CHAPTER II
THEORY AND LITERATURE REVIEW

2.1 Fundamentals of tissue engineering

Medical device development began in 1950s with the introduction of devices and materials derived from synthetic polymers. This field has progressed to the point where devices now used cell and biopolymers made using cultured or genetically engineered cell. Physicians have treated organ or tissue failure by transplanting organ from one individual into another, performing surgical reconstruction using corresponding tissue, or using mechanical devices such as blood oxygenators [6]. Although these approaches are used daily to save lives, they fall shot to begin available for everyone at a reasonable cost. Mechanical devices fail to provide all the biological functions required for normal homeostasis. Therefore, the replacement of tissue and organ structure and functions requires a combination of biological components engineered into a device.

Tissues are integral and fundamental components of organs. Tissues consist of specific cells and of extracellular matrix that is formed and maintained by the chemicals compounds synthesized by cells. For example, bone consists of osteoblasts, osteocytes, and osteoclasts. These cells reside in a matrix that is a composite of an inorganic and an organic phase.

The field of tissue engineering involves the applications of principles of engineering and life sciences toward the development of biological substitutes and restore, maintain, or improve tissue function. The approaches envisioned to be included in tissue engineering include use of isolated cells and tissues, and cell in combination with matrixes that are either in immunological contact with host tissue or are encapsulated to prevent immunological contact.

Cells derived from external tissues, skeletal tissue, cardiovascular tissue and specialized organs have been used in this approach. In this case of external tissue, tissue engineering of skin has progressed rapidly and repaired of skeletal tissues including bones and cartilage repair using tissue engineered materials has received much attention. Cardiovascular tissue engineering has focused on understanding the behavior of the endothelial cell and creation of small diameter vessels, and specialized organs including the pancreas and liver, are still engineering challenges. Figure 2.1

demonstrates the various approached used of isolated cells from both soft and hard tissue i.e. bone, muscle, tendon, cartilage.

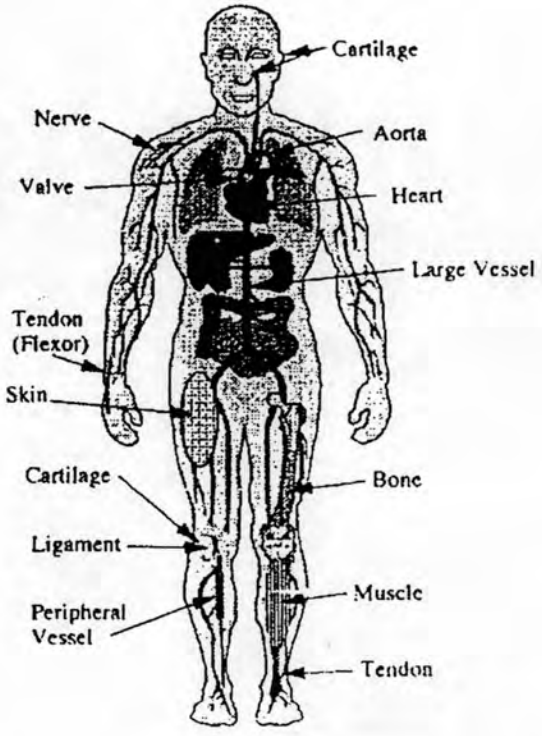


Figure 2.1 Tissue engineering approaches.

Tissue engineering can be divided into two broad categories:

(1) *In vitro* construction of bioartificial tissue from isolated by enzymatic dissociation of donor tissue, and

(2) *In vivo* alteration of cell growth and function.

The first category of applications includes bioartificial tissues (i.e, tissue which are composed of natural and synthetic substances) to be used as an alternative to organ transplantation. Besides their potential clinical use, reconstructed organs may also be used as tools to study complex tissue functions and morphogenesis in vitro [7]. For tissue engineering in vivo, the objective is to alter the growth and function of cell in situ, an example being the used of implanted polymeric tubes to promote and growth and reconnection of damaged nerves. The following are examples of applications of tissue engineering currently are listed in Table 2.1

Table 2.1 Applications of tissue engineering

Application	Examples
Implantable device Extracorporeal device Cell production *In situ* tissue growth and repair	Endothelialized vascular grafts Bone and cartilage implants Bioartificial skin Bioartificial pancreatic islets Bioartificial livers Nerve regeneration

In the optimal case, a tissue construct can be generated from autologous (i.e. the patient's own) cells. Therefore, cells have to be isolated from healthy tissue of patient. As the cell are more or less strongly connected which each other in single tissue, the tissue have to be chopped up mechanically and after that have to be separated from their extracellular

The ability of cell to grow into tissue and maintain tissue–specific function depends critically on many factors, such as the cell-tissue and cell-cell interaction and the extracellular matrix. In natural tissue environment, each cell has its individual cell biological interaction with the surrounding extracellular matrix. Thus, when choosing material to create an artificial matrix, it is absolutely crucial to find out first whether the chosen biomaterial meets the requirements for the desired tissue construct in all respects.

2.1.1 Control of cell function by the extracellular matrix

Different materials are available to serve as a base for cell attachment. In general, there are three routes that can be taken:

(1) use of extracellular matrix created from animal or human tissue,

(2) use of polymers generated from pure chemical substances, and

(3) use of composite materials fabricated from different biological and/or chemical components.

To create three-dimensional fiber construction, two dimension matrices are settled first and, following rolling-up, then made into three dimension structures (Figure 2.2). Another possibility is offered by three-dimension polymers in which living cell can settle in the pores of fiber spaces or the superstructures create [8].

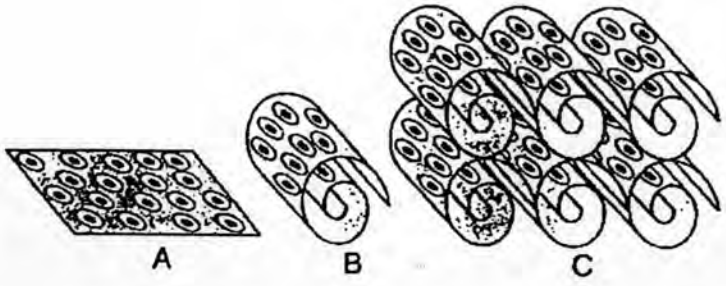


Figure 2.2 Three dimension matrix structure. (A) plane matrices can be settled by cells. (B) and then roll up (C) form a three dimension structure.

The selection of matrix primarily depends on the tissue to be created. For example, with epithelial tissue, the surfaces have to be settled, and the cultivated cells must not detach from the surface and must resist rheological stress. With connective tissue, the inners spaces of the scaffold must be settled. In this case, the biomaterial used has to support the synthesis of extracellular solid material during the generation of cartilage and bone, so mechanically stressable structures can be develop. With neural tissue, the material used is also settled three dimensionally. However, in this case, the growing dendrites and axons have to guide by the matrix in such a way that directed growth of structure results and contact via synapses can develop. With muscle tissue, in turn, the matrix must be composed of flexible material so that the structures created can make contract. For this reasons there is no matrix is equally well suitable for all of the different tissues with their individual specializations.

Since the vast majority of mammalian cells are anchorages dependent and spread on a substrate to proliferate and function normally. Cell adhesion is generally mediated by extracellular matrix protein such as fibronectin, vitronectin, laminin and collagen as well as various glycosaminoglycans [9]. Adsorption of these proteins to surface and the conformation of the adsorbed protein appear to be important factors influencing cell attachment and growth on synthetic substrates. Small sequences of the cell binding region of these proteins can also be covalently attached to surface to promote cell-substrate adhesion.

In general, seeding density is important for normal cell function, especially if cell-cell communications must be established, either by direct cell-cell contacts or via the secretion of trophic factors by the cell. Efficient cell seeding will mainly depend on:

(1) the affinity of certain cell surface proteins for extracellular matrix components,

(2) the density of cell-substrates binding sides on the material surface, and

(3) the presence or absence of certain nutritional factors.

While the first two factors can be controlled independently the numbers of cells placed on the surface, the last issue can be problematic because attempts at seeding large number of cell can significantly deplete nutrients, with the result that less cell than expected will attach. After seeding, the cell spread on the surface and reach a stable shape. The final morphology of the cells depends on three factors that are:

(1) adherence of the substrate for the cell, which is a function of the affinity and number of adhesion sites,

(2) rigidity of the substrate (i.e. ability to resist cell-generated tractional force) and the toxicity of the substrate

(3) cell-cell adherence.

Cell-substrate and cell-cell interactions are central to many biologic phenomena. Understanding of these interactions helps explain cell behavior *in vitro* and *in vivo*. Ability to manipulate these interactions improves the design of medical devices.

2.1.2 Scaffold and natural material for tissue engineering

As shown in Figure 2.3, the primary function of the scaffold is tissue conduction and therefore, it must allow cell attachment, migration onto or within the scaffold, cell proliferation and differentiation. It must also provide an environment in which the cells can maintain their phenotype and synthesize required proteins and molecules [10]. The scaffolds must have the following characteristics:

(1) Controllable degradation rate

(2) Biocompatibility

(3) Degradation products that are excreted by normal physiologic metabolic pathways

(4) Large surface area to volume ratio

(5) Easy processability into three-dimensional shapes of complex geometry

(6) Mechanical properties capable of withstanding stresses in specific applications

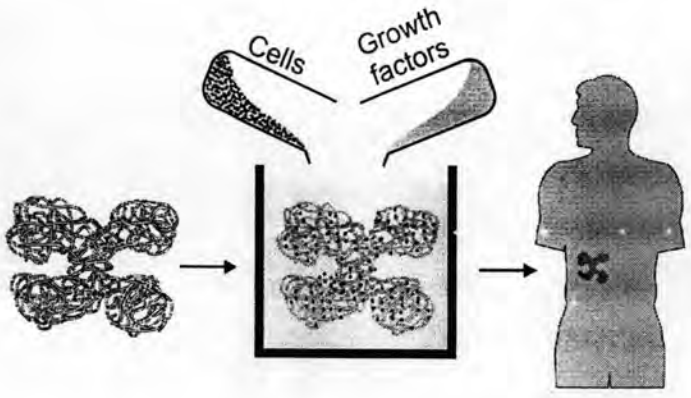


Figure 2.3 Scaffold-guided tissue regeneration

Selection of tissue engineering substrate includes a choice between absorbable and nonabsorbable materials. The most common synthetic polymers used for fibrous meshes and porous scaffolds include synthetic and natural material. A notable disadvantage of synthetic material is their lack of cell-recognition signals. In contrast, natural materials have certain advantages over synthetic polymers. For example, naturally derived material provides a natural surface for better cell attachment and greater capacity of promoting cell differentiation and low chronic inflammatory. Examples of natural materials include collagen, chitosan, gelatin, and alginates.

Biodegradable materials are required for repair or remodeling, not necessarily long-term stability. Manufacturing feasibility, the ability to form final product design, short-term mechanical properties with negligible toxicity from degradation products are prerequisite essentials for these materials. In 2005, Nikolovski and Mooney [11] fabricated poly glycolic acid (PGA), poly (L-lactic acid) (PLLA) and copolymers of poly (lactic-*co*-glycolic acid) (PLGA) as a scaffold for tissue engineering application. In this study, they demonstrated the various specific proteins adsorb onto these polymers from serum-containing medium in vitro and that smooth muscle cells utilize specific integrin receptors to bind to these proteins. Cell adhesion to synthetic polymer implants *in vivo* and to culture surfaces *in vitro* is typically dependent upon surface-adsorbed bronectin and vitronectin. This result confirmed that the cell adhesion events play a crucial role in the long-term gene expression.

Presently, because of merits of nanofibers which have trend to apply in biomedical material. Many researchers investigated electrospinning nanofibers to study the effects of various parameters on morphology and diameter of obtained nanofiber. Based on their researches, although synthetic polymers can be easily spun into nanofiber but sometime it is quite difficult to use for biomedical application. Therefore, the natural polymers which have biocompatible and biodegradable properties have drawn a lot of attention in order to apply in biomedical field. For example, PLLA is a promising scaffold material due to its biocompatibility and biodegradation. Yang et al. [12] fabricated aligned PLLA fibrous scaffolds by an electrospinning technique under optimum condition and the diameter of the electrospun fibers can easily be tailored by adjusting the concentration of polymer solution. The representative SEM micrographs of neural stem cells (NSCs) cultured for 2 days on the PLLA scaffolds indicates that the cell body had an apparent bipolar elongated morphology with the outgrowing neuritis. Both cell elongation and neuritis outgrowth follow the same direction of PLLA nanofibers. They also show the interaction between the NSCs and aligned nanofibers. However, they have not observed any signal to show the filament-like structure of NSCs on the random fibers, which implies that the fiber alignment may have considerable effects in mediating the interaction between the NSCs and the scaffolds.

2.2 Electrospinning fiber

2.2.1 Principle of electrospinning

The recently fast developing "electrospinning" technology is a unique way to produce novel polymer nanofibers with diameters typically in the range from 10 nm to 100 nm. Electrospinning is the process of using electrostatic forces to distort a pendant droplet of polymer solution into a fine filament to be deposited onto a substrate. The advantages of electrospinning are due to its ability to produce novel synthetic fibers of unusually small diameter and good mechanical properties [13]. This leads to fiber mats with high surface area to volume ratio and the ability to control pore size. Another interesting aspect of using nanofibers is that it is feasible to modify not only their morphology and their (internal bulk) content but also their surface structure to carry various functionalities. Nanofibers can be easily post-synthetically functionalized (for example by chemical or physical vapour deposition). Furthermore, it is even feasible to control secondary

structures of nanofibers in order to prepare nanofibers with core/sheath structures, nanofibers with hollow interiors and nanofibers with porous structures.

Figure 2.4 shows the schematic of the electrospinning setup. In the electrospinning process a high voltage is used to create an electrically charged jet of polymer solution or melt, which dries or solidifies to leave a polymer fiber. One electrode is placed into the spinning solution/melt and the other attached to a collector. Electric field is subjected to the end of a capillary tube that contains the polymer fluid held by its surface tension. This induces a charge on the surface of the liquid. Mutual charge repulsion causes a force directly opposite to the surface tension. As the intensity of the electric field is increased, the hemispherical surface of the fluid at the tip of the capillary tube elongates to form a conical shape known as the Taylor cone [3]. With increasing field, a critical value is attained when the repulsive electrostatic force overcomes the surface tension and a charged jet of fluid is ejected from the tip of the Taylor cone. The discharged polymer solution jet undergoes a whipping process wherein the solvent evaporates, leaving behind a charged polymer fiber, which lays itself randomly on a grounded collecting metal screen. In the case of the melt the discharged jet solidifies when it travels in the air and is collected on the grounded metal screen.

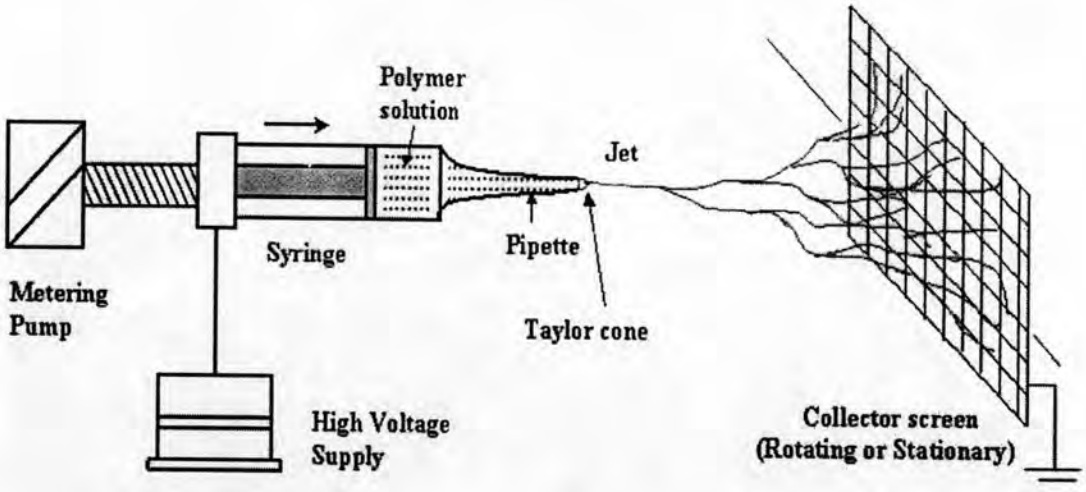


Figure 2.4 Schematic of the electrospinning setup

The polymer solution or melt is contained in a glass tube, usually a pipette that is connected to a syringe like apparatus. A metering pump attached to the plunger of the syringe generates a constant pressure and flow of the fluid through the pipette.

The driving force is provided by a high voltage source through a wire immersed in the solution. The high voltage source can generate up to 30 kV, and the setup can be run on either positive or negative polarity. Adjusting the flow of the fluid and the magnitude of the electric field controls the spinning rate. Many parameters can influence the transformation of polymer solutions into nanofibers through electrospinning. These parameters include:

(1) system parameters such as polymer molecular weight, molecular weight distribution and the solution properties (e.g. viscosity, elasticity, conductivity, and surface tension),

(2) process parameters, such as flow rate, electric potential, distance between the tips and the collecting screen, motion of collector, and

(3) ambient parameters such as solution temperature, humidity, and air velocity in the electrospinning chamber

Figure 2.5 shows a nanofiber which has a diameter in the range of nanometers to over 1 μm and a high porosity. Another interesting aspect of using nanofibers is that it is feasible to modify not only their morphology and their (internal bulk) content but also their surface structure to carry various functionalities [14]. Nanofibers can be easily post-synthetically functionalized (for example by chemical or physical vapour deposition). Furthermore, it is even feasible to control secondary structures of nanofibers in order to prepare nanofibers with core/sheath structures, nanofibers with hollow interiors and nanofibers with porous structures.

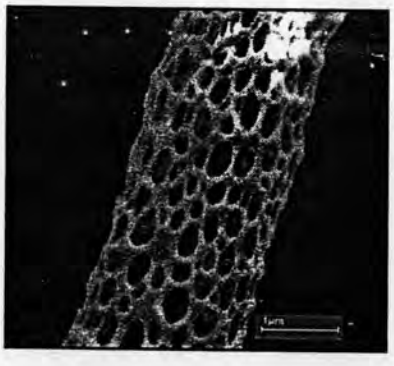


Figure 2.5 Nanofibers with specific topology

2.2.2 Electrospinning for biomedical applications

From a biological viewpoint, almost all of the human tissues and organs can be deposited on nanofibrous forms or structures. Examples include: bone, dentin, collagen, cartilage, and skin. All of them are characterized by well organized fibrous structures realigning in nanometer scale. As such, current research in electrospun polymer nanofibers has focused one of their major applications on bioengineering [14]. From this view point, one can easily find their promising potential in various biomedical areas. Some examples are listed later.

Medical prostheses

Polymer nanofibers fabricated via electrospinning have been proposed for a number of soft tissue prostheses applications such as blood vessel, vascular, breast, etc. In addition, electrospun biocompatible polymer nanofibers can also be deposited as a thin porous film onto a hard tissue prosthetic device designed to be implanted into the human body. This coating film with gradient fibrous structure works as an interphase between the prosthetic device and the host tissues, and is expected to efficiently reduce the stiffness mismatch at the tissue/device interphase and hence prevent the device failure after the implantation.

Cosmetics

The current skin care masks applied as topical creams, lotions or ointments may include dusts or liquid sprays which may be more likely than fibrous materials to migrate into sensitive areas of the body such as the nose and eyes where the skin mask is being applied to the face. Electrospun polymer nanofibers have been attempted as a cosmetic skin care mask for the treatment of skin healing, skin cleansing, or other therapeutical or medical properties with or without various additives. This nanofibrous skin mask with very small interstices and high surface area can facilitate far greater utilization and speed up the rate of transfer of the additives to the skin for the fullest potential of the additive. The cosmetic skin mask from the electrospun nanofibers can be applied gently and painlessly as well as directly to the three-dimensional topography of the skin to provide healing or care treatment to the skin.

Wound dressing

Polymer nanofibers can also be used for the treatment of wounds or burns of a human skin, as well as designed for hemostatic devices with some unique characteristics. With the aid of electric field, fine fibers of biodegradable polymers can be directly sprayed/spun onto the injured location of skin to form a fibrous mat dressing, which can let wounds heal by encouraging the formation of normal skin growth and eliminate the formation of scar tissue which would occur in a traditional treatment. Non-woven nanofibrous membrane mats for wound dressing usually have pore sizes ranging from 500 nm to 1 mm, small enough to protect the wound from bacterial penetration via aerosol particle capturing mechanisms. High surface area of 5–100 m2/g is extremely efficient for fluid absorption and dermal delivery.

Drug delivery

In general, the smaller the dimensions of the drug and the coating material required to encapsulate the drug, the better the drug to be absorbed by human being. Drug delivery with polymer nanofibers is based on the principle that dissolution rate of a particulate drug increases with increasing surface area of both the drug and the corresponding carrier if needed. The electrospun polymer nanofibers for drug delivery, which can be designed to provide rapid, immediate, delayed, or modified dissolution, such as sustained and/or pulsatile release characteristics. As the drug and carrier materials can be mixed together for the drug in the resulting nanostructed products are: (1) drug as particles attached to the surface of the carrier which is in the form of nanofibers, (2) both drug and carrier are nanofiber-form, hence the end product will be the two kinds of nanofibers interact together, (3) the blend of drug and carrier materials integrated into one kind of fibers containing both components, and (4) the carrier material is electrospun into a tubular form in which the drug particles are encapsulated.

Tissue engineering

The primary focus in electrospinning has been on the production of nanofibers due to their resemblance scale-wise to native extracellular matrix (ECM). Polymer meshes comprised of fibers with diameters as low as tens of nanometers, and exhibiting porosities as high as 90% and pore sizes as low as 100 nm. Cells seeded onto nanofiber matrixes tend to spread, attaching at multiple focal points, and in some

cases extend filopodia along the length of the fiber. Several studies suggest the ability of attached cells to push against nanofibers and migrate into the nanofiber matrix [2] however, culture of cells on nanofiber matrics also results in a monolayer of cells and ECM, thus limiting their potential for 3D tissue engineering applications. It has been suggested that smaller fibers can inhibit cellular infiltration. The reduced cellular infiltration into the depths of the scaffold has been attributed to the pore diameters being smaller than that of cell. For cell migration or infiltration to occur, the pore size of a scaffold should at least be the size of a cell, a value of 10 μm has been suggested as necessary for cellular infiltration. Although microfiber scaffolds are not on the same size scale as extracellular matrix components, they could be potentially advantageous because they are comprised of larger pores as compared to nanofiber scaffolds. These larger pore sizes scaffolds could allow or facilitate cellular infiltration and/or diffusion of nutrients during in vitro culture.

2.2.3 Poly (ε -caprolactone) (PCL)

Poly (ε -caprolactone) (PCL) is biodegradable polyester that has been extensively investigated as potential biomaterials. PCL is linear polyester manufactured by ring-opening polymerization of ε-caprolactone. ε-caprolactone, a cyclic ester, which can be prepared by the Baeyer-villiger reaction for the oxidation of cyclic ketones and lactones [15]. ε -caprolactone can be polymerized by either a cationic and or an anionic mechanism. Molecular structure of PCL is presented in Figure 2.6. PCL is commercially available in a variety of molecular weight, which is usually controlled by used of dry monomer and addition of a specific amount of active-hydrogen initiator. It is a hydrophobic and semicrystalline polymer with a degree of crystallinity around 50%. It has rather low glass transition temperature and melting point. The PCL chain is flexible and exhibits high elongation at break and low modulus. Its physical properties and commercial availability make it very attractive not only as a substitute material for nondegradable polymers for commodity applications but also as a specific plastic of medicine and agricultural areas. The main drawback of PCL is its low melting point (65 ^{0}C) which can be overcome by blending it with other polymers or by radiation cross-linking processes resulting in enhanced properties for wide rage of application.

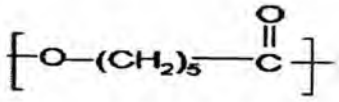


Figure 2.6 Molecular structure of PCL

Poly (ε-caprolactone) is degraded very slowly, much more slowly than poly (α-hydroxy acids), *in vivo* to yield ε-hydroxycaporic acid. Its *in vivo* degradation is initiated by non-enzymatic ester hydrolysis in the extracellular matrix. The final stage of degrade-action however was found to involve phagocytosis of polymer fragments by macrophages and giant cells, and degradation with these cells by lysosome derived enzymes [16]. In vitro studied on PCL degradation have established its sensitive to microbial enzyme and, as expected, increased degradability of amorphous relative to the crystalline phase.

A large number of polymeric biomaterials, including non-biodegradable and biodegradable polymers, have been tested and analyzed for tissue engineering applications. Since non-biodegradable polymers would interfere with tissue turnover and remodeling, the current trend is to use biodegradable polymers in tissue engineering, although the non-biodegradable polymers have the advantage that their properties, both chemical and mechanical, are less affected by the cellular and tissue environmental. On the other hand, polymer biodegradation via the combined effect of enzymatic and hydrolytic activities generates space within the scaffold to allow for cell proliferation and the deposition of newly synthesized ECM. Ideally, optimal tissue regeneration occurs upon complete biodegradation of the polymeric matrix followed by restoration of biological functions. Among various types of biomaterials, PCL has many advantages because of its biocompatibility, low cost, easy processability and slow hydrolytic degradation rate. It has been used for the reconstruction of various tissues such as bone, skin, nerve and retina. Recently, there were many report successfully fabricated PCL into membranes of less than 10 nm in thickness with different mechanical properties [17-19]. The aim was to minimize the long-term host tissue response by reducing the volume of scaffold material required for tissue engineering applications. This ultra-thin PCL membrane provides promising applications in soft tissue engineering. However, being a synthetic biomaterial, PCL has a hydrophobic surface and lacks functional groups. Thus, it is not a good substrate

for cell adhesion. There were various researches dedicated to modify the PCL surface. Recently, more technologies have been devoted to emulate the fibrillar topology, using an electrospinning process where high voltage electric field was used to spin various biomaterials into nanofibers. Hereafter is described some work already published

Williamson et al. [20] fabricated the PCL fibers by ''gravity spinning'' from solutions of the polymer and The proliferation rate of Swiss 3T3 mouse fibroblasts and C2C12 mouse myoblasts on as-spun, 500% cold-drawn and gelatin-modified PCL fibers. They found that proliferation of both cell types was consistently higher on gelatin-coated fiber relative to as-spun fiber at time points below 7 days. Fibroblast growth rates on cold-drawn PCL fibers exceeded those on as-spun fibers but myoblasts proliferation was similar on both substrates. Moreover, After 1 day in culture, both cell types had spread and coalesced on the fibers to form a cell layer, which conformed closely to the underlying topography.

Yoshimoto et al. [21] assess the potential of electrostatic poly (ε-caprolactone) fiber spinning, as an alternative scaffold fabrication technique to engineer bone in vitro using rotational oxygen-permeable bioreactor system (ROBS) to provide optimal oxygen tension and mechanical stresses to cell-polymer constructs in culture. The results show the cells migrated inside the scaffold and produced an extracellular matrix of collagen throughout the scaffold. The cell-polymer constructs maintained the size and shape of the original scaffolds. In particular, no shrinkage was observed. After 4 weeks, the cell-polymer constructs had become noticeably harder. Moreover, SEM showed that the surfaces of the cell-polymer constructs were covered with cell multilayer after 4 weeks.

Chen et al. [22] mimic the architecture of the natural extracellular matrix and create nano topography for enhanced cellular attachment. The results showed that uniform nanofibrous topology were successfully achieved on the surface of the PCL nanofibrous membrane, with increased roughness (more than 17times) and surface area. This nanofibrous topology induced capillary effects after sodium hydroxide (NaOH) treatment, causing the water contact angle to drop to almost zero. Changes in cell morphology were viewed on the plain PCL before and after NaOH treatment; while cell morphologies were totally different between the nanofibrous membranes before and after NaOH treatment. An alamarblue assay indicated that 3T3 fibroblast cells proliferated well on the nanofibrous membrane.

Venugopal et al. [23] aimed to modify the PCL with collagen, to increase the mechanical strength of nanofibers in tissue engineering and to allow the cells to secrete their own ECM to form natural blood vessels useful for the replacement of diseased vessels. This study proved that PCL nanofibers supporting cell growth need collagen support for migration of cells inside the nano fibrous matrices. Collagen nanofibers showed cells attached to the nanofibrous matrices and normal cell morphology leading to tissue-like architecture after 72 h. These observations confirm that PCL nanofibrous matrices coated with collagen can definitely support cell adhesion and migration inside the nanofibrous matrices to form a rigid tissue-like architecture.

In the same year, Venugopal et al. [2] aimed to fabricate PCL and PCL-blended collagen nanofibrous membrane by electrospinning for wound dressing, cell adhesion and proliferation, and cell matrix interaction, to produce allogeneic cultured dermal substitute (CDS) for skin defects and burn wounds. They showed the dermal fibroblast proliferation decreased in PCL nanofibrous membrane compared to PCL-blended collagen and collagen nanofibrous membrane because of the absence of collagen. The collagen provided cell binding sites to nanofibers for the attachment of human dermal fibroblasts (HDFs) and proliferation to form the dermal substitute. These results prove that the PCL blended collagen nanofibrous membrane provided mechanical stability, slow degradation (hydrophilic), and suitability for cell attachment and proliferation to design the dermal substitute for skin regeneration.

2.2.4 Surface treatment of electrospun fibers with polyelectrolyte multilayer (PEMs)

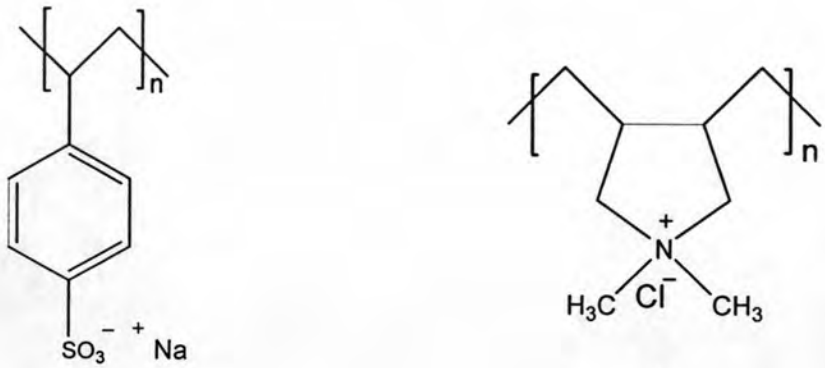
There is an increasing interest in developing new coating to improve biocompatibility electrospun fiber and to prepare biomaterial surface that can either be resistant or enhance cellular adhesion. Being the focus of many researches, the most important step toward this end concerns the improvement at the nano-scale and micro-scale of materials which surface properties can be turned to thickness or roughness. Many kinds of coating material have been used for the fabrication of films coating on nanofiber such as leaching of glass, sol-gel deposition, chemical vapor deposition (CDV), sputtering, grafting and layer-by-layer (LBL) self assemble method. Compare to other strategies, electrostatic self-assembly approach offers several applications such as light-emitting diodes, electrochromic devices, nonlinear

optical devices, optical sensors, and biomedical application [4] From this view point, Ding et al.[24] studied the electrospun cellulose acetate (CA) nanofibrous mats as support because their negative charges, low fiber density, and good water insolubility. The LBL self-assembly technique is applied to coat CA nanofibers with oppositely charged poly (allylamine hydrochloride) (PAH) and poly (acrylic acid) (PAA). The results showed that the formation of PEMs films on CA nanofibers was strongly influenced by the pH value of polyelectrolyte solutions. Moreover, they was observed that the thickness of PAH/PAA films coated on CA nanofibers was controllable by regulating the number of PEMs bilayers. The results of AFM indicated that the PEMs films coated fibers have a rougher surface than the uncoated. In the same year, Ding et al.[25] fabricated PEI/polyoxometalate ($H_3PMo_{12}O_{40}$) ultrathin tubular films on electrospun cellulose acetate (CA) nanofibers via the electrostatic layer-by-layer (LBL). The results showed that the formation and morphology of the LBL films containing polyoxometalates (POM) on CA nanofibers was strongly influenced by the pH value and ionic strength of polyelectrolyte solutions, the number of coating bilayers, and the concentration of POM. The spaces among the adjacent fibers in fibrous mats can be blocked by the quickly growing PEI/$H_3PMo_{12}O_{40}$ films. Moreover, the results also indicated that the porous LBL films coated fibers can be fabricated with increasing the ionic strength of polyelectrolyte solutions by adding NaCl.

2.3 Polyelectrolyte multilayer thin films

2.3.1 Definition and general description of polyelectrolyte

The term "polyelectrolyte" is employed for polymer systems consisting of a "macroion," i.e., a macromolecule carrying covalently bound anionic or cationic groups. Examples of anionic and cationic polyelectrolyte (PEL) are presented in Figure 2.7.



(a) (b)

Figue2.7 Structure of (a) PSS as an anionic polyelectrolyte and (b) PDADMAC as an cationic polyelectrolytte

Both polystyrene sulfonate and poly (diallyldimethylammonium chloride) are dissociated into macroion and counterions in aqueous solution in the total pH range between 0 and 14 [26]. Also polymers like poly(acrylic acid) or poly(ethylene imine) are usually classified as polyelectrolytes, in spite of the fact that they form a polyion-counterion system only in a limited pH range, and remain as an undissociated polyacid in the acid range or an undissociated polybasic in the alkaline range as shown in Figure 2.8 This is a behavior typical for weak polyelectrolytes and quite analogous to weak low molecular electrolytes.

(a)

(b)

Figure 2.8 Dissociation equilibrium of the weak polyelectrolytes
(a) Poly (acrylic acid) and (b) Poly (ethylene imine)

On the other hand, a polymer like cellulose capable of dissociating partially into cellulosed anions and counterions at extremely alkaline conditions (pH > 14) cannot be classified as a polyelectrolyte, as in the conventional pH range of dilute aqueous systems the OH groups of polymer are not ionized.

A special case of polyelectrolytes, the "polyampholytes" carrying both anionic and cationic groups covalently bound to the macromolecule, are represented in nature by an abundant number of proteins but can also be obtained by various synthetic routes. An example is presented in Figure 2.9 as a typical polyampholyte, this copolymer carries cationic charges in an acid and anionic charges in an alkaline medium, while at the so-called "isoelectric point," in the example pH 4, no free net charge exists in the macromolecule.

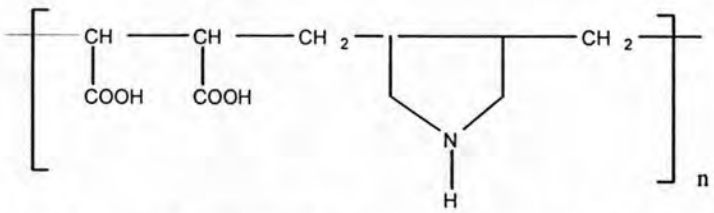


Figure 2.9 Chemical structure of a maleic acid-diallylamine copolymer

In principle, any macromolecular chemical structure can be transformed into a polyelectrolyte structure by covalently attaching a reasonable number of ionic groups to the polymer backbone, with linear or branched macromolecules at a compound soluble in an aqueous medium of appropriate pH after a sufficient number of ionic groups, while in the case of a cross linked polymer its swell ability in aqueous media is enhanced by transferring into a polyelectrolyte. Limiting our further considerations to linear and branched structures, a vast number of polyelectrolyte classes are known today, a section of which is listed in Table 2.2

Table 2.2 Selected classes of polyelectrolytes

Anionic and cationic polysaccharides and polysaccharidic derivatives
Nucleic acids
Gelatin
Lignosulfonic acids
Polyacrylic and polymethacrylic acid and its copolymers
Maleic acid anhydride copolymers
Polystyrene sulfonic acid
Polyethylene imine
Polyamines and polyamidamines
Ionenes
Poly(diallyldimethylammonium chloride)
Homo-and copolymers of cationic acrylic acid esters

Today's commercial polyelectrolytes are predominantly obtained by a polymerization, polycondensation, or polyaddition process. Also numerous important polyelectrolyte also originate from nature, such as gelatin, as a representative of the widespread class of proteins or pectin belonging to the group of anionic polysaccharides. Furthermore, some polyelectrolytes of practical importance result from a chemical modification of nonionic natural polymers such as cellulose or starch [27]. From these relevant articles from polyelectrolyte multilayers were successfully constructed and then some published articles were studied about the parameters controlling the growth of PEM as follow.

Dubas and Schlenoff [28] studied the factors controlling the growth of polyelectrolyte multilayers and the dependence of polyelectrolyte multilayer thickness on salt concentration, salt type, solvent quality, deposition time, and polymer concentration is evaluated. Polymers are deposited on spinning silicon wafers. For the strong polycation/polyanion pair studied, film thickness is approximately proportional to the number of layers and the salt concentration. The irreversibility of overall molecule adsorption is indicated by the lack of exchange of surface (radiolabeled) for solution polymer. The hydrophobic nature of the driving force for polymer sorption is illustrated by the choice of salt counterion or solvent. Salt, competing with polymer segments for the surface, permits localized rearrangements. In the mechanism

proposed, excess polymer is accommodated within several layers, rather than in one layer of loops and tails. Steric barriers coupled with slow conformational changes are responsible for long-term polymer adsorption. Considering the disorder and interpenetration, multilayer buildup has much in common with solution phase or coprecipitated polyelectrolyte complexes. Surface hydrophobicity can be enhanced using fluorinated surfactants as counterions.

Dubas and Schlenoff [29] then studied polyelectrolyte multilayer containing a weak polyacid: construction and deconstruction and found that the growth of multilayer made from a combination of a weak polyacid and a strongly dissociated polycation is studied as a function of salt concentration and molecular weight. Film thickness reaches a maximum at around 0.3 M salt and then decreases quickly. Preformed multilayer are shown to decompose rapidly and, for high molecular weights, completely when exposed to aqueous solutions of NaCl of concentration>0.6 M. The apparent dissociation of multilayer polyelectrolyte complexes is due to competition for polymer/polymer ion pairs by external salt ions. Similar experiments aimed at decomposing multilayers by protonating the weak acid, thus decreasing polymer/polymer interactions, lead to incomplete loss of polymer, probably due to additional hydrogen bonding from the protonated weak acid. A model based on ion exchange/swelling of multilayers is used to explain their stability and permeability as well as the dependence of film thickness on salt concentration and type.

2.3.2 Formation of polyelectrolyte multilayer thin films

Polyelectrolyte multilayer films created via Layer-by-Layer (LbL) deposition are currently used to modify the surface properties of materials. These polyelectrolyte based films are capable of self-organization. The self-organization process of polyelectrolyte films, also referred to as electrostatic self-assembly (ESA), has been well documented over the past ten years.

Starting in the early 1990s, Decher's group began work on the realistic method for the ESA of nanolayers over charged substrate [30]. The process developed by Decher has increased in popularity since its introduction. This is a result of the method's simplicity and the fact that polyelectrolytes as well as charged nano objects can be deposited in a controlled manner. Biological compounds, conducting and light emitting polymers, and dyes have also been deposited onto suitable substrates via ESA.

The LbL process is based on the alternating adsorption of charged cationic and anionic species. The process begins by properly charging a substrate. The charged substrate is then primed by adsorbing a layer of a polyelectrolyte with an opposite charge sign to that imparted to the substrate. Once the substrate is primed, it is then dipped into a solution of a counterions polyelectrolyte. A rinse step is included between the two adsorption processes to remove excess as well as to prevent cross-contamination of the polyelectrolyte solutions. These simple steps complete the LbL deposition of the nanolayers. Multiple layers can be created by simply dipping the substrate in alternating anionic and cationic baths.

From Figure 2.10, the top diagram shows the simplified molecular concept of the first two adsorption steps depicting film deposition starting with a positively charged substrate. The polyion conformation and layer interpenetration are an idealization of the surface charge reversal with each adsorption step which is the basis of the electrostatically driven multilayer build up depicted here. Counterions are omitted for clarity. The bottom diagram of Figure 2.10 demonstrates the schematic of film deposition process using glass slides and beakers. Steps 1 and 3 represent the adsorption of a polyanion and polycation respectively, and steps 2 and 4 are washing steps. The four steps are the basic buildup sequence for the simplest film architecture (A/B)*n* where *n* is the number of deposition cycles. The construction of more complex film architectures requires additional beakers and an extended deposition sequence.

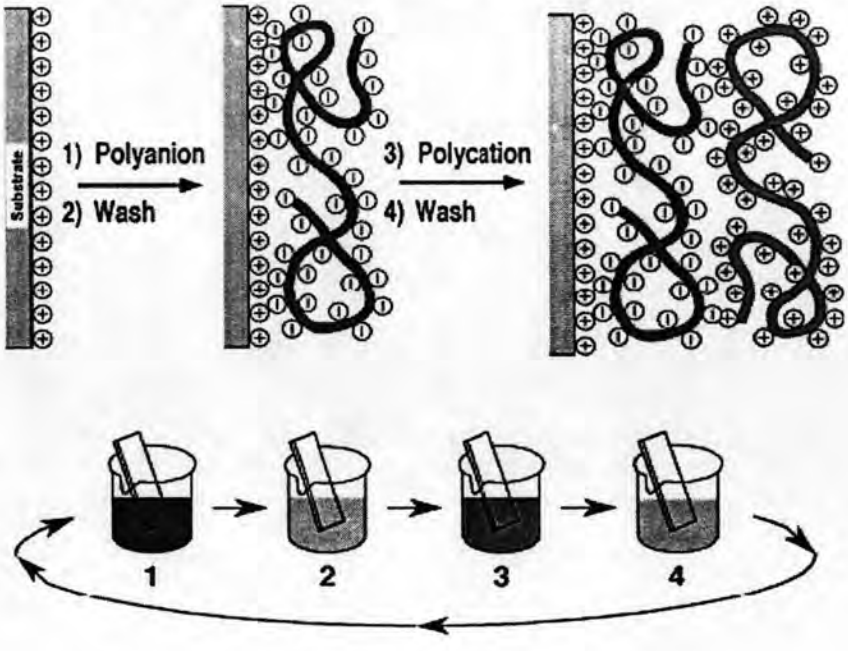


Figure 2.10 Schematic of the electrostatic self-assembly (ESA)

Given the large set of materials which are easily incorporated into multilayer films, layer-by-layer deposition is a rather general approach for the fabrication of complex surface coatings. It combines several advantages as shown in Table 2.3 It is possible to coat almost any solvent-accessible surface starting with sub-micron objects up to the inside of tubing or even objects with a surface of several square meters [27]. Like a chemical reaction, the precise structure of each layer depends on a set of control parameters such as, Salt concentration, number of layers, pH, or polyelectrolyte concentration but in general the processing window is rather broad.

Table 2.3 Parameters controlling the growth of PEMs

Major parameters	Minor parameter
Salt concentration	Polyelectrolyte concentration
Number of layers	Deposition time
pH	Surface roughness
	Surface charge

2.3.3 Application of polyelectrolyte multilayer for cell adhesion

Surface modification using polyelectrolyte multilayer (PEMs) to develop biocompatible materials has attracted attention lately due to the ease of synthesis and cost-effectiveness of the Layer-by-Layer technique. Surface properties ranging from hydrophobic to hydrophilic, charged to uncharged, and smooth to rough can be generated using a variety of parameters. Because proteins play an important role in the adhesion, spreading and growth of cells, considerable effort has been expended in developing polyelectrolyte thin films with properties that make the surface adhesive or resistant to protein adsorption. Although an understanding of PEMs–protein adsorption is necessary to intelligently engineer cell-biomaterial interaction. It is difficult to predict PEMs–cells biocompatibility from simple measurements of protein adsorption. Recent investigations using cultured cells revealed PEMs properties important for cell biocompatibility. These investigations have demonstrated that surface can be rendered cytophilic or cytophobic by embedding or attaching proteins to the multilayer and turning the pH used for multilayer buildup. Boura et al. [31] use of polyelectrolyte films has been suggested as a new versatile technique of surface

modification aimed at tissue engineering. In this study, they evaluate the adhesion properties of endothelial cells (ECs) on two types of polyelectrolyte films ending either by poly(D-lysine) (PDL), or poly(allylamine hydrochloride) (PAH), and compared them to data obtained on PDL or PAH monolayers. The seeding of ECs on polyelectrolyte films showed a good morphology, allowing ECs to resist physiological shear stress better compared to ECs seeded on glass or fibronectin (Fn)-coated glass. Moreover, the interactions of ECs with ~PSS ~PAH ending films do not disturb the transduction of the adhesion signal, confirming the biocompatibility of such a film type.

Mhamdi et al [32] prepared three different types of film: a thin dense film made of PSS/poly (allylamine hydrochloride) (PSS/PAH), thick film of hyaluronan/poly (L-lysine) (HA/PLL) and poly (L-glutamicacid)/poly (L-lysine) (PGA/PLL) films for test the adhesion, proliferation and cell morphology of primary human fibroblasts on top of the different films. They found correlation only between cell adhesion rate and the polar components of the PEM surface. In parallel, surface hydrophobicity and roughness were found to be unfavorable for both adhesion and proliferation. Adhesion and proliferation were found not to be correlated, the best adhesion being observed for the (PGA/PLL) films whereas the best proliferation was found to be for the (PSS/PAH) film. A particular poor proliferation rate was found with HA based films

Generally, animal cell usually carry negatively charge and therefore give rise to adhesion of cationic polyelectrolyte as the first step of interaction, while usually no columbic interaction takes place with polyanion. The adhesion steps of cationic compounds can be followed by further step of interaction as a disordering and destruction of the cell membranes, and subsequent reactions with cell components with eventually lethal consequences for living cell. Other modifications such as chemical cross linking have improved the PEMs stability and cell adhesion. Certain surface such as polysaccharide films made by Layer-by-Layer buildup has been investigated for used as cell attachment and bioactive cell surface coating.

2.3.4 Natural polyelectrolyte for cell adhesion

Relevant classes of natural polymers for preparing water-soluble polyelectrolyte are polysaccharides, protein, collagen, gelatin, chitosan etc.

Depending on the starting material and polyelectrolyte structure intended, polyelectrolyte can be obtained from these biopolymers by several routes:

(1) isolation of a preformed polyelectrolyte from the moiety of the natural product by an extraction / precipitation technique,

(2) isolation by a combination of extraction and chemical modification in order to liberate a preformed ionogenic group and/or to degrade the natural products for obtaining a soluble polyelectrolyte, and

(3) derivatization of an isolated nonionic polymer to an anionic or cationic polyelectrolyte.

A general problem in preparing polyelectrolyte from natural polymer arises from the species dependent composition of latter, which can be more or less pronounced, but always has to be taken into account with regard to reproducibility of end-product properties [26]. In this section the synthesis and characterization of renewable sources based natural polymers and their properties are summarized.

2.3.4.1 Gelatin

Gelatin (also called gelatine) is prepared by the thermal denaturation of collagen, isolated from animal skin and bones, with very dilute acid. It can also be extracted from fish skins. Gelatin contains a large number of glycine (almost 1 in 3 residues, arranged every third residue), proline and 4-hydroxyproline residues. A typical structure is Ala–Gly–Pro–Arg–Gly–Glu–4Hyp–Gly–Pro– [33]. The molecular structure of gelatin is presented Fig 2.11. Gelatin is a heterogeneous mixture of single or multistranded polypeptides, each with extended left-handed proline helix conformations and containing between 300- 400 amino acids. The triple helix of collagen extracted from skin and bones, as a source for gelatin, is composed of two a1(I) and one a2(I) chains, each with molecular mass ~95 kD, width ~1.5 nm and length ~0.3 mm. Gelatin consists of mixtures of these strands together with their oligomers and breakdown (and other) polypeptides. Solutions undergo coil-helix transition followed by aggregation of the helices by the formation of collagen-like right-handed triple-helical proline/hydroxyproline rich junction zones. Higher levels of these pyrrolidines result in stronger gels. Each of the three strands in the triple helix require 25 residues to complete one turn; typically there would be between one and two turns per junction zone. Chemical cross-links can be introduced to alter the gel properties, using transglutaminase to link lysine to glutamine residues or by use of

glutaraldehyde to link lysine to lysine. There are two types of gelatin dependent on whether or not the preparation involves an alkaline pretreatment, which converts asparagine and glutamine residues to their respective acids and results in higher viscosity [34]. Acid pretreatment (Type A gelatin) utilizes pigskin processed. Whereas alkaline treatment (Type B gelatin) makes use of cattle hides and bone. Gelatin is primarily used as a gelling agent forming transparent elastic thermoreversible gels on cooling below about 35^0C, which dissolve at low temperature to give 'melt in the mouth' products with useful favor-release. In addition, the amphiphilic nature of the molecules endow them with useful emulsification (e.g. whipped cream) and foam-stabilizing properties (e.g. mallow foam). On dehydration, irreversible conformational changes take place that may be utilized in the formation of surface films. Such films are strongest when they contain greater triple-helix content. Gelatin is also used as a fining agent to clarify wine and fruit juice.

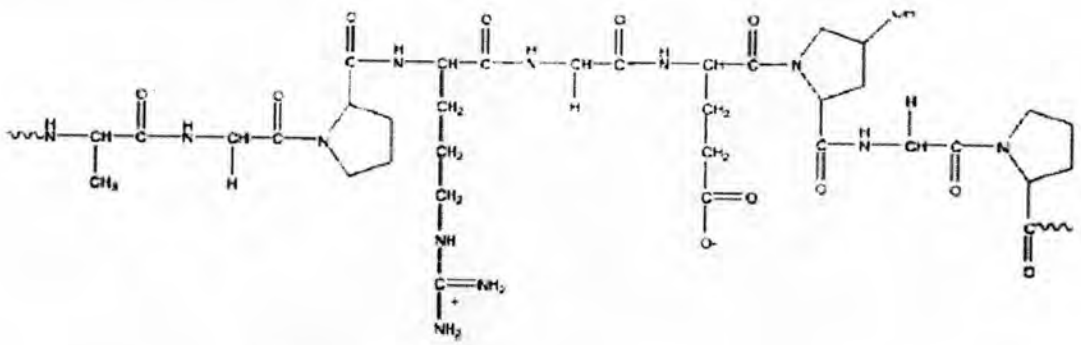


Figure 2.11 The general structure of gelatin

The amphoteric character of gelatin is due to the amino acids functional groups and the terminal amino and carboxyl groups created during hydrolysis. In strongly acid solution, the gelatin is positively charged and migrates as a cation in the electric field. In strongly alkaline solution, it is negatively charged and migrated as an anion. The intermediate point, where not charge is zero and no migration occurs, is known as the isoelectric point (IEP) and is designated in pH units. A related property, the isoelectric point, can be determined by utilizing a mixed-bed ion-exchange resin to remove all nongelatin cations and anions. The resulting pH of gelatin solution is the isoelectric point and is expressed in pH units. The isoelectric point is reproducible, whereas the isoelectric point depends on the salts present [33]. Type A gelatin has a broad isoionic region between pH 7 – 10 ; type B is in a lower, more reproducible

region, reaching an isoelectric point of pH 5.2 after 4 weeks of liming, which drops to 4.8 after prolonged or more vigorous liming processes. The isoelectric point can also be estimated by determining a pH value at which a gelatin solution exhibits maximum turbidity. Many isoionic point references are recorded as isoelectric points even though the letter is defined as a pH at which gelatin has net charge of zero and thus shows no movement in the electric field.

Coating a surface with gelatin can enhance cell attachment to that surface. Through experiments carried out by Fibrogen and multiple collaborators, Fibrogen's recombinant human gelatins (types A and B) have been proven to perform as a potent substrate for cell attachment of various cell types. Thus, fibrogen's recombinant human collagens are appropriate for use in applications where enhanced cell attachment is desired. Collagen has been also used as a hemostat for decades to stop bleeding and to facilitate the wound healing process, and to reduce the risk of post-surgical complications. Type A gelatin appears first in a wound and initiates the haemostatic process. However, type B gelatin is the most readily available type in commercial quantities. With its unique recombinant technology, fibrogen can produce pure type B collagen, offering a consistent product with superior homeostatic properties compared to animal-derived gelatin. In addition, 3-D matrix formulations (e.g., sponges) of recombinant human type A gelatin demonstrate superior mechanical integrity, larger surface area, and higher homeostatic activity than bovine gelatin in experimental models.

Lin et al. [35] investigated surface modification of Poly (L-lactic acid) (PLLA) by gelatin at different pH values through electrostatic self-assembly. X-ray photoelectron spectroscopy (XPS) and water contact angle data indicated that the gelatin adsorption at pH = 3.4 resulted in much higher surface coverage by gelatin than at pH = 7.4. Furthermore, the PLLA/PSS/gelatin (gelatin used as positively charged) exhibited better cell compatibility than the PLLA/PAH/gelatin (gelatin used as negatively charged), exceeding that of TCPS, mainly because of its higher gelatin coverage on the surface. These results clearly indicate that the solution pH is critical in the application of gelatin as a modifier via electrostatic assembly to improve the cell compatibility of synthetic polymers.

Cai et al. [36] improved the surface biocompatibility of titanium films based on the polyelectrolyte-mediated electrostatic adsorption of chitosan and gelatin. The results showed that titanium films could be modified with chitosan/gelatin which may

affect the biocompatibility of the modified titanium films. Moreover, cells adhered to LBL-modified titanium films displayed a significant difference compared to the control sample. The osteoblasts cultured in TCPS showed higher viability comparable to that of uncoated titanium in this study. Foe cell morphology, compared with that of control titanium film, osteoblasts attached on LBL-modified titanium films were fully spread.

2.3.4.2 Chitosan

Chitosan, poly-β (1,4)-2-amino-2-deoxy-D-glucose, is the deacetylated product of chitin, poly (N-acetyl-D-glucosamine), a natural polymer found in the exoskeletons of crustaceans and insects and in the cell wall of fungi and microorganisms. The structure of chitosan is presented in Figure 2.12. Chitin with a deacetylation degree (DD) of 75% or above is generally known as chitosan, which can be considered as a copolymer composed of glucosamine and N-acetylglucosamine units and dissolves in dilute organic acids, providing clear, homogeneous, and viscous solution [37]. Thus, the chemically active groups in the chitosan structure are the free amine groups, located in the C_2 position of the glucose residue in the polysaccharide chain, and the hydroxyl groups, with both being susceptible to modification. As a primary aliphatic polyamine, chitosan is involved in all of the reactions typical of amines. Most of the applications of chitosan are based on the polyelectrolyte nature and chelating ability of the amine group of the macromolecules, and such properties are mainly governed by the acidity of the $-NH^+_3$ group. The weak-base anion-exchange ability of pure chitosan has been applied in the development of surface-modified sensors for anion detection, based on the casting of chitosan films onto the surface of glassy carbon electrodes. Nevertheless, these devices lack of long-term stability properly because of alteration of the characteristics of chitosan films.

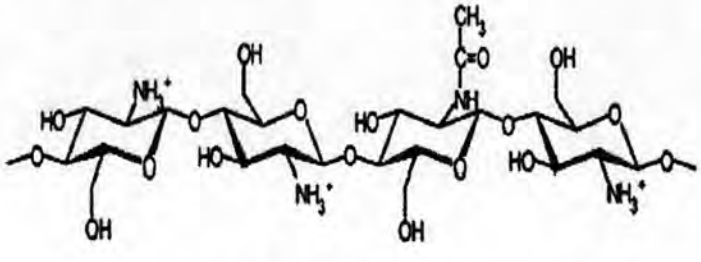


Figure 2.12 Molecular structure of Chitosan

Swelling is mainly influenced by ionic interactions between chitosan chains, which depend on the crosslinking density set during the formation of the network. An increase in crosslinking density induces a decrease in swelling and pH-sensitivity, by improving the stability of the network [38]. However, in ionically crosslinked the crosslinking density is further modified by external conditions after administration, mainly by the pH of the application medium. It influences the global charge densities of chitosan and crosslinker, which directly determine the crosslinking density, interactions and swelling. Ionically crosslinked cannot only swell in acidic but also in basic conditions, which extends their potential applications. If the pH decreases, the charge density of the crosslinker and therefore the crosslinking density decrease, which leads to swelling. Moreover, swelling is favoured by the protonation and repulsion of chitosan free ammonium groups. If the pH increases, the protonation of chitosan decreases and induces a decrease of the crosslinking density, allowing swelling. If the pH becomes too high, amino groups of chitosan are neutralized and ionic crosslinking is inhibited.

Chitosan is currently receiving a great deal of attention for medical and pharmaceutical applications [43, 44]. The main reasons for this increasing interest are undoubtedly due to its appealing intrinsic properties. Indeed, chitosan is known for its biocompatibility allowing its use in various medical applications such as topical ocular application, implantation or injection. Moreover, chitosan is metabolized by certain human enzymes, e.g. lysozyme, and can be considered as biodegradable. In addition, it has been reported that chitosan acts as a penetration enhancer by opening epithelial tight-junctions. Due to its positive charges at physiological pH, chitosan is also bioadhesive, which increases retention at the site of application. Chitosan also promotes wound-healing and has bacteriostatic effects. Finally, chitosan is abundant in nature, and its production is of low cost and is ecologically interesting.