CHAPTER II

THEORY AND LITERATURE REVIEW

2.1 Polycaprolactone

Polycaprolactone (PCL) is aliphatic polyester (Figure 2.1). It is a semicrystalline polymer having a melting temperature (T_m) of 60°C and a glass transition temperature (T_g) of -60°C [1]. PCL is a U.S. Food and Drug Administration (FDA)–approved bioresorbable, biocompatible polymer, and has good mechanical properties when biaxially stretched. Due to its biodegradability and biocompatibility, it has been suggested for wide biomedical applications such as tissue-engineered skin [2,3], drug delivery system [4] and scaffolds for supporting fibroblasts and osteoblasts growth [5] etc.

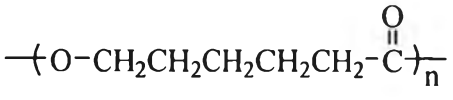


Figure 2.1 Structure of polycaprolactone

2.2 Surface Modification of Biomaterial by Introduction of Hydrophilic Group

Hydrophilicity is one of the most important factors that affect the cytocompatibility of biomaterials. The adhesion and growth of cells on a surface are considered to be strongly influenced by the balance of hydrophilicity/hydrophobicity, frequently described as wettability. Many works have demonstrated that cells prefer to attach on hydrophilic surface than on hydrophobic surface [6]. Because surface hydrophilicity plays such important roles in cytocompatibility of biomaterials, many methods such as plasma- or photo-induced grafting of hydrophilic polymers [1,7] and aminolysis [8,9] have been widely used to introduce hydrophilic groups onto the hydrophobic synthetic biomaterials.

2.2.1 Graft Copolymerization [10]

Graft copolymerization, by analogy to the botanical term, refers to the growth of a branch of different chemical composition on the backbone of the linear macromolecule. The important of this type of polymer structures is due basically to the fact that polymer chains of different chemical structure, which are normally incompatible and form separate phase, are chemically bonded to each other.

Graft copolymerization by free radical reactions has been the most widely applied system for the formation of graft copolymers, as it provides the simplest method and can be used with the wide variety of polymers and monomers.

Irradiation has been most widely used to provide active sites for graft copolymerization. This is done with ultraviolet or visible radiation, with or without added photosensitizer, or with ionizing radiation, particularly the latter. Types of UV irradiation are listed in Table 2.1. The one that is commonly used for polymerization is the UVB having a wavelength in the range of 280-315 nm. Free radical reactions are involved in all cases.

Photochemical or photo initiations occur when radicals are produced by ultraviolet and visible light irradiation of a reaction system. In general, light absorption results in radical production by either of two pathways:

1. Some compound in the system undergoes excitation by energy absorption and subsequence decomposition into radicals.
2. Some compound undergoes excitation and excited species interacts with a second compound (by either energy transfer or redox reaction) to form radicals derived from the latter and/ or formed compound(s).

Table 2.1 Purposes of ultraviolet light [11]

Types of ultraviolet light	Purpose
UVA (315-400 nm)	Blacklight. Used for low energy UV polymerization reactions, also for fluorescent inspection purposes.
UVB (280-315 nm)	Used with UVA for polymerization and accelerated light aging materials. It is the light responsible for sun tanning.
UVC (200-280 nm)	Used for rapid surface cure of UV inks and lacquers, also for sterilization and germicidal applications.
VUV (100-200 nm)	Vacuum UV can only be used in a vacuum and therefore is of minor commercial importance.

2.2.2 Aminolysis [8]

In polyester molecules, there exist abundant ester groups (-COO-). These ester groups can be hydrolyzed to carboxylic acid under alkaline condition. In addition, it is possible that the amino groups can be introduced onto the polyester surface by a reaction with diamine, providing that one amino group reacts with the -COO- group to form a covalent amide bond, -CONH-, while the other amino group is unreacted and free to react with biomolecules as shown in Figure 2.2.

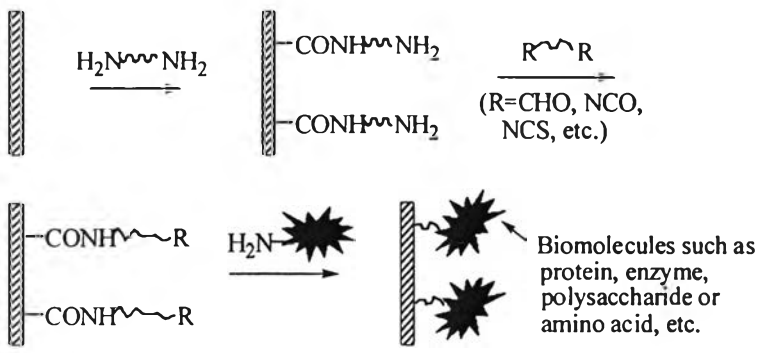


Figure 2.2 Schematic representation of aminolysis and further immobilization of biomolecules on polyester membrane

Some advantages in tissue engineering can be expected by the steady introduction of these amino groups: (1) nontoxic to cells or tissues; (2) decreasing the

surface hydrophobicity; (3) neutralizing the acid generated during the scaffold degradation and reducing the inflammation around the implanted scaffold; (4) applying to three-dimensional (3-D) porous polyester scaffolds; (5) providing active sites through which other biomolecules such as collagen, gelatin, or RGD peptides can be further immobilized, obtaining cytocompatible surface on which cells can grow well.

2.3 Surface Modification of Biomaterial by Immobilization of Biomolecules [12, 13]

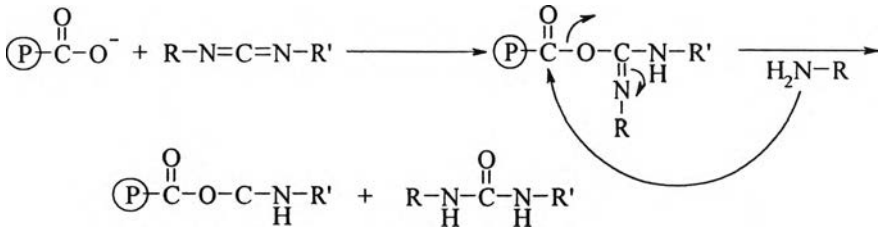
A current trend to enhance biocompatibility consists of chemical modification of the biomaterial surface by the grafting of biologically active molecules such as peptides, proteins, antibodies and biomolecules. This procedure offers the advantage of improving surface properties with respect to biocompatibility without adverse effect on the bulk properties of the system. Immobilization of such molecules can be achieved by a variety of different techniques that exploit either physical adsorption (through Van der Waals, hydrophobic, or electrostatic forces) or chemical binding. Both approaches have advantages and disadvantages. Physical adsorption processes are generally experimentally simple and often allow retention of the biomolecular activity. However, the adsorption is often reversible, with target molecules being removed by certain buffers or detergents or replaced by other molecules in solution. In contrast, chemical immobilization involves the covalent bonding (or complexation) of the target molecule to the solid phase. This method is experimentally more difficult and often exposes the molecule to a harsher environment. However, the resultant irreversible binding which can be produced with high levels of surface coverage makes this approach more popular, although in some cases chemical binding can alter the conformational structure and active center of the molecule, causing a reduction in activity. Some aspects of the physical adsorption and chemical binding are summarized in Table 2.2.

Table 2.2 Properties of physical adsorption and chemical binding

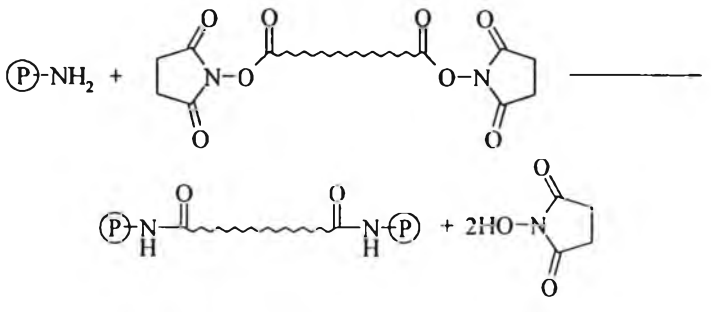
	Physical adsorption	Chemical binding
Principles	- Van der Waals forces - hydrophobic forces - electrostatic interactions	- covalent bond - complexation - coordination
Advantages	- easy to prepare under mild experimental conditions - adsorption reversible - biological activity retained	- controlled coverage - stable in physiological conditions and for multiple uses
Disadvantages	- not stable under all physiological conditions - one time use - poor reproducibility	- stringent reaction conditions - some biological activity may be lost

The concept of cross-linking and conjugation originally stems from protein and peptide chemistry. Chemical cross-linking involves joining of two molecular components by a covalent bond achieved through the use of cross-linking reagents. The components may be proteins, drugs, nucleic acids, or solid substrates. The chemical cross-linkers are bifunctional reagents containing two reactive functional groups derived from classical chemical modification agents. The reagents are capable of reacting with the side chains of the amino acids of proteins. They may be classified into homobifunctional, heterobifunctional, and zero-length crosslinkers. The zero-length crosslinkers are essentially group-activating reagents which cause the formation of a covalent bond between the components without incorporation of any extrinsic atoms. The homobifunctional reagents consist of two identical functional groups and the heterobifunctional reagents contain two different types of reactive functional moieties. Model reactions for the three kinds of crosslinkers are shown below.

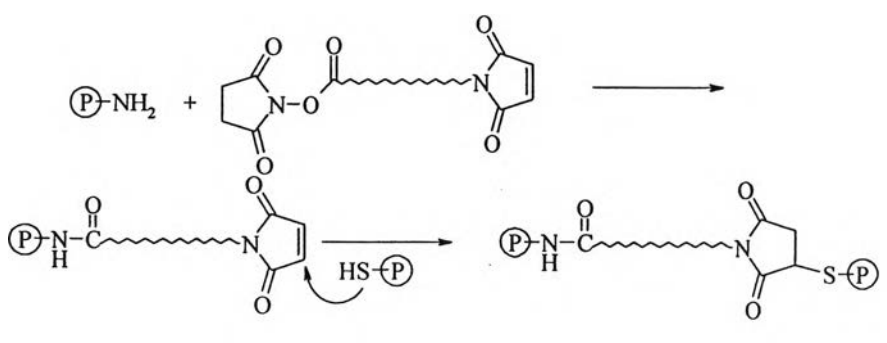
a) Zero-length crosslinkers e.g. carbodiimides



b) Homobifunctional crosslinkers e.g. bifunctional succinimidyl esters



c) Heterobifunctional crosslinkers e.g. succinimidyl ester- and maleimidyl groups



Biomolecules react *via* their amino groups with different groups on polymers, employing a two step procedure (Figure 2.3). First, the functional group on polymer surface is activated by coupling agent: polymers that contain carboxyl groups can be preactivated with a dicarbodiimide and *N*-hydroxysuccinimide (NHS) to generate an active ester, polymers containing amino groups can be preactivated with *N,N'*-disuccinimidyl carbonate (DSC) dicarbodiimide and polymers containing hydroxyl groups can be preactivated as *p*-nitropenyl carbonate. Second, the activated functional groups are coupled with biomolecules.

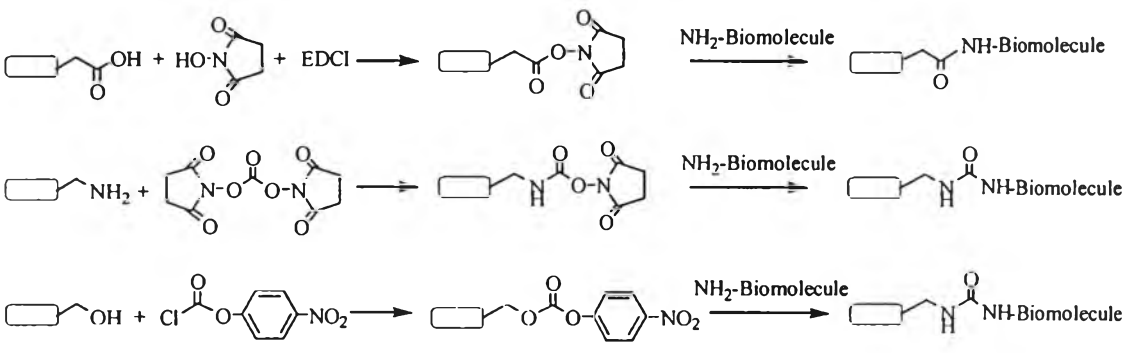


Figure 2.3 Biomolecules react *via* their amino groups with different active groups on polymers

2.4 Biomolecules

2.4.1 Chitosan

Chitosan is a deacetylated derivative of chitin, a high molecular weight, second most abundant natural biopolymer commonly found in shells of marine crustaceans and cell walls of fungi. Chitosan is a linear polysaccharide, composed of glucosamine and *N*-acetyl glucosamine linked in a β(1-4) manner; the glucosamine/*N*-acetyl glucosamine ratio being referred as the degree of deacetylation. Depending on the source and preparation procedure, its molecular weight may range from 300 to over 1000kD with a degree of deacetylation from 30% to 95%. In its crystalline form, chitosan is normally insoluble in aqueous solutions above pH 7; however, in dilute acids (pH<6.0), the protonated free amino groups on glucosamine facilitate solubility of the molecule [14].

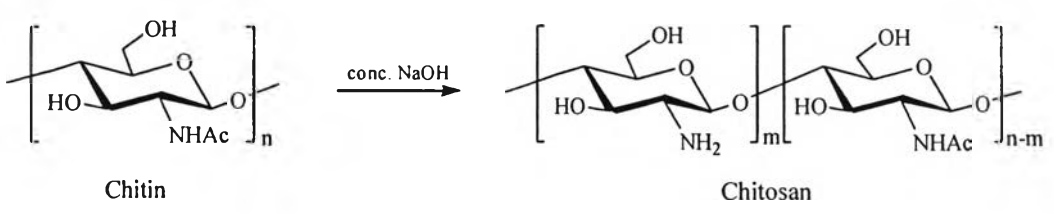


Figure 2.4 Structures of chitin and chitosan

The cationic nature of chitosan is primarily responsible for electrostatic interactions with anionic glycosaminoglycans (GAG), proteoglycans and other

negatively charged molecules. This property is of great interest because a large number of cytokines/growth factors are linked to GAG (mostly with heparin and heparin sulphate). Moreover, the presence of the *N*-acetylglucosamine moiety on chitosan also suggests related bioactivities.

2.4.2 Collagen [15,16]

The collagens are a large family of proteins, containing at least 19 different members, found in all muticellular animals. They are secreted by connective tissue cells, as well as by a variety of another cell types. As a major component of skin and bone, they are the most abundant proteins in mammals, constituting 25% of the total protein mass in these animals.

The primary feature of a typical collagen molecule is its long, stiff, triple-stranded helical structure, in which three collagen polypeptide chains, called *α chains*, are wound around one another in a ropelike superhelix (Figure 2.5). The triple helix domains of the collagens consist of repeats of the amino acid sequence Gly-X-Y. A glycine or Gly (the smallest amino acid, with a side chain consisting only of a hydrogen) is required in every third position in order for the polypeptide chains to pack together close enough to form the collagen triple helix. Proline is frequently found in the X position and hydroxyproline in the Y position; because of their ring structure, these amino acid stabilize the helical conformations of the polypeptide chains.

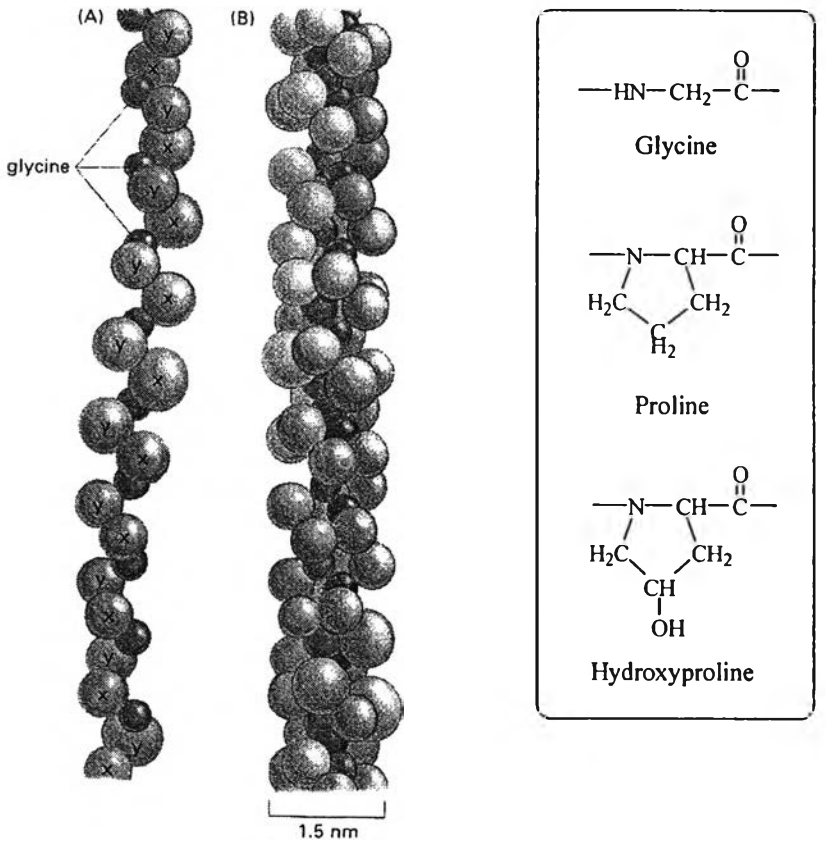


Figure 2.5 Structure of a typical collagen molecule

Despite the rather high structural diversity among the different collagen types, all members of the collagen family have one characteristic feature: a right-handed triple helix composed of three α-chains. These might be formed by three identical chains (homotrimers) as in collagens II, III, VII, VIII, X, and others or by two or more different chains (heterotrimers) as in collagen types I, IV, V, VI, IX, and XI. Each of the three α-chains within the molecule forms an extended left-handed helix with a pitch of 18 amino acids per turn. Based on their structure and supramolecular organization, they can be grouped into fibril-forming collagens, fibril-associated collagens (FACIT), network-forming collagens, anchoring fibrils, transmembrane collagens, basement membrane collagens and others with unique functions (see Table 2.3).

Table 2.3 Some types of collagen and their properties [17]

Collagen class	Type	Tissue distribution
Fibril-formation collagens	I	Bone, skin (dermis), tendon, ligaments, cornea, internal organs (90% of body collagen)
	II	Cartilage, vitreous body, nucleus pulposus
	III	Skin, blood vessel, reticular fibers of most tissues (lungs, liver, spleen, etc.)
	V	Lung, cornea, bone, fetal membrane; together with type I collagen
	XI	Cartilage, vitreous body
Basement membrane collagens	IV	Basement membranes (basal lamina)
Microfibrillar collagens	VI	Widespread: dermis, cartilage, placenta, lungs, vessel walls, intervertebral disc
Anchoring fibrils collagen	VII	Skin, dermal-epidermial junctions: oral mucosa, cervix
Hexagonal network-forming collagens	VIII	Endothelial cells
	X	Hypertrophic cartilage
FACIT collagens	IX	Cartilage, vitreous humor, cornea
	XII	Perichondrium, ligaments, tendon
	XIV	Desmis, tendon, vessel wall, placenta, lungs, liver
	XIX	Human rhabdomyosarcoma
	XX	Corneal epithelium, embryonic skin, sternal catilagr, tendon
	XXI	Blood vessel wall
Transmembrane collagens	XIII	Epidermis, hair follicle, endomysium, instestine, chondrocytes, lungs, liver
	XVII	Dermal-epidermal junctions
Multiplexins	XV	Fibroblasts, smooth muscle cells, kidney, pancreas
	XVI	Fibroblasts, amnion, keratinocytes
	XVIII	Lungs, liver

2.4.3 Immobilization of Biomolecules on Polymer

In 2002, Zhu, *et al.* [7] reported the grafting copolymerization of hydrophilic poly(methacrylic acid) (PMAA) onto PCL membrane surface under UV irradiation and the further immobilization of gelatin using 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide (EDCI) as the condensing agent. The endothelial cell (EC) culture proved that the PCL membrane slightly modified with suitable amount of PMAA or gelatin had better cytocompatibility than control PCL or PCL membrane heavily modified with PMAA or gelatin.

In the same year, Zhu, *et al.* [8] introduced amino groups on PCL surface by aminolysis of 1,6-hexamethylenediamine and further immobilized chitosan, collagen or gelatin *via* a cross-linking agent, glutaraldehyde. After biomolecule immobilization, the EC attachment and proliferation ratios were improved. The cells showed a similar morphology to those on tissue culture polystyrene and secreted von Willebrand factor (vWF) verified the endothelial function. Hence, a better EC-compatible PCL was produced.

Ma, *et al.* [18] immobilized collagen type I or gelatin on surfaces of poly-L-lactic acid (PLLA) film grafted with poly(hydroxyethyl methacrylate) (PHEMA) or poly(methacrylic acid) (PMAA) *via* photo-induced grafting. The hydroxyl groups in PLLA-*g*-PHEMA were activated with methyl sulfonyl chloride and the carboxyl groups in PLLA-*g*-PMAA were activated with EDCI before immobilization of collagen and gelatin. ATR-IR and XPS measurements confirmed the occurrence of the grafting and surface wettability of the modified films was improved.

In 2003, Wang, *et al.* [19] immobilized chitosan and heparin on surface of poly (lactic acid-*co*-glycolic acid) (PLGA) using EDCI/*N*-hydroxysuccinimide (NHS) in a 2-morpholinoethane sulfonic acid (MES) buffer system. The water contact angle of chitosan and chitosan/heparin immobilized PLGA film was greatly decreased and the element content on the surface of the films changed correspondingly. Platelet adhesion assay showed that blood compatibility of the modified film was improved while hepatocyte culture indicated that the cell compatibility of the modified film was enhanced.

In 2004, Zhu, *et al.* [20] introduced free amino groups onto the polyurethane (PU) membrane and the vascular scaffold surface through an aminolyzing reaction with 1,6-hexanediamine to immobilize gelatin, chitosan or collagen onto PU surface by employing glutaraldehyde as a coupling agent. The human umbilical vein endothelial cells (HUVECs) cell proliferation ratio of both the aminolyzed and the biomolecules-immobilized PU membranes was improved greatly comparing with the control PU. The gelatin-immobilized PU vascular scaffold had formed a monolayer of endothelial intima on its luminal surface after HUVECs were cultured for 6 d. Therefore, the aminolysis and the following biomolecule immobilization is a promising way to enhance the cell–PU interaction that can accelerate the endothelium regeneration, which is crucial for blood vessel tissue engineering.

Cheng and Teoh [1] reported the surface modification of an ultra-thin PCL film produced by biaxial stretching with collagen. PCL was firstly modified by UV-induced graft polymerization with acrylic acid and then collagen immobilization was carried out by using EDCI as a coupling agent. The hydrophilicity of the surface has improved significantly after surface modification. Human dermal fibroblasts and myoblasts attachment and proliferation were improved remarkably on the modified surface. The films showed excellent cell attachment and proliferation rate.

2.5 Skin, Wound Healing and Artificial skin [21-22]

The skin is the largest connective tissue in the body. It makes up about 10% of the total body weight.

2.5.1 Functions of the Skin

- Protection – the skin is a blockage from the external surrounding. It acts as a barrier against such things as water loss/entry, chemicals, bacteria and fungi as well as against minor trauma.
- Touch – sense organs for touch, pressure, pain and temperature.
- Regulation of the physiological surrounding – the skin helps the body maintain a normal body temperature, accumulate water, and get rid of toxic materials in the process of sweating.

- Vitamin-D synthesis in response to sun exposure
- Wound Healing

2.5.2 Layers of the Skin

The skin is composed of three main layers; epidermis, dermis and hypodermis. Apart from these layers, the basement membrane lies between the epidermis and the dermis.

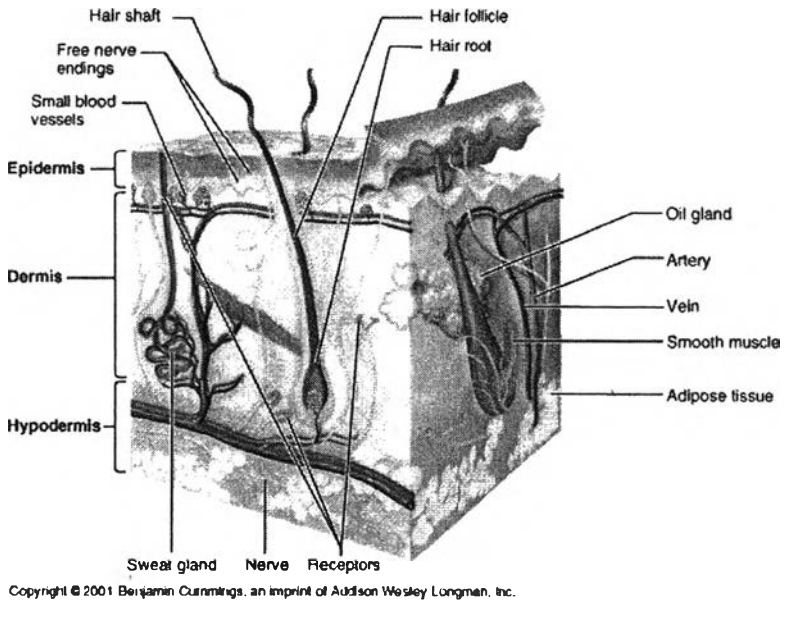


Figure 2.6 Structure of skin

1) Epidermis

The epidermis forms the external surface of the skin and is mainly composed of keratinocyte cells which differentiate to form 5 layers (Figure 2.7):

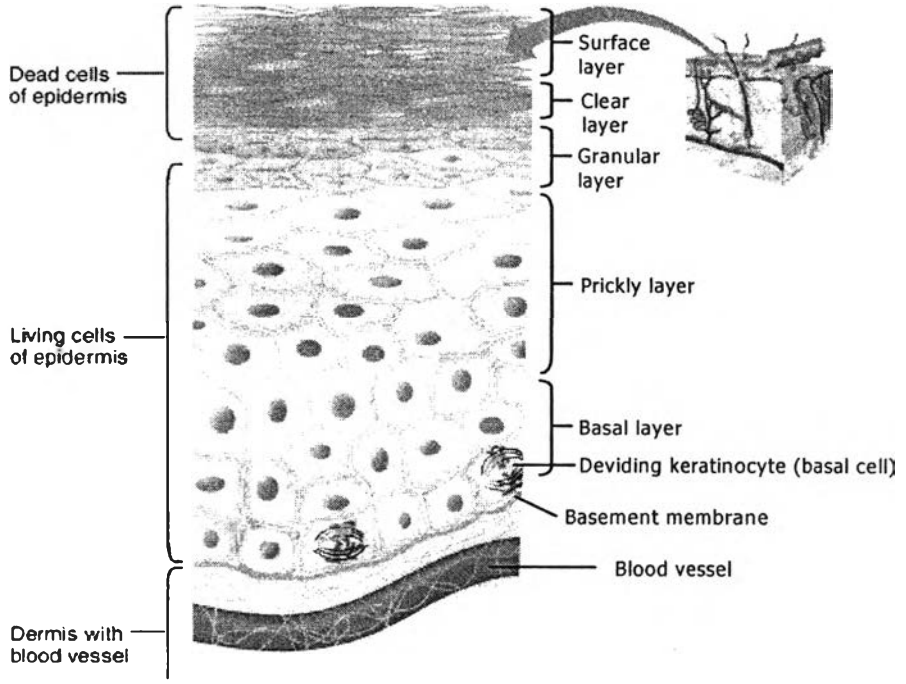


Figure 2.7 Five layers of the epidermis

1) Stratum Basale (basal layer): The basal layer, the deepest layer of the epidermis, consists of 1 cell layer of cuboidal cells attached to a thin basement membrane which separates it from the underlying dermis.
2) Stratum Spinosum (spinous or prickly layer): above the basal layer is the spinous or prickle cell layer so named because of the prickly appearance at high magnification due to the fine cell processes containing desmosomes attaching one polyhedral-shaped cell to another. Active protein synthesis occurs in this layer.
3) Stratum Granulosum (granular layer): above the spinous layer is the granular layer, in which each keratinocyte contains basophilic keratohyalin granules.
4) Stratum Lucidum (clear layer): the stratum lucidum is normally only well seen in thick epidermis and represents a transition from the stratum. granulosum to the stratum corneum.

5) Stratum Corneum (surface or cornified layer): the outer layer of the epidermis is the corneal layer which consists of fully keratinized, flat, fused cells bound together by lipids synthesized in the granular layer.

The basal or deepest epidermal cells are anchored to the basement membrane by adhesion molecules (or glue), namely fibronectin. These immature cells are continually divided and migrated toward the surface to replace lost surface cells e.g. after an injury. As the cells mature and migrate to the surface they form keratin which becomes an effective barrier to environmental hazards such as infection and to excess water evaporation. Replacement of the epidermal layer by this regenerative process takes 2-3 weeks. Cues and biologic stimuli at the wound surface are necessary to direct proper orientation and mitotic response of the epidermal cells. Many of the cues come from dermal elements, especially the matrix proteins and matrix glycosaminoglycan.

2) Basement membrane

The basement membrane technically forms part of the extracellular matrix. The basement membrane consists of two parts, the basal lamina and the reticular lamina. The basal lamina is a sheet-like structure which is mainly composed of collagen type IV, a glycoprotein called laminin and proteoglycans. The reticular lamina consists of thin reticular fibers which assist in anchoring the basal lamina to the extracellular matrix. Functionally, the main purpose of the basement membrane is to assist in binding the cell to the extracellular matrix. Other functions including acting as a filter to control the types of molecules which pass between the cell and the extracellular matrix.

3) Dermis

The dermis is separated from the epidermis by the basement membrane. It is divided into 2 layers, the papillary dermis and the reticular dermis. The papillary dermis is the major factory for the proteins providing direction for epidermal replication. The upper dermis (reticular dermis) also contains the highest blood flow. The primary cell type is the fibroblast which produces the key structural extracellular matrix proteins collagen (collagen is the predominant protein in the dermis layer,

mainly collagen Type I) and elastin as well as matrix or ground substance. In addition these cells produce the key adhesion proteins used to attach epidermal cells to the basement membrane and for used epidermal cell migration and replication. Fibronectin is a key fibroblast derived signal protein for orchestration of healing. The ground substance or matrix is made up of complex polysaccharide-protein complex known as glycosaminoglycan or the GAG component as well as hyaluronic acid. The matrix provides a semi fluid which allows for cell and connective tissue orientation as well as nutrient diffusion to the cells and a scaffold for cell migration. Macrophages, other cells in this layer, are normally present in tissue but increase in number after injury, attracted by chemical messages released by the activation of inflammation. The long lived cells release the protein chemical messages, growth factors and growth stimulants which orchestrate healing in an organized fashion.

4) Hypodermis

This layer, often called the Subcutaneous Layer, consists of loose connective tissue and fat. The ends of the sebaceous, sweat and hair glands anchor in this layer and stretch out through the dermis and epidermis.

2.5.3 Wound Healing

Wound repair is a complex process involving an integrated response by many different cell types controlled by a variety of growth factors. During the initial inflammatory phase, fibroblasts start to enter the wound where they synthesize and later remodel new extracellular matrix material, of which collagen is the main component. The dermal response is only one aspect of cutaneous wound repair however, the outermost and vital barrier layer, the epidermis which is composed of several layers of keratinocytes, must also be restored. In injured skin, basal layer keratinocytes migrate from the wound edge and from injured epidermal appendages (hair follicles and sweat glands) into the defect, moving over the newly formed dermal scaffolding. They proliferate, stratify and differentiate to produce a neoepidermis to cover the wound and restore the skin's barrier function [23].

A large variety of "polypeptide growth factors" involved in wound healing are shown in Table 2.4. Although each has a predominant function on a specific cell, all growth factors have a multitude of actions. Epidermal growth factor (EGF) is a key component for re-epithelialization of a partial-thickness burn, and addition of EGF to the wound surface increases re-epithelialization. Keratinocyte growth factor (KGF) is an important fibroblast derived stimulant for epithelialization. Macrophages are thought to be the main producers of growth factors; however, all skin cells, including fibroblasts and keratinocytes, play an important role in secreting growth factors. The initial stimulus requires the onset of wound inflammation, and once activated, further production continues until the wound is healed.

Table 2.4 Growth factors involved in wound healing

Molecule	Source	Action
Fibroblast growth factor	Keratinocytes, macrophages	Stimulates angiogenesis
Epidermal growth factor	Platelets	Stimulates epidermal cell proliferation
Keratinocyte growth factor	Fibroblasts	Stimulates epidermal cell growth
Interleukin -1	Macrophages, epidermal	Stimulates epidermal growth and motility
Platelet-derived growth factor	Platelets, macrophages	Stimulates epidermal growth, fibroblast proliferation
Transforming growth factor-B	Fibroblasts, platelets	Fibrosis and increased tensile strength

2.5.4 Artificial Skin

Artificial skin, dressing material, is the fake skin made by humans to place over an area of damaged skin (e.g. burnt skin) to start healing. Skin substitutes and dressings for burns have varied from Aloe vera gel, potato peels to sponge and artificial skin. Many natural materials which show excellent biocompatibility, biodegradability and cell adhesion, such as fibronectin, laminnin, collagen, gelatin or chitosan, [24-27] and synthetic polymer, such as polycaprolactone (PCL) [1,28-29], poly(L-lactide) [30], polyvinyl alcohol (PVA) [31], polyurethane (PU) [32], are used as biomaterials in tissue engineering to create artificial skin that can facilitate the healing of wounds.

2.6 Cell Surface and Cell Adhesion [15]

Adhesive mechanisms are grouped into two major categories: cell-cell and cell-extracellular matrix (ECM) adhesion.

2.6.1 Cell-Cell Adhesion

Three possibilities of how cells bind together are illustrated in Figure 2.8: (1) in *homophilic binding*, molecules on one cell bind to other molecules of the same kind on adjacent cells; (2) in *heterophilic binding*, the molecules on one cell bind to molecule of a different kind on adjacent cells; (3) in *linker-dependent binding*, cell-surface receptors on adjacent cells are linked to one another by secreted multivalent linker molecule. Although all of these mechanisms can operate in animals, the one that depends on an extracellular matrix linker molecule seems to be the least common.

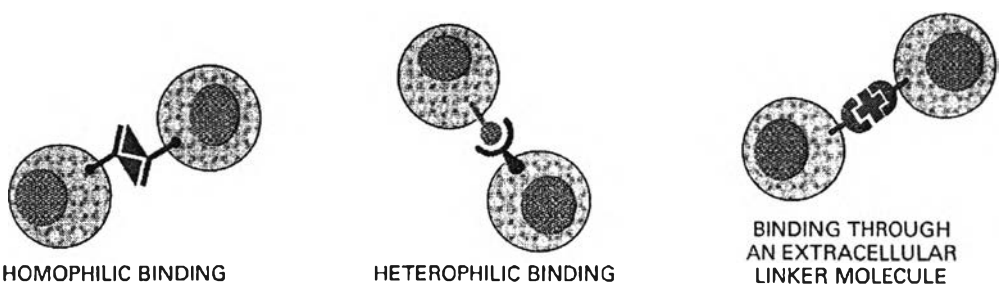


Figure 2.8 Three mechanisms by which cell-surface molecules can mediate cell-cell adhesion

2.6.2 Cell-Extracellular matrix (ECM) Adhesion

Many of cells in tissues of multicellular organisms are embedded in an extracellular matrix consisting of secreted proteins and polysaccharide. The extracellular matrix fills the spaces between cells and tissues together.

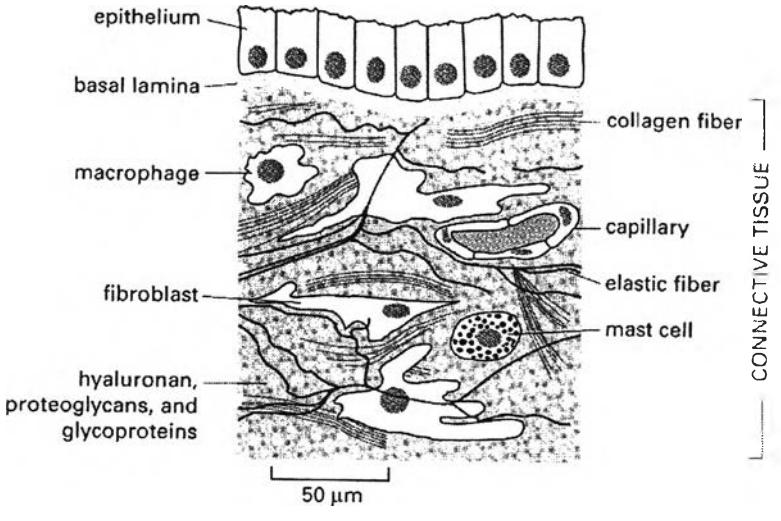


Figure 2.9 Example of extracellular matrix: the connective tissue underlying an epithelium

The extracellular matrix can influence the organization of a cell's cytoskeleton. Most cells need to attach to the extracellular matrix (cell-ECM adhesion) to grow and proliferate–and, in many cases, even to survive. This dependence of cell growth, proliferation, and survival on attachment to a substratum is known as anchorage dependence, and it is mediated mainly by integrins and the extracellular signals they generate. The integrins function as cellular receptors for many ECM glycoproteins. They provide a transmembrane linkage between the ECM and the cytoskeleton. The ECM also plays an indirect role in cell-cell adhesion by providing an intercellular "scaffold" to which cells adhere. The physical spreading of a cell on the matrix also has a strong influence on intracellular events. Cells that are forced to spread over a large surface area survive better and proliferate faster than cells that are not spread out.

2.7 Characterization Techniques

2.7.1 Attenuated Total Reflectance-Fourier Transform Infrared Spectroscopy (ATR-FTIR)

The IR beam from the spectrometer is focused onto the beveled edge of an internal reflection element (IRE) where the sample is placed in close contact with the beam is then reflected through the IRE crystal, and directed to a detector (Figure 2.10).

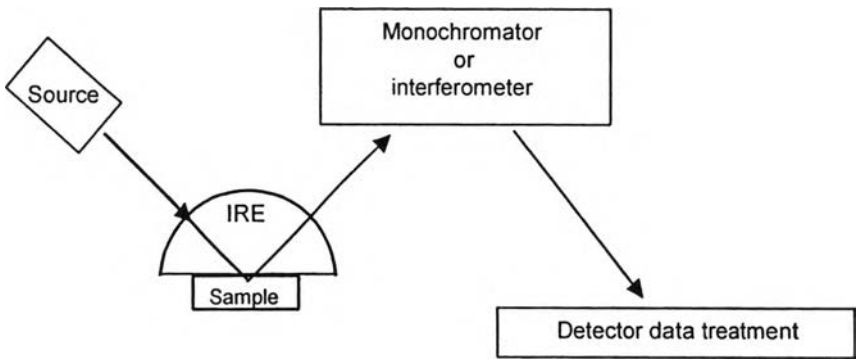


Figure 2.10 Diagram of ATR-FTIR

Typical materials used for ATR prism are Ge, Si and ZnSe. The infrared radiation can penetrate a short distance into the sample, thus interacts with any functionalities existing within that depth in the same manner as in conventional infrared spectroscopy. The depth of penetration (d_p, defined as the distance from the IRE-sample interface where the intensity of the evanescent wave decays to 1/e of its original value) can be calculated using the formula in the following equation:

$$d_p = \frac{\lambda}{2\pi n_p(\sin^2\theta - n_{sp}^2)^{1/2}} \qquad \text{.....(2.1)}$$

where λ = wavelength of the radiation in the IRE, θ = angle of incidence, n_{sp} = ratio of the refractive indices of the sample vs. IRE, and n_p = refractive index of the IRE. Sampling depth of characterization is 1-2 μm.

2.7.2 Contact Angle Measurements

Contact angle measurements are often used to assess changes in the wetting characteristics of a surface and hence indicate a change in surface energy. The technique is based on the three-phase boundary equilibrium described by Young's equation, (Figure 2.11)

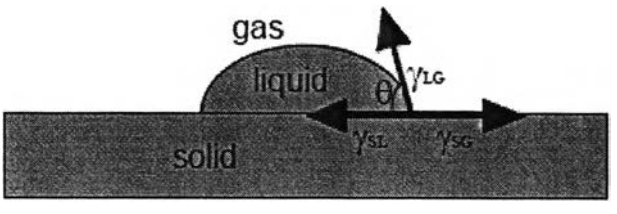


Figure 2.11 Schematic representation of the Young's equation

$$\gamma_{LG}\cos\theta = \gamma_{SG} - \gamma_{SL} \quad \text{.....(2.2)}$$

where γ_{ij} is the interfacial tension between the phases i and j, with subscripts L, G, S corresponding to liquid, gas, and solid phase, respectively and θ refers to the equilibrium contact angle.

The Young's equation applies for a perfectly homogeneous atomically flat and rigid surface and therefore supposes many simplifications. In the case of real surfaces, the contact angle value is affected by surface roughness, heterogeneity, vapor spreading pressure, and chemical contamination of the wetting liquid. Although the technique to measure contact angles is easy, data interpretation is not straightforward and the nature of different contributions to the surface is a matter of discussion. Generally, one can define the complete wetting, wetting, partial wetting, and nonwetting according to Figure 2.12.

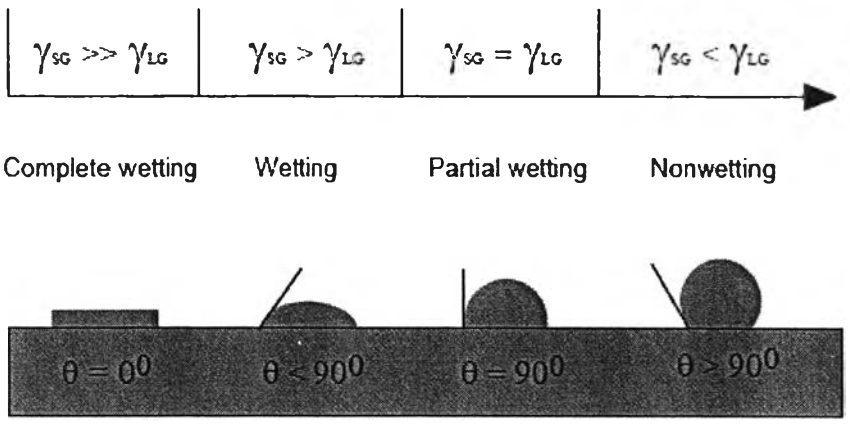


Figure 2.12 Schematic representation of wettability

2.7.3 X-ray Photoelectron Spectroscopy (XPS)

This technique is sometimes known as ESCA which is an acronym for Electron Spectroscopy for Chemical Analysis. XPS is a surface analysis method that provides information on atomic composition of the first 10 nm layer of material surface. In general, the sample is put inside a high-vacuum chamber (pressure 10^{-10} - 10^{-8} Torr), and irradiated with a beam of (monochromatic) X-rays produced by electron bombardment of an anode material (Al, Mg, Si). When the X-rays interact with the sample, core-level electrons (such as 1s, 2s, etc.) are emitted from a surface as shown in Figure 2.13. Photoelectrons are collected and analysed by the instrument to produce a spectrum of emission intensity versus electron binding energy. The binding energies, E_B, of the photoelectron are obtained via the Einstein relation:

$$E_B = h\nu - E_K - \phi \qquad \text{.....(2.3)}$$

where E_K is the measured electron kinetic energy, $h\nu$ is the energy of the exciting radiation, and ϕ is the spectrometer work function.

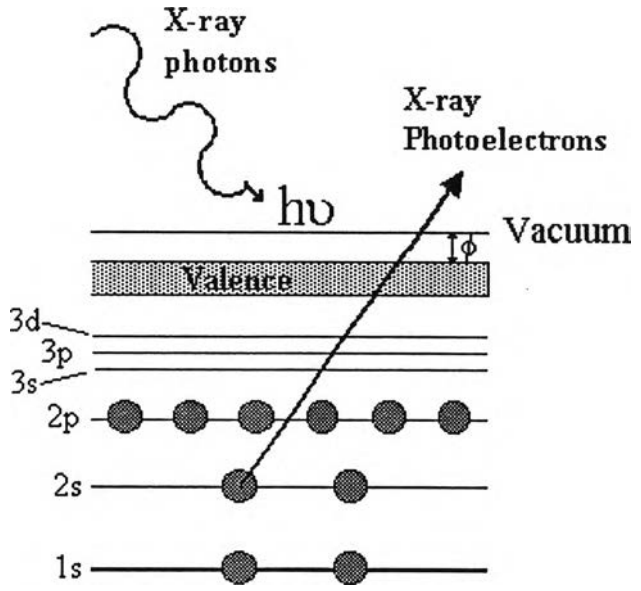


Figure 2.13 Schematic diagram of the x-ray photoelectron emission process

In general, the binding energies of the photoelectrons are characteristic of the element from which they are emanated so that the spectra can be used for surface elemental analysis.

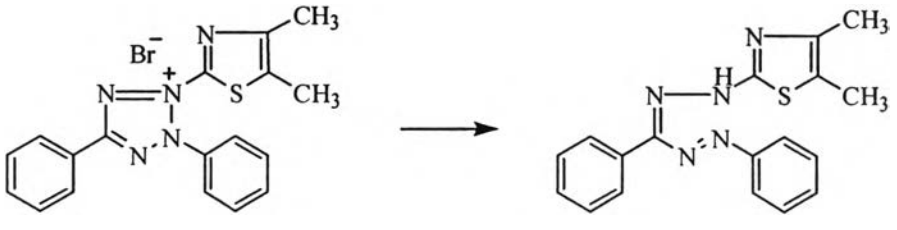
XPS can provide the following information:

1. Elemental identification. Because the number of protons increases as we progress through the periodic table, the electron binding energies for a fixed core level (such as the 1s level) will increase monotonically; thus, measuring the electron kinetic energy is equivalent to determining which elements are present on the surface.
2. Oxidation states for any given elements. There will be small shifts in the binding energies due to changes in oxidation states; higher oxidation states generally have higher binding energies, and emit electrons with lower kinetic energies.
3. Quantitative analyses through curve fitting and calculation of atomic concentrations because the photoelectron intensity is directly related to the atomic concentrations of the photoemitting atoms.
4. Depth profiling when combined with ion etching (sputtering) techniques.

5. Images or maps showing the distribution of the elements or their chemical states over the surface. Modern instruments can have a spatial resolution down to a few microns.

2.7.4 MTT Reduction Assay [33]

The MTT reduction assay is a simple, rapid, inexpensive and reliable method for estimating the percentage of viable cell, depending on the ability of metabolically active cells to reduce the tetrazolium salt (3[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, MTT) to formazan.



MTT tetrazolium salt
yellow

MTT formazan
purple, 540 nm

Figure 2.14 Reduction of the MTT tetrazolium salt to formazan

MTT is a yellow water-soluble tetrazolium salt. The dye is converted to a water-insoluble purple formazan on the reductive cleavage of its tetrazolium ring by the succinate dehydrogenase system of the active mitochondria (Figure 2.14). Thus, the amount of formazan formed can be determined spectrophotometrically and serves as an estimate of the number of mitochondria and hence the number of living cells in the sample. The MTT assay was used in many studies to evaluate the viability of different cells because this test is fast, many samples (up to 10) can be examined at the same time and many replications of each sample can be tested simultaneously.