CHAPTER II

THEORY

2.1 Skin

2.1.1 Anatomy of skin [10, 11]

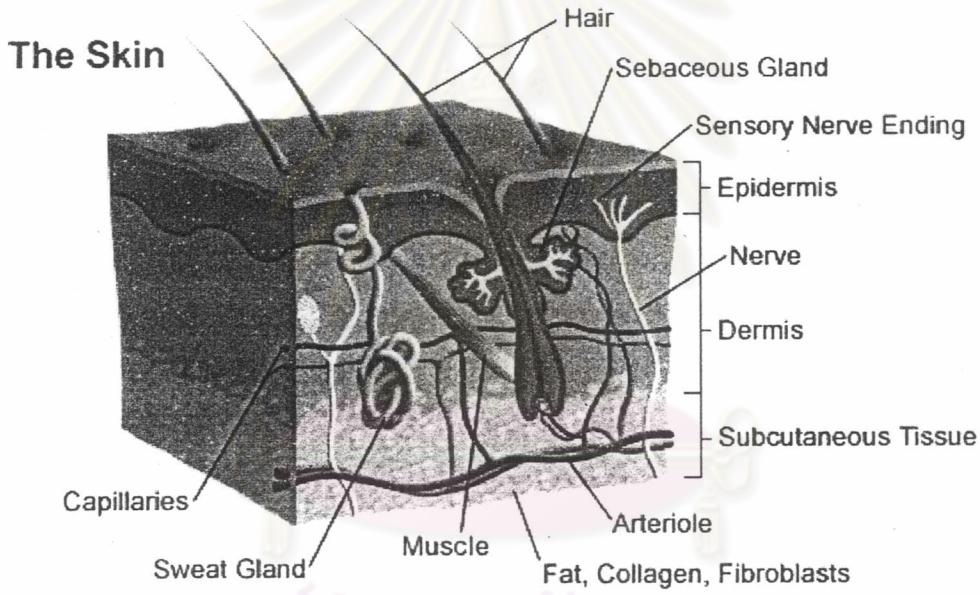

Figure 2.1 Diagram of the skin made up of three layers with each layer performing specific functions [11].

The skin is a stratified epithelial tissue, which is derived from the ectoderm layer of the embryo. It consists of three layers, which are epidermis, dermis, and subcutaneous tissue as shown in Figure 2.1. The epidermis classified as fully regenerative organ is the upper layer of the skin, which provides the body with its first line of defence. The epidermis consists of the following three parts:

- Stratum corneum (horny layer): This layer consists of fully mature keratinocytes which contain fibrous proteins (keratins). The outermost

layer is continuously shed. The stratum corneum prevents the entry of most foreign substances as well as the loss of fluid from the body.

- Keratinocytes (squamous cells): This layer, just beneath the stratum corneum, contains living keratinocytes (squamous cells), which form the stratum corneum when they are mature.
- Basal layer: The basal layer is the deepest layer of the epidermis, containing basal cells. Basal cells continuously divide, forming new keratinocytes to replace the old ones that are shed from the skin.

The epidermis also contains melanocytes, the cells that produce melanin (skin pigment). The basal layer of keratinocytes is located adjacent to the basement membrane that comprises the dermo-epidermal junction. The rapidly dividing stem cells are responsible for the generation of keratinocyte that differentiate and mature as they move outwards.

The middle layer of the skin is called dermis which is non-regenerative organ containing the following components:

- Blood vessels
- Lymph vessels
- Hair follicles
- Sweat glands
- Collagen bundles
- Fibroblasts
- Nerves

The dermis is held together by a protein called collagen. This layer also contains pain and touch receptors. Of necessity, cell turnover is rapid, as cells must be readily replaced when they are lost. It is differentiated into various components such as sebaceous glands, sweat glands, nerves and hair follicles. While the differentiation is difficult to replicate during repair after a severe injury, many cell types are not essential to the correct functioning of skin. The essential dermal cell type is fibroblast, which is responsible for the production and maintenance of the structural elements of

skin. These elements, including collagen and elastin, combine with non-fibrous substances such as glycosaminoglycans (GAGs) to form the extracellular matrix (ECM). The ECM also supports the basement membrane, ensuring the integrity of the dermo-epidermal junction. Organized tissue renewal depends on the ECM. Normally, turnover of collagen is low, but it occurs at a higher rate during damage repair. The vascular network, which is difficult to replace, is quite critical to skin regeneration. Without an adequate blood supply, repair is inhibited, and if revascularization cannot be achieved, scar tissue is the only tissue type that can be supported. Scar tissue has a number of faults as follows:

- Fully crosslinked, it only has 70% of the tensile strength of the tissue it replaces.
- It is not fully functional, and is undifferentiated.
- Scar tissue is aesthetically disfiguring.

The subcutaneous tissue is the deepest layer of skin. The subcutaneous tissue, consisting of a network of collagen and fat cells, helps conserve the body's heat and protects the body from injury by acting as a shock absorber.

2.1.2 The function of skin [12]

Anatomically and functionally, skin has two layers. The superficial epidermal layer provides a barrier against infection and moisture loss. The deeper dermal layer is responsible for the elasticity and mechanical integrity of the skin, and contains the blood vessels that are responsible for the nutrition of the epidermal layer. Appendages, such as hair follicles or sweat glands, breach the epidermal and dermal layers. Cutaneous sensory nerves pass through the dermal tissue into the epidermal tissue. Regeneration of the epidermis relies on residues of epidermal cells that lie deep within dermal structures. Ingrowth from the edges of a wound will be insufficient when the wound is more than a few centimeters across. Skin substitute requires a material to restore the epidermal barrier function and incorporate into the healing

wound. Materials used for skin substitute rely on the ingrowth of granulation tissue for adhesion.

2.1.3 Extracellular matrix [10]

Primarily, the extracellular matrix (ECM) provides a scaffold to which fibroblasts can attach. The ECM has been shown to be useful as a carrier of cytokines. When considering desirable properties for the ECM, it must be realised that the artificial ECM has an important role as guidance for fibroblast infiltration. The initial structure of artificial ECM will be replaced by synthesized ECM secreted from fibroblasts. The synthesized ECM does not necessarily have to have the structure of natural skin. However, it has been found that the repair is promoted by an ECM structure similar to that of the original. Choices of materials from which the ECM can be created are both natural and synthetic. It is generally easier to control the properties of synthetic materials, such as strength, degradation and permeability rates. Natural materials such as collagen have decisive advantage since it contains RGD groups binding with cell integrin receptors. The ECM is typically constructed from a combination of collagen and glycosaminoglycans (GAGs). Collagen provides the template with structure and mechanical integrity, while the GAGs slow down in vivo degradation and encourage proper biological activities. It has been suggested that hyaluronic acid, the largest GAG, acts as a transport media for growth factors to be more stable.

In order to have a skin replacement as similar as natural skin, the physical requirements must be considered in addition to the biological requirements. The affinity of the skin for the wound bed must exceed the affinity of air for the wound bed; otherwise pockets of air will build up in the interface. This is not only bad for healing, but also for wound exposure to potential sources of infection. In addition, the modulus of elasticity of the skin replacement must be close to that of natural skin so that the skin replacement can flex with the natural movement of the body without pulling away from the wound. Typically, once the modulus of elasticity is right (this is dictated by the material of construction) the desired flexibility is achieved through

manipulation of the graft thickness. The balance between growth and differentiation is affected by the hardness of the ECM. Compliant gels promote differentiation, whereas stiffer gels support growth. Thus a gel that is initially hard and becomes softer as it degrades is desirable for complete healing.

2.2 Wound

2.2.1 Classification of wound [13, 14]

One of the most painful injuries that one can ever experience is a burn injury. Serious burns are complex injuries. In addition to the burn injury itself, a number of other functions may be affected. Burn injuries can affect muscles, bones, nerves, and blood vessels. The respiratory system can be damaged, with possible airway obstruction, respiratory failure and respiratory arrest. Since burns injure the skin, they impair the body's normal fluid/electrolyte balance, body temperature, body thermal regulation, joint function, manual dexterity, and physical appearance. Apart from the physical damage caused by burns, patients may also suffer from emotional and psychological problems that begin at the emergency scene and could last a long time. Burns are classified according to the degrees of burn as shown in Figure 2.2.

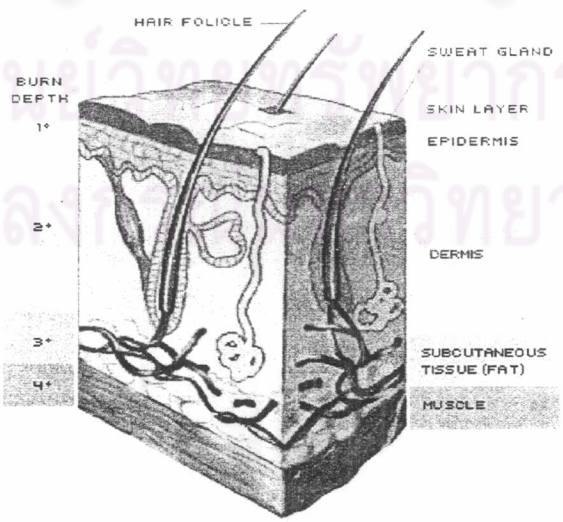

Figure 2.2 Diagram of burn depth [13].

- First degree burns are superficial injuries that involve only the epidermis or outer layer of skin. They are the most common and the least severity of all burns. The skin is reddened and extremely painful. The burn will heal itself without scarring within two to five days. There may be peeling of the skin and some temporary discoloration.
- Second degree burns occur when the first layer of skin is burned through and the second layer, the dermis, is damaged but the burn does not pass through to underlying tissues. The skin appears moist and there will be deep intense pain, reddening, blisters and a mottled appearance to the skin. Second degree burns are considered minor if they involve less than 15 percent of the body skin in adults and less than 10 percent in children. When treated with reasonable care, second degree burns will heal themselves and produce very little scarring. Healing is usually complete within three weeks.
- Third degree burns involve all the layers of skin. They are referred as full thickness burns and are the most serious comparing to other burns. These are usually charred black and include areas that are dry and white. While a third degree burn may be very painful, some patients feel little or no pain because the nerve endings have been destroyed. This type of burn may require skin grafting. As third degree burns heal, dense scars form.

2.2.2 Theoretical models of wound healing [15, 16]

An immediate response to skin injury is bleeding and the formation of a blood clot. Initially the vasculature constricts to decrease the blood flow but later it dilates and becomes more permeable allowing the blood plasma and various cell types to easily enter the wound site. During this 'inflammatory phase' of healing, many white blood cells aggregate in the wound, and these form an important source of regulatory chemicals in the later phases of repair. The upper part of the blood clot dries out to form the scab, and the epidermal layer of the skin heals by direct cell migration underneath the scab. The lower part of the blood clot provides a scaffold along which fibroblasts and other cells migrate into the wound, from surrounding and underlying

tissue, and thus changes from an acellular region to a region rich in cells and capillaries known as granulation tissue. Within the wound, fibroblasts can become actively contractile, and pull the wound edges together. This causes the tension lines familiar around a wound. The fibroblasts also break down the blood clot and replace it with collagen based scar tissue. These healing processes in the dermal layer of the skin depend on a dense network of capillaries, which form in response to injury, and are responsible for the red appearance of a healing wound.

The repair process described above can be conveniently divided into epidermal healing, changes in dermal matrix, and wound contraction.

2.2.2.1 Epidermal healing

Repair of the epidermis is usually fast and effective which occurs in healing of both deep and epidermis damaged wounds. Epidermal healing is a combination of two key processes: cell migration into the wound, and regulated cell division near the wound edge. Both processes are regulated by a wide range of growth factors. For many years, the key growth factors regulating epidermal repair were thought to be members of the epidermal growth factor family. But in the mid-1990s, the work of Werner and coworkers [17] showed that keratinocyte growth factor (KGF) plays a key role in epidermal healing, and is expressed at very high levels within 24 hours of injury. Although its effects are on epidermal keratinocytes, KGF is produced only within the dermal layer of the skin. This implies that the regeneration of epidermis cannot be occurred without dermal support.

2.2.2.2 Repair of the dermal extracellular matrix

The starting point for scar tissue formation is the blood clot, a dense random network of fibers made from the protein fibrin. As fibroblasts cells migrate into the wound from surrounding tissue, they break down the fibrin, replacing it with a provisional extracellular matrix (ECM), which in turn is converted to the new dermal tissue whose key constituent is collagen. The fibroblasts will continue to reorganize

the collagen–based ECM for many months after wounding. Typically the new dermal tissue is different from the original dermal tissue and is called a scar.

Two key features of scar tissue ECM that attracted the attention of empirical modeling are the details of collagen composition, and the orientation of the fibers. Among different types of collagen, types I and III are most abundant in the dermis, and the proportion of type III collagen is higher in scar tissue than in normal dermis. Since type III collagen decorates the surface of type I fibrils, this results in thinner collagen fibers. The balance between types I and III collagen is regulated by the different isoforms of TGF-β. Although collagen type is different in scar tissue and normal dermis, the differences in fiber orientation are more significant. In normal dermis, the collagen fibers are arranged in a random, 'basket-weave' form, whereas in a scar, there is a predominant direction of alignment. The difference in fiber alignment causes a scar to look different from the surrounding skin, and it also gives the scar poorer mechanical properties. The presence of predominant fiber orientations means that scar tissue is anisotropic. A number of inorganic materials, such as graphite, are anisotropic, and there are established methods for studying such materials mathematically. But scar tissue is much more complicated because the anisotropy varies dynamically in space and time, as fibroblasts move into the wound and fibrin is replaced by collagen.

Conventionally, if an area of dermis is removed, the wound contracts and a scar forms. Scar tissue, which is less flexible than physiologically normal dermis, can lead to restricted motion at a joint. If, instead, a synthetic matrix is used, contraction is inhibited and there is little scar formation. The formation of scar tissue is associated with wound contraction.

2.2.2.3 Wound contraction

The familiar tautness of skin around a wound is evidence of wound contraction, the process by which contractile fibroblasts within a wound pull its edges together. This occurs to a much greater extent in animal skin than in humans, because

of the absence of the *panniculus carnosus*, a muscle that connects human dermis to underlying tissue.

The contractile fibroblasts within wounds are thought by some investigators to have a separate phenotype, while others regard the traction force as part of normal fibroblast behavior. But the basic phenomenon is universally agreed. Wound contraction occurs by the following sequence of events. In normal dermis, skin fibroblasts are inactive. After wounding, fibroblasts proliferate and synthesize a new collagen matrix called granulation tissue. Migrating fibroblasts at the edges of the wound initiate contraction. As contraction continues, the resistance of the wound to further contraction increases and the contracting fibroblasts at the edges of the wound differentiate into myofibroblasts. Myofibroblasts are elongated cells with actin filament bundles (also known as stress fibers) oriented along the lines of greatest resistance, allowing them to further contract the wound. Differentiation of fibroblasts to myofibroblasts has been shown to depend at least in part on the extent to which the wound resist contraction. After the wound has healed, the fibroblast population decreases and extracellular matrix remodelling begins.

2.2.3 Treatments of burned wounds [18]

Severe burn injuries cause extensive damage of tissue and loss of body fluids. Burned wounds become seriously infected further aggravating morbidity. The main requirement in burn wound management is an economical, easy to apply, readily available dressing. The method of coverage should prevent infection as well as heat and fluid losses. Furthermore, it should provide good pain relief and promote healing. The elastic, non-antigenic, and adhesive properties are also required for spontaneous epithelialization of superficial partial thickness burns or for permanent coverage with autologous epithelium of deeper burn wounds.

Autografts from uninjured skin remain the mainstay of treatment for many patients and skin graft preservation for the purpose of delayed application is still a basic tool in burn treatment and plastic and reconstructive surgery. Autologous skin,

however, has limited availability and is associated with additional scarring. Severe burn patients invariably lack sufficient skin donor sites necessary for burn wound coverage and healing. Additional limitation to the modality of autologous partial thickness skin grafting is the creation of additional donor site wounds equivalent to second degree burns thus further increasing the total body surface area (TBSA) affected. Due to the limitation of autografts, methods for handling burn wounds have developed in recent decades as follows.

2.2.3.1 Amniotic membrane

Since 1910, allogenic amnion has been used as a biological wound dressing. Amniotic membrane is a thin semi-transparent tissue forming the innermost layer of the fetal membrane. It has an avascular stroma and a thick continuous basement membrane with a full complement of collagen types IV and V as a laminin. It also contains several proteinase inhibitors. It is widely used in developing countries particularly where religious barriers preclude the acceptance of bovine and porcine skin or cadaveric skin. It has been claimed that it is one of the most effective biological dressings ever used in wound treatment. In fact, efficiency of amniotic membrane in preserving excised wound bed and maintaining low bacterial count is the same as that of human skin allograft dressings. Contrary to human skin allograft, it has a fragile structure which is more difficult to handle. Advantages of human amniotic membranes in burn wound management are reduction of protein, body fluids, and heat losses as well as inhibition of infection and antibiotic administration. The amniotic membrane can avoid bulky dressings and minimize both pain and analgesia associated with dressing changes. Moreover, the method can accelerate epithelial regeneration reducing length of hospitalization.

Human amnion is primarily used to cover debrided second degree burns until complete healing, which varies depending upon the extent and depth of the wound and the amount of exudates, is achieved. It may also be used as a temporary coverage of full thickness wounds for skin transplantation. Human amniotic membranes can

provide a useful cover for microskin grafts as well or an overlay of widely meshed autografts promoting early epithelialization and rapid wound healing.

2.2.3.2 Human cadaver allografts and xenografts

When skin donor sites are limited or when the immediate coverage of excised burn wounds with autologous skin is not possible, there may still be a clinical need for human cadaver allograft skin (HCAS) for a temporary biologic dressing. Similar to allogenic amnion, HCAS may also be used as a dressing to cover widely meshed autografts in extensive burns. It must be noted that the usage of allograft skin has been associated with slightly operative procedures per percent of TBSA burn. As the demand for skin allografts has increased, an adequate supply of good quality material is necessarily considerable. The responsibility for processing, storage and evaluation of graft performance of preserved skin has become an important issue. Tissue banking organizations are established to supply good quality skin grafts, especially, when cell viability in allograft skin may be an essential consideration for clinical repair of wounds in which cytokine activity or dermal integration are desirable. Nonetheless, serious problems are associated with HCAS including limited supply, variable and occasionally poor quality, inconvenience of harvesting skin in the mortuary and ultimate immune rejection.

On the other hand, xenografts have been used for hundred years as temporary replacement for skin loss. Donor species include frog, lizard, rabbit, dog and pig. Although these grafts provide a biologically active dermal matrix, the immunologic disparities prevent engraftment and predetermine rejection over time. It must be noticed that xenografts and allografts, are only temporary burn wound cover except human skin allografts in patients taking the usual dosages of immunosuppressants for renal transplantation. In such category of patients, skin allografts seem to survive with minimal repopulation of skin allografts by autogenous keratinocytes and fibroblasts. In case of discontinuation of immunosuppression, the skin allograft does not reject acutely. It persists clinically and the allograft cells are destroyed and replaced slowly with autogenous cells.

2.2.3.3 Keratinocyte culture

Cell therapy is an emerging therapeutic strategy aimed at replacing or repairing severely damaged tissues with cultured cells. The culture and transplantation of keratinocytes are definitely a major and important progress in the treatment of severe burns. Epidermal regeneration obtained with autologous cultured keratinocytes (cultured epithelial autografts (CEAs)) can be life-saving for patients suffering from massive full thickness burns. In 1975, serial subculture of human keratinocytes was first reported. Clinical application of this discovery was made possible after the preparation of these cells into epithelial sheets. In 1981, the earliest application of cultured autologous epithelia was made for the treatment of extensive third degree burns.

The most important advantage of cultured keratinocyte allografts is the large surface area obtained from a relatively small biopsy of healthy skin from the patient. A major disadvantage, nevertheless, is the time-consuming process to provide cultured keratinocyte sheets for clinical application. Fragility and difficult handling of the grafts, and extremely high costs are other major disadvantages.

2.2.3.4 Cultured dermal substitutes—fibroblast cultures

An *in vivo* study of cultured artificial dermal substitutes showed that an artificial dermis containing autologous cultured fibroblasts enhances the re-epithelialization of a full-thickness skin defect compared to an acellular dermal substitute scaffold. This implies the importance of incorporating fibroblasts in any bioengineered skin replacement and healing. Allogeneic cultured dermal substitute or artificial skin, can be prepared by culturing fibroblasts on a specially designed scaffold such as two-layered spongy matrix of hyaluronic acid and atelocollagen. A biodegradable salt-leached porous gelatinous scaffold is also appropriate to seed cultured fibroblasts. It has been demonstrated that the fibroblasts cultured under such conditions mainly attaches on the surface of the pores in the scaffold, whereas cells seeded on freeze dried scaffolds are only widely distributed and aggregated on the top

and the bottom of the scaffold. After 14 days of culturing, the fibroblasts exhibit a good affinity and proliferation on the gelatin scaffolds without showing any signs of biodegradation.

2.2.3.5 Dermal regeneration templates (artificial skin substitutes)

The recovery of skin function is the goal of each burn surgeon. Autograft treatment of full thickness skin defects, a widely used standard treatment method, leads to scar formation which is often vulnerable and unstable. It frequently leads to severe debilitating contractures due to a lack of adequate dermal support. The clinical use of artificial skin substitutes has been celebrated enthusiastically as an improvement in burn therapy over the last two decades. It has been successfully used to permanently replace skin destroyed by burn ranging from 10 to over 95% TBSA. Artificial skin is a bilaminar membrane made of dermal and epidermal portions. An example of the dermal portion is a porous fibrous matrix arranged in a three-dimensional pattern closely resembling the fiber pattern of normal dermis. A thin silastic covering serves as a temporary epidermis. When grafted on an excised wound, it takes some time to be populated by the patient's own fibroblasts. An autogenous ''neodermis'' is thus produced as fibroblasts and vessels migrate from the wound bed into the artificial dermal template. Using the artificial fibers as a scaffolding, migrating autologous fibroblasts synthesize new connective tissue in the collagen fiber pattern of normal dermis rather than the pattern of scar, while slowly biodegrading the artificial fibers. The replaced dermis functions as normal dermis, not a scar tissue. In practice, the rate of dermal tissue formation and scarring is influenced directly by the rate of scaffold angiogenesis, degradation, and host response induced by the scaffold materials. The patient's own epidermal cells, subsequently seeded or grafted on the neodermis, grow into a confluent epidermal layer producing a permanent skin.

Various artificial skin substitutes are available commercially. They are effective for management of contractures and chronic wounds. They may decrease the risk of donor area morbidity, which is more difficult to treat in children. In addition,

they can be used in conjunction with autologous cultured epithelium applications. Decellularization of porcine skin to produce an acellular dermal matrix (ADM) for biomedical applications has also been described. The practical application of ADM in the management of burn wounds still needs to be clarified.

2.2.4 The role of skin substitutes [19]

Obviously, what is required from the substitute will be different according to the nature of the wounds treated. Perhaps the general functions of individual substitutes can be identified as following.

Protection

Many substitutes can be used to provide an impermeable barrier at the wound surface. They have a benefit in restricting fluid loss and providing a barrier to wound colonization. Generally, an impermeable (occlusive) dressing may not be wholly desirable because the difficult management of exudate is then encultured. Impermeable membranes are often designed to have a measurable rate of vapor transmission although they will restrict access to the wound by microorganisms. If pooling of exudate occurs and accesses from the edge of the dressing, these dressings may cause problems in the management of subsequent wound colonization and infection.

Procrastination

Procrastination is perhaps not an obviously beneficial property. If autografts or other autologous materials are not readily available, the skin substitute will provide a stable situation in the wound bed until skin replacements become available. Skin substitute must be taken to protect the wound bed until the wound can be finally closed.

Promotion

A skin substitute should have the ability to provide a suitable environment to promote biological activities involved in the replacement of lost tissue and the salvage of damaged tissue. Substitutes may supply matrix components, cell growth, and growth factors to promote the rate of wound healing.

Provision

Provision of new elements should be incorporated into the healed wound. A skin substitute should supply an organized structure that forms the basis of a stable wound. Cells may be seeded to accelerate the process of tissue replacement or repair. Materials supplied may be designed to persist in the healed wound or to provide a substrate for neodermis remodeling.

2.3 Biodegradable materials [9, 20]

The choice of biodegradable material is also critical to the success of such tissue engineering approaches in dermal wound repair. A variety of biomaterials, naturally occurring and synthetic biodegradable, have been introduced as potential cell carrier substances for dermal wound repair. Natural biomaterials include type I collagen, chitosan, chitin, or alginate in forms of scaffold, film, gels, or composite gels. Synthetic biomaterials include polycaprolactone (PCL), polyglycolic acid (PGA) and polylactic acid (PLA), and their composites. In skin tissue engineering, PGA, PLA, and PGA-PLA copolymers have been studied for their efficiency as fibroblast-delivering scaffolds *in vitro* and *in vivo*. However, the acidic degradation products of the classical polyesters; PLA, PGA, PCL, and their copolymers, have been implicated in adverse tissue reactions, particularly in wound sites. This, somehow, limits the range of physical properties [21].

Many natural polymers and their synthetic analogues are used as biomaterials, but the characteristics of collagen as a biomaterial are distinct from those of synthetic

polymers mainly due to its interaction in the body. Collagen plays an important role in the formation of tissues and organs, and is involved in various functional expressions of cells. Collagen is a good surface-active agent and demonstrates its ability to penetrate a lipid-free interface. Collagen exhibits biodegradability, weak antigenicity and superior biocompatibility compared with other natural polymers, such as albumin, gelatin, alginate, and cellulose. Due to its excellent biocompatibility and safety, the use of collagen in biomedical application has been rapidly growing and widely expanding to bioengineering areas. However, some disadvantages of collagen-based systems arise from the difficulty of assuring adequate supplies, fast biodegradation rate, and ineffectiveness in the management of infected sites. Improvement of the physical, chemical and biological properties is necessary to address some of drawbacks in collagen-based applications. It has been shown that dermal specific extracellular matrix (ECM) components such as type I collagen and glycosaminoglycan (GAG) play a critical role in regulating expression of the fibroblast phenotype both *in vitro* and *in vivo*.

Given the importance of GAGs in stimulating the fibroblast behavior, use of GAGs or GAG analogs as components of a dermal tissue scaffold appears to be a logical approach for enhancing fibroblast genesis. One such candidate is chitosan, a partially deacetylated derivative of chitin, found in arthropod exoskeletons. Structurally, chitosan is a linear polysaccharide consisting of β(1→4) linked D-glucosamine residues with a variable number of randomly located *N*-acetyl-glucosamine groups. It thus shares some characteristics with various GAGs presented in dermal templates, as shown in Figure 2.3.

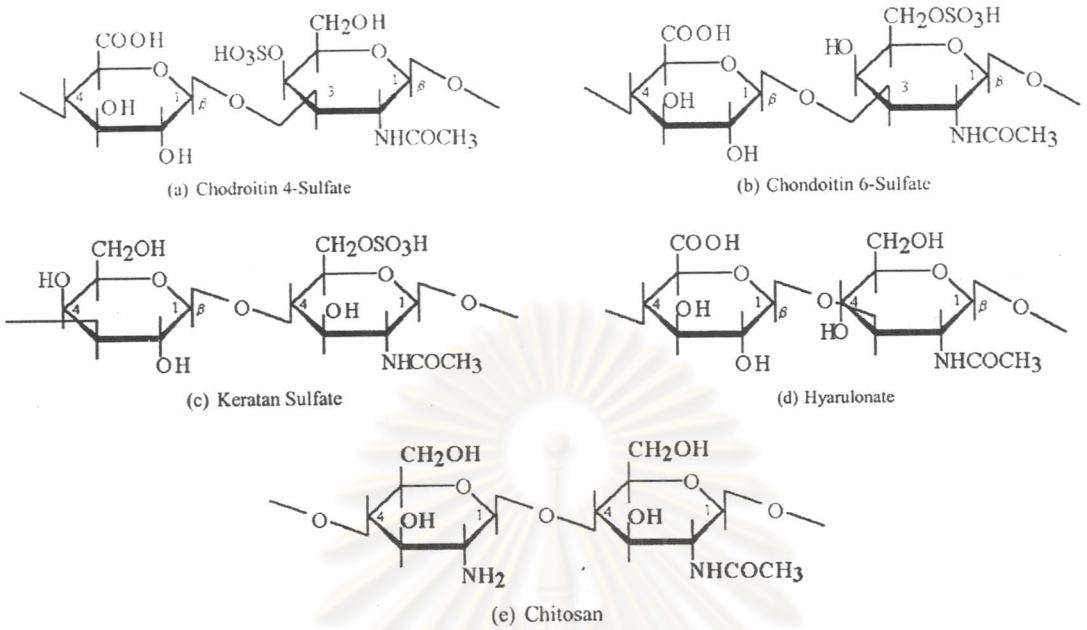

Figure 2.3 Molecular structures of the polysaccharide repeating units [9].

2.3.1 Collagen [22-24]

The use of collagen as a biomaterial is currently undergoing a renaissance in the tissue engineering field. The biotechnological applications focus on the aspects of cellular growth or delivery of proteins capable of stimulating cellular response. The use of collagen in the form of tendons as suture material goes back millennia and could hold its ground with catgut which is still representing a useful suture material in surgery. Collagen can be derived from different sources such as bovine, porcine, or fish scales. Due to the long historic use of collagen, the term collagen is generally applied and represented individual molecules, native fibril, aggregated, or bulky forms of unspecified nature. Collagen represents the chief structural protein accounting for approximately 30% of all vertebrate body protein. More than 90% of the extracellular protein in the tendon and bone and more than 50% in the skin consist of collagen. Connective tissue derives prominent features such as mechanical strength and activation of the blood clotting cascade from the ubiquitous scleroprotein collagen and its architectural arrangement.

2.3.1.1 Amino acid composition and sequence

Amino acid composition

Approximately 1/3 of the total amino acid residues is composed of glycine and 2/9 are imino acids, i.e. proline and hydroxyproline. Hydrophilic amino acid residues are very few. Tryptophan is totally absent and a few tyrosine residues are presented only in the telopeptide regions (non-helical regions of the molecule). In addition, telopeptide regions are denoted for regions of 9–26 amino acids at the amino and carboxyl terminal chain ends of the molecule that are not incorporated into the helical structure. Table 2.1 represents the amino acid composition of α-chains in bovine collagen. Moreover, type I collagen is a glycoprotein with a carbohydrate content of less than 1%. The sugar components are either a single galactose unit or a disaccharide of galactose and glucose O-glycosidically attached via hydroxylysine residues.

Amino acid sequence

Primary structure of collagen is characterized by the present of Gly-X-Y repeating sequence. The presence of glycine in every three residues is essential for the formation helical structure. X and Y represent other amino acid residues. The frequency of amino acids in these positions is different in different species of collagen. For type I collagen, proline and hydroxyproline exist in X and Y, respectively. Such a regular sequence is not found in the telopeptide regions.

Table 2.1 Amino acid composition of bovine collagen α-chains [23, 24].

Amino acid	Residues/1,000 residues			
	α1(I)	α2(I)	α1(II)	α1(III)
3-Hydroxyproline	1	-	2	-
4-Hydroxyproline	85	85	91	127
Aspartic acid	45	47	43	48
Threonine	16	17	22	14
Serine	34	24	26	44
Glutamic acid	77	71	87	71
Proline	135	120	129	106
Glycine	327	328	333	366
Alanine	120	101	102	82
Cysteine	-	-	-	2
Valine	18	34	17	12
Methionine	7	4	11	7
Isoleucine	9	17	9	11
Leucine	21	34	26	15
Tyrosine	4	3	1	3
Phenylalanine	12	16	14	9
Hydroxylysine	5	11	23	7
Lysine	32	21	15	25
Histidine	3	8	2	8
Arginine	50	57	51	44

2.3.1.2 Classification of collagen

Different collagen types are necessary to confer distinct biological features to the various types of connective tissues in the body. Collagen comprises a family of generically distinct molecules which have a unique triple-helix configuration of three polypeptide subunits known as α-chains which will be described in following section. Currently at least 13 types listed in Table 2.2 have been isolated which vary in the

amino acid composition, the length of the helix, the nature and size of the non-helical portions.

Table 2.2 Chain composition and body distribution of collagen types [22].

Collagen type	Chain composition	Tissue distribution
I	$(\alpha1(I))_2\alpha2(I)$ or trimer$(\alpha1(I))_3$	Skin, tendon, bone, cornea, dentin, fibrocartilage, large vessels, intestine, uterus, dentin, dermis, tendon
II	$(\alpha1(II))_3$	Hyaline cartilage, vitreous, nucleus pulposus, notochord
III	$(\alpha1(III))_3$	Large vessels, uterine wall, dermis, intestine, heart valve, gingiva (usually coexists with type I except in bone, tendon, cornea)
IV	$(\alpha1(IV))_2\alpha2(IV)$	Basement membranes
V	$\alpha1(V)\alpha2(V)\alpha3(V)$ or $(\alpha1(V))_2\alpha2(V)$ or $(\alpha1(V))_3$	Cornea, placental membranes, bone, large vessels, hyaline cartilage, gingiva
VI	$\alpha1(VI)\alpha2(VI)\alpha3(VI)$	Descemet's membrane, skin, nucleus pulposus, heart muscle
VII	$(\alpha1(VII))_3$	Skin, placenta, lung, cartilage, cornea
VIII	α1(VIII) α2(VIII) chain organization of unknown helix	Produced by endothelial cells, Descemet's membrane
IX	$\alpha1(IX)\alpha2(IX)\alpha3(IX)$	Cartilage
X	$(\alpha1(X))_3$	Hypertrophic and mineralizing cartilage
XI	$\alpha1(XI)\alpha2(XI)\alpha3(XI)$	Cartilage, intervertebral disc, vitreous humour
XII	$(a1(XII))_3$	Chicken embryo tendon, bovine periodontal ligament
XIII	Unknown	Cetal skin, bone, intestinal mucosa

2.3.1.3 Three dimensional structure of type I collagen

Type I collagen is predominant in higher order animals especially in the skin, tendon, and bone where extreme forces are transmitted. It is a compound of three chains, two of which are identical, termed α1(I), and one α2(I) chain with different amino acid composition or it can rarely represent a trimer built of three α1(I) chains. The α-chains form a helical structure by rotating counterclockwise with 3.3 amino acid residues per rotation. Helical structure is presented at the center and relaxed structure is presented at both ends of the collagen molecule as shown in Figure 2.4a. The telopeptide regions are still remained for type I collagen extracted from acid or neutral salt soluble process and discarded for that from enzyme solubilized procsess. Each of the α-chains is composed of about 1,000 amino acid residues with Gly-X-Y repeating sequence as shown in Figure 2.4b. For type I collagen, X and Y residues are proline and hydroxyproline in every six residues. Furthermore, the three α-chains as a whole form a helical structure by rotating clockwise with 30-45 residues per rotation. When the triple helix is denature and becomes gelatin, polypeptides are separated to one α-chain, two β-chains, and three γ-chains.

Helical region of collagen is cylindrical structure. Therefore, a collagen molecule which is consisted of three polypeptide chains of 1,000 amino acid residues can be considered as a cylinder of 3,000 Å in length and 15 Å in width. Only glycine residue is facing toward the center of the cylinder and the other amino acid residues are all facing outside. This is because of the steric hindrance caused by the large side chains of amino acids other than glycine. Different from other proteins, hydrophobic amino acids can also face outside. This is related closely to the low solubility of collagen in water.

Denaturing temperature of collagen from various animal species is different and it is related to the temperature of the environment surrounding the cells. For example, the denaturing temperature of human or higher order animals is 40°C, whereas that of the fish in Antarctic is about 5°C. Furthermore, it is known that collagen in different tissues of the same animal has different denaturing temperature.

Hydroxyproline plays an important role in heat stability of the collagen molecule. Introduction of —OH group in proline facilitates the formation of hydrogen bonds within the molecule through water molecules. Thus, the denaturing temperature is high in collagen molecules which have high hydroxyproline content.

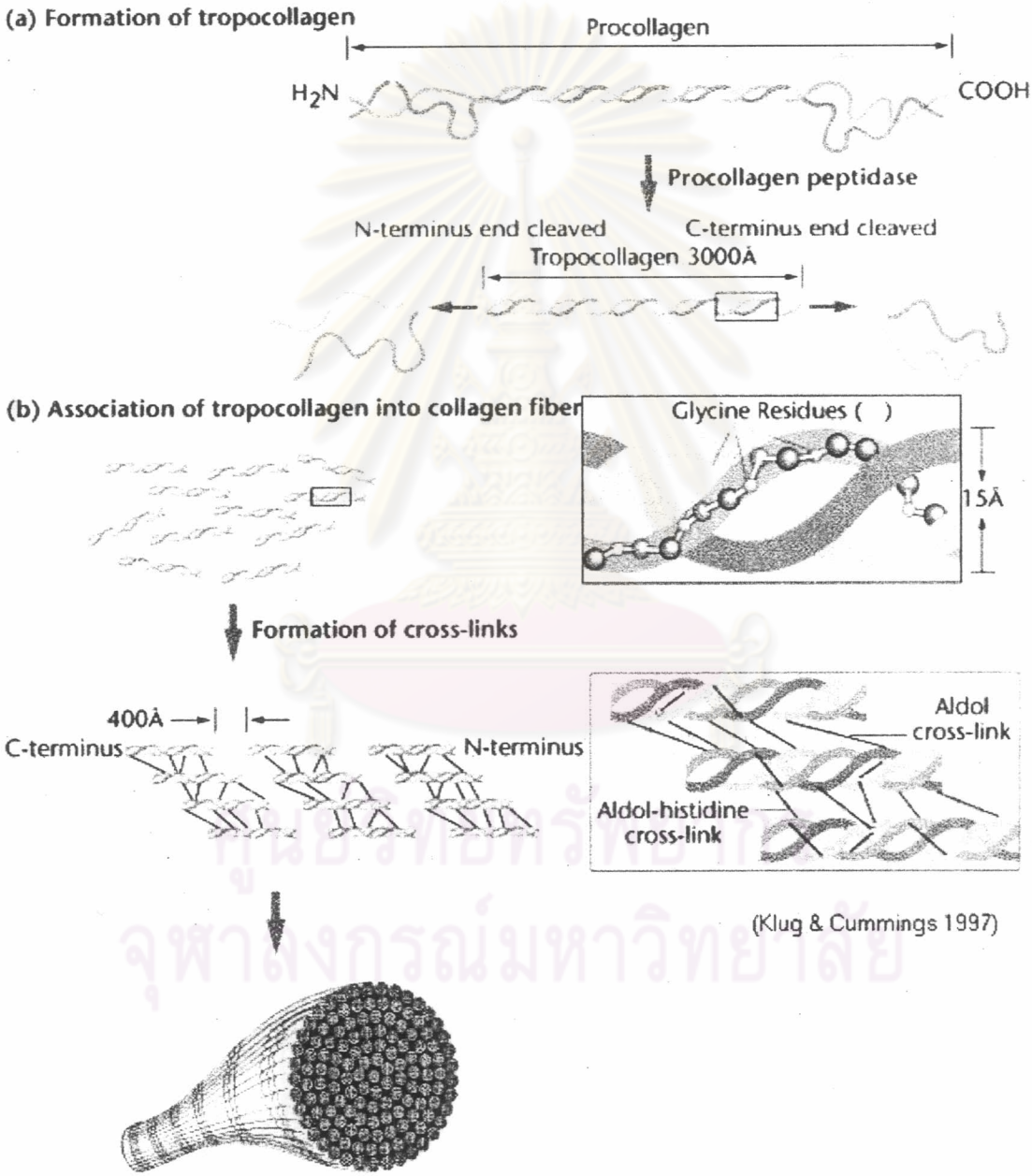

Figure 2.4 Chemical structure of type I collagen [25].

2.3.1.4 Collagen as medical materials

Recently, attention has been focused on the use of collagen as medical materials. Different types of collagen used are suitable for different purposes. It is classified into two types, i.e. native collagen retaining the original tissue structure and solubilized collagen. Examples of native collagen are the use of bovine carotide artery as artificial blood vessels and the use of porcine skin in treatment of burn. Solubilized collagen is processed and fabricated for uses in most applications as listed in Table 2.3.

Table 2.3 Applications of collagen [20].

Form of collagen	Applications
Solution	Cell culture media for transplant development
	Plasma expander
	Soft tissue augmentation
Gel	Vitreous body
Power	Hemostatic agent
Film (membrane)	Corneal replacement
	Hemodialysis membrane
	Valve prosthesis
	Membrane oxygenarators
	Wound dressing
	Tissue repair or reinforcement
Thread	Sutures
Sponge	Hemostatic agents
	Wound dressing
	Surgical tampons
Tubing	Vessel prosthesis
	Reconstruction or reinforcement of hollow organs (vasculature, esophagus, and trachea)
	Nerve resection acids

The reason why collagen is suitable as medical materials is explained as follows. Firstly, antigenicity of collagen is very low compared with other proteins. Antigenic determinant of collagen is mainly presented in the telopeptide regions. Since enzyme solubilized collagen is devoid of these regions, the antigenicity of collagen molecule is almost negligible. It is also known that the introduction of crosslinks by either chemical or physical techniques in processing of fibrils or membranes rendering the antigenicity is very low. Secondly, collagen plays a role as substratum for various types of cells. Since the affinity of collagen to tissues is very good, collagen allows the cells to grow on it when applied in the body as a medical material. The affinity of collagen to the body is much better than other synthetic high molecular weight materials. Lastly, the nature of collagen can be controlled by chemical modification of the molecule. For example, introduction of crosslinks lowers the degradation of collagen in the body. Methylation of collagen renders it thrombotic and succinylation of collagen renders it antithrombotic.

2.3.2 Chitosan [26]

Chitin and chitosan are known biodegradable natural polymers based on polysaccarides, which are extracted from various animals and plants. Chitin exists widely in cell walls of some microorganisms such as fungi, molds and yeasts and in the cuticular and exoskeletons of invertebrates such as crustaceans, mollusks, crabs, shrimps, lobster, squid and insects (for example beetles). Chitosan exists naturally only in a few species of fungi. Chitin and chitosan are copolymers consisting of 2-acetamido-2-deoxy-β- D-glucose (or N-acetyl-glucosamine) and 2-amino-2-deoxy-β-D-glucose (or N-glucosamine) as repeating units. The repeating units are randomly or block distributed throughout the biopolymer chains depending on the processing method used to derive the biopolymer (Figure 2.5). When the number of N-acetyl-glucosamine units is lower than 50%, the biopolymer is referred as chitosan. The term "degree of deacetylation" is used to represent the amount of N-glucosamine units in the molecules. If the degree of deacetylation is higher than 50%, it will be classified as chitosan. In contrast, when the number of N-glucosamine units is lower than 50%, the term chitin is used. On the other hand, chitosan is the N-acetylated form of chitin.

Figure 2.6 shows the deacetylation reaction of chitin. The degree of acetylation is one of the most important structural parameters in chitosan. Many methods have been developed for determining the degree of acetylation, including from elemental analysis, infrared spectroscopy, colloidal titration, circular dichroism, pyrolysis-gas chromatography, gel permeation chromatography, thermal analysis, HPLC, and NMR (nuclear magnetic resonance) and X-ray diffraction methods.

There are two advantages of chitosan over chitin. Firstly, in order to dissolve chitin, highly toxic cosolvents such as lithium chloride and dimethylacetamide (DMAc) must be used whereas chitosan is readily dissolved in agents such as 1-10% (v/v) aqueous acetic acid. The second advantage of chitosan is the presence of the free amine group that not only renders a polyelectrolytic effect to the polymer backbone, but also presents an active site upon which many chemical reactions may be applied.

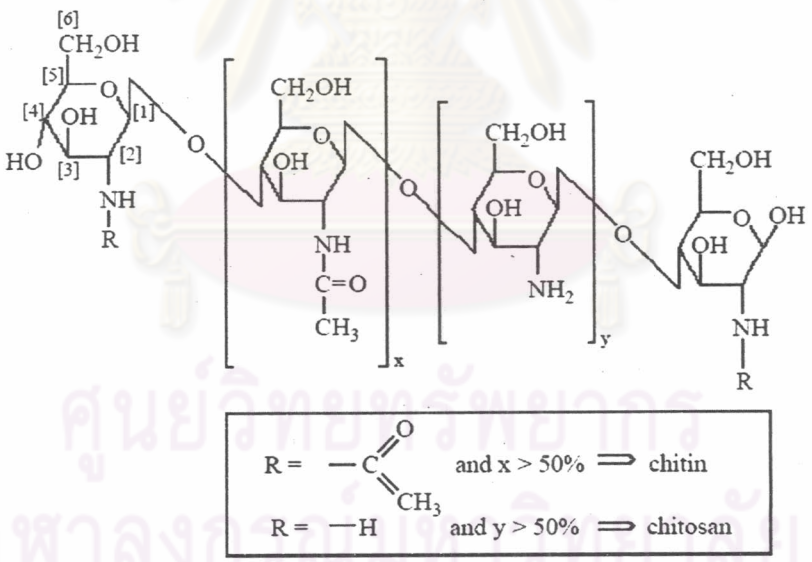

Figure 2.5 Schematic representation of the chitin and chitosan depicting the copolymer character of the biopolymers [9].

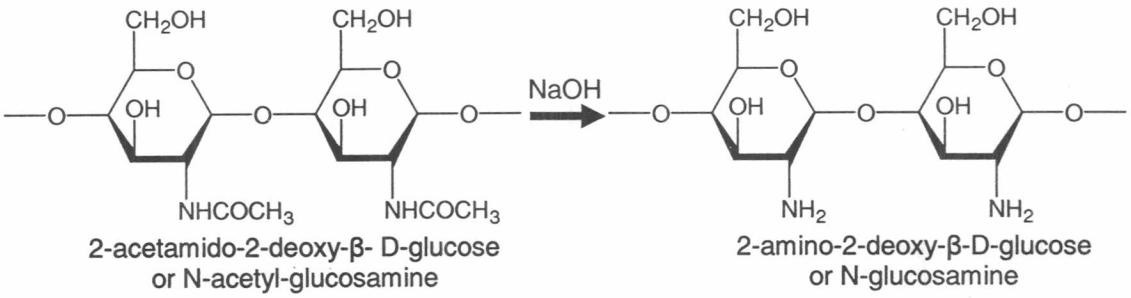

Figure 2.6. The chitosan production with deacetylation of chitin [26].

Apart of the solubility of chitosan in acidic solvent below pH 6, organic acid such as acetic, formic, and lactic acids are used for dissolving chitosan and most commonly used is 1% acetic acid solution (pH around 4.0). Solubility in inorganic acids is quite limited. Chitosan is soluble in 1% hydrochloric acid but insoluble in sulfuric and phosphoric acids. The solubility of chitosan solution is poor above about pH 7. At higher pH, precipitation or gelation will occur. Chitosan solution forms polycation complex with anionic hydrocolloid and provides gel. Chitosan and their derivatives can be used in many applications as listed in Table 2.4. The uses of chitosan in medical applications have been interested because of its advantages as follows:

- Natural renewable resources
- Abundant polymeric material in the earth
- Biocompatibility
- Biodegradability
- Availability
- Nontoxicity
- Extendability into three-dimensional structures (knitted,woven, and nonwoven fabrics)
- Biofunctionalities (antitrombogenic, hemostatic, immunity enhancing, antitumor activity, immunoadjuvant activity, acceleration of wound healing, antimicrobial activity)
- Molecular affinity
- Polyelectrolyte-forming

Table 2.4 Applications of chitosan and its derivatives [26].

Wastewater Treatment	Removal of Metal Ions Flocculant/Coagulant Protein Dye Amino Acids
Food Industry	Removal of Dye, Suspended solids, etc. Preservative Animal Feed Additive
Medical	Bandages Blood Cholestrol Control Controlled Release of Drugs Skin Burn
Agriculture	Seed Coating Fertilizer Controlled Agrochemical Release
Cosmetics	Moisturizer Face, Hand and Body Creams Bath Lotion
Biotechnology	Enzyme Immobilization Protein Separation Cell Recovery Chromatography Cell Immobilization
Pulp and Papers	Surface Treatment Photographic Paper
Membrane	Permeability Control Reverse Osmosis

2.4 Fabrication of scaffolds

2.4.1 Principles of freeze drying [27, 28]

Freeze drying has been used in a number of applications for many years, most commonly in food and pharmaceutical industries. There are also some other uses for the processes including the stabilization of living materials such as microbial cultures, preservation of whole animal specimens for museum display, restoration of books and other items damaged by water, and recovery of reaction products. Freeze drying involves the removal of water or other solvent from a frozen sample by a process called sublimation. Sublimation occurs when a frozen liquid goes directly to gas phase without passing through liquid phase. In contrast, drying at ambient temperatures from the liquid phase usually results in changes in the product, and may be suitable only for some materials. The scaffolds used in this research were fabricated using a freeze drying (lyophilization) process where a suspension of collagen and chitosan is solidified (frozen), leaving the collagen/chitosan coprecipitating between the growing ice crystals. The process produces a continuous, interpenetrating network of ice and the coprecipitate. Sublimation of ice crystals forms the highly porous scaffold structure. The pore volume fraction and the size of the pores can be controlled by the volume fraction of the precipitate in the suspension and by the underlying freezing processes with typical pore volume fractions above 99%. The formation of the ice crystals in the collagen/chitosan suspension is influenced by the nucleation rate, heat rate, and protein diffusion. These processes can primarily controlled by the final freezing temperature and the heat transfer processes associated with freezing. Furthermore, the homogeneity of ice nucleation can be produced by a constant cooling rate technique.

The freeze drying process consists of three stages involving prefreezing, primary drying, and secondary drying.

2.4.1.1 Prefreezing

Since freeze drying is a state change from solid to gas phase, material to be freeze dried must first be adequately frozen. The method of prefreezing and the final temperature of the frozen sample have a direct effect on freeze dried material. Rapid cooling rate results in small ice crystals, useful in preserving structures to be examined microscopically, but more difficult to freeze dry. Slower cooling rate results in larger ice crystals and lesser restrictive channels in the matrix during the drying process. A low final freezing temperature can increase the rate of cooling as well as the rate of ice crystal nucleation and decreases the rate of heat and protein diffusion, leading to small ice crystals. Samples that are subjected to freeze drying consist primarily of water or solvent, materials dissolved or suspended in water or solvent. Most samples used to be freeze dried are eutectics which are a mixture of substances that freeze at lower temperatures than surrounding water. When the aqueous suspension is cooled, changes occur in the solute concentrations of the matrix. As cooling proceeds, water is separated from solutes as it changes to ice, creating more concentrated areas of solute. These pockets of concentrated materials have a lower freezing temperature than water. Although a product may be frozen because of all the ice presented, it is not completely frozen until all of the solute in the suspension is frozen. It is very important in freeze drying to freeze the sample to below the eutectic temperature before beginning the freeze drying process. Small pockets of unfrozen material remaining in the sample expand and compromise the structural stability of the freeze dried sample.

2.4.1.2 Primary drying

Several factors can affect the ability to freeze dry a frozen suspension. While these factors can be discussed independently, it must be remembered that they interact in a dynamic system, and it is delicate balance between these factors that results in a properly freeze dried sample. After prefreezing the product, conditions must be established in which ice can be removed from the frozen sample via sublimation, resulting in a dry, structurally intact sample. This requires very careful control of two

parameters, temperature and pressure. The rate of sublimation of ice from a frozen sample depends upon a difference in the vapor pressure of the product compared to the vapor pressure of the ice collector. Molecules migrate from a higher pressure area to a lower pressure area. Since vapor pressure is related to temperature, it is necessary that the sample temperature is warmer than the cold trap (ice collector) temperature. The balance between the temperature that maintains the frozen integrity and the temperatre that maximizes the vapor pressure of the sample is extremely important, since it is the key to optimum drying. The typical phase diagram shown in Figure 2.7 illustrates this point. Most samples are frozen well below their eutectic temperature (Point A), and then the temperature is raised to just below this critical temperature (Point B) and they are subjected to a reduced pressure. At this point the freeze drying process is started.

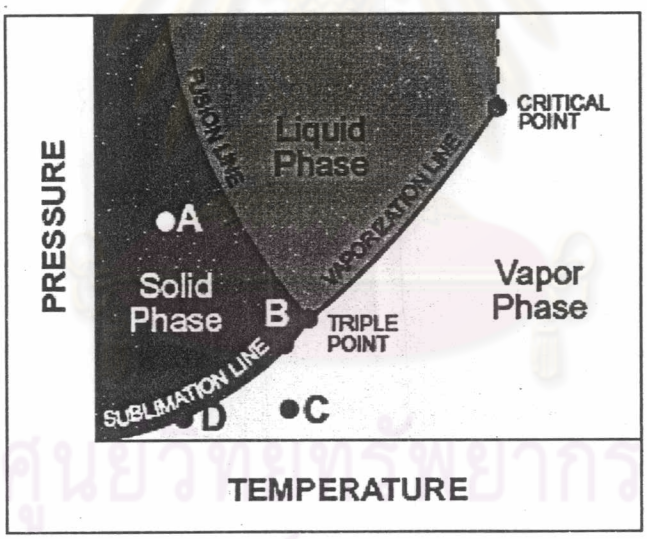

Figure 2.7 A typical phase diagram [28].

Freeze drying conditions must be created to encourage the free flow of water molecules from the sample. Therefore, a vacuum part is essentially needed to lower the pressure of the environment (to Point C). Another essential part is a collecting system, which is a cold trap used to collect moisture that leaves the frozen sample. The collector condenses all condensable gases, i.e. water, and the vacuum part removes all non-condensable gases. It is important to understand that the vapor pressure of the sample forces the sublimation of the water vapor molecules from the

frozen sample matrix to the collector. The molecules have a natural affinity to move toward the collector because its vapor pressure is lower than that of the sample. Therefore, the collector temperature (Point D) must be significantly lower than the sample temperature. In addition, raising the sample temperature has more effect on the vapor pressure than lowering the collector temperature. The relationships between vapor pressure and temperature are shown in Table 2.5.

Table 2.5 Relationships between vapor pressure and temperature [28].

Vapor Pressure (mBar)	Temperature (°C)
6.104	0
2.599	-10
1.034	-20
0.381	-30
0.129	-40
0.036	-50
0.011	-60
0.0025	-70
0.0005	-80

The last essential part in a freeze drying system is energy. Energy is supplied in form of heat. Energy required to sublime a gram of water from the frozen to gas sphase is almost 10 times greater than that required to freeze a gram of water. Therefore, with all other conditions being adequate, heat must be applied to the sample to encourage the removal of water in the form of vapor from the frozen sample. The heat must be very carefully controlled, since applying more heat than the evaporative cooling in the system can warm the sample above its eutectic temperature. Heat can be applied by several methods. One is to apply heat directly through a thermal conductor shelf such as in tray drying. Another method is to use ambient heat as in manifold drying.

2.4.1.3 Secondary drying

After primary freeze drying is complete and all ice has sublimed, bound moisture is still presented in the sample. The residual moisture content may be as high as 7-8%. Continued drying is necessary at a higher temperature to reduce the residual moisture content to optimum values. This process is called isothermal desorption as the bound water is desorbed from the sample.

Secondary drying is normally operated at a sample temperature higher than ambient but compatible with the sensitivity of the sample. All other conditions, such as pressure and collector temperature, remain the same. Secondary drying is usually carried out for approximately 1/3 to 1/2 the time required for primary drying.

2.4.2 Crosslinking treatments

Natural crosslinking gives high tensile strength and proteolytic resistance to collagen. Due to dissociation of crosslinks isolation processes, reconstituted forms of collagen such as films, fibers, or sponges can lack sufficient strength and may disintegrate upon handling or collapse under the pressure from surrounding tissue *in vivo*. Furthermore, the rate of biodegradation has to be customized based on the specific applications. For example, an implant scaffold has to maintain its structure while it is gradually replaced by host collagen. Thus, it is often necessary to confer mechanical property and collagenase resistance by introduction of exogeneous crosslinking into the molecular structure. Crosslinking of the collagen matrix is the primary technique by which the mechanical properties and degradation rate of the matrix can be controlled. Crosslinks stabilize the conformation of collagen fibers and may be induced by either chemical or physical methods.

A major handicap of chemical crosslinking agents is the potential toxic effect of residual molecules and/or compounds formed during *in vivo* degradation. Glutaraldehyde (GTA) is the most widely used crosslinking agent. However, while GTA polymerizes in solution, the release of GTA monomer with time is cytotoxic at

concentrations as low as 10 ppm. Therefore, alternative physical methods are pursued, including a dehydrothermal treatment (DHT), and an exposure to ultraviolet or γ-irradiation. Both ultraviolet and γ-irradiation increase the collagen shrinkage temperature, the resistance to collagenolytic degradation, and the durability under load in collagenase. However, collagen becomes partially denatured by these physical treatments. Formation of crosslinks during ultraviolet and γ-irradiation is thought to be initiated by free radicals formed on aromatic amino acid residues which indicate a limited maximum degree of crosslinking due to a small number of tyrosine and phenylalanine residues in collagen. Exposure times can therefore be kept short since crosslinking density soon reaches its limits, otherwise, triple-helices may be cleaved. In order to minimize the degradation of the triple-helices, it is crucial for DHT treatment to reduce the water content via vacuum prior to heating. Even small amounts of residual moisture can cause breakdown of the helical structures and proteolysis. Severe dehydration itself induces amide formation and esterification of carboxyl, free amino, and hydroxyl groups, respectively. Typical temperature for DHT treatment is 105°C for several hours up to a few days. The sensitivity of collagen material to trypsin, pepsin, and lysozyme is increased after DHT treatment. In addition, physical crosslinks of collagen chains can reduce immunogenic response from the host, since crosslinks mostly occur between the non-helical ends of the chains. It is these non-helical ends that generate an immunogenic response.

2.5 Characterization of polymers

2.5.1 Molecular weight determinations

2.5.1.1 Viscosity-averaged molecular weight determination [29, 30]

It was known that small amount of dissolved polymer could cause tremendous increases in viscosity. Larger molecules will impede flow more than smaller ones and give a higher solution viscosity. The solution viscosity also depends on the solvent viscosity, temperature, solute concentration, and particular polymers, because the interactions between the polymer and solvent influence the conformation of the

polymer molecules, and entanglements between the polymer molecules. The dependence of the solution viscosity can be presented as follows:

$$\eta = \eta\ (\eta_0, \text{T, polymer, solvent, c, entanglements, M}) \qquad (2.1)$$

where η and η_0 represent the solution viscosity and solvent viscosity, respectively.

The effect of solvent viscosity can be normalized by calculating the fractional increase in viscosity caused by solute. The value is called the specific viscosity, η_{sp} :

$$\eta_{sp} = \frac{(\eta - \eta_0)}{\eta_0} = \frac{\eta}{\eta_0} - 1 = \eta_0 - 1 \qquad (2.2)$$

where $\eta_r = \eta / \eta_0$ is known as the relative viscosity. Similarly, the specific viscosity can be normalized by the concentration to get the reduced viscosity, η_{red} :

$$\eta_{red} = \frac{\eta_{sp}}{c} = \frac{(\eta/\eta_0 - 1)}{c} \qquad (2.3)$$

To get rid of the influence of the entanglements on viscosity, the reduced viscosity is extrapolated to zero concentration to get the intrinsic viscosity, $[\eta]$:

$$[\eta] = \lim_{c \to o} \frac{(\eta/\eta_0 - 1)}{c} \qquad (2.4)$$

The intrinsic viscosity is a function of the molecular weight of the polymer, the solvent, and the temperature. If the viscosity of the particular polymer is determined at a constant temperature using a specified solvent, it should be quantitatively related to the molecular weight of the polymer.

Huggins proposed a relationship between reduced viscosity and concentration for dilute polymer solutions ($\eta_r < 2$):

$$\frac{\eta_{sp}}{c} = [\eta] + k' [\eta]^2 c \quad \text{(Huggins equation)} \qquad (2.5)$$

By expanding the natural logarithm in a power series, an equivalent form of the Huggins equation can be obtained as follows:

$$\eta_i = \ln \frac{\eta_r}{c} = [\eta] + k'' [\eta]^2 c \qquad (2.6)$$

where η_i reprsents the inherent viscosity and k″ equals to k′ - 0.5. From Equation 2.6, an alternative definition of intrinsic viscosity can be written as:

$$[\eta] = \lim_{c \to o} \eta_i = \lim_{c \to o} [\ln(\eta/\eta_0)] \quad (2.7)$$

Plots of the reduced and inherent viscosities are linear with concentration, at least at low concentration and the common intercept at zero concentration is the intrinsic viscosity (Figure 2.8).

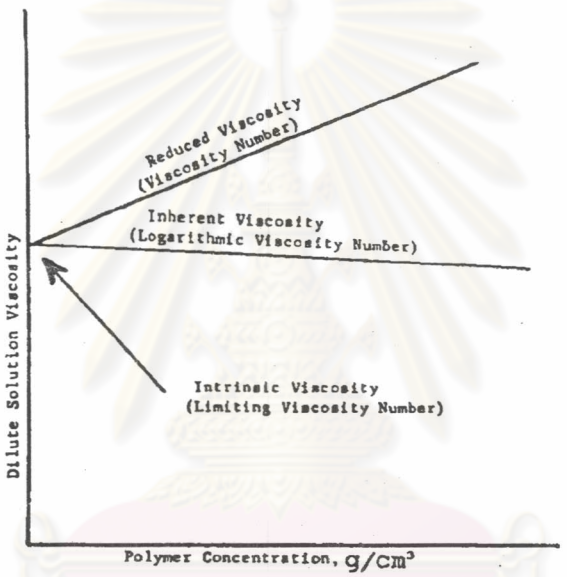

Figure 2.8 Example plot to determine intrinsic viscosity [30].

To relate the intrinsic viscosity and molecular weight of polymers, many studies on the intrinsic viscosity of monodisperse polymer fractions whose molecular weights have been established by one of the absolute methods indicate a rather simple relation :

$$[\eta]_x = K (M_x)^a \quad (0.5 < a < 1) \quad (2.8)$$

where the subscript x refers to a monodisperse sample of a particular molecular weight. Equation 2.8 is known as the Mark-Houwink relation.

To determine the viscosity-averaged molecular weight, the viscosity of the polymer is usually measured using a glass capillary viscometer, in which the solution flows through a capillary under its own head. Two common types of viscometers, the

Ostwald and Ubbelohde, are sketched in Figure 2.9. The Ubbelohde viscometer has the distinct advantage that the driving fluid head is independent of the amount of solution, hence, dilution can be carried out right in the instrument. Flow time, t, can be related to the viscosity of the solution by Equation 2.9:

$$\frac{\eta}{\rho} = \nu = at + \frac{b}{t} \tag{2.9}$$

where a and b are instrument constant, ρ is the solution density, and ν is the kinematic viscosity. The last term, the kinetic energy correction, is generally negligible for flow time for over minute. Since the density of the dilute polymer solution does not much differ from that of the solvent, the relationship between the ratio of solution viscosity (η) and solvent viscosity (η_0) and the ratio of solution flow time (t) and solvent flow time (t_0) can be written as follows:

$$\frac{\eta}{\eta_0} \approx \frac{t}{t_0} \tag{2.10}$$

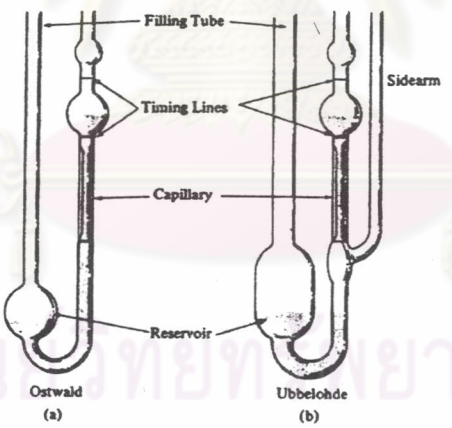

Figure 2.9 Dilute-solution viscometer (a) Ostwald (b) Ubbelohde [29].

2.5.1.2 Size exclusion chromatography (SEC) or gel permeation chromatography (GPC) [29]

Size exclusion chromatography (SEC) or gel permeation chromatography (GPC) has been established as a method for determining molecular weight averages and distributions. SEC makes use of a column, or series of columns, packed with particles of porous substrate. The term gel in gel permeation chromatography refers to

a crosslinked polymer that is swollen by the solvent used. This is perhaps the most common type of substrate. Other common substrates are porous glass beads. The column is maintained at a constant temperature, and solvent is passed through it at a constant rate. To analyze the molecular weight of the polymer, a small amount of polymer solution is injected just ahead of the column. The solvent flow carries the polymer through the column. The smaller molecules in the solution have easy access to the substrate pores and diffuse in and out the pores, following a circuitous route as they progress through the column. The large molecules simply cannot fit into the pores, and are swept more or less directly through the interstices in the packing. Thus, a separation is obtained, the largest molecules being washed through the column first, followed by successively smaller ones.

A concentration-sensitive detector is placed at the outlet of the column. The most common detector is a differential refractometer, which measures the difference in refractive index between pure solvent and the polymer solution leaving the column, a sensitive measure of polymer concentration. UV or IR detectors can also be used if the polymer has some functional groups that absorb the radiation. Regardless of the type of detector, some relative quantities proportional to the mass concentration of polymer at the column outlet are measured. An example of SEC curve is shown in Figure 2.10. A SEC curve consists of a plot of quantitative measurement versus GPC count no. which can be either volume that passed through the detector, called elution volume or time that consumed for polymer moving through the column since sample injection, called elution or retention time. For an example shown in Figure 2.10, GPC count no. may be blipped every 5 cm^3 of volume.

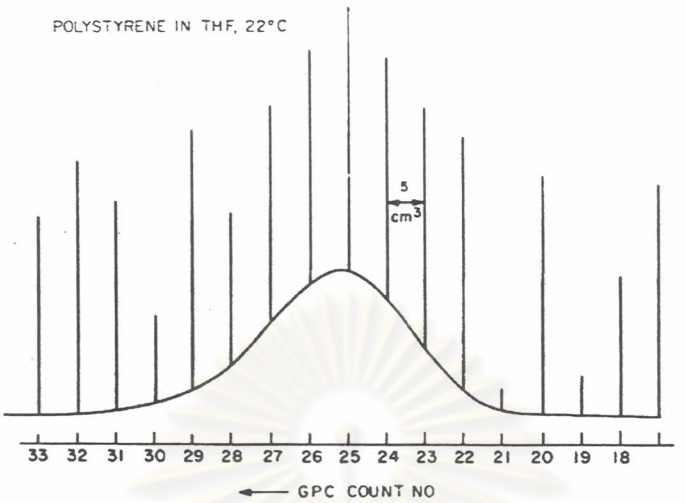

Figure 2.10 SEC curve for polystyrene in tetrahydrofuran solvent [29].

Conventional SEC is a relative method. To provide quantitative results, the relation between molecular weight and elution or retention volume must be established by the calibration using monodisperse polymer standards. A calibration curve is shown in Figure 2.11. Typically, a linear plot of log M vs. GPC count no. is obtained, but sometimes the slope turns up sharply at low v (high M).

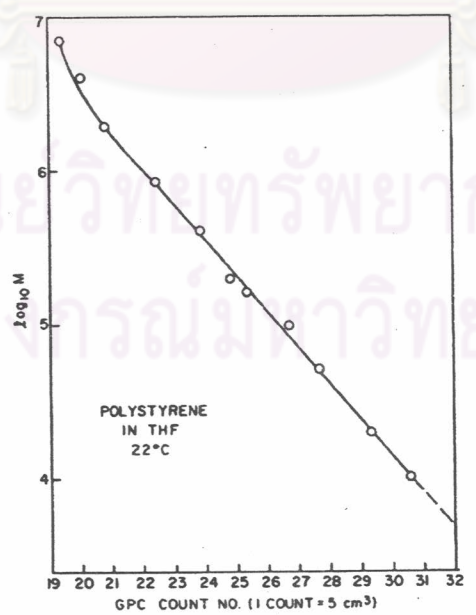

Figure 2.11 SEC calibration. Narrow-polydispersity (M_w/M_n) < 1.1 polystyrene samples in tetrahydrofuran at 22°C [29].

2.5.2 Fourier transform infrared (FT-IR) spectroscopy [31-34]

Infrared spectroscopy is a widely used technique providing the specific information of molecular level. When infrared interacts with a matter, it can be absorbed and caused the vibrations of chemical bonds consisted in the material. There are two kinds of fundamental vibrations for a molecule: stretching and bending. Stretching is the movement along the bond axis (i.e., symmetric and asymmetric types). In the contrary, bending vibrations arise from changes in bond angle between two atoms and movement of an atomic group. It relates to the remainder of the molecule. As a result, molecule adsorbs a certain energy that is equivalent to raise the vibrational energy level and additional exchange its electronic dipole moment. Absorption peaks can be noticed at the wavenumber of IR absorbed. Various stretching and bending vibrations occur at specific frequencies. Bending vibrations normally require less energy and occur at longer wavelength than those with stretching vibrations. Vibrational frequencies are not only affected by the structures of specific atomic groups, but also shifted by either greater or lesser degree by steric hindrance effects by other parts of molecules. Therefore, different molecules represent different vibration patterns due to different compositions in molecular structure.

Since molecular vibrations readily reflect chemical feature of molecule, such as a rearrangement of nuclei and chemical bonds within the molecule, infrared spectroscopy contributes considerably to not only molecular identification but also studies of the molecular structure. As a consequence, infrared spectroscopy has become a useful technique to determine molecular interactions. The infrared spectrum can be divided into three categories, i.e. the far-infrared (<400 cm^{-1}), the mid-infrared (400-4000 cm^{-1}), and the near-infrared (4000-13000 cm^{-1}) regions. Most infrared applications employ the mid-infrared region since it contains fundamental mode of vibration of molecules. It is used to establish the identity of a compound. However, the near- and far-infrared regions can also provide specific information about materials. The near-infrared regions consist mostly of overtone or combination bands of fundamental modes appearing in the mid-infrared region. The far-infrared region can provide such information regarding lattice vibrations.

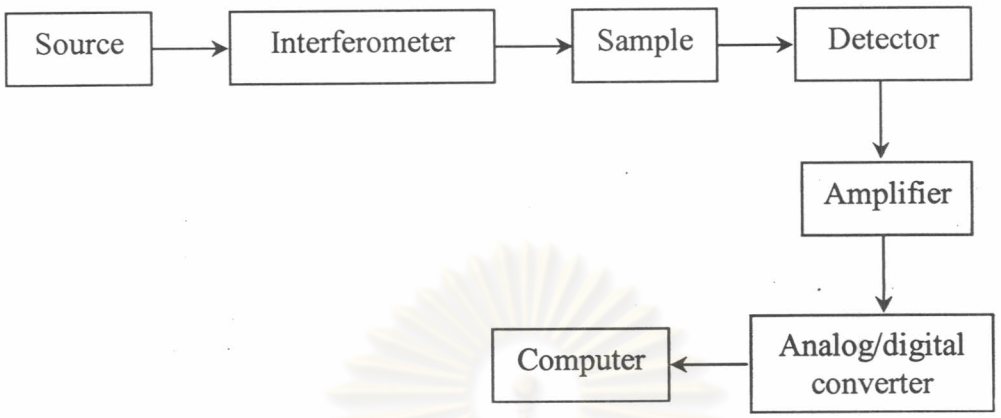

Figure 2.12 Schematic of a typical FT-IR converter spectrometer [33].

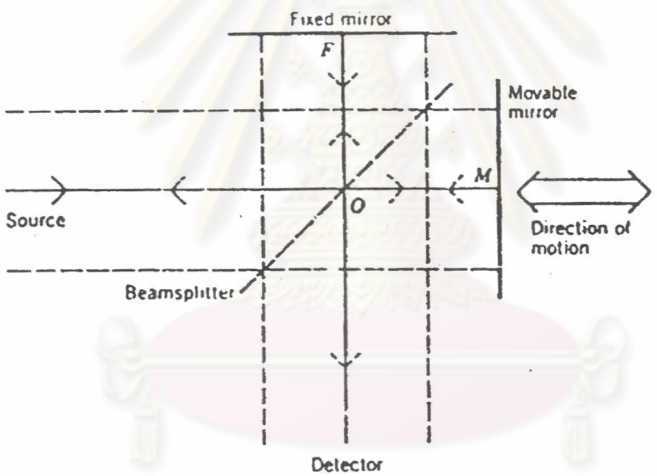

Figure 2.13 Schematic diagram of Michelson interferometer [33].

FT-IR spectrometer is based on the interference of two beams, producing an interferogram. Time and frequency domains are interconvertible by the Fourier transform method. The basic components of FT-IR spectrometer are shown schematically in Figure 2.12. The radiation generated by the IR source passes through an interferometer. Michelson interferometer is the mostly widely used instead of prisms and slits in conventional dispersive infrared spectrometer. Figure 2.13 shows the schematic diagram of a Michelson interferometer. Beam splitter separates an incident beam into two paths and then recombines them. The intensity variations of the exit beam can be measured by a detector as a function of travel path differences.

The signal produced as a function of changes between the two beams. The exit beam is absorbed by a sample before reaching a detector. Upon the signal amplification, in which high-frequency contributions have been eliminated by a filter, the data are converted into a digital from by an analog-to-digital converter. Then they are transferred to the computer for Fourier transformation. The finally FT-IR spectrum is obtained from the ratio of a single beam of the sample against that of the reference.

2.5.3 Equilibrium swelling properties [35]

Consider a polymer in contact with a solvent. Diffusion takes place in both directions, the polymer into solvent, and vice versa. However, the rate of diffusion of the solvent, being a small molecule, is much faster.

The theoretical extent of swelling is predicted by the Flory-Rehner theory on the basis of the crosslinking density and the attractive forces between the solvent and the polymer. The swelling ratio, E_s, is defined by

$$E_s = \frac{(W_s - W_d)}{W_d} \qquad (2.11)$$

where W_s is the weight of swollen sample, and W_d is the dry weight.

2.5.4 Biodegradation in lysozyme [36]

Lysozyme was first accidentally discovered by Sir Alexander Fleming in 1921. While he was suffering from a cold, a drop of nasal mucus fell onto an agar plate where large colonies of contaminant had grown and lysozyme was discovered. He made his important discovery because when he saw that the colonies of the contaminants were fading, his mind went straight to the cause of the phenomenon he was observing that the drop from the nose contained a lytic substance. It was found that nasal mucus contained a large amount of lysozyme. It was later found that tears and sputum were very potent in the lytic action, and this property was possessed by a

very large number of tissues and organs in the body. Moreover, lysozyme was widely found in animals and also in plants.

More than seventy years after Fleming's discovery of lysozyme, this enzyme is subjected to study in biochemistry and biology. When the word of lysozyme is currently used, hen egg-white lysozyme is generally meant: it is the classic representative of this enzyme family and the related enzymes are called chicken-type or conventional type lysozymes (c-type). The lysozyme of hen egg-white is remarkable in many ways. However, with the development of the studies devoted to lysozyme, it rapidly became evident that besides the c-type lysozymes other distinct types of lysozymes exist, e.g. lysozyme of birds, phages, bacteria, fungi, invertebrates and plants. These distinct types of lysozyme differ on the basis of structural, physicochemical and immunological criteria. Only the specificity of all these enzymes is the same. They cleave a β-glycosidic bond between the C-1 of N-acetylmuramic acid and the C-4 of N-acetylglucosamine of the peptidoglycan. Hence, the natural substrate for lysozyme is a high molecular weight, insoluble peptidoglycan polymer, which is a gigantic polymer of polysaccharide strands crosslinked through peptide bridges.

2.6 In vitro cell culture

2.6.1 Introduction [37]

Until animal cell culture emerged as a valuable research tool in the 1940s and 1950s, experimental approaches to the study of cellular physiology were limited to either studies in the whole animal or short term studies with isolated cells or slices from various tissues. The development of cell culture led to a new experimental approach to cellular physiology. Functionally differentiated cells could be maintained in culture under conditions that allowed direct manipulations of the environment and measurement of the resulting changes in the function of a single cell type. Today, many aspects of research and development involve the use of animal cell as *in vitro*

model systems, substrates for virus replication, and in the production of diagnostic and therapeutic products.

2.6.2 Types of cell cultures [37]

2.6.2.1 Primary cell cultures

The *in vitro* cultivation of animal tissues was first shown to be possible in 1907 and the first human cell line was obtained in 1952. However, it is only in the last 20 – 25 years that reproducible and reliable large scale culture of mammalian cells has been achieved. The process of initiating a culture from cells, tissues, or organs taken directly from an animal and before the first subculture is known as primary culture. Primary culture is the source of all cells in culture. There are a number of advantages and applications associated with primary culture:

- To provide issue for surgery, e.g. primary culture of skin samples in skin grafting and plastic surgery.
- To optimize drug treatment, especially anti-cancer drug treatments, using samples from each individual patient.
- To produce cells that have the potential to become cell lines. This allows animals to be replaced as a model and improves the reproducibility of experiments.

2.6.2.2 Finite life-span cell cultures

Certain primary cultures may be passaged for a finite number of population doublings before senescence occurs. The number of population doublings can vary significantly between different cell types. As high as 70 population doublings can be obtained with human diploid cell strains but usually the number of doublings is more limited in adult-derived or differentiated cell types. Normal tissues usually give cultures with finite life-span.

2.6.2.3 Continuous cell lines

After a number of subcultures, a cell line will either die or a population of cells can transform to become a continuous cell line. This occurs frequently in rodent cell cultures, but rarely in normal human cells (as opposed to cultures derived from tumors). The resulting cell lines will generally exhibit a combination of the following characteristics: smaller cell size, less adherence and more rounded, increased growth rate and higher cloning efficiency, increased tumorigenicity, a reduction in their serum dependency, variable chromosome complement, divergence from the donor phenotype, and loss of tissue-specific markers. It is not clear in all cases whether the stem line of a continuous culture pre-exists, masked by a finite population or arises during serial propagation. Lines of transformed cells can also be obtained from normal primary cell cultures by infecting them with oncogenic viruses or treating them with a carcinogenic chemical. It is very difficult to obtain a normal human cell line from a culture of normal tissue. In contrast, neoplasms from humans have been generated into many cell lines. It appears that the possession of a cancerous phenotype allows for the easier adaptation to cell culture, which may be due to the fact that cancer cells are aneuploid. There are two main sources of cell lines:

- Cell lines established in one's own laboratory, from tissue cultures.
- Cell lines obtained commercially, e.g. from the ATCC (American Type Culture Collection) or the ECACC (European Collection of Animal Cell culture).

2.6.3 Mitochondrial activity or MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) assay for cell viability [38-40]

MTT is a water soluble tetrazolium salt yielding a yellowish solution when prepared in media or salt solutions lacking phenol red. Dissolved MTT is converted to an insoluble purple formazan by cleavage of the tetrazolium ring by dehydrogenase enzyme from mitochondria. The yellow tetrazolium MTT (3-(4,5-dimethylthiazolyl-2)-2, 5-diphenyl tetrazolium bromide) is reduced by metabolically active cells (see

Figure 2.14), in part by the action of dehydrogenase enzymes, to generate reducing equivalents such as NADH and NADPH. The resulting intracellular purple formazan can be solubilized using isopropanol or other solvents such as dimethyl sulfoxide (DMSO) and the dissolved material is measured spectrophotochemically yielding absorbance as a function of concentration of converted dye.

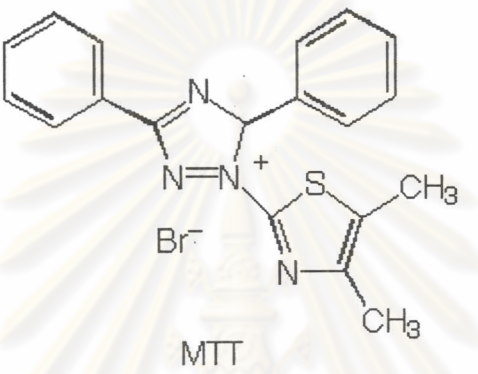

Figure 2.14 Molecular structure of MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyl tetrazolium bromide).

The cleavage and conversion of the soluble yellow dye to the insoluble purple formazan has been used to develop as assay system alternative to the conventional H-thymidine uptake and other assays for measurement of cell proliferation. Active mitochondrial dehydrogenases of living cells will cause this conversion. Dead cells do not cause this change. This has been mainly applied to measure the cytotoxicity and the number of cells. The MTT cell proliferation assay is also used to measure the cell proliferation rate as well as cell viability. The number of assay steps has been minimized as much as possible to expedite sample processing. The MTT reagent yields low background absorbance values in the absence of cells. For each cell type the linear relationship between cell number and signal produced is established, thus allowing an accurate quantification of changes in the rate of cell proliferation.