CHAPTER II

RELEVANT THEORY AND LITERATURE REVIEWS

2.1 Materials for scaffold fabrication (Ikada Y., 2006; Biondi, 2008)

Scaffold material has a significant effect on cellular activity. Depending on the tissue of interest and the specific application, the required scaffold material and its properties will be different. In general, a biologically active scaffold should provide

- well-defined porous structure with an interconnected pore network
- mechanical properties to temporarily give the biomechanical structural characteristics for the replaced tissue
- non-toxic, bioabsorbable substrate with a controllable absorption rate to match cell and tissue growth in vivo, eventually leaving no foreign materials within the replaced tissues

2.1.1 Synthetic polymers

Synthetic materials have been applied for the replacement of tissues and organs, fulfilling some auxiliary functions. Further possibilities exist now for synthetic materials to create tissues and organs with controlled mechanical properties and well-defined biological behavior. In biomaterial area, there are two kinds of synthetic polymers, non-absorbable and absorbable. Absorbable polymers have been used as key materials for artificial organs, implants, and other medical devices because provides many advantages over non-absorbable materials. Non-absorbable polymers are not adequate as the major component of permanent devices. The removal of non-absorbable implants is not satisfactory to both patients and economy.

2.1.2 Natural polymers

The sources of naturally occurring polymers are human, animals, or plants. Materials from natural sources such as collagen derived from animal tissues have been considered to be advantageous because of their inherent properties of biological recognition. However, the biologically derived materials have several concerns, especially complexities associated with purification, sustainable production, immunogenicity, and pathogen transmission. Apart from this fact, medical applications of absorbable natural polymers are limited, because their mechanical strength is not strong enough when hydrated. One exception is chitin (and chitosan) that is a crystalline polymer. Most of natural polymers are soluble in aqueous media or hydrophilic. Because water-soluble polymers are not appropriate as scaffolds, they should be converted into water-insoluble materials by physical or chemical reactions. In this study, silk and gelatin are introduced as natural polymers due to their attractive properties.

2.1.2.1 Silk (Gandhi M., 2006)

Silks are generally defined as protein polymers that are spun into fibers by Lepidoptera larvae such as silkworms, spiders, scorpions, mites and flies. The most widely interested silks are silkworm *Bombyx Mori* and spider *Nephila clavipes*. Silk is a natural protein fiber containing about 70-75% of actual fiber fibroin secreated from two salivary glands in the head of the silkworm larva, and about 25-30% sericin, a gum which cements the two filaments together as shown in Figure 2.1.

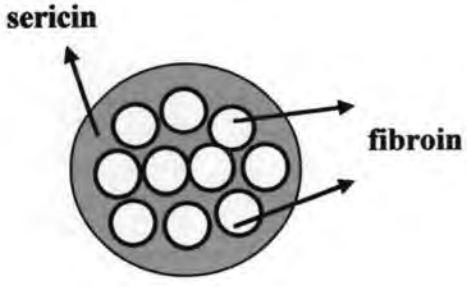

Figure 2.1 Structure of silk fiber

Structure of silk fiber

•Silk sericin

Sericin is the group of gummy protein that binds the fibroin filaments. It is a yellow, brittle, and inelastic substance. Sericin is insoluble in cold water, however, it is easily hydrolyzed, resulting in smaller fractions of molecules, which are easily solubilised in hot water (Mondal M. *et.al.*, 2007).

•Silk fibroin

A major constituent of raw silk fiber is silk fibroin. The structure of silk is generally β-sheet due to the dominance of hydrophobic domains of short side chain amino acids in the primary sequence. This structure permits tight packing of stacked sheets of hydrogen bonded anti-parallel chains of the protein (Figure 2.2). Large hydrophobic domains interspaced with smaller hydrophilic domains foster the assembly of silk and the strength and resiliency of silk fiber (Bini E. *et.al.*, 2004). Silk fibroin fibers are about 10 – 25 mm in diameter and consist of two proteins: light chain (~26k Da) and heavy chain (~390k Da), linked by a single disulfide bond.

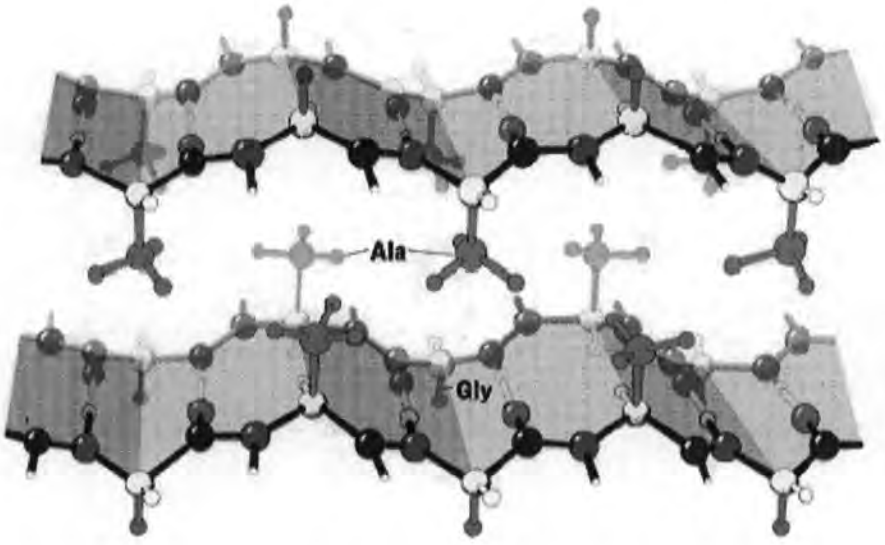

Figure 2.2 Structure of fibroin (http://www.cem.msu.edu)

The heavy chain forms the crystalline regions in silk fibers and make up 94% of the sequence. It consists of glycine-X repeats, with X being alanine, serine, threonine and valine. Each domain consists of sub-domain hexaapeptides including:

GAGAGS, GAGAGY, GAGAGA or GAGYGA where G is glycine, A is alanine, S is serine and Y is tyrosine (Figure 2.3). The repeat GAGAGS is the most frequently (70%) occurring hexapeptide repeat sequence. These sub-domains end with tetrapeptides such as GAAS or GAGS. The less crystalline forming regions of the fibroin heavy chain, known as linkers. All the linkers have non-repetitive sequence, which is composed of charged amino acids not found in the crystalline region.

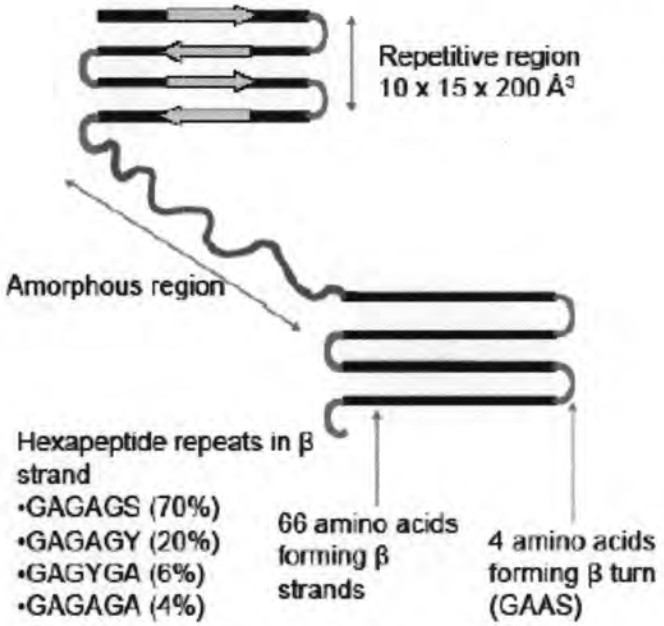

Figure 2.3 Schematic structure of Bombyx mori silk fiber protein (Gandhi M., 2006)

The amino acid composition of silk fibroin from *Bombyx Mori* is summarized in Table 2.1. The isoelectric point (IEP) is around 3.

Table 2.1 Amino acid compositions of *Bombyx mori* silk fibroin (Ayutsede, 2005)

Amino Acid	Symbol	Charge	Hydrophobic/ Hydrophilic	Amount (g/100 g silk fibroin)
Alanine	Ala	neutral	hydrophobic	32.4
Glycine	Gly	neutral	hydrophilic	42.8
Tyrosine	Tyr	neutral	hydrophilic	11.8
Serine	Ser	neutral	hydrophilic	14.7
Aspartate	Asp	negative	hydrophilic	1.73

Arginine	Arg	positive	hydrophilic	0.90
Histidine	His	positive	hydrophilic	0.32
Glutamate	Glu	negative	hydrophilic	1.74
Lysine	Lys	positive	hydrophilic	0.45
Valine	Val	neutral	hydrophobic	3.03
Leucine	Leu	neutral	hydrophobic	0.68
Isoleucine	Ile	neutral	hydrophobic	0.87
Phenylalanine	Phe	neutral	hydrophobic	1.15
Proline	Pro	neutral	hydrophobic	0.63
Threonine	Thr	neutral	hydrophilic	1.51
Methionine	Met	neutral	hydrophobic	0.10
Cysteine	Cys	neutral	hydrophobic	0.03
Trytophan	Trp	neutral	hydrophilic	0.36

Thai silk (http://www.moac.go.th/builder/mu/index.php)

Thai silk is one of *Bombyx mori* silkworms. Thai silk is domesticly produced in the northern and north-eastern parts of Thailand for textile industry. Yellow color and coarse filaments are the main characteristics of Thai silk. It contains more silk gum (up to 38%) than other normal *Bombyx mori* silk (20 – 25%)

Properties of silk

Silk is an excellent combination of lightweight with the density of 4.5 g/d in dry basis and 2.8-4.0 g/d in wet basis. Silk is insoluble in most solvents, including water, dilute acid or dilute alkaline solutions. Silk is thermally stable up to 250°C, allowing processing over a wide range of temperature. Silk is a poor conductor of electricity. Silk is biocompatible and biodegradability, good oxygen and water vapor permeability, and minimal inflammatory reaction.

Applications of silk (Ikada Y., 2006)

Silk is used in many applications including textile industry, cosmetics and medical applications. The commercialized product of silk has been employed for

medical fields. Furthermore, silk has been found to uses as artificial skin, blood vessels, and tendons. A non-woven cloth called Silk Sheet helps to keep fruit and vegetables fresh, with a significant economic benefit. Attempts are now being made to use silk to clean rivers polluted by household and industrial waste water. The pollutants are absorbed and broken down with bacteria cultured in gaps between scrap cocoons. Examples of silk applications are shown in Figure 2.4.

Figure 2.4 Applications of silk (http://en.wikipedia.org/wiki/Silk)

2.1.2.2 Gelatin (Tabata Y. *et.al.*, 1998)

Gelatin is prepared by the thermal denaturation of collagen, isolated from animal skin and bones, with very dilute acid. Its random coil structure can be readily soluble in water. Gelatin forms thermo-reversible gel, with the gel point below 30°C (depending on viscosity). Gelatin is commonly used for pharmaceutical and medical applications because of its solubility, biodegradability and biocompatibility in physiological environments.

Type of gelatin

Gelatin can be divided into 2 types depending on the production process as shown in Figure 2.5.

- Acidic gelatin

Acidic gelatin (Type B gelatin) is derived from alkaline process. The alkaline process, targets the amide groups of asparagines and glutamine, and hydrolyses them into carboxyl groups. Therefore, the converting of these residues to aspartate and glutamate yields gelatin with a high density of carboxyl groups, which makes the gelatin negatively charged. This type of gelatin has a lower IEP about 4-5.

●Basic gelatin

Basic gelatin (Type A gelatin) is derived from acid treatment of collagen. Basic gelatin produced from a milder condition, the products consist of higher amount of amino group. It contains more positive charges than that of type B gelatin. Type A gelatin has IEP at 9. Specifications for both types of gelatin are summarized in Table 2.2.

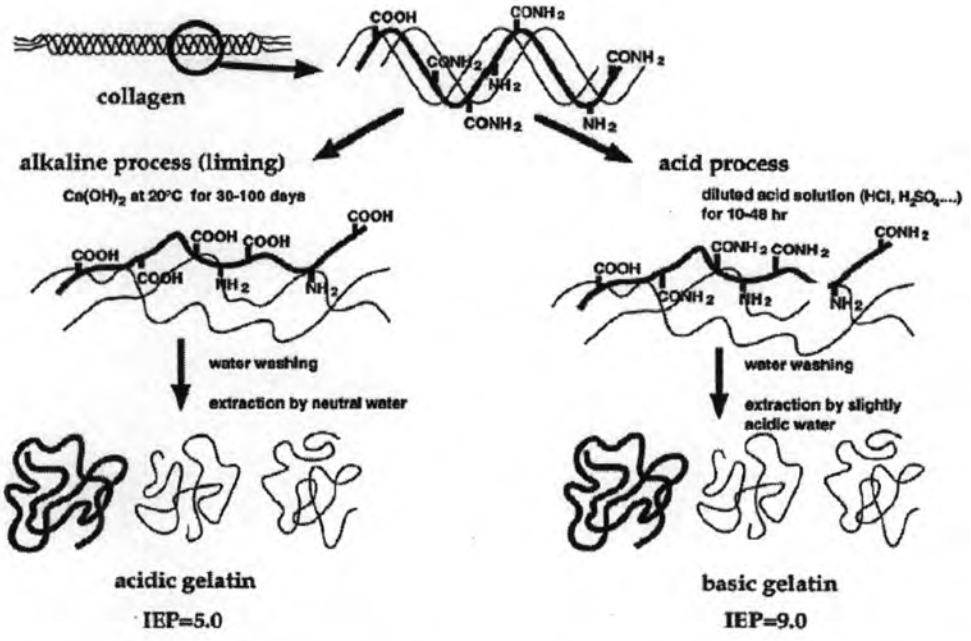

Figure 2.5 Preparation processes for acidic and basic gelatins from collagen (Tabata Y. *et.al.*, 1998)

Table 2.2 Specifications for type A and B gelatin (http://www.gelatin-gmia.com/html/rawmaterials_app.html)

	Type A	**Type B**
pH	3.8-5.5	5.0-7.5
Isoelectric Point	7.0-9.0	4.7-6.0
Gel strength (bloom)	50-300	50-300
Viscosity (mps)	15-75	20-75
Ash (%)	0.3-2.0	0.5-2.0

Amino acid compositions of gelatin

The amino acid composition of gelatin contains many glycine (almost 1 in 3 residues, arranged every third residue), proline and 4-hydroxyproline (4-Hyp) residues. A typical structure is –Ala-Gly-Pro-Arg-Gly-Glu-4Hyp-Gly-Pro- as shown in Figure 2.6. Amino acid composition of gelatin depends on the preparation process as shown Table 2.3.

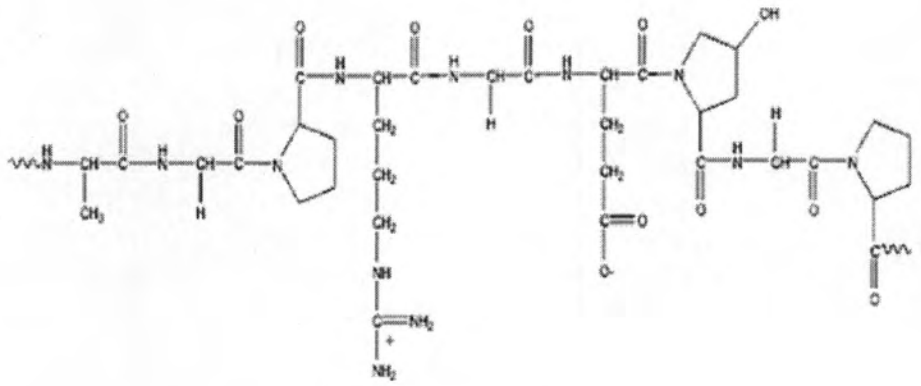

Figure 2.6 Structure of gelatin
(http://www.madehow.com/Volume-5/Gelatin.html)

Table 2.3 Amino acid composition of gelatin – per 1000 residues
(Philips G.O. and Williams P.A., 2000)

Amino acid	Type A gelatin	Type B gelatin
Arginine	49	48
Glutamic acid	25	72
Histidine	4	4
Hydroxyproline	91	93
Leucine	24	24
Methionine	4	4
Proline	132	124
Theronine	18	18
Valine	26	22

Alanine	112	117
Asperic acid	29	46
Glycine	330	335
Hydroxylysine	6	4
Isoleucine	10	11
Lycine	24	24
Phenylanine	14	14
Serine	35	33
Tyrosine	3	1

Properties of gelatin

Gelatin is translucent, colorless or slightly yellow. It is amphoteric and neither acidic nor alkali depending on the production process. Gelatin swells and absorbs 5 – 10 times its weight of water to form a gel in aqueous solutions at low temperature. Gelatin is soluble in hot water, glycerol, and acetic acid, and insoluble in organic solvents.

Applications of gelatin (Ikada Y., 2006)

Gelatin is used in a wide array of applications, most typically in edible/foods, pharmaceutical, photographic and technical products. Frozen foods, dessert confectionary, bakery filling and wine also have edible gelatin as an ingredient. Pharmaceutical gelatins are used in two-piece hard capsules, soft elastic capsules, tableting and micro-encapsulation. Gelatin is also used as photographical. Gelatin for photographic use is primarily Type B for emulsion preparation due to gelatin type A has limited application for coating and subbing. In addition, gelatin has also applied in paper manufacturing, printing processes and adhesives etc. Examples of gelatin applications are shown in Figure 2.7.

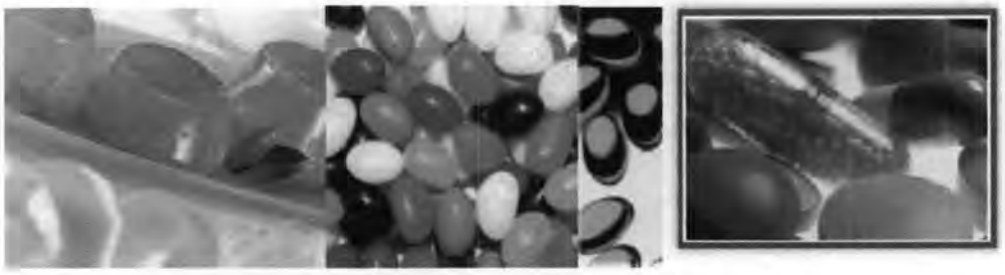

Figure 2.7 Applications of gelatin.
(http://www.gelatin-gmia.com/html/rawmaterials_app.html)

2.2 Fabrication of scaffolds (Ikada Y., 2006)

2.2.1 Freeze drying

Freeze drying is one of the most extensively used methods that produce matrices with porosity greater than 90%. Freeze drying process is allowed to induce phase separation of polymer and solvent. After removal of solvent, the space originally by the solvent becomes pores in the polymer matrix. The pore sizes depend on the growth rate of ice crystals during the freeze drying process.

2.2.2 Porogen leaching

Porogen leaching is a technique that has been used to fabricate porous scaffolds, because of easy operation and accurately controlling pore size and porosity. The particles leaching technique consists of adding particles to a polymer solution. The overall porosity and level of pore connectivity are regulated by the ratio of polymer/particles and the size of the particles. After drying, the incorporated particle is finally leached out using water to leave behind a water-insoluble polymer. Monosodium glutamate, alginate hydrogel, glucose and salt can be used as porogen particles.

2.2.3 Electrospinning (Subbiah T. *et.al.*, 2005; Kaplan D. *et.al.*, 2006)

Production of synthetic filaments using electrostatic forces has been known for more than one hundred years. This process is known as electrospinning. It has gained much attention in the last decade not only due to its versatility in spinning a wide variety of polymeric fibers but also due to its consistency in producing fibers in the submicron range. The ultrafine fibers from this electrospinning process exhibit several interesting characteristics, for examples, large surface area to mass or volume ratio, small pore size between depositing fibers of the electrospun mat, and flexibility for surface functionalization. These fibers have enormous applications in nanocatalysis, tissue scaffolds, protective clothing, filtration, and optical electronics.

2.2.3.1 Apparatus

The apparatus used for electrospinning is simple in construction, which consists of a high voltage electric source with positive or negative polarity, a syringe pump with capillaries or tubes to carry the solution from the syringe to a spinnerette, which is connected to high voltage power supply via a high voltage side or ground side, and a conducting collector like aluminum foil, which is connected to high voltage power supply as a counter electrode to the spinnerette. The collector can be made in any shape according to the requirements, like a flat plate, rotating drum, etc. A schematic of the electrospinning process is shown in Figure 2.8

2.2.3.2 Mechanism of electrospinning (Kleinmeyer J. *et.al.*, 2001; Subbiah T. *et.al.*, 2005)

Electrospinning utilizes a high voltage source to induce surface charge of a certain polarity on an outer surface of polymer droplet at the spinneret, which is then accelerated toward a collector of opposite polarity. The electrostatic repulsions between like charges at the surface of polymer solution become stronger the leading edge of the solution changes from a rounded meniscus to a cone (the Taylor cone). A fiber jet is eventually ejected from the Taylor cone as the electric field strength

exceeds the surface tension of the solution. As the charged jet initiated from the tip of the cone, it undergoes a electrically driven bending instability and travels towards the collector because of an electrostatic attraction. While the fiber jet travels through the atmosphere, the solvent evaporates, thus leading to the solidification of polymer fibers on the collector.

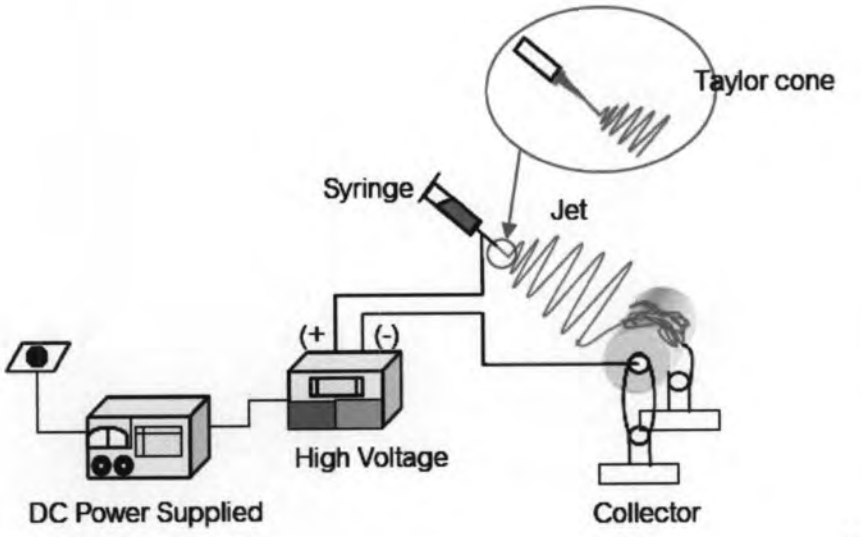

Figure 2.8 The schematic of electrospinning process
(Jeeratawatchai H., 2008)

2.2.3.3 Electrospinning parameters

Various instability modes that occur during the fiber forming process are expected to occur by the combined effect of both process and solution parameters.

1. Process parameters

- Applied voltage

In electrospinning, applied voltage used to created the electric field between spinneret and the collector . Increasing or decreasing applied voltage will increase or decrease the electric field strength that the polymer solution is subjected to in similar fashion, leading to change in surface charge density on the polymer droplet (at the initiation step) and the ejected charged jet (during fiber

spinning step), which in turn, influences polymer charged jet elongation and fiber size. The applied voltage also affects the mass flow rate of the charged jet at the spinning site, i.e. at the tip of the cone, which in turn affects the fiber size.

●Collecting distance

Collecting distance affect both fiber morphology and structure. At the same applied voltage, the electric field in the spinning system with a larger distance is lower than that with shorter distance since the electric field strength is a function of the potential difference (applied voltage) and inversely proportion to the distance between electrodes. The larger distance also provides the charged fiber jet in liquid stage sufficient time and space for solvent evaporation and formation solid fiber.

●Polymer flow rate

The flow rate of the polymer from the syringe is influences the material transfer rate and fiber uniformity.

●Spinning environment

Environmental conditions around the spinneret, like the surrounding air, its relative humidity, etc, influence the fiber structure and morphology of electrospun fibers.

2. Solution parameters

●Solution viscosity

Viscosity of polymer solution is the most important parameter in the electrospinning process along with the electric field strength of the system. In general, for each polymer-solvent pair, there is an optimum range of the solution viscosity which is suitable for fiber formation. Solution with too low viscosity does not have sufficient entanglement of polymer chain and leads to a break up of charged polymer jet in flight due to the capillary instability. The solution with too high viscosity requires much larger force to initiate and elongate resulting the formation of a large drop, non uniform fiber or no fiber at all. Only at the optimum viscosity range, where the restrain that one will get continuous fiber.

●Volatility of solvent

As electrospinning involves rapid solvent evaporation and

phase separation due to jet thinning, solvent vapor pressure determines the evaporation rate and the drying time. Solvent volatility plays a major role in the formation of nanostructures by influencing the phase separation process.

The effects of parameters on fiber morphology were summarized in Table 2.4.

Table 2.4 Effects of electrospinning parameters on fiber morphology (Still T.J. *et.al.*, 2008)

Parameter	Effect on fiber morphology
Increasing in applied voltage	Fiber diameter decreases initially, then increases.
Increasing in flow rate	Fiber diameter increases (beads occur if the flow rate is too high)
Increasing in distance (between needle and collector)	Fiber diameter decreases
Increasing polymer concentration (viscosity)	Fiber diameter increases (within optimal range)
Increasing in solution conductivity	Fiber diameter decreases (board diameter distribution)

2.2.3.4 Application of electrospun fibers

- Catalytic electrospun fibers

Chemical reactions employing enzyme catalyst are important in chemical process due to their high selectivity and mild reaction conditions. Nanomaterials are of recent interest as catalyst substrates due to their large surface area per unit mass and the feasibility for high catalyst loading. Nanofibrous catalysts could substitute catalytic nanoparticles in order to overcome the limitations of catalyst recovery.

- Filtration

The potential for using electrospun fiber webs as a filtering medium is highly promising. Knowing that the essential properties of protective clothing are high

moisture vapor transport, increased fabric breathability, and enhanced toxic chemical resistance, electrospun nanofiber membrane have been found to be good candidates for these applications. The highly porous electrospun membrane surfaces help in moisture vapor transmission.

- Tissue scaffolds

Electrospun fibers with high surface area and porosity have enormous scope for applications in engineering due to mechanically stable and biologically functional tissue scaffolds. The tissue scaffolding material must be selected carefully to ensure its biocompatibility with the body cells. The biocompatibility depends on the surface chemistry of the scaffolds, which is influenced by the material properties.

- Controlled drug delivery

Electrospun fibers mats also have an application as drug carriers for the drug delivery system. Either biodegradable or non-degradable materials can be use to control drug released via diffusion alone or diffusion and scaffold degradation. Additionally, due to the flexibility in material selection, a number of drugs can be delivered including antibiotics, anticancer drugs, proteins and DNA. Using the various electrospinning techniques, a number of different drug loading methods can also be utilized: coatings, embedded drug and encapsulated. These techniques can be used to give finer control over drug release kinetics.

2.3 Controlled drug delivery (Leong K.W. *et.al.*, 1987; Fan L.T., 1989)

It has long been recognized that simple pills and injections may not be the best mode of drug administration. To improve methods of drug administration, increasing efforts have been devoted to designing effective delivery systems. With a collaborative effort between polymer scientists, pharmacologists, engineers, chemists and medical researchers, significant accomplishments on controlled delivery have been obtained since research started several decades ago.

Controlled release may be defined as a technique by which active chemical are made available to a target site at a rate and duration so as to produce a desired effect. The types of polymer used for controlled release can be biodegradable and non-biodegradable. As drug-carriers, these polymers exist in the form of microspheres, matrices and membranes. The advantages are more efficient utilization of the active agent, possibility of targeting, less frequent administration and reduction in side effects.

The basic controlled release formulation consists of an active agent (drug, protein, fertilizer etc.) and a carrier (commonly a polymeric material) arranged so as to allow the active agent to be released at the target over a period of time at a controlled rate.

For non-biodegradable matrix and membrane devices, release is by diffusion and is driven by the concentration gradient. It can also be driven by osmotic pressure or matrix swelling. For matrices which are biodegradable or contain drug conjugates, release is controlled by the hydrolytic or enzymatic cleavage of the relevant chemical bonds, although diffusion of the reactants and the liberated drug molecules may sill be rate-limiting step.

2.3.1 Physical controlled release mechanism

2.3.1.1 The diffusion controlled release systems

Under this category, the release may be controlled by drug diffusion or solvent penetration. The diffusion controlled systems can be distinguished into reservoir and matrix devices.

- **Reservoir devices**

The drug reservoir is encapsulated by a polymeric membrane. The drug core can be in the solid or the liquid state, whereas the membrane can be microporous or non-porous. If the drug core is maintained in a saturated state, the

transport of drug molecules across the membrane will be kept constant, as the driving force is unchanged. Such a constant or zero-order-release would require the drug core to remain in a solid or suspension state. The saturated state would be difficult to maintain if the drug has high water solubility. Even if all the requirements for constant release are met, the release is generally not constant in the initial and end periods. When the device is placed in a releasing medium, it takes a certain time for the system to reach a steady state. Depending on the device, a lag time or a burst effect may be observed. If the membrane is devoid of drug molecules at the time of release, an induction period is needed to saturate the membrane. If, on the other hand, the drug molecules have been accumulating in the membrane as a result of fabrication or storage, the initial release rate will be higher than the steady-state value. Toward the end of the release period, the drug concentration in the core will drop below the saturation value and the release rate will decrease. If a non-perfect sink situation exists, which may occur in many in vivo situations, the increased mass-transfer resistance across the membrane will slow down the release.

- **Matrix devices**

The simplest and most widely used devices are the matrix system in which the drug is dissolved or dispersed in the polymer. One characteristic of these systems is a release rate decreasing with time as a result of increasing diffusion distance for the drug solutes to travel from the core to the surface. The amount of drug released is often proportional to the square root of elapsed time. In addition to permeation through the polymer phase, the drug molecules can also diffuse through channels created by dissolution of the drug phase. Release of macromolecules which have low permeability generally occurs via these pores. Since the extant and size of pores and channels created are determined by the drug incorporated, the loading level and particle size of the drug solutes have a profound influence on release kinetics. Below a critical loading level, some of the isolated drug particles may be trapped inside the matrix.

The release behavior of these diffusion-controlled systems is highly dependent on the physical properties of the drug. In addition to loading level and particle size of the solutes, the drug solubility in the polymer and drug diffusivity in

the polymer phase are both important parameters. The shape of the device, which determines the surface area available and the path length for diffusion, is also critical.

2.3.1.2 Solvent-controlled systems

The release of drug is regulated by the permeation of water through the polymer. An osmotic pump is constructed by enclosing a drug core with a semi-permeable membrane equipped with an orifice. When placed in water, the device will deliver a volume of drug solution or suspension equal to the water influx. The release rate is then determined by the nature of the membrane and the osmotic activity of the drug core. A constant release rate is obtained if the osmotic pressure across the membrane is kept constant by maintaining a saturated drug core.

Another solvent-activated system depends on the relaxation of the polymer as a result of water absorption. The drug is incorporated into a hydrogel, which in the dry state is glassy. In the presence of water, the hydrogel relaxes into an elastic state which presents little resistance to the diffusion of the drug solutes. The release rate is therefore mainly related to the swelling phenomenon. The important parameters are the hydrophilicity and crosslinking densities of the hydrogel.

2.3.2 Chemically controlled release mechanism

The two common chemically controlled systems are a biodegradable matrix in which the drug is dispersed, and a polymer-drug conjugation in which the drug molecules are linked to the side chains of the polymer. The dissolution of the matrix is effected by hydrolytic or enzymatic cleavage of the backbone of polymer. The cleavage can also occur in the crosslinking bonds, rendering soluble an initially crosslinked polymer. Alternatively, the dissolution originates from hydrolysis, ionization or protonation of the side chains of the polymer. If drug release for these systems is controlled solely by the biodegradation of the matrix, a constant release rate will be obtained, provided the surface area of the device is maintained constant. However, that represents an idealized case. In practice the drug molecules can also diffuse through the matrix. The release is commonly intermediate between zero-order

and first-order kinetics. This system has many advantages over the non-biodegradable systems.

The release rate of the polymer-drug conjugated is dependent on the cleavage of the polymer-bond. If the drug is attached to the polymer via a spacer, the hydrolysis of the polymer spacer and the spacer-drug bonds are both relevant. The spacer approach provides an effective mean of controlling the release rate. Although penetration of water into the matrix and outward diffusion of the cleaved drug molecules constitute part of the rate barrier, the cleavage of the polymer-drug bond as the rate-determining step is preferred for better control. Generally, the release rate drops with time as the drug concentration decreases.

2.3.3 Advantages of controlled release (Wnek G.E. *et.al.*, 2004)

- Reduce toxicity associated with bolus oral, injection.
- Reduce systemic toxicity by providing localized delivery.
- Provide precise timing in delivery.
- Bypass various barriers to drug delivery, such as cell membranes or aggressive digestion environments.
- Protect drugs from in-vivo metabolism allowing higher stability of the drug and longer periods of efficacy.
- Enhance delivery of poorly soluble drugs.

2.4 Literature reviews

2.4.1 Electrospinning of silk fibroin and gelatin

In 2005, Ayutsede J *et.al.* prepared silk fibroin solution for electrospinning by dissolving silk fiber in 50% aqueous $CaCl_2$ and dialyzed against deionized water. Then the obtained solution was frozen for 24h at -20°C and air-dried at room temperature. The regenerated silk fibroin was dissolved in formic acid (98-100%) to obtain 9-15%w/w concentration. The influence of the concentration of silk fibroin solution was investigated. Field emission environmental scanning electron microscope (FESEM) was used to determine average fiber diameter and distribution. Raman spectroscopy (RS), Fourier transform infrared (FTIR) spectroscopy and wide angle X-ray diffraction (WAXD) was used to characterize the secondary structure, chemical composition and crystalinity of silk fibroin at each stage of the electrospinning process. The experiments demonstrated that concentration of 9% could be spun into nanofiber with diameters ranging from 8-223 nm. The increased concentration resulted in an increase in the average diameter of silk fibroin electrospun fiber. The secondary conformation changed from random coil to β-sheet after electrospinning. Formic acid promoted the β-sheet crystallization and removal of formic acid from the fibroin led to a further increase in the β-sheet content.

In 2006, Chen C. *et.al.* prepared non-woven mats from stable regenerated silk fibroin aqueous solution at concentration ranging from 28-37wt%. No droplets or beaded fibers were observed at the concentration of 34-37% due to the appropriate viscosity to be spun and the droplet of solution suspended at the end of the syringe needle dried readily in the electrospinning process. The solution with a concentration of 34wt% was chosen to fabricate non-woven mats. The fiber had a belt-like morphology instead of general wire morphology. The as-spun fiber had a random coil conformation, after methanol treatment, the conformation changed to β-sheet.

In 2006, Li C. *et.al.* prepared 5 groups of electrospun silk fibroin scaffolds. For group 1, the silk/poly ethylene oxide (PEO) blend solution was prepared. For

group 2, similar solution as group 1 was prepared but PEO was removed by leaching in deionized water. For group 3, bone morphogenetic protein-2 (BMP-2) was added into silk fibroin/PEO solution. For group 4, nano-hydroxyapatite particles (nHAP) were added into silk fibroin/PEO solution. For group 5, similar solution as group 4 was prepared and BMP-2 was added at the last step. All five groups of electrospun fibers were treated with 100% methanol for 5 min. For group 2, the removal of PEO did not affect fiber morphology, which indicates that fiber integrity depended on the silk fibroin. The nHAP particles were embedded in the silk/PEO electrospun fiber scaffold with some aggregate. Mesenchymal stem cell (hMSCs) were seed on all five groups of electrospun scaffolds. Calcium content and total DNA on five different scaffolds were determined. In group 1, the scaffold showed the lowest calcium content and highest level of DNA. It might reflect the inverse relationship between proliferation and differentiation. The differentiation level on group 1 scaffold was lower compared to the other four groups. Significantly, higher calcium deposition was found for group 3-4. The PEO-extracted scaffold (group 2) showed a three-fold increase in calcium deposition. The incorporation of BMP-2(group 3) and nHAP(group 4) in the scaffolds resulted an increase in calcium deposition when compared to the silk/PEO scaffolds. The group 5, the scaffold contained the highest levels of calcium deposition.

In 2007, Meechaisue C. *et.al.* spun silk fibroin solution in formic acid and studied the effects of the increased concentration of the solution. Since either beaded or smooth fibers could only be prepared from the silk fibroin solutions with the concentration of 20-40%(w/v). The electrospun fiber mats from 35%(w/v) silk fibroin solution were used to culture mouse osteoblast-like cells (MC3T3-E1). Quantification of the viable cell on the surface of silk fibroin scaffold at each seeding time point (2, 4and 8 h) was similar. The proliferation of the cells on scaffold was determined at day 1, 3, 5. The viability of the proliferated cell was found to increase from that of the attachment period of 8 h. Since the pore size of the silk fibroin fibrous scaffolds was much smaller than that of the cells, cells could only attach and propagate on the surface of the scaffolds and fully cover the surface of the scaffold at day 5 after cell culture.

In 2008, Songchotikunpun P. *et.al.* extracted gelatin from fish skin and fabricated into fiber mat by electrospinning method. The effects of solvent (acetic acid and formic acid) and concentration of solvent (ranging from 10-100% (v/v)) were investigated. It was observed that for both types of acid solvent, shear viscosity increased with increasing the acid concentration. That was explained by free amino groups of some acid residues binded with dissociated protons causing the chains to carry positive charges and led to the expansion of the coil-like structure of the gelatin chains. A high acid concentration (40% acetic acid and 80% formic acid), the smooth fiber mats without bead were obtained. The smooth fiber mats were prepared from 20% and 23%(w/v) gelatin solution. It was found that after gluteraldehyde vapor crosslinking method, slight shrinkage from original dimension was observed, with an evidence of fibers fusing to one another at touching points.

In 2008, Guibo Y. *et.al.* aimed to modify the mechanical and biological properties of silk fibroin(SF) nanofibers with gelatin and investigate the feasibility for engineering blood vessel scaffolds. It has been found that the addition of gelatin into SF solution improve the spinnability of blend solution. When the blend proportion was kept at 70/30, the solution had the best spinnability. The obtained nanofibers were homogeneous, nonbeaded and continuous with the average fiber diameter of 83.9 nm. The electrospun fiber mats were immersed into methanol for 10 min to form β-sheet conformation of SF. The IR spectra and DTA curve showed that methanol could convert SF conformation from random coil to β-sheet conformation while gelatin electrospun fiber mats exhibited a mixture of α-helical and random coil conformation. The comparison of SF air dried membrane, SF and SF/gelatin nanofiber on cell culture were investigated. The result showed that number of the cell on SF and SF/gelatin nanofiber were significantly higher than that of SF air dried membrane. However, the cells seeded on SF/gelatin nanofibers were shuttle shape, which demonstrated that the spreading of cell on SF/gelatin nanofibers were definitely better than that on the SF nanofiber because of the addition of gelatin.

2.4.2 Studies of silk fibroin and gelatin systems

In 2001, Um I.C. *et.al.* compared structural characteristics, thermal and solution properties of the regenerated silk fibroin (SF) casted from formic acid with those of SF casted from distillated water. With an increase of SF concentration, turbidity of aqueous SF solution markedly increased. On the other hand, turbidity of SF formic acid solution unchanged with an increase of concentration and remained transparent. The shear viscosity of SF formic acid solution was nearly unchanged for 4 days, indicating that SF molecular chains were stable in formic acid without any severe degradation. After methanol-induced, SF solution in water has more crystallinity than SF solution in formic acid. Since methanol-induced crystallization of SF solution in formic acid suppressed by the precrystallized region induced by formic acid while that of SF solution in distillated water occurred without any restriction. Long-range ordered crystallites are predominantly formed in case of methanol treatment while short-range ordered structures are formed in case of formic acid.

In 2004, Bini E. *et.al.* fabricated silk scaffolds by freeze drying, salt leaching and gas foaming methods. Freeze drying scaffold was obtained by blending aqueous silk solution with methanol or propanol. The scaffold formed highly interconnected and porous structures with pore diameters of 50±20 μm. The higher the concentration of silk fibroin in solution, the smaller the pores. With 2-proponal, a more leaf morphology was observed, whereas with the methanol, smaller spherical pores were formed. The scaffold formed from methanol were friable and easily broken, unlike the scaffolds formed from 2-propanol. The pore size of salt leaching scaffold was 202±112 μm with highly interconnected while the scaffold formed by gas foaming had a highly interconnected open pore with diameters of 155±114 μm. The effect of solvent induced β-sheet conformation was analyzed by using methanol, 2-propanol and 1-butanol. The results showed methanol promoted β-sheet conformation, while 1-butanol and 2-propanol resulted slight β-sheet and no β-sheet conformation, respectively.

In 2006, Gil E. *et.al.* prepared protein blends by mixing gelatin (G) with silk fibroin (SF) and used aqueous methanol (MeOH) to post-induce SF crystallization. Both aqueous solution were prepared at same concentration of 4wt% and was casted into film. The dried film appeared homogeneous. Upon subsequent exposure to aqueous MeOH (75/25 w/w MeOH/water), SF undergoes a conformation change from random coil to β-sheet. This transformation occurs in pure SF, as well as in the G/SF blends. The SF conformational transition occurs in the presence of the triple-helix of G indicating that G remains unaffected by exposure to aqueous MeOH and does not constrain the SF chains as they undergo their solvent-induced conformational change. The formation of the β-sheet structure promotes thermal stability of pure SF and SF-containing blends at elevated temperatures. Crystallization of SF enhances the mechanical properties, such as tensile modulus, elongation and tensile strength of G/SF blends.

In 2008, Patel Z. *et.al.* prepared gelatin microparticles and studied the effects of gelatin crosslinking (glutaraldehyde concentration of 10 and 40 mM), growth factor dose (6 and 60 ng mg^{-1} of dry microparticles), release medium (phosphate buffer saline (PBS) and collagenase containing PBS (Coll)) and gelatin type (acidic and basic gelatin) on bone morphogenetic protein-2 (BMP-2) release. Increasing crosslinking extent, dose and acidic gelatin resulted in decreased release, while the addition of collagenase to the buffer caused increased BMP-2 release. The addition of collagenase can be confirmed that gelatin undergoes enzymatic degradation. In collagenase containing PBS, acidic gelatin microparticles crosslinked with 10mM gluteraldehyde were completely degraded by day 9, exhibiting 100% release of the BMP-2. However, acidic gelatin microparticles crosslinked with 40 mM gluteraldehyde did not show any visible degradation and also had less than 32% cumulative release over 28 days in collagenase containing PBS. The role of gelatin type on BMP-2 release was clear. BMP-2 would have lower release from acidic gelatin microparticles crosslinked with 40mM compared to basic gelatin due to stronger electrostatic attractions.

In 2009, Mandal B *et.al.* investigated the release profile of three model compounds from silk fibroin/gelatin (SF/G) multilayered films. The influence of the SF/G blend ratio (1:1, 2:1 and 4:1) and number of layers (one, three and five layers) on the degradation rate and release profile were studied. They have found that in case of fibroin/gelatin with ratio 1:1, degradation was more as compared to the other ratios. Furthermore they have found that on increasing the number of layers, degradation rate slowed down. The three different molecular weight of model compound (trypan blue (961Da), FITC-inulin (3.9kDa) and FITC-BSA (66kDa)) was loaded into SF/G blend solution and casted onto second layered-film. It was observed that in case of multi-layer's composed of pure fibroin, the release percentage is very low (8-10%) over period of 28 days. The higher the content of gelatin, faster was the release of model compounds. This due to the increasing in gelatin dissolution in samples with higher gelatin content, thus giving rise to more void volume for the release of model compound. The increasing in layered-film can slow the release rate which lead to the controllable release behavior. The different molecular weight in three model compound resulted in different release rates. The trypan blue with the lowest molecular weight showed the maximum release percentage while the FITC-BSA with the highest molecular weight showed the minimum release percentage. This result revealed that the small size of molecule (trypan blue) can be diffuse through the void of the layered-film faster than the big size of molecule (FITC-BSA). From these points, it concluded that the desirable degradation and release rate can be determined by SF/G blended ratio and number of layers.

2.4.3 Studies of the controlled release of electrospun fiber mats

In 2009, Maretschek S. *et.al.* have studied controlled release profile of fiber mats fabricated via electrospinning process. Cytochrome C was used as model drug release and loaded into poly(L-lactide) (PLLA) solution for further electrospinning. To optimize loading method, transmission electron microscopy (TEM) was used. TEM revealed crystallite structures of cytochrome C outside the fibers. Native PAGE gel electrophoresis was used to check whether the electrospinning process would have a negative effect on the protein. The resulted proved that neither degradation nor

aggregation of the protein occurred during electrospinning and electrospun proteins are stable and retain their bioactivity. The influence of the polymer concentration in chloroform on the release profile was investigated. The fiber mats from 1wt%, 2wt% or 3wt% PLLA solution were immersed in PBS solutions and the amount of released protein was determined via BCA assays. The fiber mats from 2wt% and 3wt% solution showed no burst release, implying the perfect inclusion of the protein within fiber mats while fiber mats from 1wt% solution showed a slightly increased release. The reason is their highly hydrophobic surface characteristics which led to very slow wetting. To clarify the effect of wettability on the protein release, Tween 20 was added to release media. Higher concentrations of Tween 20 led to a faster release of the incorporated protein. This proved that the wettability of fiber mats response for the release rate. Major part of cytochrome C was encapsulated within the fiber mats but not within the fiber itself. The addition of hydrophilic polymers was used to reduce hydrophobic surface of fiber mats. With increasing amount of hydrophilic polymers the hydrophobicity of fiber mats was reduced and the protein release rate was increased.