CHAPTER I
INTRODUCTION

Nowadays, many of surgical treatments have required tissue and organ substitutes to repair or replace damaged or diseased organs or tissues. A number of tissues engineering approaches have been developed to generate new, natural tissue replacements by creating implantable devices composed of tissue-specific cells on biodegradable polymer scaffolds (Langer, 1993). Polymer scaffolds offer the advantage of being able to use either allogenic cells or autologous transplants (Vacanti, 1993). Scaffold-guided tissue regeneration involves seeding highly porous biodegradable scaffolds with donor cells and/or growth factors, then culturing and implanting the scaffolds to induce and direct the growth of new tissue. The goal is for the cells to attach to the scaffold then replicate, differentiate and organize into normal healthy tissue as the scaffold degrades. Scaffolds must posses many key characteristics such as high porosity and surface area, structural strength, and specific three-dimensional shape. Biodegradable and biocompatible polymers have been attractive candidates for scaffolding materials since they facilitate cell attachment and maintenance of differentiation function (Yannas *et al.*, 1981). In addition, they degrade as the new tissues are formed, eventually without inflammatory reaction or toxic degradation (Ma *et al.*, 2001). A number of synthetic and natural degradable polymers such as poly(glycolic acid) (PGA) (Cooper *et al.*, 1991), poly(lactic acid) (PLLA) (Bostman, 1991), poly(DL-lactic-co-glycolic acid) (PLGA) (Murphy *et al.*, 2000), collagen (Yannas, 1988), and chitosan (Elein *et al.*, 1998;Hutmacher *et al.*, 2000) are currently being used as scaffolds. These polymers meet the requirements and have been used in the tissue engineering of cartilage (Cao *et al*, 1997), bone (Ishaug *et al.*, 1997), skin (Zacchi *et al.*, 1998), ligament (Lin *et al.*, 1999), bladder (Oberpenning *et al.*, 1999), and liver (Cusick *et al.*, 1997).

Chitosan is an aminopolysaccharide derived from chitin by deacetylation under alkali condition. It is a copolymer consisting of β(1→4)-linked 2-acetamido-D-glucose unit and β(1→4)-linked 2-amino-D-glucose unit. Chitosan has been applied to conduct the extracellular matrix (ECM) formation in tissue regenerative

therapy (Cima *et al.*, 1991). The superior tissue compatibility of chitosan may primarily be attributed to its structural similarity to glycosaminoglycan in the extracellular matrix (Hollinger *et al.*, 1986). Chitosan has been reported to stimulate the activity of growth factors of skin (Peluso *et al.*, 1994). In vitro studies have clarified the contribution of chitosan in wound healing through its activation of fibrogenic mediators such as growth factors. Increased expression of growth factors enhanced fibroblastic activity and promoted fibrous tissue synthesis (Usami *et al.*, 1994). In addition, one of chitosan's most promising features is its excellent ability to be processed into porous structures for use in cell transplantation and tissue regeneration (Madihally *et al.*, 1999).

Chitin and chitosan derivatives have been evaluated to overcome their limited solubility. To obtain water-soluble chitin and chitosan derivatives, one of the chemical modification techniques is carboxymethylation of chitin and chitosan under alkaline condition to give carboxymethylchitin (CM-chitin) and carboxymethylchitosan (CM-Chitosan) (Muzzaraelli *et al.*, 1988). CM-chitin is a polyelectrolyte and soluble not only in acidic media but at any pHs. CM-chitin has been considered to be one of advanced carriers for the polymeric drug since CM-chitin was reported as highly biodegradable and non-toxic mucopolysaccharide in animal body (Nishimura *et al.*, 1984). In addition, intraperitoneal injection of CM-chitin was reported to activate mouse peritoneal macrophages for short periods and to induce the mitogenic activity very faintly (Nishimura *et al.*, 1986). CM-chitosan, a water-soluble chitosan derivative, has unique chemical, physical and biological properties such as high viscosity, large hydrodynamic volume, non toxicity, biocompatibility, and film- and gel-forming capabilities. CM-chitosan has been reported for its unique excellent moisture retention ability (Chen *et al.*, 2002). Moreover, it can be easily being dissolved in the cell cultured media of skin fibroblast and play an important role in wound healing and it also stimulates the extracellular lyzozyme activity of skin fibroblast. As a result, CM-chitosan has been widely investigated as biomaterial for medical applications including tissue engineering (Krause *et al.*, 2001; Chen *et al.*, 2002).

Fabrications of porous scaffolds have been achieved in several ways, in particularly by freeze-drying. This gentle drying method is favorable for alleviating

the denature degree of nature molecules and obtain uniformly porous scaffold with interconnected pore using only water (Kang *et al.*, 1999). Accordingly, freeze-drying technique has been used to prepare various polymer scaffolds such as gelatin (Kang *et al.*, 1999), alginate (Shapiro *et al.*, 1997), chitosan (Madihally *et al.*, 1999), and so on.

Crosslinking to form a polymer network is useful for improving mechanical properties and reducing solubility in good solvents of a polymer. Both physical and chemical approaches for crosslinking of chitosan and its derivatives have been reported, such as thermal heating (Ma *et al.*, 1996), ultraviolet irradiation (Weadock *et al.*, 1995), steam treatment (Janvikul *et al.*, 2003), and treatment with glutaraldehyde (Osborne *et al.*, 1999) or carbodiimide (Zeeman *et al.*, 1999). To avoid introducing potential cytotoxic chemical residuals and sustain the excellent biocompatibility of biomaterials, steam treatment has been employed as a promising mean to crosslink polymer scaffolds because this technique is simple, economical, non-toxic, and to achieve sterilization for medical applications.

In this study, porous scaffolds of chitosan, carboxymethylchitin (CM-chitin), and carboxymethylchitosan (CM-chitosan) were fabricated via freeze-drying technique and further crosslinked by using steam treatment in an autoclave. The effects of steaming temperatures on crosslinking of the polymers were investigated. The degrees of swelling, mechanical properties, and morphology of the steamed scaffolds were evaluated.

1.1 Theoretical Background

1.1.1 Scaffold

The goal of tissue engineering is to develop materials and approaches which can be used to facilitate repair, regeneration, or replacement of damaged of diseased tissue.

Scaffold-guided tissue regeneration involves seeding highly porous biodegradable scaffolds with donor cells and/or growth factors, then culturing and implanting the scaffolds to induce and direct the growth of new tissue.

Scaffold is porous three-dimensional temporary matrix, which acts as a substrate to promote cell adhesion, maintenance of differentiated function without hindering proliferation, template to organize and direct the growth of cell and help in the formation of extracellular matrix.

In order to achieve restoration of tissue architecture, the tissue scaffold may be required to perform a variety of tasks.

- Highly porosity is needed for cell seeding or migration throughout the material (typical porosities are greater than 90%). Pore size plays a critical role in both tissue ingrowth and the internal surface area available for cell attachment. The pore size must be within a critical range (usually 100-200 μm): the lower bound is controlled by the size of the cells (~20 μm) while the upper bound is related to the specific surface area through the availability of binding sites. The porosity must be interconnected to allow ingrowth of cells, vascularization and diffusion of nutrients, sufficient to replace or restore organ function can be cultured.
- Large surface to volume ratio
- Biodegradability allows the gradual and orderly replacement of the scaffold with functional tissue.
- Non-toxicity
- Biocompatibility
- Sterilizability
- Processability to be formed into desired shape

- Including specific interaction with or mimic of ECM, growth factors, or cell surface receptors.
- Sufficient mechanical integrity to resist handling during implantation and *in vivo* loading

1.1.2 Polymer Scaffold Processing

Polymer scaffolds must posses many keys characteristics, including high porosity and surface area, structural strength, and specific three-dimensional shapes, to be useful as materials for tissue engineering. These characteristics are determined by the scaffold fabrication technique, which must be developed such that it does not adversely affect biocompatibility of the material of construction. The processing technique should allow the manufacture of scaffolds with controlled porosity and pore size, both important in organ regeneration and also affect the mechanical properties of scaffold. Several of fabrication techniques are described as following. Each technique has its advantages, but none can be considered as an ideal method of scaffold fabrication to be employed for all tissues.

a) Freeze drying

Freeze drying is a novel method which employs water present in swollen hydrogels as a porogen for shape template was suggested for preparing porous materials. After freezing the swollen hydrogels, the ice formed within the hydrogel network was sublimated by freeze-drying. This simple method produced porous hydrogels. Irrespective of any rinsing and washing processes, water was homogenously distributed into the hydrogel network; allow the hydrogel networks to uniform enlarge and the ice acts as a porogen during the freezing processes. The porosity of dried hydrogels can be controlled by the size of ice crystals formed during freezing. The present freeze-drying procedure is a bio-clean method for formulating biodegradable sponges of different of different pore structures without use of any additives and organic solvents (Mao, *et al*., 2003). Typical pore sizes are in the range of 100-200 μm while typical porosities are in the range of 90-99%.

2) Fiber bonding

It has been used to make matrices for attachment of liver cells. A non-bonded structure of PGA fibers is immersed in a solution of PLLA (which is not a solvent of PGA). The solvent is evaporated, leaving an interpenetrating network of PGA and PLLA which is then heated to above the melting temperature of PGA bond the fibres at their junctions. The PLLA is then dissolved in methylene chloride to give a porous scaffold of PGA.

3) Solvent casting and particulate leaching

Sieved NaCl particles are combinded with PLLA or PLGA powder in a solution of chloroform or methylchloride. The solvent is evaporated (NaCl is insoluble in PLLA or PLGA). The remaining solid is then heated to above the melting point of the polymer to distribute it uniformly. After cooling, the material is immersed in water to leach out the salt, leaving a porous structure. Porosities in the range of 20-93% and pore sizes in the range of 30-120 μm have been achieved with this technique.

4) Membrane lamination

Membrane lamination offers a means of constructing highly porous biomaterials with anatomical shapes. However, the lamination procedure is useful only if it preserves the uniform porous structure of the original membranes. Also, the boundary between two layers must be indistinguishable the bulk of device. The methodology to process biodegradable polymer scaffolds with anatomical shapes involves the construction of a contour plot of the particular three-dimensional shape. The shapes of contours are cut from highly porous biodegradable membranes prepared using the solvent casting and particulate leaching. A small quantity of chloroform is then coated onto the contacting surfaces of the adjacent membranes and a bond is formed. Thus, the desired three-dimensional shape is constructed layer by layer.

5) Melt molding

Melt molding, an alternative method of constructing three-dimensional scaffolds, has any advantages over the membrane lamination. PLGA scaffolds have been produced by leaching PLGA/gelatin microsphere composites formed using a molding technique. A fine PLGA powder is mixed with previously

sieved gelatin microspheres and poured into a Teflon mold, which heated above the glass transition temperature of the polymer. The PLGA/gelatin microsphere composites is subsequently removed from the mold and placed in distilled-deionized water. The gelatin, which is soluble in water, is leached out, leaving a porous PLGA scaffold with geometry identical to the shape of the mold.

6) Extrusion

It has not been widely used for the fabrication of polymer scaffolds in tissue engineering applications Widmer *et al.*, (1998), however, developed an extrusion process in combination with the solvent casting technique to fabricate porous, biodegradable tubular conduits for the purpose of peripheral nerve regeneration. Briefly, PLGA or PLLA polymers are first fabricated into composites wafers with NaCl as a porogen by the solvent casting technique. The wafers are cut to the desired length and placed in a customized extrusion tool. The tool is mounted on a hydraulic press and heated to the appropriate processing temperature. Pressure is then applied between the nozzle and the piston of the extrusion tool and the polymer/salt composite is extruded as a tube. After cooling, the salt is leached out by immersion in water and the entire scaffold is then vacuum dried.

7) Three-dimensional printing

This technique can also be used to create porous structures. A powder of PLA or PGA is mixed with NaCl and a layer of the powder is then spread into a powder beds. A printhead nozzle is then used to deposit chloroform solvent over the layer; the chloroform acts as a binder by partially dissolving the polymer. After the nozzle passes over the first layer, the powder bed is lowered; a second layer of powder is placed in the bed and bonded by the deposition of more chloroform. After the desired size of the sample is built up by repeating this process, any remaining chloroform is removed by drying and the salt is leached out by immersion in water. Porosities up to 95% can be achieved by this technique. The pore size, controlled by the size of the NaCl particles, is typically 100 μm.

8) Gas foaming

This method has been developed to create porous matrices utilizing a gas foaming technique to avoid the use of organic solvents. Carbon dioxide gas is dissolved in a polymer under high pressure (800 psi and 25°C) and then expanded to

form bubbles by releasing the pressure. Scaffolds with a porosity of 93% and pore sizes of roughly 100 μm have been made this way.

9) Phase separation

The ability to deliver bioactive molecules from a degrading polymer scaffold to cells within or surrounding the scaffold is an attractive one because it could potentially allow manipulation of tissue growth and cell function. In order to achieve this goal, the scaffold manufacturing process must lend itself to incorporation of bioactive molecules and must not cause any loss of drug activity due to exposure to harsh chemical or thermal environments. These are the following phase-sepation technique; first, the polymer is dissolved in a solvent (molten phenol or naphthalene) at a low temperature (55°C for phenol and 85°C naphthalene). The bioactive molecule is then dissolved or dispersed in the resulting homogenous solution, which is then cooled in a controlled fashion until liquid-liquid phase separation is induced. The resulting biocontinuous polymer and solvent phases are then quenched to create a two-phase solid. The solidified solvent is then removed by sublimation, leaving a porous polymer scaffold with bioactive molecules incorporated within the polymers.

10) Polymer/ceramic composite foams

An alternative method is widely utilized in bone regeneration. Hydoxyapatite short fibres and a porogen (either gelatin microspheres or salt particles) are first dispersed in a PLGA/dichloromethane solution. The result, after solvent evaporation and dring, is a composite material consisting hydroxyapatite fibres and a porogen embedded in a PLGA polymer membrane. The required three-dimensional shape is then achieved by compression molding the composite material. Subsequent leaching of the porogen then leaves a porous composite scaffold of PLGA reinforced with short hydroxyapatite fibres. Within a range of fiber contents, these scaffolds have superior compressive strength compared to nonreinforced PLGA scaffolds of the same porosity.

1.1.3 Chitin and chitosan

Chitin is a naturally occurring polysaccharide found in the supporting material of crustaceans, insects, etc. It is a (1→4)-β-linked glycan composed of 2-acetamido-2-deoxy-D-glucose (N-acetylglucosamine) (Figure 2.1). It is a highly insoluble material resembling cellulose in its solubility and low chemical reactivity. It may be regarded as cellulose with hydroxyl at position C-2 replaced by an acetamido group. Like cellulose, it functions naturally as a structural polysaccharide. Chitin is white, hard, non-elastic, nitrogenous polysaccharide and the major source of surface pollution in coastal areas. In nature, chitin serves as 'glue' for chemical components making up the delicate wings of insects and the crunchy integuments of crustaceans such as crabs and shrimps. To obtain chitin from crustacean shell waste, it is usually ground and mixed with a dilute aqueous sodium hydroxide solution to dissolve protein. The residual material is then treated with a dilute aqueous hydrochloric acid solution to dissolve calcium carbonate, leaving behind chitin as a white powder.

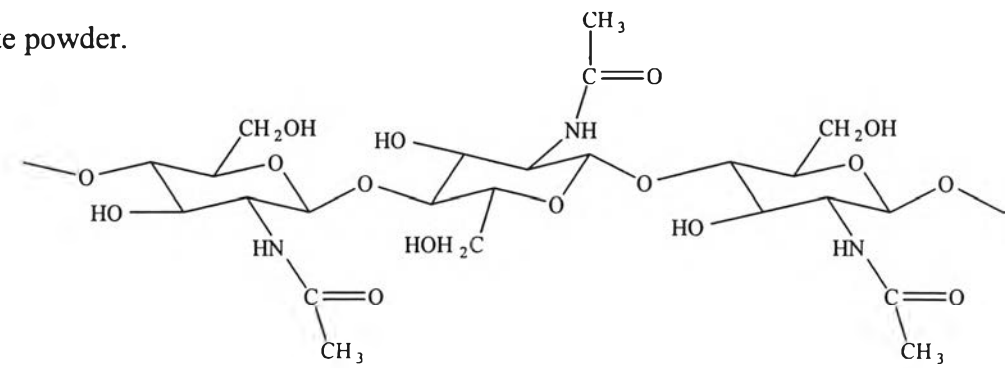

Figure 1.1 Chemical structure of chitin.

Chitosan, a polyaminosaccharide, [β(1→4)-2-amino-2-deoxy,β-D-glucan], is the N-deacetylated derivative of chitin, although this N-deacetylation is almost never complete. The structure of chitosan is shown in Fig.1.2.

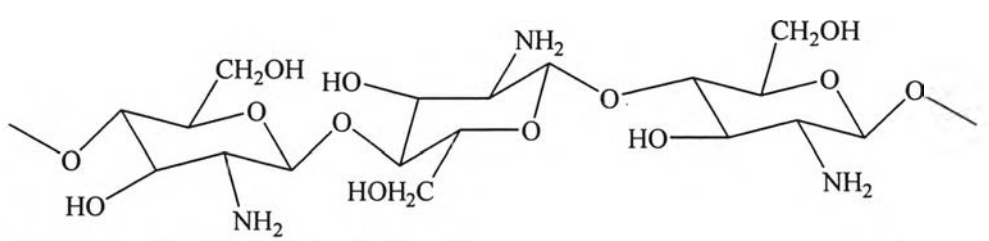

Figure 1.2 Chemical structure of chitosan.

Chitosan is a natural polycationic polymer which is biodegradable, which makes to be degraded by the action of enzymes from microorganisms, biocompatibility, which is compatible to living tissues, non-toxicity with its degradation products being known natural metabolites, ability to improve wound healing and/or clot blood, ability to absorb liquids and to form protective coatings, nonantigenicity, and selective binding of acidic lipid, thereby lowering serum cholesterol level. It also can be processed into powders, film, gels, beads, tablets, fibers, sponge, and fabric formats. As a result, chitosan has been utilized in a number of biomedical applications such as wound dressing, suture, drug delivery and controlled release system etc. Moreover, chitosan is hydrolysable by lysozyme and chitosanase. Lysozyme is a very important enzyme present in the human body fluid and acts as an endohydrolase and catalyzes hydrolysis of N-acetylglucosaminide (1→4)-β linkages. The N-acetylglucosamine moiety in chitosan is a structural feature also found in the GAGs (glycosaminoglycan). Since GAGs properties include many specific interactions with growth factors, receptors, and adhesion proteins, this suggests that the analogous structure in chitosan may also have related bioactivities. It was shown that in the area of wound healing chitosan can reduce the scar tissue (fibroplasia) by inhibiting the formation of fibrin in the wounds. Also, chitosan and chitosan derivatives affect macrophage activity, which will influence the wound healing process. It has been reported to stimulate the activity of growth factors. In vivo studies have clarified the contribution of chitosan in wound healing through its activation of fibrogenic mediators such as growth factors enhanced fibroblastic activity and promoted fibrous tissue synthesis (Usami *et al.*, 1994). Chitosan can promote tissue growth and differentiation in tissue culture and play the role of a biological primer for cell-tissue proliferation and reconstruction. As a result, all these interesting properties of chitosan make this natural polymer suitable for making scaffold (Francis *et al.*, 2000). In addition, one of chitosan's most promising features is its excellent ability to be processed into porous structures for use in cell transplantation and tissue regeneration (Madihally, 1999)

1.1.4 CM-chitin and CM-chitosan

To obtain water-soluble chitosan derivatives, one of the chemical modification techniques is carboxymethylation of chitin and chitosan under alkaline condition to give CM-chitin (carboxymethylchitin) and CM-chitosan (carboxymethylchitosan), respectively. (Muzzaraelli *et al.*, 1988). The structure of CM-chitin and CM-chitosan are shown in Fig.1.3 and 1.4.

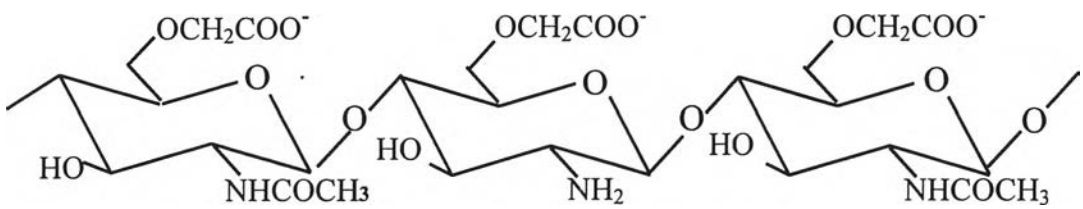

Figure 1.3 Chemical structure of CM-chitin.

Carboxymethyl-chitin (CM-chitin), a water-soluble chitin derivative and one of hydrogel polymers can be achieved by carboxymethylation of chitin under alkaline conditions. CM-chitin is a polyelectrolyte with properties resembling those of carboxymethyl-cellulose (CMC). CM-chitin is soluble not only in acidic media but at any pHs. CM-chitin has been considered to be one of advanced carriers for the polymeric drug, since CM-chitin was reported as highly biodegradable and non-toxic mucopolysaccharide in animal body (Nishimura *et al.*, 1984). In addition, intraperitoneal injection of CM-chitin was reported to activate mouse peritoneal macrophages for short periods and to induce the mitogenic activity very faintly (Nishimura *et al.*, 1986).

CM-chitosan is not only soluble to both acidic and basic physiology circumstances, but also has unique chemical, physical and biological properties such as high viscosity, large hydrodynamic volume, non toxicity, biocompatbility and film, gel-forming capabilities. It is unique for its excellent moisture retention ability. Moreover, it can be easily be dissolved in the cell cultured media of skin fibroblast and play an important role in wound healing and it also stimulates the extracellular lyzozyme activity of skin fibroblast (Chen *et al.*, 2002). As a result, CM-chitosan

has been widely used as biomaterial in medical applications such as tissue engineering (Krause *et al.*, 2001; Chen *et al.*, 2002).

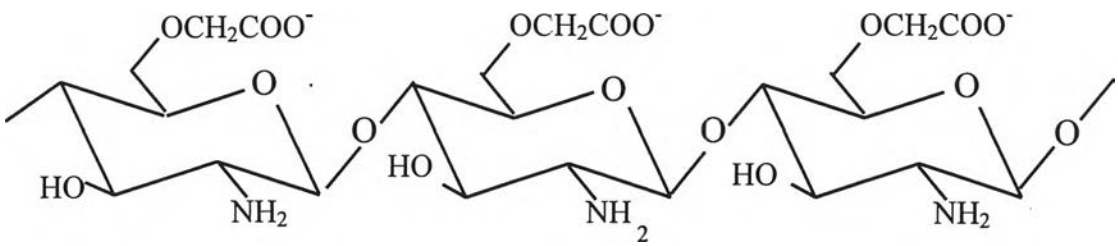

Figure 1.4 Chemical structure of CM-chitosan.