CHAPTER II
LITERATURE REVIEW

2.1 Tissue Engineering

Tissue engineering is a technique which studies the growth of new tissues or organs from cells on the biological substitute. It involoves the study of the seeding and attachment of cells on the scaffold. The main target of tissue engineering can produce new tissues or organs which will restore, maintain or improve tissue function. Currently, biopolymer is a one way to used as a scaffold because it can be degraded and does not toxic by-products in the human body. Furthermore, the patients have not suffered from secondary surgery.

2.2 Poly(caprolactone)

Polycaprolactone (PCL) is a linear polyester and synthesized from the ring-opening polymerization of ε-caprolactone. It is one of the biodegradable polyester which is widely used in the biomedical applications. The biodegradation occurs through the hydrolysis of its aliphatic ester linkage. It is a semicrystalline polymer with a melting point (T_m) of (60°C) and a glass transition temperature (T_g) of (-60°C) (Mattanavee, et al., 2009). PCL is currently used as a part of wound dressing, degradable staple and in long-term drug delivery devices because it is regarded as a nontoxic, tissue-compatible material and slow degradation, which is approved by the US Food and Drug Administration (FDA). The drawback of PCL is hydrophobicity that is unfavorable to cell adhesion and growth (Lin & Lu, 2002). It can be copolymer with other polymers such as glycolide, lactide, δ-valerolactone, ε-decalactone and poly(ethylene oxide), or blending with other biodegradable polymers such as PHB, PLA and starch (Ewa Rudnik, 2008).

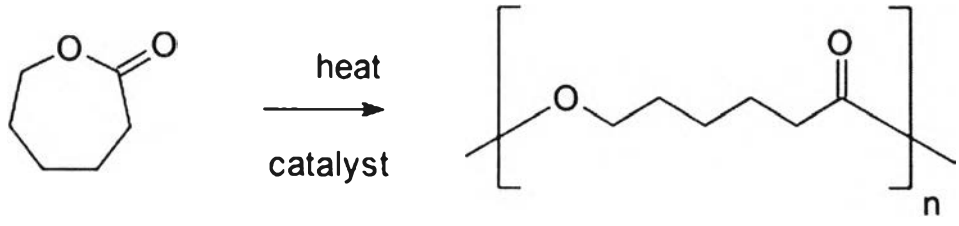

Figure 2.1 Schematic route to polycaprolactone.

2.3 Bovine Serum Albumin (BSA)

Albumin is the main protein in human blood and the key to regulating the osmotic pressure of blood. It helps move many small molecules through the blood, such as bilirubin, calcium, progesterone.

Bovine serum albumin (or called BSA) is a large globular protein with a molecular weight of 66,430 Da. BSA is a polypeptide chain that composed of 583 amino acid sequence. It is good water solubility and the most abundant protein found in plasma or circulatory system in the pH ranges from 5-7. The important property of BSA is the ability to bind to various ligands. Therefore, BSA has been used as a component of cell media to regenerate plants from cells.

2.4 Collagen

Collagen is the major insoluble fibrous protein in the extracellular matrix and connective tissue. It is a main component in skin and bone. There are at least 16 types of collagen but 80-90 percent of the collagen in the body consists of type I, II and III. These collagen molecules pack together, their structures formed as long thin fibrils and triple helix similar structure.

Each type of collagen has its characteristic amino acid sequence. Each collagen molecule consists of three individual polypeptides known as α-chains. Each α-chain has a left-handed helical secondary structure around its axis. Three α-chains are coiled around each other into a right-handed superhelix. This triple-helical

conformation is unique to collagen (Uitto *et al.* 1981). Proline and Glycine is the major amino acid that found in collagen. The ring structure of proline stabilizes the helical conformation of α chain. Glycine is the smallest amino acid, it allows three α chains pack closely together. Each individual collagen polypeptide chains are produced from ribosomes and injected to endoplasmic reticulum (ER) as pro-α chains. Each pro-α chains are H-bonded together in endoplasmic reticulum to form triple helix molecule called procollagen. Proteolytic enzyme converts procollagen into collagen molecules by the propeptide cleavage at the end of procollagen molecule outside the cell.

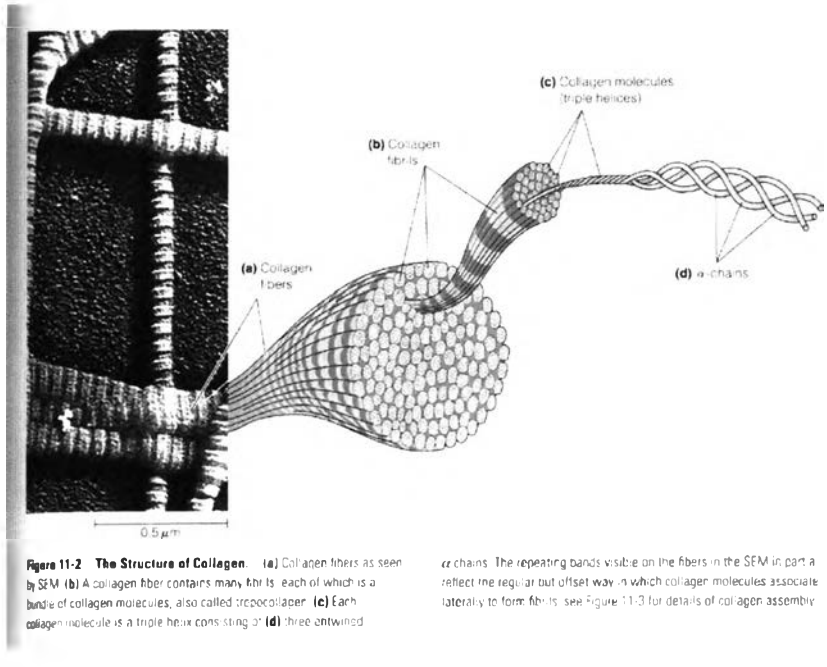

Figure 11-2 The Structure of Collagen. (a) Collagen fibers as seen by SEM. (b) A collagen fiber contains many fibrils, each of which is a bundle of collagen molecules, also called tropocollagen. (c) Each collagen molecule is a triple helix consisting of (d) three entwined α chains. The repeating bands visible on the fibers in the SEM in part a reflect the regular but offset way in which collagen molecules associate laterally to form fibrils. see Figure 11-3 for details of collagen assembly.

Figure 2.2 The structure of collagen.
(http://greatcourse.cnu.edu.cn/xbfzswx/wlkc/kcxx/4English%287565312Bytes%29.htm)

2.5 Protein

Protein is constructed from a long chain of amino acids. Each amino acid is linked to one another through a covalent bond called peptide bond. As a result, protein is also known as polypeptide. Amino acid in the chain determines the shape

of protein. There are 20 types of amino acids found in protein. Different amino acid has different properties due to different side chains.

The long chain of protein can fold and be stabilized by many weak non-covalent bonds between different parts of the chain. Types of weak bond associated with protein folding are hydrogen bonds, ionic bonds and van der waals attractions. Noncovalent bond alone is much weaker than covalent bond. However, many weak bonds can hold two region of polypeptide chain together.

Another weak force that affects the shape of protein folding is hydrophobic force. The amino acid with non-polar side chain is considered hydrophobic. When the hydrophobic molecules are in aqueous environment, they tend to stay together to minimize the effect of hydrogen bond with water molecules. Therefore, the major factor contributing to the folding of the protein is governed by the distribution of polar and non-polar amino acids. The amino acids with non-polar or hydrophobic side chains tend to cluster together to form the hydrophobic core in the interior of molecule in order to avoid the contact with the surrounded water molecule. On the other hand, the amino acids with polar or hydrophilic side chains tend to arrange themselves outside the molecules so that they can form hydrogen bond with water molecules easily.

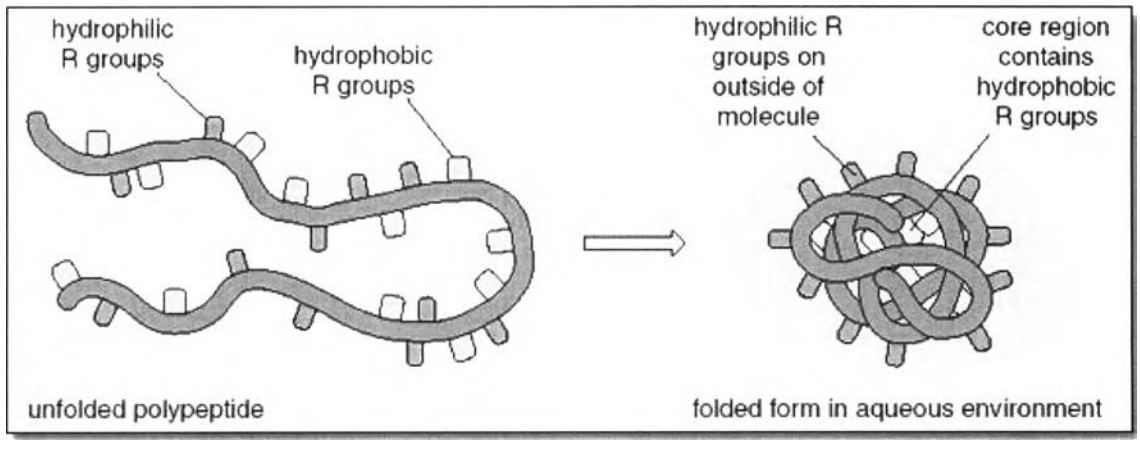

Figure 2.3 Protein conformation in water system.
(http://labspace.open.ac.uk/mod/resource/view.php/id=388704)

Each protein has a specific three-dimensional structure. The final conformation is usually the one with the lowest free-energy. Upon the adsorption on the solid surface with different hydrophobicity, protein can undergo conformational change.

2.6 Protein function

Each protein has its own amino acid sequence which determines its unique three-dimensional conformation. The particular protein shape allows it to bind to other selected molecules, for example, antibodies bind to viruses for destruction. The substance that binds to protein is called a ligand. The ability of protein to bind to a ligand depends on a set of non-covalent bonds. The position on the protein which binds to ligand is called binding site.

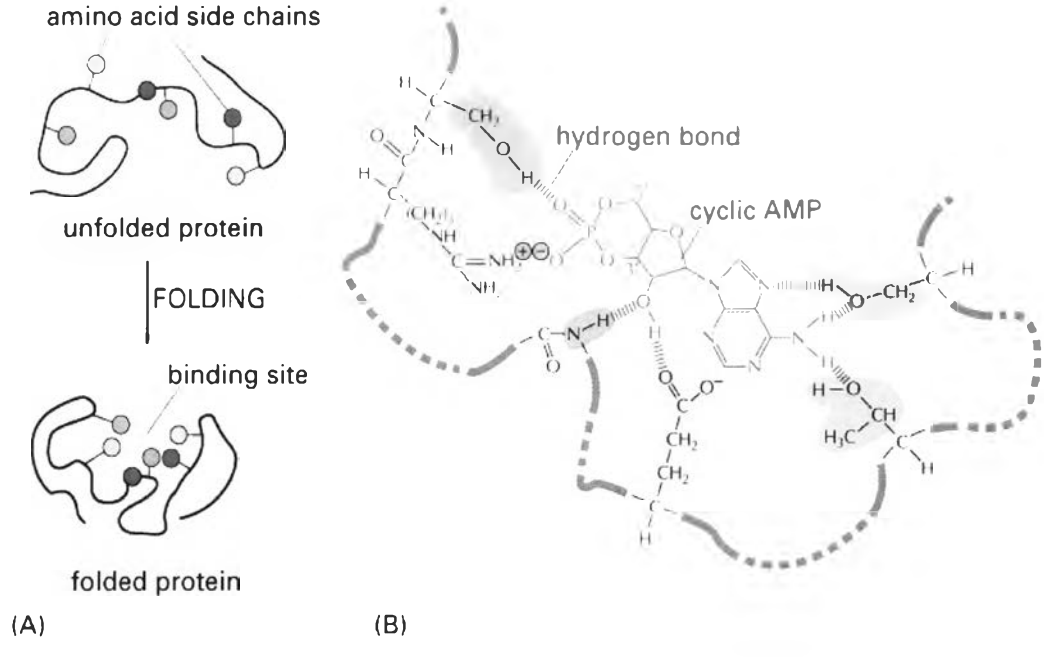

Figure 2.4 Protein binding site.
(http://www.accessexcellence.org/RC/VL/GG/ecb/protein_binding_site.php)

Binding sites allow a protein to interact with specific ligands (A). The folding of the polypeptide chain typically creates a cavity on the protein surface. This cavity contains a set of amino acid side chains that they can bind ligands by noncovalent bonds. (B) Close-up view of an actual binding site showing the hydrogen bonds and ionic interactions formed between a protein and its ligand (in this example, the bound ligand is cyclic AMP, shown in pink).

2.7 Extracellular matrix

The extracellular matrix (ECM) composed of a various proteins and polysaccharides, it is the part of the tissue outside the cell. The network of secreted extracellular macromolecules has many functions. Extracellular matrix provides an organized environment and support. The cell can move and communicate with one another. Organism is made from different types of tissue.

The function of the extracellular matrix as the scaffold to stabilize the structure of the tissue, it has a role in controlling cell behavior, proliferation and shape.

The components of the extracellular matrix are

- Collagen: The fibrous proteins of the matrix.
- Proteoglycans (PGs): Protein-polysaccharide complex with a core protein attached to Glycosaminoglycan (GAG) chains.
- Noncollagen components of the ECM: Fibronectin, Laminin, and other proteins of the ECM bind to collagen or PGs, or bind cells to the ECM.

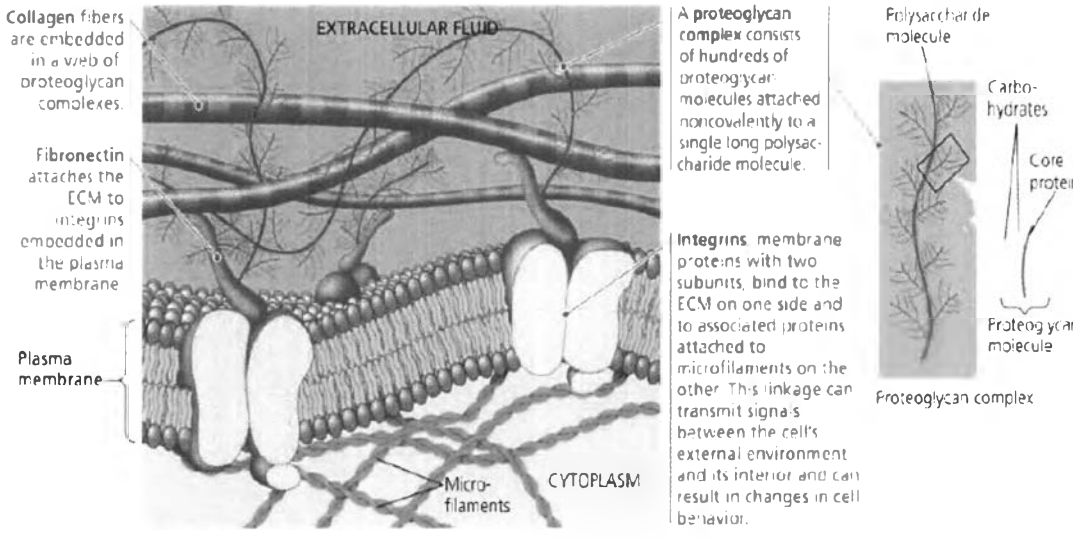

Figure 2.5 Extracellular matrix (ECM) of an animal cell. The molecular composition and structure of the ECM varies from one cell type to another, In this example, three different types of glycoproteins are present: proteoglycans, collagen, and fibronectin (Campbell, Book).

2.8 RGD sequence

The Arg-Gly-Asp (or called RGD) sequence is a part of the recognition sequence, it has a role in many recognition systems involved in cell-to-cell and cell-to-matrix adhesion. According to it can be recognized by a family of cell-surface receptors called integrin. Bovine serum albumin (BSA) itself does not contain integrin binding site, Ponik and coworker reported that osteoblasts were cultured on BSA for 5 hours could secrete fibronectin or extracellular matrix proteins that contain binding site for focal adhesion (Ponik & Pavalko, 2004).

2.9 Integrin

Integrin is the main receptor protein in which cell uses to bind and respond to extracellular matrix. Integrin consists of two subunits called α and β, it is non-covalent bond held together.

Binding of integrin to extracellular matrix protein, extracellular matrix proteins contain RGD sequence, such as fibronectin. It can be recognized by integrin which has the specific binding site in this tripeptide sequence.

Clustering of integrin is occurred by transmembrane adhesion protein of integrin, which can form a structure called focal adhesion. Focal adhesion binds cell to extracellular matrix and allows cell to pull on the substratum.

2.10 Surface topography

2.10.1 Measurement of the nanoscale roughness

The study of objects and phenomena at very small area is measured by atomic force microscopy (AFM). AFM provides a 3D profile on a naniscale, by measuring forces between a sharp probe (radius less than 10 nm) and surface at very short distance (0.2-10 nm probe-sample separation). The probe is supported on a flexible cantilever and the AFM tip gently touches the surface and records the small force between the probe and the surface. Force involved in the tip-sample interaction affect how the probe interacts with the sample.

There are three primary imaging modes in AFM:

- Contact mode: the probe-surface-separation is less than 0.5 nm. This imaging mode is fast scanning, good for rough surface and used for hard surface (shown in Figure 2.6).
- Intermittent or tapping mode: range of the probe-surface-separation is 0.5 to 2 nm. The imaging mode is well-suited for soft biological specimen and for poor surface adhesion sample (DNA and carbon nanotubes) (shown in Figure 2.7).
- Non-contact mode: the probe-surface-separation is 0.1 to 10 nm. The imaging mode is suitable for imaging soft surfaces (shown in Figure 2.8).

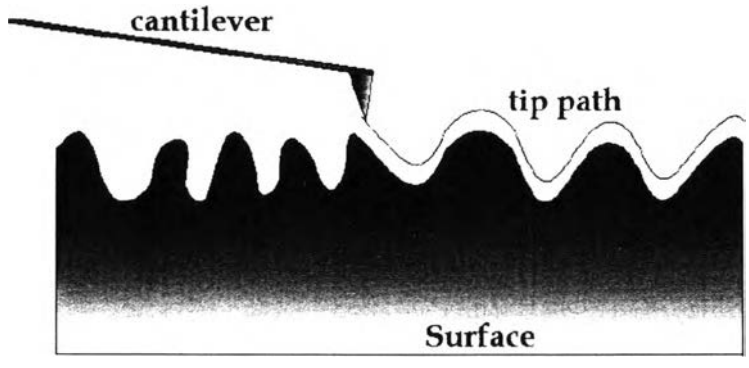

Figure 2.6 Contact mode.

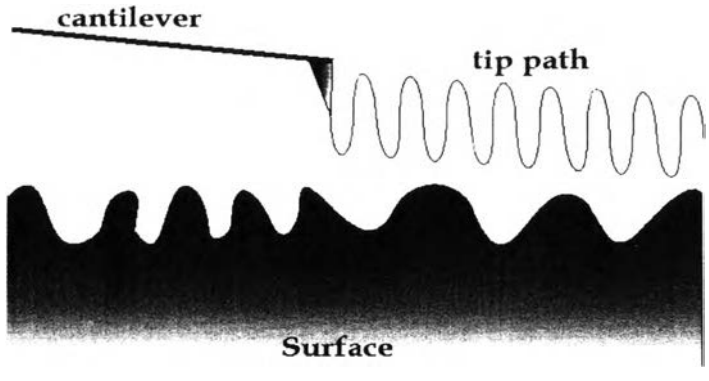

Figure 2.7 Intermittent-contact mode or tapping mode.

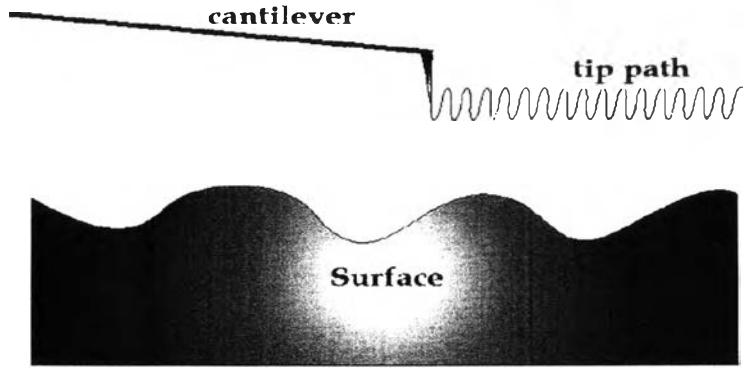

Figure 2.8 Non-contact mode.

The roughness can be characterized by several parameters and function. Among height parameters, the roughness average (R_a) is the most widely and a simple parameter to obtain when compared to other samples. Another parameter is the root mean square (RMS), the RMS roughness of a surface is more sensitive to peaks and valleys than the average roughness due to the squaring of the amplitude in its calculation.

$$Ra = \frac{1}{L}\int_0^L |Z(x)|dz \tag{1}$$

$$Rq = \sqrt{\frac{1}{L}\int_0^L |Z^2(x)|dx} \tag{2}$$

Where Z(x) is the function that describes the surface profile analyzed in terms of height (Z) and position (x) of the sample over the evaluation length "L" (Figure 2.9) (De Oliveira R.R.L., et.al, 2012).

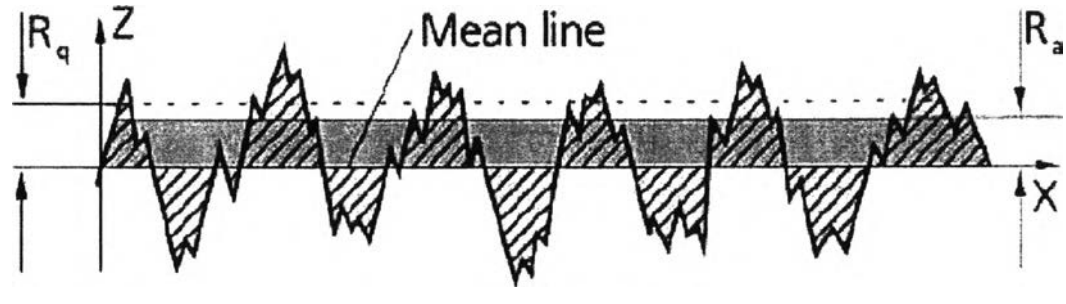

Figure 2.9 Profile of a surface (Z). It represents the average roughness R_a and R_q is the RMS roughness based on the mean line.

2.10.2 The influence of different preparation to surface topology

PCL film can be produced many technique. The simplest and cheapest method is solvent-casting by phase separation. One is by melting using compression moulding and the other is acetone-water phase separation. Morphology of PCL is prepared by acetone-water phase separation provide porous surface.

Furthermore, it is important for cell attachment and growth that suitable support for human osteoblasts.

2.10.3 The effect of different solvents to surface topology

The different types of solvents affect to surface topographies. Choosing solvent to dissolve polymer as solvent or solvent/non-solvent mixtures considers difference of solubility parameters. The solvent can dissolve polymer, it needs the similar value of solubility parameter, called good solvent. On the other hand, the value of solubility parameter is different, called poor solvent.

Polycaprolactone was dissolved in many solvent systems such as chloroform, tetrahydrofurane, acetone and ethyl acetate, and cast onto glass Petri dishes. Hydrophilicity of PCL was determined using contact angle. The functional groups of PCL were identified using attenuated total reflection-Fourier transform infared spectroscopy (ATR-FTIR) in different solvent systems, the ATR-FTIR spectra of all solvent systems were similar. In good solvent systems showed morphological surface in term of the aggregates during phase separation. While in poor solvent systems showed filamentous structure. Cell culture experiments, the cells favoured the surface of ethyle acetate cast PCL films that it induced the finest pore size and low contact angle, and promoted fibroblast grew on the PCL surface.

2.11 Protein adsorption on various polymer surface topography

The biological processes occuring on the surface when foreign material is inserted into the body are protein adsorption, cell attachment and the formation of tissue. The living cells cannot interact directly with foreign materials. Therefore, in vivo or in vitro the cells start to adsorb layer of biomolecules (e.g. proteins), induce attachment and spreading of cells, and communication between cells will lead to the formation of tissue. These phenomena are occurred on material interface.

In the recent year, many research studied in difference of surface topology to attachment and proliferation of cells. In 2005, the researchers studied effect of fiber diameter on spreading, proliferation and differentiation of osteoblastic cells on

Electrospun poly(lactic acid) substrates. Osteogenic factors affected to cell density on the smooth surface, cell density increased with fiber diameter.

On the smooth fiber, cells had a smaller projected area. Fiber diameter (2.1 μm) exhibited a high cell aspect ratio for attachment to substrate because this fiber diameter was observed to extend lamellapodia along individual fibers. Moreover, this research demonstrated that fiber diameters of 0.14-2.1 μm affect cell morphology and cell proliferation (Badami, et al., 2005).

Cell culture experiments with Mouse-Calvaria-Derived preosteoblastic cells (MC3T3-E1) responds to culture on electrospun fibers. Electrospun fibers are fabricated from blending of 50/50 w/w polycaprolactone (PCL) and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV). This research indicated that the cell preferred the smooth surface of 10% PCL/PHBV fibrous substrate, exhibited the lowest static water contact angles of ~85° and obtained average fiber diameter of ~0.8 μm. These results are the best promoting the attachment, proliferation and differentiation of the cell cultured MC3T3-E1 on this substrate (K-hasuwan, et al., 2011).

Improving hydrophilicity of PCL scaffolds by immobilizing the polar group on the surface of PCL. Mattanavee and et al. studied the immobilization of biomolecules on PCL surface. The PCL film was aminolyzed by 1,6-hexanediamine (HMD). HMD is a chemical treatment to introduce the amino groups on the surface. The aminolyzed PCL was immersed in *N,N'*-disuccinimidyl carbonate (DSC) to activate the aminolyzed PCL. Then, the activated aminolyzed PCL was transferred to collagen or chitosan solution to immobilize biomolecules on the surface.

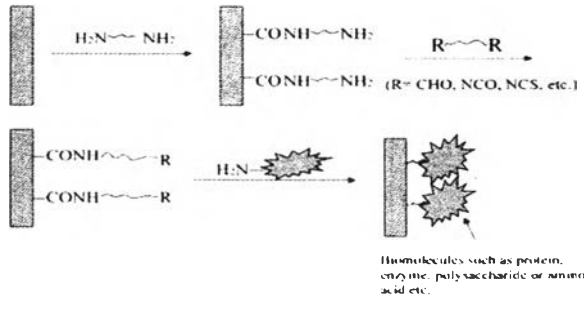

Figure 2.10 The aminolysis and immobilization of biomolecules on polyester surface.

Characterization on the surface using ATR-FTIR confirms functional groups and using water contact angle measures hydrophilicity on their surface. Mouse fibroblasts (L929) cells were cultured on these PCL fibrous scaffolds, results indicated that the neat and the modified PCL fibrous scaffolds release no substances harmful to the cells. Type I collagen showed the greatest ability to support the attachment and proliferation of all cell types (Mattanavee, et al., 2009).

In the systems of competitive adsorption between bovine serum albumin (BSA) and collagen using silicone wafer as substrate. Adsorption of BSA on the hydrophobic surface was higher than collagen type I. On the other hand, adsorption of BSA on the hydrophilic surface was less than collagen type I. It explained that BSA is flexible protein which easily denatured after adsorption, it formed a globular conformation. Therefore, BSA is easier to make a conformation rearrangement to interact on the hydrophobic surface, as the results that the hydrophobic interaction of BSA is more than collagen type I. Therefore, adsorption of BSA is also higher than collagen type I on the hydrophobic surface.

On the hydrophilic surface, the results showed that collagen type I is stronger adsorption on hydrophilic than BSA because collagen molecule is a helical coil of three peptides, it is non-flexible and rigid. Therefore, adsorption of collagen type I involves H-bonding and electrostatic interaction between hydrophilic surface and proteins. The rearrangement or orientation of adsorbed molecules might take place near the surface of substrate (silica), this orientation enables the molecule to bind tightly on the surface. Therefore, adsorption of collagen is higher than BSA on the hydrophilic surface (Yong Yu, et al., 2004).