CHAPTER II

LITERATURE REVIEW

1. Melanoma

Melanoma is a type of skin cancer which originates from melanocytes. Melanocytes are found in the epidermis and they contain melanin, the pigment that expresses the human skin color and protects the skin from sunlight damage. Men most often get melanoma on the trunk, the area of the body between the shoulders and hips or on the head or neck and women most often get melanoma on the arms and legs. Melanoma is the most serious form of skin cancer. However, if it is recognized and treated early, it is nearly 100 percent curable. But if it is not, the cancer can advance and spread to other parts of the body, where it becomes hard to treat and can be fatal. The incidence of melanoma and the death rate from melanoma has steadily increased annually. The American Cancer Society estimates that in 2007, approximately 59,940 new cases of melanoma in the United States were diagnosed and almost 8,110 deaths were occurred (Jemal et al., 2007). In Thailand, the incidence of melanoma is low. However, the risk of melanoma for Thai people may increase because of increasing in sunlight exposure. There are many factors that can cause melanoma. Sun exposure is the most important risk factor. Ultraviolet A and B in sunlight are dangerous to the skin, and can induce skin cancer. Sunburns in early childhood and cumulative exposure increase risk. People who live in locations that get more sunlight may get more skin cancer. The others risk factors are unusual mole. Individuals with atypical moles (dysplastic nevi) have a higher risk of developing the cancer. Atypical moles are often large, flat, and asymmetrical with irregular borders. The risk of melanoma is also correlated with the number of dysplastic nevi. The fairer skin type, family and personal history of melanoma and weakened immune system may increase risk.

Classifications for melanomas are called stages. The stage refers to the thickness, depth of penetration, and the degree to which the melanoma has spread.

The staging of melanoma can be determined on histologic examination by the thickness of the tumor, known as Breslow's classification, and/or the anatomic level of local invasion, known as Clark's classification (The Skin Cancer Foundation, 2007; National Cancer Institute, 2003). Breslow's thickness measures in millimeters the distance between the upper layer of the epidermis and the deepest point of the tumor's penetration. The thinner the melanoma, the better the chance of a cure. *In situ* melanoma remains confined to the epidermis, very thin tumors are less than 1.0 mm, thin tumors are 1.0-2.0 mm, intermediate tumors are 2.0-4.0 mm, and thick melanomas are 4.00 mm or more. Very thin tumors are classified according to Clark's level of invasion, based on the number of layers of skin penetrated by the tumour as the following (The Skin Cancer Foundation, 2007)

- Clark's level I. The melanoma occupies only the epidermis.
- Clark's level II. The melanoma penetrates to the layer under the epidermis, the papillary dermis.
- Clark's level III. The melanoma fills the papillary dermis and invades into the reticular dermis, the next layer down.
- Clark's level IV. The melanoma penetrates into the reticular or deep dermis.
- Clark's level V. The melanoma invades the subcutaneous fat.

The degree to which the melanoma has spread is one of the most important indicators to determine melanoma stages. The initial spread is most likely to be in the lymph node closest to the primary tumor, known as the sentinel node. This is identified by lymphoscintigraphy, a procedure based on injecting a small amount of radioactive substance to trace a blue dye through the lymphatic fluid. The dye is taken by the sentinel node. This is then excised, called sentinel node biopsy, and studied in the laboratory for the presence of melanoma cells. If they are found, the other nodes in the region are also excised. Metastasis is a factor to be considered in all tumors more than 1.0 mm in thickness or when a thinner tumor shows evidence of ulceration (The Skin Cancer Foundation, 2007). Early melanomas, composing of stages I and II, are localized. Advanced melanomas, composing of Stages III and IV, have spread or

metastasized to other parts of the body. There are also subdivisions within stages (The Skin Cancer Foundation, 2007).

Stage I melanoma is subdivided based on the thickness of the primary original tumour.

- Stage Ia: The tumour is less than 1.0 mm in Breslow's thickness without ulceration and is in Clark's level II or III.
- Stage Ib: The tumour is less than 1.0 mm in Breslow's thickness with ulceration and/or Clark's level III or IV, or it is 1.0 - 2.0 mm in thickness without ulceration, or has spread to the closest lymph nodes.

Stage II melanoma is also subdivided based on the levels in thickness and/or depth, the presence or absence of ulceration, and regional lymph node metastases.

- Stage IIa: The tumour is 1.0 - 2.0 mm in Breslow's thickness with ulceration, or is 2.0 - 4.0 mm in thickness without ulceration.
- Stage IIb: The tumour is 2.0 - 4.0 mm in Breslow's thickness with ulceration, or is greater than 4.0 mm in thickness without ulceration.
- Stage IIc: The tumour is greater than 4.0 mm in Breslow's thickness with ulceration.

Stage III, melanoma has metastasized or spread through the lymph system. This can be determined by examining a biopsy of the node closest the tumor, called the sentinel node. The biopsy is done when a tumor is more than 1 mm in thickness, or when a thinner melanoma shows the ulceration.

Stage IV, melanoma has metastasized to lymph nodes far away from the primary tumor or to internal organs, most often the lung, liver, brain, bone and gastrointestinal tract.

Melanomas are highly curable if it is recognized early and have not spread beyond the site where they developed. Treatment for melanoma depends on the stage, location of the tumour, the patient's age and general health. Surgery is the standard treatment for all stages of melanoma. Additional or adjuvant therapy may be given

after surgery for high risk melanomas to destroy any cancer cells that may have escaped from the primary site. Chemotherapy, radiation therapy, immunotherapy, or combinations of these treatments are adjuvant therapies for melanoma (The Skin Cancer Foundation, 2007). Surgery involves the removal of the tumour and a surrounding area of normal skin. The goal of surgery in early stage melanoma is to remove the tumour completely, before it has a chance to spread. Surgery for patients with later stage disease may involve the removal of cancerous tumours or lymph nodes that have spread to other areas of the body.

Chemotherapy is the use of anticancer drugs, given orally or by injection, to kill cancer cells throughout the body. It is an adjuvant treatment sometimes used for stage IV disease and recurrent melanoma, and for lower stages if surgery cannot be performed. Chemotherapy drugs for the treatment of melanoma may be administered in single or in combination, or in combination with immunotherapy, drugs that act on the immune system.

Radiation therapy is the use of high energy rays, including x-rays, to damage cancer cells and stop them from growing. It is generally used for advanced melanoma where surgery is not possible or may be complicated. Radiation is used to relieve symptoms of melanoma that has metastasized to areas such as the brain or bone. Radiation may also be targeted to the regional lymph node to prevent cancer recurrence after surgery.

Biological therapy, or biotherapy, also called immunotherapy is a treatment that uses the natural and manufactured substances derived from the body helps the body's immune system to fight cancer. Interferon alpha and interleukin-2 are the most active agents used as immunotherapy for melanoma.

The other treatment has been studying for melanoma treatment is gene therapy which is the introduction of new genetic material to damaged genes or cancer cells. The goal of gene therapy is to replace damaged cells with healthy ones, or to make cancer cells more sensitive to the effects of immunotherapy and chemotherapy.

2. Dacarbazine

Dacarbazine (or dimethyl-triazeno-imidazole carboxamide), also known as DTIC, was approved by US Food and Drug Administration for treatment of melanoma. Its mechanism of action is methylation of nucleic acids or direct DNA damage resulting in arrest of cell growth or cell death. When used as a single agent, an approximately 20% objective response rate can be achieved with median response duration of 5 to 6 months and complete response rates of 5% (Flaherty, 2006). Several chemotherapy drugs have been combining with dacarbazine in order to improve responsiveness in melanoma treatment such as 'Dartmouth' regiment, which consist of dacarbazine, cisplatin, carmustine and tamoxifen. Combinations of immunotherapy drugs, such as interferon and interleukin with dacarbazine also were evaluated. However, those may not improve response and may cause severe side effect (Eggermont et al., 2004). New chemotherapy drugs used for melanoma also have been developing. Temozolomide, a prodrug that degrades to the active agent methyltriazenoimidazole carboxamide (MTIC) which is also the active metabolite of DTIC, has the advantages of oral administration and better penetration into the central nervous system. In the last decade, researchers have been focusing on the signal pathway in cancer cell. The alteration of some signal pathway in melanoma cell provides the development of agents directed on molecular targets such as protein-kinase inhibitors (sorafenib or BAY 43-9006, imatinib mesylate, temsirolimus), pro-apoptotic oligonucleotides (oblimersen), and anti-angiogenic agents (bevacizumab) (Queirolo et al., 2006). Whereas new chemotherapy drugs used for melanoma have been discovering, dacarbazine may be developed in order to improve effectiveness.

Dacarbazine is a synthetic compound that functions as an alkylating agent following metabolic activation by liver microsomal enzymes via oxidative *N*-demethylation to the monomethyl derivative. As a class, the alkylating agents exert their cytotoxic effects via transfer of their alkyl groups to various cellular constituents. Alkylations of DNA within the nucleus probably represent the major interactions that lead to cell death. However, these drugs react chemically with sulfhydryl, amino, hydroxyl,

carboxyl, and phosphate groups of other cellular nucleophiles as well. The general mechanism of action of these drugs involves intramolecular cyclization to form an ethyleneimonium ion that may directly or through formation of a carbonium ion transfer an alkyl group to a cellular constituent. Dacarbazine is a structural analogue of 5-amino-imidazole-4-carboxamide (Figure 1) which is an intermediate in purine biosynthesis. Dacarbazine can be metabolized to generate the reactive compound, methyltriazenoimidazole carboxamide (MTIC), which is responsible for the alkylation of nucleic acids. The cytotoxicity of MTIC is thought to be due primarily to the formation of methylcarbonium ions that attack nucleophilic groups in DNA. Dacarbazine may also inhibit DNA and RNA synthesis by acting as a purine analogue. Dacarbazine is cell cycle phase non-specific.

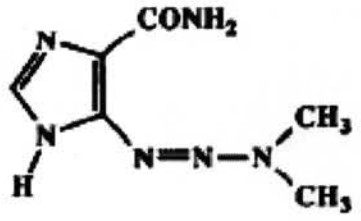

Figure 1 Structure of dacarbazine (Shetty et al., 1992)

Dacarbazine is poorly absorbed from the gastrointestinal tract, which could result in unpredictable tumour responses and possible increased toxicity. Therefore the drug is recommended for intravenous administration only. Plasma concentrations of dacarbazine appear to decline in a biphasic manner. The initial phase half life ($t_{1/2\alpha}$) is very short, with one study reporting $t_{1/2\alpha}$ as 2.9 minutes. The terminal phase half life ($t_{1/2\beta}$) is about 35 minutes. Dacarbazine is metabolized by cytochrome P450 in the liver into active metabolite, methyltriazenoimidazole carboxamide (MTIC) (Figure 2) and the major inactive metabolites, amino imidazole carboxamide (AIC) which is then excreted in the urine. About half of the drug remains unchanged and is rapidly excreted in the urine within 6 hours.

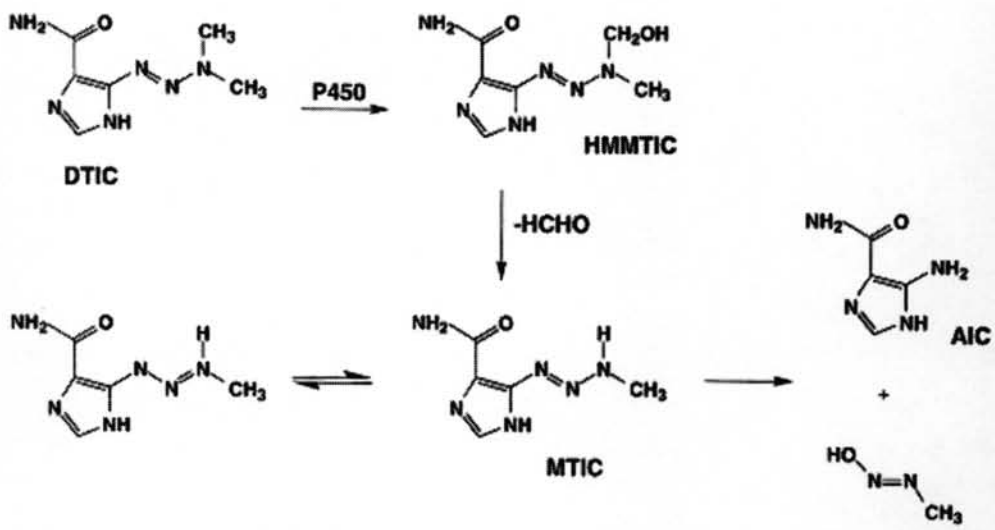

Figure 2 DTIC metabolic pathway (Safgren et al., 2001)

The recommended dosage of dacarbazine is 2 to 4.5 mg/kg/day for 10 days, repeated at 3 week intervals. An alternate recommended dosage is 200 to 250 mg/square meter body surface/day as an intravenous injection for 5 days every 3 to 4 weeks. As an alternative to an intravenous bolus injection, dacarbazine can be mixed in 250-500 mL normal saline solution (NSS) or 5% dextrose (D5W) and administered as a short-term infusion over 15 - 30 minutes. Intermittent intravenous infusion typically less painful than direct intravenous injection.

The effectiveness of the cancer treatment is directly related to the ability to target and to kill the cancer cells while affecting as few healthy cells as possible. The degree of change in the patient's quality of life and eventual life expectancy is directly related to this targeting ability of the treatment. The bolus administration of anticancer drugs always causes some systematic side effects and sometimes the side effects are so intense that the patient must discontinue therapy before the drugs have a chance to eradicate the cancer. Infusional chemotherapy has been increasingly used on the basis of the observations that some anticancer drugs have a relatively short half-life following bolus exposure and that steady level of the drug concentration over time may maximize the antitumor effect. In addition, the infusion schedule may reduce the

acute and chronic toxicities commonly associated with high peak levels (Williams and Lokich, 1992).

Dacarbazine also has a short half life which causes cancer cell exposed to the drug for only short time results in low effectiveness in melanoma therapy. Thus, the administration by continuous infusion of low concentration may provide more effectiveness than high concentration bolus (Sewell and Hong, 1997). Unfortunately, long-term continuous infusion of dacarbazine may fail because of its short stability in aqueous solution (Shetty et al., 1992). The commercial formulation of dacarbazine also contains citric acid and mannitol in order to improve drug solubility. After reconstitution, the resulting solution contains 10 mg/mL of dacarbazine having a pH of 3.0 to 4.0 and shows the shelf-life of only 8 hours at room temperature and up to 72 hours at 4°C. If the reconstituted solution is further diluted in 5% dextrose injection or sodium chloride injection, the resulting solution may be stored up to 8 hours at normal room conditions or up to 24 hours at 4°C. The stability of dacarbazine is also affected by light and temperature. Some study shown that dacarbazine solutions exposed to direct and indirect sunlight rapidly degrade within 3-4 hours. The maximal stability of dacarbazine under exposure to fluorescent light at room temperature was 24 hours. Temperature greatly influences the stability of dacarbazine in solution. When protected from light, dacarbazine solutions remain stable (<10% loss) for up to 48 hours at 20°C, for 24 hours at 25 °C, and for 8 hours at 35 °C (Williams and Lokich, 1992). In order to improve the stability in aqueous solution of dacarbazine, nanoparticles were prepared in this investigation.

3. Nanoparticles

Nanoparticles are solid colloidal particles in size less than 1 micron. Nanoparticles can be divided into two forms, nanosphere which the drug is uniformly dispersed throughout the particles, and nanocapsule which the drug is entrapped in the inner core. Drug can be bound to the nanoparticles either by production in the presence of the drug or by adsorption after the preparation of blank nanoparticles. Production in the presence of the drug lead to covalent coupling to the polymer, or it

may produce the solid solution or the solid dispersion of the drug in the polymer matrices. The addition of the drug to previously prepared blank nanoparticles results in covalently coupling or the sorption of the drug to the polymer. The sorption can lead to either to the diffusion of the drug into the polymer matrices and to the formation of a solid solution or to surface adsorption of the drug (Kreuter, 1994).

The numerous protocols exist for preparation of nanoparticles based on the type of drug used and the desired delivery route. Once a protocol is chosen, the parameters must be controlled to create the best characteristics for the nanoparticles. The most important characteristics of nanoparticles are size, zeta potential (surface charge), encapsulation efficiency, and release characteristics. The molecular weight and concentration of the polymer used will also affect the nanoparticles. The molecular weight of the polymer has opposite effects on nanoparticles size and encapsulation efficiency. Smaller size nanoparticles, approximately 100 nm, can be prepared with lower molecular weight polymer but provided low drug encapsulation efficiency. On the other hand, an increase in polymer concentration increases encapsulation efficiency and the size of the nanoparticles (Hans and Lowman, 2002). The routine and the fastest method which is used for size measurement is photon correlation spectroscopy or dynamic light scattering (Kreuter, 1994). Photon correlation spectroscopy determines the hydrodynamic diameter of the nanoparticles via Brownian motion. Thus, the size measurement will be influenced by the interaction of the particles with the surrounding liquid medium. For this reason, it is important to verify the size of obtained nanoparticles by the other method such as electron microscopy (Kreuter, 1994). There are two types of electron microscopy have been used, scanning electron microscopy (SEM) and transmission electron microscopy (TEM).

The zeta potential is a measure of the charge of the particle, as such the larger the absolute value of the zeta potential the larger the amount of charge of the surface. The zeta potential represents an index for particle stability. For the case of charged particles, as the zeta potential increases, the repulsive interactions will be larger leading to the formation of more stable particles with a more uniform size

distribution. A physically stable nano-suspension stabilized by electrostatic repulsion will have a minimum zeta potential of ±30 mV. This stability is important in preventing aggregation.

A successful nanoparticulate system may have a high loading capacity to reduce the quantity of the carrier required for administration. Drug loading into the nanoparticles is achieved by two methods, one is by incorporating the drug at the time of nanoparticles production or secondly, by adsorbing the drug after the formation of nanoparticles by incubating them in the drug solution. It is thus evident that a large amount of drug can be entrapped by the incorporation method when compared to the adsorption (Soppimath et al., 2001). The incorporation of drugs to the polymer matrices can be done through the various techniques depends upon the physicochemical properties of the drug molecules and the polymers. Nanoparticulate system can be used to provide targeted delivery of drugs, to sustain drug effect in site, to improve oral bioavailability and stability of therapeutic agents against enzymatic degradation. Nanoparticles have a further advantage over larger-size particles, because they are better suited for intravenous delivery and their sterilization may be simply done by filtration. The smallest capillaries in the body are 5–6 μm in diameter. The size of particles being distributed into the bloodstream must be significantly smaller than 5 μm, without forming aggregates, to ensure that the particles do not form an embolism (Hans and Lowman, 2002). Nanoparticles have been widely used for delivery and targeting of various drugs, especially in cancer therapy (Peppas and Blanchette, 2004; Jain, 2005). These increase antitumor drug efficacy while reducing side effects and toxicities. Nanosize range of particles makes them more pass into and accumulates at the required target by passive targeting. In addition, modifying these nanocarriers with targeting moiety, the passage and accumulation may increase by active targeting. Incorporating hydrophilic polymer into nanocarriers makes them escape from rapidly clearance by mononuclear phagocytic system (MPS), provides them long circulation in blood system resulting in more chance for drug exposure to the target tissue. In addition, nanoparticles can be used to improve the stability of various agents (Saxena et al., 2004; Perugini et al., 2002).

4. Polymeric nanoparticles

Over the past few decades, there has been considerable interested in developing biodegradable nanoparticles as effective drug delivery devices. Various polymers have been used in drug delivery research as they can effectively deliver the drug to a target site and thus increase the therapeutic benefit, while minimizing side effects. The controlled release of pharmacologically active agents to the specific site of action at the therapeutically optimal rate and dose regimen has been a major goal in designing such devices. Liposomes have been used as potential drug carriers instead of conventional dosage forms because of their unique advantages which include ability to protect drugs from degradation, target the drug to the site of action and reduce the toxicity or side effects. However, development of work on liposomes has been limited due to the problems such as low encapsulation efficiency, rapid leakage of water-soluble drug in the presence of blood components and poor storage stability. On the other hand, polymeric nanoparticles offer some specific advantages over liposomes. For instance, nanoparticles help to increase the stability of drugs / proteins and possess useful controlled release properties (Soppimath et al., 2001).

Polymeric nanoparticles become an important area of research in the field of drug delivery due to their biocompatibility and biodegradability. The three key advantages that polymeric drug delivery system can offer are localized delivery of drug. The product can be implanted directly at the site where drug action is needed and hence systemic exposure of the drug can be reduced (Sinha and Khosla, 1998). This becomes especially important for toxic drugs which are related to various systemic side effects (such as the chemotherapeutic drugs). The second advantage is sustained delivery of drug to varying areas of the body (Langer et al., 1998; Zhang and Kosaraju, 2007). The drug encapsulated is released over extended periods and hence eliminates the need for multiple injections. This feature can improve patient compliance especially for drugs for chronic indications, requiring frequent injections. The third advantage is stabilization of the drug by reducing direct contact of the drugs to their environment such as aqueous media, temperature and light. The polymer can protect the drug from the physiological environment and hence improve its stability *in*

vivo. This particular feature makes this technology attractive for the delivery of labile drugs such as proteins (Gombotz and Pettit, 1995).

Polymeric nanoparticles are prepared from natural or synthetic polymers. The examples of synthetic polymer are poly (glycolide) (PGA), poly (lactide) (PLA), and poly (lactide-co-glycolide) (PLGA) (Hans and Lowman, 2002; Soppimath et al., 2001), and the examples of natural polymer are sodium alginate and chitosan. A great deal of attention has been directed to polymeric colloidal nanoparticulate formulations obtained with the natural polymers. The major advantage of natural polymers includes their low cost and compatibility with the wide range of drugs, with minimal use of organic solvents. Their bioadhesion property, stability, safety and their approval for human use by the US Food and Drug Administration are additional advantages. A wide range of compounds have been successfully formulated into natural carrier systems such as antituberculosis drug (Ahmad et al., 2006), antifungal drug (Ahmad et al., 2007), amphotericin B (Sangeetha et al., 2007), oral insulin (Sarmento et al., 2006) and antisense oligonucleotide (Ferreiro et al., 2002). Chitosan and alginate are two biopolymers that have received much attention and have been extensively studied for such use.

5. Alginates

Alginate is a linear, anionic polysaccharide of (1–4)-linked β-D-mannuronic acid (M) and α-L-guluronic acid (G) residues. The residues may widely vary in composition and sequence and are arranged in a pattern of blocks along the chain. These homopolymeric regions of β-D-mannuronic acid blocks and α-L-guluronic acid blocks are interdispersed with regions of alternating structures (β-D-mannuronic acid-α-L-guluronic acid blocks). The composition and extent of the sequences and the molecular weight determine the physical properties of the alginates. Alginate have been widely used as a food and pharmaceutical additives, for instance as a tablet disintegrant, thickening agent, gelling agent and a colloidal stabilizer. Alginates possess a number of characteristics that makes it useful as a formulation excipient, both as a conventional excipient and more specifically as a tool in polymeric-

controlled drug delivery. Alginates are naturally occurring polymers isolated from brown seaweed. The seaweed is extracted with a dilute alkaline solution which solubilizes the alginic acid present. Free alginic acid is obtained on treatment of the resulting thick and viscous mass with mineral acids. The alginic acid then be converted to a salt of which sodium alginate is the major form currently used. Alginic acid is a linear polymer consisting of D-mannuronic acid and L-guluronic acid residues that are arranged in the polymer chain in blocks. These homogeneous blocks are separated by blocks made of random or alternating units of mannuronic and guluronic acids. Alginates have been reported to undergo proton-catalyzed hydrolysis, which is dependent on time, pH, and temperature. Alginates from different sources are varied in their proportions of blocks. Hydration of alginic acid leads to the formation of a high-viscosity ''acid gel'' due to intermolecular binding. After gelation the water molecules are physically entrapped inside the alginate matrix, but are still free to migrate. This is of great importance in many applications (e.g., alginate gels for cell immobilization/encapsulation). The water-holding capacity of the gel is due to capillary forces. Monovalent metal ions form soluble salts with alginate whereas divalent and multivalent cations (except Mg^{2+}) form insoluble salts or gels. The various cations show different affinity for alginate, and selective ion binding is the basis for the ability of alginate to form ionotropic hydrogels. Alginates with a high content of guluronic acid blocks give gels of considerably higher strength compared to alginates rich in mannuronate, as the G residues exhibit a stronger affinity for divalent ions than the M residues (Tønnesen and Karlsen, 2002) as shown in Figure 3. Alginate is chemically very stable at pH values between 5 and 10.

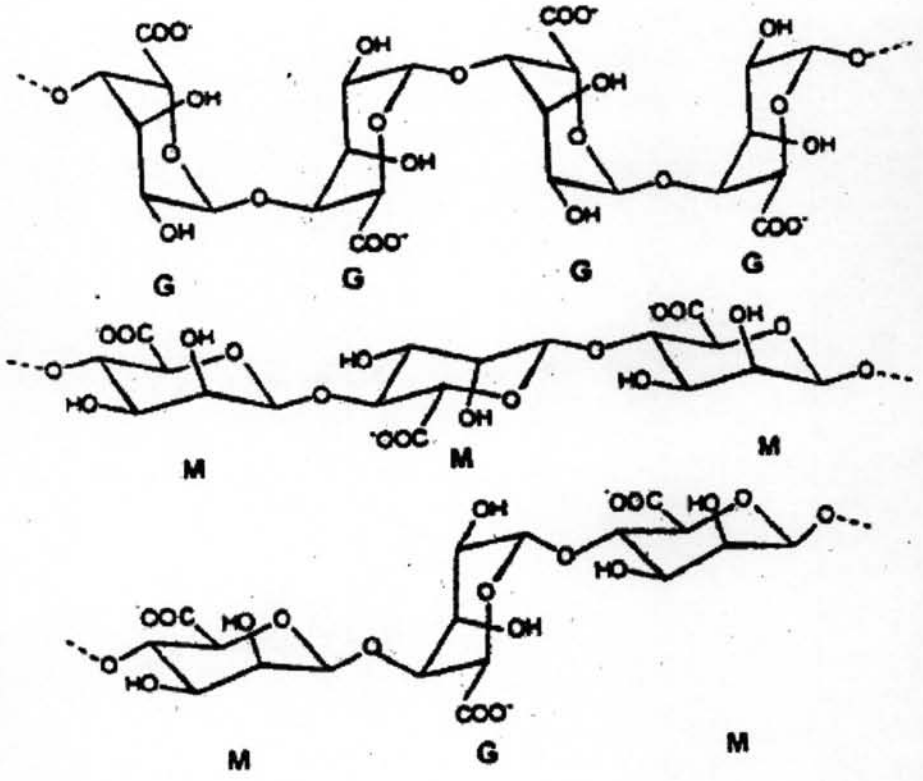

Figure 3 Alginate block types: G is guluronic acid, M is mannuronic acid (Tønnesen and Karlsen, 2002).

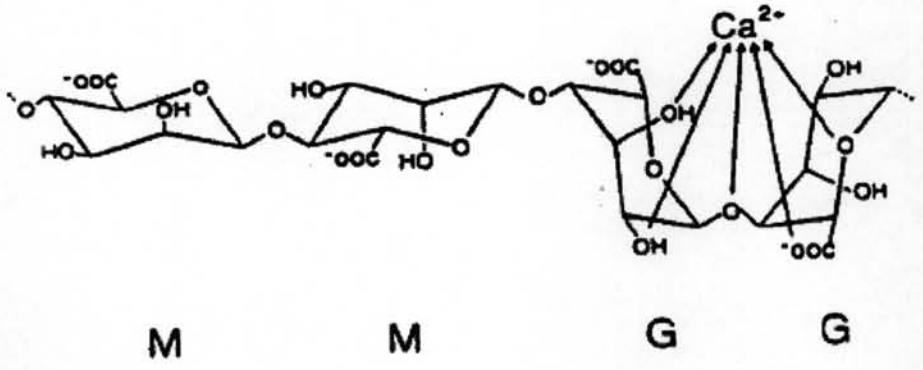

Figure 4 Probable binding mode between the calcium ion and two G residues (Tønnesen and Karlsen, 2002).

Swelling and viscoelasticity of alginate gel membranes are highly affected by the M/G ratio as shown in Figure 4. Calcium alginate gels are the most extensively studied. The binding was made between calcium ion and two G residues. The physicochemical properties of the polymer system and the swelling process to activate the release of drugs will be dependent on the type of gel formed. Alginic acid and its sodium and calcium salts are regarded as generally non-toxic and biocompatible. These products are commercially available and over 200 different alginate grades. However, since alginates are obtained from a natural source, a variety of impurities may potentially be present. These include heavy metals, proteins, and endotoxins. For pharmaceutical applications, particularly for parenteral administration, these impurities should be removed. Compounds used as excipients will often find more than one application which generally depends on the thickening, gel-forming, and stabilizing properties. As examples it can be mentioned that sodium alginate can be used as a binding and disintegrating agent in tablets, as a suspending and thickening agent in water-miscible gels, lotions, and creams, as a stabilizer for emulsions and in the development of alginate-controlled drug delivery systems (Tønnesen and Karlsen, 2002).

Alginate also has several unique properties that have enabled it to be used as a matrix for entrapment and/or delivery of a variety of proteins and cells. These properties include a relatively inert aqueous environment within the matrix, a mild room temperature encapsulation process free of organic solvents, a high gel porosity which allows for high diffusion rates of macromolecules, the ability to control this porosity with simple coating procedures and dissolution and biodegradation of the system under normal physiological conditions.

Degradation of a cation (Ca^{2+}, Zn^{2+}) crosslinked alginate gel can occur by removal of the cations. This can be accomplished by the use of a chelating agent or by a high concentration of ions such as Na^{+} or Mg^{2+}. As the cations are removed, the crosslinking in the gel decreases and the gels are destabilized. This can lead to leakage of entrapped material and solubilization of the high molecular weight alginate polymers (Raj and Sharma, 2003).

Alginate is commonly used and forms a matrix for various delivery systems including gels, film, beads, microparticles and nanoparticles. Alginate based nanoparticles were reported to deliver and controlled release of various drugs and agents such as antituberculosis drug (Ahmad et al., 2006), antifungal drug (Ahmad et al., 2007), Amphotericin B (Sangeetha et al., 2007) and antisense oligonucleotide (Ferreiro et al., 2002). Alginate nanoparticle forms in the present of multivalent cation in alginate solution. Salts of alginate are formed when metal ions react with the guluronic acid and mannuronic acid residues (De and Robinson, 2003). Alginate salts undergo an aqueous sol-gel transformation to water-insoluble salts due to the addition of divalent ions such as, calcium, strontium, and barium. Although strontium and barium alginate forms stronger insoluble matrices, calcium is the most widely used. Calcium ions initially react with repeating guluronic acid units to form an 'egg box'-shaped structure that stack upon each other. Additional calcium ions then interact with unreacted guluronic and mannuronic acid residues to form a calcium alginate complex.

Cationic polymers are widely used to increase stability of alginate nanoparticles by forming strong complex with negative charge of calcium alginate complex. Chitosan is a commonly used cationic polymer for this purpose.

6. Chitosans

Chitosan is a naturally occurring polysaccharides consisting of glucosamine and N-acetyl glucosamine with polycation characteristics. Chitosan is the deacetylated form of chitin, a natural biopolymer derived from crustacean shells such as crabs, shrimps and lobsters. The primary unit in the chitin polymer is 2-deoxy-2-(acetylamino) glucose. These units combined by β-(1, 4) glycosidic linkages as shown in Figure 5, forming a long chain linear polymer (Tiyaboonchai, 2003).

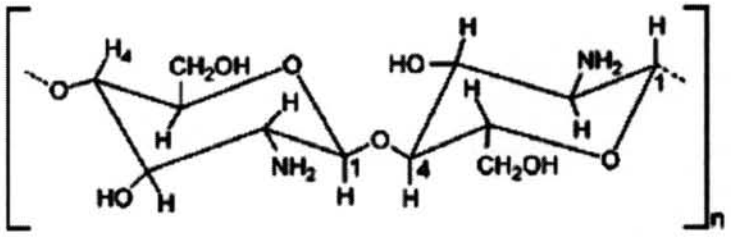

Figure 5 The chemical structure of chitosan (George and Abraham, 2006)

Although chitin is insoluble in most solvents, chitosan is soluble in most organic acidic solutions at pH less than 6.5 including formic, acetic, tartaric, and citric acid. It is insoluble in phosphoric and sulfuric acid. Chitosan is available in a wide range of molecular weight and degree of deacetylation. Molecular weight and degree of deacetylation are the main factors affecting the particle size, particles formation and particles aggregation (Tiyaboonchai, 2003).

In recent years, both chitin and chitosan have received great attention as biologically active substances. Chitin can be converted to chitosan or chitooligosaccharide by chemical process or enzymatic preparation. Chemical methods have several defects such as low product yields and chemical modifications of glucose ring, but are used extensively for commercial purpose of chitosan preparation because of low cost and suitability to mass production. Enzymatic methods can minimize alterations in the chemical nature of the reaction products.

Chitosan has been used widely in the food and pharmaceutical industries, for instance, as a dietary supplement, a wound healing biomaterial and a pharmaceutical excipient (Illum et al., 1998). Chitosan and its derivatives have been used in a wide variety of applications, but the effectiveness of these materials has been found to be dependent upon their molecular and degree of deacetylation.

Chitosan is a cationic polymer has been used for the production of microspheres and nanoparticles as drug delivery systems for drugs (Agnihotri et al. 2004; Janes et al., 2001b; Tiyaboonchai, 2003), proteins (Janes et al., 2001a), DNA and other oligonucleotides (Leong et al., 1998) with encouraging results. There are many

chitosan–polyanion complexes that have been investigated as drug delivery systems. The polycationic of chitosan forms strong complex with negatively charged polymer by ionotropic gelation. Among the various types of chitosan–polyanion complexes reported in the literature, the combination of chitosan and sodium alginate is the most interesting for colloidal carrier systems.

There has been increasing interest in the study of alginate and chitosan for various applications because of their non-toxic, biocompatible and biodegradable properties. Chitosan–alginate microspheres have been widely studied for the encapsulation of several drugs (González-Rodríguez et al., 2002; Pandey and Khuller, 2004) with promising results. Despite the attractive properties offered by chitosan–alginate system, its development and application in the submicron scale has been studied (Motwani et al., 1998; Ahmad et al., 2006). Alginate and chitosan have been used for preparation of polymeric nanoparticles. The interaction between biodegradable cationic and anionic polymers leads to the formation of polyionic hydrogels, which have exhibited favorable characteristics for drug entrapment and delivery. Numerous studies show the achievement when using alginate-chitosan nanoparticles to improve drug properties. Both alginate and chitosan are used together to form a strong complex, resulting in more stable nanoparticles. Chitosan–alginate polyionic complexes are formed through the ionic gelation via interaction between the carboxyl groups of alginate and the amine groups of chitosan. The complex protects the encapsulant, has biocompatible and biodegradable characteristics, and limits the release of encapsulated materials more effectively than either alginate or chitosan alone. A further advantage of this delivery system is its non-toxicity permitting the repeated administration of therapeutic agents.

7. Chitosan-coated alginate nanoparticles

Chitosan-coated alginate nanoparticles were prepared from dilute alginate solution containing drug by inducing an ionotropic pre-gel with calcium counter ions, followed by polyelectrolyte complex coating with chitosan. The interaction between

alginate in dilute solution with Ca^{2+} occurs at a certain ion concentration (De and Robinson, 2003). A pre-gel state results with stirring, avoiding the gel point and forming a continuous system. Subsequent addition of an aqueous polycationic chitosan solution results in a polyelectrolyte complex, stabilizing the alginate pre-gel nucleus into individual nanoparticles. The relative mass ratios of sodium alginate, calcium chloride, and chitosan are critical to form nanoparticles. Specifically, a calcium chloride to sodium alginate mass ratio less than 0.2 was necessary to maintain the pre-gel state essential for the preparation of nanoparticles, as was the addition of chitosan. Based on particle size, particle density, and polydispersity measurements, the optimal conditions for the preparation of chitosan–alginate nanoparticles occurred when calcium chloride to sodium alginate ratio was about 0.2 and chitosan to sodium alginate ratio was 0.1 (De and Robinson, 2003). The factors, such as concentration and molecular weight of alginate or chitosan, N-acetylation degree of chitosan, the mannuronic/guluronic acid ratio of alginate, pH value in preparation, and preparation procedures may influence the nanoparticles properties.

Chitosan-coated alginate nanoparticles have been successfully formulated as the carrier for delivery of various drug such as oral insulin (Sarmento et al., 2007), antituberculosis drug (Ahmad et al., 2006) and azole antifungal drug (Ahmad et al., 2007).

8. Freeze-drying

The major limit of the nanoparticles development when they are stored as aqueous suspensions is due to the physical instability such as aggregation and particle fusion, the chemical instability such as hydrolysis of polymer materials forming the nanoparticles, drug leakage of nanoparticles and/or other component degradation, chemical reactivity of drug during the storage and microbiological growth. The time of contact with water also influence the amount of drug incorporated into the nanoparticles, especially the drug which degrades in an aqueous environment. In order to improve the physical and chemical stability of nanoparticles, water has to be removed in this system. The most commonly used process which converts solutions

or suspensions into solids of sufficient stability in the pharmaceutical field is freeze-drying. Freeze-drying, also known as lyophilization, is a process which waters are removed from a frozen sample, followed by sublimation and desorption under vacuum (Abdelwahed et al., 2006; Schwarz and Mehnert, 1997). Freeze-drying as a drying method has many applications for nanoparticles technology. The literature contains many examples of such applications. The main use of freeze-drying is essential for improving long term nanoparticles stability. The transformation of colloidal suspension into solid form has the advantage of preventing particles aggregation, also the degradation of polymer forming nanoparticles and the leakage of encapsulated drug out of nanoparticles. Furthermore, freeze-drying could be transformed into another solid dosage form intended for different administration routes such as parenteral, oral, nasal, and pulmonary (Abdelwahed et al., 2006).

This process generates various stresses during freezing and drying steps. Thus, protectants are usually added to the formulation to protect the nanoparticles from freezing and desiccation stresses. It has been reported that additives such as saccharides can be used as cryoprotection of the nanoparticles in the freeze-drying process. These saccharides may act as a spacing matrix to prevent particles aggregation. In the literature, there are a few papers on freeze-drying process of polymeric nanoparticles compared to other colloidal systems such liposomes. Furthermore most often, the investigations into nanoparticles freeze-drying have been carried out by a trial and error without studying the scientific principles of this complex process. For some nanoparticles formulations, it was possible to freeze-dry nanoparticles without adding cryoprotectant or lyoprotectant. Such polymer layer formed at the nanoparticles surface can stabilize the nanoparticles and improve their freezing resistance. Because of the applications and procedure of freeze-drying, this process could affect the nanoparticles properties such as size, the release behavior and the drug pharmacokinetics (Soppimath et al., 2001).

9. HPLC Method

The High Performance Liquid Chromatography (HPLC) is the commonly used technique for qualitative and quantitative analysis. In the analytical method, method validation is the process by which it is established, by laboratory studies, that the performance characteristics of the method meet the requirements for the intended analytical applications. The USP analytical characteristics used in method validation are accuracy, precision, specificity, detection limit, quantitation limit, linearity, range and robustness (USP 29). Typically, accuracy, precision, specificity, linearity and range are needed first for analysis of active ingredients in pharmaceutical products.

The accuracy of an analytical procedure is the closeness of test results obtained by that procedure to the true value. Accuracy is calculated as the percentage of recovery. The International Conference on Harmonization (ICH) documents recommend that accuracy should be assessed using a minimum of nine determinations over a minimum of three concentration levels (three concentrations and three replicates of each concentration). Precision can be defined as the degree of agreement among individual test results when the procedure is applied repeatedly to multiple sampling of a homogenous sample. The precision is usually expresses as the standard deviation or relative standard deviation of a series of measurements. The ICH divides precision into three types: repeatability, intermediate precision, and reproducibility. The precision assessment for initial method validation often requires first two of them. Repeatability refers to the use of analytical procedure within a laboratory over a short period of time using the same analyst with the same equipment. The ICH recommends that repeatability should be assessed using a minimum of nine determinations covering the specified range for the procedure (three concentrations and three replicates of each concentration). Intermediate precision is expressed within laboratory variation, as on different days, or with different analysts or equipment within the same laboratory. The linearity of a method is a measure of how well a calibration plot of response and concentration nearly a straight line. A linearity correlation coefficient above 0.999 is acceptable for most method. The ICH

recommends that a minimum of five concentrations normally be used for establishment of linearity. The range of a method can be defined as the lower and upper concentrations for which the analytical method has adequate accuracy, precision, and linearity. System suitability also used in analytical method to verify that the detection sensitivity, resolution, and reproducibility of the chromatographic system is adequate for the analysis to be done. This must be established before sample analysis. The example parameters for system suitability are reproducibility and tailing factor. Reproducibility is the % relative standard deviation calculated from peak area of five replicate injections of a standard solution. The obtained result should be less than 2%. The tailing factor is a measurement of peak symmetry. The accuracy of quantitation decreases if the peak tailing increases. The tailing factor of each five peaks from five replicate injections of a standard solution should be less than 2.

10. The stability of the drug

Stability is defined as the time during which a reconstituted drug retains its integrity in terms of quantity and chemical identity. The reconstituted drug is considered to be stable when the drug concentration remains within acceptable limits, i.e., when the drug concentration(s) on any day of analysis is not less than 90% of its initial concentration (Williams and Lokich, 1992).

Once the drug has been reconstituted in an appropriate infusion fluid, the rate of drug degradation usually increases dramatically. Environmental factors such as temperature, pH, light, air, and the type of container used can affect the stability of the final solution. The most important factor affecting drug stability is pH, which can have a dramatic effect on the stability of labile drugs.

The second most important factor that can influence the rate of degradation and substance stability is temperature. An increase of 10°C in the storage temperature can enhance chemical degradation by a factor of 2-5. The loss of drug potency due to chemical degradation in infusion fluids usually results from hydrolysis, oxidation, or

photolysis. Hydrolysis is the most frequently encountered type of chemical reaction responsible for drug degradation in aqueous solutions and is usually pH-dependent. Control of the pH can optimize the stability of the infusion. Oxidation reactions proceed through a mechanism involving oxygen and can be catalyzed by light, pH, and metal ions. Some of the antineoplastic drugs most susceptible to oxidation include doxorubicin, dacarbazine, methotrexate, and leucovorin (Williams and Lokich, 1992). Oxidation reactions in infusion solutions can be controlled by pH and protection from light. Photolysis describes the light-catalyzed degradation of substances, which is dependent not only on the wavelength of the light but also on its intensity and on the pH of the solution.

Polymer-based delivery systems may provide an increased physical and chemical stability of nanodispersed formulations containing active compounds over a period of storage time. They have been developed to improve the stability of various agents (Saxena et al., 2004; Perugini et al., 2002). The protective effect of nanoparticles seemed to be due to the polymeric-envelop which protected the entrapped drug by isolating it from the surrounding environment. PLGA nanoparticles was achieved to provide efficient aqueous-stability, photo-stability and thermal stability to indocyanine green (Saxena et al., 2004). Also 2-ethylhexyl-p-methoxycinnamate, a sunscreen agent, PLGA nanoparticles reduced the photodegradation of this agent in emulsion vehicles (Perugini et al., 2002).