CHAPTER II

RELEVANT THEORY

2.1 Skin

2.1.1 Skin biology and structure [2],[3]

Skin covers the external surface of the body. It is the largest organ of human body, about one-sixth of total body weight. It consists of two main layers which are epidermis and dermis. The basal layer of epidermis is located adjacent to the basement membrane that comprises the dermo-epidermal junction. The layer below dermis is a layer of subcutaneous fat. Skin biology and structure are shown in Figure 2.1.

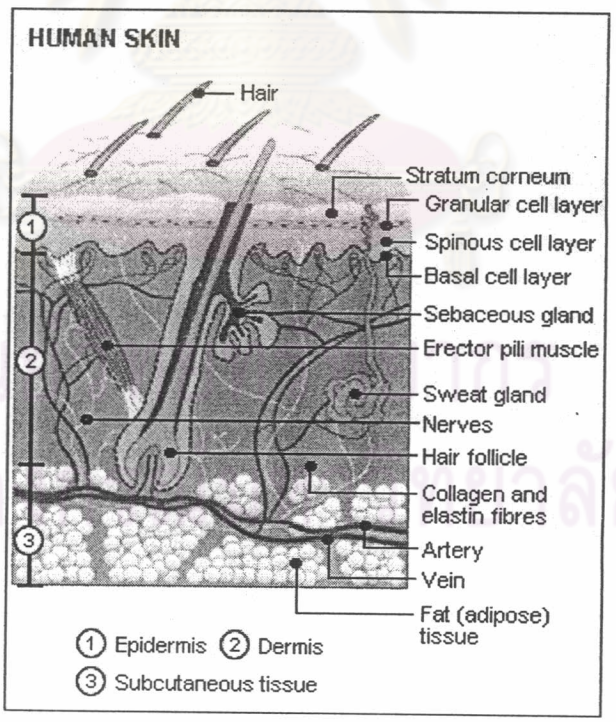


Figure 2.1: Layers & structures of the skin [4].

2.1.1.1 Epidermis

The epidermis, approximate 1 mm in thickness, is the upper layer of the skin providing the body with its first line of defence. It consists of five morphological layers, as following: *Basal cell layer*, the germinal layer; *Spinous or Prickle cell layer*, named for the prickly appearance of cells at high magnification; *Granular layer*, consisting of intracellular granules that contribute to the process of keratinization; *Stratum lucidum*, found only in extremely thick skin such as in fingertips, palms, and soles of the feet; and *Horny layer or Stratum corneum*, comprising flattened and fused cells which are ready to be removed and replaced with the below younger cells.

The epidermis can be represented as a cell maturation gradient along which cells move continuously. Cells called keratinocyte in the basal cell layer are continuously dividing to produce new skin tissue and moving along the layers toward the outside, and finally pushed upward by the dividing cells below them. A process of cell movement with a period of 25-50 days is established along the maturation gradient. Stabilization of the epidermis is afforded by a meshwork of filaments inside the cytoplasm that serves to anchor neighboring cells to each other. The result is a mechanically stable keratinizing epithelium that withstands the constant abrasion, desiccation and the variety of biological and physico-chemical assaults which the body is continuously exposed to. In addition, substantial mechanical stabilization is supplied to the epidermis by the underlying thick dermis, which is below the basal cell layer. Besides providing the mechanical stabilization, blood vessels in the dermis supply nutrients to support the active growth of new keratinocyte cells.

2.1.1.2 Dermis

The dermis is the inner layer of skin and consists of two layers, papillary and reticular layers. The papillary layer is the top layer of the dermis that attached to the epidermis. It comprises relatively thin loosely packed collagen fibers and sensory nerves that end at the epidermis. The main bulk of the dermis is the reticular layer. It comprises highly interacting collagen fibers that are thicker and more closely packed than those in the papillary layer. The collagen and elastin fibers provide strength and

flexibility to the skin. The dermis is the living layer that acts as a substrate and a support network for the epidermis. It is differentiated into various components such as sebaceous glands, sweat glands, nerves and hair follicles. Here are some details of these components that enhance the function of skin.

Blood vessels supply nutrients to the dividing cells in the basal layer and remove any waste products. They also help maintaining body temperature by carrying more blood when the body needs to lose heat from its surface. They narrow and carry less blood when the body needs to limit the amount of heat lost at its surface.

Specialized nerves in the dermis detect heat, cold, pain, pressure and touch and relay this information to the brain.

Hair follicles are embedded in the dermis and occur all over the body, except on the soles, palms and lips. Each hair follicle has a layer of cells at its base that continuously divides, pushing overlying cells upwards inside the follicle. These cells become keratinized and die, like the cells in the epidermis.

A sebaceous gland opens into each hair follicle and produces sebum, a lubricant for the hair and skin that helps repelling water, damaging chemicals and microorganisms.

Sweat glands occur on all skin areas. When the body needs to lose heat, these glands produce sweat which is a mixture of water, salts and some waste material such as urea. Sweat moves to the surface of skin via the sweat duct, and the evaporation of water from the skin has a cooling effect on the body.

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. Fibroblast, which is the essential dermal cell, is responsible for the production and maintenance of the structural elements of skin, including collagen and elastin combined with non-fibrous substances such as glycosaminoglycans (GAGs) to form an extracellular matrix (ECM). The ECM supports the basement membrane, ensuring the integrity of the dermo-epidermal junction. Also, organized tissue renewal depends on the ECM. For collagen, its turnover is normally low but it occurs at a higher rate during damage repair. Furthermore, 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, the result of healing is scar tissue.

2.1.1.3 Subcutaneous fat

The innermost layer of the skin is the layer of subcutaneous fat, and its thickness varies in different regions of the body. The fat stored in this layer represents an energy source for the body and helps to insulate the body against changes in the outside temperature.

2.1.2 Function of skin

2.1.2.1 Function of epidermis

The epidermis protects the organism against dehydration and acts as a physical barrier against invasion by microorganisms. It also protects against diverse insults, including those of mechanical, thermal, chemical, and ultraviolet origin.

2.1.2.2 Function of dermis

The dermis supports the epidermis in two vital ways. First, it provides a tough base that can repeatedly absorb substantial mechanical forces of various types, including shear, tensile, and compressive forces. Second, it incorporates a rich vascular system that is required for the metabolic support of the avascular epidermis. The blood supply of the dermis becomes intimately available to the epidermis at the papillary layer. In addition, the dermis is the largest sensory organ in the body and contains receptors for touch, pressure, pain, and temperature. It also thermoregulates the body by the presence of hair and subcutaneous fat. Heat loss is facilitated by the evaporation of sweat from the skin surface and by an increase in blood flow through the rich vascular network of the dermis.

2.1.3 Regeneration of skin

Epidermis, the regenerative tissue, is spontaneously synthesized by keratinocytes. In contrast, the dermis does not spontaneously regenerate. This can be observed in the response to a severe injury, such as full-thickness wound. The resulting wound closes spontaneously by contraction of edges and synthesis of epithelialized scar. The epidermis of scar is thinner and there are few undulations in

basement membrane. In the subepidermal region of scar, collagen fibers with their axes oriented in a relatively random array, are absent. The connective tissue layer of scar or dermal scar is largely avascular, rarely has nerve endings, and the collagen fibers are packed tightly with their axes oriented largely in the plane of the epidermis. The characteristics of non-preferable scar tissue can be concluded as follows.

a) Fully crosslinked, having only 70% of the tensile strength of the tissue it replaces.

b) It is not fully functional and is undifferentiated.

c) Scar tissue is aesthetically disfiguring.

2.2 Wounds

2.2.1 Types of burn

Burns are classified by severity as first, second or third-degree categories [5].

2.2.1.1 First-degree burns, the results of mild to moderate sunburn, only affect the epidermis as shown in Figure 2.2. They can be healed rapidly, and generally do not require medical attention.

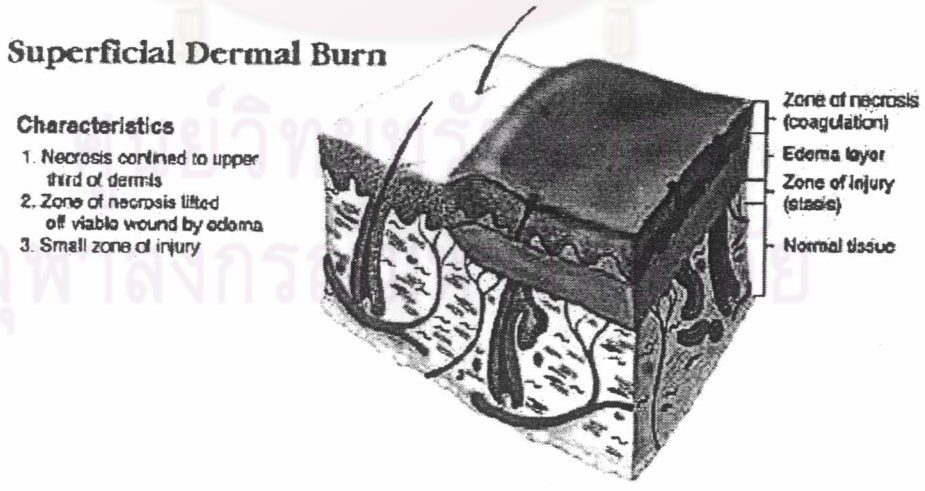


Figure 2.2: First-degree burn [6].

2.2.1.2 Second-degree burns, the results of direct contact with flames, cause damage to both the epidermis and the dermis that can be dangerous to health as shown in Figure 2.3.

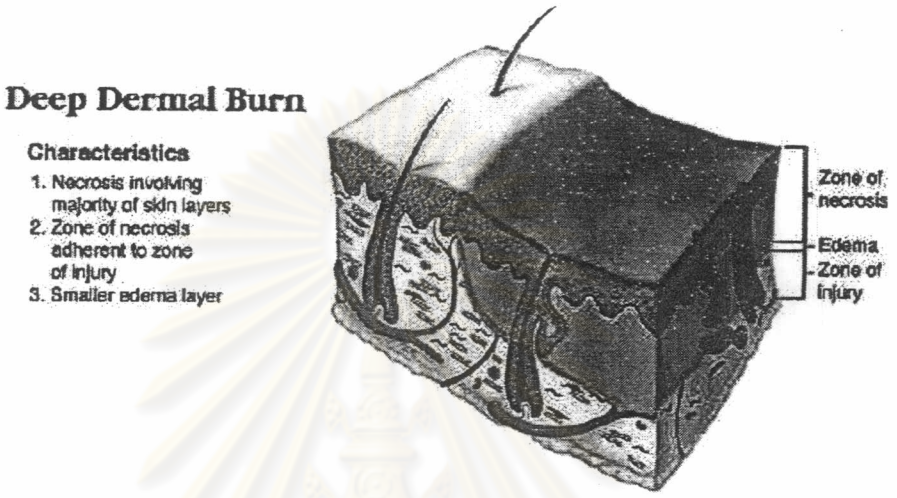


Figure 2.3: Second-degree burn [6].

2.2.1.3 Third-degree, or Full thickness burns, the results of flames or explosion with very high temperature, are burns for which the skin has been damaged or destroyed to its full depth, and may also involve damage to underlying tissues such as fat, muscle, and connective tissues as shown in Figure 2.4.

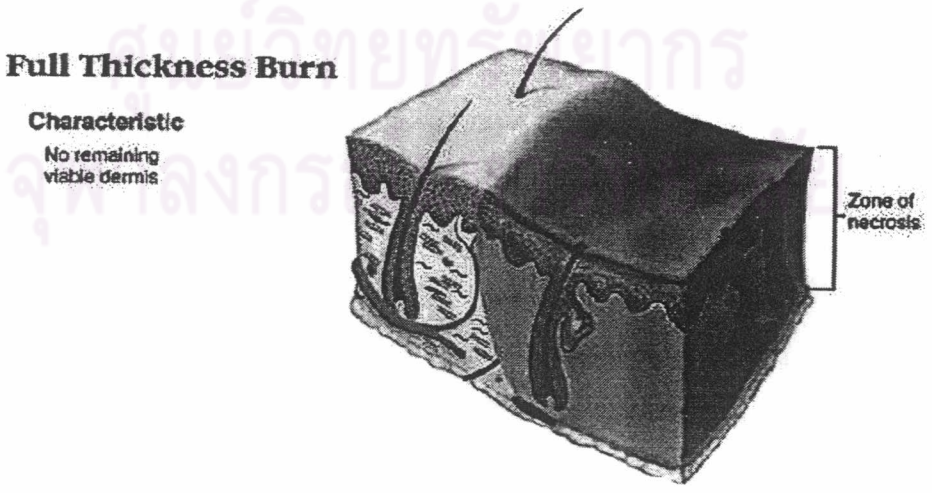


Figure 2.4: Third-degree burn [6].

2.2.2 Mechanism of spontaneous wound healing

Natural wound healing typically consists of three phases:

- Inflammation and debridement,
- Repair or fibroplasia,
- Maturation or tissue remodeling.

From Figure 2.5, there is considerable overlap among these phases.

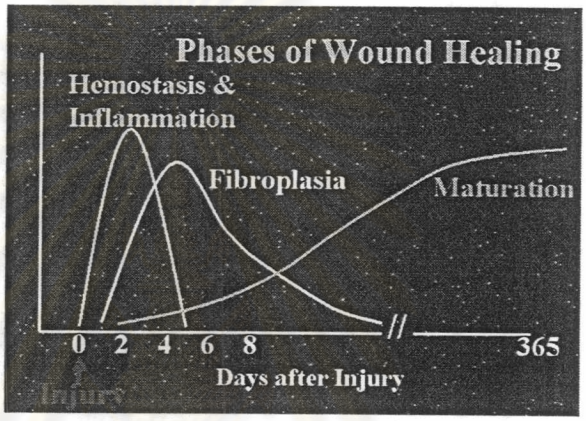


Figure 2.5: Phases of wound healing [7].

Inflammation and debridement

Immediately a wound is sustained, the body enters healing process. Platelets in the blood are activated by contacting with collagen outside the blood vessels, which causes them to adhere to each other (the blood coagulates). They also release chemicals, which encourage both vascular constrictions to stem blood flow and platelet aggregation to enlarge clot and plug the wound. White blood cells are mobilized to fight sources of infection as they enter the wound, so capillaries surrounding the wound become engorged. This engorgement has the effect of increasing the permeability of the capillary walls. Lytic enzymes remove dead tissue to speed the healing process. The length of the inflammation and debridement phase is prolonged by infection, lack of blood supply to the wound site, and the obstruction by necrotic tissue. Inflammation and debridement phases are shown in Figure 2.6.

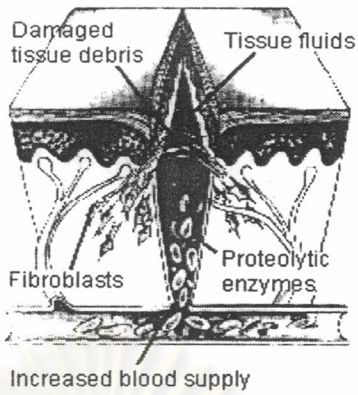


Figure 2.6: Phase I – Inflammation and debridement [7].

Repair or fibroplasia

The repair phase normally begins within twelve hours of the wound occurrence. This involves granulation, fibrification and epithelialisation. Granulation involves the formation of a scab, which acts as a scaffold for new cells to attach to. Depending upon the severity of the wound, fibrification may be required. Fibroblasts, which produce fibrous tissue, enter the wound site during fibrification. Fibrous tissue acts as a barrier against infection, and is required for wound contraction, during which intact tissue around the wound contracts to bring the sides of the wound together. Revascularization occurs to some extent during fibrification. Once a scaffold is in place, epithelialisation can take place: the migration and multiplication of cells to form new tissue. The presence of infection, too much granulation, and insufficient blood supply are all factors that protract the repair phase. Repair or fibroplasias phase is shown in Figure 2.7.

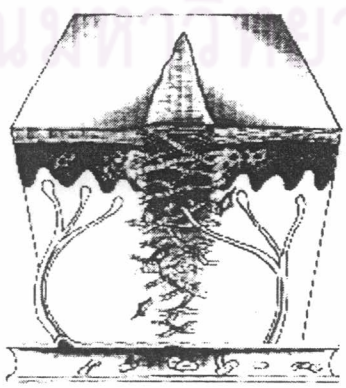


Figure 2.7: Phase II – Repair or fibroplasia [7].

Maturation or tissue remodeling

Depending upon the severity of the wound, maturation takes between 14 days and 12 months to complete. The fibroblast concentration at the wound site is reduced, and fibroclasps enter the site. Fibroclasps produce lytic enzymes that remove the irregularly arranged collagen laid down during the initial phases. The wound site is then strengthened through regeneration of the ECM. This is achieved by the laying down and crosslinking of correctly oriented collagen fibers by fibroblasts. Scarring occurs when the damage is extensive so that an adequate support system cannot be regenerated. The blood vessel network is not replaced. Scars typically exhibit an inverted triangular cross-section, as epidermal tissue encroaches from each side above the necrotic dermis, leaving a thin line of scar tissue visible at the surface. Maturation or tissue remodeling phase is shown in Figure 2.8.

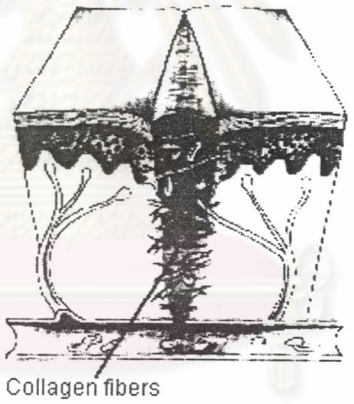


Figure 2.8: Phase III – Maturation or tissue remodeling [7].

The mechanism of wound healing is illustrated in Figure 2.9.

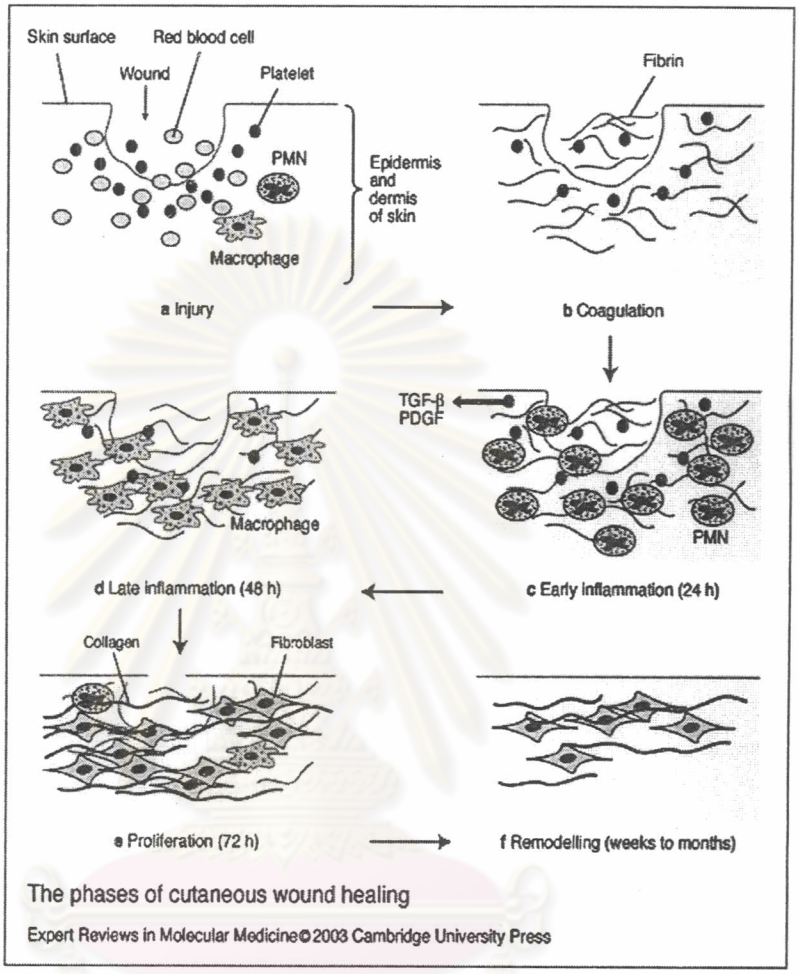


Figure 2.9: Mechanism of wound healing [8].

2.2.3 The irreversibility of wound

Fetal skin wound healing has commonly provided the most common model of scarless healing. The transition in wound healing from fetal to adult is apparently characterized by at least three major changes: increasing in the expression of the fibroblast phenotype associated with contraction of granulation tissue, decreasing levels of hyaluronic acid synthesis, as well as increasing importance of closure by contraction rather than by regeneration with increasing development. These changings bring adults to response to chronic and acute injuries by repairing the injured anatomical site. Therefore, adults always face the problems of scar formation and loss

of tissue function more than infants do. The problems can be solved by changing healing process from repair to regeneration.

Skin repair is an adaptation to the loss of normal tissue mass and leads to restoration of the interrupted continuity by synthesis of scar tissue without restoration of normal tissue. In contrast, skin regeneration restores the interrupted continuity by synthesis of the missing tissue mass at the original anatomical site. Therefore, skin regeneration is distinct from skin repair as an endpoint of a healing process following injury. Regeneration restores the normal structure and the function of the tissue while repair does not. Skin regeneration may take place unaided by the spontaneous regeneration or it may be deliberately provoked using induced regeneration template such as skin substitute.

Induced regeneration is the recovery of physiological structure and function of nonregenerative tissues such as dermis. Tissue regeneration may be induced in an adult by the use of external templates or scaffolds. Some additives such as cells and cytokines can be incorporated into scaffolds to accelerate the regenerative process. Various types of induced regeneration templates will be described in the next section.

2.3 Skin substitute

2.3.1 History of skin graft [9]

The technique of skin harvesting and transplantation was initially described approximately 2500-3000 years ago with the Hindu Tilemaker Caste, in which skin grafting was used to reconstruct noses that were amputated as a means of judicial punishment. More modern uses of skin grafting were described in the mid-to-late nineteenth century, including Reverdin's use of the pinch graft in 1869; Ollier's and Thiersch's uses of the split-thickness graft in 1872 and 1886, respectively; and Wolfe's and Krause's use of the full-thickness graft in 1875 and 1893, respectively. Today, skin grafting is commonly used in dermatologic surgery.

2.3.2 Types of skin graft [10]

Five approaches have been used to solve the problem of nonregenerative tissue.

2.3.2.1 Autografting

A mass of similar or identical tissue from the patient is surgically removed and used to treat the area of loss. The approach can be considered to be spectacularly successful until one considers the long-term cost incurred by the patient. An example is the use of sheet autograft to treat extensive areas of full-thickness skin loss. Although the patient incorporates the autograft fully with excellent recovery of function, the donor site used to harvest the autograft remains scarred. The autograft is not available in cases of burns extending over more than 30% of body surface area.

2.3.2.2 Allografting

The donor tissue is typically harvested from a cadaver. Allograft is harvested within a day after death and is cryopreserved at liquid nitrogen temperature. While allograft skin will ultimately require excision and autologous regrafting, it may adhere well to the wound for up to several weeks before clinically obvious rejection occurs. Nevertheless, although results with cadaver skin for burn coverage are good, problems with availability, expense, potential disease transmission and questions about the detrimental effects of the cryopreservation process have limited its use.

2.3.2.3 Xenografting

Xenograft skin is harvested from other animal species, most often porcine or bovine. It has also been tested extensively over the years in order to be used instead of autograft and allograft. Results are similar to those with allograft that there are the ultimate rejection and the requirement for removal within a few days to a few weeks.

2.3.2.4 Grafting of cultured cells

Cultured cells graft is based on efforts to synthesize tissues *in vitro* using autologous cells from the patient. This approach has yield so far a cultured epidermis,

a tissue which regenerates spontaneously, about 2-3 weeks after the time when the patient was injured. *In vitro* synthesis of dermis, a tissue which does not regenerate, has not been accomplished so far.

2.3.2.5 Synthetic skin substitutes

This approach is based on the discovery an analog of ECM that induces partial regeneration of the dermis in full-thickness skin wounds in adult mammals. Both synthetic and natural material can be used to produce the ECM scaffolds or synthetic skin substitutes.

2.3.3 Reasons for synthetic skin substitutes

The increasing of severe burn patients, who have insufficient sources of autologous skin for grafting, brings a high cost for hospitalizations. The annual incidence of severe burn in the USA is estimated at 70,000, the prevalence of venous leg ulcers is between 600,000 and 1,500,000, and 15-20% of people with diabetes eventually suffer a chronic foot wound. The direct cost for recovery from acute injuries range from US$36,000 to 117,000 per patient or over $5 billion per year [11].

In addition, there are many disadvantages of some traditional treatments, allograft, and xenograft. These treatments bring with them a viral infection. Furthermore, they are very expensive and time-consuming. Therefore, the synthesis of skin substitute from natural and synthetic materials, a novel method, has been developed instead of the traditional treatments.

2.3.4 Available synthetic skin substitutes [12]

2.3.4.1 Skin substitutes for wound cover

Biobrane is a bilaminate membrane consisting of nylon mesh fabric attached to a thin layer of silicone. The nylon mesh is coated with peptides derived from porcine type I collagen, in order to enhance cell attachment and growth, while the silicone serves as a semi-permeable membrane. Biobrane is recommended for use on superficial partial-thickness burns within the first 6 hours of injury, as it is best

reserved for clean wounds, also, it has been used as temporary cover for freshly excised full-thickness wounds. Healing process is expected within 10-14 days.

Transcyte is a fibriblast-seeded Biobrane so its healing properties can be improved. Because nylon is not biodegradable, this material can not act as a dermal substitute. The role for Trancyte is currently evolving to target partial-thickness burns. It adheres rapidly to a wound surface and stimulates epithelialisation. The results of healing indicate that wounds treated with Transcyte heal with less scarring.

Dermagraft is a cryopreserved living dermal structure, manufactured by cultivating fibroblasts on a polymer scaffold. The fibroblasts become confluent within the polymer mesh, secreting growth factors and dermal matrix proteins. Dermagraft facilitates healing by stimulating the ingrowth of fibrovascular tissue from the wound bed and re-epithelialisation from the wound edges. It is marketed for stimulating the healing of chronic lesion rather than for closing burn wounds.

2.3.4.2 Skin substitutes for wound closure

Alloderm is processed human cadaveric skin from which the epidermis has been removed and the cellular components of the dermis have been extracted prior to cryopreservation in order to avoid a specific immune response. Its role is as a template for dermal regeneration. It is reported to have good take rates and to reduce subsequent scarring of full-thickness wounds.

Integra is currently the most widely accepted synthetic skin substitute to be developed for use in burn patients, and was described originally by Yannas *et al* [13]. Integra, as shown in Figure 2.10, has a bilaminar structure, consisting of crosslinked bovine collagen and glycosaminoglycan, coated on one side with a silicone membrane that provides epidermal function. Following application to a freshly excised wound, the collagen layer is biointegrated with the wound to form a vascular neodermis, a process that takes approximately 3-6 weeks. Once this stage has been reached, the silicone layer can be removed and an ultra-thin split-skin graft is then applied. The disadvantage to the use of this product is that it is relatively expensive when compared with cadaveric allograft skin. The advantages are that it

provides improved elasticity and cosmesis, as well as, a fast healing with less scarring. Also, it reduces the risks of cross infection.

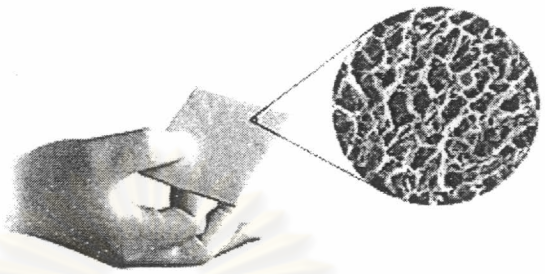


Figure 2.10: INTEGRA synthetic skin substitute [14]

2.4 Extracellular matrix scaffold as a synthetic skin substitute

2.4.1 Scaffold : Skin regeneration template [2]

A scaffold is made to mimic the structure and composition of the natural ECM in dermis, which is mainly composed of type I collagen and glycosaminoglycans (GAGs). The first skin regeneration template is called INTEGRA by Yannas, as previously mentioned.

The synthetic scaffold was designed to approximate the supporting layer of protein and carbohydrate normally secreted by dermal cells. Unexpectedly, Burke and Yannas found that when the synthetic scaffold was applied to a wound site, dermal cells around the wound site migrate into to the artificial matrix and attach to the collagen fibers. The bovine collagen is slowly degraded and replaced with authentic human collagen which synthesized by the dermal cells. Blood vessels grow into the wound to vascularize the new tissue. After the dermal layer has had a chance to repair itself, the outer membrane can be removed and replaced with very thin epidermal transplant that providing a natural moisture seal.

To have a collagen matrix that will attract dermal cells to colonize it, the matrix must have a specific surface which involves the density of attachment sites for cells. The rate at which the matrix degrades is also very important. The matrix must

persist while the inflammation rages on, but ultimately it must be degraded so that it can be replaced by authentic human collagen.

For a severe burn, the protein matrix itself is severely damaged, and the original cells in the wound have died. The burn site itself is temporarily occupied with immune system cells, like macrophages and lymphocytes, which keep infection from spreading. There are no appropriate cells left to replace the matrix correctly, and there is no matrix left to organize the tissue. Skin regeneration template solves the problem by providing a synthetic matrix, or scaffold, on which new tissue can arrange itself. Integra claims to have excellent results at healing burns, even in older patients, whose skin is already thin and brittle with age. Integra is now conducting clinical trials to expand its use to cosmetic plastic surgery to treat scarring caused by previous wounds or burns. This is a larger market than the original indication.

2.4.2 Characteristics of extracellular matrix scaffold [15]

Growing cells in three-dimensional scaffold has been of great interest. In this approach, scaffold plays an important role. It guides cells to grow, synthesizes extracellular matrix and other biological molecules, and facilitates the formation of functional tissues.

Ideally a scaffold should possess the following characteristics to bring about the desired biologic responses [16]:

a) Three-dimensional and highly porous with an interconnected pore network for cell/tissue growth and flow transport of nutrients and metabolic waste

b) Biodegradable or bioresorbable with a controllable degradation and resorption rate to match cell/tissue growth *in vitro* and/or *in vivo*

c) Suitable surface chemistry for cell attachment, proliferation and differentiation

d) Mechanical properties to match those of tissues at the site of implantation

e) Easily processed to form a variety of shapes and sizes

2.4.2.1 Pore size and curvature

Scaffold pores can be divided into two categories based on size as micro (diameter < 100μm) and macro (diameter > 100μm). For colonization of macropores to occur, the minimum pore size in which bone will form is claimed to be approximately 100μm [17]. Some works have created scaffolds with pore sizes of between 150-300μm and 500-710μm to promote bone formation [18]. The pore size employed may also be dependent on the tissue type. For example scaffolds with pore sizes less than 150μm have been successfully used for regeneration of skin in burn patients. Osteoblasts appear to exhibit greater cellular response when pore sizes of 200-400μm are employed [17]. This may be due to the curvature of the pore which may provide optimum compression and tension on the cells mechanoreceptors and allow them to migrate into the pores.

The interaction of the host tissue with the scaffold and transplanted cells can be controlled by both the geometry of the scaffold and the internal structure. The number of inflammatory cells and cellular enzyme activity around implanted polymeric scaffold has been found to depend on the geometry of the scaffold. The scaffold geometry that contains the curvature has reported to have the greatest response [10].

The pore structure of a scaffold dictates the interaction of the scaffold and transplanted cells with the host tissue. The pore structure is characterized in terms of the size, size distribution, and continuity of the individual pores within the scaffold. The pore size of a scaffold can be classified as presented in Table 2.1.

Table 2.1: Pore size of a scaffold and the penetrable molecules [10].

Type	Pore diameter	Penetrable molecules
Microporous	d < 2 nm	small molecules
Mesoporous	2 nm < d < 50 nm	small proteins
Macroporous	d > 50nm	large proteins and cells

If the pores are large enough ($d>10^4$nm), cells are capable of migrating through the pores of the scaffold. The proper design of a scaffold can allow desirable

signals to be passed to transplanted cells. Fibrovascular tissue can invade a scaffold if the pores are larger than approximately 10 μm, and the rate of invasion increases with the pore size and the total porosity of a scaffold.

It is important to realize that many materials do not have a unimodal pore size distribution or a continuous pore structure, and the ability of molecules or cells to be transported through such a scaffold will often be limited by such bottle necks in the pore structure. In addition, the pore structure of a scaffold may change over time in a biological environment.

2.4.2.2 Interconnectivity, macroporosity and microporosity

A scaffold should provide an open porous networked structure allowing for easier vascularisation, which is important for the maintenance of penetrating cells from surrounding tissues and the development of new tissue *in vivo*. The more the macroporosity the easier it is for vascularisation to occur. Chang *et al.*[17] proposed that the degree of interconnectivity has a greater influence on osteoconduction rather than the actual pore size. Interconnectivity is a physical characteristic that aids in the delivery of nutrients and removal of metabolic waste products. When the pore size used is too small, pore occlusion can occur by cells preventing further cell penetration [19]. It is essential that a scaffold possess a high degree of interconnectivity in conjunction with a suitable pore size, in order to minimize diffusion limitations and pore occlusion. The incorporation of microporosity within the scaffold material may have additional advantages with regard to nutrient delivery and cellular response.

2.4.2.3 Physical Attributes [20]

The scaffold with the following physical attributes makes it ideal for use as a matrix for soft tissue repair:

a) Flexibility

b) Tensile strength

c) Suture Retention

d) Excellent handling properties

2.4.2.4 Biological Attributes

In addition to favorable physical properties, the acellular matrix provides a biochemical and extracellular environment that contains the following biological characteristics:

a) Minimal antigenicity

b) Allows revascularization

c) Allows cellular repopulation

d) Conversion to host tissue

2.4.3 Biomaterials for scaffold fabrication

The three main material types which have been successfully used in developing scaffolds include:

a) Natural polymers, such as collagen, gelatin, glycosaminoglycan, starch, chitin and chitosan.

b) Synthetic polymers, based on polylactic acid (PLA), polyglycolic acid (PGA) and their co-polymers (PLGA).

c) Ceramics, such as hydroxyapatite(HA) and β-tricalcium phosphate (β-TCP).

Synthetic materials can be easily processed into various structures and can be produced cheaply and reproducibly. Moreover, it is possible to tightly control various properties such as the mechanical strength, hydrophobicity, and degradation rate. Natural biomaterials must be isolated from plant, animal, or human tissue. They are typically expensive and suffer from large batch-to-batch variations. Furthermore natural materials sometimes exhibit a limited range of physical properties and can be difficult to isolate and process. Although they have some limitations, there are some important advantages of using natural materials, for example, they do have specific biological activity and generally do not elicit unfavorable host tissue response. Some synthetic polymers, in contrast, can elicit a long-term inflammatory response from the host tissue. Ceramics have also been widely used, due to their high biocompatibility and resemblance to the natural inorganic component of bone and teeth [17]. Ceramics are inherently brittle so that their applicability in soft tissue engineering is limited.

Beside of the materials, fabrication techniques are also another factor that presents many challenges in obtaining specific physical and biological properties of the scaffolds. Many choices of fabrication techniques lead to get the desired scaffold properties.

2.4.4 Scaffold fabrication techniques

Several techniques have been developed to fabricate scaffolds. These include solvent casting and particulate leaching, gas foaming, fiber meshes/fiber bonding, phase separation, melt moulding, emulsion freeze drying, and freeze drying as shown in Table 2.2.

Table 2.2: Conventional scaffold processing techniques for tissue engineering[16]

Process	Advantages	Disadvantages
Solvent casting and particulate leaching	Large range of pore sizes	Limited membrane thickness (3mm)
	Independent control of porosity and pore size	Limited interconnectivity
	Crystallinity can be tailored	Residual porogens
	Highly porous structures	Poor control over internal architecture
Fibre bonding	High porosity	Limited range of polymers
		Residual solvents
		Lack of mechanical strength
Phase separation	Highly porous structures	Poor control over internal architecture
	Permits incorporation of bioactive agents	Limited range of pore sizes
Melt moulding	Independent control of porosity and pore size	High temperature required for nonamorphous polymer
	Macro shape control	Residual porogens
Membrane Lamination	Macro shape control	Lack of mechanical strength
	Independent control of porosity and pore size	Limited interconnectivity
Polymer/ceramic fibre composite foam	Independent control of porosity and pore size	Problems with residual solvent
	Superior compressive strength	Residual porogens
High-pressure processing	No organic solvents	Nonporous external surface
		Closed-pore structure
Freeze drying	Highly porous structures	Limited to small pore sizes
	High pore interconnectivity	
Hydrocarbon templating	No thickness limitation	Residual solvents
	Independent control of porosity and pore size	Residual porogens

2.4.4.1 Freeze drying technique

Freeze drying or lyophilization is an ideal method of preserving products, and active ingredients such as proteins, enzymes, microorganisms, chemical and natural products. Freeze drying is the only way that water can be successfully removed from an organic substance or material without damaging the cell structure and losing of volatile components.

Procedure and theory [21]

The first step in the lyophilization of a product is to convert the product into a frozen state. It is important that during the freezing process, the solvent or water is crystallized. The formation of ice crystals results in a separation of the solutes and the solvent. Since the concentration of the solvent is generally greater than that of the solutes, the formation of ice forces the solutes into a region between the crystals known as the interstitial. Thus the second function of the freezing process is to cause a separation of the solutes and the solvent.

The second step is sublimation. Once frozen, the product is placed under vacuum and gradually heated without melting the product. This process, called sublimation, transforms the ice directly into water vapor, without first passing through the liquid state. Because the process of sublimation occurred at atmospheric pressures is quite slow, we can increase the sublimation rate by decreasing the pressure above the ice surface. This is accomplished by placing the frozen material into a chamber and removing the gases to create a vacuum. The water vapor given off by the product in the sublimation phase condenses as ice on a collection trap, known as a condenser, within the vacuum chamber of the lyophilizer. The lyophilized product should contain 3% or less of its original moisture content and should be properly sealed

The result of these two steps is the porous scaffold. Pore size, porosity, and interconnected pore of this scaffold depend directly on concentration of solution, and freezing rate.

The key components of a lyophilizer and each component's function are along with the five significant stages of processing. Figure 2.11 summarizes the procedures of lyophilization. Examples of lyophilizers are shown in Figure 2.12.

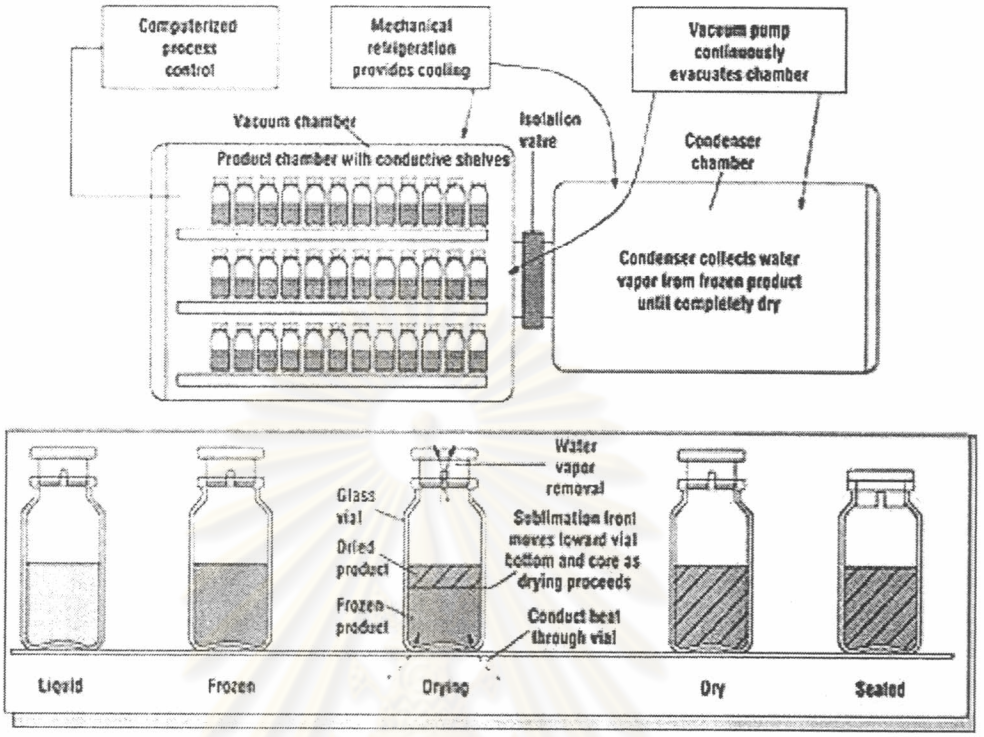


Figure 2.11: Steps of lyophilization [21]

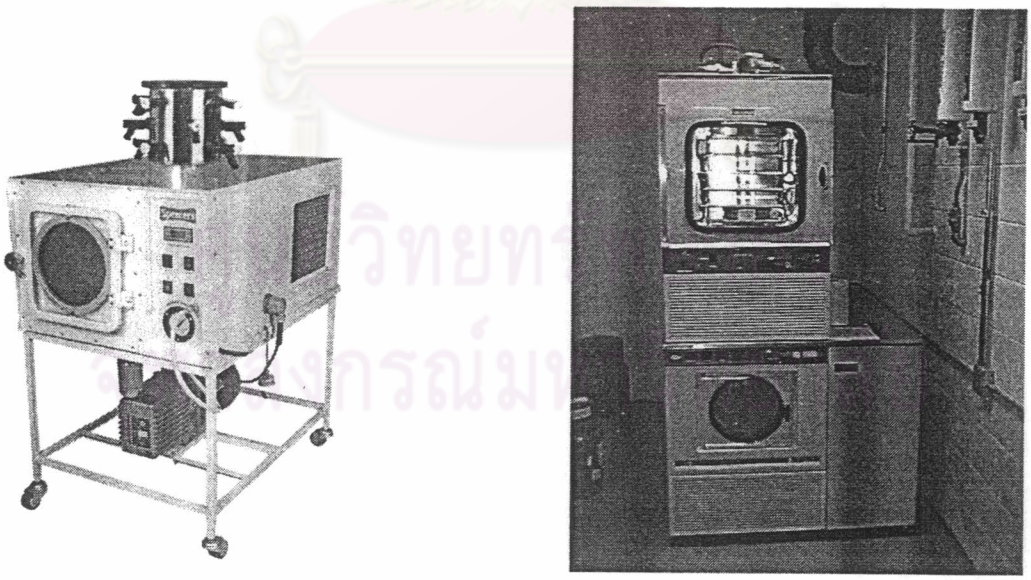


Figure 2.12: Examples of lyophilizers [22]

2.4.4.2 Crosslinking methods [23]

To enhance mechanical properties and decrease biodegradation rate, the scaffolds have to be crosslinked. Crosslinking can be done by various methods such as chemical treatment, dehydrothermal treatment, ultraviolet irradiation, and electron beam irradiation. The crosslinking density of scaffolds can be controlled by the time of treatment and UV irradiation or by the dose of electron beam irradiation.

a) Chemical crosslinking

Among chemical crosslinking agents used, glutaraldehyde is the most widely used due to its high efficiency in the stabilization of collagenous materials. Crosslinking of collagenous samples with glutaraldehyde involves the reaction of free amino groups of lysine or hydroxylysine residues of polypeptide chains with the aldehyde groups of glutaraldehyde. It is likely that glutaraldehyde can crosslink between two amino residues of collagen chains. Moreover, other chemicals such as formaldehyde and 1-ethyl-3-(3-dimethyl aminopropyl)carbodiimide (EDC) are mostly found as the crosslinking agents. The disadvantage of chemical crosslinking is that some chemical agents, which are toxic to cells, can be left over in the scaffold and can cause the irritation to patient when using the scaffolds as the skin substitutes.

b) Dehydrothermal crosslinking

The scaffold can be crosslinked by the method of dehydrothermal(DHT) treatment in a vacuum oven, as show in Figure 2.13, at temperature above 100°C. Dehydrothermal treatment generates chemical bonding between the amino and carboxyl groups of collagen molecules due to thermal dehydration. Dehydrothermal crosslinking can occur only if the amino and carboxyl groups are close to each other. Thus, it is believed that dehydrothermal treatment allows scaffolds to crosslink to a less extent than chemical treatment.

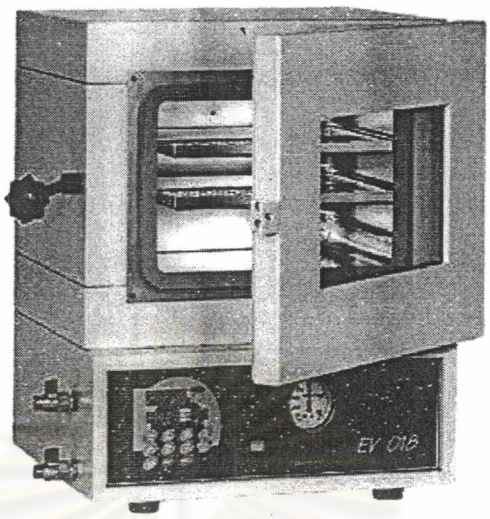


Figure 2.13: Vacuum oven for dehydrothermal treatment [24]

c) Ultraviolet irradiation

UV irradiation generates radicals at the aromatic residues of gelatin 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. When the irradiation time is short, UV irradiation will enable gelatin to crosslink intermolecularly. However, it is possible that irradiation for longer time preferably acts on the chain scission of gelatin molecules. A balance of the crosslinking and chain scission will result in unchanged density of crosslinking.

d) 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.

Comparing four crosslinking methods, chemical treatment is the best for gelatin crosslinking. Unfortunately, many chemicals like glutaraldehyde and formaldehyde are toxic to cells and sometimes change the color of scaffolds. Thus,

dehydrothermal treatment is chosen in this study, and the crosslinking density can be improved by increasing treatment time. With dehydrothermal treatment, there is no cells toxicity problem.

2.5 Biomaterials

2.5.1 Gelatin

2.5.1.1 History of gelatin [25]

Gelatin has been used in food industries for hundreds year. The earliest commercial manufacture of gelatin was in Holland in the mid 17th century. Not long after (around 1700) it also began in England. During the Napoleonic era, claims were made on the food value of gelatin. This possibly led to its manufacture in France in the late 18th century. It was first made in North America in 1808. Today, gelatin is made worldwide, and used in many industries.

2.5.1.2 The nature of gelatin

Gelatin is a protein which does not occur in nature. It is derived from collagen, a main component found in connective tissue, skin and bone of human and animals such as fish, bovine and porcine. Gelatin can be obtained by partial hydrolysis of collagen with subsequent purification, concentration, and drying operations.

Gelatin is a polypeptide, i.e. a series of amino acids joined together by peptide bonds as shown in Figure 2.14.

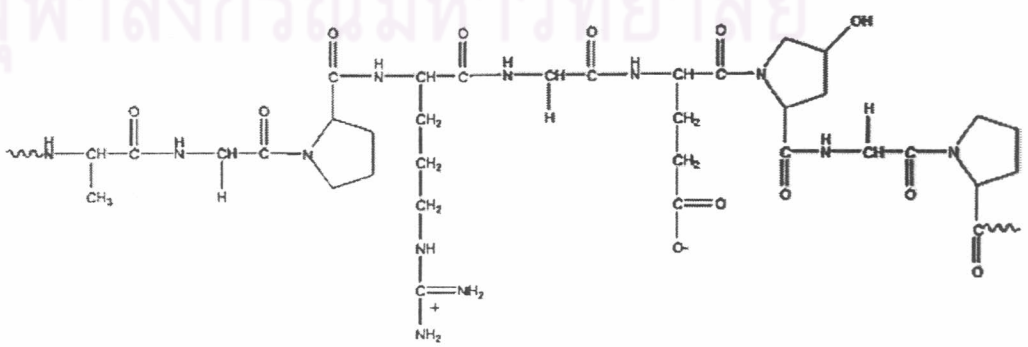


Figure 2.14: Molecular structure of gelatin [26]

2.5.1.3 Types of gelatin

Gelatin can be divided into 2 types depending on the production process.

a) Type A gelatin is produced from an acid process. This process is mainly applied to porcine skin, in which the collagen molecule is young. The isoelectric points (pI) of type A gelatin are in the range of 6-9. High gel strength (bloom strength) gelatins normally have the higher pI and the low bloom strength gelatins have a pI closer to 6.

b) Type B gelatin is formed form an alkaline process. It is mainly applied to cattle skin and bone, in which the triple helix collagen molecule is more densely crosslinked and complex. Type B gelatin has a pI value around 5.

The differences in properties and nutritional information of both gelatin types are shown in Table 2.3 and 2.4.

Table 2.3: Properties of type A and type B gelatin [27]

	Type A	**Type B**
pH	3.8 - 5.5	5.0 - 7.5
Isoelectric point (pI)	7.0 – 9.0	4.7 – 5.4
Gel strength (bloom)	50 -300	50 – 300
Viscosity (cp)	15 -75	20 – 75
Ash (%)	0.3 -2.0	0.5 – 2.0

Table 2.4: Nutritional information of type A and type B gelatin [27]

	Type A	**Type B**
Moisture (%)	10.5 +/- 1.5	10.5 +/- 1.5
Fat (%)	0	0
Carbohydrates (%)	0	0
Ash (%)	0.5 +/- 0.4	1.5 +/- 0.5

	Type A	Type B
Sodium (ppm)	500 +/- 200	3600 +/- 1400
Phosphorous (ppm)	1 +/- 0.8	-
Iron (ppm)	4 +/- 2	15 +/- 10
Lead (ppm)	0.002 +/- 0.002	0.005 +/- 0.002
Zinc (ppm)	1.5 +/- 0.5	5 +/- 3
Calcium (ppm)	90 +/- 30	900 +/- 100
Potassium (ppm)	125 +/- 50	330 +/- 50

2.5.1.4 Properties of gelatin

Gelatin is nearly tasteless and odorless. It is colorless or slightly yellow, transparent and brittle. It is soluble in hot water, glycerol, and acetic acid, and insoluble in organic solvents. Gelatin swells and absorbs 5-10 times its weight of water to form a gel in aqueous solutions at low temperature. The viscosity of the gel increases under stress (thixotrophic) and the gelation are thermally reversible. Gelatin has a unique protein structure that provides a wide range of functional properties. These proteins can form a triple helix in aqueous solution.

Gelatin is amphoteric, meaning that it is neither acidic nor alkali, depending on the nature of the solution. The pH at which charge of gelatin in solution is neutral is known as the isoelectric point (pI).

The isoelectric point (pI) of a protein is the pH at which the protein will not migrate in an electric field. This is due to the fact that at that pH the molecule carries an equality of positive and negative charges. Gelatin, is rather unique as it can have an isoelectric point anywhere between pH 9 and pH 5, depending upon the source and method of production.

The properties of gelatin from various sources can be different, for example, fish gelatin is distinguished from bovine or porcine gelatin by its low melting point, low gelation temperature, and high solution viscosity.

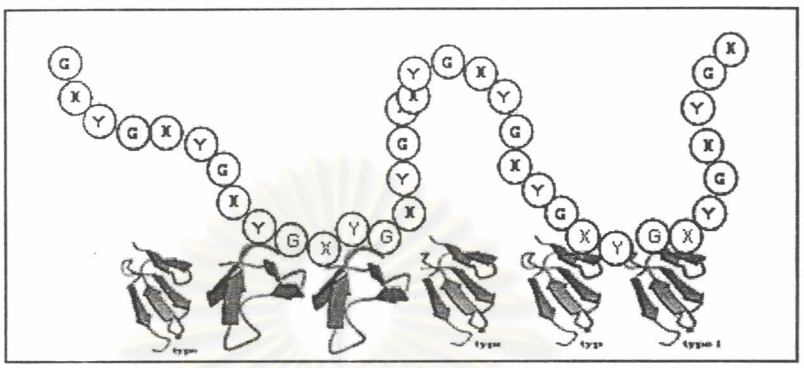
2.5.1.5 Amino acid composition

Gelatin is a heterogeneous mixture of water-soluble proteins of high molecular weight. On a dry weight basis, gelatin consists of 98 to 99% of protein. The molecular weight of these large protein structures typically ranges between 20,000 and 250,000, with some aggregates weighing in the millions. Coils of amino acids are joined together by peptide bonds. As a result, gelatin contains relatively high levels of amino acids.

Table 2.5: Amino acid composition in gelatin

Amino Acid	**%**
Alanine	8.9%
Arginine	7.8%
Asperic acid	6.0%
Glutamic acid	10.0%
Glycine	21.4%
Histidine	0.8%
Hydoxylysine	1.0%
Hydroxyproline	11.9%
Isoleucine	1.5%
Leucine	3.3%
Lycine	3.5%
Methionine	0.7%
Phenylanine	2.4%
Proline	12.4%
Serine	3.6%
Theronine	2.1%
Tyrosine	0.5%
Valine	2.2%
Total	100%

Amino acid sequence of gelatin is shown in Figure 2.15. The predominant amino acid sequence is Glycine-Proline-Hydroxyproline(Gly-Pro-Hyp).



$(Glycine\text{-}X\text{-}Y)_n$

where X is frequently Proline

Y is frequently Hydroxyproline

Figure 2.15: Amino acid sequence of gelatin [28]

2.5.1.6 Characteristics and applications of gelatin

Gelatin combines all the following characteristics in a single product:

a) Exemplary water binding characteristics
b) Excellent foaming stability
c) Viscosity modification
d) Gelation
e) Emulsifying properties
f) Binding properties
g) Elasticity
h) Film forming properties
i) Protein supplement

These characteristics enable gelatin to be used in a wide range of applications and products found both in industries and in homes. In food industry, gelatin is used in many products such as gummy bears, jellies, ice cream and yoghurts. Without gelatin, the pharmaceutical and vitamin industries would be unable to produce the modern day capsules which allow for the measured dosage and

controlled release of medicines, drugs and vitamins. Furthermore, gelatin continues to find new uses. Not only application in photographic film and paper but also application in global electronic communications. The applications of gelatin are shown in Figure 2.16.

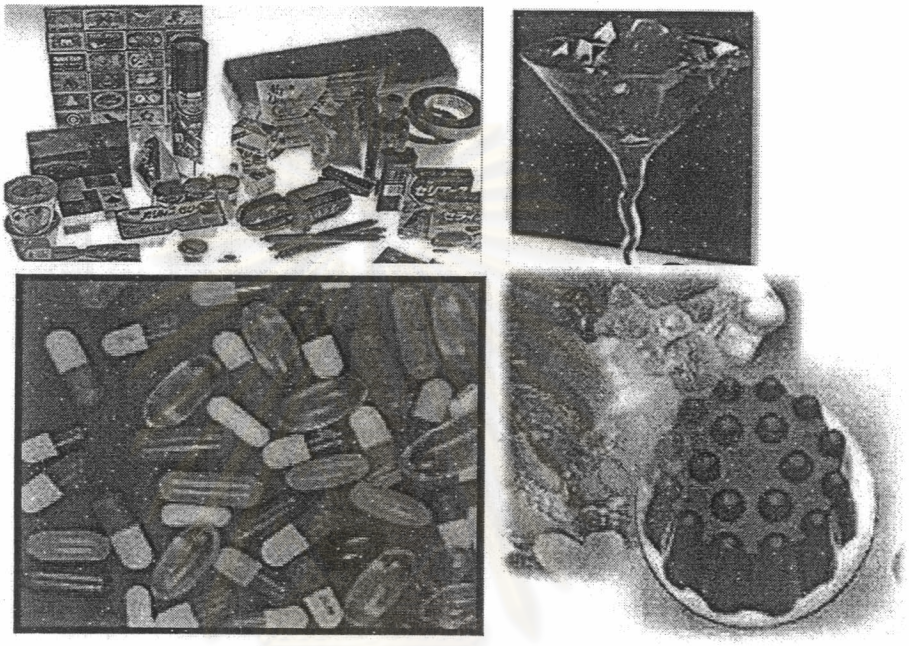


Figure 2.16: Applications of gelatin [29]

Furthermore, gelatin has become the interesting natural choice in tissue engineering application. Gelatin has many characteristics which are suitable for used as the skin substitutes:

a) Biocompatible
b) Biodegradable
c) Nonimmunogenic
d) Does not express antigenicity
e) Cheap and easy to obtain solution
f) Poor mechanical properties

2.5.2 Collagen [30]

Collagen is the most abundant protein in mammals, 25% of our total protein mass. It is found in skin, bone, teeth, tendons, cartilage, blood vessels, and connective

tissue. It is the major provider of strength and responsible for elasticity to tissues. In addition, it is the main component of ligaments and tendons. Collagen has an unusual amino acid composition, containing about one-third glycine and one-third proline and 4-hydroxyproline. The arrangement of the amino acids in the polypeptide chain gives collagen its strong secondary structure that is three left-handed polypeptide helices twisted together to form a triple helix structure called tropocollagen as shown in Figure 2.17.

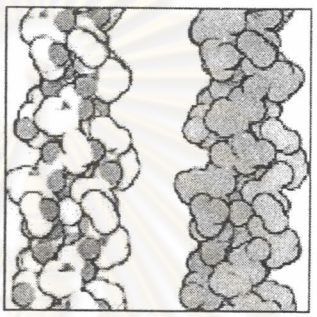


Figure 2.17: The collagen triple helix [31]

2.5.2.1 Types of collagen [32]

Collagen can be found in many parts throughout the body, and it is classified in different forms known as types:

a) Type I collagen molecule

Type I collagen molecule is secreted from cells as a soluble precursor, type I procollagen. Procollagen is processed to collagen in the extracellular spaces of connective tissues. Type I collagen itself is virtually insoluble in tissue fluids, and the molecules within a fiber are chemical crosslinked to each other. This chemical crosslinking is another important phase of collagen maturation.

The procollagen consists of three pro-α-chains. Each pro-α-chains may be considered in terms of three domains. The first domain is an amino-terminal propeptide of 139 amino acids, most of which are in a non-triplet sequence, but there is a short triplet sequence near the joint with the second domain. The second domain is the α-chains, and the third domain is a carboxy-terminal propeptide of 246 residues consisting of a non-triplet sequence.

The structure of the procollagen molecule shows the triple-helical central collagenous domain and the amino- and carboxyl-terminal propeptides. Both the N- and C-terminal regions are cleaved to form the collagen molecule. Each collagen molecule consists of three distinct long polypeptide chains, containing about 1056 amino acids called alpha(α) chains. Each of these α-chains is twisted into a left-handed helix with about three amino acids per turn. The three helical α-chains are then coiled around each other into a right-handed super-triple helix to form a rigid structure like that of a rope. The sequence of the predominant part of these chains is characteristic and consists of repeating tripeptides of glycine-X-Y where X and Y represent amino acids other than glycine. The X is often proline and the Y residue is often hydroxyproline. In the α-chains of type I collagen there are 338 glycine-X-Y triplets repeated in sequence. The additional 32 amino acids flank the long triplets sequence at each end. They are known as telopeptides. There is both an amino-terminal($-NH_2$) and a carboxy-terminal(-COOH) telopeptide.

Type I collagen molecule is a heterotrimer. Two of the α-chains are identical and the third is distinct. The molecular composition can therefore be written as $(\alpha 1)_2(\alpha 2)$. For type II collagen, the molecule is a homotrimer with three identical α-chains, each similar to the α1-chains of type I collagen.

The three α-chains in each collagen molecule are wrapped round each other to form a long rigid triple-helical molecule 297 nm in length. The short telopeptide sequences at the ends of the α-chains remain free. It is the repeating Gly-X-Y triplet in each chain that allows the three α-chains to form the triple-helical structure. Glycine is the smallest amino acid and its replacement by any other larger amino acid residue inhibits triple-helical formation. There is simply not enough space. The three chains can no longer be tightly wound round each other because they are not in the normal triple helix structure. This structure of collagen also depends critically on the presence of high amounts of proline and hydroxyproline in the α-chains. Both amino acids differ from the other α-amino acids of proteins because they are amino acids with a rigid cyclical structure, this rigid structure limiting rotation of the polypeptide chain back bone. Their presence is essential for the stability of the triple helix. Thus in type I collagen, about 100 of the X residues are proline and 100

of the Y residues are hydroxyproline. Type I collagen α-chains that lack hydroxyproline can form a triple helix at low temperature, but the triple helix falls apart at body temperature.

b) Other collagen molecules

All molecules classified as a collagen contain a structural triple-helical region similar to that which forms the predominant portion of the type I collagen molecule. However, they differ in their α-chains composition and in the length or number of helical regions. Types I, II and III collagen are closely related molecules in which there is a long uninterrupted helical domain of about 300 nm in length. These three molecules can form collagen fibers. They constitute the bulk of connective tissue collagen. For other collagen molecules, the helical domain is separated by non-helical segments. This renders the molecules highly flexible. They aggregate by end-to-end rather than by lateral association, forming open mesh-like arrays.

Types of collagen and their mostly found location in the body were presented in Table 2.6

Table 2.6: Types of collagen and location in the body

Type	Location
1. Type I collagen	Skin, tendons and the organic part of bone
2. Type II collagen	Articular cartilage
3. Type III collagen	collagen of granulation tissue
4. Type IV collagen	Basal lamina
5. Type V collagen	most interstitial tissue
6. Type VI collagen	most interstitial tissue,
7. Type VII collagen	epithelia
8. Type VIII collagen	some endothelial cells
9. Type IX collagen	cartilage
10. Type X collagen	hypertrophic and mineralizing cartilage
11. Type XI collagen	cartilage

2.5.2.2 Amino acid composition

Amino acid compositions of type I collagen from various animal sources are shown in Table 2.7. It is noted that there is a slight difference in amino acid composition of type I collagen obtained from various sources.

Table 2.7: Amino acid compositions of type I collagen from various animal species [33]

Amino acid	Residuals/1000 residues				
	Bird feet	**Bovine skin**	**Frog skin**	**Pig skin**	**Shark skin**
Alanine	57.1	40.3	34.3	34.6	29.3
Arginine	16.0	10.9	14.9	12.0	12.5
Asperic acid	6.2	2.4	2.1	2.0	1.4
Glutamic acid	46.5	18.4	12.1	9.9	13.4
Glycine	42.9	49.8	49.6	52.0	44.1
Histidine	382.1	411.8	389.0	396.8	402.7
Hydoxylysine	127.8	146.6	162.3	153.5	155.0
Hydroxyproline	1.7	2.4	4.1	3.1	4.3
Isoleucine	14.6	17.1	15.9	20.6	18.3
Leucine	8.0	12.1	11.9	10.2	20.1
Lycine	23.9	26.8	32.2	27.8	43.8
Methionine	37.3	37.1	40.3	42.8	34.3
Phenylanine	1.1	2.7	5.9	4.2	2.3
Proline	12.2	11.7	14.2	13.4	11.8
Serine	9.1	10.1	11.1	12.8	12.1
Theronine	49.3	55.2	54.1	63.6	51.1
Tyrosine	23.8	26.1	25.2	26.9	26.5
Valine	136.6	29.1	117..5	125.4	97.8

2.5.3 Chondroitin sulfate [34, 35]

Chondroitin sulfate (CS) belongs to a family of heteropolysaccharides called glycosaminoglycans or GAGs. GAGs were formerly known as mucopolysaccharides. GAGs in the form of proteoglycans comprise the ground substance in the extracellular matrix of connective tissue. CS is made up of linear repeating units containing D-galactosamine and D-glucuronic acid. The molecular weight of CS ranges from 5,000 to 50,000 daltons and contains about 15 to 150 basic units of D-galactosamine and D-glucuronic acid. It is represented by the following structural formula:

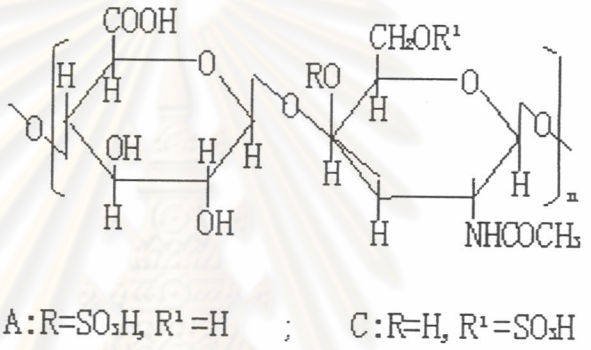


Figure 2.18: Structure of CS [36]

CS, which is found in humans in cartilage, bone, cornea, skin and the arterial wall, is sometimes referred to as chondroitin 4-sulfate. The amino group of galactosamines in the basic unit of chondroitin 4-sulfate is acetylated, yielding N-acetyl-galactosamine; there is a sulfate group esterified to the 4-position in N-acetyl-galactosamine, as demonstrated in Figure 2.19.

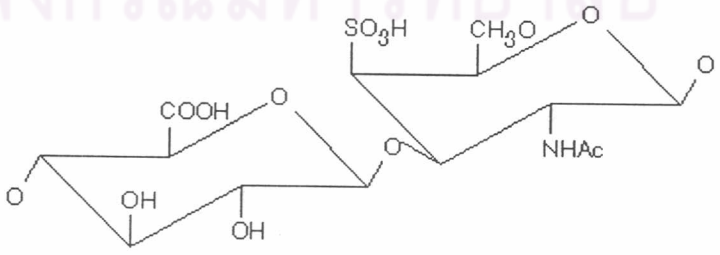


Figure 2.19: Structure of chondroitin 4-sulfate [37]

Chondroitin 6-sulfate, primarily found in fish and shark cartilage, is also made up of linear repeating units of D-galactosamine and D-glucuronic acid. The amino group of D-galactosamine is acetylated to give N-acetyl-galactosamine, and, in the case of chondroitin sulfate C, the sulfate group is esterified to the 6-position in N-acetyl-galactosamine, as demonstrated in Figure 2.20.

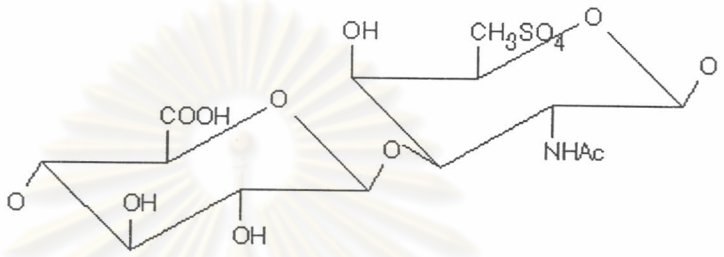


Figure 2.20: Structure of chondroitin 6-sulfate [37]

CS and hyaluronic acid are vital for the structure and function of articular cartilage. CS and hyaluronic acid are fundamental components of aggrecan found in articular cartilage. Aggrecan confers upon articular cartilage shock-absorbing properties. It does this by providing cartilage with a swelling pressure that is restrained by the tensile force of collagen fibers. This balance confers upon articular cartilage the deformable resilience vital to its function. Hyaluronic acid, which is also found in synovial fluid, has lubricating properties for the joint.

One of the earliest wound dressing based on the principles of wound healing was that of Yannas and Burke [38] which consisted of a collagen/CS scaffold. CS was included in collagen to introduce a more porous structure in the scaffolds, to increase its elastic modulus as well as to impart resistance to degradation by enzyme. Similar scaffolds were developed later by a modification of the method of Yannas by Matsuda et al [39], [40]. They reported that the addition of CS reinforced the mechanical properties of collagen but decreased cell proliferation.

2.6 *In vitro* cell culture

2.6.1 Nature of cells [41]

Two types of cell culture should be distinguished:

2.6.1.1 Primary cultures, which are obtained directly from an animal, can keep the differentiated state for a short period, days to weeks. Functionally differentiated primary cell cultures have a limited life span, and although maintenance of the differentiated properties has been improved by additives to the culture medium, components of the extracellular matrix or by different forms of co-culture, cell specific functions will eventually decline.

2.6.1.2 Permanent cultures or cell lines cultures, which have an unlimited proliferation capacity, is originated from embryos, tumors or transformed cells. Examples of cell lines are L929 mouse skin fibroblast, HeLa, 3T3, MDCK etc.

Cells can proliferate and/or differentiate, both with different limitations, depending on the cell type studied. Numerous publications provide protocols for the isolation of different cell types, their culture conditions, and for the evaluation of the degree of differentiation.

L929 is a fibroblast-like cell line cloned from strain L. The parent strain was derived from normal subartaneous areolar and adipose tissue of a male C3H/An mouse. Figure 2.18 shows L929 cells after four days incubation at 37°C.

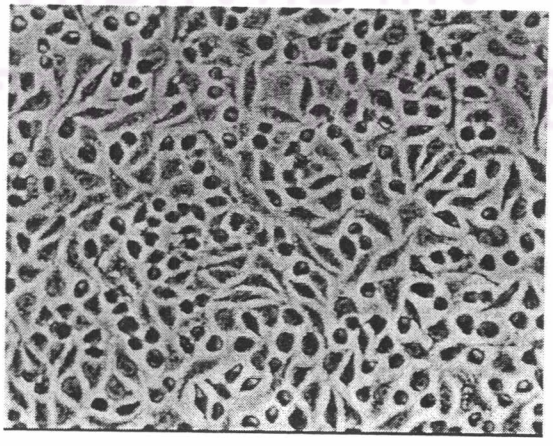


Figure 2.21: L929 cells after four days incubation at 37°C [42].

2.6.2 Cell culture techniques [43]

2.6.2.1 Work area and equipments

a) Laminar flow hoods

There are two types of laminar flow hoods; vertical and horizontal. The vertical hood, also known as a biology safety cabinet, is best for working with hazardous organisms since the aerosols that are generated in the hood are filtered out before they are released into the surrounding environment. Horizontal hoods are designed such that the air flows directly at the operator hence they are not useful for working with hazardous organisms but are the best protection for your cultures. Both types of hoods have continuous displacement of air that passes through a HEPA (high efficiency particle) filter that removes particulates from the air. In a vertical hood, the filtered air blows down from the top of the cabinet, but in a horizontal hood, the filtered air blows out at the operator in a horizontal fashion. The hoods are equipped with a short-wave UV light that can be turned on for a few min to sterilize the surfaces of the hood.

b) CO_2 Incubators

The cells are grown in an atmosphere of 5% CO_2 because the medium used is buffered with sodium bicarbonate/carbonic acid and the pH must be strictly maintained. Culture flasks should have loosened caps to allow for sufficient gas exchange. The humidity must also be maintained for those cells growing in tissue culture dishes so a pan of water is kept filled at all times.

c) Microscopes

Inverted phase contrast microscopes are used for visualizing the cells.

d) Vessels

Anchorage dependent cells require a nontoxic, biologically inert, and optically transparent surface that will allow cells to attach and allow movement for growth. The most convenient vessels are specially treated polystyrene plastic that are supplied sterile and are disposable. These include petri dishes, multi-well plates, roller

bottles, and screwcap flasks. Suspension cells are shaken, stirred, or grown in vessels identical to those used for anchorage-dependent cells.

2.6.2.2 Preservation and storage

Liquid N_2 is used to preserve tissue culture cells, either in the liquid phase (-196°C) or in the vapor phase (-156°C). Freezing can be lethal to cells due to the effects of damage by ice crystals, alterations in the concentration of electrolytes, dehydration, and changes in pH. To minimize the effects of freezing, several precautions are taken. First, a cryoprotective agent which lowers the freezing point, such as glycerol or DMSO, is added. A typical freezing medium is 90% serum, 10% DMSO. In addition, it is best to use healthy cells and to replace the medium 24 hours before freezing. Also, the cells are slowly cooled from room temperature to -80°C to allow the water to move out of the cells before it freezes. The optimal rate of cooling is 1-3°C per min. Some labs have fancy freezing chambers to regulate the freezing at the optimal rate by periodically pulsing in liquid nitrogen. Cells are stored at liquid nitrogen temperatures because the growth of ice crystals is retarded below -130°C. To maximize recovery of the cells when thawing, the cells are warmed very quickly by placing the tube directly from the liquid nitrogen container into a 37°C water bath with moderate shaking. As soon as the last ice crystal is melted, the cells are immediately diluted into prewarmed medium.

2.6.2.3 Maintenance

Cultures should be examined daily, observing the morphology, the color of the medium and the density of the cells.

a) Growth pattern

Cells will initially go through a quiescent or lag phase that depends on the cell type, the seeding density, the media components, and previous handling. The cells will then go into exponential growth where they have the highest metabolic activity. The cells will then enter into stationary phase where the number of cells is constant;

this is the characteristic of a confluent population, where all growth surfaces are covered.

b) Harvesting

Cells are harvested when the cells have reached a population density which suppresses growth. Ideally, cells are harvested when they are in a semi-confluent state. Cells that are not passaged and are allowed to grow to a confluent state can possibly lag for a long period of time and some may never recover.

- *Suspension culture*: suspension cultures are fed by dilution into fresh medium.
- *Adherent cultures*: adherent cultures that do not need to be divided can simply be fed by removing the old medium and replacing it with fresh medium.

When the cells become semi-confluent, proteolytic enzymes is used to remove the cells from the growing surface so that they can be diluted. Trypsin, collagenase, or pronase, usually in combination with EDTA, causes cells to detach from the growth surface. This method is fast and reliable but can damage the cell surface by digesting exposed cell surface proteins. The proteolysis reaction can be quickly terminated by the addition of complete medium containing serum

c) Media and growth requirements

c.1) *Physiological parameters*

- Temperature 37°C is necessary for cells.
- pH 7.2-7.5 and osmolality of medium must be maintained.
- Humidity is required.
- Visible light can have an adverse effect on cells. Light induced production of toxic compounds can occur in some media, therefore cells should be cultured in the dark and exposed to room light as little as possible.

c.2) *Medium requirements*

- Bulk ions: Na, K, Ca, Mg, Cl, P, Bicarb or CO_2
- Trace elements: iron, zinc, selenium

- Sugars: glucose is the most common
- Amino acids: 13 essential amino acids
- Vitamins: B, etc.
- Serum: contains a large number of growth promoting activities. It has undefined effects on the interaction between cells and substrate, and contains peptide hormones or hormone-like growth factors that promote healthy growth.
- Antibiotics: although not required for cell growth, antibiotics are often used to control the growth of bacterial and fungal contaminants.

c.3) *Feeding: 2-3 times/week*

c.4) *Measurement of growth and viability*

The viability of cells can be observed visually using an inverted phase contrast microscope. Live cells are phase bright; suspension cells are typically rounded and somewhat symmetrical; adherent cells will form projections when they attach to the growth surface. Viability can also be assessed using the vital dye, trypan blue, which is excluded by live cells but accumulates in dead cells. Cell numbers are determined using a hemocytometer.

2.6.2.4 Cell culture procedures

During maintaining the cells, these cells should be monitored daily for morphology and growth characteristics, fed every 2 to 3 days, and subcultured when necessary. Each time the cells are subcultured, a viable cell count should be done and the subculture dilutions should be noted. As soon as the amounts of cells are adequate, several vials should be frozen away and stored in liquid N_2. One vial from each freezing should be thawed 1-2 weeks after freezing to check for viability. These frozen stocks will be proved to be vital if any of the cell cultures become contaminated.

Procedures

1) *Media preparation*

2) *Growth and morphology*

Cells have to be visually inspected frequently. Frequent feeding is important for maintaining the pH balance of the medium and for eliminating waste products. Cells do not typically like to be too confluent so they should be subcultured when they are in a semi-confluent state.

3) *Cell feeding*

Feeding and subculturing suspension cultures are done simultaneously. About every 2-3 days, the cells must be diluted into fresh media. The dilution depends on the density of the cells and how quickly they divide. Typically 1:4 to 1:20 dilutions are appropriate for most cell lines.

4) *Subculturing adherent cells*

When adherent cells become semi-confluent, they have to be subcultured using trypsin-EDTA. The procedures are as follows.

- Remove medium from culture dish and wash cells in a balanced salt solution without Ca^{++} or Mg^{++} and remove the wash solution.
- Add enough trypsin-EDTA solution to cover the bottom of the culture vessel and then pour off the excess.
- Place culture in the 37°C incubator for 2 min.
- Monitor cells under microscope. Cells are beginning to detach when they appear rounded.
- As soon as cells are in suspension, immediately add culture medium containing serum. Wash cells once with serum containing medium and dilute as appropriate (generally 4-20 fold).

5) *Thawing frozen cells*

- Remove cells from frozen storage and quickly thaw in a 37°C water bath by gently agitating vial.
- As soon as the ice crystals melt, pipette gently into a culture flask containing prewarmed growth medium.

6) *Freezing cells*

- Harvest cells as usual and wash once with complete medium.
- Resuspend cells in complete medium and determine cell count/viability.

- Centrifuge and resuspend in ice-cold freezing medium: 90% calf serum/10% DMSO, at 106 - 107 cells/ml. Keep cells on ice.
- Transfer 1 ml aliquots to freezer vials on ice.
- Place in a container that is at room temperature and that has sufficient isopropanol.
- Place the container in the -70°C freezer overnight. Cells should be exposed to freezing medium for as little time as possible prior to freezing.
- Next day, transfer to liquid nitrogen.

7) *Viable cell counts*

Hemocytometer is used to determine total cell counts and viable cell numbers.

2.6.3 MTT cell proliferation assay [44]

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay, first described by Mosmann in 1983, is based on the ability of a mitochondrial dehydrogenase enzyme from viable cells to cleave the tetrazolium rings of the pale yellow MTT and form a dark blue formazan crystals which is largely impermeable to cell membranes, thus resulting in its accumulation within healthy cells. Solubilization of the cells by the addition of a detergent results in the liberation of the crystals which are solubilized. The number of surviving cells is directly proportional to the level of the formazan product created. The color can then be quantified using a simple colorimetric assay. The results can be read on a spectrophotometer.

Procedures [45]

1) Make a solution of 5 mg/ml MTT dissolved in DMEM without phenol red.
2) After 5 hours of the cell incubation, remove media and then wash with PBS.
3) Add MTT solution from step one to each well containing cells.
4) Incubate the plate in a CO_2 incubator at 37°C for 30 min.

5) Remove MTT solution.

6) Add DMSO to each well and pipette up and down to dissolve crystals.

7) Transfer to the cuvettes and measure the absorbance at 570nm.