CHAPTER II

RELEVANT THEORY

2.1 Three-dimensional scaffolds [1, 5]

Three-dimensional scaffolds are required in tissue engineering to support the formation of tissue relevant mimics as well as to promote cellular migration, adherence, formation of new extracellular matrix, tissue ingrowth and to foster the transport of nutrients and metabolic wastes. Ideally, scaffolds should:

- support cell attachment, migration, cell–cell interactions, cell proliferation and differentiation;
- be biocompatible to the host immune system where the engineered tissue will be implanted;
- biodegrade at a controlled rate to match the rate of neotissue growth and facilitate the integration of engineered tissue into the surrounding host tissue;
- provide structural support for cells and neotissue formed in the scaffold during the initial stages of post-implantation, and
- have versatile processing options to alter structure and morphology related to tissue-specific needs.

2.2 Biomaterials for scaffold fabrication [13]

There are many natural, synthetic polymers and ceramics which have high potential as biocompatible and biodegradable materials for scaffolding.

2.2.1 Synthetic polymers

Poly(glycolic acid) (PGA), poly(lactic acid) (PLA), and their copolymers poly(lactic acid-co-glycolic acid) (PLGA) are a family of linear aliphatic polyesters, which are most frequently used in tissue engineering. These polymers degrade through hydrolysis of ester bonds. There are other linear aliphatic polyesters, such as poly(ε-caprolactone) (PCL) and poly(hydroxy butyrate) (PHB), which are also used in tissue engineering.

2.2.2 Inorganic materials or ceramics

These materials can be categorized as porous bioactive glasses and calcium phosphates. Within the calcium phosphates, β-tricalcium phosphate (β-TCP), hydroxyapatite (HAp) and its derivatives, and their combinations are the most frequently used. In many natural material such as bone, cartilage, shells, and skin, are composed of inorganic minerals and organic molecules. These inorganic materials are widely considered to be osteoconductive (their surface properties support osteoblastic cells adhesion, growth, and differentiation). However, these inorganic materials are often difficult to process into highly porous structures and are mechanically brittle. To overcome these disadvantages, composite materials with synthetic or natural polymers have been explored for bone tissue engineering.

2.2.3 Natural macromolecules

Natural polymers, such as proteins and polysaccharides, have also been used for tissue engineering applications. Collagen is a fibrous protein and a major natural extracellular matrix component. It has been used for various tissue regeneration applications. Another category of well-known natural fibrous proteins is silk. Silkworm silk has been used in textile production for centuries, and has been used as nondegradable sutures for decades because of its excellent tensile mechanical property. Polysaccharides such as alginate, chitosan, and hyaluronate are another class

of natural polymers that have been used as porous solid-state tissue engineering scaffolds.

2.2.3.1 Silk [14-15]

Silk is generally defined as a protein polymer that is spun into fibers by some lepidoptera larvae such as silkworms, spiders, scorpions, mites and flies. 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)*.

Fibrous proteins, such as silks and collagens, are characterized by a highly repetitive primary sequence that leads to significant homogeneity in secondary structure, i.e., triple helices in the case of collagens and β-sheets (Figure 2.1) in the case of many types of silks.

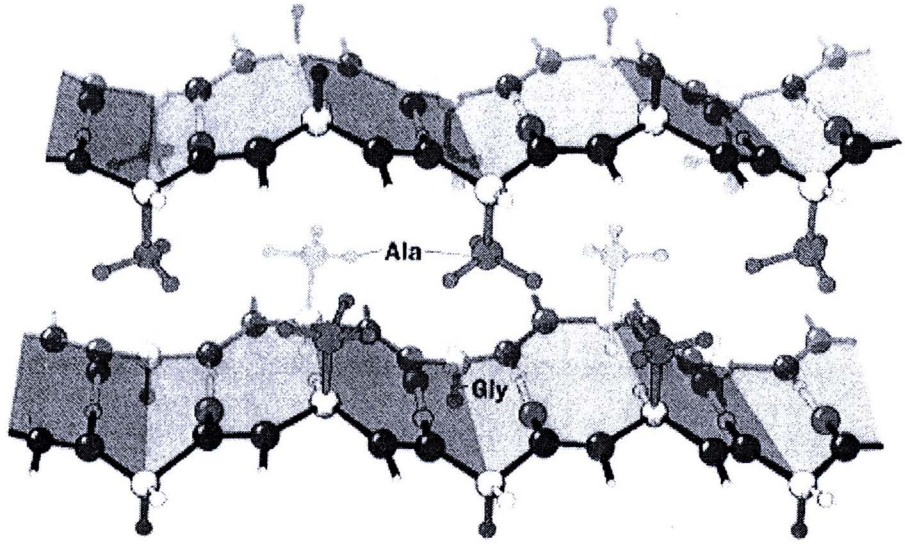


Figure 2.1 β-sheet secondary structure of silk [16]

Silk consists of two main parts called silk sericin and silk fibroin.

(a) Silk sericin

Silk sericin or silk gum is a minor component of fiber (i.e. 20-25 wt% of raw silk) and it also has some impurities such as waxes, fats, and pigments. Sericin is a yellow, brittle, and inelastic substance. It acts as an adhesive for the twin silk fibroin filaments and covers the luster of silk fibroin as shown in Figure 2.2. Sericin is known as an amorphous structure. It can be dissolved in a hot soap solution.

(b) Silk fibroin

A major constituent of raw silk fiber, about 75-80 wt%, is silk fibroin is an insoluble protein in most solvents, including water, dilute acid, and alkaline.

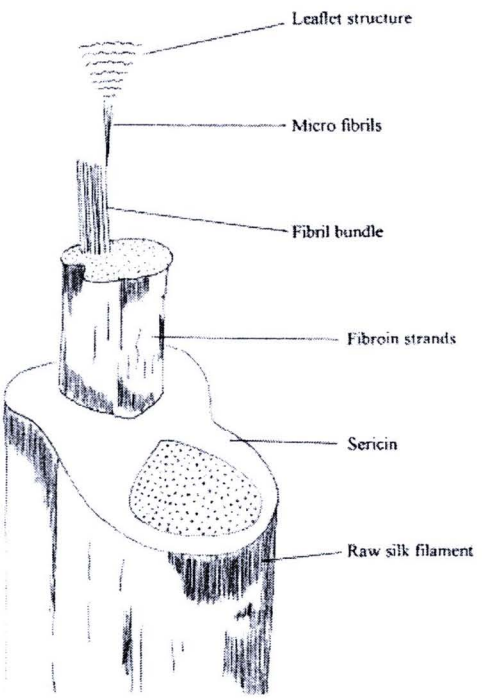


Figure 2.2 Structure of raw silk fiber [6]

Structure of silk fibroin

Silk fibroin contains at least two major proteins, light and heavy chains, 25 and 325 kDa, respectively. The heavy chain protein consists of 12 repetitive regions called crystalline regions and 11 non-repetitive interspaced regions called amorphous regions. The crystalline regions of the domestic silk proteins contain several

repetitions of the basic sequence –(Gly-Ala-Gly-X)$_n$-, with X: Ser or Tyr. The amorphous regions contain most of the amino acid residues with bulky and polar side chains. Silk fibroin has a highly oriented and crystalline structure.

Amino acid compositions of silk fibroin

Silk fibroin is composed of 18 amino acids (Table 2.1). The isoelectric point is around 5.

Table 2.1 Amino Acid Compositions of *Bombyx mori* silk fibroin [17]

Amino Acid	Symbol	Charge	Hydrophobic/ Hydrophilic	Amount (g/100 g silk fiboin)
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

Characteristics of silk fibroin

Characteristics of silk fibroin as for industrial materials can be summarized as follows.

- Pure silk proteins can be obtained from silkworms.
- Silk fibers from *Bombyx mori* silkworms can be dissolved in concentrated neutral salt solutions. After dialyzing against water a pure silk fibroin solution can be obtained.
- Various forms of silk proteins, such as powder, gel and film can be produced by controlling the cast-dry speed of the silk fibroin solution.
- Silk protein can be made insoluble by immersing it in alcohol solution. This technique is interesting for the application of silk fibroin as a biomaterial since this agent is not harmful to living tissues.

Applications of silk fibroin as a biomaterial

Silk fibers have been used as sutures for a long time in the surgical field, due to the biocompatibility of silk fibroin fibers with human living tissues and its good tensile properties. In addition, it has been demonstrated that silk fibroin can be used as a substrate for enzyme immobilization in biosensors and as a blood-compatible material. A more complete understanding of silk structure provided the possibility to exploit silk fibroin for new uses, such as the production of oxygen-permeable membranes and biocompatible materials for various medical applications.

Degradation of silk fiber

Treatment of silk fibers with acid or alkaline substances causes hydrolysis of peptide linkages. The degree of hydrolysis is based on pH factor, which is at minimum between 4 and 8. Degradation of the fiber is exhibited by loss of tensile strength or change in the viscosity of the solution.

Hydrolysis by acid is more extensive than alkaline. Acid hydrolysis occurs at linkages widely distributed along protein chains, whereas the early stages of alkaline hydrolysis happens at the end of chains. Hydrochloric acid readily dissolves silk fibroin especially when heated. Hot concentrated sulphuric acid, while rapidly

dissolving and hydrolyzing silk fibroin, also causes sulphation tyrosine. Nitric acid readily decomposes silk fibroin, due to its powerful oxidizing properties and concurrently causes nitration of the benzene nuclei. Organic acids have few effects at room temperature when diluted, but in a concentrated form, silk fibroin may be dissolved, along with a certain amount of decomposition.

Proteolytic enzymes do not apparently attack silk fibroin in fibrous form because the protein chains in silk are densely packed without bulky side chains. Serious degradation may be caused by water or steam at 100°C.

Silk protein processing [6, 18]

(a) Degummed process

As mentioned before, sericin layers contain some impurities. Therefore, sericin layers must be removed to receive purified fibroin fiber. Sericin removal affected surface of fiber as shown in Figure 2.3. This process is called degumming. The degummed processes are classified into five methods as follows:

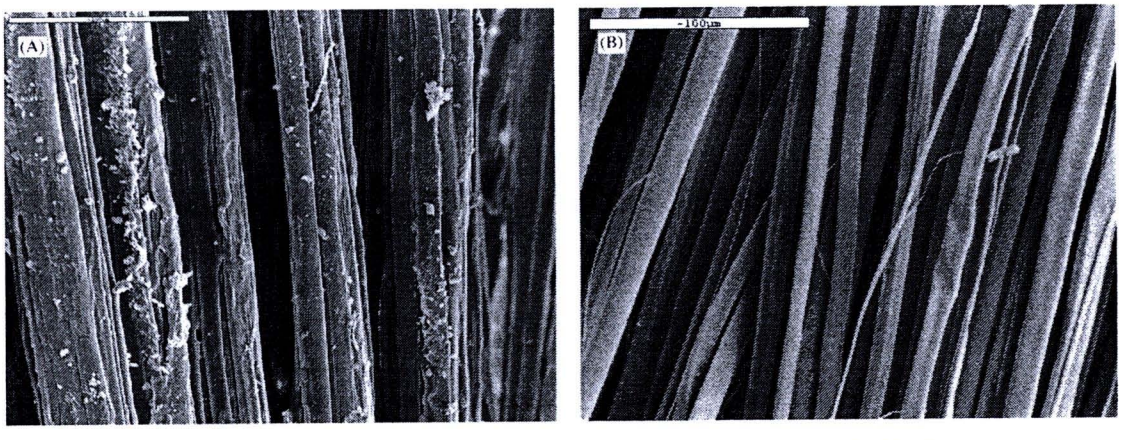


Figure 2.3 SEM micrographs of fiber (a) cocoon raw material, and (b) degummed fiber [15].

- Degummed with hot water

Sericin can easily soluble in boiling water. However, this process gives a risk of the fibroin being damaged when the treatment time is prolonged. Another disadvantage is that this process gives incomplete degumming.

• Degummed with soap

Marseilles soap, olive oil soap, is a favorable soap for degumming. For example, this process may be carried out using 10-20g/l soap at 92-98°C for 2-4 hours. Significantly, this process should use a soft water to avoid the formation of calcium soap.

• Degummed with synthetic detergent

Synthetic detergents have some advantages over soap such as reduction of the treatment time. This process is carried out using 2.5g/l non-ionic synthetic detergent for 30-40 min at 98°C.

• Degummed with acid

Some acids such as sulphuric, hydrochloric, tartaric, and citric acid can be used as degummed agent. This process is not favorable because acid conditions are more harmful to fibroin than alkaline conditions.

• Degummed with enzymes

Trypsin, papain, and bacterial enzymes are the main types of enzymes for silk degummed. These enzymes are called proteases because they degrade proteins and their degradation products are polypeptides, peptides and other substances. This process is a very slow reaction compared to alkaline soap degumming.

(b) Dialysis process

Dialysis was also used in the laboratory in the 1950s and 1960s, mainly to purify biological solutions or to fractionate macromolecules. Now the major application of dialysis is the artificial kidney more than 100 million of these devices are used annually. The dialysis processes divide into many types such as Donnan dialysis, diffusion dialysis and piezodialysis are described in the following section:

• Donnan dialysis and diffusion dialysis

One dialysis process for which the membrane does have sufficient selectivity to achieve useful separations is Donnan dialysis. Salt solutions are separated by a membrane permeable only one charge ion, such as a cation exchange membrane

containing fixed negatively charged groups. Donnan dialysis process is shown in Figure 2.4.

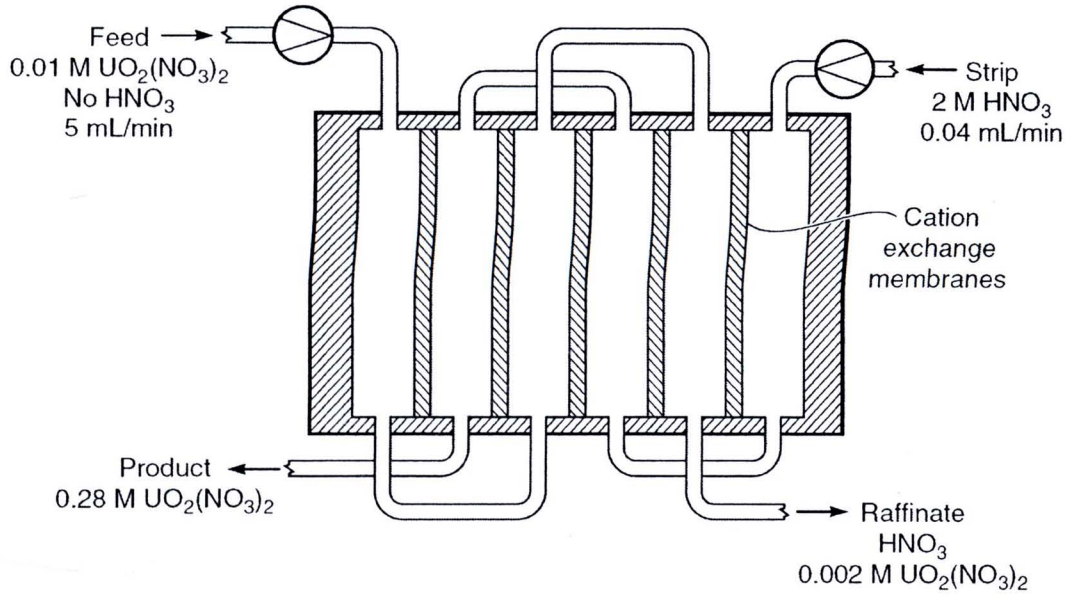


Figure 2.4. Illustration of a Donnan dialysis experiment to separate and concentrate uranyl nitrate, $UO_2(NO_3)_2$ [18].

• Charge Mosaic Membranes and Piezodialysis

Ion exchange membranes consisting of separated small domains of anionic and cationic membranes, which would be permeable to both anions and cations. These membranes are now called charge mosaic membranes. The concept is illustrated in Figure 2.5. Cations permeate the cationic membrane domain; anions permeate the anionic domain.

Thai silk [19]

Thai silk is the type of *Bombyx mori* silkworm silks. It is mainly produced by domestic industries in the northern and north-eastern parts of Thailand. Yellow color and coarse filaments are the main characteristics of Thai silk. It also contains more silk gum (e.g. up to 38%) than other types of *Bombyx mori* silk (e.g. 20-25%). There are many species of Thai silk such as, Nangnoi Srisaket, and Nangnoi Sakolnakorn including other species of blended-Thai silk such as blended-Sakolnakorn and blended-Ubonratchathani, cultivated all years.

(a) Nangnoi Srisaket

This specie is easily cultivated. The life cycle is short (about 18 days). The color of the cocoon is dark yellow.

(b) Nangnoi Sakolnakorn

The color of the cocoon is light yellow. Cocoon produced by this specie is more than that by Nangnoi Srisaket about 5-8 kilograms.

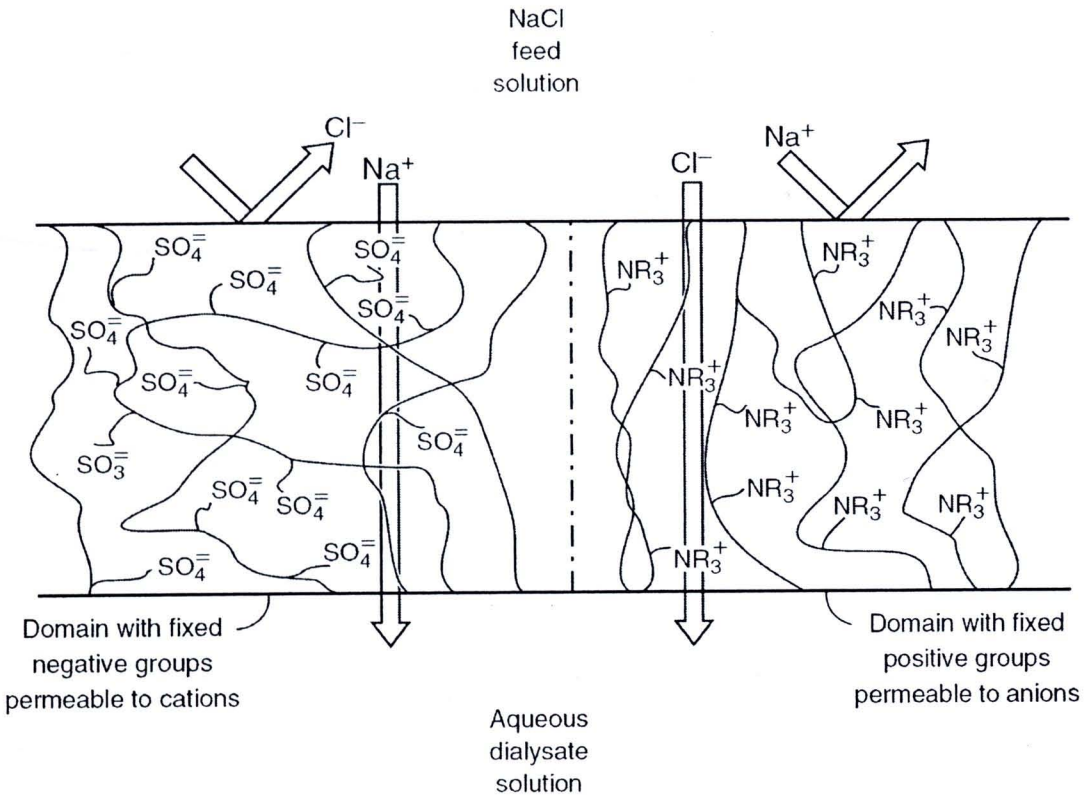


Figure 2.5. Charge mosaic membranes, consisting of finely dispersed domains containing fixed negatively and fixed positively charged groups, are salt permeable [18].

2.2.3.2 Gelatin [20-21]

Gelatin, also called gelatine, is prepared by the thermal and enzymatic denaturation of collagen, isolated from animal skin, bones, cartilage, and ligaments.

Gelatin is a vitreous, brittle solid that is faintly yellow to white and nearly tasteless and odorless.

There are two types of gelatin dependent on preparation method. Type A, with isoionic point of 7 to 9, is derived from acid treatment of collagen. Type B, with isoionic point of 4.8 to 5.2, is the result of an alkaline treatment, such as caustic lime or sodium carbonate, of collagen.

Typical specifications for type A and B gelatin are shown in Table 2.2.

Table 2.2 Typical specifications for gelatins [22]

	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

Structure and composition

Gelatin contains a large number of glycine (almost 1 in 3 residues, arranged every third residue), proline and 4-hydroxyproline residues. A typical structure is -Ala-Gly-Pro-Arg-Gly-Glu-4Hyp-Gly-Pro- as shown in Figure 2.6.

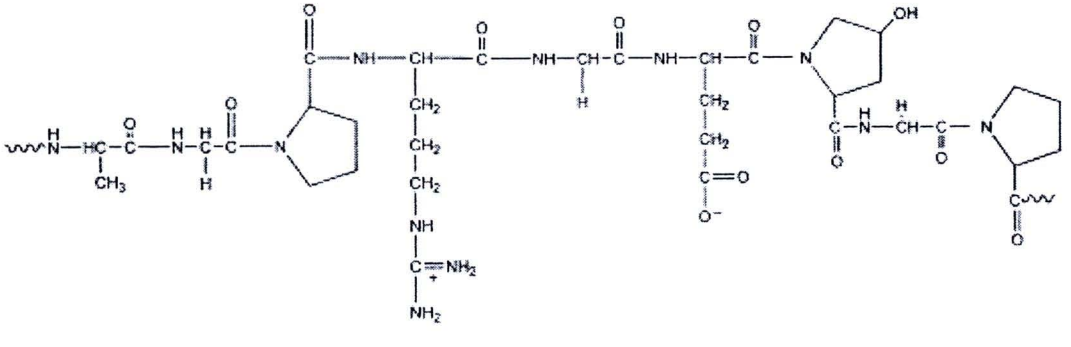


Figure 2.6 The structural unit of gelatin. [23]

Gelatin is composed of 9 amino acids essential for humans as shown in Table 2.3. Gelatin contains 84-90% protein, 1-2% mineral salts and 8-15% water. The natural molecular bonds between individual collagen strands are broken down into a form that rearranges more easily. Gelatin melts when heated and solidifies when cooled again. Together with water it forms a semi-solid colloidal gel.

Table 2.3 Amino acids essential for humans [24]

Amino Acid	g amino acids per 100 g pure protein
Alanine	11.3
Arginine *	9.0
Aspartic Acid	6.7
Glutamic Acid	11.6
Glycine	27.2
Histidine *	0.7
Proline	15.5
Hydroxyproline	13.3
Hydroxylysine	0.8
Isoleucine *	1.6
Leucine *	3.5
Lysine *	4.4
Methionine *	0.6
Phenylalanine	2.5
Serine	3.7
Threonine *	2.4
Tryptophan *	0.0
Tyrosine	0.2
Valine	2.8

* Essential Amino Acid

Applications of gelatin

Gelatin is used extensively in food industries including cake preparation, jelly, aspic, ice cream, and nougat confectionaries etc (see Figure 2.7). Gelatin is also used to make hard and soft capsules in pharmaceutical and medical applications because of its biodegradability and biocompatibility in physiological environments. Therefore, gelatin has become the interesting natural choice in tissue engineering application.

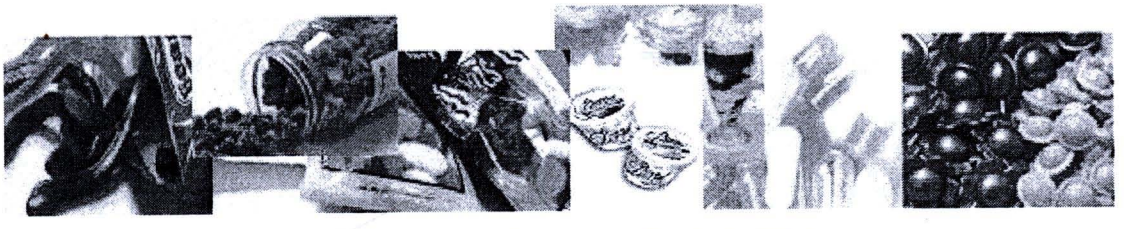


Figure 2.7 Applications of gelatin [25]

2.3 Scaffold fabrication techniques [26-27]

In the body, tissues are organized into three-dimensional structures as functional organs and organ systems. To engineer functional tissues and organs successfully, the scaffolds have to be designed to facilitate cell distribution and guide tissue regeneration in three dimensions. Many methods to prepare porous three-dimensional biodegradable scaffolds have been developed in tissue engineering as shown in Figure 2.8.

2.3.1 Particulate leaching

Particulate leaching is a technique that has been widely used to fabricate scaffolds for tissue engineering applications. Salt is first ground into small particles and those of the desired size are transferred into a mold. A polymer solution is then cast into the salt-filled mold. After the evaporation of the solvent, the salt crystals are leached away using water to form the pores of the scaffold. The pore size can be controlled by the size of the salt crystals and the porosity by the salt/polymer solution ratio. Another particle is used such as monosodium glutamate, alginate hydrogel.

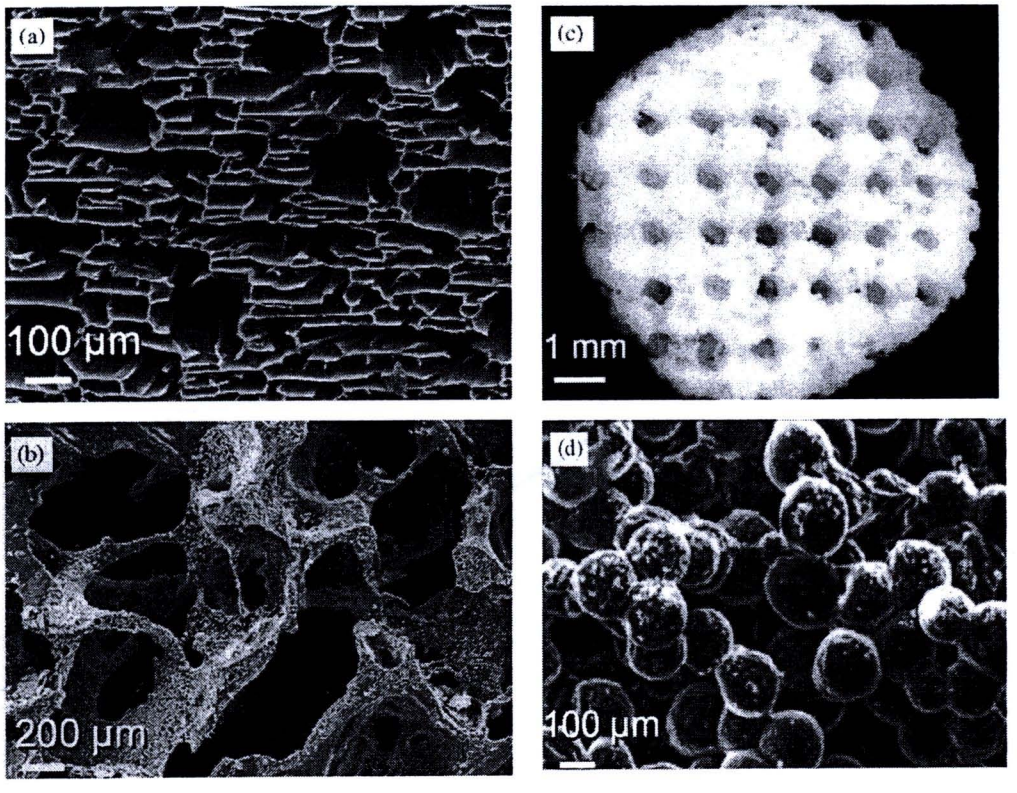


Figure 2.8 Porous polymer foams produced by different techniques. (a) phase separation, (b) particulate leaching, (c) solid freeform fabrication technique, and (d) microsphere sintering [28].

2.3.2 Gas foaming

A biodegradable polymer, such as poly(lactic-co-glycolic acid) (PLGA) is saturated with carbon dioxide (CO_2) at high pressures. The solubility of the gas in the polymer is then decreased rapidly by bringing CO_2 pressure back to atmospheric level. This results in nucleation and growth of gas bubbles, or cells, with sizes ranging between 100-500µm in the polymer.

2.3.3 Fiber meshes/fiber bonding

Fibers, produced by textile technology, have been used to make non-woven scaffolds from polyglycolic acid (PGA) and poly-L-lactides (PLLA). The lack of structural stability of these non-woven scaffolds, often resulted in significant deformation due to contractile forces of the cells that have been seeded on the

scaffold. This led to the development of a fiber bonding technique to increase the mechanical properties of the scaffolds. This is achieved by dissolving PLLA in methylene chloride and casting over the PGA mesh. The solvent is allowed to evaporate and the construct is then heated above the melting point of PGA. Once the PGA-PLLA construct has cooled, the PLLA is removed by dissolving in methylene chloride again. This treatment results in a mesh of PGA fibers joined at the cross-points.

2.3.4 Phase separation

A biodegradable synthetic polymer is dissolved in molten phenol or naphthalene and biologically active molecules such as alkaline phosphates can be added to the solution. The temperature is then lowered to produce a liquid-liquid phase separation and quenched to form a two-phase solid. The solvent is removed by sublimation to give a porous scaffold with bioactive molecules incorporated in the structure.

2.3.5 Melt molding

This process involves filling a Teflon mould with PLGA powder and gelatin microspheres and then heating the mould above the glass-transition temperature of PLGA while applying pressure to the mixture. This treatment causes the PLGA particles to bond together. Once the mold is removed, the gelatin component is leached out by immersing in water and the scaffold is then dried. Scaffolds produced this way assume the shape of the mould. The melt molding process was modified to incorporate short fibers of hydroxyapatite (HAp). A uniform distribution of HAp fibers throughout the PLGA scaffold could only be accomplished by using a solvent-casting technique to prepare a composite material of HAp fibers, PLGA matrix and gelatin or salt porogen, which was then used in the melt molding process.

2.3.6 Freeze drying

Synthetic polymers, such as PLGA, are dissolved in glacial acetic acid or benzene. The resultant solution is then frozen and freeze-dried to yield porous matrices. Similarly, collagen scaffolds have been made by freezing a dispersion or solution of collagen and then freeze-drying. Freezing the dispersion or solution results in the formation of ice crystals that force and aggregate the collagen molecules into the interstitial spaces. The ice crystals are then removed by freeze-drying. The pore size can be controlled by the freezing rate and pH. A fast freezing rate produces smaller pores.

2.4 Crosslinking techniques [29-30]

Scaffolds can be crosslink to increase mechanical properties and reduce degradation rate. Crosslinking can be done by various methods such as ultraviolet irradiation, electron beam irradiation, dehydrothermal treatment, and chemical treatment.

2.4.1 Ultraviolet irradiation

UV irradiation generates radicals at the aromatic residues of amino acids, such as tyrosin and phenylalanine. The binding of these radicals will react to each other, resulting in crosslinking formation. The crosslinking density largely changes depending on UV irradiation time. However, it is possible that irradiation for longer time preferably acts on the chain scission of molecules. A balance of the crosslinking and chain scission will result in unchanged density of crosslinking.

2.4.2 Electron beam irradiation

Electron beam irradiation also produces radicals. The number of crosslinks is not large and the water content does not decrease very much. This is because the chain scission by the over dose of electron beam also occurs.

2.4.3 Dehydrothermal crosslinking

Scaffolds can be crosslinked by the method of dehydrothermal (DHT) treatment in a vacuum oven at temperature above 100°C. Dehydrothermal treatment brings about chemical bonding between the amino and carboxyl groups within molecule of polypeptide. Dehydrothermal crosslinking can occur only if the amino and carboxyl groups are close to each other. Therefore, it is believed that dehydrothermal treatment have crosslink extent less than chemical treatment.

2.4.4 Chemical crosslinking

There are many types of chemical crosslinking agents such as, glutaraldehyde, formaldehyde, and carbodiimide solution (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) with N-hydroxy-succinimide (NHS)).

The EDC crosslinking mechanism is showed in Figure 2.9. EDC activates the carboxyl group of glutamic or aspartic acid to form an active O-acylisourea intermediate. This intermediate can then react with an amino group to form an isopeptide bond or with a water molecule to regenerate the original COOH. Crosslinks are formed after nucleophilic attack by free amine groups of lysine or hydroxylysine. Addition of the nucleophile NHS to the EDC containing solution was very effective in increasing the number of crosslinked matrix. In addition, water can act as a nucleophile, resulting in the hydrolysis of the O-acylisourea group to give the substituted urea. The crosslink reaction is usually performed between pH 4.5-5. The disadvantage of chemical crosslinking is the toxicity to cells. Therefore, the scaffolds should be rinsed thoroughly to remove overall agents.

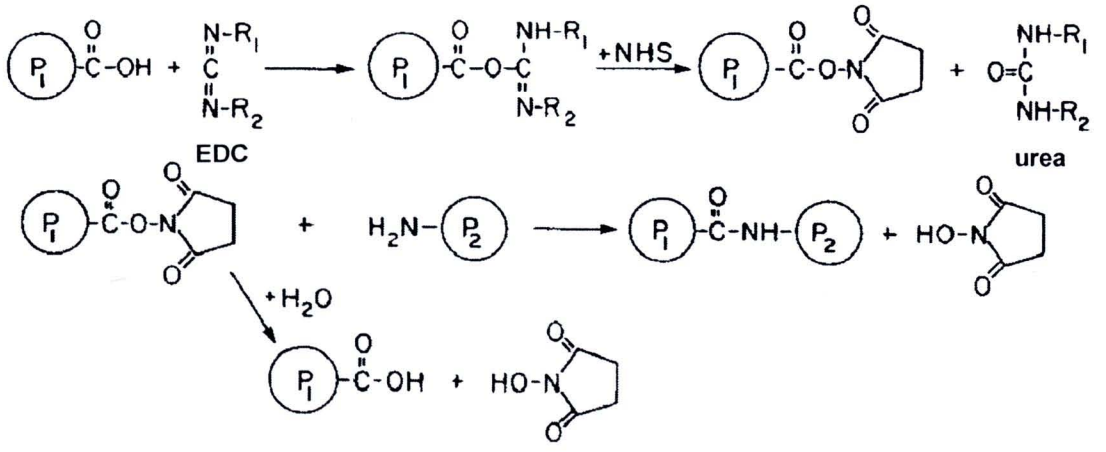


Figure 2.9 Crosslinking of protein with EDC and NHS [31].

2.5 *In vitro* cell culture

2.5.1 Types of cell cultures [5, 32-34]

Types of cell cultures are classified into two types as follow:

2.5.1.1 Primary cell cultures

Primary cell cultures are obtained directly from multiple species including mouse, guinea pig, rat, rabbit, dog, horse, and human. These cells can be kept at the differentiated state for a short period.

Mesenchymal stem cells (MSCs), one type of primary cells, can be isolated from a wide variety of tissues including bone marrow, periosteum, synovium, muscle, adipose tissue, lung, bone, deciduous teeth, dermis, and articular cartilage. Among these, bone marrow is the major source of MSCs. MSCs can be expanded and differentiated into cells of different connective tissue lineages including bone, cartilage, fat, and muscle upon proper stimulation.

These cells also have the potential for a wide range of therapeutic applications through autologous, allogeneic or xenogeneic stem cell transplantation. Bone marrow-derived MSCs have been used to treat a variety of defects and diseases, including

critical size segmental bone defects, full thickness cartilage defects, tendon defects, myocardial infarction and even nerve defects.

2.5.1.2 Permanent cultures or cell lines cultures

Cell lines cultures have an unlimited proliferation capacity. They are derived from embryos, tumors or transformed cells. Examples of cell lines are L929 mouse skin fibroblast, MC3T3-E1 mouse osteoblast, HeLa, MDCK, etc.

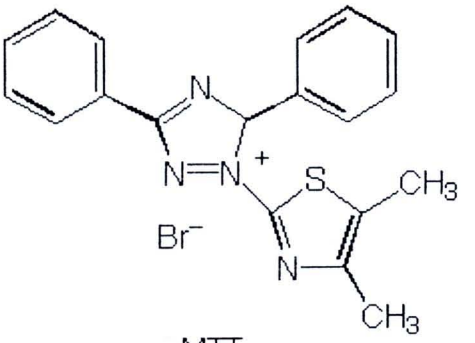
Proliferation and differentiation depending on the cell type. Numerous publications [35-37] provide protocols for the isolation of different cell types, their culture conditions, and for the evaluation of the degree of differentiation.

MC3T3-E1, preosteoblastic cell line, was derived from mouse calvaria. Culture conditions can be induced to undergo a developmental sequence leading to the formation of multilayered bone nodules. This sequence is characterized by the replication of preosteoblasts followed by growth arrest and expression of mature osteoblastic characteristics such as matrix maturation and eventual formation of multilayered nodules with a mineralized extracellular matrix. This cell line has become the standard *in vitro* model of osteogenesis and has found widespread use in studies examining many aspects and applications of osteogenesis, including transcriptional regulation, mineralization and tissue engineering.

2.5.2 MTT assay for cell viability [38]

MTT assay is a quantitative colorimetric assay for mammalian cell survival and cell proliferation. It is an indirect method for assessing cell growth and proliferation. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Figure 2.10) is a water soluble tetrazolium salt yielding a yellowish solution when prepared in media without phenol red. MTT solution is converted to an insoluble purple formazan by cleavage of the tetrazolium ring by dehydrogenase enzyme from mitochondria. The yellow tetrazolium MTT is reduced by metabolically active cells. The insoluble purple formazan can be solubilized using isopropanol or other solvents

such as dimethyl sulfoxide (DMSO). The absorbance of the resulting solution can be measured and used to quantify the number of cells using a spectrophotometer.



MTT

Figure 2.10 Molecular structure of MTT (3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl tetrazolium bromide) [39].