-treated non-crosslinked SELP-47K films had a wrinkled surface likely due to the coagulation effects of the methanol treatment. SEM analysis further revealed that
GTA-crosslinking of methanol-treated SELP-47K films induced some changes, although subtle, in their microstructure and surface morphology (Figure 2.8.C).

Figure 2.7. Surface morphology of SELP-47K films examined by SEM: non-treated (A); methanol-treated non-crosslinked (B); and methanol-treated and then glutaraldehyde-crosslinked (C).
While mechanical preconditioning is often used to stabilize the microstructures of soft materials in order to obtain more consistent mechanical properties, the underlying conformational and microstructural changes induced by this processing have rarely been evaluated [74]. We examined mechanically preconditioned SELP-47K films by SEM to determine if microstructural changes would correlate with the mechanical changes observed. Methanol-treated non-crosslinked SELP-47K films revealed the deformation-induced, anisotropic structural changes of mechanical preconditioning. In contrast to the isotropic surface morphology of SELP-47K films (Figure 2.7.B), a fiber-like texture appeared on the film surface after mechanical preconditioning and the texture was aligned along the direction of the applied strain (Figure 2.8.A). It is likely that mechanical preconditioning induced molecular alignment and led to the formation of the anisotropic microstructures. Interestingly, less anisotropic surface morphology was observed for crosslinked SELP-47K films after mechanical preconditioning (Figure 2.8.B). In this case, GTA-crosslinking likely reduced the molecular alignment and enhancement of intermolecular hydrogen bonding possible between molecules along the direction of the preconditioning strain.
Figure 2.8. Surface morphology of methanol-treated non-crosslinked (A) and methanol-treated glutaraldehyde-crosslinked (B) SELP-47K films after mechanical preconditioning. The direction of the preconditioning strain is indicated by the arrows.

2.3.6. SEM analysis suggested ductile fracture as a failure mode for SELP-47K films

The fracture surfaces of methanol-treated non-crosslinked SELP-47K films were also analyzed by SEM (Figure 2.9.A-2.9.C). Consistent with the high deformability revealed by the tensile analysis, the rough fracture surfaces suggest ductile fracture as a failure mode for the fully hydrated SELP-47K films (Figure 2.9.B, 2.9.C). A close examination of the SEM images suggests that a crack was likely initiated in region B (Figure 2.9.A). The accumulated strain energy may have induced the disruption of the crystallized physical crosslinks and the pulling-out of polymer chains and hard phases, resulting in the formation of a rough, dimpled fracture surface (Figure 2.9.B). As the crack propagated and the strain energy was gradually released, the pulling-out of polymer chains and hard phases became less feasible and, as a consequence, relatively smoother fracture surfaces were observed (Figure 2.9.C).
2.4. Conclusions

Recombinant silk-elastinlike protein copolymer SELP-47K films displayed excellent elasticity after mechanical preconditioning, shown by uniaxial tensile stress-strain, creep, and stress relaxation analyses. In particular, the mechanical properties of
methanol-treated non-crosslinked SELP-47K films, including Young’s modulus, resilience, deformability, and tensile strength, closely match those of native aortic elastin and elastin fibers from bovine ligaments. The Young’s modulus of SELP-47K films was further doubled by glutaraldehyde crosslinking.
CHAPTER 3: SECONDARY STRUCTURE TRANSITIONS

3.1 Introduction

It is well established that β-sheet crystals are responsible for the high tensile strength and fracture toughness of silk [38, 75]. However, native silk in spider’s and insect’s glands exists in a metastable silk I form, the conformation of which has not been well defined [76-77]. Under shear or elongation flow, silk molecules extend and align enhancing intra- and intermolecular interactions, and silk I is converted into the β-sheet dominated Silk II structure [76]. In the natural silk spinning process, charge suppression by adjusting the pH allows tight molecular packing and β-sheet conversion necessary for fiber formation [78]. Likewise, metallic ions, which may interact with proteins and alter their secondary structures, are another important determinant in silk conformational changes and fiber formation [78-79]. Evident by in vitro studies, alcohol such as methanol is very effective in disrupting hydrogen bonds between protein and water, consequently promoting the formation of intra/inter-molecular hydrogen bonding, conformational changes, and water removal. In order to elucidate conformational inter-conversion and silk spinning mechanisms, model peptides comprised of poly(alanine) and poly(glycylalanine) repeats that are found in spider dragline silk [80] and GAGAGS repeating motifs found in silkworm silk [81] have been used [82-85]. While β-sheet crystals give rise to the high strength and toughness of silk, plausible deformation mechanisms (e.g., longitudinal and lateral shearing) of β-sheet structures may result in irreversible conformational changes and thus poor resilience of silk [86].
In contrast, elastin-like proteins (ELP) consisting of poly(VPGVG) display excellent resilience but low mechanical strength, originating from their less-ordered conformers such as β-turn and β-sheetlike structure. Evident by Raman spectroscopy [87], NMR [88] and X-ray diffraction [89], the PG dipeptides form repetitive β(II)-turns, which are found between VG segments. These flexible VG segments undergo large-amplitude librations and the reduction of their librational entropy upon chain extension may provide the restoring force for return to the relaxed state [90]. Secondary structural analysis of native elastin by Debelle et al. revealed around 35% β-strands and 55% unordered conformations, although random coil and β-turn were not distinguished [91]. Likely, changes in the conformational entropy of unordered structures are also responsible for the elasticity of elastin and ELP, as suggested by Hoeve and Flory [92]. In addition to the entropy change of elastin backbones, hydrophobic hydration was suggested to be another important factor contributing to elasticity [93-94]. When elastin is stretched, its hydrophobic domains are more exposed to water and the enhanced polymer-water interaction reduces the orientational entropy of the hydrophobic hydration layer, providing the driving force for the recoiling of the elastin chain. Similar to silk, the micro-phase separation and mechanical behavior of ELP greatly depend on its processing conditions [66, 95].

Therefore, it is expected that secondary structure has profound influences on the mechanical properties of silk, elastin, and their derivatives. By combining the silklike sequence GAGAGS and the elastinlike sequence GVGVP, recombinant SELP microfibers display high deformability and high tensile strength and SELP films possess
excellent resilience [52, 54]. In this study, Raman and Fourier transform infrared spectroscopy were used to analyze the secondary structure of SELP-47K protein films. In particular, the influences of chemical (i.e., methanol) treatments and mechanical preconditioning on the secondary structure of SELP-47K films were investigated. Additionally, lysine residues present in the SELPs design allow glutaraldehyde crosslinking of the elastinlike blocks and enhancement of the mechanical properties of SELPs. Accordingly, changes in the secondary structure of SELP-47K films due to glutaraldehyde crosslinking were examined. A better understanding of the molecular basis for the deformation behavior of SELP films is essential for the design of biomimetic protein polymers with tailored mechanical properties.

3.2. Materials and Methods

3.2.1. Raman Spectroscopy

Raman spectra of SELP-47K films were recorded on a Thermo Nicolet Almega microRaman system (Thermo Scientific). A solid-state laser with wavelength of 532 nm was used as the spectroscopy excitation source. Three types of SELP-47K films, including the non-treated films, methanol-treated films, and methanol-treated after 10 cycles of mechanical preconditioning, were analyzed using Raman spectroscopy. The Raman spectrum of methanol-treated glutaraldehyde-crosslinked films was not obtained due to heavy fluorescence.
3.2.2. ATR-FTIR Spectroscopy

Four-types of SELP-47K films, including non-treated, MeOH-treated, MeOH-treated and preconditioned, and MeOH-GTA-treated films were analyzed for their FTIR spectra. A Magna-IR 560 Nicolet spectrometer (Madison, WI) equipped with a CsI beam splitter, DTGS-detector and OMNIC processing software was used to record FTIR spectra. Dry air free of CO$_2$ was used to continuously purge the spectrometer for eliminating the strong absorbance from atmospheric CO$_2$ and H$_2$O. For each sample, 400 scans were collected and averaged over the spectral range of 4000–650 cm$^{-1}$ at a resolution of 4 cm$^{-1}$.

3.2.3. Quantitative FTIR analysis

FTIR spectra of SELP-47K films in the spectral range of 1720-1580 cm$^{-1}$ were smoothed with a 9-point smooth Savitzky-Golay function on GRAMS 8.0 and fitted with Gaussian band profiles. A baseline subtraction was also performed on GRAMS 8.0, and all the FTIR spectra were normalized by the areas of the amide I bands. Because the broadened amide I band complicated the secondary structural analysis, by following a procedure established by Taddei and Monti [85], the secondary-derivative and self-deconvolution methods were used to identify individual characteristic bands underlying the broadened amide I bands. All types of protein polymer films displayed the same individual characteristic bands. Areas under individual bands normalized by the total area of the amide I band represent the percentage contents of secondary structures of SELP-47K films. Specifically, the band at 1616 cm$^{-1}$ was assigned to aggregated strands, while
bands at 1624, 1635, 1675, and 1695 cm\(^{-1}\) were assigned to \(\beta\)-sheet and sheet-like structure [85]. It is well established that anti-parallel \(\beta\)-sheets formed by inter-molecular and intra-molecular hydrogen bonding display a strong band at 1630–1640 cm\(^{-1}\), and a weak band at around 1675 cm\(^{-1}\) which is attributed to transition dipole coupling [96-97]. Bands at 1662 and 1684 cm\(^{-1}\) were assigned to \(\beta\)-turns. Bands at 1646 and 1653 cm\(^{-1}\) were assigned to irregular structures, including random coils and extended chains.

3.3. Results and Discussions

3.3.1. Raman and FTIR spectroscopy are complementary in the secondary structural determination of SELP-47K films.

The Raman and FTIR spectra of SELP-47K films are provided in Figure 3.1., and the band assignment is detailed in Table 3.1. In the 4000–2600 cm\(^{-1}\) spectral region SELP-47K displayed very intensive methyl CH stretching bands at 2927 and 2875 cm\(^{-1}\) and an anti-symmetric stretching band of CH\(_2\) groups at 2935 cm\(^{-1}\) in the Raman spectra. However, the IR methyl and methylene CH stretching doublets were very weak. Krimm and Bandekar reported that both the antiparallel-chain rippled sheet (APRS) (Gly)_n and the antiparallel-chain pleated sheet (APPS) \(\beta\)-(Ala)_n and \(\beta\)-(GlyAla)_n displayed strong methyl and methylene CH stretching in Raman but weak counterparts in IR and that the opposite was observed in helical \(\alpha\)-(Ala)_n [98]. Likely, SELP-47K films largely adopt antiparallel-sheet and/or sheet-like structure. Two weak bands at 2771 and 2729 cm\(^{-1}\), attributable to the stretching vibration of C-(CH\(_3\))\(_2\) groups of valine and to the CH-CH\(_3\) group of alanine, were observed in the Raman but not the FTIR spectra. In addition to
amide A bands at around 3300 cm\(^{-1}\), some spectral features including weak FTIR amide B bands at 3070 cm\(^{-1}\) and the Raman stretching vibrations of the free OH groups at around 3500 and 2500 cm\(^{-1}\). In the 1800-400 cm\(^{-1}\) region, while no features were observed in the FTIR spectra under 800 cm\(^{-1}\) the Raman spectra still displayed a few bands in the same region.
3.3.2. Methanol treatment induced crystallization of the silklike blocks of SELP-47K films.

Non-solvents such as methanol are often used as a coagulant in artificial silk fiber spinning to remove water and to induce the irreversible formation of β-sheet crystals [99]. A Raman analysis of the non-treated films in the 1700-800 cm$^{-1}$ spectral region revealed Raman marker bands of silk I structure, including 1248 cm$^{-1}$ (amide III [100]), 1102 cm$^{-1}$ (CC skeletal stretching [101] and C(CH$_3$)$_2$ rocking [102]), 954 cm$^{-1}$ (CH$_3$ rocking [84, 103]), and 856 cm$^{-1}$ (CC and CN stretching [83-84]) (Figure 3.2.). Upon methanol treatment, SELP-47K films displayed Raman marker bands of silk II structure, such as 1230 cm$^{-1}$, 1085 cm$^{-1}$, 971 cm$^{-1}$, and 878 cm$^{-1}$ [76, 100]. It is well established that silk fibroins (SF) exist in two distinct crystalline forms: silk I and silk II. While silk II is largely comprised of antiparallel β-sheets, the conformation of silk I has not been well understood. Since the sequence analysis of the B. mori fibroin [104] revealed that the crystalline regions of silk fibroins contain a highly repetitive GAGAGS sequence, poly(Ala-Gly) copolymers have long been used as a model for study of the silk I structure [105]. Based on solid-state NMR analyses of isotopically labeled poly(Ala-Gly) copolymers, Asakura and colleagues proposed a repeated type II β-turn structure for silk I [77, 106]. A solid-state NMR analysis further suggested that poly(GAGAGS) has a stronger propensity than poly(Ala-Gly) to form the silk II structure after various treatments including air drying [107]. However, our Raman spectral analysis here
indicates that air-dried SELP-47K films exist predominantly as silk I. Because SELP-47K has a monomer structure of \((S)_4(E)_4(EK)(E)_3\), it is likely that the large elastinlike block \((E)_4(EK)(E)_3\) prevents the 4 repeats of the silklike sequence GAGAGS from being fully crystallized into insoluble silk II. A similar disruptive effect on the formation of silk II structure of poly(GAGAGS) was reported when other sequences with hydrophobic side chains (e.g., Y, V) were incorporated [107]. Nevertheless, methanol treatment of SELP-47K films induced a conformational conversion from Silk I to insoluble Silk II, which greatly impacted the material stability and mechanical properties of the SELP-47K films.

In contrast, mechanical preconditioning did not induce any appreciable changes in the Raman spectra of methanol-treated non-crosslinked SELP-47K films. We were not able to assess the possible changes in conformation, if any, after chemical crosslinking because strong fluorescent effects likely induced by the formation of C≡N bonds due to glutaraldehyde (GTA) crosslinking prevented the collection of a meaningful Raman spectrum of crosslinked SELP-47K films. It is also worthy of mentioning that the shift of the peaks in the Raman spectrum of SELP-47K after methanol treatment is not due to the effects of any methanol remaining. Mammone et al. reported that methanol displayed very strong anti- and symmetric CH$_3$ stretching bands at 2940 cm$^{-1}$ and 2832 cm$^{-1}$ [108]. In contrast, the anti- and symmetric CH$_3$ stretching bands of large molecules such as SELP-47K appeared at 2972 cm$^{-1}$ and 2875 cm$^{-1}$, which were observed in the Raman spectra of methanol-treated SELP-47K films. In addition, the strong C-O stretching band of methanol at 1033 cm$^{-1}$ did not show in the Raman spectra of SELP-47K films. Thus, it
is crystal structural change, not methanol remaining, that induced the shift of the Raman spectra of SELP-47K films.

![Graph](image)

Figure 3.2. Methanol treatment induced conformational conversion of SELP-47K films from silk I to silk II structure: Raman spectra of non-treated films (a); methanol-treated non-crosslinked films (b); and methanol-treated and then mechanically preconditioned films (c). The spectra were normalized to the absorbance of the methylene bending band at 1450 cm\(^{-1}\), which is insensitive to changes in secondary structure. Raman marker bands of silk I and silk II are marked by blue and red arrows, respectively.

3.3.3. Methanol treatment induced aggregation of β-strands in SELP-47K films.

In contrast to the unambiguous detection of the unambiguous detection of the silk II conversion by Raman spectroscopy, FTIR analysis of SELP-47K films was less distinctive (Figure 3.3.). The amide I band of non-treated SELP-47K films appeared at
1630 cm\(^{-1}\), typical of antiparallel \(\beta\)-sheets. Still, the breadth of the amide I band indicated the coexistence of other conformations. The amide I band of the methanol-treated SELP-47K films at 1617 cm\(^{-1}\) suggested the formation of aggregated strands, likely a consequence of strong inter-molecular hydrogen bonding [97]. It has been reported that silk I displays amide I, II, and III bands at 1654, 1540, and 1240 cm\(^{-1}\) while silk II shows these amide bands at 1700–1626, 1534, and 1264–1234 cm\(^{-1}\) [85]. All four types of SELP-47K films showed amide I bands at 1630 or 1617 cm\(^{-1}\), amide II at 1518 or 1511 cm\(^{-1}\) and amide III at 1230 cm\(^{-1}\), seemingly distinct from the traditional classification of the FTIR spectra for silk I and II. Relatively low amide II frequencies were also observed in the APRS (Gly)\(_n\) and the APPS \(\beta\)-(Ala)\(_n\) [98]. Nevertheless, infrared bands also characteristic of silk I at 1410 cm\(_1\) (C\(_{\alpha}\)H\(_2\) stretching) and 1330 cm\(^{-1}\) (CH\(_3\) symmetric stretching) identified by Taddei and Monti [85], were displayed by non-treated SELP-47K protein films and disappeared or weakened after methanol treatment (Figure 3.3.B). A doublet at 1390 and 1370 cm\(^{-1}\) was likely attributed to the CH\(_3\) stretching of valine residues [109]. Taddei and Monti identified a silk I marker band at 1387 cm\(^{-1}\), ascribed to CH\(_3\) stretching [85]. The CH\(_3\) stretching doublet of SELP-47K films is less sensitive to conformational changes. In contrast, the band intensity at 1070 cm\(^{-1}\) typical of silk II increased after methanol treatment. Therefore, FTIR spectroscopic analysis not only confirms the silk II conversion in SELP-47K films, but also suggests the aggregation of \(\beta\)-strands induced by methanol treatment. It is expected that the formation of aggregated strands will have particular implication to the mechanical behavior of SELP-47K films.
3.3.4. Mechanical preconditioning disrupted β-sheets in SELP-47K films

NH and OH stretching bands are extremely sensitive to changes in hydrogen bonding, and have recently used to study pH-induced conformational transitions in the poly-L-lysine model system [110]. In the Raman spectra of non-treated SELP-47K films (Figure 3.4.A), a broad band at 3308 cm\(^{-1}\) is assigned to the stretching of free and hydrogen-bonded NH groups (amide A band). The stretching vibration of hydrogen-
bonded and free OH groups of serine (S) residues displayed a weak band at 3410 and a shoulder at 3510 cm$^{-1}$, respectively. Upon methanol treatment, the amide A band shifted to 3285 cm$^{-1}$, suggesting stronger intra-intermolecular hydrogen bonding. Interestingly, the amide A band was further narrowed after mechanical preconditioning, likely due to the formation of more ordered structure. Furthermore, the stretching vibration of the free OH group at 3510 cm$^{-1}$ intensified, indicating the disruption of hydrogen bonds involving the OH groups of serine residues. A similar but less pronounced effect was observed in the 3700-3000 cm$^{-1}$ region of the FTIR spectra of SELP-47K films (Figure 3.4.B). We presume that mechanical preconditioning induces greater alignment of molecules along the direction of the applied strain which enhances intermolecular hydrogen bonding. To accommodate this molecular realignment, however, some preexisting ordered structures in the direction perpendicular to the preconditioning strain are undoubtedly disrupted and some hydrogen bonds are broken, explaining the partial freeing of the OH groups of serine residues. While mechanical preconditioning has been shown to alter the microstructures of soft tissues/materials, allowing more consistent mechanical properties to be obtained, the underlying conformational changes induced by this processing have rarely been examined [111].
Figure 3.4. The Raman (A) and FTIR (B) amide A spectra of SELP-47K films
The direct FTIR spectroscopy (Figure 3.4.B) revealed the emergence of the weak 1260 cm\(^{-1}\) band after mechanical preconditioning, attributable to disrupted β-sheets. Consistent with the Raman amide A analysis, mechanical preconditioning disrupted some preexisting, hydrogen-bonded structures like beta sheets and promoted aggregation of beta strands along the preconditioning strain direction. The positive 1230 cm\(^{-1}\) band in the FTIR difference spectrum might be due to the enhanced β-strand aggregation.

3.3.5. The glutaraldehyde crosslinking influences the secondary structure of SELP-47K films

The presence of lysine residues in the SELP-47K protein chain allows glutaraldehyde (GTA) crosslinking of the films, which is expected to influence the secondary structure of the polymer and the mechanical behavior of the film to some extent. In particular, the FTIR spectrum of glutaraldehyde-crosslinked films was obtained, but the strong fluorescence indicated by its sloping baseline prevented collecting Raman spectrum. While the FTIR spectral changes in the amide A (Figure 3.4.B), I (Figure 3.3.A), and III (Figure 3.3.B) after GTA-crosslinking were very subtle, if not negligible, spectral changes were more pronounced in the regions of the CC skeletal stretching vibrations (Figure 3.3.C). In particular, bands of ordered helix at 928 and 912 cm\(^{-1}\) nearly disappeared, indicating that GTA crosslinking disrupted ordered helical conformations of SELP-47K films. Additionally, a band at 1070 cm\(^{-1}\) attributable to the CC skeletal stretching of silk II, which was intensified after a methanol treatment, was weakened after GTA crosslinking. In contrast, GTA crosslinking intensified a band at 1060 cm\(^{-1}\),
which was observed in silk I [85]. Likely, GTA crosslinking slightly reversed the silk II conversion induced by methanol treatment.

3.3.6. Quantitative conformational analysis

Quantitative structural information of SELP-47K films were derived by curve fitting of the FTIR amide I bands with Gaussian band profiles (Figure 3.5.). Approximate assignments of the Gaussian bands are listed in Table 3.1. Although Taddei and Monti did not differentiate aggregated strands from β-sheet for silk fibroin [85], our recent mechanical analysis of SELP-47K films suggests that it is useful to distinguish the two conformations [54]. Specifically, non-treated SELP-47K films did not display significant mechanical strength and were unstable when re-hydrated in water, while methanol-treated films possessed excellent mechanical strength and retained material integrity in water. Indeed, the mechanical treatment increased the content of aggregated strands from 22% to 38%, at the expense of β-sheet and α-helix conformations (Table 3.1.). Aggregated β-strands, which are more extensively hydrogen-bonded, but are not necessarily more ordered, than antiparallel β-sheets, likely provide more robust physical cross-links and thus higher tensile strength to SELP-47K films.
Figure 3.5. FTIR amide I spectra fitted with Gaussian band profiles. A: non-treated films; B: MeOH-treated films; C: MeOH-treated and then mechanically preconditioned films; D: MeOH-treated and then GTA-crosslinked films.
Table 3.1. Percentages of individual peaks as determined by curve fitting of the FTIR amide I band

<table>
<thead>
<tr>
<th>frequency (cm⁻¹)</th>
<th>a</th>
<th>b</th>
<th>c</th>
<th>d</th>
<th>approximate assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1616</td>
<td>22.0</td>
<td>38.1</td>
<td>47.7</td>
<td>40.4</td>
<td>aggregated strands</td>
</tr>
<tr>
<td>1624</td>
<td>7.9</td>
<td>12.1</td>
<td>2.5</td>
<td>13.7</td>
<td>β-sheet and sheet-like structure</td>
</tr>
<tr>
<td>1635</td>
<td>19.0</td>
<td>8.5</td>
<td>6.3</td>
<td>2.6</td>
<td>β-sheet and sheet-like structure</td>
</tr>
<tr>
<td>1646</td>
<td>9.3</td>
<td>10.8</td>
<td>12.2</td>
<td>13.5</td>
<td>unordered structure</td>
</tr>
<tr>
<td>1653</td>
<td>6.2</td>
<td>2.3</td>
<td>1.8</td>
<td>1.7</td>
<td>α-helix</td>
</tr>
<tr>
<td>1662</td>
<td>13.8</td>
<td>11.2</td>
<td>15.1</td>
<td>12.4</td>
<td>β-turn</td>
</tr>
<tr>
<td>1675</td>
<td>12.8</td>
<td>7.3</td>
<td>4.5</td>
<td>6.6</td>
<td>β-sheet and sheet-like structure</td>
</tr>
<tr>
<td>1675</td>
<td>12.8</td>
<td>7.3</td>
<td>4.5</td>
<td>6.6</td>
<td>β-sheet and sheet-like structure</td>
</tr>
<tr>
<td>1684</td>
<td>1.9</td>
<td>1.5</td>
<td>4.0</td>
<td>2.2</td>
<td>β-turn</td>
</tr>
<tr>
<td>1695/7</td>
<td>6.9</td>
<td>8.3</td>
<td>5.9</td>
<td>6.9</td>
<td>β-turn</td>
</tr>
</tbody>
</table>

a: non-treated films; b: MeOH-treated films; c: MeOH-treated and then mechanically preconditioned films; d: MeOH-treated and then GTA-crosslinked films.

Consistent with the qualitative analysis of the FTIR and Raman spectra, the β-sheet/sheet-like structures of methanol-treated SELP-47K films were partially disrupted by mechanical preconditioning, down from 27.9% to 13.3% (Table 3.1). In contrast, after mechanical preconditioning the conformational contents of β-turns and aggregated strands increased from 21% to 25% and from 38.1% to 47.7%, respectively. Little effect of mechanical preconditioning on unordered and α-helical structure was observed. Clearly, the disrupted β-sheets by mechanical preconditioning partially formed aggregated β-strands, the conformation of which has not been well defined. The enhanced aggregation of β-strands is also consistent with the Raman amide A spectra.
(Figure 3.4.A), which suggests that mechanical preconditioning disrupted some hydrogen bonds involving the OH groups of serine residues and promoted the formation of more extensively hydrogen-bonded structures. Interestingly, our mechanical analysis revealed that mechanical preconditioning can enhance the resilience of methanol-treated SELP-47K films from 66 ± 4% to 86 ± 4%. It has been well established that β-sheet dominated silk possesses poor resilience while elastin with β-turn and/or β-sheetlike conformation is very resilient. Thus, the quantitative FTIR analysis provides a secondary structural basis for the understanding of the mechanical behavior of SELPs.

A slight disruption of the β-sheet/sheetlike structure was also observed in methanol-treated SELP-47K films upon glutaraldehyde crosslinking. The β-sheet content decreased from 27.9% to 22.0%. Still, a slight gain in aggregated β-strands was offset by an increase of unordered structure after glutaraldehyde crosslinking (Table 3.1). This was consistent with the qualitative analysis of the weak FTIR spectra ascribed to the CC skeletal stretching (Figure 3.3.C), which suggested a slight increase in Silk I at the expense of silk II. However, the disappearance of the FTIR bands of ordered helix at 928 and 912 cm⁻¹ indicating a decrease of the helix content was largely unconfirmed by the quantitative analysis of the FTIR amide I spectra. The helix content of methanol-treated films decreased from 2.3% to 1.7% after glutaraldehyde crosslinking. Indeed, the curve fitting of the FTIR amide I spectra led to an estimation of low helix contents.
3.4. Conclusions

The influences of chemical treatments and mechanical preconditioning on the secondary structure of recombinant silk-elastinlike protein copolymer SELP-47K cast films were analyzed using Raman and FTIR spectroscopy. SELP-47K dry films largely adopt silk I structure, which may include $\beta$-turn, $\beta$-sheetlike, and unordered conformers. Methanol treatment converted a significant portion of Silk I structure of SELP-47K films into silk II form characterized by extended, strongly hydrogen-bonded $\beta$-strand aggregation. The analysis of Raman amide A spectra suggested that mechanical preconditioning may disrupt some hydrogen bonds involving the OH groups of serine residues but likely strengthens other hydrogen-bonded structures. Indeed, the quantitative analysis of FTIR amide I spectra revealed a large decrease in the $\beta$-sheet and sheet-like content, which was accompanied by increases in the $\beta$-turn and aggregated strand contents. Likewise, the glutaraldehyde crosslinking of SELP-47K films resulted in subtle changes of the protein secondary structure. Significantly, the spectroscopic studies may provide a secondary structure basis for the understanding of the mechanical behavior of recombinant SELPs and for the design of new protein-based materials.
CHAPTER 4: OPTICAL AND SURFACE CHARACTERIZATION

4.1. Introduction

Optically transparent polymers have many important applications. For instance, poly(hydroxyethyl methacrylate) is the primary polymer used for contact lenses fabrication. Additionally, poly(dimethylsiloxane) (PDMS) has been widely used for fabricating microfluidic devices, due to its optical transparency, mechanical robustness, and ease of processing [112-113]. PDMS [114] and silicon rubber [115-116] have been used as cell culture substrates as well; their optical transparency permits real-time observations and analyses of the cellular and sub-cellular processes. However, for these applications because they are biologically inert, PDMS and silicone rubber substrates need to be coated with extracellular matrix (ECM) proteins in order to promote cell attachment. In contrast, recombinant protein polymers often display enhanced biocompatibility, promoting cell-material interactions. Moreover, specific functional groups, such as the RGD and CS5 cell binding domains of fibronectin, can be readily incorporated in to silklike [117] and elastinlike [118] protein polymers. However, recombinant protein polymers have been rarely reported as optically transparent. Interestingly, silk fibroin proteins from silkworms were recently processed into transparent thin films, although silk fiber is not transparent [119].

Recombinant protein polymer materials composed of silklike blocks and elastinlike blocks may be rendered optically transparent by controlling or limiting the size and the chemical nature of their inter-chain crosslinks. In this study, the optical properties
of the SELP-47K protein polymer were investigated. Effects of the covalent and non-covalent crosslinking on the light transmittance of SELP-47K films were also examined. In addition, the cytocompatibility of SELP-47K films was analyzed too.

4.2. Materials and Methods

4.2.1. Optical Characterization

SELP-47K films of 20 to 100 µm in thickness were prepared by casting the protein solution on coverslips. The solvent was evaporated at room temperature under ambient conditions. Film thickness was controlled by the amount of protein solution placed on top surface of coverslips and measured by a Dektak 150 Surface Profiler (Veeco). Following a protocol detailed elsewhere [56, 120-121], three types of SELP-47K films, including non-treated, MeOH-treated, and MeOH-GTA-treated samples, were optically analyzed. Briefly, a dry or hydrated sample was taped to a sample holder with the sample facing incident beam, and transmittance was measured using a Cary 5000 UV/Vis-NIR spectrometer (Varian). The transmittance of glass coverslips without any films samples was also measured. A method was established to subtract the effect of the coverslip from the transmittance measurement of SELP-47K films. Hydrated samples were prepared by wetting thin films in DI water, equilibrating overnight, and blotting away any excess water before measurement.

SELP-47K thin films (5 µm thick) were also cast on SiO₂ wafer surface under ambient conditions. The refractive index (RI) of non-treated, MeOH-treated, and MeOH-GTA-treated films was determined using a Metricon prism coupler (Metricon) at
wavelength of 532, 632.8, and 1554 nm, respectively [122]. SELP-47K films on SiO₂ wafer were brought into close contact against the base of the prism to ensure the coupling between the sample and prism surface. The measurements were performed on three to five replicates of each type of SELP-47K film.

4.2.2. Water Contact Angles

To evaluate the hydrophilicity of SELP-47K films, contact angles were obtained by placing a small droplet of DI water (5 µL) using Easy Drop DSA20B (Kruss) [123], and determined by analyzing the optical images of the droplets using the sessile drop fitting algorithm [124].

4.2.3. Surface Roughness

Because surface roughness can greatly affect contact angle measurements [125], atomic force microscopy (AFM) was performed to determine the surface roughness of SELP-47K films. The AFM images were acquired under tapping mode using a MultiMode AFM (Digital Instruments) equipped with a NSC-15 tapping mode cantilever (Figure 4.1.). The associated AFM software NanoScope was used to calculate the mean surface roughness of each sample (Table 4.1.).
Figure 4.1. AFM images of non- (A), MeOH- (B), and MeOH-GTA-treated SELP-47K films (C).

Table 4.1. Mean surface roughness values of non-, MeOH-, and MeOH-GTA-treated films

<table>
<thead>
<tr>
<th></th>
<th>non-treated</th>
<th>MeOH-treated</th>
<th>MeOH-GTA-treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean surface roughness (nm)</td>
<td>1.485 ± 0.245</td>
<td>1.421 ± 0.271</td>
<td>1.300 ± 0.145</td>
</tr>
</tbody>
</table>

4.2.4. Equilibrium Swelling

The swelling behavior of free-standing MeOH- and MeOH-GTA-treated SELP-47K films was evaluated in DI water containing 0.2 mg/ml NaN₃ to prevent biological contamination. The DI water was changed several times over a time period of 72 hrs, ensuring the removal of the dissolved protein polymer. After gently removing excess water, the weight of swollen films ($W_s$) was measured every 24 hrs until samples reach equilibrium (<1% change in weight in 24 hrs). Dry films were obtained by placing swollen films in a vacuum oven at 60 °C. After being equilibrated to room temperature in a dessicator, dry films were weighted and placed back in the oven. The weight of dry films ($W_d$) was measured every 24 hrs until the difference between two consecutive
measurements was less than 1%. The equilibrium swelling ratio (q) and water content (H) of the SELP-47K films were determined using the following formulas [126-128],

\[ q = \frac{W_s}{W_d} \]  \hspace{1cm} (1)

\[ H = (1 - \frac{W_d}{W_s}) \times 100\% \]  \hspace{1cm} (2)

4.2.5. Cell Viability

Both MeOH- and MeOH-GTA-treated SELP-47K films along with coverslips were sterilized by immersion in 70% ethanol overnight. Following a protocol detailed elsewhere [53], 230 µL of NIH/3T3 fibroblast suspension in DMEM containing 10% FBS at a cell density of 300 cells/µL was added onto each film, and incubated in an incubator at 37 °C with 5% CO₂ until cells reached 100% confluence. Fresh media was exchanged each 3 days. On day 5, the viability of cells grown on the MeOH- and MeOH-GTA-treated films was examined using a LIVE/DEAD Viability/Cytotoxicity Kit (Invitrogen).

4.2.6. Cell Proliferation.

The proliferation of cells cultured on the MeOH- and MeOH-GTA-treated SELP-47K films with coverslips was analyzed by the MTS assay (Promega) on days 1, 3, 5, and 7. The initial cell seeding density was 10,000 cells/cm². Live cells react with a tetrazolium salt in the MTS reagent, producing a soluble formazan dye, which has an absorbance at a wavelength of 490 nm. Within the linear region of the absorbance curve, the number of cells is proportional to the absorbance intensity, measured using a
Nanodrop UV-Vis spectrophotometer (Thermo Scientific). After each SELP-47K film
with cells was retrieved, it was placed into 200 µL of medium containing 40 µL of MTS
reagent, and incubated for 2 hrs prior to measurement of the absorbance intensity at 490
nm. As a control, cells were also cultured in a tissue culture polystyrene (TCPS) well
without SELP-47K film.

4.2.7. Statistical Analysis

Cell viability and proliferation were analyzed in at least triplicate, and
measurements were expressed as mean ± standard deviation (SD). Student's t-test
(SigmaPlot) was employed to assess statistical significance of the results. Differences
were considered statistically significant at p < 0.05.

4.3. Results and Discussions

4.3.1. SELP-47K thin films are transparent to visible light

UV-vis spectroscopy reveals that SELP-47K thin films (30 µm thickness) are
optically transparent to visible light but opaque to ultraviolet (UV) light (Figure 4.2.).
Transmittances of non- and MeOH-treated films decrease slightly from 95% at 800 nm to
92% and 90% at 350 nm, respectively. Transmittances of MeOH-GTA-treated films
decrease from 96% at 800 nm to 91% at 450 nm, and then to 75% at 350 nm. Considering
variation in sample thickness and experimental error, all three types of SELP-47K thin
films display comparable transparency at wavelengths of 800~450 nm. The low optical
transparency of SELP-47K films at wavelengths below 350 nm is likely due to strong UV
absorption by the SELP-47K polypeptide. Indeed, UV absorption at 280 nm is routinely used to quantify protein concentration. GTA crosslinking intensifies the UV absorption of SELP-47K films. GTA is known to react with and modify the amines of collagen and other proteins, leading to UV absorption at 300 and 325 nm [129-130]. This likely explains the lower transparency of MeOH-GTA-treated films in the near-UV region, as compared to that of non- and MeOH-treated films.

![Transmittance graph](image)

Figure 4.2. Transmittance of non- (▽), MeOH- (○), and MeOH-GTA-treated (◇) SELP-47K dry films of 30 µm thickness. Measurements were done in triplicate. Insert: photo images of SELP-47K films cast on coverslips.

The influence of film thickness on the optical transparency of non- and covalent crosslinking SELP-47K films was assessed using thicker samples (average thickness of 92 µm; Figure 4.3.). Non-treated films displayed transmittances of 90% at 800 nm, 88%
at 450 nm, and 85% at 350 nm. At the same wavelengths, MeOH-treated films possessed transmittances of 93%, 90%, and 88%, while MeOH-GTA-treated films showed transmittances of 77%, 66%, and 37%, respectively. Again in these thicker films, non-covalent crosslinking via methanol does not compromise the optical transparency of SELP-47K films. In contrast, considerable reduction (i.e., about 20%) in the transmittance of MeOH-treated SELP-47K films is induced by covalent crosslinking using GTA. Moreover, MeOH-GTA-treated films appear to be slightly yellowish (Figure 4.2, insert).

4.3.2. Hydration slightly improves the optical transparency of SELP-47K films.

The physical properties of hydrated recombinant protein polymers are more relevant than dry ones to many biomedical applications. Interestingly, hydration slightly improves the optical transparency of all three types of SELP-47K films to visible light, resulting in a 2~6% increase in their transmittance (Figure 4.3.). Hydrated non- and MeOH-treated films display nearly identical transmittances at all wavelengths from 800 to 300 nm. Although the underlying mechanism still remains poorly understood, hydration was reported to greatly enhance the optical transparency of upper cortices of *L. pulmonaria* [131]. In contrast, the anticancer drug Tamoxifen, which induced dehydration in the lens of the eye, impaired lens clarity [132]. We speculate that hydration changes the interfacial properties of protein microstructures including our SELP-47K films, thereby attenuating light scattering and reflection. In our previous study, scanning electron microscopy (SEM) revealed the microfibrillar structures of
SELP-47K films, which were formed by protein globules of micrometer in size [54]. According to the hierarchical self-assembly model proposed by Jin and Kaplan for silk [38], those micrometer protein globules may be formed by micelles of 100-200 nm in size. The micro/nanofibrils and microgobules undoubtedly create numerous micro-interfaces within SELP-47K films. Light deflection and scattering at these interfaces may account for the slight reduction of optical transparency of SELP-47K films. This is analogous to reduction in the optical transparency of glass by internal cracks. However, hydration changes the micro-interfaces of SELP-47K films from air/protein into water/protein, probably leading to reduction in light deflection/scattering and improvement in film transparency.

Figure 4.3. Hydration effects on the optical transparency of non-(▼▼), MeOH- (○●), and MeOH-GTA-treated (◇◆) SELP-47K films of 92 µm thickness (dry film thickness). Measurements were done in triplicate. Dry samples (▼○◇); hydrated samples (▼●◆).
4.3.3. The swelling ratio of SELP-47K films is much lower than SELP hydrogels

The equilibrium swelling ratio and the extent of hydration of MeOH- and MeOH-GTA-treated SELP-47K films were analyzed in DI water (Table 4.2). When fully hydrated, a MeOH-treated film absorbed approximately 100% of its weight of water, while a MeOH-GTA-treated film absorbed water up to 62% of its weight. As a result, hydrated MeOH- and MeOH-GTA-treated films contained 51% (w/w) and 38% (w/w) water, respectively. It is worth noting that hydration only marginally increased the dimensions of SELP-47K films, e.g., less than 5% in length and width. Likely, most of the absorbed water filled the existing micro and nanopores of the dry SELP-47K films. The swelling ratios of SELP-47K films are much lower than SELP-47K hydrogels (e.g., 8 to 9) [128]. The major difference between SELP-47K films and hydrogels resides in the fabrication process and their resulting microstructures. When casting films, the complete evaporation of solvent (e.g., DI water) enhances the interactions between the polymer chains, leading to the formation of intra- and intermolecular hydrogen bonds and the irreversible crystallization of the silklike blocks that greatly stabilizes the films. In contrast, the solvent retained when SELP-47K hydrogels are cured results in less crystallization of the silklike blocks. This would explain the significant differences between the swelling ratios of SELP-47K films and hydrogels.

Table 4.2. The equilibrium swelling ratio ($q$) and water content ($H$) of SELP-47K films.

<table>
<thead>
<tr>
<th></th>
<th>MeOH-treated</th>
<th>MeOH-GTA-treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>$Q$</td>
<td>2.06 ± 0.09</td>
<td>1.62 ± 0.12</td>
</tr>
<tr>
<td>$H$</td>
<td>51.35 ± 2.13</td>
<td>38.02 ± 4.74</td>
</tr>
</tbody>
</table>
4.3.4. The contact angles of SELP-47K films is close to silicone-based contact lenses

Non-treated SELP-47K films are not mechanically stable after hydration in DI water [54]. Indeed, droplets placed on the non-treated films for contact angle measurement were quickly absorbed by the film surface. As a result, the contact angle of non-treated films was initially 36.2 ± 4.4°, but quickly reduced to 11.9 ± 2.2° when the droplets stabilized on the sample surface after about 1 minute. In contrast, MeOH- and MeOH-GTA-treated films were stable in water. Their contact angles were 67.8 ± 2.2° and 64.1 ± 3.7°, respectively, which are similar to those of contact lens materials such as Asmoficon (71.2 ± 1.5°), Balafison (71.5 ± 1.1°) and Enfilcon (68.3 ± 1.5°) [133].

4.3.5. The optical transparency of SELP-47K films displayed weak thickness dependence

When the film thickness increased from 18 µm to 97 µm, a moderate reduction of 3% in transmittance was observed in hydrated MeOH-treated films (Figure 4.4.). Increasing the thickness of non-treated films resulted in a similar reduction in transmittance. MeOH-GTA-treated films showed a stronger dependence of optical transparency on thickness. When their thicknesses increased from 16 µm to 92 µm, a 17% loss of optical transparency was observed. This is consistent with the fact that GTA crosslinking induced more absorbance of SELP-47K films at all wavelengths. The thickness-dependent transmittance (T) of SELP-47K films can be described by Beer’s
law, $T = 10^{-\alpha l}$, where $\alpha$ is the attenuation coefficient and $l$ is the film thickness. Linear regression was used to obtain the attenuation coefficients of hydrated SELP-47K films (Table 4.3.). Non- and MeOH-treated films displayed comparable attenuation coefficients at 450 and 350 nm. The attenuation coefficient of MeOH-GTA-treated films was much higher than that of non- and MeOH-treated films at the same wavelengths, suggesting a more pronounced reduction in transparency when their thickness increased.

![Transmittance of hydrated MeOH-treated SELP-47K films of various thicknesses, 18, 30, 56, and 97 µm (from top to bottom).](image)

<table>
<thead>
<tr>
<th>Wavelength</th>
<th>Non-treated</th>
<th>MeOH-treated</th>
<th>MeOH-GTA-treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>at 450 nm</td>
<td>1.58</td>
<td>1.30</td>
<td>12.89</td>
</tr>
<tr>
<td>at 350 nm</td>
<td>1.51</td>
<td>1.58</td>
<td>25.02</td>
</tr>
</tbody>
</table>

Table 4.4. Refractive indices of SELP-47K films measured at different wavelengths.
<table>
<thead>
<tr>
<th></th>
<th>532 nm</th>
<th>633 nm</th>
<th>1554 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-treated</td>
<td>1.5345±0.0025</td>
<td>1.5300±0.002</td>
<td>1.5128±0.0018</td>
</tr>
<tr>
<td>MeOH-treated</td>
<td>1.5366±0.0047</td>
<td>1.5345±0.0039</td>
<td>1.5181±0.0031</td>
</tr>
<tr>
<td>MeOH-GTA-treated</td>
<td>1.5413±0.0021</td>
<td>1.5399±0.0043</td>
<td>1.5264±0.0024</td>
</tr>
</tbody>
</table>

Listed in Table 4.4 are the refractive indices (RI) of non-, MeOH-, and MeOH-GTA-treated films measured at 532, 632.8, and 1552 nm, respectively. The RI of SELP-47K films were similar to those of PMMA (1.490 ± 0.001) [134], Etafilcon A (1.412 ± 0.0017) [135] and Lotrafilcon B (1.424 ± 0.0003) [135], which were measured at 589 nm.

4.3.6. Cell can adhere and grow continuously on SELP-47K films

An ideal biomaterial should be biocompatible with cell attachment, proliferation, and other cellular functions. In this study, the NIH/3T3 fibroblasts were used as a model system to evaluate the in vitro cytocompatibility of SELP-47K films. Cell viability was assessed using the LIVE/DEAD assay. After staining, live cells produce an intense green fluorescence while dead cells emit a bright red fluorescence. The cell viability assay revealed that the cells seeded and grown on both types of SELP-47K films for 5 days were substantially viable (Figure 4.5A, 4.5B). Cell viability was quantified by counting the number of live cells versus the total cells in ten digital fluorescence images of stained cells on each type of film. The percent viabilities of 3T3 fibroblasts on MeOH- and MeOH-GTA treated films were 97.2 ± 2.0% and 99.6 ± 0.5%, respectively.
Figure 4.5. Fluorescent staining for cell viability of NIH/3T3 fibroblasts grown on MeOH- (A) and MeOH-GTA-treated (B) SELP-47K films on day 5. Living cells are in green and dead cells are in red. Scale bars: 100 µm.

To evaluate the ability of SELP-47K films to support cell proliferation, NIH/3T3 fibroblasts were seeded at the same density on MeOH- and MeOH-GTA-treated films and in TCPS wells as a control. Their proliferation profiles were assessed using the MTS assay. During a period of 7 days, fibroblasts cultured on SELP-47K films continuously grew in number (Figure 4.6.). Both scaffolds demonstrated cell proliferation comparable to the culture control on TCPS. Together with the viability assays, the proliferation study shows that SELP-47K films display excellent cytocompatibility.
Figure 4.6. Proliferation profiles of 3T3 fibroblasts grown on TCPS wells (▽, as control), MeOH-treated (○) and GTA-crosslinked (◇) SELP-47K films up to 7 days. Data and error bars represent measurements conducted on triplicate culture wells.

4.4. Conclusions

The genetically engineered silk-elastinlike protein polymer SELP-47K in the form of thin films displays excellent transmittance in the wavelength range of 350~800 nm. While GTA crosslinking of the films results in a moderate reduction in transmittance, non-covalent crosslinking via MeOH treatment retains the high optical transparency of SELP-47K films to visible light. When fully hydrated, a MeOH-treated film of 97 µm thickness possesses a transmittance of 96%. This verifies that recombinant protein polymers containing the silklike sequence can be formed in thin films with good optical transparency. Last, the LIVE/DEAD and cell proliferation assays demonstrate the high in vitro cytocompatibility of SELP-47K films.
5.1. Introduction

The design of drug release devices capable of providing safe, sustained delivery to the precorneal regions of the eye is attracting paramount interest. Currently, topical eye drop administration remains the most commonly used method for approximately 90% of ophthalmic medications on the market [136-137], but it is very inefficient and fails to maintain the prolonged period of effective therapeutic drug concentration for various ocular diseases. The drug availability of topical delivery tends to be very low, as once applied to the surface of the eye, the drop is diluted and washed away by reflex tearing; and dispersed by the blinking. The overdosing of ocular drug solutions through topical administration could be effective, however, leading to decreased patient compliance, increased side effects, toxicity, etc. The concept of using hydrophilic polymer hydrogel as ocular drug delivery systems was introduced as early as 1960 with the aim to improve drug retention on the ocular surface [138]. Since then, several researchers have designed the hydrogel-based ocular drug delivery systems [139-145]. Nevertheless, drug release kinetics from these systems is characterized by a burst of drug delivered during the first few hours, followed by declining, subtherapeutic drug levels in the subsequent hours. Very little drug is eluted by the second day of use. As these hydrogel-based ocular systems can only deliver drugs effectively for a few hours after implantation, achieving even more sustained drug release could be beneficial for many ophthalmic applications.
Polymers have long been employed as the material for design sustained drug release systems to effectively regulate the release of drug molecules with defined kinetics so as to maintain specific therapeutic concentrations. In recent years, there has been considerable interest for genetically engineered protein polymers used as matrices for controlled drug delivery [146-148]. In contrast to chemically synthesized polymers, genetic engineering of recombinant proteins has recently enabled the material scientists to design protein polymer biomaterials with uniform composition, molecular weight, and precisely controlled polypeptide sequences [149-151]. Consequently, SELPs may be fabricated into controlled drug release devices for a variety of biomedical applications [55, 57, 146, 152-154]. However, to our best knowledge, the application of SELPs for ocular drug delivery systems has never been reported.

The physicochemical properties of a drug carrier, SELP-47K protein film in this study, such as equilibrium weight swelling ratio in the physiological environment, the nature of crosslinking, and crosslinking density of the polymers influence the rate and extent of drug release. In addition, possible drug-carrier interactions may have a profound effect on the drug transport from the drug release matrices as well. Thus in order for the recombinant protein polymers to be used as controlled release vehicles, it is critical to understand the relationship between the polymeric network and drug release kinetics.

In present study, we explored the potential ophthalmic application of SELPs to be fabricated as various complete protein-based drug release devices. The casted SELPs thin films were stabilized via EtOH or MeOH vapor treatments to induce the crystallization of silk-like sequences. The resultant films were characterized by optical transmittance,
followed by soluble fraction study and swelling measurement with the aim to determine the physical crosslinking density of the polymer chain network. The secondary structures of the films with different treatments were assessed by Fourier transform infrared (FTIR) and Raman spectroscopy. The contact angle and surface roughness were quantified as well in order to examine the surface properties of the films. As ciprofloxacin is a representative broad-spectrum antibiotic typically found in eye drop formulations, it is used as the model drug incorporated into the drug eluting vehicles with/without coatings for release study. Meanwhile, a physics-inspired model was developed to elucidate the mechanism of ciprofloxacin transport from the drug delivery systems.

5.2. Materials and Methods

5.2.1. Solvent Casting Drug-Protein Films

The lyophilized SELP-47K protein powder was dissolved in DI water at a concentration of 5% (w/w). The ciprofloxacin (Sigma) was incorporated into the protein solution with a mass loading ratio of 1:10. The ciprofloxacin-loaded protein solution was then poured into a custom-made PDMS well with diameter of 6 mm. The solvent evaporation was carried out in a fume hood with laminar air flow overnight at room temperature. The air-dried films were denoted as non-treated drug-protein films. Some of the non-treated films were stabilized in either the MeOH vapor for 24 h, denoted as MeOH-treated drug-protein films, or the EtOH vapor environment respectively for 48 h in a tightly sealed dessicator which were denoted as EtOH-treated drug-protein films.
5.2.2. Encapsulating Drug-Protein Films

The three types of the drug-protein films, including non-, EtOH-, and MeOH-treated drug-protein films prepared according to the above protocol were further embedded in the SELP-47K protein films. To fabricate the drug-protein composite with drug-protein films as the inner core and pure protein films as the outer coating [55, 137], the 5% (w/w) protein solution was poured into another PDMS mold with diameter of 8 mm. It formed the bottom layer of the composite after the solvent has been completely evaporated. The non-, MeOH-, and EtOH-treated drug-protein films were then manually pressed against the dried bottom layer. They were subsequently sealed by filling with protein solution. The outer coating of the resultant drug-protein composites were stabilized in either MeOH vapor for 24 h or the EtOH vapor conditions for 48 h. The final composite consists of a thin drug-protein film core, which were untreated or treated with EtOH, or MeOH, and outer protein coating with EtOH-, and MeOH-treatment. Eight types of resultant drug release devices were abbreviated for conciseness according to their core and coating treatments and were described in great detail in Table 5.1.

5.2.3. Drug Release Studies

The aforementioned eight types of ciprofloxacin-loaded protein films with/without protein coatings were placed in elution tubes that have been prewarmed to 34°C. 2 ml aliquot of prewarmed (34°C) PBS was immediately dispensed to the tube and incubated at 34°C for the ciprofloxacin release study. At predetermined time intervals,
the elution tubes were removed from incubator, agitated by inversion, then 1 ml aliquot was withdrawn from the release medium and replaced by an equal volume of fresh PBS. The amount of ciprofloxacin released into PBS was measured with a UV/Vis NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE) at a wavelength of 270 nm. The concentration and mass of the released ciprofloxacin were calculated based on a calibration curve prepared with known ciprofloxacin concentrations. The loading amount of ciprofloxacin was adjusted such that 100% release would allow the reading within the linear measurement range of the spectrophotometer.

5.2.4. Model simulation of drug release

A simple, three-parameter, physics-inspired drug release model that considers reversible drug-carrier interaction and first-order kinetics was successfully developed in our group [155]. In this model, the drug concentration change due to mass convection/diffusion follows the first-order kinetics:

\[
\frac{dm}{dt} = \frac{d(V \cdot c)}{dt} = -Ak_1c
\]

where \( m \) and \( c \) are the mass and average drug concentration in the matrix. \( V \) and \( A \) are the volume and outer surface area of the drug carrier, and \( k_1 \) is the rate constant. Rearranging the above equation results in the following form:

\[
\frac{dc}{dt} = -k_sc
\]

Here, \( k_S = Ak_1/V \) can be considered as convection/diffusion rate constant.
In addition to convection/diffusion mechanism, the drug-matrix interaction is another important mechanism affecting the drug release kinetics. Simply assuming the drug-carrier interaction is reversible, the concentrations of free and bound drug molecules, $c_F$ and $c_B$ can be derived as follows by taking the convection/diffusion mechanism into consideration:

$$\frac{d}{dt} \begin{pmatrix} c_F \\ c_B \end{pmatrix} = \begin{pmatrix} k_s + k_{on} & k_{off} \\ k_{on} & -k_{off} \end{pmatrix} \begin{pmatrix} c_F \\ c_B \end{pmatrix}$$

Here, $k_{on}$ and $k_{off}$ are rate constants of association, and dissociation processes. When the association and dissociation reach equilibrium at $t = 0$, the initial amounts of free and bound drugs is determined by the free energy difference between the two states, $\Delta G = -k_B T \ln \left( \frac{k_{on}}{k_{off}} \right) = -k_B T \ln \left( \frac{c_B(0)}{c_F(0)} \right)$. Here, $k_B$ is the Boltzmann’s constant, and $T$ is the absolute temperature (assumed to be 300 K). By normalizing the above two equations with initial drug concentration $c_0 = c_F(0) + c_B(0)$, one may obtain:

$$\frac{d}{dt} \begin{pmatrix} c_F \\ c_B \\ c_0 \\ c_0 \end{pmatrix} = \begin{pmatrix} k_s + k_{on} & k_{off} & 0 & 0 \\ k_{on} & -k_{off} & 0 & 0 \\ 0 & 0 & 1 & 0 \\ 0 & 0 & 0 & 1 \end{pmatrix} \begin{pmatrix} c_F \\ c_B \\ c_0 \\ c_0 \end{pmatrix}$$

The above linear ordinary differential equations can be readily solved analytically, giving the following expression:

$$\begin{pmatrix} c_F \\ c_0 \\ c_B \\ c_0 \end{pmatrix} = \frac{k_{off}}{k_{on} + k_{off}} \begin{pmatrix} \lambda_1 - \lambda_2 & \lambda_1 - \lambda_2 \\ \lambda_1 - \lambda_2 & \lambda_1 - \lambda_2 \\ \lambda_1 - \lambda_2 & \lambda_1 - \lambda_2 \\ \lambda_1 - \lambda_2 & \lambda_1 - \lambda_2 \end{pmatrix} \begin{pmatrix} e^{-\lambda_1 t} \\ e^{-\lambda_2 t} \end{pmatrix}$$
where \( \lambda_{1,2} = \left( k_s + k_m + k_{\text{off}} \pm \sqrt{(k_s + k_m + k_{\text{off}})^2 - 4k_s k_{\text{off}}} \right)/2 \), and \(-\lambda_{1,2}\) are eigenvalues of the linear system of differential equations.

Thus the cumulative drug release \( M_t = V(c_0 - c_F - c_B) \) which is normalized by the initial total loaded drug amount \( (M_0 = Vc_0) \) can be simulated by solving the linear ordinary differential equations:

\[
\frac{M_t}{M_0} = 1 - \frac{c_F - c_B}{c_0} = \frac{\lambda_2 (k_s - \lambda_2)}{(k_m + k_{\text{off}})(\lambda_1 - \lambda_2)} (1 - e^{-\lambda_2 t}) + \frac{\lambda_1 (\lambda_1 - k_s)}{(k_m + k_{\text{off}})(\lambda_1 - \lambda_2)} (1 - e^{-\lambda_1 t})
\]

In the model simulation, three parameters, \( \Delta G \), \( k_s \), and \( k_{\text{off}} \) are used to fit the experimental drug release data on the cumulative release of ciprofloxacin from the drug release devices.

5.2.5. Ciprofloxacin antimicrobial assay

The antibacterial effectiveness of the eluted ciprofloxacin samples were tested against a ciprofloxacin sensitive \( E. coli \) 136 and a ciprofloxacin resistant \( E. coli \) 132. The ciprofloxacin resistant strain was tested to make sure the bacterial killing is due to the ciprofloxacin not to any unknown inhibitory agent. The two \( E. coli \) strains (EC136\(^S\) and EC132\(^R\)) were cultured in Muller-Hinton medium. Before the experiment, the bacteria were grown in medium to a log phase and diluted to appropriate concentrations for the assay. 0.1 ml of the eluted ciprofloxacin solution in 24 hours was added into the 1ml aliquot of the diluted bacteria in the 1.5 ml centrifuge tube. The tubes were incubated in an orbital shaker (BarnStead Inc) at 300 RPM and 37 °C. At predetermined time intervals, the concentration of the bacteria was measured in a spectrophotometer (NanoDrop 2000) by observing the absorbance at 600 nm.
5.2.6. Statistical analysis

All studies were performed in triplicate unless otherwise noted. Results were presented as mean ± standard deviation (SD).

5.3. Results and Discussions

5.3.1. Influence of physical crosslinking on release kinetics

The release of ciprofloxacin from eight types of drug eluting systems under sink conditions at 34 °C was studied. The release profile was characterized by two parameters according to the first-order kinetics, one is the cumulative final release amount which describes the percentage of eluted ciprofloxacin from total incorporated drug; the other one is the time to achieve 50% final release amount which indicates the drug release rates. The final cumulative release and the time required for 50% release from each type of drug release devices are summarized in Table 5.1.

Table 5.1. Final release amount and time required achieving 50% release of various devices and parameters used for model fit

<table>
<thead>
<tr>
<th>Sample types</th>
<th>Final release</th>
<th>Time to 50% release</th>
<th>Model parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>$k_s$ (hr$^{-1}$)</td>
</tr>
<tr>
<td>E</td>
<td>84.0 ± 2.6%</td>
<td>10 h</td>
<td>0.080</td>
</tr>
<tr>
<td>M</td>
<td>71.2 ± 3.2%</td>
<td>15 h</td>
<td>0.050</td>
</tr>
<tr>
<td>DE</td>
<td>85.2 ± 4.3%</td>
<td>22 h</td>
<td>0.034</td>
</tr>
<tr>
<td>EE</td>
<td>73.0 ± 1.9%</td>
<td>18 h</td>
<td>0.040</td>
</tr>
<tr>
<td>ME</td>
<td>66.3 ± 1.7%</td>
<td>20.5 h</td>
<td>0.040</td>
</tr>
<tr>
<td></td>
<td>Drug Release Rate</td>
<td>Time</td>
<td>P-value</td>
</tr>
<tr>
<td>-----</td>
<td>------------------</td>
<td>-------</td>
<td>---------</td>
</tr>
<tr>
<td>DM</td>
<td>73.9 ± 4.4%</td>
<td>31.5 h</td>
<td>0.028</td>
</tr>
<tr>
<td>EM</td>
<td>69.8 ± 2.0%</td>
<td>48 h</td>
<td>0.015</td>
</tr>
<tr>
<td>MM</td>
<td>56.4 ± 4.3%</td>
<td>46 h</td>
<td>0.018</td>
</tr>
</tbody>
</table>


The uncoated drug-protein films, devices E and M showed the drug release with first-order kinetics for 108 h (Figure 5.1.). The device E, EtOH-treated drug-protein films without coating released ciprofloxacin a higher rate than that of device M, which is MeOH-treated. The time to 50% release for bare EtOH- and MeOH-treated drug-protein films are 10 h and 15 h, respectively. The cumulative ciprofloxacin release reaches 84.0 ± 2.6% and 71.2 ± 3.2% after 108 h for EtOH-, and MeOH-treated drug-protein films, with less than 1% release per day thereafter. The reduced release rate of ciprofloxacin from MeOH-treated drug-protein films is likely due to their increased physical crosslinking density and enhanced stability when compared to the EtOH-treated films which are supported by their soluble fraction analysis and swelling measurement. As the density of physical crosslinking gets higher, the micro/nano-pores or channels formed inside the films become lesser and smaller which would retard the drug molecules transfer to the exterior. Meanwhile, ciprofloxacin molecules might bond to SELP-47K polymer chains to form intermediate drug-carrier complexes which cannot be easily delivered due to large molecule weight and chain entanglements. Therefore, for the lower cumulative
ciprofloxacin release magnitude from device M, we hypothesize this could be resulted from the fact that the intermediate complexes have higher density and stronger interactions than those formed in device E.

Figure. 5.1. Cumulative ciprofloxacin release from uncoated EtOH- (▽), and MeOH-treated (□) drug-protein films. Solid lines represent the model fit results.

5.3.2. Influence of coating properties on release kinetics

With the aim towards the sustained release, we further fabricated the protein coated drug release devices which consist of a thin drug-protein film core with a protein
layer on two sides. There are six different types of coated drug release devices, including non-, EtOH-, and MeOH-treated drug-protein film cores with EtOH-, and MeOH-treated protein coatings which were abbreviated as DE, EE, ME, DM, EM, and MM. The ciprofloxacin release patterns of these drug eluting systems were grouped together in order to investigate the influence of different coatings and cores on the drug release kinetics.

Figure 5.2. Cumulative release of ciprofloxacin from non-treated drug-protein film core with EtOH- (▽), and MeOH-treated (□) protein coatings. Solid lines represent the model fit results.
Either EtOH- or MeOH-treated protein coating on non-treated drug-protein films core has significantly improved the stability of the drug release systems, whereas the non-treated protein films solely were not water stable, thus it is not used as the release matrix without coating. Both coated DE, DM devices have the first-order ciprofloxacin release patterns which continued for about 132 h (Figure 5.2), at which time there are $85.2 \pm 4.3\%$ and $73.9 \pm 4.4\%$ of total encapsulated drug was eluted. In addition, although DE and DM systems have completely the same interior, the time for them to achieve 50% release was differed by about 10 h.

![Figure 5.3](image)

Figure 5.3. Cumulative release of ciprofloxacin from EtOH-treated drug-protein film core with no (◇), EtOH- (▽), and MeOH-treated (□) protein coatings (left panel), and MeOH-treated drug-protein film core with no (◇), EtOH- (▽), and MeOH-treated (□) protein coatings (right panel). Solid lines represent the model fit results.

The effect of different coatings was further illustrated in Figure 5.3 where ciprofloxacin release from E, EE, EM (Figure 5.3, left panel) and M, ME, MM (Figure 5.3, right panel) was studies. As discussed extensively in previous sections, the coating
will slower down the ciprofloxacin release due to its barrier nature on the way for the drug transports into the exterior environment. Furthermore, as the MeOH-treated coatings have higher physical crosslinking density than EtOH-treated ones, their sustained-release character became more obvious than they counterparts. The barrier effect of the coatings should be obviously reflected from the prolonged 50% release time. Meanwhile, the cumulative release should achieve the same level if there is no interaction between ciprofloxacin molecules and coating polymer chains at all. Interestingly, the time required for 50% release and ultimate ciprofloxacin release for devices E, EE, and EM were recorded as 10 h, 18 h, and 48 h, 84.0 ± 2.6%, 73.0 ± 1.9%, and 69.8 ± 2.0%, respectively. Likewise, the 50% release times of devices M, ME, and MM are 15 h, 20.5 h, and 46 h. And eventually, there are 71.2 ± 3.2%, 66.3 ± 1.7%, and 56.4 ± 4.3% of total ciprofloxacin released from them, respectively. The extended 50% release time is undoubtedly consistent with previous speculation, while the unexpected reduction in cumulative release from these devices demonstrated that the ciprofloxacin could interact with the SELP-47K protein polymer chains on their delivery pathways.

5.3.3. Influence of core properties on release kinetics

The ciprofloxacin release from DE, EE, and ME (Figure 5.4, left panel) and DM, EM, and MM (Figure 5.4, right panel) was also investigated in order to elucidate the influence of core physical stability on the release kinetics. The physical crosslinking of air dried non-treated protein film is not strong enough to keep the film intact in water, it is still water soluble. While the EtOH-, MeOH-treatment have significantly improved the
stability of the SELP-47K protein films, they won’t break down once rehydrated in water.

In addition, the MeOH-treated SELP-47K protein films are more stable than EtOH-treated ones as concluded from the soluble fraction study. The ciprofloxacin release profile from devices DE, EE, and ME and DM, EM, and MM indeed followed this trend. The final cumulative ciprofloxacin release was evaluated to be 85.2 ± 4.3%, 73.0 ± 1.9%, and 66.3 ± 1.7% for DE, EE, and ME. Similarly, the final drug release achieved at 73.9 ± 4.4%, 69.8 ± 2.0%, and 56.4 ± 4.3% for DM, EM, and MM. Interestingly, although the final release extent showed a decrease trend, the time to achieve 50% release didn’t increase. The 50% release for DE, EE, and ME was obtained to be 22 h, 18 h, and 20.5 h; for DM, EM, and MM to be 31.5 h, 48 h, and 46 h. Thus the parameter 50% release time is not able to capture the release features. It is also worth noting that the drug release from devices EM and MM showed the kinetics clearly deviate from the first-order. With the objective to clarify the underlying drug release mechanism, a physics-based model was developed for simulation to capture the full essences of the release patterns.

Figure 5.4. Ciprofloxacin release kinetics from non- (○), EtOH- (△), and MeOH-treated (□) drug-protein film cores with EtOH-treated protein coating (left panel), and non- (○),
EtOH- (▼), and MeOH-treated (□) drug-protein film cores with MeOH-treated protein coating (right panel). Solid lines represent the model fit results.

5.3.4. Model simulation of drug release

A physics-inspired model was constructed to gain new insights into the influence of physical crosslinking, protein coatings and cores on the release of ciprofloxacin. The comparison of model fit (solid lines) with the experimental ciprofloxacin release measurements (dash lines) is presented in Figure 5.1 to Figure 5.4. The parameters used in the simulations are listed in Table 5.1. Notably, the model fit well captures the ciprofloxacin release from all the drug delivery system studied. In particular, both $k_S$ and $\Delta G$ values fitted for M are smaller than those of E, suggesting MeOH-treatment not only induces less permeability of the SELP-47K protein polymer chain network which accounts for the lower convection rate constant $k_S$ but also increases the probability of ciprofloxacin to interact with the chain network. The result agrees very well with that MeOH-treatment generates higher physical crosslinking density than that of EtOH does which was determined from the soluble fraction study and swelling test.

For group DE and DM, the model fit indicates both $k_S$ and $\Delta G$ decreases while $k_{off}$ increases when changing the EtOH-treated SELP-47K protein coatings to MeOH-treated counterparts. While for E, EE, and EM, $k_S$ and $\Delta G$ derived from simulation decreases once the bare drug-protein films are embedded in the EtOH-treated, and MeOH-treated protein polymer coatings. The same trend for $k_S$ and $\Delta G$ has been observed for M, ME, and MM. Thus clearly, both types of coatings retard convection of
ciprofloxacin from the drug release system to extra-system buffer solution when compared to the non coated devices. And MeOH-treated protein coatings further slower down the drug convection rate than that of EtOH-treated coatings. In addition to affecting $k_s$, extra coatings increase the interaction between ciprofloxacin drug molecules and polymer chain network when comparing to the devices without coating. And as the MeOH-treated protein coatings have a higher density of crosslinker than EtOH-treated ones, they may further add up the interaction probability.

For group DE, EE, and ME, $\Delta G$ simulated from the physics-model decreases while $k_s$ remains at the comparable level. Model fit of DM, EM, and MM revealed a same tendency for free energy difference $\Delta G$ and convection rate constant $k_s$. These suggest that although the group of drug release devices has different cores, the ciprofloxacin can convect/diffuse out of the systems at similar rates presumably due to the fact that the protein coatings may have a more inhibitive effect on convection/diffusion of the drug than the cores do. The decrease in $\Delta G$ which was observed from same core groups and same coating groups can be interpreted as it is the combined effect from drug-protein cores and protein coatings, which both can intensify the interaction between ciprofloxacin molecules and SELP-47K protein polymer chain network while ciprofloxacin travels from the interior to medium surrounding the drug eluting systems.

5.3.5. Ciprofloxacin antimicrobial assay

Antimicrobial activity of ciprofloxacin eluted from drug-protein was tested to ensure that the effective was not impaired by the processing steps involved such as
physical crosslinking, extended presence in buffer solution at 34 °C. The released ciprofloxacin solution in 24 h was collected and tested against EC136 Cipro\textsuperscript{R} and EC 132 Cipro\textsuperscript{S} grown in Muller-Hinton medium. In particular, a complete inhibition of ciprofloxacin sensitive E. coli 136 growth was observed, comparable to that induced by fresh ciprofloxacin (Figure 5.5, left panel). For comparison, the ciprofloxacin resistant E. coli 132 exposed to ciprofloxacin continuously grew as shown in right panel of Figure 5.5. For both E. coli strains, their growth without any presence of ciprofloxacin was used as control.

![Graph](image)

Figure 5.5. Ciprofloxacin-sensitive \textit{E. coli} strain EC 136 Cipro\textsuperscript{S} (left-panel) and Ciprofloxacin-resistance \textit{E. coli} strain EC 132 Cipro\textsuperscript{R} growth (right panel) with the presence of ciprofloxacin fresh prepared or released from the drug release system.

5.4. Conclusions

The potential of optically transparent protein polymer SELP-47K films in various configurations for the sustained release of a common ocular antibiotic, ciprofloxacin was explored. The drug release kinetics can be modulated by the treatment of the films with
EtOH and MeOH, which alters their physical crosslinking density and, possibly, their drug/polymer chain interactions, as measured experimentally and simulated using a physics-based mathematical model. SELP-47K film specimens released ciprofloxacin over a period of up to 220 hours, with near-first order kinetics. The rate of release was affected by the degree of physical crosslinking of the films and the presence and treatment of the SELP-47K coating. Treating the specimens with ethanol or methanol vapor controlled the degree of crosslinking of the film while not altering the chemical structure of the polymer or the drug. The incorporation of ciprofloxacin in and its release from the protein polymer films did not affect its antimicrobial activity. Thus, the optically transparent recombinant protein polymer SELP-47K could well serve as an ophthalmic material to sustainably deliver drugs of considerable therapeutic interest.
CHAPTER 6: WATER VAPOR ANNEALING TO STABILIZE PROTEIN POLYMER STRUCTURE

6.1. Introduction

Recombinant protein-based biopolymers have been extensively investigated as they can be engineered to mimic the properties of naturally available counterparts, or to create novel materials which material properties are naturally unattainable [40, 151]. However, organic chemicals usually are used to stabilize protein structures to improve the mechanical strength of the materials for biomedical applications. For example, Elastin-like polypeptides (ELPs) are often crosslinked with chemicals, like glutaraldehyde [63], bis(sulfosuccinimidyl) suberate [64]. Similarly, silk-elastinlike protein polymers (SELPs) are often treated with methanol and glutaraldehyde for stabilized structures [54]. Due to the toxic nature of these chemicals, extra processes, washing, and rinsing, are usually needed to remove excessive or un-reacted chemicals. Nevertheless, despite the extensive rinsing, there might be a small amount of remaining chemicals on protein polymers, which might cause unexpected problems for later studies. Therefore, there is a great need to develop a chemical-free “green” method to stabilize protein structure. Among various proposed methods, water vapor annealing might be an appealing option [156]. It is a simple and effective method to obtain controlled molecular structure of silk-based biomaterials.
6.2. Materials and Methods

6.2.1. Water vapor annealing process

The cast dry SELP-47E protein polymer films were placed into a sealed humidified chamber at room temperature for water vapor treatment. At predetermined time intervals, the water vapor annealed protein polymer films were taken out of the water vapor chamber to stop the annealing process. The annealed protein films were air-dried prior to following studies.

6.2.2. Soluble fraction analysis

The soluble fraction of water vapor annealed SELP-47E films was evaluated in 2 ml of phosphate buffered saline (PBS) containing 0.2 mg/ml NaN₃ to prevent biological contamination [128, 157]. Prior to the study, the weight of the films was measured. At predetermined time points, the buffered saline was aliquot, and the amount of dissolved protein polymers contained in PBS was measured with a UV/Vis NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE) at a wavelength of 230 nm. The concentration and mass of dissolved protein polymers were calculated from a standard calibration curve prepared with known SELP-47E concentrations.

6.2.3. Fourier-transform infrared spectroscopy (FTIR)

A thin layer of protein polymer film was cast on FTIR windows made of ZnSe. The cast thin SELP-47E protein films on ZnSe windows were treated with water vapor at room temperature for different times, and they were air-dried in a dessicator prior to
FTIR analysis. Briefly, the spectra of water vapor annealed protein polymer films were recorded on a Magna-IR 560 Nicolet spectrometer (Madison, WI) in transmission mode. For each spectrum, 400 scans were collected and averaged over the spectral range of 4000-650 cm⁻¹ at a resolution of 4 cm⁻¹. The spectrometer was continuously purged with CO₂ free dry air to eliminate CO₂ and H₂O absorbance.

6.2.4. Optical microscopy and scanning electron microscopy (SEM)

The surface morphology of water vapor annealed protein films was examined with optical microscope and scanning electron microscope, respectively.

6.3. Results and Discussion

6.3.1. Protein polymer remaining fraction analysis

The soluble fraction represents the percentage of polymer chains in the initial aqueous solution that does not participate in network formation [128]. For solvent cast dry films, which are not water vapor annealed, they could readily dissolve in PBS showing no remaining fraction in Figure 6.1. The remaining fraction of SELP-47E protein polymer films gradually increases once the water vapor annealing time is increased from 0 hrs to 24 hrs. SELP-47E protein films, which are water vapor annealed for 24 hrs, have more than 50% remaining weight after extensive immersion in 1X PBS. These results suggest that water vapor annealing process could induce more polymer chain interactions, thus the higher extent of physical crosslinking density of the protein polymer chain network, in contrast to air-dried films that fully dissolve in PBS.
Figure 6.1. Remaining fraction of SELP-47E films with water vapor annealing for different times.

6.3.2. Secondary structure analysis

FTIR spectra analysis was used to examine the changes in secondary structures of SELP-47E films induced by water vapor annealing processing (Figure 6.2.). The amide I of SELP-47E protein films showed a peak band at 1662 cm\(^{-1}\), which is a marker band for \(\beta\)-turn structure. Upon increasing the water vapor annealing time, the band at 1626 cm\(^{-1}\),
which is a typical band for β-sheet structure, becomes more distinct. The gradual protrusion at peak 1626 cm\(^{-1}\) suggests that the water vapor annealing process would increase the content of β-sheet structure. Thus, qualitative FTIR analysis demonstrated water vapor-annealing induced a partial conformational conversion from less ordered β-turn structure to well packed, and more ordered β-sheets in SELP-47E protein films. This conversion provides the molecular basis for the water vapor annealing process which can be used to stabilize recombinant protein structures.

Figure 6.2. FTIR Amide I band of water vapor annealed SELP-47K films.
6.3.3. Surface morphology

The surface of cast dry SELP-47E protein polymer films was very flat, there are no observable features under conventional inverted optical microscope. Upon water vapor annealing treatment, micro-protrusions about a few hundred microns in size started to appear on SELP-47E protein film surface. A close examination on surface between micro-protrusions showed that there were micro-aggregates developed.

Figure 6.3. SEM images of water vapor annealed SELP-47E protein films

During the water vapor annealing process, water vapor molecules may play two roles for enhanced material integrity of SELP-47E protein films. First, they may penetrate into SELP-47E protein chain networks and act as plasticizers to lower the glass-transition temperature of SELPs. This would give SELPs protein polymer higher chain mobility for long range movement. Second, the water vapor molecules which bring enough thermal energy would make the long range protein polymer chain movement possible from the energy point of view.
6.4. Conclusion

Water vapor annealing of SELP-47E protein polymer films induces a partial conversion from less ordered $\beta$-turn to well packed $\beta$-sheet structure. This provides the molecular basis for water vapor annealing to be used as a chemical-free “green” process to stabilize the structure of recombinant protein polymers.
CHAPTER 7: CONCLUSIONS AND FUTURE WORK

7.1. Conclusions

Recombinant protein-based biopolymers may be utilized as a new model system for understanding the relationship between materials properties and molecular structure. In this work, a silk-elastinlike protein polymer, SELP-47K, which contain the peptide sequence from native silk and elastin was processed into film form by solvent casting method. The cast protein films were treated with methanol to induce the crystallization of silklike block, thus to significantly enhance their mechanical properties. The lysine residues introduced into the polymer chain permit the chemical crosslinking via glutaraldehyde inter-molecularly and intra-molecularly.

The resultant SELP-47K protein films were fully characterization in terms of their mechanical property, secondary structures, surface morphology, optical, surface, physical properties etc. In addition, the SELP-47K protein films were fabricated into various drug release devices to sustainably deliver a common ocular antibiotic.

The cast dry film is not water stable, it can readily dissolve back into water when rehydrated. However, its mechanical property was significantly improved upon methanol treatment. More interestingly, the methanol-treated protein films displayed mechanical properties which is closely match that of native elastin derived from bovine ligament. Glutaraldehyde crosslinking further enhanced the mechanical properties of SELP-47K protein films.
To elucidate the molecular basis for the obtained mechanical properties of SELP-47K films, Raman and FTIR spectra were recorded and analyzed for protein secondary structure. Qualitatively, the marker bands from Raman spectra showed that methanol treatment would induce a partial conversion from less ordered silk I to well packed silk II structure. Similarly, the typical bands on FTIR spectra suggested the crystallization and aggregation of silklike blocks upon methanol treatment. Quantitatively, the amide I band from FTIR spectra were deconvoluted using Gaussian band profiles. The individual Gaussian bands derived from curve fitting were used to estimate the protein secondary structure content. Indeed, the aggregated strands content increased by about 16% after methanol treatment which is completely consistent with the qualitatively spectra analysis.

Besides characterization, SELP-47K protein films were processed into various drug release devices for ophthalmic applications. In particular, the fabricated SELP-47K devices can sustainably released ciprofloxacin over a period of up to 220 hours, with near-first order kinetics. The rate of release can be controlled by the degree of physical crosslinking of the films and the presence and treatment of the SELP-47K coating. These results may have implications for silk-elastinlike protein polymers as a class of appealing novel biomaterials for medical applications.

7.2. Future Work

Despite the considerable work has been done in this dissertation and in other groups for silk-elastinlike protein polymers, there are a lot of research areas for this type of protein-based polymers to be explored in great detail.
Figure 7.1. AFM images of self-assembled nanofibers from SELP-815K.

Figure 7.2. Self-assembled nanofibers from SELP-815K (left) to globules from SELP-37K (right).
Although SELPs have been widely fabricated into various materials for the applications in drug delivery and tissue engineering, little is known about the fundamental self-assembly characteristics of these protein polymers [158-159]. Due to its amphiphilic nature, SELPs molecules may be able to self-assembly into various nanostructures (Figure 7.1). Atomic force microscopy (AFM) could be used to examine the self-assembled nanostructures and the associated kinetics. A variety of factors, like molecular composition, environmental stimuli may affect the nanostructure formation. Thus, it is possible through precise tuning of the ratio of silklike blocks to elastinlike block and other factors to generate various structures including nanoparticles, nanogels, and nanofibers. This self-assembly process might provide opportunities to generate innovative smart materials for biosensors, tissue engineering, and drug delivery. Furthermore, it may even have significant implications for protein aggregation related diseases, like Alzheimer’s disease.
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