CHAPTER II
LITERATURE REVIEW

2.1 Silk Fibroin

Silks are generally defined as protein polymers that are spun into fibers by some lepidoptera larvae such as silkworms, spiders, scorpions, mites and flies (Kaplan *et al.*, 1992, 1994, & 1998). Silk proteins are usually produced within specialized glands after biosynthesis in epithelial cells, followed by secretion into the lumen of these glands where the proteins are stored prior to spinning into fibers. Silks differ widely in composition, structure and properties depending on the specific source. The most extensively characterized silks are from the domesticated silkworm, *Bombyx mori*, and from spiders (*Nephila clavipes* and *Araneus diadematus*). Many of the more evolutionarily advanced spiders synthesize different types of silks. Each of these different silks has a different amino acid composition and exhibits mechanical properties tailored to their specific functions: reproduction as cocoon capsular structures, lines for prey capture, lifeline support (dragline), web construction and adhesion (Altman *et al.*, 2003).

The silk from the cocoon of *Bombyx mori* contains at least two major fibroin proteins, light and heavy chains, 25 and 325 kDa, respectively. These core fibers are encased in a sericin coat, a family of glue-like proteins that holds two fibroin fibers together to form the composite fibers of the cocoon case to protect the growing worm. This structural arrangement contrasts with spider silks where these glue-like proteins are generally absent. Silkworm cocoon silk production, known as sericulture, produces high yields since the larvae can be maintained in high densities. The core sequence repeats in the fibroin heavy chain from *Bombyx mori* include alanine–glycine repeats with serine or tyrosine (Altman *et al.*, 2003).

Nowadays silk is currently being explored for further biomaterial utility as the regenerated materials to form films, fiber, and scaffolds. The regenerated materials can be obtained by four major steps; degumming, dissolving, dialyzing, and fabricating. In the degumming step, the fibers are boiled (100°C) in an aqueous solution of Na_2CO_3 to remove the sericin proteins. Then, the degummed fibers can be

dissolved in various types of solvents such as an aqueous (9.3 M) Lithium Bromide (LiBr) solution (Jin *et al.*, 2005), aqueous saturated (9 M) lithium thiocyanate (LiCNS) solution (Yamada *et al.*, 2004), or $CaCl_2$:Ethanol:H_2O (1:2:8 molar ratio) (Min et al., 2004). After dissolution, the solution is then dialyzed against water to remove all salt molecules. Finally, it is depended on a desired structure and morphology e.g., solution-casting or freeze-drying technique was used to produce films or sponges, respectively.

Normally, the regenerated silk fibroin materials have to be subsequently stabilized with an aqueous alcohol solution (aqueous methanol solution is most widely used) to induce the transition of a random coil conformation to β-sheet structure. The conformation of the fibroin proteins can be classified into three forms; random coil, silk I, and β-sheet structure. Typically, the formation of β-sheet structure can be induced by treating with organic solvents such as aqueous alcohol solution (Nam *et al.*, 2001), by physical stretching (Jin *et al.*, 2005), or by heat treatment (Saitoh *et al.*, 2004) resulting in water-insoluble materials. The simple techniques used to characterize crystal structures of silk fibroin were FTIR spectroscopy (Magoshi *et al.*, 1979; Asakura *et al.*, 1985) and XRD (Asakura *et al.*, 1985; Jin *et al.*, 2005). However, Asakura *et al.* suggested that FTIR spectroscopy could not distinguish conformation difference between random coil and silk I forms.

2.2 Tissue Engineering

Annually, millions of individuals are affected by end-stage organ failure or tissue loss. In the United States alone, at least eight million surgical procedures are performed each year to address these disorders, requiring 40 to 90 million hospital days at total national health care cost exceeding $40 billion per annum (Langer & Vacanti, 1993).

Surgical strategies that have evolved to deal with tissue loss include organ transplantation from one individual to another, tissue transfer from a healthy to an affected site in the same individual, and of tissue function with mechanical devices

(such as prosthetic valves/joints and dialysis machines). Additional medical strategies include pharmacologic supplementation of the metabolic products of absent or non-functional tissue. While these strategies incorporate monumental advances in the field of medicine, they have a number of inherent limitations. Organ transplantation is constrained by the number of available donors. Currently, 50,000 people are on transplant waiting lists in the United States; many of these individuals will die before transplant material becomes available for them (Vacanti, Josef & Charles, 1997). Lifelong immunosuppression in organ recipients carries additional morbidity and mortality. Transferring tissue from one site to in the same individual often entails an imperfect match for the reconstructive need, as well as donor site morbidity and the potential for complications in the transferred tissue. Finally, mechanical devices have limited durability, lack the biological mechanisms of self-repair, may incur inflammation and/or infection, may necessitate anti-coagulation will not grow as the recipient grows (Marler *et al.*, 1998).

The goal of tissue engineering consists of activating regenerative abilities of the body that have come to a standstill and, if necessary, replacing damaged tissues with tissue implants. Due the special technical difficulties, the generation of tissue constructs requires the particularly close cooperation of medical doctors, cell biologists, material scientists and engineers. The spectrum of tissue engineering covers all kinds of tissue present in the body (Minuth, Strehl, & Schumacher, 2005). Formally, tissue engineering has been defined as the application of scientific principles to the design, construction, modification, growth, and maintenance of living tissue (Palsson *et al.*, 2003).

2.2.1 Cell Theraphy and Tissue Creation

Typically, isolated cells from donor tissues alone or in combination of an artificial extracellular matrix (ECM) to construct the bioartificial tissue or organ can be used for regenerative purposes. The former is termed cell therapy.

Figure 2.1 Principle of cell therapy. In cell therapy, cells from the patient are cultivated and injected into the damaged tissue or organ. In the therapy of burns and ulcers, the cultivated cells are laid, pipetted or sprayed (Minuth, Strehl, & Schumacher, 2005).

In cell therapy, a suspension of single cells is injected into sick or damaged tissue areas or placed onto them (see Figure 2.1). In this way the body's own regeneration should be promoted and supported. The cells used are in a relatively immature state at the time of implantation and should not develop completely without the influence of the surrounding tissue within the patient's body. The development of a functional tissue and its integration should take place within the surrounding areas. The cells required for this kind of therapy can be isolated from the patient's body (autologous) and multiplied *in vitro* before the implantation. In the future, totipotent or pluripotent stem cells may become available (Minuth, Strehl, & Schumacher, 2005).

In tissue creation, there are great advantages for regeneration and healing if whole functional tissues or their mature precursors, instead of isolated cells, can be used with patients. To do this, cells are isolated from the patient, e.g. from unburdened cartilage (see Figure 2.2), and multiplied in culture to reach a sufficient cell mass. Alternatively, stem cells can be used here. When the desired cell mass is reached, the cells are transferred onto an artificial ECM. Within or on top of this scaffold, the cells already start to develop a functional tissue in culture. Consequently, at the time of implantation, a partly mature tissue is already available, through which the risk of an undesired development is reduced and the duration of the healing process is shortened (Minuth, Strehl, & Schumacher, 2005).

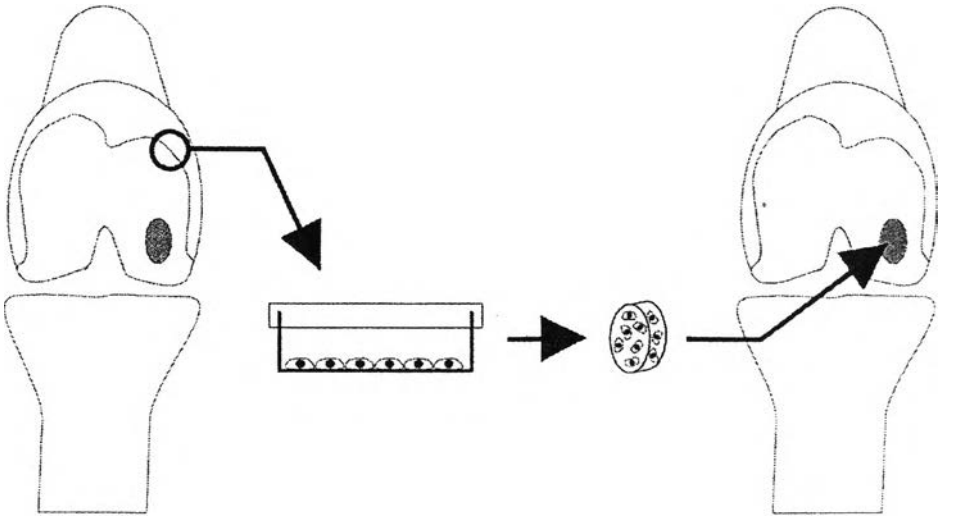

Figure 2.2 Principle of artificial tissue creation. The creation of a cartilage construct with cultivated cells and an artificial ECM (scaffold) for therapy of joint damage is shown (Minuth, Strehl, & Schumacher, 2005).

Most of the time, the handling and the mechanical resistance of such a construct are better than with cell therapy. Today, limitations of this technique are set by insufficient knowledge in the area of tissue development. Often the constructs do not reach the intended differentiation or level of function needed (Minuth, Strehl, & Schumacher, 2005).

2.2.2 Tissue Types

In order to develop the biological substitutes or activate the regenerative abilities of tissue or organ, basic knowledge about structure of the tissue must be considered and understood.

The cellular networks in complex organs reflect in the structural and functional characteristics of tissues. Tissue is not only an accumulation of individual cells, but consists of defined cellular and specific extracellular structures. Both parts are functional irreplaceable. Surprisingly, humans only possess four different kinds of basic tissue i.e. epithelia, connective tissue, muscle tissue and nervous tissue. From these come four completely different functions, such as the division of the organism from other compartments, the connection of structures, movement and

control. No organ of the body consists of only one basic tissue. Nearly all need each of the four tissues in a particular arrangement in order for each special function to become effective (Minuth, Strehl, & Schumacher, 2005).

2.2.3 Artificial ECM or Scaffold

Most cells produce not only their own organelles, but also proteins of the surrounding ECM. This is an interactive scaffold that provides mechanical stability and cell anchorage, and is also able to control cell functions. In building the ECM, cells synthesize mainly high-molecular-weight fibrous proteins, which are secreted out of the cell and built up in the surrounding environment to form an insoluble network. In epithelia or muscle cells this is a leaf-like basement membrane, whereas connective tissue cells form a three-dimensional network, called the pericellular or ECM. The basement membrane and the pericellular matrix consist mostly of the same protein families; however, due to the varied amino acid sequences, the individual components are differently interconnected. Components of the ECM include the various collagens, laminin, fibronectin and individual proteoglycans. In many tissues, the ECM is soft and elastic, whereas mechanically strong structures are formed in tendon, cartilage and bone (Minuth, Strehl, & Schumacher, 2005).

According to the structure of the tissue that consisting of the ECM in combination with individual cells to form a functional tissue or organ, a synthetic matrix which mimics the body's own ECM onto which cells can attach, migrate, multiply and function is used as an artificial ECM for scaffolding the seeding and/or migrating cells. Such a material is called a scaffold (Freyman *et al.*, 2001).

Basically, matrix material that used for producing a scaffold must satisfy a number of requirements. The solid phase must be biocompatible and promote cell growth and adhesion. Over time, as the cells produce their own natural ECM, the artificial matrix should degrade into non-toxic components that can be eliminated from the body. The processing technique must be able to produce irregular shapes to match those of the tissue to be replaced. The cellular structure must also be designed to satisfy several requirements. High porosity is needed for cell seeding and ingrowth (typical porosities are greater than 90%). The pore size

must be within a critical range (usually 100-200 μm): the lower bound is controlled by the size of the cells (~20 μm) while the upper bound is related to the specific surface area through the availability of binding sites. The porosity must be interconnected to allow ingrowth of cells, vascularization and diffusion of nutrients. And the material has to have sufficient mechanical integrity to resist handling during implantation and *in vivo* loading. Today, porous scaffolds are currently being developed for regeneration of skin, cartilage, bone, nerve, and liver (Freyman *et al.*, 2001).

2.3 Three-Dimensional Fabrications of Silk Fibroin for Tissue Engineering

As mentioned above, biomaterials are used as part of the engineered tissue, i.e., as scaffolds, providing physical support for cell attachment and growth. The use of scaffolds provides three-dimensional environments and brings the cells in close proximity to enable self-assembly and formation of various components that are associated with the tissue microenvironment. Ideally, the material will degrade as cells deposit their extracellular matrix molecules (Levenberg & Langer, 2004). Three-dimensional structure of scaffolds can be designed to several approaches depending on their morphology and shape. For example, scaffolds shaped into tubes are used for engineering tubular tissues such as engineered blood vessel (Lovett et al., 2007) or nerve guidance conduit (Yang et al., 2007). Scaffolds can also be constructed into the final required shape of soft cartilagenious tissue as in the nose or ear (Shastri et al., 2000). A viable pore structure of scaffolds sometimes is required to control the engineered tissue structure such as engineered bone-like structure (Hofmann et al., 2007). Up to now, techniques that are used for fabricating the three-dimensional structure of silk fibroin to achieve a various of forms and designs from simple to more complicated structure can be self-gelation, salt leaching, freeze drying, electrospinning, layer by layer depositing, knitting, enwinding, or in a combination of two techniques. Here, self-gelation, salt leaching, and freeze-drying technique will be discussed below.

2.3.1 Self-gelation

It is advantageous to use an aqueous-based system to form a desirable scaffold because essential components (such as a growth factor or cells) might be incorporated without losing their functions. The induction of self-gelation of silk fibroin is an irreversible process, and can be used directly from an aqueous silk fibroin solution after adjusting a desired concentration. The gelation rate is influenced by several parameters such as temperature, polymer concentration, pH, and calcium ion. For example, the 6 wt% silk fibroin solution can be kept for about 2 months before gelation occurred by refrigeration at 4°C while at room temperature within a week the solution of fibroin proteins were no longer mobile. The lower in the polymer concentration, pH, temperature, and/or higher of calcium ion concentration led to the longer gelation time (Kim et al., 2004). Recently, Wang et al (2008) utilize this gelation behavior in couple with ultrasonication treatment allowing rapid formation of the silk fibroin gels from only minutes to hours depending on power output and treatment time. These sonicated-silk gels can provide a three-dimensional environment in which the human bone marrow derived mesenchymal stem cells (hMSCs) are embedded with survival and sustained their functions. Thus these hMSCs-encapsulated silk gels are considered useful scaffolds for tissue regeneration. Moreover, this ultrasonication technique in couple with the self-gelation might be applied for drug-delivery system by injecting the suitable sonicated-solution of silk fibroin into targeted site and the gel would occurred within a desirable time (Cappello et al., 1998).

2.3.2 Sponge-like Structure

Some scaffolds are made into sponge-like structures, in which the pore size can be determined through the fabrication procedure. The size of the pores can affect how close the cells are at the initial stages of cultivation (allowing for cell–cell communication in three dimensions), but also influences how much space the cells have for self three-dimensional organization in later stages. Cell seeding in the center of the scaffold and feeding the inner surfaces of the scaffolds are limited when the pores are too small, whereas larger pores affect the stability of the scaffold and its ability to provide physical support for the seeded cells (Levenberg & Langer, 2004).

Silk fibroin scaffolds can be fabricated into sponge-like structure by either salt-leaching (Nazarov et al., 2004; Kim et al., 2005a & 2005b) or freeze-drying techniques (Wongpanit et al., 2007). The salt-leached silk fibroin sponges can be produced using aqueous-derived or organic solvent-derived methods (Nazarov et al., 2004; Kim et al., 2005a & 2005b). Generally speaking, salt used in salt-leaching technique should not be dissolved by the solvent of the polymer. Firstly, the process is started by adding salt particles into a polymer solution. The size and distribution of salt particle determine the pore size and pore distribution of the sponges. Sometimes the mixture of salt and solution might be kept in a period of time prior to evaporating the solvent out of the system in order to provide sufficient time for homogeneous distribution of the solution. Then salt particles in solidified polymer would be removed by immersion in water for several days. Finally, a highly porous sponge would be formed.

However, solvent of silk fibroin of aqueous-derived method, i.e., water can be used because even surface of salt crystal (NaCl) dissolved in silk fibroin solution, most of salt retained as solid particles due to saturation of solution. In addition, the sponges were stable in water without using a crosslinking agent because the hydrophobic interaction that occurred during the process act as a physical crosslinking forming β-sheet structure (Li et al., 2001; Kim et al., 2005b) while organic-derived method after the step of evaporation of organic solvent, the materials needed to be treated with aqueous methanol solution to induce the transition of a random coil to β-sheet structure (Nazarov et al., 2004).

Kim et al., (2005b) prepared the silk fibroin sponges by salt leaching techniques in order to compare between aqueous-derived or organic solvent-derived methods. Note that the organic solvent for organic solvent-derived method was hexafluoroisopropanol (HFIP). It was found that three-dimensional porous aqueous-based sponges exhibited a more uniform morphology compared with the HFIP-based sponges. The heterogeneity in the HFIP-based sponges is likely due to the evaporation of the solvent. For mechanical properties, even though the aqueous-based sponges have larger pores and required a lower concentration of silk fibroin in the preparation, the mechanical properties of aqueous-based sponges were similar to the HFIP-based silk sponges in term of the compressive strength but were superior in

term of compressive modulus. Additionally, the aqueous-based sponges almost fully degraded upon exposure to protease during 21 days. In contrast, HFIP-based sponges remained at least 70% of their initial weight.

Although the aqueous-based sponges offered essential properties for scaffolding materials as well as excellent supported adult mesenchymal stem cells (hMSCs) to express their phenotype of chondogenesis like native articular cartilage tissue (Wang et al., 2005), recently the use of HFIP-based method had been received considerable popularity to prepare the sponge-like structure of silk fibroin for both in vitro (Meinel et al., 2004a, 2004b, 2005 & 2006; Karageorgiou et al., 2006; Marolt et al., 2006; Chang et al., 2007; Hofmann et al., 2007; Mauney et al., 2007); and in vivo (Meinel et al., 2005 & 2006; Karageorgiou et al., 2006; Mauney et al., 2007) study. This is because the use of this organic solvent-based technique offer a wider range of pore size structure and controllable of pore geometry. For example, the HFIP-based method can produce pore size of the sponges over a range between >0 to 1400 μm (Kim et al., 2005a) while the lowest average pore size of aqueous-based sponges was ca. 500 μm due to the limitation of salt dissolution in the polymer solution. Typically, the pore size of scaffolds must be within a critical range. The lower bound is controlled by the size of the cells (~20 μm) while the upper bound is related to the specific surface area through the availability of binding sites (Freyman et al., 2001). In the case of aqueous-based method, it produced the sponges having a quite large average pore size which might not suit in practical whereas the HFIP-based method produced the adjustable pore size structure by varying a size of salt particles as well as concentration of silk fibroin. Furthermore, because of the pore structure of the sponges was governed by the size of salt particles, Hofmann et al., (2007) used salt leaching technique with HFIP-based method to control the pore geometry in the silk fibroin sponges by putting a smaller salt particle diameter range on the container before adding a larger salt particle diameter range on top. Subsequently, the 17% (w/v) silk fibroin solution in HFIP was added at a ratio of 20:1 (NaCl/silk fibroin). By using this technique, the sponges exhibited one side having a smaller pore and the other having a larger pore in a single material. In nature, a viable morphology on a single bone tissue was observed. Thus, the viable pore geometry in a single sponge enabled to control the structure of engineered tissue.

Another sponge-like structure producing technique is a freeze-drying. Usually, this technique has been used by mean of an aqueous-based system. Commercially, type I Collagen- Glycosaminoglycan (CG) sponges are successfully being used to regenerate skin in burn patients; this material received FDA approval for clinical use in 1996. Porous CG sponges have been primarily fabricated using a freeze-drying technique (Freyman et al., 2001). The structure of the sponge is controlled by the final temperature of freezing and concentration of the polymer solution. Lower the final temperature of freezing and higher concentration of the polymer solution, smaller ice crystals are formed, leading to a sponge with a smaller average pore diameter following sublimation (Li et al., 2001). The freeze-dried silk fibroin sponges must be treated with an aqueous methanol solution to induce the β-sheet formation to achieve the sponges insolubility in water. Additionally, the direction of heat transfer and the speed of heat transfer influence the shape of ice crystals, i.e., the existence of a predominant direction of heat transfer leads to the formation of columnar ice crystals with the major axis aligned in the predominant direction of heat transfer (Loree et al., 1989). Creation of a scaffold with an equiaxed pore structure requires removing predominant direction of heat transfer from the freezing process (O'Brien et al., 2004).