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Fabrication of 3D Ultrafine Fibrous Protein Structures via Freeze-Drying

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FABRICATION OF 3D ULTRAFINE FIBROUS PROTEIN STRUCTURES
VIA FREEZE-DRYING

by

Yiling Huang

A THESIS

Presented to the Faculty of

The Graduate College at the University of Nebraska

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Lincoln, Nebraska

November, 2014
In this thesis, ultrafine fibrous 3D matrices were fabricated using three different proteins (soy protein, wool keratin, and chicken feather keratin) via freeze-drying. Protein matrices are preferable for tissue engineering compared to matrices made from synthetic material because of their similarity to native extracellular matrices. Due to their cell-binding motifs, natural proteins are also recognized as more biocompatible compared. Freeze-drying, which is a simple method used to produce 3D sponge matrices, was employed in this study to fabricate 3D fibrous matrices in a controlled manner. The inner structures of the 3D matrices fabricated ranged from film to fibers, and the diameters of the fibers ranged from the micro scale down to the nano scale. This controlled fabrication of protein matrices was achieved by individually varying protein concentration, SDS concentration, and freezing time. The techniques developed in this study to fabricate ultrafine fibrous 3D protein matrices could potentially be applied to other proteins and be used in tissue engineering applications.
Acknowledgement

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CHAPTER 1. INTRODUCTION

1.1 Tissue Engineering

Native organ or tissue loss from an injury or disease cannot be restored through the natural process of regeneration in the vast majority of cases. Therefore, tissue engineering plays an irreplaceable role in medicine for the development of appropriate biological substitutes in order to restore, replace or assist in the endogenous regeneration of defective tissue (Langer R, 1993; S Ramakrishna, 2005).

1.2 Biomedical Scaffolds

Scaffolds made of different biomaterials can mimic the extracellular matrices (ECMs) and serve as structural support to guide tissue development and also act as an adhesive substrate for implanted cell growth (Langer R, 1993). Scaffolds have been intensively studied for a long time. For efficient function, scaffolds should meet certain requirements. First, proper architecture and geometry of the scaffolds are needed for tissue or organ replacement. Second, adequate pore size and high porosity will allow deep and even distribution of cells through the whole structure, sufficient diffusion of cell nutrients and expressed products as well as transportation of metabolites (S Ramakrishna, 2005). Furthermore, water stability of the scaffolds is necessary to maintain the three dimensional architecture during the implantation. Scaffolds should also be made of biocompatible materials that have bio-signaling moieties to facilitate cell attachment and proliferation (Chen GP, 2002). Moreover, biodegradability of the scaffolds is an important factor
that allows scaffolds to be absorbed by native tissue instead of surgical removal. The degradation rate of the materials should also match the growth rate of cells. When the newly formed cells are fabricating their own ECM, the scaffold should eventually break down when it is no longer needed. Lastly, scaffolds should be cost effective and fabricated in a controlled and reproducible manner.

To meet these requirements, scaffolds can be designed by following structural concepts. Three-dimensional (3D) scaffolds are preferred over two-dimensional (2D) ones, because they structurally emulate the native ECMs to facilitate cellular growth and differentiation following the patterns of native organ (Cai SB, 2013). Fibrous structures are more favorable than other types of structures such as sponge-like (Gavenis K, 2006), film-like (Wang HJ, 2009), and hydrogel (Annabi N, 2009) structures because they are the most similar to extracellular matrices, which are built from collagen fibers with diameters ranging from 50-500nm (Liu XH, 2009). Scaffolds with fibrous structures have high porosity and interconnection. These properties provide transportation of oxygen, nutrients, and metabolic products from cells; they also assist in cell migration, adhesion and proliferation (Wei GB, 2008; Sill TJ, 2008). Therefore, scaffolds designed with 3D fibrous structures have been developed for biomedical applications (Zhang XH, 2008; Cai SB, 2013).

1.3 Materials for Fabricating Biomedical Scaffolds

Biocompatible materials such as polymers, metals, and ceramics have been widely used as surgical implantation (Chen GP, 2002). Among them polymer materials including synthetic polymers and natural polymers have been extensively made into scaffolds for tissue engineering due to the ability to vary their degradability and processability (Chen GP, 2002). Scaffolds made from synthetic polymers have demonstrated good mechanical properties (Pathiraja A, 2003).
However, due to their lack of bio-signaling motifs, cells do not tend to adhere well and proliferate as desired in these synthetic polymer-based scaffolds. The advantages of natural materials, such as natural polymers such as proteins, over that of synthetic materials are their preferable biocompatibility and biodegradability. Natural polymers such as collagen, gelatin, hyaluronic acid, and chitosan can well approach cell differentiation and expansion (Nazemi K, 2014). However, these materials have disadvantages such as poor mechanical properties and a fast rate of degradation, which must be overcome by crosslinking with other chemicals (Nazemi K, 2014). In terms of molecular structure, protein-based scaffolds are the most similar to native tissues and organs. Due to this similarity, protein scaffolds have the potential to facilitate biological functions and reactions and to be degraded by proteolysis. Furthermore, proteins can also serve as carriers of other molecules, such as growth factors and drugs, providing additional functionality to the fabricated scaffolds (Liu XH, 2009; MaHam A, 2009).

1.4 Methods for Fabricating Biomedical Scaffolds

Despite the large variety of techniques that have been developed for fabricating scaffolds, the methods of controlling the structure of 3D fibrous scaffolds are very limited. Currently, only three methods have been developed for fabricating 3D fibrous scaffolds: molecular self-assembly, electrospinning, 3D printing, and freeze-drying (Smith LA, 2008).

Molecular self-assembly is a method that can fabricate supramolecular architectures with ordered structures and stable arrangements chemical bonds via a spontaneous process (Decher G, 1997). Collagen scaffolds with diameters ranging from 50-500nm have also been fabricated by this method (Smith LA, 2008). Although this method can produce fibers with diameters at the nano
scale, the control of important factors for cell migration and proliferation such as pore sizes and pore structures is not well understood (Smith LA, 2008).

Electrospinning is a method that can fabricate scaffolds with long and uniform fibers by extruding them from a polymer solution using an electric field (Reneker DH, 1996). This method has long been used for fabricating 2D fibrous structures with materials such as PEO (Son WK, 2004), collagen (Dong B, 2009), chitosan (Geng XY, 2005), and silk protein (Li CM, 2006). The diameters of fibers can be controlled from the micro to the nano scale by changing the solution concentration, and the alignments of the fibers can also be controlled by rotating the grounded target (Smith LA, 2008). Recently, 3D fibrous scaffolds have been fabricated by electrospinning with materials such as zein and soy protein (Cai SB, 2013). However, electrospinning has difficulties in fabricating 3D fibrous structures with controllable shape due to its unique way to collecting fibers. There is also currently no literature on how to control fiber alignment in 3D fibrous scaffolds using electrospinning. The use of electrospinning places strict requirements on the spinnability of polymer solutions, and it is ineffective at producing scaffolds in large quantities.

3D printing technique is also a promising method to fabricate 3D fibrous structures. However, this method is more preferable for generating structures larger than the nano scale (Lam CXF, 2002).

Freeze-drying, or thermally induced phase separation, has been used for the purposes for producing 3D porous scaffolds for many years (Haugh MG 2010; Chen GP, 2002). Freeze-drying produces 3D porous structure by removing moisture from frozen materials. Solvent crystals and polymer will be phase separated by freezing polymer solution. A network of polymer structure will form and remain after freeze-drying; this process is also known as sublimation. During the
freeze-drying process, the frozen material is reduced by the surrounding pressure, and ice in the material is sublimated from the solid phase to the gaseous phase directly. In this process, freezing temperature is the key factor to the structures of scaffolds, because it induces a polymer solution to undergo a phase separation into a polymer-rich phase and a polymer lean phase (Smith LA, 2008). By using different materials, solvents, polymer concentrations, freezing temperatures, different inner structures and morphologies of scaffolds can be achieved (Smith LA, 2008). Due to its mold-based technology, the architecture of scaffolds is also controllable and can be produced easily into relatively desirable shapes (Smith LA, 2004).

In addition to being an easy and efficient method, freeze-drying can also produce 3D porous scaffolds with controllable alignment and shapes in large quantities and in a cost effective manner. Freeze-drying is also recognized as a green and sustainable method that the water worked as solvent is easily approachable and environmental friendly (Lei Q, 2010). Although freeze-drying has been traditionally used to produce scaffolds with sponge-like structures, fibrous scaffolds that mimic the fibrous structure of natural type I collagen have also been developed. However, the materials used in for making these scaffolds are limited to such as Poly (L-lactic acid) (PLLA) (Ma PX, 2006; Ma PX, 1999), gelatin (Liu XH, 2009), and chitosan (Kim MY, 2011). In the fabrication process, freeze-drying is combined with additional complicated procedures such as casting, gelation, solvent exchange (Liu XH, 2009; Ma PX, 2006; Ma PX, 1999), and electrospaying (Kim MY, 2011). In this study, only the simple process of freeze-drying was employed to fabricate 3D fibrous structures with protein solution that can be easily prepared.
1.5 Formation of Ice Crystals during Freezing Process

The formation of ice crystal plays a crucial role in determining the structure of the resulting matrix after freeze-drying as the structure mirrors that of the ice crystals. Controlling the formation of ice crystals and the factors that influence the structure of ice crystals may be a feasible approach to fabricate matrices with desirable structures.

Nucleation which is the initial process of crystalline formation in the solution, is defined as the atomic or molecular rearrangement into a nucleus that has the ability to grow into large-sized crystals (Cubillas P, 2010). Primary nucleation is divided into homogeneous and heterogeneous by the presence of foreign particles in the solution (Cubillas P, 2010). Secondary nucleation will also be induced based on the existence of crystals in the same substance (Cubillas P, 2010). Nucleation and the growth of ice crystals are driven by supersaturation events which form at the interface between solute and ice crystals (Cubillas P, 2010).

During the formation of ice crystals, solute are moved onto the surface of crystals (Cubillas P, 2010). Many factors can affect ice crystal growth kinetics (Pawelec KM, 2014). Decreasing the temperature increases the number of crystals generally increases the growth rate (Pawelec KM, 2014; Hallett J, 1964). Higher molecular weight of the solute decreases the growth rate (Pawelec KM; Blond G, 1988). Increasing solute concentration generally decreases growth rate (Pawelec KM, 2014), and finally increasing the viscosity can increase growth rate (Pawelec KM; Blond G, 1988). Crystal growth kinetics determines the final structure of the solid. For example, when the rate of ice crystal formation increases, the spacing between fibrous structures has been shown to decrease in ceramic scaffolds (Deville S, 2006). It has also been demonstrated that when the temperature gradient is high, crystals grow along the direction of the temperature gradient.
regardless of favorable crystal orientations (Deville S, 2011). If the ice front velocity is too low, uniformly oriented structures do not form (Bareggi A, 2011).
2.1 Natural Protein Structures

In terms of material, natural polymer could be more preferable than synthetic polymer as biomaterial, because synthetic materials may have potential to cause inflammation and produce toxic products.

It is also known that keratin-based materials are preferred due to their biocompatibility, mechanical durability, and biodegradability (Rouse JG, 2010). In Tachibaba’s study (Tachibana A, 2002), wool keratin sponge scaffolds were produced via freeze-drying. The resulting structures showed high-density and long-term cell growth, most likely due to the presence of cell binding motifs RGD and LDV that are important for cell adhesion and proliferation. Keratin from chicken feather was also fabricated into water-stable 3D fibrous structures using electrospinning method in Xu’s study (Xu HL, 2014). The structures made from chicken feather keratin are promising scaffold candidates for tissue engineering due to their desirable properties in cell growth and development. The application of keratin from chicken feather (a waste product from the poultry industry) in the biomedical field also solves an environmental disposal problem for abandoned waste products (Yin XC, 2013).

Soy protein, which is natural and abundant resource, has been attractive in the biomedical field as an alternative to animal-derived protein (Karen B., 2012). In Xu’s study, water-stable 3D ultrafine fibrous soy protein scaffolds were fabricated for soft tissue engineering (Xu HL, 2014). The soy protein structures without extensive crosslinking showed good water stability, uniform distribution and allowed for the differentiation of stem cells. In Chien’s study (Chien KB, 2013),
3D porous soy protein scaffolds were produced using freeze-drying and 3D printing. The resulting soy protein scaffolds were also adaptable for use as implant for tissue regeneration.

The natural protein gelatin was also fabricated into 3D structures for tissue engineering (Liu XH, 2009). However, extensive crosslinking was required to improve its water stability.

2.2 Fibrous Structures via Freeze-Drying

Recent advances have been achieved in fabricating 3D fibrous scaffolds using freeze-drying.

Early in 1980, Walter Mahler and Max F. Bechtold fabricated freeze formed silica fibers (Mahler W, 1980). Directional freezing of liquid solution produced a variety of microstructures composed of silica fibers.

Studies from Peter X. Ma and Ruiyun Zhang showed that materials such as Poly (L-lactic acid) (PLLA) and Poly (D-L-lactic acid) (PDLLA) can be fabricated into nano-scale fibrous synthetic extracellular matrices (160-170nm) by applying freeze-drying (Ma PX, 1999).

Chen, Smith and Ma produced 3D nano-fibrous scaffolds composed of poly (L-lactic acid) (PLLA) using reverse solid freeform fabrication and thermal phase separation for bone tissue engineering. In this work, internal structures, pore sizes, and external scaffold shapes were controlled using computed-tomography scans and histological sections. (Chen VJ, 2006).
In addition to synthetic materials, 3D fibrous scaffolds made from the protein gelatin have also been produced (Liu XH, 2009). The fibers in these scaffolds were thin (around 157nm in diameter), and the fiber lengths were around 497nm (Liu XH, 2009).

Studies from Kim and Lee showed that fibrous structures made of chitosan from nonwoven fabrics could be fabricated by freeze-drying. The nanoparticle solution for fabricating fibrous structures was prepared by electro-spraying particle suspensions of chitosan at low concentration (Kim MY, 2011).

2.3 Ice-Template

Freeze-templating is a novel approach that produces porous structures by templating and freezing solvent (Deville S, 2008). Template-free strategy is more preferable than template-dependent due to its low cost and the ability to produce in large quantities (Xie X, 2013). Different 3D porous structures such as sponge-like, film-like, and fibrous have been fabricated using this method with different materials.

Orientated of ice crystal growth leads to the directional formation of fibers in the matrix. To approach the desired structure, controlling the heat flow during the freezing process is necessary. Many studies made efforts on producing directional structures. For example, in Stefan Flauder and Thomas Heinze’s research (Flauder S, 2013), cellulose scaffolds were fabricated using ice-templating. The aligned cellulose network was fabricated by freezing from bottom of the mold, which allowed for heat flow in only one direction. By controlling the rate in which the solution is
immersed in liquid nitrogen, different structures were produced because the rate of immersion is correlates with the rate of cooling (Park SH, 2013).

Freezing temperature is recognized as one of the most important factors for controlling the range of structures. In Zhao’s work (Zhao K, 2011), decreasing lamellar spacing were observed in the fabricated hydroxyapatite (HA) scaffolds with aligned channels by increasing the freezing rate via ice-templating. In Kim’s work (Kim JW, 2008), 7 wt. % of PLLA/dehydrated 1, 4-dioxane solution was frozen in liquid nitrogen at different rates. The fabricated honeycomb structures demonstrated that as the freezing rate increased the density of tube increased and average tube diameters and thickness of the walls decreased. 0.1 wt. % chitosan solution were frozen at -20 Celsius degree and -196 Celsius degree and freeze-dried in the Lei’s study (Lei Q, 2010). At -20 Celsius, random macroporous structures were obtained and at -196 Celsius, nanofibrous structures were obtained.

The concentration of the solute is also another crucial factor that can control the structures of the matrix using ice-templating method. In Lee’s study (Lee J, 2011), cellulose microfibril porous foams were produced using unidirectional freezing method. Fibrous structures and channels were influenced by the concentration of microfibrils in the suspension. The results showed that increasing the concentration of the content leads to the structural transition from fibrous crosslinked network to lamellar channel structure, and further increase of concentration increases the wall thickness. In Kim’s study (Kim JW, 2008), Honeycomb structures were produced with PLLA solution by putting it at a constant rate in the liquid nitrogen. After changing the PLLA concentration from 10 wt. % to 3 wt. %, the tube diameter of the honeycomb structures and their wall thickness decreased while the number of tubes increased. PVA and SCMC dilute
solution were frozen in the liquid nitrogen, and fabricated into nanofibrous structures in Lei’s study (Lei Q, 2009). Changing the solute concentration from 0.5 wt. % to 0.05 wt. %, allowed fibrous structures to form instead of film-like structures.

Particle size is also a factor due to its influence on ice nucleation and growth during ice-templating (Deville S, 2010). In Deville’s study, it was proposed that nucleation and growth of ice crystals occur at relatively higher freezing temperatures for smaller size particles because the surface of the particles can act as nucleation sites (Deville S, 2010).
CHAPTER 3. OBJECTIVE

To fabricate 3D fibrous protein scaffolds by freeze-drying.
CHAPTER 4. MATERIALS AND METHODS

Table 1. Sample preparation parameters

<table>
<thead>
<tr>
<th>Varying protein concentration</th>
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<td>Freezing temperature</td>
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<table>
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<table>
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<tr>
<th>Varying freezing temperature</th>
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<tbody>
<tr>
<td>Protein concentration</td>
</tr>
<tr>
<td>0.025%</td>
</tr>
</tbody>
</table>

4.1 Materials

Chicken feathers, wool, and soy protein were used as raw materials for this experiment. Urea and cysteine were used to extract proteins from chicken feather and wool. Hydrochloric acid
and sodium sulfate were used for precipitation after the extracting process. SDS, cysteine, and buffer were used for dissolving proteins and acetone was used for removing SDS in the scaffolds.

4.2 Pretreatment

Keratin was extracted from chicken feathers and wool. Raw materials were dissolved in 8 molar urea solution containing cysteine as the reductive agents to induce thiol-disulfide exchange. After 24 hours, hydrochloric acid and sodium sulfate were used to precipitate the protein. The resulting proteins were washed with distilled water and dried in the oven.

4.3 Methods

![Protein SDS Cysteine Buffer → Freezing → Freeze drying → Washing (Acetone)](image)

Figure 1. The procedures of protein scaffold fabrications

For fabricating fibrous scaffolds from soy protein, keratin from chicken feathers and wool, proteins were dissolved with 10 wt. % based on the weight of proteins and six different protein concentrations (0.025 wt. %, 0.05 wt. %, 0.075 wt. %, 0.1 wt. %, 0.25 wt. %, 0.5 wt. %) and 4 different SDS concentrations (50 wt. %, 100 wt. %, 200 wt. %, 300 wt. %) with buffer at 70°C for 2 hours. The protein solution was frozen under three different temperatures (-20°C, -80°C, -196°C).
The frozen protein solution was then put into the freeze-drying machine until all moistures were removed. The resulting scaffolds were washed in 60% acetone.

4.4 Morphology Observation

The images of the bulk structures of matrices after freeze drying and fibers trapped in ice during freezing process were taken by digital camera. The detailed morphologies of structures after freeze dried were observed using scanning electron microscope (SEM) at different magnifications from 100x to 4500x.

4.5 Fiber Diameter

Fiber diameters were measured using software Image J by counting 100 fibers under each condition in their respective SEM pictures. Film-like structures were not measured.

4.6 Molecular Weight

Molecular weights of proteins (soy protein, keratin from wool and chicken feather) were tested by Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).
5.1 Molecular Weight

Figure 2. SDS-PAGE of proteins (Lane 1: standard protein maker, lane 2: soy protein, lane 3: keratin from chicken feather, and lane 4: keratin from wool)

SDS-PAGE showed that all three kinds of proteins (soy protein, keratin from wool and chicken feather) contain peptides larger than 188 kDa. It can be observed in lane 2 in Figure 1 that the major bands of soy protein are at 188 kDa, 62 kDa, 37 kDa and 20 kDa. Keratin from chicken feather and wool were extracted by a pretreatment process, which could lead to breaks in the
disulfide bonds compared to soy protein. Molecular weight of keratin from chicken feather are around 188 kDa, 20 kDa and 10 kDa which are shown in Figure 2 (lane 3). Molecular weight of keratin from wool are around 4~188 kDa Figure 2, lane 4.

5.2 Fibrous Structures of Protein Matrices

5.2.1 Morphology and Structure of Fibrous Protein Matrix

Figure 3. Image of the bulk structure of soy protein matrix after freeze-drying (Left); SEM image of the bulk of fibrous structure of soy protein matrix after freeze-drying (Right)

The architecture of protein matrix just after freeze-drying fabricated under low protein concentration and low freezing temperature Figure 3 (left), the protein matrix was fabricated in the same shape as the container filled with frozen protein solution. Controllable architectures of matrices could be obtained with the different desirable shapes by choosing different molds. The
inner structure of protein matrix is shown in Figure 3 (right), which was fibrous with diameters on the micro scale and highly interconnected.

### 5.2.2 Orientation of Fibers in the Matrix Formed in Freezing Process

<table>
<thead>
<tr>
<th>Freezing time</th>
<th>Initial freezing</th>
<th>A few minutes later</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.025% dyed gelatin solution at -20 °C</td>
<td><img src="imageA.png" alt="Image A" /></td>
<td><img src="imageB.png" alt="Image B" /></td>
</tr>
</tbody>
</table>

Figure 4. Images of freezing of 0.025% dyed gelatin solution at -20 °C taken at two different times: initial freezing (A) and a few minutes later (B).

To understand fiber orientation in the matrix, dyed gelatin solution with 0.025% protein concentration was frozen at -20 Degree Celsius. From Figure 4 (A and B), we can see that the solution started to freeze from its surface, which is the first point of contact for heat exchange in the refrigerator. The solution gradually froze into the center of the cup, the orientation of the fibers is the same as the direction of ice crystal growth. Ice crystals grow in the solution, and as a result, water molecules in the solution accumulate onto the ice crystals while excluding protein molecules. Front view and cross section of frozen dyed gelatin solution at -20 Degree Celsius in the cylinder
are shown in Figure 5. The fibers were oriented towards the center of the cylinder. This suggests that fiber formation mirrors the direction of ice crystal growth, which is along the temperature gradient (Xie X, 2013).

Figure 5. Images of frozen dyed gelatin solution (Frozen at -20 Degree Celsius in the cylinder)

Fiber orientation is marked with white arrows in both the front view and the cross section.
5.2.3 Fiber Formation of Matrix via Freezing

Figure 6. Image of cross section of frozen dyed gelatin solution

To understand fiber formation in the ice, an image of the cross section of frozen dyed gelatin solution was taken (Figure 6). As shown in Figure 5, the fine gelatin fibers (dyed) were aligned with the ice, and air bubbles were also excluded and trapped between the fibrous and extruded structures.
Figure 7. Schematic diagram of fiber formation during the freezing process for protein solutions at relatively low protein concentration (Dark blue: ice crystal; Light blue: solution; White: excluded or phase separated protein)

A schematic diagram of fiber formation is shown in Figure 7. Crystallization occurs during the freezing of protein solution. When a low protein concentration solution freezes in the refrigerator, nuclei are produced and grows into ice crystals which are projected from points of nucleation. As a result, protein molecules are excluded due to local supersaturation within the freezing solution and will be gradually accumulate and get trapped in the gaps of the surrounding projecting crystals. The trapped protein is phase-separated from the ice crystals and forms fibers.

During freezing process, fiber formation and extrusion could be influenced by the following: force of ice compression and created concentration gradient. The force of ice compression is created by the increasing volume of ice crystals, which are surround by the trapped protein molecules. The concentration of protein trapped in between growing ice crystals is higher
than the overall protein solution; therefore, a protein concentration gradient is created, and protein molecules move from areas of higher concentration to areas of lower concentration in the solution. This movement could lead to fiber extrusion during fiber formation. Another force helps fiber extrusion is created by the ice concentration gradient. As ice crystals grow in the solution, proteins near ice crystals will gradually increase.
5.3 Effects of Protein Concentration on the Structures of Freeze-Drying Matrices

5.3.1 Morphologies of Protein Matrices (Soy Protein, Keratin from Chicken Feather and Wool) under Different Protein Concentrations

**Soy Protein**

<table>
<thead>
<tr>
<th>Protein Concentration</th>
<th>Magnification</th>
<th>Magnification</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>X100</td>
<td>X 350</td>
</tr>
<tr>
<td>0.5%</td>
<td><img src="image" alt="Image" /></td>
<td><img src="image" alt="Image" /></td>
</tr>
<tr>
<td>0.25%</td>
<td><img src="image" alt="Image" /></td>
<td><img src="image" alt="Image" /></td>
</tr>
</tbody>
</table>
Figure 8 shows that the concentration of the soy protein solution influenced the structures of freeze-dried matrices. At 0.5 wt. % protein concentration, the matrix formed film-like structures with no fiber in it. At 0.25 wt. % protein concentration still no fiber formed, but film-like structures became smaller and were films at 0.5 wt. % concentration divided into several small pieces in length direction. This trend was more pronounced with decreasing protein concentrations as the film structures disappeared and fibers formed. At below 0.075 wt. % protein concentration, most of the structures were fibrous, and as protein concentration lowered, fibers became uniform and finer. At 0.025 wt. % protein concentration, the fibers were three-dimensionally interconnected with high porosity.
### Keratin from Chicken Feather

<table>
<thead>
<tr>
<th>Protein Concentration</th>
<th>Magnification X100</th>
<th>Magnification X 350</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5%</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
</tr>
<tr>
<td>0.25%</td>
<td><img src="image3.png" alt="Image" /></td>
<td><img src="image4.png" alt="Image" /></td>
</tr>
<tr>
<td>0.1%</td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
</tr>
</tbody>
</table>
Figure 9. Morphologies of chicken feather keratin matrices under different protein concentrations (SEM). Chicken feather protein matrices were freeze dried at different protein concentrations of 0.5 wt. %, 0.25 wt. %, 0.1 wt. %, 0.075 wt. %, 0.05 wt. %, 0.025 wt. % (Magnification: Left, 100x; Right, 350x)
Figure 9 demonstrated that concentration of chicken feather keratin also affected the formation of structures in the matrices. At 0.5 wt. % protein concentration, all structures were films which were bigger than films at 0.25% wt. protein concentration. At 0.1% wt. protein concentration, fiber structures started to form along with films; and at 0.075% wt. % chicken feather keratin concentration, most of the structures were fibers. As the protein concentration lowers, fibers became finer and more uniform.
## Keratin from Wool

<table>
<thead>
<tr>
<th>Protein Concentration</th>
<th>Magnification X100</th>
<th>Magnification X 350</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5%</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
</tr>
<tr>
<td>0.25%</td>
<td><img src="image3.png" alt="Image" /></td>
<td><img src="image4.png" alt="Image" /></td>
</tr>
<tr>
<td>0.1%</td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
</tr>
<tr>
<td>0.075%</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
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<tr>
<td>-------</td>
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</tr>
<tr>
<td>0.05%</td>
<td><img src="image3.png" alt="Image" /></td>
<td><img src="image4.png" alt="Image" /></td>
</tr>
<tr>
<td>0.025%</td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
</tr>
</tbody>
</table>

Figure 10. Morphologies of wool keratin matrices under different protein concentrations (SEM).

Wool protein matrices were freeze dried at different protein concentrations of 0.5 wt. %, 0.25 wt. %, 0.1 wt. %, 0.075 wt. %, 0.05 wt. %, 0.025 wt. % (Magnification :Left, 100x; Right, 350x).
As shown in **Figure 10**, morphologies of wool protein matrices also showed the same trend with respect to structural changes as observed for soy protein and chicken feather keratin as its protein concentration was varied. At 0.5 wt. % and 0.25 wt. % wool protein concentration, structures of matrices were films. At a 0.1 wt. % protein concentration, most of the structures were fibers, and fibers became finer and uniform with lower protein concentrations. Fibers were easily observed at protein concentration from 0.1 wt. %.
5.3.2 Diameters of Fibers from Matrices (Soy protein, Keratin from Chicken Feather and Wool) under Different Protein Concentrations

Figure 11. Diameters of fibers from proteins (soy protein, keratin from chicken feather and wool) freeze-dried matrices formed under different protein concentrations
As shown in the **Figure 11**, protein concentration had a great effect on the thickness of fibers from protein matrices. No fiber formed at protein concentrations greater than 0.1 wt. %. Fibers from each protein became finer with decreasing of the protein concentration. Diameters of fibers from chicken feather matrices showed the greatest amount of decrease compared to the other two proteins. Diameters of fibers from soy protein matrices decreased slightly from 4.51µm at 0.1 wt. % to 1.80 wt. % at 0.025 wt. %. Among the three proteins, diameters of wool keratin fibers reached the finest which was about 1.30µm. The large error bars in the chart indicates that the diameters of fibers were not uniform. As the protein concentration decreased, fibers became more uniform; nonetheless the diameters of fibers still had a wide range at low protein concentrations.
Figure 12. Schematic diagrams of fiber formation at high protein concentration (Left) and at low protein concentration (Right)

As shown in **Figure 8, Figure 9 and Figure 10**, structural changes from films to fibers and coarse fibers to fine fibers can be observed with continuous decrease in protein concentrations. This demonstrates that protein concentration played an important role in the formation of fibrous structures. The structural changes caused by varying protein concentration may be due to the following. First, when the protein concentration is low in the solution, the number of excluded protein molecules are relatively less than at higher protein concentration. As a result, fiber formation is finer. Second, at low protein concentrations, there is a relatively higher volume of total ice formation and higher compression pressure on the excluded or trapped protein between the ice crystals, leading to the formation of thinner fibers. Third, from the protein concentration gradient aspect, it is also possible that at low protein concentrations, it is easier for protein molecules to move to low protein concentration areas from areas of high protein concentration and in the same direction as ice crystal growth during freezing. The gradient created at a low protein
concentration may be higher relatively than the gradient created at a high protein concentration leading to the formation of finer fibers at low protein concentration. The schematic diagrams are represented in the Figure 12.
5.4 Effects of SDS Concentration on the Structures of Protein Freeze-Drying Matrices (Soy Protein, Keratin from Chicken Feather and Wool)

5.4.1 Morphologies of Protein Matrices (Soy protein, Keratin from Chicken Feather and Wool) with Different SDS Concentrations

Soy Protein

<table>
<thead>
<tr>
<th>SDS Concentration</th>
<th>Magnification</th>
<th>Magnification</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>350 X</td>
<td>1000 X</td>
</tr>
</tbody>
</table>

50 wt. %

100 wt. %
Figure 13. Morphologies of soy protein matrices under different SDS concentrations (SEM). Soy protein matrices were freeze dried at different SDS concentrations of 50 wt. %, 100 wt. %, 200 wt. %, 300 wt. %, (Magnification: Left, 350x; Right, 1000x)

**Figure 13** shown the morphologies of soy protein matrices at different SDS concentrations. At 50 wt. % SDS concentration all of the structures were films. At 100 wt. %, there were some fibers formed along with film structure. As the SDS concentration increased, more fibers were generated with less films and beads in the matrices. Structures of the matrices also became clearer with uniform, finer and longer fibers. At 300%, nearly all structures were fibers.
**Keratin from Chicken Feather**

<table>
<thead>
<tr>
<th>SDS Concentration</th>
<th>Magnification</th>
<th>Magnification</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 wt. %</td>
<td>350X</td>
<td>1000X</td>
</tr>
<tr>
<td>100 wt. %</td>
<td>350X</td>
<td>1000X</td>
</tr>
<tr>
<td>200 wt. %</td>
<td>350X</td>
<td>1000X</td>
</tr>
</tbody>
</table>
Figure 14. Morphologies of chicken feather keratin matrices under different SDS concentrations (SEM). Matrices were produced at 0.025 wt. % protein concentration and frozen at -20 Degree Celsius with different SDS concentrations of 50 wt. %, 100 wt. %, 200 wt. %, 300 wt. %.

(Magnification: Left, 350x; Right, 1000x)

As shown in Figure 14, the structures of chicken feather keratin matrices changed from fuzzy pieces of films into fibers with increasing SDS concentration. At 100 wt. % SDS concentration, fine fibers appeared along with film structures. When SDS concentration increased to 200 wt. %, film structures completely disappeared and coarse fibers dominated the matrix. As the SDS concentration increased to 300 wt. %, more and more fine fibers were fabricated.
## Keratin from Wool

<table>
<thead>
<tr>
<th>SDS Concentration</th>
<th>Magnification X350</th>
<th>Magnification X1000</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 wt. %</td>
<td><img src="image1.jpg" alt="Image" /></td>
<td><img src="image2.jpg" alt="Image" /></td>
</tr>
<tr>
<td>100 wt. %</td>
<td><img src="image3.jpg" alt="Image" /></td>
<td><img src="image4.jpg" alt="Image" /></td>
</tr>
<tr>
<td>200 wt. %</td>
<td><img src="image5.jpg" alt="Image" /></td>
<td><img src="image6.jpg" alt="Image" /></td>
</tr>
</tbody>
</table>
Figure 15. Morphologies of wool keratin matrices under different SDS concentrations (SEM).

Matrices were produced under conditions at 0.025 wt. % protein concentration and frozen at -20 Degree Celsius with different SDS concentrations of 50 wt. %, 100 wt. %, 200 wt. %, 300 wt. %,

(Magnification: Left, 350x; Right, 1000x)

**Figure 15** demonstrates that SDS concentration had influence on the structural changes of wool keratin matrices. These structural changes were similar with the previous changes observed from matrices made from soy protein and chicken feather keratin. At 50 wt. % and 100 wt. % SDS concentrations, both structures (film and fiber) were present. As SDS concentration increased, more fibers formed relative to at 50 wt. % SDS concentration. At 200 wt. % and 300 wt. % SDS concentrations, no film structure could be observed. There were also no significant changes with further increasing SDS concentration.
5.4.2 Diameters of Fibers from Protein Matrices (Soy Protein, Keratin from Chicken Feather and Wool) with Different SDS Concentrations

Figure 16. Diameters of fibers from protein (soy protein, keratin from chicken feather and wool) freeze dried matrices with different SDS concentrations

**Figure 16** demonstrates that SDS concentration played an important role on the diameter changes of freeze dried protein matrices. Similar diameter changes were observed for the three different proteins. At 50% wt. SDS concentration, fiber was observed only in matrices made from wool keratin. The diameters of soy protein and chicken feather keratin fibers were not measured.
since no fiber was observed at 50 wt. % SDS concentration. The average diameter of wool keratin fibers increased slightly and then decreased again as SDS concentration increased from 100 wt. % 300 wt. %. This pattern also occurred in the diameter changes of fibers with keratin from chicken feather. This result was due to the trend that thin fibers were formed at low SDS concentration alongside films structures; and then as SDS concentration increased coarse fibers were formed without film; further increasing of SDS concentration led to a decrease in the diameters of fibers without film structure.

SDS is a surfactant and helps dissolve proteins. When protein concentration and freezing temperature were set as constant, increasing the SDS concentration could allow more protein to be dissolved in the solution. When SDS concentration is low, the partially dissolved protein molecules may form aggregates and are excluded out from solution to form film structures. At the same time, some fully dissolved protein molecules may form fine fibers. This could be the reason that images in Figure 13, Figure 14, and Figure 15 show a mixture of films and fibers at low protein concentration and high freezing temperature. When SDS concentration is high, it is possible that more protein molecules were fully dissolved and distributed in the solution, which led to the production of finer and more uniform fibers. It is also possible that higher SDS concentration (up to a certain level) led to more stable protein molecules and higher ability for fiber extrusion in the solution. This may also contribute to the formation of finer fibers compared to a lower SDS concentration.
5.5 Effects of Freezing Temperature on the Structures of Protein Freeze-Drying Matrices

5.5.1 Morphologies of Protein Matrices (Soy Protein, Keratin from Chicken Feather and Wool) with Different Freezing Temperatures

**Soy Protein**

<table>
<thead>
<tr>
<th>Freezing Temperatures</th>
<th>Low magnification</th>
<th>High magnification</th>
</tr>
</thead>
<tbody>
<tr>
<td>-20°C</td>
<td><img src="image1" alt="Image" /></td>
<td><img src="image2" alt="Image" /></td>
</tr>
<tr>
<td></td>
<td>350x</td>
<td>1000x</td>
</tr>
<tr>
<td>-80°C</td>
<td><img src="image3" alt="Image" /></td>
<td><img src="image4" alt="Image" /></td>
</tr>
<tr>
<td></td>
<td>350x</td>
<td>1000x</td>
</tr>
</tbody>
</table>
Soy protein matrices were freeze dried at different freeze temperatures of -20 °C (Magnification: Left, 350x; Right, 1000x), -80°C (Magnification: Left, 350x; Right, 1000x), -196°C (Magnification: Left, 350x; Right, 4500x)

As shown in **Figure 17**, the freezing temperature had a drastic effect on structures of matrices. Fibers became finer with decreasing freezing temperature. At -20°C, fibers were coarse, long and flat; at -80°C, fibers became finer and more uniform, and at -196°C, fibers were short, coiled, and highly interconnected.
## Keratin from Chicken Feather

<table>
<thead>
<tr>
<th>Freezing Temperatures</th>
<th>Low magnification</th>
<th>High magnification</th>
</tr>
</thead>
<tbody>
<tr>
<td>-20°C</td>
<td>350x</td>
<td>1000x</td>
</tr>
<tr>
<td>-80°C</td>
<td>350x</td>
<td>1000x</td>
</tr>
<tr>
<td>-196°C</td>
<td>350x</td>
<td>6000x</td>
</tr>
</tbody>
</table>
Figure 18. Morphologies of chicken feather keratin matrices under different freezing temperatures (SEM). Matrices made from chicken feather keratin were freeze dried at different freeze temperatures of -20 °C (Left, 350x; Right, 1000x), -80°C (Left, 350x; Right, 1000x), -196°C (Left, 350x; Right, 6000x)

As it is shown in Figure 16, at -20 Degree Celsius freezing temperature fibers from chicken feather keratin were coarse and flat. The diameters of fibers decreased as freezing temperature lowered. At -196 Degree Celsius, fibers were fine with diameters on the nano scale and highly interconnected. The structures also had a high degree of orientation.
## Keratin from Wool

<table>
<thead>
<tr>
<th>Freezing Temperatures</th>
<th>Low magnification</th>
<th>High magnification</th>
</tr>
</thead>
<tbody>
<tr>
<td>-20°C</td>
<td>![Image] 350x</td>
<td>![Image] 1000x</td>
</tr>
<tr>
<td>-80°C</td>
<td>![Image] 350x</td>
<td>![Image] 1000x</td>
</tr>
<tr>
<td>-196°C</td>
<td>![Image] 350x</td>
<td>![Image] 6000x</td>
</tr>
</tbody>
</table>
Figure 19. Morphologies of wool keratin matrices under different freezing temperatures (SEM).

Matrices made from keratin from wool were freeze dried at different freeze temperatures of -20 °C (Left, 350x; Right, 1000x), -80°C (Left, 350x; Right, 1000x), -196°C (Left, 350x; Right, 4500x)

**Figure 19** shows that structural changes on the matrices made from wool keratin followed the same patterns of matrices from chicken feather keratin and soy protein, fibers became finer with decreasing freezing temperature. Fiber structures at -80 Degree Celsius freezing temperature were finer, longer and more uniform than fibers at -20 Degree Celsius.
5.5.2 Diameters of Fibers from Protein Matrices (Soy Protein, Keratin from Chicken Feather and Wool) with Different Freezing Temperatures

![Bar chart showing diameters of fibers from different proteins at varying temperatures.](chart.png)

Figure 20. Diameters of fibers from protein (soy protein, keratin from chicken feather and wool) freeze dried matrices controlled by freezing temperature

- Soy protein
- Keratin from chicken feather
- Keratin from wool
As it shown in **figure 20**, temperature had a great effect on diameters of fibers. Fibers became finer with decreasing freezing temperature. At all of these freezing temperatures (-20°C, -80°C, -196°C) with 0.025 wt. % protein concentration and 300 wt. % SDS concentration, there were no film structures observed in the matrices. At -20°C freezing temperature diameters of fibers from wool keratin were at 5.69µm on average and diameters of fibers from keratin from chicken feather and soy protein were around 3.5µm. At -80°C, diameters of fibers from those three proteins were around 1.5µm. At -196°C freezing temperature diameters of fibers from the matrices made from three different proteins were all fine which was a jump to the nano scale from the micro scale and were similar to the diameters of the natural collagen fibrils in ECM (50 to 500nm) (Kumar A, 2013). Notably, diameters of fibers of soy protein matrices reached to nearly 0.39µm.

![Figure 21](image.jpg)  
*High freezing temperature*  
*Low freezing temperature*  

**Figure 21.** Schematic diagram of fiber formation affected by freezing temperature
As shown in the SEM images of matrices (Figures 17, 18, and 19), the diameters of fibers fabricated by freeze-drying were significantly lowered with decreasing freezing temperature. This result indicates that freezing temperature greatly influences the formation of fibers especially fiber width. Fibers were formed during the freezing process and fiber structures were related to the formation of ice crystals. To develop desirable fibrous structures instead of film structures, triple interfaces among three ice phases could be more favorable than between two phases (Kim MY, 2011). Therefore, the formation of numerous thin ice crystal columns, which are mainly produced at a fast freezing rate (Kim MY, 2011) are needed. It is also possible that at low freezing temperatures there are numerous nuclei of ice crystals formed, and the fast cooling rate lead to a rapid extraction of heat during crystallization resulting in the inhabitation of large ice crystals (Kang HW, 1999).

When freezing temperature is high, fewer nuclei will be created. Due to the slow rate of freezing, water molecules on the protein will gradually move onto the nuclei, resulting in the formation of large ice crystals. Protein molecules that have lost water will aggregate to form large-diameter fibers or film structure. In the Xie’s study (Xie X, 2013), it was shown that the solidification direction was along with the lamellar ice crystals produced at a relatively high freezing temperature, at which the shape of ice crystal growth had a more dominant effect than the number of nuclei.

When freezing temperature is very low, the rate of freezing is fast, leading to the creation of a large number of nuclei. Due to the numerous nuclei created, crystals can form in both water-water interface and water-protein interface. Not like the aggregated protein at higher freezing temperature, the proteins molecules will be separated from each other more frequently and more
finely spaced due to the smaller size of ice crystals formed at the protein-water interface. It has also been shown that at low freezing temperatures, the dominant effect is nucleation, and the fast freezing rate of the solution at low freezing temperatures leads to the instantaneously production of nuclei throughout the solution (Xie X, 2013). During this process, there is not enough time and space to allow for the growth of ice crystals into lamellar structure (Xie X, 2013).
CHAPTER 6. CONCLUSIONS

In summary, 3D fibrous matrices were fabricated using freeze-drying with three different proteins (soy protein, chicken feather keratin, and wool keratin). Matrices with simple architecture can be fabricated into the same shapes as their molds. Centrally oriented fibers were obtained by simply exposing the solution in the refrigerator. The production of thin fibers can be achieved by decreasing the protein concentration, increasing the SDS concentration or decreasing the freezing temperature.
CHAPTER 7. LITERATURE CITED


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