CHAPTER II

LITERATURE REVIEW

1. Nanotechnology

1.1 Definition

Nanotechnology literally means any technology performed on a nanoscale that has applications in the real world (Bhushan, 2004). In a broad sense, nanotechnology can be defined as the science and technology involved in the design, syntheses, characterization, and application of materials and devices whose smallest functional organization in at least one dimension is on the nanometer scale, one billionth of a meter, ranging from a few to several hundred nanometer (Silva, 2004).

1.2 Application

Nanotechnology encompasses the production and application of physical, chemical, and biological systems at scales ranging from individual atom or molecule to submicron dimensions, as well as the integration of the resulting nanostructure into larger systems. A variety of nanosystems, nanocomponents, nanodevices, and nanomaterials are used in many industrials, for example, nanomagnetic particles in magnetic media, nanowires, carbon nanotubes as electrodes in fuel cells, nanoscopic lasers, sharp nanotips for atomic force microscopy (AFM), etc., including biomedical applications (Bhushan, 2004)

For biomedical applications, nanomaterials and nanodevices can be designed to interact with cells and tissues at a molecular or subcellular level with a high degree of functional specificity (Silva, 2004). Several nanoscale devices that could find practical implementation in medicine have been described in the literature.

For instance, artificial mechanical red blood cells, artificial cell membranes, artificial nanostructures that can interact with and replace natural biological materials (Bogunia-Kubik and Sugisaka, 2002). Novel drug delivery systems, especially for the blood brain barrier in some cases, using nanoparticles are included. Another class of applications being developed is chemically functionalized dendrimers, highly branched molecules with a tree-like branching structure that can be used as molecular building blocks for gene therapy agents or as magnetic resonance imaging (MRI) contrast agents. In addition, much research is going into biologically inspired functional nanodevices, such as the development of DNA polymerase chain reaction or protein-based molecular computers, which encode information in the nucleotide sequences of DNA molecules or in the tertiary structures of proteins (Silva, 2004).

2. Nanoparticles

2.1 Definition

Nanoparticles are defined as small spherical particles in submicron size, generally vary from 10 to 1,000 nm. In this system, the therapeutic agent is dissolved, entrapped, encapsulated, or attached to the nanoparticle matrix (Bogunia-Kubik and Sugisaka, 2002).

The two basic types of nanoparticles are nanocapsules and nanospheres. Nanocapsules are defined as vesicular systems in which the drug is encapsulated within the cavity, which is an oil or aqueous core, surrounded by a unique polymer membrane. Whereas, nanospheres are defined as matrix systems in which the drug is physically and uniformly dispersed throughout the particles (Allémann *et al.*, 1996). The schematic diagram of cross-section nanoparticles is shown in Figure 2-1.

A variety of therapeutic agents including low molecular weight lipophilic or hydrophilic drugs and high molecular weight DNA or antisense can be encapsulated in nanoparticles (Murakami *et al.*, 1999). Drug-containing nanoparticles can be obtained through the incorporation of a drug substance during or after the preparation (Bodmeier and Maincent, 1996). On the other hand, the drug content in nanoparticles may be released by one or combination of following processes; desorption of surface-bound drug, diffusion through the nanoparticle matrix, diffusion through the polymer wall of nanocapsules, nanoparticle matrix erosion, and combined erosion diffusion process (Kreuter, 1988).

2-1a 2-1b

Figure 2-1. Schematic cross-section diagrams illustrating nanoparticles: nanocapsules (2-1a), and nanospheres (2-1b).

2.2 Application

Nanoparticles have been investigated and found to have potential as effective delivery devices in many therapeutic applications as described below.

2.2.1 Gene delivery

The therapeutic efficacy of nanoparticles could be due to their ability to protect the content from lysosomal enzymatic degradation. Following intracellular uptake and endothelial lysosomal escape, nanoparticles could release the encapsulated DNA at a sustained rate resulting in suatained gene expression in the

target tissue. Rapid escape of nanoparticles from the degradative endo-lysosomal compartment to the cytoplasmic compartment and their intracellular retention suggest that nanoparticles containing plasmid DNA could serve as an efficient sustained release gene delivery system (Panyam *et al.*, 2003).

2.2.2 Antisense delivery

Antisense oligonucleotides have emerged as potential gene-specific therapeutic agents and are currently undergoing evaluation in clinical trails for a variety of diseases, such as advanced carcinoma and non-Hodgkin's lymphoma. Antisense oligonucleotides are molecules that are able to inhibit gene expression, being therefore, potentially active for the treatment of viral inflections or cancer. However, the problems such as the poor stability of antisense oligonucleotides versus nuclease activity *in vitro* and *in vivo*, and their low intracellular penetration have limited their use in therapeutics. In order to increase their stability, to improve cell penetration, and also to avoid non-specific aptameric effects (leading to non-specific binding of antisense oligonucleotides), the use of particulate carriers such as nanoparticles, has been considered (Bogunia-Kubik and Sugisaka, 2002).

2.2.3 Peptide and protein delivery

The systems of delivery of peptides and proteins by the oral route have attracted considerable attention as being much easier and more acceptable than the injection. Unfortunately, there is limitation due to both the degradation within the intestine and poor uptake across the intestinal wall. To overcome these problems, it is possible to utilize the uptake mechanism of vitamin B_{12} to enhance the oral uptake of various peptide and protein pharmaceuticals. To maximize the potential of the delivery system, the peptide or protein is being incorporated within biodegradable nanoparticles, and coated with vitamin B_{12} (Russell-Jones *et al.*, 1999).

2.2.4 Other applications

In addition to oral and parenteral administration, the use of nanoparticles for nasal (Illum *et al.*, 2001) and ophthalmic delivery of drugs (de Campos *et al.*, 2001) has been investigated. Nanoparticles have enabled crossing the blood-brain barrier that represents an insurmountable obstacle for a large number of drugs, including antibiotics, antineoplastic agents, and a variety of central nervous system active drugs, especially neuropeptides (Kreuter, 2001; Schroeder *et al.*, 1998). Furthermore, vitamins molecules, such as vitamin A, and vitamin E, can act as nanosize carriers, having potential applications in dermatology and cosmetics (Jenning *et al.*, 2000) (Dingler *et al.*, 1999).

2.3 Polymeric nanoparticles

A number of different polymers, both synthetic polymers (e.g. poly (lactide-co-glycolide) copolymers, polyacrylates, and polycaprolactones) and natural polymers (e.g. albumin, gelatin, alginate, collagen, and chitosan) have been utilized in formulating polymeric nanoparticles. Synthetic polymers have the advantage of sustaining the release of the encapsulated therapeutic agent over a period of days to several weeks compared to natural polymers, which have a relatively short duration of drug release (Murakami *et al.*, 1999).

Over the past few decades, there has been considerable interest in developing biodegradable polymeric nanoparticles as effective drug delivery devices. Firstly, the increasing attention has been paid to injectable drug carriers, which would enable a long systemic circulation. Biodegradable polymeric nanoparticles offer some specific advantages over liposomes. For instance, nanoparticles have higher stability than liposome, and can help to improve the stability of drugs or proteins. Nowadays, biodegradable polymeric nanoparticles are attracted considerable attention as potential

drug delivery devices for the controlled release of drugs and drug targeting to particular organs/tissues. They have also been investigated as carriers of DNA in gene therapy, and delivery of proteins, peptides and genes through an oral route of administration, and delivery other drugs via pulmonary or nasal route. The controlled release of pharmacological 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 (Soppimath *et al.*, 2001).

Various types of biodegradable 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 (Kreuter, 1994). Among these polymers, poly (α-hydroxycarboxylic acids), such as poly (lactic acid) (PLA), poly (glycolic acid) (PGA), and their copolymers, poly (d,l-lactide-*co*-glycolide) (PLGA) have been the most extensively investigated (Murakami *et al.*, 1999).

PLGA is a group of polymers, consisting of PLA and PGA monomer. The PLGA polymers are considered as biodegradable and biocompatible, which have been approved by the United States of America Food and Drug Administration (US FDA). The drug entrapped in PLGA matrix is released at a sustained rate though diffusion of the drug in the polymer matrix and by degradation of the polymer matrix PLGA copolymers undergo hydrolysis in the body, forming biologically compatible and metabolizable moieties (lactic acid and glycolic acid) that are eventually removed from the body by the citric acid cycle. These biodegradation products are formed at a very slow rate, and hence they do not affect the normal cell function. These polymers have also been tested for toxicity and safety in extensive animal studies, and are currently being used in humans for resorbable sutures, bone implants and screws, and contraceptive implants. These polymers are also used as graft materials for artificial

organs, and recently as supporting scaffolds in tissue engineering research (Murakami *et al.*, 1999).

2.4 Preparation methods

Nanoparticles can be prepared from preformed polymers or through polymerization of monomers. The preparation from preformed polymers has several advantages over the preparation through polymerization of monomers. These include the use of polymers with well characterized physicochemical properties, established safety and approval standards, the absence of residual monomers or polymerization reagents (e.g., initiators or catalysts) and the lack of possible reactions between drugs and monomers. In addition, it is not possible to obtain nanoparticles by emulsion polymerization for polymers where suitable monomers are not available (Bodmeier and Maincent, 1996). The preparation techniques of nanoparticles from preformed polymers are classified as follows.

2.4.1 Emulsification/solvent evaporation method

a) Water-immiscible organic solvent

This method is more suitable for preparing nanoparticles containing water-insoluble drugs. The drug and polymer are dissolved or dispersed in an organic solvent, such as dichloromethane or ethyl acetate and then emulsified into an aqueous phase containing surfactant or emulsifying agent, e.g. gelatin, poly (vinyl alcohol), polyxamer-188, sodium dodecylsulfate etc., to make oil in water (O/W) emulsion. After that, the O/W emulsion is homogenized to form nanospheres prior to the evaporation of organic solvent (Krause *et al.*, 1985).

The water-soluble drugs have also been prepared by using the water-in-oil-in-water (W/O/W) double emulsion technique. In this case, the drug

is dissolved in water before adding to the polymer organic solution to form water-in-oil (W/O) emulsion. The intermediate emulsion is then added to the external aqueous phase, resulting in the formation of water-in-oil-in-water (W/O/W) emulsion (Davda *et al.*, 1998; Song *et al.*, 1997; Zambaux *et al.*, 1998).

b) Water-miscible organic solvent

In this method, less toxic solvents, such as ethanol, acetone, are used to replace toxic organic solvents. Then the nanoparticles are prepared as the same method as of the water-immiscible organic solvent (Bodmerier and Maincent, 1996).

2.4.2. Spontaneous emulsification/solvent diffusion method

This method is modified from the emulsion/solvent evaporation method. In contrast, the combination of water-soluble solvent, such as acetone or methanol, and organic solvent, e.g. dichloromethane or chloroform, are used as an oil phase instead of pure organic solvent (Niwa *et al.*, 1993).

2.4.2 Salting-out method

In this method, water-in-oil (W/O) emulsion is formed by adding an electrolyte saturated, e.g. $CaCl_2$, aqueous solution containing a stabilizing and a viscosity-enhancing agent, generally poly (vinyl alcohol) (PVA), to an acetone solution of drug and polymer (organic phase) under vigorous stirring. After that, the W/O emulsion is inverted to an oil-in-water (O/W) emulsion by further addition of the aqueous phase portion (the final organic: aqueous weight ratio is 1:2). Purified water or an aqueous solution of PEG is then dropped into the O/W emulsion to allow complete diffusion of the organic solvent into the aqueous phase, thus inducing the formation of nanospheres (Ibrahim *et al.*, 1992; Perugini *et al.*, 2002).

2.4.3 Emulsification-diffusion method

This method is modified to overcome the problem of using of large amounts of salt in salting-out method, because the electrolytes sometimes cause problems of compatibility with some bioactive compounds. An aqueous gel of a stabilizing hydrocolloid (e.g. PVA, gelatin) is added to a solution of polymer dissolved in benzyl alcohol under mechanical stirring. An oil-in-water emulsion is first obtained. The final emulsion undergoes a phase inversion upon complete addition of the aqueous gel, thus leading to the precipitation of the polymer as nanospheres (Allémann *et al.*, 1996).

2.4.4 Interfacial deposition method

In this method, the polymer is dissolved in a water-miscible solvent, generally acetone. A lipophilic drug is then added to this solution along with a phospholipids mixture, e.g. lecithin, and benzyl benzoate. The resulting solution is poured into water containing a surfactant, such as poloxamer 188, under mild magnetic agitation. Consequently nanocapsules containing the drug in the inner core are formed. Finally, the acetone is removed (Allémann *et al.*, 1996; Cauchetier *et al.*, 2003).

The choice of a particular preparation method and a suitable polymer depends on the physicochemical properties of the drug substance, the desired release characteristic, the therapeutic goal, the route of administration, etc. The successful selection of a preparation method is determined by the ability to achieve high drug loading, high encapsulation efficiency, and high product yield (Bodmeier *et al.*, 1996).

2.5 Purification methods

Since most nanoparticles of PLGA are design for parenteral administration, a purification of the raw nanoparticulate suspensions has to be considered for eliminating the potentially toxic and/or undesirable preparation additives, e.g. organic solvents, stabilizers, and large polymer aggregates. Purification is also needed to separate free drug from the drug bound to the particles in order to avoid a burst effect at the time of injection. Polymer aggregation in the micrometer range is usually easily discarded by filtration through sintered glass filters. Organic solvents, especially chlorinated solvents, must absolutely be eliminated, to be below pharmacopoeia limits (Allémann *et al.*, 1996). The purification methods available are as follows.

2.5.1 Ultracentrifugation

This method is also used for purifying raw nanoparticulate suspensions although it is widely used for separating free from bound drug during in the vitro release studies. After ultracentrifugation, the supernatant is discarded, and the particles are resuspended in an appropriate solvent, usually deionized or distrilled water. This procedure is repeated four or five times to quantitatively remove the undesired preparation additives. Ultracentrifugation is a time-consuming process only suitable for laboratory-scale batches. Up-scaling of this method is not currently possible.

2.5.2 Centrifugal ultrafiltration

It requires a device that is mainly based on a centrifuge tube separated from an enclosed tube by an ultrafiltration membrane. During centrifugation, this device allows separation of nanoparticles from the dispersion

medium. This technique is also appropriate for the purification of small amounts of nanoparticles.

2.5.3 Cross-flow filtration

To overcome the problem of scale-up, a cross-flow filtration (CFF) procedure has been developed. In CFF, the fluid to be purified is directed tangentially to the surface of the membranes to prevent clogging of the filters. In practice, the raw nanoparticulate suspension is pumped into a CFF device. The filtrate, which contains the soluble undesired additives, is discarded and the suspension is recirculated several times in the CFF device. The nanospheres are maintained in suspension by adding distilled water from the feed tank at the same rate as the filtration rate. This step is named diafiltration. The advantages of this purification technique are that it is fast, it does not alter the particle size, and the process up-scaling is feasible by enlarging the filtering surface.

2.5.4 Gel permeation

With the use of fractogel® HW55 (Merck, Darmstadt, Germany) or Sephacryl® S-200 (Farmacia,Freiburg, Germany), even high molecular weight compound (e.g. polyxamers) molecules can be extracted from the raw suspensions. This technique has also been used by Beck et al., (51) with Sephadex® G50 (Farmacia) gel filtration media to separate free from particle-bound drug.

2.5.5 Dialysis

Dialysis may be a suitable purification method for laboratory scale batches. This process is efficient but requires a quite long time for running, e.g. 24 hours to purify 500 mg of particles. Moreover, to quantitatively eliminate the

undesired additives, the solution of the receptor compartment must be changed two or three times during the procedure.

2.6 Stabilization

Degradation of polymer, drug leakage, and/or drug degradation may occur if nanoparticles are kept in an aqueous medium during storage. In order to improve the physicochemical stability of the nanoparticles, a drying step is generally required. Heat-drying may be harmful to thermolabile active compounds. Therefore, freeze-drying represents the most applicable technique.

For freeze-drying, the purified or washed nanoparticles are resuspended with deionized water and then frozen at temperatures ranging from -40 to -60°C or in liquid nitrogen. Subsequently, they are freeze-dried for periods ranging from 24 to 90 hours, at the pressure below 10 Pascal.

Ease of redispersion of freeze-dried nanoparticles should be concerned. Nanoparticles prepared in the presence of poloxamer, polysorbate, PVA, or Sodium dodecylsulfate are readily redispersible in water since residues of these stabilizers or surfactants display the cryoprotective effect. However, addition of a cryoprotective agent may be necessary if freeze-dried nanoparticles are not readily redispersible. Dextrose, lactose, trehalose, sucrose, and mannitol are the most common (Allémanne *et al.*, 1996).

2.7 Characterization

2.7.1 Particle size, morphology, and surface charge

Nanoparticles are generally characterized by the average particle diameter, size distribution, and charge. These parameters can affect, for example, the physical stability and the in vivo distribution of nanoparticles (Bodmeier *et al.*, 1996). In addition, modification of size significantly influences the release rate of an incorporated substance. The release rate tends to be decrease from increasing of the particle size (Allémann *et al.*, 1996).

The polydispersity index (PI) is necessary to characterize the particle size distribution of nanoparticles. The PI value of monodisperse system is zero or relatively small, e.g. less than 0.06. The small value of PI, e.g. 0.1, corresponding a narrow size distribution, whereas the larger PI values indicate a broad size distribution (Bodmeier and Maincent, 1996)

The surface charge of nanoparticles is expressed as the zeta potential, which are usually negative values ranging from about -100 to -5 mV. High surface charges are also responsible for the physical stability of the colloidal polymer dispersions since the repulsive forces can prevent aggregation upon aging (Bodmeier *et al.*, 1996).

There are several technique used for the objective of particle size and size distribution, morphology, and surface charge of nanoparticles (Allémann and Leroux, 1996; Bodmeier and Maincent, 1996).

a) Laser diffraction techniques

This technique relies on the fact that diffraction angle is inversely proportional to particle size. One method is the low angle laser light

scattering (LALLS), suitable for measuring particle sizes ranging from 0.1 – 3000 μ m. Another method is photon correlation spectroscopy (PCS), based on dynamic laser light scattering, this method allows the determination of particle size in the range of 5 nm - 5μm. The size distribution can also be determined. These methods are reproducible, rapid, accurate, and nondestructive. In addition, the sample preparation is easy. The particles only need to be sufficiently diluted in a solvent, which is generally filtered water.

b) Electron microscopy techniques

Microscopic technique allows determination of the particle size and size distribution, including the particle shape and morphology.

Scanning electron microscopy (SEM) is widely used since it has high resolution. The specimen is scanned with a focused fine beam of electrons and the surface characteristic is obtained from secondary electrons emitted from the specimen surface. This technique is performed in high vacuum. For visualization, the particles have to be dry and coated with gold. The mean particle size can also be obtained by SEM, with comparable to the results obtained by PCS.

Transmission electron microscopy (TEM) is a technique based on the transmission of electrons through thin specimens. It is not as widely used as PCS and SEM for particle size analysis, but it is still the powerful method for determining the particle morphology.

Atomic force microscopy (AFM) is a technique enables the visualization of nanoparticles at atmospheric pressure without gold coating. Thus, any modification of the original aspect of the particles from high vacuum and gold coating can be eliminated (Allémann *et al.*, 1996). AFM has been widely applied to provide surface-dependent information in three dimensions on a nanometer scale. It is capable

of resolving surface details down to the atomic level and can give morphological images in high resolution (MU *et al.*, 2002).

2.7.2 Nanoparticle recovery, drug content, and entrapment efficiency

It is a prime of importance to determine whether the preparation procedure used for incorporating a compound into the nanoparticles is efficient, especially if it considers with costly substances. The parameters used in representing the effectiveness of formulations are the nanoparticle recovery, the drug content, and the entrapment efficiency (Allémann and Leroux, 1996).

The nanoparticles recovery can be calculated from Equation 2-1

$$\textbf{Recovery (\%)} = \frac{\textbf{Amount of nanoparticles}}{\textbf{Amount of raw materials}} \times 100 \qquad \text{(Equation 2-1)}$$

The drug content is calculated from Equation 2-2

$$\textbf{Content (\% w/w)} = \frac{\textbf{Amount of drug in nanoparticles}}{\textbf{Amount of nanoparticle}} \times 100 \qquad \text{(Equation 2-2)}$$

In order to determine drug content, separation of free from bound drug has to be accomplished firstly. The analysis can be assessed after ultracentrifugation of a nanoparticles suspension and dissolution of the sediment in an appropriate solvent. Additional analysis of the free drug in the supernatant may be used to confirm the results. On the other hand, the freeze-dried nanoparticles can be directly dissolved in an adequate solvent before analysis. Methods such as spectrophotometry, or high-performance liquid chromatography (HPLC) can be used in analysis.

Drug entrapment efficiency, which is defined as encapsulation efficiency in case of nanocapsules, can be calculated from Equation 2-3.

Entrapment efficiency (%)

$$= \frac{\textbf{Amount of drug in nanoparticles}}{\textbf{Amount of drug used in formulation}} \textbf{ x 100} \quad \text{(Equation 2-3)}$$

2.8 *In vitro* Release Study

In vitro release study, in principle, should be useful for quality control as well as for the prediction of in vivo kinetics of nanoparticulate systems. In addition, it can be very useful for evaluation of the influences of process parameters on the release rate of active compounds from nanoparticles. There are several methods used for *in vitro* release study (Allémann *et al.*, 1996).

2.8.1 Dialysis

In this method, the nanoparticles are suspended in a small volume of release medium and separated from a large bulk sink medium by a dialysis membrane, which is permeable to the drug. The released drug in the receptor phase is assayed. Nevertheless, the appearance rate of the drug in the sampling compartment depends on experimental factors, such as drug excipient interactions, drug membrane interactions, micelles, and osmosis. Therefore, dialysis methods are often not appropriate for determining the release characteristics.

2.8.2 *In Situ* Method

The nanoparticles are dispersed in a buffer solution and the drug is directly assayed by spectrophotometrical method in a buffer solution with out

separating the free from the particle-bound drug. To do this, some characteristics (e.g., spectroscopic) of the drug in solution must be different from the particle-bound drug. The disadvantages inherent to the dialysis method are therefore avoided. Unfortunately, most active compounds do not show significant differences in physicochemical characteristics between the free states.

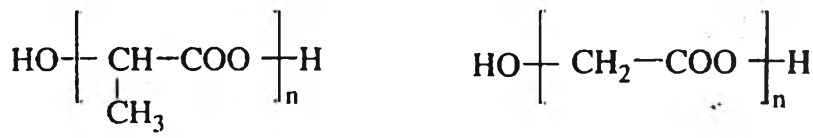
2.8.3 Sample and separation technique

The nanoparticles are suspended into a sink medium maintained at a constant temperature under agitation. Ideally, the sink medium should be a buffer solution with a pH of 7.4, but some active substances require other pH values or the addition of a surfactant to reach sink conditions. Usually, the working temperature is 37° C. Sampling is carried out at various time intervals. The continuous phase is then separated from the dispersed phase by ultracentrifugation or centrifugal filtration, and the release drug is assayed.

2.8.4 Ultrafiltration at low pressure

This method is based on direct suspending of the nanoparticles in the release medium, in a sufficient amount to provide sink conditions. The amount of drug released from the carrier is determined by sampling the filtrate, which is collected at set time intervals from the filtration at a slow rate and at low pressure (less than 0.5 bars).

3. Poly (d,l-lactide-*co*-glycolide) copolymers (PLGA)



2-2a 2-2b

Figure 2-2. Chemical structure of poly (lactic acid) (PLA) (2-2a), and poly (glycolic acid) (PGA) (2-2b)

3.1 Physicochemical properties of PLGA

Poly (l-lactide) (l-PLA) is found to be semicrystalline in nature due to high regularity of its polymer chain while poly (d,l-lactide) (d,l-PLA) is an amorphous polymer because of irregularities in its polymer chain structure. Hence the use of d,l-PLA is preferred over l-PLA since it enables more homogeneous dispersion of the drug in the polymer matrix. Poly (glycolic acid) (PGA) is highly crystalline because it lacks the methyl side groups as of the PLA.

The physical properties such as the molecular weight and the polydispersity index have a deep impact on the mechanical strength of the polymer and its ability to be formulated as a drug delivery device. Also these properties may control the polymer biodegradation rate and hydrolysis. The commercially available PLGA copolymers are usually characterized in terms of intrinsic viscosity, which is directly related to their molecular weights. In addition, the mechanical strength, swelling behavior, capacity to undergo hydrolysis, and subsequently the biodegradation rate are directly influenced by the crystallinity of the PLGA copolymer. The crystallinity of the PLGA copolymer is dependent on the type and the molar ratio of the individual monomer (lactide and glycolide) components in the copolymer chain. Glycolic acid is more hydrophilic than lactic acid and hence PGA-

rich PLGA copolymers are more hydrophilic, absorb more water, and subsequently degrade more quickly. For example, PLGA copolymers, containing 50:50 ratio of lactic and glycolic acids, are hydrolyzed much faster than those containing higher proportion of either of the two monomers, e.g. 75:25, 65:35, and 85:15 (Jain, 2000).

3.2 Biological properties of PLGA

Both, in vitro and in vivo the PLGA copolymer undergoes degradation in an aqueous environment (hydrolytic degradation or biodegradation), through the cleavage of its backbone ester linkages. The polymer chains undergo bulk degradation and the degradation occurs at uniform rate throughout the PLGA matrix.

The biodegraded products of PLGA polymer are lactic and glycolic acids. Lactic acid enters the tricarboxylic acid cycle and is metabolized and subsequently eliminated from the body as carbon dioxide and water. In a study conducted using C-labeled PLA implant, it was concluded that lactic acid is eliminated through respiration as carbon dioxide. Glycolic acid is either excreted unchanged in the kidney or enters the tricarboxylic acid cycle and eventually eliminated as carbon dioxide and water (Jain, 2000).

3.3 Advantages for pharmaceutical applications

Polyglycolic acid (PGA), polylactic acid (PLA), and copolymers of lactic acid and glycolic acid (PLGA) are used in variety of medical applications, because of their biodegradability, biocompatibility, and non toxicity both local and systemic. They degrade slowly by hydrolysis to lactic acid and glycolic acid, which are our body metabolites (Brophy *et al.*, 1994).

PLGA copolymers also possess the strength and mechanical properties required for processing into a variety of forms, such as drug encapsulation, and matrix systems. There are many studies focusing on the preparation and evaluation of various types of drug loaded PLGA nanoparticles. The results indicated that PLGA nanoparticles can improve the photostability of sunscreen agent, *trans*-2-ethylhexyl-*p*-methoxycinnamate (*trans*-EHMC) (Perugini et al., 2002) and can prolong drug effect and protect the encapsulated agent from enzymatic degradation (Davda *et al.*, 2002).

Consequently, various drugs have been formulated in PLGA systems with duration of release varying from days to years. Several dosage forms have made with PLGA, including subcutaneous injectables and implantables, which are normally in the form of microcapsules or rods, respectively. Several fertility-regulating drugs, steroids, antimalarials, and narcotic antagonists have been studied in PLGA systems. Another application of these polymers is in the veterinary area for the manufacture of a bolus for ruminants to delivery agents that improve feed efficiency and rate of weight gain.

3.4 PLGA nanoparticles

Degradation of the PLGA copolymers can be varied by changing block copolymer composition and molecular weight, and hence, the release of encapsulated therapeutic agent can be altered from days to months. Therefore, by modulating polymer characteristics, the degradation, rate, controlled release property, and the release profile of a therapeutic agent from nanoparticles can be adjusted to achieve desired therapeutic level in target tissue and required duration for optimal therapeutic efficacy (Panyam *et al.*, 2003). Since the structures of PLA and PGA are semicrystalline, they exhibit much more slower degradation rate than PLGA, which is amporphous. This is ascribed to the more hydrophilic and more permeable nature of

copolymer. Depending on the intended application, PLGA might be used rather than PLA or PGA to avoid the risk of polymer accumulation in the body.

Encapsulation efficiency, physicochemical properties (particle size, size distribution, and particle morphology), and overall stability of PLGA nanopraticles are also depending upon many factors including preparation methods, molecular weight of polymers, type/concentration of emulsifying agents or stabilizers, etc. (Allé mann,*et al.* 1996).

For the nanoparticles prepared from poly (D, L-lactide-co-glycolide) (PLGA) copolymers, the polymerization technique is not proper because of the limitation of polymer molecular weight (Allémann *et al.*, 1996). Hence, the technique used preformed polymers seems to be better.

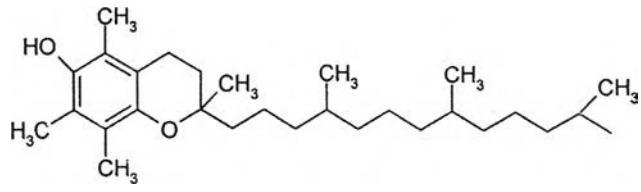
Under such circumstances, various methods have been proposed for the preparation of PLGA nanoparticles. One promising technique is the spontaneous emulsification solvent diffusion (SESD) method (Niwa *et al.*, 1993), in which nanoparticles of PLGA can be effectively obtained by pouring the polymeric organic solution into an aqueous phase with moderate mechanical stirring. One unique technical characteristic of this method is the use of a binary mixture of a water-miscible organic solvent such as acetone and a water-immiscible solvent such as dichloromethane as the solvent of the polymeric solution, and the particles are formed via an emulsification process and a subsequent solvent-evaporation process.

This process seems rational, based on physicochemical interesting phenomena. However, due to a considerable amount of residual dichloromethane, the particles are likely to aggregate during the solvent-evaporation process at high fed amount of polymers. Furthermore, it is necessary to avoid the use of a chlorinated solvent such as dichloromethane because of its toxicity. Therefore, Murakami *et al.* (1999) modified this technique and named the new technique as the modified-SESD

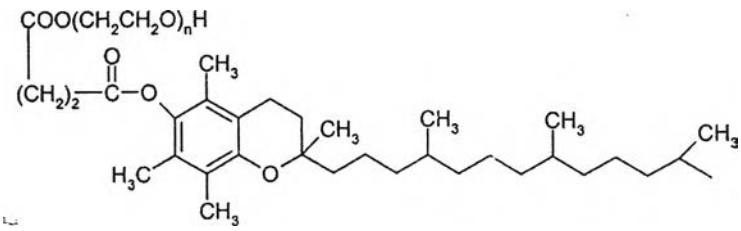
method. In the modified-SESD process, the mixture of two water-miscible organic solvents, such as ethanol or methanol, and acetone or acetonitrile was employed, instead of using the mixture of dichloromethane and acetone. This alteration could prevent the aggregation of particles even at a high fed amount of polymeric solution, resulting in improvement of yield. This alteration also provided some additional advantages; for instance, the use of a toxic solvent such as dichloromethane could be avoided; and uniform nanoparticles dispersion could always be attained by even mild agitation.

Polyvinyl alcohol (PVA) has been the most commonly used stabilizer for the formulation of PLGA nanoparticles, because the particles formed by using this stabilizer are relatively uniform and smaller in size, and are easy to redisperse in aqueous medium (Panyam *et al.*, 2003). However, because of its difficulty for complete removing from the obtained nanoparticles, PVA may exert some side effects to therapeutic formulations. Natural surfactants, such as polysaccharides, phospholipids, cholesterol, and vitamins, or nonionic surfactants may have less side effects and better performance in preparation. The study of Vandervort *et al.*, (2002) indicated that poloxamer could be considered as a potentially alternative stabilize for PVA.

Interestingly, a new type of emulsifying agent, d-α-tocopheryl polyethylene glycol 1000 succinate (vitamin E TPGS or TPGS) would be served as an alternative stabilizer for PVA. Vitamin E TPGS is a water-soluble derivative of natural vitamin E. Its molecule comprises both lipophilicity and hydrophilicy, resulting in amphiphilic properties. The lipophilic alkyl tail and hydrophilic polar head portion are bulky and have large surface area. These characteristics make it to be an ideal and effective stabilizer (Mu *et al.*, 2002).



2-3a



2-3b

Figure 2-3. Chemical structures of vitamin E (2-3a) and vitamin E TPGS (2-3b)

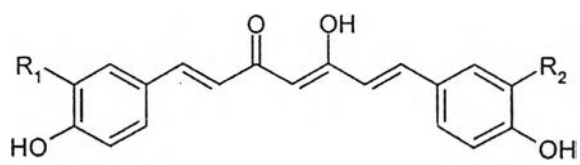
After preparation, PLGA nanoparticles are needed to be purified for separating potentially toxic and/or undersirable preparation additives, such as organic solvents, surfactants, stabilizer, large polymer aggregrates, and also free drug from the raw nanoparticles suspension (Allémann, *et al.* 1996). There are many methods for nanoparticle purification; ultracentrifugation (Fonseca *et al.*, 2002), centrifugal ultrafiltration (Murakami *et al.*, 1999), cross-flow filtration (Allémann, *et al.* 1993), gel permeation (Pirker *et al.*, 1996), and dialysis (Perugini *et al.*, 2002).

The stability of PLGA nanoparticles is depending on storage conditions, e.g. storage temperatures and mediums. The degradation rate increased as an increasing of temperature. The overall stability of PLGA nanoparticles tend to increase when they are stored in buffer solution or freeze-drying form, compared to the PLGA nanoparticles which are stored in water, with the following increasing orders: freeze-drying > buffer solution > water (Lemoine *et al.*, 1996). As the result,

drying is required for improving the physicochemical stability of PLGA nanoparticles. Heat drying may be harmful to thermolabile active compounds. Moreover, the elevated temperature may induce fusion or aggregation of PLGA nanoparticles because of their low glass transition temperature. Therefore, freeze-drying appears to be one of the most suitable methods to stabilize and facilitate the handling of PLGA nanoparticles (Allémann, *et al.* 1996).

4. Curcuminoids

Curcuminoids are composed of three diarylhepyanoids; curcumin (diferuloylmethane), demethoxycurcumin (*p*-hydroxycinnamoyl feruloymethane) and bisdemethoxycurcumin (di-*p*-hydroxycinnamoylmethane) (Figure 2-4). The main constituent of curcuminoids is curcumin (Jayaprakasha *et al.*, 2002).



Curcumin	R_1: OCH_3	R_2: OCH_3	MW: 368.4
Demethoxycurcumin	R_1: OCH_3	R_2: H	MW: 338.3
Bisdemethoxycurcumin	R_1: H	R_2: H	MW: 308.1

Figure 2-4. Chemical structures of curcuminoids

4.1 Biological activities of curcuminoids

Curcuminoids and each of its constituents; curcumin, demethoxy-curcumin, and bisdemethoxycurcumin, have been exhibited various biological activities in many research. These biological activities, including anti-inflammatory, antioxidant, anti-carcinogenic, antitumorigenic, chemopreventive, hepatoprotective, antipsoriatic, and anti-atherogenic, are as follows.

4.1.1 Anti-inflammatory activity

Brouet *et al.* (1995) showed that curcumin inhibited the inducting of nitric oxide synthase (NOS) in macrophage at the lowest concentration (IC_{50} = 6μ M) among various non-steroidal anti-inflammatory agents: aspirin, sodium salicylate, piroxicam, indomethacin, ibuprofen, and sulindac. From the study of Loe *et al.* (1997) additional feeding of curcumin (30 mg/kg bw/day) along with dietary lipid decreased the incidence, delayed the onset and reduced the extent of inflammation of mycobacterial adjuvant-induced arthritis in rats. Chuang *et al.* (2000) found that gavage administration of 200 mg of curcumin suppressed diethyl nitrosamine-induced inflammation and hyperplasia in rats, as shown by histopathological examination. Curcumin also protected against inflammation-related changes in the liver prostanoids in an animal model of alcohol-caused hepatic injury, which linked to an increased activity in serum of the enzymes aspartate transaminase and alkaline phosphatase. When the diet of the ethanol-consuming rats was supplemented with curcumin, not only the activity of those serum enzymes was decreased but there was also a reduction in the abnormally raised levels of prostaglandins E1 and E2 in liver as well as in kidney and brain (Jayadeep *et al.*, 2000). Moreover, curcumin (0.5 and 20 μM) inhibited the expression of cytokines (TNF-α, IL-1β, and IL-8) in dose-dependent manner, in lipopolysaccharide (LPS) stimulated neonatal lung inflammatory cells *in vitro* (Literat *et al.*, 2001)

4.1.2 Antioxidant activity

The study of Grinberg *et al.* (1996) demonstrated that curcumin acted an antioxidant against iron-catalyzed oxidative damage of (β-thalassemia) red blood cell (THRBC), and hydrogen peroxide (H_2O_2)-induced damage of normal red blood cell (RBC). Bonté *et al.*, 1997, indicated that curcuminoids could protect normal human keratinocytes from hypoxanthine/xanthine oxidase injury. The proposed mechanism was a decrease in superoxide radical formation, leading to lower levels of cytotoxic hydrogen peroxide radical. Moreover, pure curcumin was found to be less effective than the curcuminoid mixture, suggesting a synergistic effect between the compounds. In addition, Das *et al.* (2002) determined the reactive oxygen species (ROS) scavenging or quenching properties of curcumin by electron paramagnetic resonance (EPR) spin-trapping technique, a reliable technique for detecting activated ROS in aqueous solution. They found that curcumin was an dose-dependent effective oxygen quencher in aqueous solutions at biologically relevant concentrations.

4.1.3 Anti-carcinogenic and anti-tumorigenic activity

Dietary administration of curcumin possessed anti-carcinegenic activity against 7, 12- dimethylbenz(*a*)anthracene (DMBA)-induced initiation and tetradecanoylphorbol-13-acetate (TPA)-induced promotion mouse skin carcinogenesis (Limtrakul *et al.*, 1997). The study of Huang *et al.* (1997) on the effects of curcumin on TPA-induced tumor promotion indicated that topical application of 100 nmol of curcumin together with 5 nmol TPA twice a week for 18 weeks markedly inhibited TPA-induced tumor promotion. Inano *et al.* (2002) studied the anti-tumorigenic action of curcumin in a standard model of radiation-induced tumor in rat mammary gland. After whole body γ-irradiation, a high incidence (70.3%) of mammary tumorigenesis was seen in the control group receiving standard diet, whereas, the incidence of

tumors was lower (18.5%) in the rats receiving 1 % curcumin in the diet during the initiation stage. The appearance of the first palpable tumor was delayed by 6 months in the curcumin-fed group and a histological examination showed a 50% decrease in the proportion of tumors in the curcumin fed animals in comparison to the controls.

4.1.4 Chemopreventive effect

Khafif *et al.*, (1998) demonstrated that curcumin expressed chemopreventive effect in normal, premalignant, and malignant human oral epithelial cells. It was effective both in single treatment and in combination with (-)-epigallocatechin-3-gallate (EGCG), which is the major constituent of green tea. In addition, Perkins *et al.* (2002) found that 2% dietary curcumin, which equates to about 300 mg/kg, prevented or retarded adenoma formation in the Min/+ mouse model. The susceptibility depends on the location along the intestinal tract and the size of adenoma.

4.1.5 Hepatoprotective effect

Song *et al.* (2001) showed that curcumin, demethoxycurcumin, and bisdemethoxycurcumin exhibited hepatoprotective effect on tacrine-induced cytotoxicity in human hepatoma Help G_2 cells, with EC_{50} values of 86.9, 70.7, and 50.2 μM, respectively.

4.1.6 Antipsoriatic effects

A study by Heng *et al.* (2000) on patients with active psoriasis showed that topical trearment with curcumin resulted in resolution of the psoriatic activity as assessed by clinical, histological and immunological criteria.

4.1.7 Anti-atherogenic effects

The work of Ramirez-Tortosa *et al.* (1999) indicated that hydro-alcaholic extract of C. longa (1.6 mg/kg bw) decreased the susceptibility of LDL to $CuSO_4$ mediated lipid peroxidation in rabbits. The rabbits in the groups treated with oral turmeric extract had lower levels of total plasma cholesterol than the control group.

Other than the advantage of several biological activities of curcuminoids, it was found that curcuminoids have no toxicity in the long-term administration. Sharma *el al.*, (2001) exhibited that when patients with advanced colorectal cancer ingesting Curcuma extract capsules containing 40 to 200 mg curcuminoids for 4 months, they well tolerated at all dose levels with no severe toxicity. However, two adverse effects were found; nausea in one patient taking 120 mg of curcuminoids daily during the first month (National Cancer Institute toxicity grade 1) and diarrhea in two patients taking 80 and 200 mg of curcuminoids daily in the fourth month and first month, respectively (National Cancer Institute toxicity grade 2 and 1, respectively)

4.2 Limitation of use of curcuminoids

Although curcuminoids have advantages, due to their various biological activities and no severe toxicity, they also posses some disadvantages due to their limitation of use, not only as a food supplement but also as a therapeutic agent. The limitations are listed as follows.

4.2.1 Poor water solubility

Curcumin is insoluble in water at acidic or neutral pH, however, it is soluble in ethanol and aqueous alkali solution (THP I, 1995; Tønnesen *et al.*, 2002).

4.2.2 Rapid decomposition in neutral-basic pH solution

Tønnesen *et al.*, (2002) stated that the pKa values for the dissociation of the three protons in curcumin have previously been determined to 7.8, 8.5 and 9.0 respectively. At pH above neutral, curcumin undergoes a rapid hydrolytic degradation. The main decomposition products are feruloyl methane, ferulic acid, and vanillin. The study of Wang *et al.,* (1997) indicated that more than 90% of curcumin decomposed rapidly in neutral-basic pH conditions of buffer systems at 37 C.

4.2.3 Photodegradation

In organic solvent, curcumin decomposed when exposed to sunlight (Tønnesen *et al.*, (2002). The recommended storage condition in the Thai Herbal Pharmacopoeia (Volume I, 1995) is cool, dry and light protection.

4.2.4 Low biovailability

Oral administration curcumin was detectable only in the feces of patients with colorectal cancer. Neither curcumin nor its metabolites; curcumin glucuronide, curcumin sulfate, hexahydrocurcumin, or hexahydrocurcuminol, was found in the plasma or urine at up to 29 days of daily treatment at doses between 440 and 2200 mg of *Curcuma* extract (containing between 40 and 200 mg curcuminoids) (Sharma *et al.*, 2001). Moreover, curcumin disappeared relatively rapidly from rodent

tissues, including the target tissue, once treatment discontinued after intraperitoneal (i.p.) administration (Perkins *et al.*, 2002).

4.2.5 Short circulation half-life

Ireson *et al.*, (2001) demonstrated that curcumin and its conjugates disappeared rapidly from the plasma of rats, after intravenous (i.v.) administration.

The limitations of curcuminoids result in the difficulties on its formulation and, in turn, it cannot be effectively used as a preventive or therapeutic agent. Therefore, an effective delivery device is required to overcome such problems.

5. The Box-Behnken design of experiment

Box and Behnken (1960) have proposed some three-level designs for filling response surface, which is as the Box-Behnken design. These designs are response surface method used to examine the relationship between one or more response variables and a set of quantitative experimental parameters. They are formed by combining 2^k factorials with incomplete block designs. The resulting designs are usually efficient in term of the number of required runs, and they are either rotatable or nearly rotatable.

The three-factor, three-level Box-Behnken design is suitable for constructing second order polynomial models. This design consists of the set of points lying at the midpoints of each edge of the multidimensional cube that defines the region of interest (Montgomery 2001). The design model is shown geometrically in Figure 2-5.

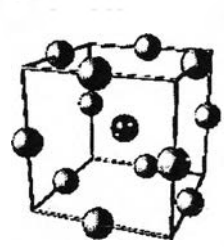


Figure 2-5. Schematic diagram illustrating a Box-Behnken design for three factors and three levels experiment

The inclusion of three replicates at the center point provides a more precise estimate of experimental error and a measurement for the adequacy of the model (lack of fit). It also enabled the determination of the significance of the main, the interaction, and the quadratic effects, for example, the second order model in which coefficients were significantly non-zero (Ragonese *et al.*, 2002). Therefore, 15 design points are required to complete the three- factor, three-level Box-Behnken design as shown in Table 2-1.

Table 2-1. A three-factor, three-level Box-Behnken design

Run	Factor		
	X_1	X_2	X_3
1	-1	-1	0
2	-1	1	0
3	1	-1	0
4	1	1	0
5	-1	0	-1
6	-1	0	1
7	1	0	-1
8	1	0	1
9	0	-1	-1
10	0	-1	1
11	0	1	-1
12	0	1	1
13	0	0	0
14	0	0	0
15	0	0	0

The non-linear quadratic model generated by the design is of the forms:

$$Y = A_0 + A_1X_1 + A_2X_2 + A_3X_3 + A_4X_1X_2 + A_5X_2X_3 + A_6X_1X_3 + A_7X_1^2 + A_8X_2^2 + A_9X_3^2 + E \quad \text{(Equation 2-4)}$$

Where Y is the measured response associated with each factor level combination

A_0 is an intercept

A1 – A9 are the regression coefficients

X_1, X_2 and X_3 are the factors studied

and E is the error term

The Box-Behnken design does not contain any points at the vertices of the cubic region created by the upper and lower limits for each variable factor. This could be advantageous when the points on the corner of the cube present factor-level combinations that are prohibitively expensive or impossible to test (Montogomery, 2001). However, this may be a disadvantage in some case since the effect of each factor on the response cannot be evaluated (Zeaiter et al., 2004).

Smith (1996) demonstrated that the central composite face-centered (CCF), Box-Behnken (BB), and the three-level full factorial (FF-3) designs for determining the effects of three factors were quite similar. This indicates that the BB is more cost-effective since it requires fewer runs, 12 runs plus 3 replicates of center point, whereas the CCF and the FF-3 require 14 runs plus 3 replicates of center point, and 27 runs, respectively.

The Box-Benhken design has been usded in the optimization of drug analysis (Ragonese et al., 2002), and pharmaceutical formulation development (De La Maza et al., 1995, Karnachi and Khan, 1996; Sastry et al., 1997; Nazzal et al., 2002). In addition, the design have been reported to be for robustness testing of analylical methods (Ragonese et al., 2002) and industrial processes (Zeaiter et al., 2004).