CHAPTER II

THEORY AND LITERATURE REVIEWS

2.1 Tissue engineering

The term 'tissue engineering' was officially coined at a National Science Foundation (NSF) workshop in 1988. It was defined as the application of principles and methods of engineering and life sciences toward fundamental understanding of structure-function relationships in normal and pathological mammalian tissues and the development of biological substitutes to restore, maintain or improve (human) tissue function [1].

The tissue engineering paradigm is to isolate specific cells through a small biopsy from a patient, to grow them on a three-dimensional scaffold under precisely controlled culture conditions, to deliver the construct to the desired site in the patient's body, and to direct new tissue formation into the scaffold that can be degraded over time. The aims of tissue engineering are to regenerate natural tissues and create new tissues using biological cells, biomaterials, biotechnology, and clinical medicine. Tissue engineering involves seeding a three dimensional scaffold with cells, expanding the cell population, and then implanting the engineered tissue construct in vivo. There are three approaches for using materials in tissue engineering [2]:

(1) The use of isolated cells or cell substitutes to replace those cells that supply the needed function.

(2) The delivery of tissue inducing substances, such as growth and differentiation factors, to targeted locations.

(3) Growing cells in three-dimensional scaffolds.

2.1.1 Biomaterials used in tissue engineering

The National Institute of Health Consensus Development Conference of November 1982 defined a biomaterials as "Any substance (other than a drug) or combination of substance, synthetic or natural in origin which can be used for any period of time, as a whole or as a part of a system which treats, augments, or replaces any tissue, organ or function of the body" [3]. In a practical sense, biomaterials are metals (e.g.

metallic alloy, titanium and titanium alloy), ceramics (e.g. alumina, hydroxyapatite), synthetic polymer (e.g. poly(α-hydroxy acid), family of polymers (e.g.poly (lactic-co-glycolic acid) (PLGA)]), and natural macromolecules (biopolymers) (e.g.collagen, fibrin, elastin, alginate, chitosan, silk fibroin and hyaluronic acid), that are manufactured or processed to be suitable for use in or as a medical device that comes into intimate contact with proteins, cells, tissues, organs, and organ systems. These materials may be degradable or non-degradable materials. The degradable materials are referred to degradation after implantation with complete excretion of the by-products. Some of the disadvantages of non-degradable materials have been the release of wear particles or other materials into the surrounding tissue causing the need for a second surgical procedure for implant removal and revision surgery. The objective of biodegradable materials is to provide initial fixation strength and scaffolding while allowing degradation and replacement by host tissue.

2.1.1.1 The biomaterial scaffold

The biomaterial plays an important role in most tissue engineering strategies. Materials are critically important for tissue engineering in designing temporary, artificial extracellular matrices (scaffolds), which support three-dimensional tissue formation. There are many natural and synthetic materials that are biocompatible and biodegradable scaffold materials. Scaffold materials as biomaterials for making matricies for tissue engineering may be either of synthetic polymers or natural polymers.

- Synthetic Polymers [4] are used in a broad variety of biomedical applications. A number of biodegradable synthetic polymers are poly(α-hydroxyester) family (e.g. poly(glycolide) (PGA), poly(L-lactide) (PLLA) and their copolymers of poly(lactide-co-glycolide) (PLAGA), poly(ε-caprolactone), poly(ortho ester), copoly(ether-ester), poly(carbonate), poly(imino carbonate), polyanhydride, polyphosphazene, trimethylene carbonate, poly(ß-hydroxybutyrate), poly(g-ethyl glutamate), and poly(dioxanone)). The polylactic acid, polyglycolic acid, and copolymers FDA-approved polymers are currently used as suture materials. However, they are also being examined for uses such as bone,

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skin, and liver substitutes. These polymers are broken down in the body hydrolytically to produce lactic acid and glycolic acid, respectively.

In 2004, S.R. Bhattari *et al.* [5] fabricated the nanofibrous matrix from novel biodegradable poly(p-dioxanone-co-L-lactide)-block-poly(ethylene glycol) (PPDO/PL LA-b-PEG) copolymer by electrospinning. They analyzed cell proliferation and cell-matrix interaction of the nanofibrous matrix by seeding NIH3T3 fibroblast cells onto matrix. Good capability of the nanofibrous structure for supporting the cell attachment and proliferation are observed. This novel biodegradable scaffold will be applicable for tissue engineering based upon its unique architecture, which acts to support and guide cell growth.

In 2006, Wutticharoenmongkol *et al.* [6] showed that bone scaffolding materials can be fabricated by electrospinning from polycarprolactone (PCl) solution containing nanoparticles of calcium carbonate ($CaCO_3$) or hydroxyapatite (HA) as filler. They evaluated the ability of as-electrospun mat PCl, PCl/$CaCO_3$ and PCl/HA as bone scaffold by an indirect cytotoxicity test using human osteoblasts (SaOS2) and mouse fibroblasts as reference cells. The results showed that all of the fibrous scaffold can be used as bone scaffolds.

- **Natural Polymers** or polymers derived from living creatures. Natural biomaterials include such materials as protein polymers, polysaccharide polymers, and lipids. Of these materials, collagen, chitin, silk fibroin, and hyaluronic acid (HA) are the most commonly used for tissue engineering devices. Typically, the desirable scaffold requirement are :

- Biodegradable
- Non-toxic or non-inflammatory
- Mechanically similar to the tissue to be replaced
- Highly porous
- Encouraging of cell attachment and growth
- Easy and cheap to manufacture
- Biocompatibility

Natural polymers often easily fulfill these requirements. Therefore, these materials have been investigated extensively. In 2003, L.Ma *et al.* [7] fabricated porous scaffolds for skin tissue engineering by freeze-drying the mixture of collagen and chitosan solutions. They used glutaraldehyde (GA) to treat the scaffolds to improve their biostability. They investigated cell infiltration and proliferation with seeding human fibroblasts cells onto as-prepared scaffolds. The results showed that the scaffold could sufficiently support and accelerate the fibroblasts infiltration from the surrounding tissue successfully. Immunohistochemistric assay indicated that the biodegrading behavior of the 0.25% GA treated collagen/chitosan scaffold was a long-term period. In conclusion, the GA-treated collagen/chitosan scaffold was a potential candidate for dermal equivalent with enhanced biostability and good biocompatibility.

2.1.1.2 Scaffold properties

Ideally, a scaffold should possess the following characteristics to bring about the desired biological response [8] namely,

- Three-dimensional and highly porous with an interconnected pore network for cell/tissue growth and flow transport of nutrients and metabolic waste.
- Biodegradable or bioresorbable with a controllable degradation and resorption rate to match cell/tissue growth *in vitro* and/or *in vivo*.
- Suitable surface chemistry for cell attachment, proliferation and differenttiation.
- Mechanical properties to match those of tissues at the site of implantation.
- Be easily processed to form a variety of shapes and sizes.

2.1.1.3 Scaffold fabrication techniques

The objective is to fabricate a scaffold that provides the newly regenerating tissue with a temporary site for cell attachment, proliferation, and mechanical stability. This scaffold should also be capable of withstanding the normal physiological stress found at the implant site. In present the scaffold can be fabricated in various techniques that are:

- **Textile technologies** are techniques that can prepare non-woven meshes of different polymers. The main disadvantages are related to control high porosity and regular pore size.

- **Solvent Casting and Particle Leaching (SCPL):** this technique can fabricate porous structures with regular porosity, but with a limited thickness. First, the polymer is dissolved into a suitable organic solvent (e.g. polylactic acid could be dissolved into dichloromethane), then the solution is cast into a mold filled with porogen particles. Such porogen can be an inorganic salt like sodium chloride, crystals of saccharose, gelatin spheres, or paraffin spheres. The size of the porogen particles will affect the size of the scaffold pores, while the polymer to porogen ratio is directly correlated to the amount of porosity of the final structure. Subsequently, the sovent is allowed to fully evaporate followed by immersion in a bath of a suitable liquid to remove the porogen (e.g. water is the suitable liquid for sodium chloride). Once the porogen has been fully removed a porous structure is obtained. Other than the small thickness range that can be obtained, another disadvantage of this technique is the use of organic solvents which must be fully removed to avoid any possible damage to the cells seeded on the scaffold.

- **Gas Foaming** is appeared to overcome the use of organic solvents and solid porogens. This technique uses gas as a porogen. First, disc shaped structures made of the desired polymer are prepared with means of compression molding using a heated mold and then the discs are placed in a chamber where are exposed to high pressure CO_2 for several days. The pressure inside the chamber is gradually restored to atmospheric levels. The pores of polymer are formed during this process resulted from the carbon dioxide molecules that abandon the polymer. The resulting polymer appears to be a sponge like structure. The main problems related to such a technique are caused by the excessive heat used during compression molding (which prohibits the incorporation of any temperature labile material into the polymer matrix) and by the fact that the pores do not form an interconnected structure.

- **Emulsification and Freeze-drying**: this technique does not require the use of a solid as porogen like SCPL, but the emulsion is formed and then fast freeze drying to form pores. Briefly, first a polymer is dissolved into a suitable solvent then water is added to the polymeric solution and the two liquids are mixed in order to obtain an emulsion. Before the two phases can separate, the emulsion is cast into a mold and quickly frozen

by means of immersion into liquid nitrogen. The frozen emulsion is subsequently freeze-dried to remove the dispersed water and the solvent, thus leaving a solidified, porous polymeric structure. Comparing to SCPL technique emulsification and freeze-drying allows a faster preparation if compared to SCPL, since it does not require a time consuming leaching step. Although this technique has many advantages, it still requires the use of solvents. Moreover pore size in polymer structure is relatively small and porosity is often irregular. Freeze-drying by itself is also a commonly employed technique for the fabrication of scaffolds.

In 2004, G.Guang *et al.* [9] attempted to develop scaffolds with improved biocompatibility for cell culture. In their work, hybrid scaffolds were fabricated by modifying poly(ε-caprolactone)(PCl) with silk fibroin (SF) in a prorous structure by freeze-drying technique. They assessed the scaffold biocompatibility by MTT assay, the human fibroblasts were seeded on the hybrid scaffolds. The results indicated that the hybrid scaffold was favorable for cell adhesion, spreading, and proliferation. From these reasons, the hybrid scaffold could effectively improves its biocompatibility and facilitate its application in practical tissue engineering.

- **Liquid-liquid phase separation** is similar to the previous technique but does not to need freeze-drying step. This technique requires the use of a solvent with a low melting point that is easy to sublime. Following cooling below the solvent melting point and some days of vacuum-drying to sublime the solvent a porous scaffold is obtained. Liquid-liquid phase separation presents the same problems of emulsification and freeze-drying technique.

- **CAD/CAM Technologies** this technique uses computer added to control of porosity and pore size since the above described techniques are limited in the control of porosity and pore size. Briefly, first, a three-dimensional structure is designed using CAD software, then the scaffold is realized by using ink-jet printing of polymer powders or through Fused Deposition Modeling of a polymer melt.

2.1.2 Tissue engineering applications

The tissue engineering has variety of the applications as listed below:

- Biomaterials for Genitourinary Tissue Engineering

- Engineered Blood Vessel Substitutes
- Tissue Engineering of Tendon and Ligament
- Tissue Engineering of the Cornea
- Materials Employed for Breast Augmentation and Reconstruction
- Scaffolding in Periodontal Engineering
- Tissue Engineering of Craniofacial Structure
- Hemoglobin-Based Red Blood Cell Substitutes
- Nerve Regeneration
- Functional Tissue Engineering of Cartilage and Myocardium: Bioreactor Aspects
- Stem Cells in Tissue Engineering
- Osteochondral Tissue Engineering - Regeneration of Articular Condyle from Mesenchymal Stem Cells
- Tissue Engineered Meniscal Tissue
- Tissue Engineering for Insulin Replacement in Diabetes
- 3-D Tissue Fabrication: Application to Hepatic Tissue Engineering

2.2 Electrospinning

One of the methods of producing fibers with average diameters in the ranging from several micrometers down to 100 nm or less is through the process of electrospinning. Electrospinning is a technique for producing nonwoven meshes and has received attention recently. The electrospinning process is started by applying voltage to a polymer solution or polymer melt that is being held at the tip of a syringe by its surface tension. Charges are induced into solution. When the applied voltage reaches a critical value, a protruding conical shape known as the Taylor cone is formed at the tip of the needle. Beyond this critical value, the applied voltage causes the charges to overcome the surface tension and form a charged jet of solution. As this jet travels through the air, it soldifies leaving behind a polymer fiber to be collected on a grounded metal screen. Continuous fibers are laid on the screen to form a nonwoven fiber (fabric). This mechanism is shown in Figure 2.1.

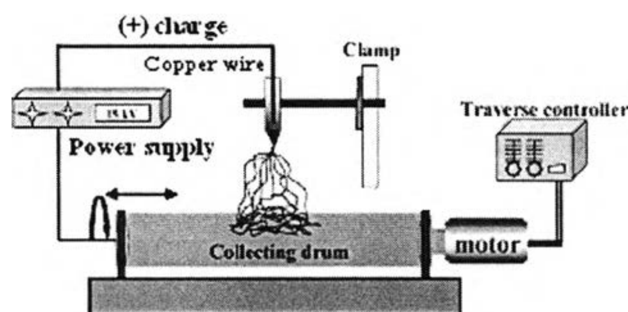

Figure 2.1 The formation of fiber by the electrospinning.

In 1998, Zarkoob *et al.* [10] first studied the electrospinning of *B. mori* cocoon silk of Nephila clavipes dragline silks. The concentration of silk solution were varied from 0.23 to 1.2 wt% in hexafluoroisopropanol. Polymeric fibers with diameters in the range of 6.5–200 nm were produced with crystallographic order equivalent to that of the original natural fibers.

In 2003, S. Sukigara *et al.* [11] studied the effect of the elecetrospinning parameters on the morphology and fiber diameter of regenerated *B. mori* silk. Threes parameters were investigated including of various electric field, tip-to-collection plate distances, and silk concentrations. Formic acid was used as a solvent. They found that the silk concentration was the most important parameter in producing uniform fiber less than 100 nm in diameter.

A number of applications of the electrospinning have been explored such as composite reinforcement, filtration application, biomedical application, cosmetic and protective clothing application [12]. This technique is always realized in biomedical application extensively. Particularly, the fabrication of highly porous biodegradable scaffolds. Because this technique is easy to use, cost-effective process, and simple equipment setting. Besides, it can produce the small fibers diameters and the porous structure of electrospun nonwoven resulting in a high specific surface area. The large surface area to volume ratio also allows cellular migration and proliferation in tissue engineered scaffolds.

In 2003, J. Zeng *et al.* [13] examined the influences of surfactants and medical drugs on the diameter size and uniformity of electrospun poly(L-lactic acid) (PLLA) fibers by adding various surfactants (cationic, anionic, and nonionic) and typical drugs

into the PLLA solution. Significant diameter reduction and uniformity improvement were observed. The results shown that the drugs were capsulated inside of the fibers and the drug release in the presence of proteinase K followed nearly zero-order kinetics due to the degradation of the PLLA fibers. Such ultrafine fiber mats containing drugs may find clinical applications in the future.

2.3 Silks

Silks are generally defined as natural protein fiber. Unlike wool, silk contains a very small amount of sulfur. Silks are spun into fibers by silkworm to form cocoon for protection own larvae. The silk fiber can also be produced by some spiders [14-15]. Unlike silkworms' fiber, the spiders' fiber cannot be commercial produced. Therefore, silk fiber often referred as silkworms' fiber. The silk fibers have been used for manufacturing sutures in biomedical application but they are found that the use of silk resulted in immunological reactions. In fact, inflammatory responses of silk sutures are the effect of sericin proteins, not the silk fibroin. Therefore, the use of silk fiber in biomedical application needs to remove sericin from the raw silk fiber. The process of removal sericin is known as *degumming*. The silk fiber can be divided from silkworm into 2 types [16] that are:

1. **Mulberry silk (*Bombyx mori*)** is produced by cultivated silkworm. The cultivated silkworm is fed with mulberry leaves.

2. **Wild silk** is produced by wild silkworm. This type often referred as **Tussah silk.** The wild silkworm is fed with oak leaves.

The wild silkworm is hard to culture and the silk fibers obtained from wild silkworm are coarser, more irregular, and brownish in appearances. While the silk fibers obtained from cultivated silkworm are fine, almost white (when degummed), and soft fiber with lusture. Moreover, wild silkworm' fiber has more silk gum (e.g. up to 38%) than *Bombyx mori* (*B. mori*) silkworm' fiber (20-25%). From these characteristic, silk fiber produced by *B. mori* silkworm is well known and has been studied extensively in biomedical applications.

Silk fiber consists of two main protein types as silk fibroin and sericin. Silk fibroin (SF) is a core structural protein and is coated by sericin (silk gum), a family of

glue-like proteins that hold the fibroin core fibers together. The sericin is a minor component of fiber (i.e. 25% of the weight of raw silk) and it also has some puritles such as waxes, fat, and pigments. Silk fibroin is a fibrous protein unlike sericin which is a globular protein. The elemental compositions in SF are listed in Table 2.1.

Table 2.1 The composition of elements in silk fibroin [17]

Element	Percentages of element
Carbon	48-49
Hydrogen	6.40-6.51
Nitrogen	17.35-18.89
Oxygen	26.00-27.90
Sulphur	slightly

The SF is a linear polypeptide whose chemical composition comprised of few types of amino acid of alternating of Glycine (Gly) residue with two thirds Alanine (Ala) and one third Serine (Ser), (i.e., Ala-Gly-Ala-Gly-Ser-Gly) and simplified as repeating squences of $(Ala\text{-}Gly)_n$ [18]. The polypeptide chains (Figure 2.1) always form anti-p^rarelle β-sheet because this structure has hydrogen bonds (H-bond) between peptide groups on adjacent beta strands and these H-bonds can stabilize structure.

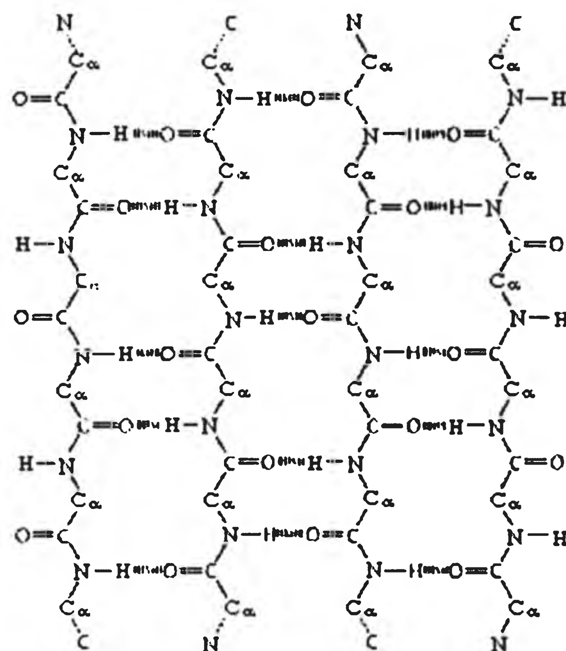

Figure 2.2 The polypeptide chain of SF

The SF can take two structures known as silk I and silk II forms. The silk II structure is identified by the ^{13}C chemical shifts that are indicative of anti-parallel β-sheet, while the silk I structure gives the chemical shifts that are related with a loose helix [19-20]. The two structures can undergo a conformational transition from silk depending on any factors such as solvent, temperature, or shear and tensile stresses [21]. The region of anti-parallel β-sheet is a crystal region which results in the stability and mechanical features of silk fibroin.

Because of the impressive mechanical properties including biological properties, biocompatibility and biodegradability, the SF has been interested in various fields not only in textile fields but also in reinforcing composites, food additive, cosmetics, and biomedical fields [22]. Particularly, SF has been researched extensively in the biomedical fields. The applications of SF in the biomedical fields are controlled drug-delivery carrying, cell culture substrate, sutures, tissue engineering matrix, and wound dressing. The SF can be prepared as regenerated SF in various forms such as gels, powders, membranes, or fibers depending on desired application. For the biomedical application, the regenerated SF in forms of film [23-24] and fiber have been interested.

In 2003, H-J. Jin *et al.* [25] investigated human bone marrow stromal cell (BMSC) responses on the electropun scaffolds which were prepared from aqueous *B.*

mori solutions and poly(ethylene oxide) (PEO) by electrospinning technique. The electrospun mats were produced from spinning solution of 8 wt% of the aqueous silk solution and 5 wt% PEO. Then, the as-electrospun mats were immersed into a 90/10 (v/v) methanol:water to induce an amorphus β-sheet and washed with water to removed PEO from mats. The addition of PEO can increase the viscosity and surface tension of the 8 wt% pure silk solution which was not high enough to maintain a stable drop at the end of the capillary tip. The results showed that PEO can supply good mechanical properties to the electrospun mats. The efficiency between the PEO extracted and non-extracted mats in cell attachment, spreading and growth (for 1, 7 and 14 days) were investigated. From these results, at 1 day after cell seeding, the PEO extracted mats had higher density of cell than non-extracted mats. After 7 and 14 days the cells can grow on two mats but the cell density between the PEO extracted and non-extracted mats maybe due to the difference in initial cell attachment caused by the existence of PEO. From the results, it can be concluded that PEO inhibited in initial cell attachment but PEO did not affect the cell growth.

Also within the same year, B.-M. Min *et al.* [26-27] prepared three forms of SF matrices, woven (microfiber), non-woven (nanofiber), and film form. The SF microfiber (SF-M) matrix was prepared by degumming silk with 0.5 wt% $NaHCO_3$ solution at 100°C. The nanofiber and film matrices were prepared from SF solutions which were prepared by dissolving the regenerated SF sponges in 98% formic acid. While the film matrix (SF-F) was prepared by casting, the nanofiber matrix (SF-N) was prepared by electrospinning. The as-prepared SF-F and SF-N matrices were crystallized and insolubilized by immersion in a 50 % (v/v) methanol solution. The effects of methanol treatment on the conformational transition of the as-prepared SF-F and SF-N matrices were characterized. The results were shown that the as-cast SF-F matrix formed a mainly β-sheet structure that was similar to the SF-M matrix, whereas the as-spun SF-N matrix had a random coil conformation as the predominant secondary structure. However, conformational transitions from random coil to β-sheet of the as-spun SF-N occurred rapidly within 10 min following aqueous methanol treatment. Cell responses of normal human oral keratinocytes (NHOK) on three forms of matrices and ECM proteins (type I collagen, fibronectin, laminin and bovine serum albumin (BSA) as control) coating were

studied. The SF matrices, alone and ECM proteins coating were seeded with NHOK to assess cell adhesion and spreading. The results showed that SF-F and SF-N have better matrix textures than SF-M in terms of cell adhesion. The type I collagen can promote cell adhesion and spreading of NHOK onto the SF-F and SF-N matrices. They concluded that the SF-N may be more preferable than SF film and SF microfiber matrices because the SF-N matrix provides a higher level of surface area for cells to attach due to its high porosity and specific surface area.

2.4 Skin

Skin [28-30] is the body's largest organ and can be divided into 2 main layers, which are the epidermis and the dermis, this skin structure is given in Figure 2.3. It makes up about 16% of body weight. The functions of skin are about protection, sensation, thermoregulation, immune system, and metabolism etc.

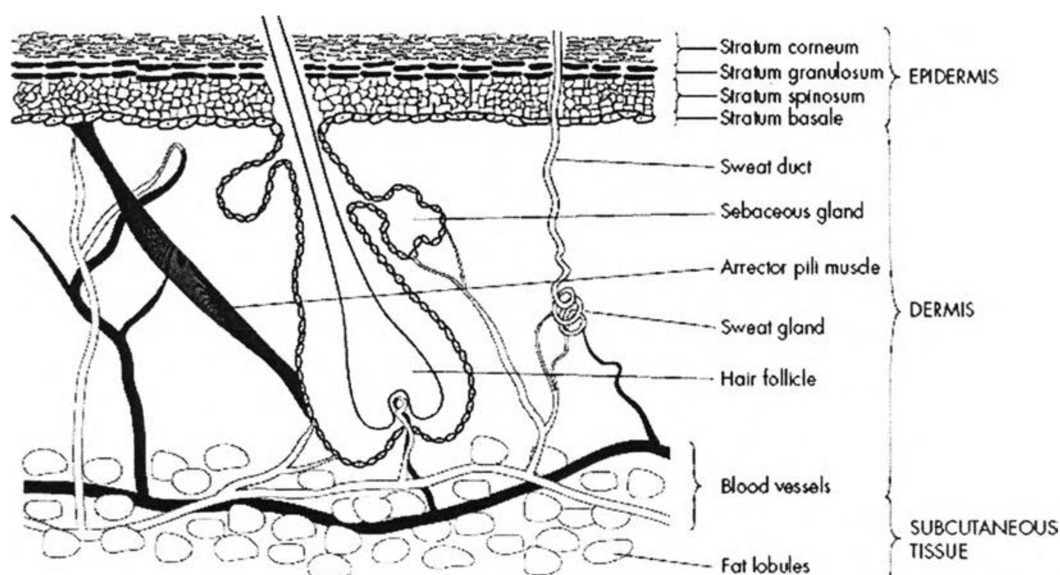

Figure 2.3 The skin structure

The epidermis is the outer layer. The thickness of epidermis is about 0.1 mm at the eyelids to nearly 1 mm or more on the palms and soles. The cell types of epidermis are composed of keratinocytes, langerhans cells, melanocytes, merkel cells. The main skin cell that makes up the epidermis is well known as the keratinocyte (85% of cells in

epidermis). It gives skin much of its resistance to physical wear and tear and makes skin waterproof.

The dermis or true skin, lies below the epidermis and gradually blends into the deeper tissues. The dermis layer is thickest at the palms, soles and back (3 mm), and least at the eyelids (0.3 mm) and penis. It contains many cells, fibers, and amorphous ground substance. The dermis is produced largely by fibroblasts showed in Figure 2.4, which during embryonic development are part of the mesenchyme. Fibroblast for synthesis of collagen, reticulin, elastin, glycosaminoglycans is the major cell in the dermis. The ground substance consists of two glycosaminoglycans: hyaluronic acid (HA) and dermatan sulphate. Other structures found in the dermis include blood vessels, lymphatics, nerves, nerve endings and receptors, dartos muscles in scrotum, appendageal glands, and their accessories e.g. arrector pili muscles.

Figure 2.4 The shape of fibroblast

The skin is the first line of defence against both infection and dehydration cause it is often damaged. The ability of the skin to repair itself following minor injury is remarkable, but when the injury is severe, medical intervention is required, both to speed the recovery of the skin itself and to protect the body from infection and fluid loss in the meantime. The mechanism of wound repair normally begins within twelve hours of the wound occurrence. This involves granulation, fibrification, and epithelialisation.

Granulation involves the formation of a scab, which acts as a scaffold for new cells to attach. Depending upon the severity of the wound, fibrification may be required. Fibroblasts, which produce fibrous tissue, enter the wound site during fibrification. Fibrous tissue acts as a barrier against infection, and is required for wound contraction, during which intact tissue around the wound contracts to bring the sides of the wound together. Revascularization occurs to some extent during fibrification. Once a scaffold is in place, epithelialisation can take place: the migration and multiplication of cells to form new tissue. The presence of infection, too much granulation, hypothermia, and insufficient blood supply are all factors that extend the repair phase. If the epidermis layer is damaged, it will regenerate spontaneously. While the dermis layer is damaged severely, it cannot regenerate. The treatment of severely wounded skin can be treated with the tissue substitution of skin which have 3 types as following:

1. Autografting is the grafting of skin harvested from undamaged part in the patient and transplanting into another part of the patient's own body.
2. Allografting is the grafting of skin harvested from tissue of other people or cadavers into the patient.
3. Xenografting is the grafting of tissue taken from an animal such as a cow or a pig into the patient.

However, all of these types have problem from the body's immune response which means that these grafts are almost inevitably rejected resulted in the infection of patient. Therefore, the treatment technique involves the implantation of a biodegradable material has been interesting for wound healing. The biodegradable material which should be similar structure to extra-cellular matrix (ECM) acting as a scaffold onto which cells can attach during wound repair.

Ideal tissue engineering as scaffolding should be mimic the structure and biological functions of the extracellular matrix (ECM) in vivo and then support cell regeneration until host cells can repopulate and resynthesize a new natural matrix. Nonwovens are structurally similar to collagen structure of ECM, in which collagen multi-fibrils of nanometer scale are composed of three-dimensional network structure together with proteoglycans.

ECM (extracellular matrix) is a complex structural entity surrounding and supporting cells that are found within mammalian tissues. The ECM can surround cells as fibrils that contact the cells on all sides, or as a sheet called the basement membrane. Cells in animals are also linked directly to each other by cell adhesion molecules at the cell surface. The ECM is often referred to as the connective tissue. The ECM is composed of 3 major classes of biomolecules as following:

1. Structural proteins that are collagen and elastin
2. Specialized proteins e.g. fibrillin, fibronectin, and laminin
3. Proteoglycans are a class of glycosylated proteins which have covalently linked sulfated glycosaminoglycans (i.e., chondroitin sulfate, dermatan sulfate, heparan sulfate, heparin, keratan sulfate).

Hyaluronic acid (HA) or hyaluronan related polysaccharides is called *glycosaminoglycans*. It is a large linear polymer consisting of repeating units of D-glucoronic acid and disaccharides of 2-acetamido-2-deoxy-D-glucose (N-acetyglucos-amine), this structure is given in Figure 2.5. Its molecular weight is typical range of 10^6-10^7. HA is present in the body, in the skin, vitreous humor, cartilage, and synovial fluid. For skin, it is found naturally in skin's extracellular matrix where it maintains the structure of soft tissue and is involved in a variety of tissue functions: skin repair, regeneration, and restoration [31]. HA has been shown to play an important role in lubrication, cell differentiation, and cell growth. It also has the high ability of water absorption, water retention, and lubrication which is often used for dermal wound healing applications as well as bone tissue engineering. The HA component of the scaffold will enhance the ability of wound healing and tissue regeneration, even in areas where scar tissue may block natural wound healing. HA is natural polymer and it is easily manufactured and modified, as well as hydrophilic and biodegradable. Therefore, it has been researched in medical applications. However, the poor biomechanical properties of this soluble, viscoelastic natural polymer and its rapid degradation in vivo preclude many biomedical applications. In order to obtain materials that are more mechanically robust and metabolically stable, a variety of hydrophobic modifications and chemical crosslinking strategies have been explored to produce insoluble or gel-like HA materials.

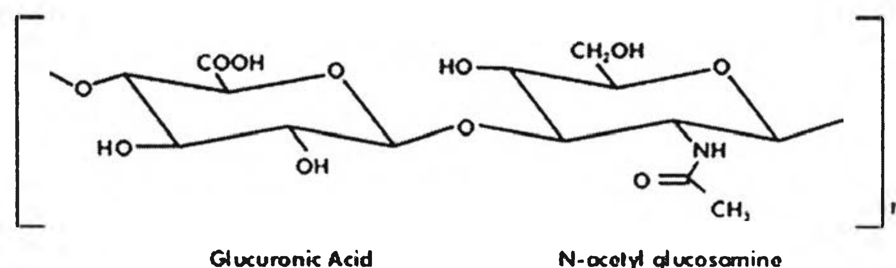

Figure 2.5 The structure of hyaluronic acid (HA) or hyaluronan

In 1997, Tomihata and Ikada [32] studied the ability of glutaraldehyde (GA) as crosslinking agent for hyaluronic acid (HA). HA was chemically crosslinked with GA to produce water-insoluble films having low water contents when brought into contact with water. They investigated the ability in crosslinking of GA by determining the swelling of the uncrosslinked hyaluronan (HA) film in phosphate buffered saline (PBS) at various concentrations of acetone, GA, and hydrochloric (HCl). The result of various concentrations of acetone was shown that the lowest water content of 63 wt % was observed at the acetone concentration between 70 and 80 vol %, the acetone concentration was always fixed to 80 vol % in the further steps. For various HCl concentrations, it was found that the absence of HCl prevented HA crosslinking even at high concentrations of GA, but HCl concentrations higher than 0.01 N retarded the crosslinking of HA. Therefore, the optimal HCl concentration for HA crosslinking should be around 0.01 N which this concentration was used in the following step. The effect of GA concentration on the water content of water-swollen HA films crosslinked in the acetone–water mixture of 80 vol % was investigated. The result showed that the crosslinking reaction did not reach equilibrium even though the reaction was allowed to proceed probably because the GA concentration was as low as 50 mM. However, the equilibrium is achieved for the crosslinking reaction within 24 h if the GA concentration is as high as 250 mM. From these results, they concluded that HA film can be crosslinked with GA in acetone–water mixtures containing 0.01 N HCl. The lowest water content of the crosslinked HA film swollen with PBS was 62 wt %. The crosslinking of HA film could be obtained with concentration of GA between 100 and 250 M in the acetone–water mixture of 80 vol % acetone. The crosslinked film remained in PBS at 37°C for 2 weeks without significant degradation.