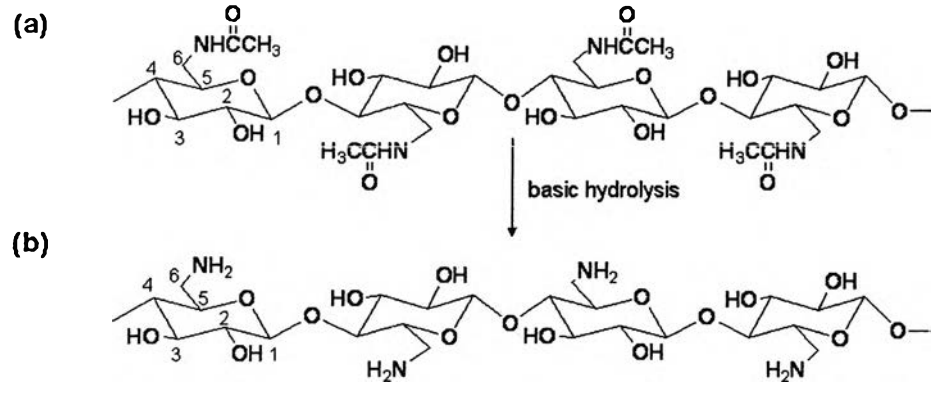
CHAPTER II
LITERATURE REVIEW

2.1 Chitin-Chitosan: The Structure and Specific Properties

Chitin is the second most abundant polysaccharide next to cellulose. Chemical structure of chitin is similar to cellulose consisting of linear β-(1-4)-linked monosaccharide. However, an important difference from cellulose is that chitin is an aminopolysaccharide with 2-acetamido-2-deoxy-β-D-glucan unit. Chitosan is obtained by basic hydrolysis acetamido group to generate amino group. The term for chitosan is when the chitosan unit or the degree of deacetylation more than 70% (Sannan *et al.*, 1976). Generally, chitin and chitosan are existed as a random copolymer (Scheme 2.1).

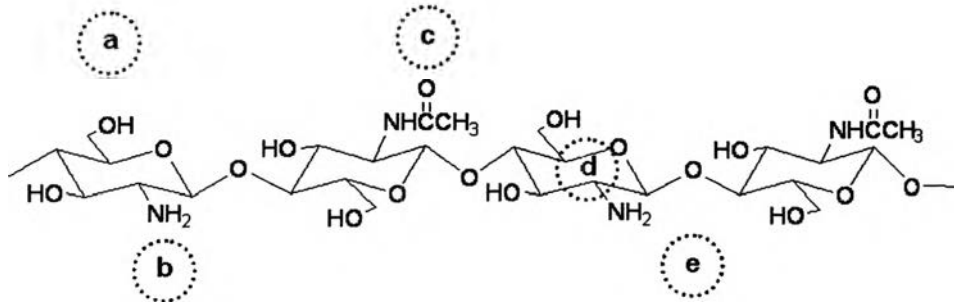
Scheme 2.1 Chemical structures of (a) chitin, and (b) chitosan



The primary amine groups render special properties that make chitosan very useful such as metal ion chelation, protonation to cationic species, including the chemical functionalizations. The known applications are such as chelating agents (Sakaguchi *et al.*, 1981), high performance adsorbents (Kurita *et al.*, 1988b), and ion exchange membranes (Pellegrino, *et al.*, 1990), etc.

Here, the structural based properties of chitin-chitosan are summarized as follows (Scheme 2.2).

Scheme 2.2 Structural based properties of chitin-chitosan



a: Hydroxyl group. Chitin-chitosan has hydroxyl groups, at C-3 and C-6 position. At C-6, hydroxyl group is more reactive than C-3 due to its primary hydroxyl groups. These hydroxyl groups impart hydrophilicity to chitosan chains and show the inclusion properties (Shimizu *et al.*, 1995). It can be, thus, formed inclusion and/or host-guest compound with ions or molecules, which is appropriate for industrial waste water treatment. The lone pair electrons of oxygen atom also form complex with metal ions, i.e. Ca^{2+}, Ni^{2+}, etc. (Nishi *et al.*, 1987). Chitin-chitosan possesses antimicrobial properties (Suzuki *et al.*, 1986) as the lone pair electrons of oxygen atom initiate the microorganism destruction.

b: Amino group. Chitosan, being a cationic polysaccharide in neutral or basic pH conditions, contains free amino groups. In acidic pH, amino groups can undergo protonation thus, making it soluble in water. Moreover, a lone pair electron of nitrogen atom tends to form bond with ions and metals. This brings the applications as wastewater treatment resin (Penniston and Johnson, U.S. Patent). The antibacterial and antiviral of chitin-chitosan are induced from the formation of ionic bond between the positively charged amino group and negative charged microorganism cell-wall, as a result the growth inhibition of bacteria and virus is achieved (Kendra and Hadwiger, 1984).

c: Acetamide group. This functional group is represented as chitin unit. Acetamido group is rather inert for the chemical reaction than amino group of chitosan. Crystal structure of chitin is dominant more than chitosan. Due to acetamide has strong hydrogen bond. This brings chitin to show poor solubility in almost all solvents.

d: Pyranose ring. Chitin-chitosan consists of pyranose ring either the *N*-acetyl–D-glucosamine or D-glucosamine. The glucosamine is reported for the detoxification and the combining with fatty acid for lowering cholesterol. Chitin-chitosan oligomer is known to activate the growth of tissue and the functions as a fibroblast for reconstruct collagen leading to an effective wound recovery. The activity of chitin-chitosan imparts the biocompatibility (Hirano *et al.*, 1988), bioactivity (Dumitriu *et al.*, 1989), and non-toxicity (Sugano *et al.*, 1978) under the structure of pyranose ring with *N*-acetyl–D-glucosamine or D-glucosamine.

e: glycoside linkage. Glycoside linkage or glucosidic bond (C-O-C) provides biodegradability (Mark *et al.*, 1985) via enzymatic hydrolysis, i.e. chitinase, chitosanase, and lyzozyme, which present in nature.

2.2 Chitin-Chitosan: Research in the Past and Future Expectation

2.2.1 Basic Applications of Chitin-Chitosan

For decades, the productions of chitosan were centered on sludge dewatering, food processing, and metal ion chelation. In addition, developments of chitosan are based on physical modifications, such as film, and membrane formations, bead preparations, gel productions, fiber extrusions, etc. Chitosan may be crosslinked by reagents such as epichlorohydrin (Wei *et al.*, 1992), diisocyanate (Welsh *et al.*, 2003) or 1,4-butanediol diglycidyl ether.(Roy *et al.*, 1998) Many chitosan hydrogel are obtained by treatment with multivalent anions such as glycerolphosphate (Chenite *et al.*, 2000), tripolyphosphate (Desai and Park, 2005). However, chitosan has limitation to dissolve in acid solution.

2.2.2 Advanced Application of Chitin-Chitosan

The present trend, in industrial applications, is toward producing high value products, such as cosmetics, drug carriers, feed derivatives, semi-permeable

membranes, and pharmaceutics. Many researchers reported on the structural modification of chitosan at molecular level to propose novel products.

2.3 Limitations and Strategies to Overcome

Considering chemical structure of chitin-chitosan, it exhibits high crystallinity through its inter- and intra- molecular hydrogen bond network. In addition the high molecular weight developed naturally, chitin-chitosan faces weak point about its solubility and reactivity. In order to overcome these limitations, structural modification at molecular level is considered; (i) reducing chitosan molecular weight to enhance the solubility, and (ii) functionalizing chitosan to elevate solubility in aqueous and/or organic solvent.

2.3.1 Reducing Chitosan Molecular Weight to Enhance the Solubility

In the past, several efforts to obtain low molecular weight chitosan (LMWC) and/or olidochitosan have been done. Chemical treatment (Allan and Peyron, 1995a and 1995b) is an easy process with low cost performance, but chemical waste and reproducibility are the main problem. Enzymatic hydrolysis (Aiba, 1994) is an effective way to achieve specific cleavage to obtain oligochitosan. However, it requires multi-steps, especially, enzyme preparation and purification. This brings a hesitation for large-scale production. Photoirradiation (Andrady *et al.*, 1996) is considered since it is easy process with a large-scale production in a single step without waste generation and no product purification required.

2.3.2 Chemical Modification to Improve Chitosan Solubility

Active functional groups on chitosan chain are hydroxyl group at C-2 and amino group at C-6. These groups are reactive to functionalize with bulky group to improve the solubility. As Nishimura *et al.*, (1991) reported N-phthaloylchitosan exhibits much improved solubility in common organic solvent such as dimethylformamide (DMF), *N,N*-dimethylacetamide (DMAc), dimethylsulfoxide (DMSO), and pyridine. The further chemical reactions of this compound are homogeneous system. Fangkangwanwong *et al.*, (2006) reported the simply method to prepare chitosan in aqueous solution by mixing chitosan with

hydroxybenzotriazole (HOBt). ^{1}H-NMR shows the complexation between chitosan and HOBt.

2.4 Chitosan Nanoparticles/ Nanospheres

In recent decades there has been increased interest in the use of nanoparticles for drug delivery applications. From literature, preparation method for chitosan nanoparticles/ nanospheres can be categorized into 4 types.

2.4.1 Covalently Crosslinked Nanoparticles.

Ohya *et al.*, (1994) reported preparation of chitosan nanospheres by water-in-oil (W/O) emulsion method followed by glutaraldehyde crosslinking of the chitosan amino group. However, the later discovery of the negative effects of glutaraldehyde crosslinking on cell viability and the integrity of macromolecular drug shifted general interest to less harsh procedures for the synthesis of nanospheres.

2.4.2 Ionically Crosslinked Nanoparticles

The cationic nature of chitosan has been conveniently exploited for the development of particulate drug delivery systems. Chitosan is not only complex formation with negative charge polyanion but also gel spontaneously on contact with specific polyanion. Among some polyanion investigated, tri-polyphosphate is mostly widely used because of non-toxic property and quick gelling property. Gan *et al.*, (2007) studied chitosan nanoparticles as protein carrier by using polyanion tripolyphosphate (TPP) as the coacervation crosslinking agent. BSA, bovine serum albumin, was applied as model protein to incorporate with nanoparticles. As a crosslink and condensing agent, TPP form further hydrogen bonds with free amine groups on both protein and chitosan molecules, resulting in more compact protein-chitosan nanoparticles. However, the difficulty of chitosan nanoparticle is controlling initial burst effect in releasing large quantities of protein molecules.

2.4.3 Dissolvation of Nanoparticles

This method was proposed to introduce the simple process to prepare chitosan microsphere by using sodium sulfate as precipitant (Berthold *et al.*, 1996)

Sodium sulfate was added dropwisely into chitosan solution under stirring and ultrasonication to dissolvate chitosan in a particular form. The amount of sodium sulfate required for microsphere formation increased with an increasing in molecular weight of chitosan. The reason is probably dependent on the number of positive charges on chitosan surface.

2.4.4 Amphiphilic Chitosan Nanoparticles via Self-assembly Process

This process has recently attracted increasing interest in pharmaceutical areas. The nanoparticles consist of hydrophobic inner core and hydrophilic outer shell in aqueous media. Compared with others method as mention previously, preparing nanoparticles through the self-assembly of amphiphilic chitosan is more simple and effective method, as it needs no additives.

Yuan *et al.,* (2006) proposed chitosan nanoparticles by grafting cholesterol as hydrophobic part. The nanoparticles were formed by self-aggregated through sonication or filtration methods. Shape of chitosan-cholesterol nanoparticles was mostly spherical as observed by TEM and diameter size was 50-200 nm. In addition, the diameter was decreased as degree substitution of cholesterol increases.

Wu *et al.,* (2005) synthesized water soluble chitosan (CS) derivatives containing polylactide (DLLA) unit by reacting DL-lactide with chitosan in DMSO solution in the presence of triethylamine. Sphere diameter from DLS measurement demonstrates that the diameter increased with an increase in DLLA/CS molar ratio, suggesting the elongation of hydrophobic polylactide side chain facilitates the growth of the hydrophobic core of polymeric micelles.

Huang *et al.,* (2006) introduced facile preparation of chitosan nanoparticles consisted of poly(butylenes glycol adipate) (PBGA) as hydrophilic side chain. The graft copolymers of chitosan with PBGA were prepared due to the esterification reaction between PBGA and 6-O-succinate-N-phthaloyl-chitosan (PHCSSA) in the presence of toluene as a swelling agent. The copolymers particles are nanoparticles with the size of a few hundred nanometers.

Kulkarni *et al.,* (2006) studied chitosan nanoaggregates by linking methoxy polyethyleneglycol (MPEG) to chitosan (PLC) in the presence of formaldehyde in a solvent of formic acid and dimethylsulfoxide (DMSO). Size of particles and zeta potential are decreased as degree substitution of MPEG increases.

2.5 Drug Encapsulation and Drug Targeting

Recently, many studies on self-assembled nanoparticles for their biomedical and pharmaceutical applications have been reported. The self-assemble nanoparticles consisted of hydrophobic moieties as a core part and an outer shell of hydrophilic groups. These bring potential of nanoparticles to act as reservoir for hydrophobic molecules. Drug loading in nanoparticles can be done by two methods, i.e., during the preparation of particles (incorporation) and after the formation of particles (incubation). In these systems, drug is physically embedded into the matrix or adsorbed onto the surface.

Yokoyama *et al.*, (1998) reported incorporation of anticancer drug, KRN 5500, into block copolymer micelles, poly(ethylene glycol)-poly(amino acid), via physical entrapment utilizing hydrophobic interaction. DMSO or DMF were used as solvent to generate homogeneous system between polymeric micelles and drug. During dialysis, micelle formation and drug incorporation into the polymeric micelle were occurred simultaneously. Interactions, mainly hydrophobic interactions, among the hydrophobic poly(amino acid) chain, KRN, and solvent are an important key to control incorporation process.

Furthermore, the advance application of polymeric nanoparticles is delivery drug to specific sites by size-dependent passive targeting or by active targeting. Active targeting has been attempted by many researchers (Rodrigues, J. *et al.*, (2003), Mitra, S. *et al.*, (2001), and Lin, C. *et al.*, (2005)) in order to gain a high degree of selectivity to a specific organ and to enhance the internalization of drug-loaded nanoparticles into the target cells. For enhancing the intracellular delivery capacity of polymeric nanoparticles to specific cells, the most widely utilized approaches is tethering cell recognizable targeting ligands, such as monoclonal antibodies, endogeneous targeting peptides, and low molecular weight compounds onto the surface of the nanoparticles.

Na *et al.*, (2003) prepared self-assembled nanoparticles (BPA) by introducing vitamin H to pullulan acetate (PA), hydrophobized polysaccharide and self-assembling via diafiltration method. Vitamin H acts as receptor of target cell. The particle size of the nanoparticles increased with degree substitution of vitamin H,

hydrophilic part, increases. The interactions between BPA and carcinoma cell line (HepG2) which is a target cell were studied by labeling nanoparticles with RITC. The interactions between the labeled nanoparticles and the HepG2 cells were quantified by a microplate-fluorescence-reader. The increasing of fluorescence intensity implied the interaction between HepG2 and nanoparticles was increased as degree substitution of vitamin H on BPA increases.

Chan *et al.*, (2007) studied conjugation of folate (FA) on water soluble chitosan (PEG-Chi) to produce non-viral carrier for tumor-targeted gene delivery. However, the solubility is decreased as grafting with folic acid. FA-PEG-Chi showed lower cytotoxicity against HEK 293 cells when compare with commercial carrier.

2.6 Scope of the Present Work

In the past our group (Yoksan *et al.*) succeeded in preparing amphiphilic chitosan nanospheres. At that time, it is well-understood that chitosan flakes in micrometer or millimeter scale can be changed to nanometer particle scale by simply introducing hydrophobic N-phthaloyl group and hydrophilic mPEG group. This simply process initiates the suspect mechanism that chitosan forms self-assembly based on the differences in hydrophobicity/hydrophilicity.

Although those successful researches bring us to develop chitosan nanoparticle and to further study on the applications for drug delivery system, the understanding of the factors related to the control of nanospheres including the drug incorporation models and the toxicity of nanospheres has to be understood in details before we can reach the ultimate goal of applications. In order to control sphere diameter, the work demonstrates effect of degree deacetylation of chitosan and chain length of mPEG.

The work extends to study how the nanosphere formation is accomplished in the details of the surface charges, the sizes and shapes related to the colloidal phenomena. As it is a question to us how chitosan nanospheres show strong negative surface charge, although copolymers has only amine group as an electrolytic group.

Hence, the work clarifies the fact behind the performance of negative charge on chitosan nanospheres.

In order to apply the merits of chitosan nanospheres obtained; incorporation of lidocaine, camptothecin, protein into chitosan nanospheres were studied as model cases to show the drug encapsulation, the efficiency of drug incorporation as well as the release performances.