CHAPTER II
THEORETICAL BACKGROUND AND LITERATURE REVIEW

2.1 General Bone Fracture Healing

Up to present, techniques for bone fracture therapies have been developed as seen in clinical treatments of autografts, allografts, xenografts, 3-dimensional porous scaffold fibrin glue whereas other artificial substitutes using metals, synthetic cements and bioceramics have been reported for the possibility to use in bone therapy (Goldberg *et al.*, 1987, Costantino *et al.*, 1994). However, each treatment has its problems and limitations, for example, autografts are associated with donor shortage and donor site morbidity whereas allografts and xenografts have the risks of disease transmission and immune responses (Costantino *et al.*, 1994). Synthetic materials wear and do not have the performance as the real bone. Currently, the possibility to use 3D porous scaffold by loading with specific living cells and/ or tissue-inducing factors to launch a tissue regeneration or replacement close to the natural system has been proposed (Ma *et al.*, 2001). The use of fibrin glue, a composite of fibrinogen and thrombin, is a good approach as the material play an important role in blood clotting and wound healing. Biological vesicles for cell transplantation are also a good candidate when considering biocompatibility, biodegradability, and binding capacity to cells (Keller *et al.*, 1985). Fibrin-stabilizing factors of fibrin glue which favors migration of undifferentiated mesenchymal stem cells (MSCs) on the highly cross-linked structure of the glue, and enhances proliferation of these cells were reported (Marktl and Rudas, 1974, Kasai *et al.*, 1983).

2.2 Advanced Bone tissue Scaffold

Nano-scaffold materials for bone tissue engineering should also be osteoconductive so that osteoprogenitor cells can adhere and migrate on the scaffolds, differentiate, and finally form new bone (Zhang, *et al.*, 1999). Biodegradable polymers, mainly polyesters such as poly(lactic acid) (PLA), poly(glycolic acid)

(PGA) and their copolymers (PLGA), have been widely used to develop porous 3D scaffolds using various fabrication techniques (Zhang, *et al.*, 1999). These synthesized materials have been demonstrated to be biocompatible and degradable into non-toxic components with a controllable degradation rate in vivo (Visscher *et al.*, 1985). Another major class of biomaterials for bone repair is ceramics such as tricalcium phosphate (TCP) (Li *et al.*, 2002). Being similar to the mineral component of natural bone, they showed good osteoconductivity and bone bonding ability (Bonfiglio *et al.*, 1972). However, the main limitation for the use of HA ceramics was their inherent brittleness and difficulty in processing (Cooke *et al.*, 1992). To combine the osteoconductivity of calcium phosphates and good biodegradability of polyesters, polymer/ ceramic composite scaffolds have been developed for bone tissue engineering either by direct mixing or by a biomimetic approach (Thomson *et al.*, 1998). As compared to plain polymer scaffolds, neo tissue matrix was formed only in the surface layer (less than 240 mm) (Ishaug *et al.*, 1997), the composite scaffolds supported cells growth and neo tissue formation throughout the scaffold including in the center of the scaffold (Ma *et al.*, 2001). Polymer/ ceramic composite scaffolds obtained mimic the natural bone to some extent. Natural bone is composed of inorganic compound mainly carbonated hydroxyapatite (HA) and organic compounds such as collagen. The nanometer size of the inorganic component (mainly bone-like apatite) in natural bone is considered to be important for the mechanical properties of the bone (Rho *et al.*, 1998). Recent research in this field also suggested that better osteoconductivity would be achieved if synthetic HA could resemble bone minerals in composition, size and morphology (Du *et al.*, 1998) (Fig. 2.1).

In addition, nano-sized HA may have other special properties due to its small size and large specific surface area. Many studies have shown a significant increasing in protein adsorption and osteoblast adhesion on the nano-sized ceramic materials compared to traditional micron-sized ceramic materials (Webster *et al.*, 2000).

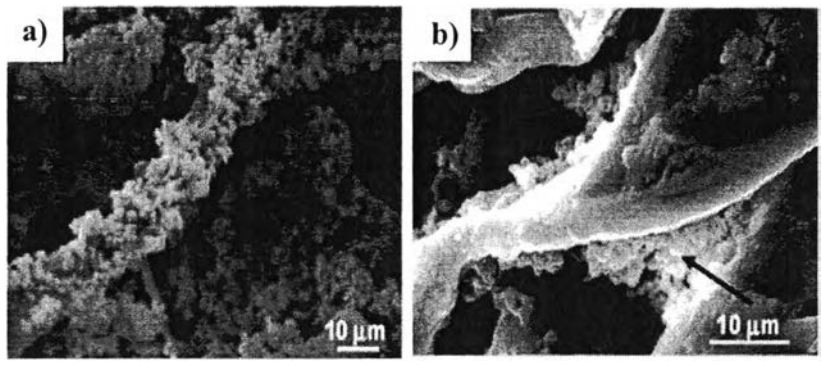
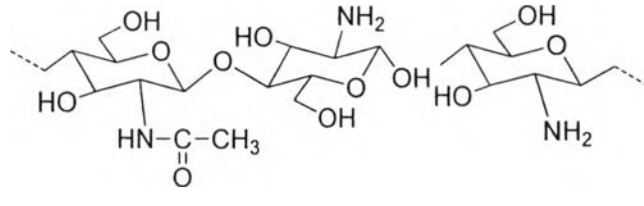


Fig. 2.1 SEM micrographs of cross-sectional cut of the mineralized scaffold. Arrow shows the apatite crystal in the pore channels and the pore walls (a), and distribution of the nano-hydroxyapatite in the gel (b) (Manjubala *et al.*, 2006).

2.3 Chitosan: Biopolymer for Tissue Engineering

Chitosan is a linear polysaccharide composed of randomly distributed β-(1-4)-linked *D*-glucosamine (deacetylated unit) and *N*-acetyl-*D*-glucosamine (acetylated unit) (Scheme. 2.1).



Scheme 2.1 Chemical structure of chitin/ chitosan.

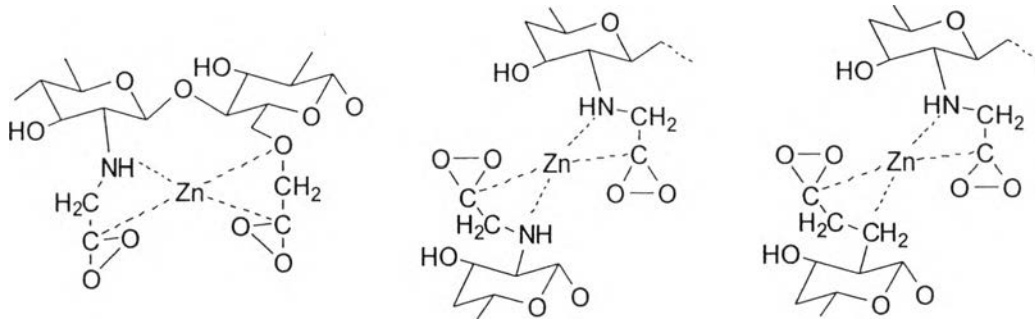
Chitosan is the most important derivative of chitin which is the second most important natural polymer in the world. Chitin is obtained from the cell walls of fungi, yeast and from the shells of crustaceans, such as crabs and shrimps (Rinaudo *et al.*, 2006, Mirko *et al.*, 2009).

Chitosan is a semicrystalline polymer and many polymorphous are reported (Mihammad *et al.*, 2010). Single crystals of chitosan were obtained using fully deacetylated chitin of low molecular weight.

Chitosan has reactive functional groups:

Hydroxyl groups (-OH), which are a primary alcohol group at C-6 and secondary hydroxyl group at C-3. In general, the primary hydroxyl group is more reactive than secondary one, and therefore most chemical reactions are occurred at C-6. These hydroxyl groups impart hydrophilicity to chitosan chains and show the inclusion properties. It can, thus, form inclusion and/ or a host-guest compound with ions or molecules, which are appropriate for industries in wastewater treatments (Shimizu *et al.*, 1995). The lone pair electrons of the oxygen atom are also reported for complexation with metal ions, e.g. Ca^{2+}, Ni^{2+}, Zn^{2+} etc. (Nishi *et al.*, 1987) (Scheme 2.2).

Amino group ($-NH_2$) of chitosan is more reactive comparing with cellulose and chitin. It can be chemically modified because chitosan has a reactive primary amino group at C-2 position. The lone pair electrons of the nitrogen atom form an interaction with metal cations or accept the proton to be a protonated species. Hence, the protonation of amino groups causes the electrostatic attraction of anionic compounds, such as anionic dyes (Guibal *et al.*, 2004).



Scheme 2.2 Chitosan complexation with metal ions.

The antibacterial and antiviral properties of chitosan are known to be formed the formation of positively charged amino group and negatively charged microorganism cell wall to result in inhibition of bacteria and virus (Kendra and Hadwiger, 1984).

Acetamide group (-NHAc) of chitosan is similar to the amino group but is rather weak in reactivity. The acetamide groups form a strong hydrogen bond network leading to the high crystallinity of chitin. The hydrogen bond via acetamide group brings about poor solubility for chitin in most solvents.

Pyranose ring of chitosan is reported for bioactivity, such as detoxification ability and cholesterol or fatty acid interaction (Muzzarelli, 1996), biocompatibility (Richardson, *et al.*, 1999), and non-toxicity (Rao and Sharma, 1992).

Glycoside linkage or glucosidic bond (C-O-C) of chitosan provides biodegradability via enzymatic hydrolysis, i.e. chitinase, chitosanase, and lysozyme. Biodegradability in nature leads to chain degradation (Yamamoto and Amaike, 1997).

Chitosan is commonly used to describe a series of chitosan polymers with average molecular weight (50 kDa $\leq M_W \leq$ 2000 kDa) and degrees of deacetylation (40% < DDA < 98%). Chitosan is not soluble in water, but it is soluble in acidic aqueous media which protonate chitosan amino groups, rendering the polymer positively charged and thereby overcoming associative force between chains. When adding a strong base to the solution, chitosan still remains in solution up to a pH in the vicinity of 6.2. If pH is more than 6.2, systematically lead to the formation of hydrogel-like precipitates. This precipitation or gel formation is occurred due to neutralization of chitosan amine groups and the consequent removal of repulsive interchain electrostatic forces which subsequently allows extensive hydrogen bonding and van der Waals force between chains. The inability to maintain chitosan in solution up to a physiological pH in the region of 7.0-7.4 has been the main obstacle to date in the development of certain biomedical applications of chitosan, for example as an encapsulating or delivery system for living cells or for pH-sensitive proteins.

Chitosan has been proposed to serve as a non-protein matrix for 3D tissue growth. Chitosan could provide the biological primer for cell-tissue proliferation and reconstruction (Manjubala *et al.*, 2006). One of the most promising features of chitosan is its excellent ability to be processed into porous structures for using in cell transplantation and tissue regeneration. In the case of tissue engineering, the porous structure of chitosan provides a scaffold for bone cells to grow in as process of bone regeneration. For rapid cell growth, the scaffold must have optimal micro architecture such as pore size, shape and specific surface area.

2.4 Chitosan-based Hydrogels

Hydrogels are three-dimensional networks which are able to retain water molecules more than 99% without dissolving. Hydrogels have received much attention for preparing biomaterials as drug delivery and tissue engineering due to their significant water uptake (Park *et al.*, 1996). Hydrogels provide the flexible texture as much as natural tissue. Thus, the use as implants can be expected for good compatibility with less irritation to surround tissues (Fig. 2.2).

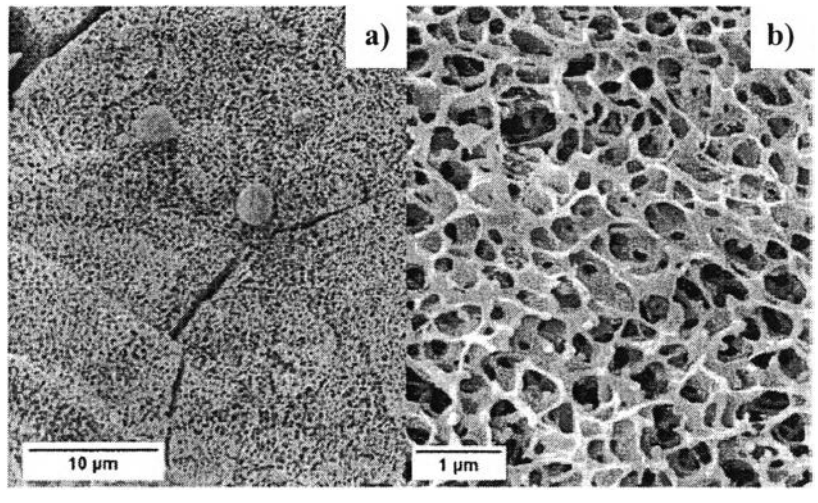


Fig. 2.2 SEM images of cross-sectional cut hydrogel formed triblock (hydrocarbon-peo-fluoro-carbon) at magnification of × 1,000 (a) and 100,000 (b) (Taribagil *et al.*, 2009).

Hydrogels prepared from chitosan and their derivatives have been reported in many literatures (Ilium *et al.*, 1998). These hydrogels can be divided in two classes; i.e., chemical hydrogels, which forms permanently cross-linked gel, and physical crosslink hydrogels, which forms reversible cross-linked gel (Lin *et al.*, 2003).

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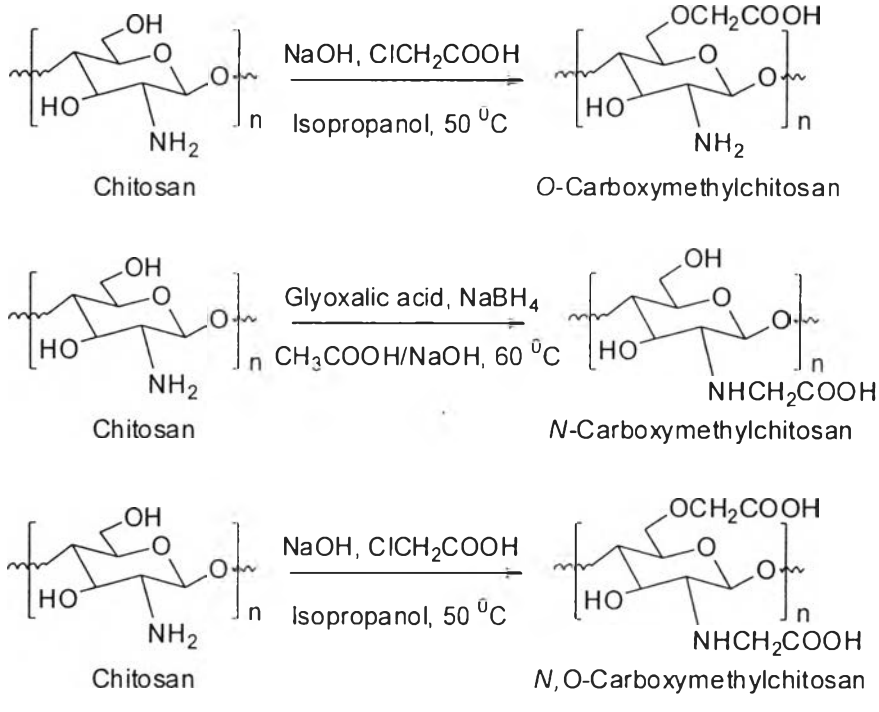
COOH, and NH_2. Hydrogels are formed via covalent linkages between polymer chains and known as materials for drug encapsulation drug, bioactive molecules. However, hydrogels might show some toxicities related to the crosslinking agent such as glyoxal, glutaraldehyde, and epichlorohydrin. To overcome this limitation of using toxic crosslinking agents, non-toxic crosslinking agents, such as diacids or diamines, and ionic crosslinking were considered as a way to form chitosan hydrogel. The key point for gelation, is that the chitosan needs to be completely dissolved in water, an if possible, at neutral pH. Several chemical modification to obtained water-based chitosan such as *O*-, *N*-carboxymethyl-chitosan, *N*-carboxymethyl-chitosan, *O*-carboxymethyl-chitosan (Scheme 2.3) (Zhou *et al.*, 2010), *N*-sulfate-chitosan (Holme *et al.*, 1997), *O*-sulfate chitosan (Focher *et al.*, 1986), *O*-butyryl-chitosan, *N*-methylene phosphonic chitosan, hydroxypropyl chitosan, *N*-trimethyl chitosan, *N*-succinyl-chitosan (Jayakumar *et al.*, 2010) are previously reported.



Scheme 2.3 Chemical modifications to obtained water-based chitosan.

Recently, a water soluble form of chitosan at neutral pH had been obtained in the presence of glycerol 2-phosphate (Chenite *et al.*, 2001, Tsai *et al.*, 2011). Stable solutions were obtained at pH 7-7.1 and room temperature, but a gel formed on

heating to about 40 °C. The sol-gel transition stage was partially reversible and the gelation temperature was depended upon experimental conditions.

2.5 Injectable Gel

Injection of in situ gel-forming biopolymers is becoming increasingly attractive for the development of therapeutic implants and vehicles (Hunter *et al.*, 1997). For example, copolymers of poly(ethylene oxide) and poly(propylene oxide) (known as Poloxamers) in aqueous solutions are well-known thermoset gel-forming materials in situ (Malmsten *et al.*, 1992). However, the gel obtained still lacks of physiological degradability and induces unexpected plasma cholesterol or triglycerides increment as found in-vivo studied on rats when injected intraperitoneally. Recently, diblock copolymers of poly(ethylene oxide) and poly(lactic acid) have been proposed as alternative materials to provide injectable drug-delivery systems because of their biodegradability and acceptance as found in-vitro case studies. A fluid material may not only fill any shape of defect but also incorporate various therapeutic agents (Fig. 2.3).

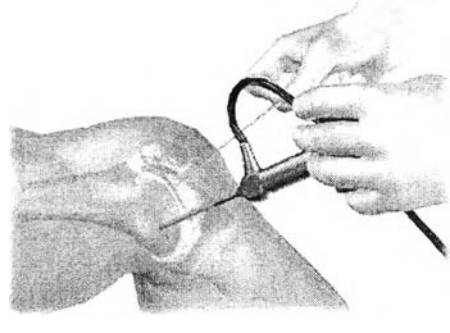


Fig. 2.3 Injectable gel for broken bone treatment (www.ekmulakatnews.com).

However, this type of material also contains residual solvents from the preformed scaffold. The advantage of the injectable gel is that there is no open surgical of the body for placement. For these reasons, several injectable biomaterials such as collagen gel (Wakitani *et al.*, 1989), polyethylene oxide (Sims *et al.*, 1996), calcium alginate (Paige et al., 1996), and fibrin glue (Silverman *et al.*, 1999; Yamada

et al., 2003) have been developed. However, several points related to degradation rates, adequate tissue penetration levels, adverse host immune responses have to be considered.

2.6 Bone Morphogenetic Proteins (BMPs)

A number of growth factors are used for inducing the formation of bone and cartilage (Dimitriou *et al.*, 2005). Members of the BMPs family are divided into at least four separate subgroups depending on their primary amino acid sequence. Group one consists of BMP-2 and BMP-4, group two includes BMP-5, -6, and -7, group three are BMP-12, BMP-13, and BMP-14, and group four includes BMP-3 (or osteogenin), and BMP-3b (Sakou, T., 1998). Studies on roles of BMPs in fracture healing in the mouse and rat have shown a variety of osteogenic effects, temporal expressions, and mitogenic capacities (Cho *et al.*, 2002). Nowadays, BMP-2 and BMP-7 are certified by Food and Drug Administration (FDA) for human clinical uses.

2.7 Our Idea on the Present Project

In the past, our group succeeded in preparing chitosan gel in water-base sytem by phthalimide chitosan reacts with epichlorohydrin and then, ring opening reaction of epoxy-*N*-phthalimide chitosan and deprotection of the amino group by using an excess amount of hydrazine (Fangkangwanwong at al., 2006). Crosslinked networks were found to occur via subsequent reactions between oxirane rings and hydrazine. The epoxy group was successfully introduced onto the hydroxyl group by reacting epichlorohydrin with *N*-phthaloylchitosan.

In this work, we propose a novel injectable material for bone healing via focusing on water-based chitosan as starting material to produce injectable gel and control gel strength by using chitosan nanoscaffold. The injectable gel is made with partial crosslinking technique. Thus, injectable gel (i) is produced via water-soluble chitosan which is modified from chitosan nanoscaffold, (ii) is studied on a viscosity for injecting material into the bone area for connecting the broken bone, (iii) is studied on gelation time and gelation temperature at pH 6.6 to 7.4, (iv) is controlled

gel strength by adding chitosan nanoscaffold, and surface modified of chitosan nanoscaffold to increase compatibility between nanofiber and gel matrix, (v) is tested on cell culture test to see toxicity and biocompatibility.