CHAPTER II

LITERATURE REVIEW

1. Gene therapy

Gene therapy is the introduction of correct genes to the target cells to replace defective genes failing to encode the essential proteins for the body. These essential proteins are useful for correcting deficiencies that lead to genetic disease, enhancing body resistance to disease or generally improving the quality of life. Gene therapy enables physicians to treat the cause of a disease rather than the symptom. Prior to this attempt, there were no alternatives to correct a genetic disorder. Therapies were developed that transiently alleviated the symptom of disease. The ability to manage protein expression in humans provides the treatment for many diseases that are currently untreatable by conventional drug therapy.

Diseases that are suitable for treatment by gene therapy can be divided into genetic and acquired disease. Genetic diseases are typically caused by a single gene mutation or deletion that impairs normal metabolic pathways, receptor function or regulation of cell cycle. Some examples of genetic disease such as severe combined immunodeficiency, hemophilia, familial hypercholesterolemia, cystic fibrosis, hemoglobinopathies, Gaucher's disease, inherited emphysema, muscular dystrophy (Sullivan, 2003). The acquired diseases are those for which no single gene has been identified as the only cause of the disease state such as cancer, neurological diseases, cardiovascular diseases, infectious diseases (Sullivan, 2003). However, expression of a single gene in the correct cell type can potentially lead to the elimination of the disease state of acquired diseases.

Gene therapy is performed with nonreproductive cells, typically known as somatic cells (Moseley and Caskey, 1993). The example of nonreproductive cells such as blood cells, skin cells, bone marrow cells, intestinal cells, or virtually any cells other than sperm cells or egg cells. The use of somatic cells provides ensurement that there is no carryover of the inserted genes to the next generation (Alcamo, 2001). The knowledge of the human genome provides a major

impetus in identifying human genes implicated in diseases. In addition, the Human Genome Project will help determine genetic markers responsible for patient response to drug therapy, drug interactions, and potential side effects. Therefore, the possible for negative side effects of gene therapy may be minimized compared to conventional treatments that typically have less specificity.

In United States about forty children are born with a disease of the immune system called severe combined immunodeficiency disease (SCID) each year. In almost half of the patients with SCID, the cells have a defective gene that cannot encode a particular enzyme. This enzyme is called adenosine deaminase (ADA). The ADA deficiency causes the body to lose the protection of both kinds of lymphocytes, therefore, the patient cannot rise a defense against infectious disease and soon dies. The gene for ADA production is located on chromosome number 20 and has 32,000 base pairs and twelve exons. In 1990, a research team from the National Institutes of Health (NIH) received approval to use gene therapy for the purpose of relieving ADA deficiency. The lymphocytes were removed from the patient and exposed the cells to billions of retroviruses carrying the genes for ADA production. This is the *ex vivo* approach compared to the *in vivo* approach where cells remain in the patient's body. On September 14, 1990, the first gene therapy was used in 4-year-old girl named Ashanti. The amount of ADA produced by the lymphocytes appeared to be increasing with time, and the ability to produce antibodies had increased substantially (Alcamo, 2001).

An extension of the success was observed in *in vivo* human gene therapy for treatment of hemophilia. Adeno-associated virus, as gene delivery vector, was delivered intramuscularly to patients lacking functional clotting Factor IX. The reduction in the number of bleeding for the treated patients was also observed (Sullivan, 2003). *In vivo* gene therapy is a more acceptable approach, because the treatment, consists of a gene and vector, is directly administered to the patient via conventional, rather than *ex vivo* approach that require isolating the patient's cells, introducing the gene into those cells, and then reintroducing the modified cells back into the patient. Thus, *ex vivo* gene therapy is not a desirable approach for pharmaceutical companies but may be a service that can be provided for individual treatment.

In the patients with cystic fibrosis (CF), the cells lining the body's organs and vessels build up the chloride ions. These ions typically pass out of cells through a channel-shaped, tunnel-like protein called cystic transmembrane conductance regulator (CTCR) protein. In person with CF, the CTCR protein is not produced because of gene defect, and the ions remain in the cells. Therefore, the water is drawn into the cells and leaves dehydrated, sticky mucus in the body's passageways, especially its airways. This condition can conduct the infection. Most CF patients die from respiratory tract and lung infections before reaching the age of thirty. The adenovirus is suitable vector because of its ability to penetrate cells of human respiratory tract (Alcamo, 2001).

In the early stages of development, gene therapy was attempted to correct inheritable disorders resulting from a single gene defect. The disease targets included adenosine deaminase deficiency in severe combined immunodeficiency disease (SCID), cystic fibrosis transmembrane conductance regulator gene mutation in cystic fibrosis, hypoxanthine-guanine phosphoribosyl transferase deficiency in Lesch-Nyhan disease and glucocerebrosidase enzyme deficiency in Gaucher's disease. Later, gene therapy was a promising treatment for a wide range of disease, including cancer, AIDS, neurological disorders such as Parkinson's disease and Alzheimer's disease and cardiovascular disorders.

In the treatment of cancer, damaged *p53* gene, tumor suppressing gene, is replaced by normal gene. The normal gene helps to repair DNA damage in the cell, but when it is in the mutated form, the gene cannot perform its function and a tumor develops (Alcamo, 2001). Gene therapy was also used to relieve the effects of acquired immunodeficiency syndrome (AIDS) by attaching genes for HIV proteins to the vector and then administer into the patients. The HIV genes stimulated the normal body cells to produce HIV proteins. Then, these proteins should stimulate the immune system to secrete anti-HIV antibodies.

2. Gene delivery system

Although gene therapy has many advantages, the process of gene therapy involves many obstacles. Poor cellular uptake and rapid degradation of DNA necessitate the use of delivery systems to facilitate cellular internalization and protect their activity. The combination of several factors such as DNA charge, size, and poor stability represent a potent barrier to cellular uptake. The negatively charged phosphate backbone of the DNA is the primary cause of its inefficient cellular association, owing to electrostatic repulsion between DNA and negatively charged cell surface. In addition, naked DNA has extremely low stability because of degradation by nuclease. Nucleases recognize the phosphodiester linkage in the DNA backbone and induce hydrolytic degradation of the DNA molecule. Nuclease degradation of DNA can be circumvented by chemical derivatization of the backbone or by using gene delivery system.

The therapeutic success of gene therapy is dependent on the development of efficient and safe gene delivery system. To deliver genes to targeted cells, the delivery system must use the carrier molecule or particle called vector. The vector is a molecule, particle, organism, or other carrier that transports genes to the targeted cells. The ideal properties of vector in gene delivery system include high transfection efficiency with a high degree of target cell specificity, low toxicity and immunogenicity, biodegradability, biocompatibility and stability of the pharmaceutical formulation. Moreover, the ideal delivery system must be simple to formulate and must lend itself to easy modification for customized DNA release, delivery, and expression.

Figure 1 represented the schematic representation of the delivery, uptake, and intracellular trafficking of DNA during delivered by gene delivery system. First of all, the complexation between DNA and vectors or the entrapment of DNA in delivery system is occurred. When DNA-vector complex is within tissue, it must diffuse efficiently between non-target cells, avoiding interactions with extracellular matrix, and then they must go to the target cells. After targeting the delivery system to the target cell, the DNA-vector complex is associated with the cell membrane and is internalized into the cytoplasm via endocytosis. In the endosomal vesicles, the DNA can be inactivated or degraded by lysosomal enzyme. If the DNA-vector

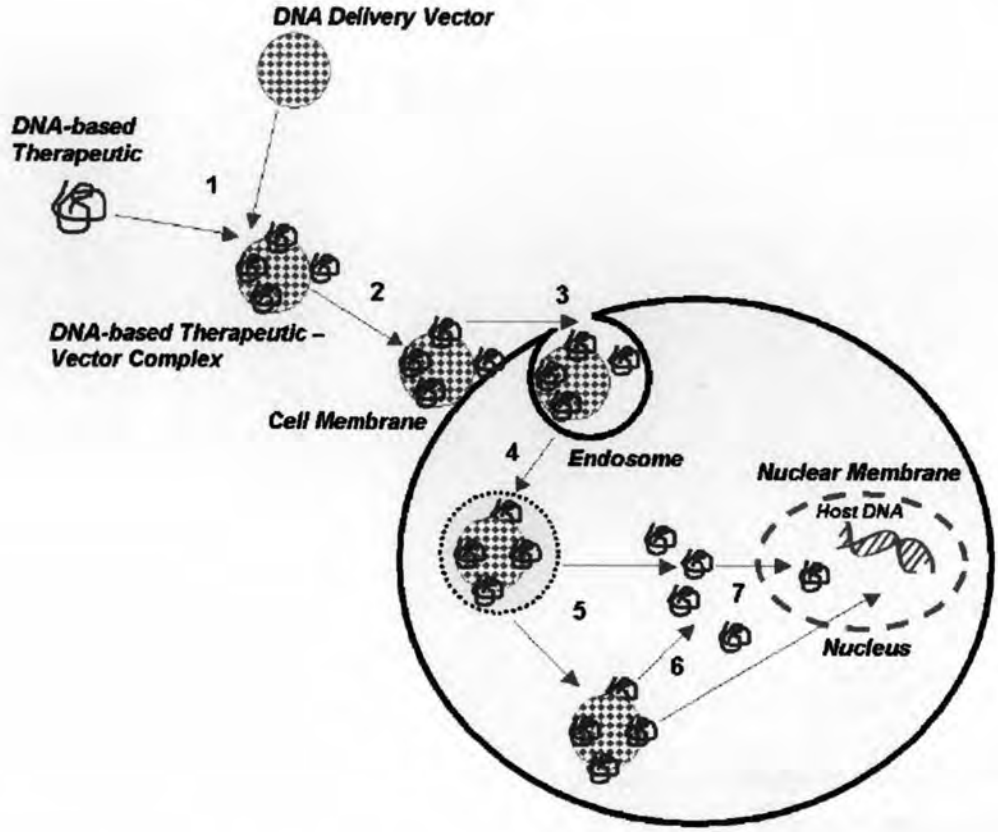


Figure 1 Schematic representation of gene delivery system. (1) complexation and/or entrapment of DNA with DNA delivery vector; (2) interaction of DNA-vector complex with cell membrane; (3) cellular internalization via endocytotic pathway; (4) endosomal breakdown; (5) cytoplasmic release of DNA-vector complex or DNA alone (cytoplasm is the site of action for antisense oligonucleotides, aptamer, ribozymes, DNAzymes, and cytoplasmic plasmid DNA expression systems); (6) dissociation of DNA from vector; (7) nuclear translocation of DNA (Nucleus is the site of action for transgenes in plasmids for gene therapy, siRNA generating plasmids, and antigene oligonucleotides.) (Patil et al., 2005).

complex escapes from the endosome, it can enter the cytoplasm of the cell. Therefore to ensuring the activity of the DNA, it is essential to ensure their rapid escape and protect the DNA from degradation in endosome because the endosome is a major site of DNA metabolism. In the cytoplasm, DNA undergoes dissociation from the vector and passes into the nucleus through the nuclear pores, or during mitosis, when the nuclear envelope is weakened. Therefore the

internalization of DNA is controlled by the pores of nuclear membrane. Lastly, when the DNA has entered the nucleus, long-lived gene expression must be ensured.

In the systemic application, particle size is the critical parameter for successful delivery playing a role in transport of DNA-vector complex through capillaries, passing out of the blood vessels (extravasation) and passing through the intertissue space. Furthermore, complex must remain stable and not aggregating in the blood. Moreover, complex should be designed not to interact with components of the blood system, such as plasma, erythrocytes, cell of the reticuloendothelial system (RES), extracellular matrix and other non-target cells (Wightman et al., 2003).

Gene delivery systems can divide into 2 major types following the type of their vector. One is viral gene delivery system, another is non-viral gene delivery system.

2.1 Viral gene delivery

Nonpathogenic attenuated viruses are used as vector for gene delivery system. The interesting gene is inserted into the viral genome and the virus uses its innate mechanism of infection to enter the target cells. The example of viral vectors, such as retroviruses (McTaggart and Al-Rubeai, 2002; Crcareva et al., 2005), parvoviruses, adenoviruses (Takahashi et al., 2000; Bramson and Parks, 2003; Bouquet et al., 2006), lentiviruses (Oh et al, 2006), adeno-associated viruses (AAV) (Martin et al., 2002; Carter, 2003; Müller et al., 2006; Wu et al., 2006) and herpes simplex virus (Selvam et al., 2006).

Gene expression using viral vectors is achieved with extremely high transfection efficiencies in a variety of human tissues (Schmid et al., 1997; Shi et al., 2004). However, there are several drawbacks over the use of viruses as vectors to deliver DNA therapeutics in humans. The major drawbacks are the toxicity of the viruses and the potential for generating immune response because of their proteinaceous capsid and not allowing repeated *in vivo* transfection using the same carrier (Timme et al., 1998; Liu et al., 1999; Favre et al., 2001; Kay et al., 2001; Raper et al., 2003). Moreover, the insertion of therapeutic genes into the host genome by the

virus takes place in a random fashion. There is no control over the exact location of the insertion that may inhibit expression of normal cellular genes or activate oncogenes. This may stimulate other problems, such as mutagenesis and carcinogenesis (Simon et al., 1993; Kamiya et al., 2001). Many other factors may limit the use of viral vectors for gene delivery. Because the viral envelope has a finite capacity, there is a limit on the size of the expression plasmid that it can incorporate.

To circumvent these drawbacks, gene delivery research is also aimed at the development of non-viral gene delivery vectors.

2.2 Non-viral gene delivery

Non-viral delivery can overcome some of the problems associated with viral vectors and are emerging as favorable alternatives to viral vectors owing to their biosafety (Pouton and Seymour, 1998) and lack of immune response. Unlike viral delivery, non-viral delivery will not insert the therapeutic genes into the host genome. Moreover, other advantages of non-viral gene delivery vectors are ease of formulation, stability and ability to be produced in large quantities. Additional, non-viral vector provide flexibility in the size and sequence of DNA that can delivered (Kreiss et al., 1999; de Jong et al., 2001). Furthermore, the low cost and consistence of production, as compared to the growth of viruses followed by purification, attract the use of non-viral delivery.

In addition, targeting ligands, in the form of peptides, carbohydrates, or antibodies, can also be attached to the complex surface to induce binding of the complexes with the target cells. The attachment of nuclear localization signal to the complexes or binding sites for transcription factors within the DNA sequence is also capable in non-viral vector.

However, non-viral gene delivery systems mediate moderate to high gene expression levels *in vitro*, but often fail to induce significant levels of gene expression *in vivo*. This is most likely the result of numerous mechanisms that the virus has evolved over millions of years to maximize transfection of the host. These mechanisms include the ability to circulate in the blood,

bind to cell surface receptors, gain entry into cell, avoid lysosomal destruction, survive degradation in the cytosol, and delivery genetic material to the nucleus (Oyewumi and Rice, 2006).

Commonly used non-viral vectors for gene delivery can be classified into 2 major types based on the nature of the synthetic material, cationic phospholipids and cationic polymers. Because of their permanent cationic charge, both types interact electrostatically with negatively charged DNA and form complexes (Tang et al., 1997; Mao et al., 2001). Then gene transfer complexes with electropositively charge bind ionically to the electronegative surface of the cells composed of proteoglycans or sialylated glycoproteins results in the internalization and ultimately the transfection of the cells.

Other types of non-viral delivery are electrical and mechanical techniques. Microinjection is highly efficient and precise, however, this method use an expense time (McAllister et al., 2000). Electroporation technique transfer DNA by using high-voltage electrical current (Regnier et al., 2000). This technique results in high cell mortality and therefore is not suitable for clinical use. Ballistic transfer of DNA-gold complexes can be achieved using equipment such as gene gun (Luo and Saltzman, 2000). However, since direct exposure of target tissues is required, this method is restricted to local expression in the dermis, muscle, or mucosal tissue, unless surgical exposure of the target is permitted. Though high transfection efficiencies have been achieved using mechanical and electrical techniques, they are extremely difficult to standardize in a clinical setting and are considered laborious, impractical, and invasive.

2.2.1 Cationic phospholipids

Cationic phospholipids have been widely used as gene delivery vectors. Liposomes are vesicles that consist of an aqueous compartment enclosed in phospholipids bilayers. The head group of cationic phospholipids is protonated affording binding to DNA, this complex is called lipoplexes. Cationic phospholipids offer several advantages over viral vector, such as low immunogenicity and ease of preparation. The phospholipids composition in the liposome bilayers can be varied, therefore liposomal delivery systems can be easily designed to yield a desired size,

surface charge, composition and morphology. Moreover, liposome can offer substantial protection to the therapeutic gene from nucleases and preserve their biological stability.

The most prevalent cationic phospholipids used in gene delivery system are N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA), 1,2-dioleoyl-3-trimethylammonium propane (DOTAP) and 3,[N-(N^1,N-dimethylehtylenediamine)-carbamoyl]cholesterol (DC-Chol), as well as several new variations such as, 2,3-dioleoyloxy-N-[2-(sperminecarboxamido)ethyl]-N,N-dimethyl-1-propanaminium chloride (DOSPA), dioctadecyl amino glycil spermine (DOFS).

Despite the success of cationic phospholipids in gene delivery, toxicity is of great concern. Cytotoxicity of cationic phospholipids has been observed in numerous *in vitro* (Lappalainen et al., 1994; Patil et al., 2004) and *in vivo* (Filion and Phillips, 1997; Dokka et al., 2000) studies (Lv et al., 2006). Additionally, the transfection efficiency of cationic phospholipids is lower than those of viral vectors. Low transfection efficiency has been attributed to the instability of complexes (Lee et al., 2001). Because the complexes are also weakly formed and thereby tend to dissociate at physiological concentrations of salt. This leads to the premature release of plasmid DNA, which is susceptible to metabolism by nuclease. Moreover, another drawback in the use of cationic phospholipids is their inactivation by the presence of serum. Some *in vivo* studies have demonstrated that gene delivery system obtained by cationic phospholipids were transient and short-lived (Liu et al., 1997).

2.2.2 Cationic polymers

Cationic polmers can easily complex with the anionic DNA molecules to form polymer-DNA complexes, also known as polyplexes. The mechanism of complexation is based on the generation of positively charged complexes owing to electrostatic interaction of cationic polymers with anionic DNA. The cationic complexes can then interact with the negatively charged cell surface to induce DNA uptake. In addition, complexation of cationic polymers with plasmid DNA can render protection against nucleases degradation to DNA.

Polymer-DNA complexes, on the other hand, are more stable than phospholipids-DNA complexes. Versatility of physicochemical properties and easy manipulation are some of the most important advantages of cationic polymers. Industrial-scale manufacturing is feasible at low cost.

However, the efficiency of gene delivery by cationic polymers is still relatively low compared to viral vectors. In order to improve transfection efficiency, the first generation of polymer-based gene delivery should be followed by a second generation that lack of drawback of the first generation. The design of new generation should attended all critical steps in the transport of DNA to the nucleus which includes the improvement of the site specific delivery, the cellular uptake, the endosomal escape capacity, the dissociation of DNA from complexes and the efficiency of the transport of the DNA into the nucleus. The dissociation of DNA is critically important. If the affinity between the DNA and cationic polymers is too low, the polyplexes will dissociate prematurely such as in the blood stream, whereas a strong affinity might prevent the release of DNA in the cells. Cationic polymers which are able both to condense large DNA molecules into smaller structure and to mask the negative DNA charges, necessities for transfecting most types of cells.

A number of the polymeric molecules have been developed. Commonly used polymers include polyethyleneimine (Weiss et al., 2006), polylysine (Yamagata et al., 2007), polyamidoamine dendrimer (Merdan et al., 2002; Choi et al., 2006) and chitosan (Borchard, 2001). Other examples of cationic polmers are polyornithine, polyarginine, histones, high-mobility group protein (HMG1), poly(2-(dimethylamino)ethyl)methacrylate (PDMAEMA), polyallylamine derivatives, diethylaminoethyl (DEAE)-dextran, poly(N-alkyl-4-vinylpyridinium) salts.

Diethylaminoethyl-dextran (DEAE-dextran) can be considered a main predecessor of the cationic polymer for gene delivery. It provides low transfection efficiency, toxicity and non-biodegradability, therefore its exploitation as gene carrier is discouraged (De Smedt et al., 1999).

Polyethyleneimine (PEI) has the highest cationic charge density, therefore is effective in DNA condensation. Every third atom on the backbone of the polymer is a nitrogen atom. All of the nitrogen atoms on linear PEI can be protonated, but in branched PEI, only two-third of nitrogen atoms can be charged. PEI possesses the ability to destabilize endosomal membranes and induce endosomal escape of the complexes. Thus, efficient gene expression has been observed with the PEI-DNA complexes. However, PEI is extremely cytotoxic due to induction of apoptosis (Florea et al., 2002). The cytotoxicity and transfection efficiency of PEI are directly proportional to its molecular weight.

Polylysine (PLL) is one of the first polycation polymers to be used for gene delivery. PLL is a biodegradable linear polypeptide of varying length of 20-1000 amino acids. The heterogeneity of chain length is commercially available and the resulting variabilities in size distribution of the polyplexes, which was the major reason for the development of polyplexes based on PLL. PLL suffers from immunogenicity and toxicity caused by its amino acid backbone. The transfection efficiency of PLL-DNA complexes increases with increasing molecular weight but the toxicity also increases. Therefore, low molecular weight PLL offers the advantage of lower toxicity and defined chemical structure and purity.

Polyamidoamine (PAMAM) dendrimers are a new class of polymers in which an amine starting material is repeatedly substituted at its amino termini to offer a branched structure. This branched spherical polymer has unique surface of primary amino groups resulting in high positive charge densities. The spherical structure of dendrimers offers synthesis control of the molecule in terms of degree and generation of branching. This control can provide polymer with a very low degree of polydispersity, which is an important advantage over other polymers such as PLL that generate highly polydispersity particles. Low polydispersity can lead to reproducible gene delivery. This polymer efficiently complex with DNA and has endosomal lytic activity, however, often cause toxicity to cells *in vitro*. Partially degraded or fractured dendrimers were found to be more efficient in transfecting cells, as compared to dendrimers, it probably because the complexes are more soluble and do not aggregate.

In contrast to the abundance of synthetic cationic polymers, there are only a small number of cationic polymers of natural origin available. Nevertheless, these polymers may offer a number of characteristics beneficial to gene delivery. Biocompatibility, low immunogenicity and minimal cytotoxicity can render these polymers a good alternative to viral or cationic phospholipids-mediated transfection. Chitosan is the example of cationic polymer from natural origin. Chitosan is cheap, biocompatible, biodegradable, non-toxic (Koping-Hoggard et al., 2001) and low immunogenic cationic polymer. Although, the transfection efficiency of chitosan is still lower than that of other polymers such as PEI, chitosan and chitosan derivatives may represent potentially safe and efficient cationic carriers for gene delivery.

A comparison of transfection efficiency of all polyplexes studied would be a huge attempt as different preparation conditions, various transfection conditions, distinct reporter plasmids and a variety of cell types were used (De Smedt et al., 2000).

The targeting of complexes to a desired cell is an important subject in the field of gene therapy. Targeting moieties, ligand, can covalently binding to the cationic polymers and allow the uptake of complexes through cell membrane via receptor-mediated endocytosis. Agents such as folates, transferrin, antibodies, or sugars such as galactose and mannose can be used as ligands for cell targeting. Many cationic polymers can be easily conjugated to ligands. Among them, polylysine has been the most extensively used for attaching targeting ligands.

3. Chitosan

Chitosan is a linear copolymer of β(1-4) linked N-acetyl-glucosamine and N-glucosamine units with different molecular weights (50-200 kDa) and degree of deacetylation (40%-98%). It is obtained by deacetylation of chitin, a polysaccharide widely distributed in nature, such as crustaceans, insects and certain fungi. Figure 2 shows the structure of chitosan. Synthesized from naturally occurring source, this polymer has been shown to be both biocompatible (Dodane and Vilivalum, 1998; Illum, 1998) and biodegradable (Onishi and Machida, 1999). It usually refers to a family of polymers that are characterized by the number of

sugar units per polymer molecule (n), which defines the molecular weight, and the degree of deacetylation (Dodane and Vilivalam, 1998).

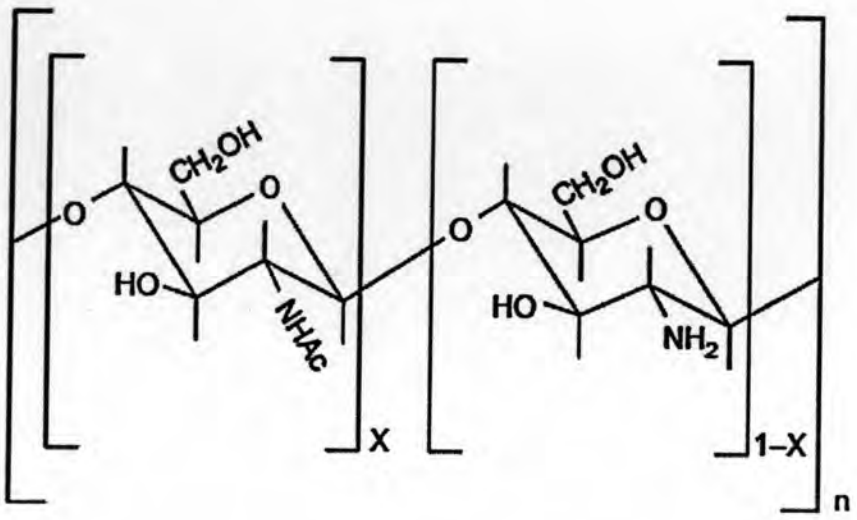


Figure 2 Structure of chitosan (Dodane and Vilivalam, 1998).

Chitosan has three reactive functional groups, an amino groups as well as both primary and secondary hydroxyl groups at the C-2, C-3, and C-6 positions, respectively. These functional groups enable chemical modifications of chitosan that include acylation, N-phthaloylation, tosylation, alkylation, Schiff base formation, reductive alkylation, O-carboxymethylation, N-carboxyalkylation, silylation, and graft copolymerization (Shi et al., 2006). Chemical modifications of these groups resulted in the synthesis of numerous derivatives for specific application.

Applications of chitin are limited compared to chitosan because of its chemically inert property and its insolubility in both water and acid, while chitosan is reactive and can be produced in various forms. Chitosan is insoluble in neutral or basic pH conditions, while soluble in acidic pH. Upon dissolution, the amine groups of chitosan are protonated and resultant soluble polysaccharide is positively charged. The solubility of chitosan depends upon the degree of deacetylation. Chitosan with low degree of deacetylation (40%) have been found to be soluble up to pH 9, whereas chitosans degree of deacetylation of about 85% have been found to be soluble only up to pH of 6.5 (Illum, 1998). Chitosan can forms salts with organic and inorganic acids

such as glutamic acid, hydrochloric acid, lactic acid and acetic acid. Additional, the solubility of chitosan is also influenced by the addition of salt into solution, the higher the ionic strength the lower the solubility (Illum, 1998).

The viscosity of a chitosan solution increases with the increase in chitosan concentration and decreases in temperature. The viscosity also increases with increasing degree of deacetylation. This attributes to the different molecular conformation of high and low degree of deacetylation. At a high degree of deacetylation, where the chitosan molecule is highly charged, chitosan has an extended conformation with a flexible chain, whereas at a lower degree of deacetylation the chitosan molecule adopts a more rod-like shape or coiled shape due to a lower charge (Illum, 1998).

Chitosan has no irritant or allergic effects and is biocompatible with both healthy and infected human skin. Lysozyme is the primary enzyme responsible for *in vivo* degradation of chitosan through hydrolysis of acetylated residues. The degradation rate of chitosan is inversely related to the degree of crystalinity, and therefore on degree of deacetylation (Shi et al., 2006). It breaks down slowly to harmless products (amino sugars), which are completely absorbed by the human body. When chitosan was administered orally in mice, the LD_{50} was found to be excess of 16 g/kg, which is higher than that of sucrose (Dodane and Vilivalam, 1998). Chitosan is proven to be safe in rats up to 10 % in diet. Toxicity tests has been performed on chitosan to include tests for effects on cilia beat frequency in guinea pigs (Aspden et al., 1997), effect on mucocilliary clearance rates on the frog palate and human nasal tissue (Aspden et al., 1995) and effect on nasal membranes in rats (Aspden, 1996). In all cases the toxicity was negligible. Many chitosan derivatives are also biocompatible and non-toxic with living tissues (Rao and Sharma, 1997; Hejazi and Amiji, 2003; Khor and Lim, 2003). These studies confirm the suitability and extensive applications of chitosan and its derivatives.

Chitosan possess the features favorable for promoting rapid dermal regeneration and accelerated wound healing (Ueno et al., 1999). Chitin and chitosan facilitate wound healing by stimulating granulation tissue formation or re-epithelization (Ishihara et al., 2002). A scientific evidence for the efficacy of chitosan in the promotion of wound healing was first reported in

1978. Furthermore, chitosan and its derivatives are also novel scaffold materials for tissue engineering (Shi et al., 2006).

Chitosan has been investigated as mucoadhesive polymer (Imai et al., 1991; Lehr et al.,1992; Berscht et al., 1994; Illum et al., 1994; Lueβen et al., 1994; Freier et al., 2005). Among various ranges of chitosans molecular weight, better mucoadhesive activity is observed for higher molecular weight, approximately 1400 kDa, compared to lower molecular weight chitosans. This characteristic of chitosan has been widely used in the development of mucoadhesive dosage forms. In addition, chitosan appears to increase cell permeability by affecting paracellular and intracellular pathways which make it a promising absorption enhancer for drug delivery *in vitro* (Martino et al., 2005) and at mucosal epithelia (Hejazi and Amiji, 2003).

Chitosan can be molded in various forms such as powder, paste, film, and fiber for different application (Shi et al., 2006). Moreover, chitosan has good film forming properties. Chitosan-coated particles have many advantages such as improvement of drug payloads, bioadhesive property and prolonged drug release properties over the uncoated particles. Chitosan acetate films, which were tough and protective, have the advantages of good oxygen permeability, high water absorption and slow enzymatic degradation, thereby avoiding the need for repeated application.

Chitosan has been widely used as a flocculant in the clarification of waste water in Japan, as a chelating agent for the detoxification of hazardous waste, for the clarification of beverages, such as fruit juices and beers (Imeri and Knorr, 1988) and for agricultural purposes such as a fungicide. Chitosan has antimicrobial activity against bacteria and fungi. In addition chitosan has been used in the cosmetic industry, the dental industry, for hair care products and for ophthalmic applications, such as for contact lens coatings or as the contact lens material itself.

For a decade, chitosan has been introduced as a material in the nutritional supplement market, especially as a weight loss aid and cholesterol lowering agent. This attribute to its effect on the lipid transport mechanism in the gut, wherein free fatty acids, cholesterol, bile salts and other components form mixed micelles that comprise an essential step in the fat absorption

process. The positively charged from chitosan can bind to free fatty acids and bile salt and consequently disrupt lipid absorption (Sugano et al., 1980).

In addition, chitosan has been considered as a pharmaceutical excipient and novel carrier material in drug delivery systems in which attention has been focused on its absorption enhancing, controlled release and bioadhesive properties.

- Oral drug delivery

The bioavailability of drugs has been improved by mucoadhesive activity. By prolonging the residence time of drug carriers at the absorption site, sustained release and improved bioavailability of drugs can be accomplished. As a pharmaceutical excipient, chitosan has been added for sustained release. An important consideration is the optimum degree of deacetylation of chitosan as it affected the release characteristics from the matrices.

- Parenteral drug delivery

In controlled release delivery system, chitosan as a biodegradable polymeric carrier offer potential advantages for the prolonged release of low molecular weight compounds to macromolecular drugs. The biodegradable activity makes chitosan an ideal drug carrier. Various sterilization methods such as ionizing radiation, heat, steam and chemical methods can be suitable adopted for sterilization of chitosan in clinical applications (Chandy and Sharma, 1990)

- Ocular drug delivery

The poor bioavailability of ophthalmic drugs implies a necessity for frequently administration to achieve therapeutic effect. This inconvenience could be overcome by a prolonged release of the drug in the corneal area. In addition to its mucoadhesive activity, chitosan is effective in retarding the rate of drug release. These give a useful approach to increase the ocular bioavailability of drugs.

- Nasal drug delivery

To increase the bioavailability for nasal delivery, various absorption enhancers have been examined, including surfactants, bile salts and cyclodextrins. However, most of these compounds

are combined with side effects, such as irreversible changes in the nasal mucosa. Several experiments have reported that chitosan appears to be safe and effective absorption enhancer for the nasal delivery drugs.

Chitosan has been widely examined in the pharmaceutical industry for its potential in the development of controlled release drug delivery systems. This attributes to its unique polymeric cationic character and its gel and film forming properties. Such system should lead to the control of the rate of drug administration and prolong the duration of the therapeutic effect. The properties of chitosan make it a versatile excipient.

4. Nanoparticulate system

The rate of clearance of gene delivery from systemic circulation will greatly reduce the transfection efficiency of gene delivery *in vivo*. Following intravenous administration, vector-DNA complexes are rapidly cleared from circulation by the reticuloendothelial system (RES). The RES is consisted of phagocytic cells that function as the body disposal mechanism for foreign particles and macromolecules. These cells are found throughout the body, either free in blood circulation or in fixed, sited such as liver, spleen, and bone marrow. Therefore, the primary site of biodistribution is the liver and spleen. The rate of clearance may be influenced by particle size, hydrophobicity, and charge of the complexes. With increasing particle size of greater than 200 nm in diameter, the spleen becomes the primary site of DNA nanoparticle biodistribution (Oyewumi and Rice, 2006). To prolong the in the circulation, the particle size should be smaller than 200 nm (Ding et al., 2006).

Several studies examined a correlation between the size of nanoparticles and their transfection efficiency in culture cells. Although, this relationship is not clear, the need to maintain a small particle size to mediate gene targeting *in vivo* is not under dispute.

Nanoparticles are solid colloidal particles ranging in size from 1 to 1000 nm. It was found that they rather represented monolithic matrix-type particles. DNA were entrapped into the polymer network or even covalently bound to the polymer (Kreuter, 1994).

Difference techniques have been used to prepare chitosan particulate systems. The selection is depends upon several factors such as particle size requirement, thermal and chemical stability of the active ingredient, stability of the final product, residual toxicity associated with the final product, nature of the active molecule as well as the type of the delivery system (Agnihotri et al., 2004).

Several methods are used to prepare chitosan nanoparticles such as ionic gelation, emulsion cross-linking, spray-drying, emulsion-droplet coalescence method, reverse micellar method and sieving method. Mostly, Chitosan-DNA nanoparticles are prepared using the complex coacervation technique (Agnihotri et al., 2004). This method is a method of spontaneous phase separation that occurred when two oppositely charge polyelectrolytes were mixed together in an aqueous solution. The electrostatic interaction between the two opposite charges resulted in the separation of coacervate. The coacervating agent was used to increase the phase separation. Drug loading in nanoparticulate systems can be done by two methods, during the preparation of particles and after the formation of particles. In these systems, drug is physically embedded into the matrix or absorbed onto the surface. The formation of nanoparticles without surfactants and organic solvents can be used without further purification.

5. Chitosan for gene delivery

Chitosan have been widely used as a vector for gene delivery for a decade. Chitosan and chitosan derivatives have been developed to improve transfection efficiency of gene delivery. PEI was graft with chitosan provided chitosan-graft-PEI to improve transfection efficiency (Jiang et al., 2007). Furthermore, chitosan salts were investigated as a non-viral gene vectors (Weecharangsan et al., 2008). For targeting the DNA to hepatocytes, lactosylated chitosan (Erbacher et al., 1998), galactosylated chitosan (Kim et al., 2004) and galactosylated chitosan-g-dextran (Park et al. 2000) were investigated for their transfection efficiencies. Other ligands such as folate (Chan et al., 2007) and trisaccharide (Issa et al., 2006) were incorporated to target the nanoparticles to specific cells.

Mainly, chitosan-DNA nanoparticles are prepared by a complex coacervation method, using sodium sulfate as a coacervating agent and yielding particle size in the range of 100 to 500 nm (Mao et al., 2001; Huang et al., 2005; Zhao et al., 2006). Other study did not added the coacervating agent and yielded particle size in the same range of that of using sodium sulfate (MacLaughlin et al., 1998). While another study used sodium chloride as a coacervating agent and had particle size ranging from 50 to 100 nm (Erbacher et al., 1998).

In the complex coacervation method, mostly used to prepare chitosan-DNA nanoparticles, the order of mixing and vortex speed of mixing play roles in influencing the size of DNA nanoparticles. After the preparation, dilution of DNA nanoparticles will not tend to decrease their particle size (Anchordoquy et al., 1997; Kwok et al., 2000).

In addition, the stability of the chitosan-DNA nanoparticles is an important parameter and depends on both the chitosan chain length and the amount of chitosan. The increase of chitosan chain length and chitosan concentration could provide more stable complexes. However, the chitosan-DNA nanoparticles which are prepared from the high molecular weight chitosan have poor physical properties such as aggregated shapes, low solubility at neutral pH, high viscosity at concentrations used for in vivo delivery and a slow dissociation and release of plasmid DNA. The more effective chitosan-DNA nanoparticles from low molecular weight chitosan were developed (Uchegbu et al., 2001; Mao et al., 2005). Moreover, depolymerized chitosan oligomers (MacLaughlin et al., 1998) and low molecular mass chitosan (Richardson et al., 1999) were investigated as carriers for gene delivery.

Moreover, chitosan nanoparticles may also be promising carriers for DNA vaccines in mucosal immnunization. Chitosan nanoparticles were formed complex pCMVArah2 DNA, encoding for a major peanut allergen, then they were orally administered in mice (Roy et al., 1999). Challenge tests showed less severe and delayed anaphylactic responses in sensitized mice immunized with chitosan-pCMVArah2 nanoparticles, than in the non-treated mice.

6. Characteristics and toxicity of acids

6.1 Acetic acid

Acetic acid is an organic acid. Its structural formula is represented as CH_3COOH. Acetic acid has molecular mass of 60.05 g/mol. Figure 3(a) shows the molecular structure of acetic acid which has a linear structure. It is weak acid with pKa of 4.76. Acetic acid is corrosive and its vapour causes irritation to the eyes, a dry and burning nose, sore throat and congestion to the lungs. It can cause severe damage to the digestive system and a potentially lethal change in the acidity of the blood. The toxicity of acetic acid to cultured rainbow trout was observed (Bullock et al., 2000).

6.2 Lactic acid

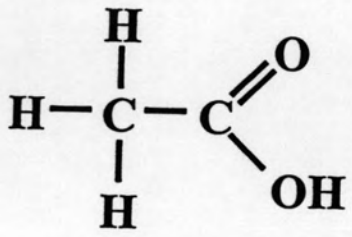
Lactic acid is an organic acid, also known as milk acid. Its structural formula is $C_3H_6O_3$. It has a hydroxyl group adjacent to the carboxyl group. Lactic acid has molecular mass of 90.08 g/mol. Figure 3(b) shows the molecular structure of lactic acid which has a branched structure. It is weak acid with pKa of 3.85. It is constantly produced from pyruvate via enzyme lactate dehydrogenase in a process of fermentation during normal metabolism and exercise. Moreover, lactic acid is found in a sour milk product such as yogurt. These indicate that lactic acid is safe. The study from Carreño-Gómez et al. (1997) concluded that lactic acid had toxicity lower than acetic acid.

6.3 Glycolic acid

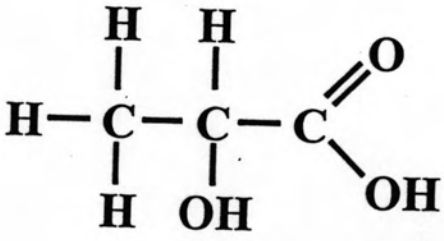
Glycolic acid is an organic acid. Its structural formula is represented as $C_2H_4O_3$. Glycolic acid has molecular mass of 76.05 g/mol. Figure 3(c) shows the molecular structure of glycolic acid which has a linear structure. It is weak acid with pKa of 3.83. Glycolic acid is isolated from natural sources. It is used I skin care products to improve the skin's appearance and

texture. Glycolic acid is non-toxic and it can enter the tricarboxylic acid cycle after which it is excreted as water and carbon dioxide.

(a)



(b)



(c)

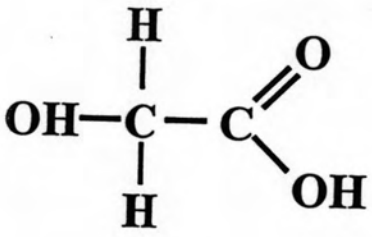


Figure 3 Molecular structure of (a) acetic acid, (b) lactic acid, (c) glycolic acid