CHAPTER 1

INTRODUCTION

1.1 Statement and significance of the problems

In the past decade, gene therapy is definitely one of the fastest developing fields of biomedical research. DNA transfer has been intensively developed using viral or non-viral carriers. Viral carriers are more efficient and specific to the target sites than non-viral carriers but some risks may exist for the host. Among the non-viral carriers, cationic polymers and cationic lipids have been investigated and the latter has been widely studied for DNA delivery. The non-viral carriers have some advantages more than the viral carriers, such as low toxicity and reduced immunogenecity (Lee and Huang,1997; Zabner, 1997; Rolland, 1998; Schatzlein, 2001). So, non-viral carriers appear to be a better alternative. Cationic liposomes have been proven to be useful tools to deliver many types of plasmid DNA and anionic drugs into the mammalian cells. The first cationic lipid N-(1-(2,3-dioleyloxy)propyl)-N,N,N-trimethyl-ammonium chloride (DOTMA) was introduced by Felgner in 1987. To date, gene therapy represents the future potential treatment of various diseases. In this therapy, many researchers have studied the therapeutic materials for manufacturing, including the target cell delivery systems. One successful approach is the use of cationic lipids. These lipids enhance the gene tranfaction due to the electrostatic interaction of cationic lipids and the negative charged nucleotides. Cationic lipids have been investigated because of the advantages of handy synthesis, low immune response, and safety. (Beiong kim, et al., 2009). Three components of cationic lipids are the positive

charge polar head group, the linker, and the lipophilic tail. Among these compositions, the cholesterol-based cationic lipid, which contains cholesterol derivatives as lipophilic tails, are widely used because of less toxicity than other cationic lipids as the main lipid of liposomal derivery for gene and chemical drug, which carry the negative charges (Hongtao Lv, et al., 2006).

1.2 Objective

The objectives of this study were to synthesize 2 cationic lipids from cholesterol and steroid (cholestranol and solasodine), which have the hydroxyl group at position 3, for the preparation of cationic liposomes. The entrapment of an anionic drug, human insulin, in the developed cationic liposomes in comparing to the neutral liposomes and other cationic liposomes, from the market available cationic lipids have also investigated.

1.3 Scope of the study

This study is divided into 6 steps which are the followings:

1. Synthesis of a cationic lipid from steroids which have hydroxy group at position 3 interact with trimethylglycine hydrochloride by DMAP/DCC esterification method.
2. Purification of the synthesized cationic lipid by column chromatography.
3. Characterization of the synthesized cationic lipid such as melting point, IR, ^{1}H NMR, LC-MS and UV-vis spectrophotometer.
4. Preparation of the cationic liposome composing of the synthesized cationic lipid.

5. Entrapment of an anionic drug (human insulin) in the liposomal formulation containing and not containing the cationic lipid.
6. Interpretation, discussion and report writing.

1.4 Literature reviews

1.4.1 Liposomes

1.4.1.1 Definition/Introduction

Liposome (Figure 1.1) is a spherical vesicle with a membrane composed of phospholipid bilayers ranging from 50 to 1000 nm in diameter. It is a useful carrier of both lipid-soluble and water soluble drugs. Liposome is from two Greek words: “Lipos” meaning fat and “Soma” meaning body. Liposomes were first produced in England in 1961 by Alec D. Bangham.

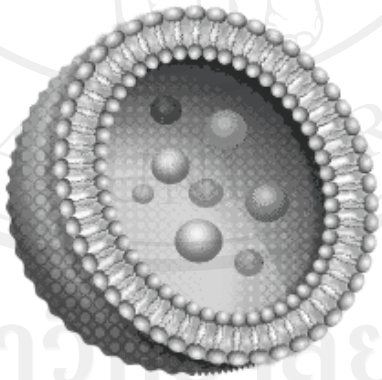


Figure 1.1 Schematic drawings of a liposome
Source: http://www.bioteach.ubc.ca/Bio-industry/Inex/

1.4.1.2 Compositions and characteristics

Phospholipids are the major material commonly used for liposome construction, which are amphiphilic molecules consisting of a glycerol backbone with two long fatty-acid chains (R-groups) and a polar portion of the molecules (a

phosphate ester groups). The phospholipids possess a hydrophobic tail structure and a hydrophilic head component. It organizes in the following manner when dissolved in water. The hydrophobic tails attract the non-polar substances, while the hydrophilic heads contact with the external and internal aqueous regions of the liposomal surface (Figure 1.2). In this way, double lipid layers are seal off to form the small vesicles, which are similar to the mamalian cells and their organelles.

Varieties of non-phospholipid lipids have been added into the liposomal formulation with the intention to alter the surface charge, fluidity, size or lamellarity of the vesicles. The common additive is cholesterol which is often used to stabilize the liposomal membranes. The ingredient such as dicetylphosphate and stearylamine are often used to impart the negative and positive charge of liposomes respectively.

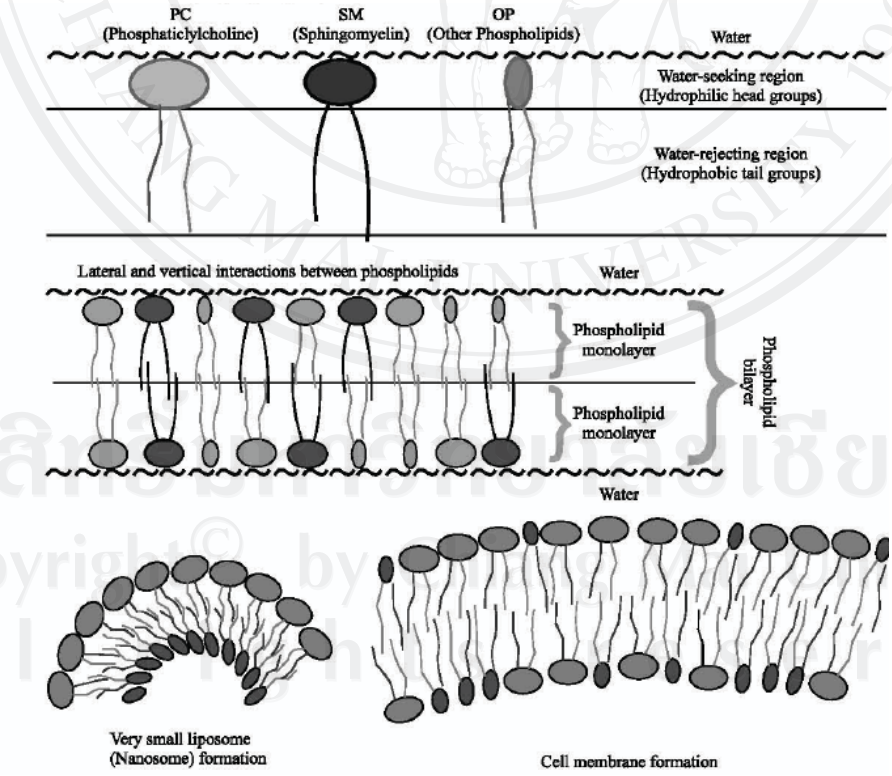


Figure 1.2 Compositional structure of liposome and the cell membrane

Source: Shaheen et al., 2006

Many physical and biological properties of liposomes, such as the amount of the entrapped volume per the given amount of lipid, leakage rates, adhesion, aggregation, stability during absorption, biological fate, and the extent of clearance from the blood depend on the liposomal sizes. The different size of liposomes are often from the different methods of manufacture. Different applications demand the different size range of liposomes.

Classification of liposomes can be identified using the different size, which is the most common index of characterization in the current use. The liposomal sizes range from 5 nm to 20 nm or above, and can be classified into three groups, which are small unillamellar vesicles (SUVs), large unilamellar vesicle (LUV) and multilamellar vesicle (MLVs) (Weiner, A.L., 1990).

Small unillamellar vesicles (SUVs) are the smallest size of liposomes (20-100 nm), depending slightly on the ionic strength of the aqueous solution and the lipid compositions.

Larg unilamellar vesicles (LUV) have the size range of 100-500 nm.

Multilamellar vesicles (MLVs) are usually consisted of the population of vesicles between 100 nm-1 μm. Each vesicle generally consists of five or more concentric lamellae.

In addition, most liposomal preparation methods give a fairly heterogeneous population of vesicles with a wide distribution of sizes, particularly towards the lower end of the range. It is very important to characterize liposome preparation according to the size in order to properly interpret their *in vivo* behaviors.

1.4.1.3. Liposome preparation methods

Various types of liposomes can be prepared by different methods which imply that there are several mechanisms to operate the formation of liposomes.

1.4.1.3.1. Hand-shaken method

Hand-shaken method or the conventional method is firstly introduced by Bangham, et al. (1995). The steps involved in this method are the preparation of lipid for hydration, hydration of lipid film by agitation and sizing of the lipid lamellar dispersion to a homogeneous distribution of the the vesicles. First, the lipids must be dissolved and mixed in an organic solvent (usually chloroform or chloroform mixed with methanol) to assure a homogeneous mixture of the lipids. Then, the solvent is removed by evaporation to form a thin lipid film on the sides of a round bottom flask, and left to dry overnight to remove the residual solvent. Hydration of the dried lipid film is simply accomplished by adding an aqueous solution, which may include drugs or active ingredients before agitating by swelling on the water bath at the temperature above the gel-liquid crystal transition temperature (Tc or Tm) of the lipids (Figure 1.3). The resulting liposome which is a large, multilamellar vesicle (LMVs) is obtained. The size of the liposome can be reduced by sonication or extrusion to obtain the required sizes.

1.4.1.3.2. Sonication method

This method is probably the most widely used method for the preparation of small unilamellar vesicles (SUVs), which has two techniques.

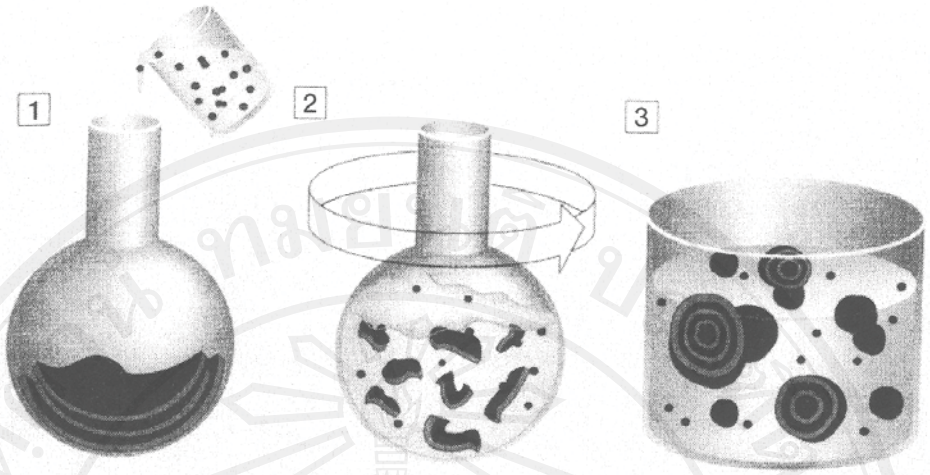


Figure 1.3 Schematic representation of the three steps of liposome preparation by hand-shaken method. 1: Addition of an aqueous phase to the dry thin lipid film. 2: Swelling and peeling of the lipid film under vigorous agitation. 3: Milky suspension of the equilibrated liposome

Source: Lasch J *et* al., 2003

Probe sonication: The tip of a sonicator is directly immersed into the liposomal dispersion. In this method, the energy input into the dispersion is very high. The dissipation of energy at the tip results in local overheating, and therefore the vessel of the dispersion must be immersed into an ice/water bath. During the sonication up to one hour, more than 5% of the lipids can be de-esterified. Also, with the probe sonicator, titanium will slough off and contaminate the dispersion. But, this contamination can be removed by centrifugation.

Bath sonication: The tube of the liposomal dispersion is placed into a bath sonicator. Controlling the temperature of the lipid dispersion is usually easier than the probe sonication method. Materials being sonicated can be kept in a sterile container or under an inert atmosphere unlike the probe units.

1.4.1.3.3. Freeze-dried rehydration method

Freeze-dried rehydration vesicles (FRVs) are formed from the preformed vesicles. Very high entrapment efficiency, even for macromolecules, can be achieved. Drying brings the lipid layers and materials to be encapsulated into close contact. Upon reswelling, the chances for entrapment of the adhered molecules are larger. Dehydration is best performed by freeze-drying. Rehydration must be done extremely carefully. The significant advantages of this method are that the drugs do not expose high temperature by solvent evaporation and lyophilization can reduce the risk of chemical degradation of many sensitive substances such as DNA and protein.

1.4.1.3.4. Reverse-phase evaporation method

Liposomes with large internal aqueous space and high capture by reverse-phase evaporation (REV) were introduced by Szoka and Papahadjopoulos in 1978. Historically, this method provided a breakthrough in liposome technology, since it allowed for the first time of the preparation of liposomes with a high aqueous space-to-lipid ratio and able to entrap a large percentage of the aqueous materials.

Reverse-phase evaporation is based on the formation of the inverted micelles. These inverted micelles are formed upon sonication of a mixture of a buffered aqueous phase, which contains the water soluble molecules to be encapsulated into the liposomes and an organic phase in which the amphiphilic molecules are solubilized. The slow removal of the organic solvent leads to the transformation of these inverted micelles into a gel-like and viscous state. At a critical point in this procedure, the gel state collapses and some of the inverted micelles disintegrate. The excess phospholipids in the environment contribute to the formation

of a complete bilayer around the remaining micelles, which results in the formation of liposomes. Liposomes by reverse phase evaporation method can be prepared from various lipid formulations and have aqueous volume-to-lipid ratios that are four times higher than multilamellar liposomes or hand-shaken liposomes.

1.4.1.3.5. Detergent depletion method

The detergent depletion method is used for the preparation of a variety of liposomal formulations. Detergents can be depleted from the mixed detergent-lipid micelles by various techniques, which lead to the formation of homogeneous liposomes. In practice, all lipids below their phase transition temperature can be used with this method. Not all detergents are suited for detergent depletion method and only a few detergents can be used for this method such as sodium cholate, alkyl (thio) glucoside and alkyloxy- polyethylenes. Mixed micelles are prepared by adding the concentrated detergent solution to multilamellar liposomes. The final concentration of the detergent should be well above the critical micelle concentration (CMC) of the detergent. Equilibrium of the mixed micelles in the aqueous phase takes quite sometimes, and does not happen during a short period. The use of different detergents results in different size distributions of the vesicles and the ratios of large unilamellar vesicles to oligolamellar vesicles and to multilamellar vesicles. Faster depletion rates can produce the smaller size liposomes.

1.4.1.3.6. Supercritical carbon dioxide method

Typically, most liposome preparation methods have the multi-step and require large amounts of organic solvent, which are toxic to human and

environments. Techniques for the preparation of liposomes have been developed in order to decrease toxicity as well as the improvement of stability and solubility. Supercritical fluid is a substance above its critical temperature (Tc) and critical pressure (Pc). At the critical point, supercritical fluids have the density as liquid and low viscosity with better flow property as gas. Carbon dioxide is a gas, which is widely used to produce supercritical fluid because of its low critical temperature (Tc = 31.1 ˚C) and pressure (Pc = 73.8 bar). It has high solvating power at the conditions near the critical point and similar to non-polar solvent. The procedure is consisted of two main parts; 1) the high-pressure part, the lipid components or cholesterol are dissolved under pressure in supercritical carbon dioxide, and 2) the low-pressure part, the homogeneous supercritical solution is expanded and simultaneously mixed with the aqueous phase to yield vesicles. Carbon dioxide is released when pressure is reduced. It is lesser toxic than an organic solvent.

1.4.1.4. Applications of liposomes

Liposomes are widly used to deliver certain vaccines, enzymes, or drugs (e.g., insulin and some cancer drugs) to human body. When used for the delivery of certain cancer drugs, liposomes help to shield healthy cells from the drugs' toxicity and prevent their concentration in vulnerable tissues (e.g., the kidneys and liver), lessening or eliminating the common side effects of nausea, fatigue and hair loss. Liposomes are especially effective in treating diseases that affect the phagocytes of the immune system because they tend to accumulate in the phagocytes, which recognize them as foreign invaders. They have also been used experimentally to carry normal genes into a cell in order to replace defective, disease causing genes.

Liposomes are usually used in cosmetics and cosmeceuticals because they have the moisturizing property and can prevent the oxidation reaction of the active ingradeints.

1.4.2 Cationic lipids

1.4.2.1. Introduction

Cationic lipids are positively charged lipids commonly contain three distinctly regions, which are the cationic region (an amine), a linker region (ester, ether, amide, etc.) and a hydrophobic domain (Figure 1.4).

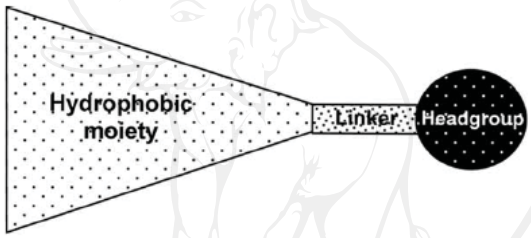


Figure 1.4 The three basic domains of a cationic lipid which are hydrophobic moiety, linker and cationic headgroup

Source: Martin et al., 2005

Cationic lipids were first introduced by Felgner et al., (1987) following early attempts to transfer DNA via encapsulation in liposomes. Thus, the first reported lipid was DOTMA (N-(1-(2, 3-dioleyloxy)propyl)-N,N,N trimethyl- ammonium chloride), which consists of a quaternary amine connected to two unsaturated aliphatic hydrocarbon chains via ether groups (Figure 1.5). Synthesis of the multivalent lipopolyamine DOGS (dioctadecyl amidoglycyl- spermine) was reported soon afterwards and the efficiency of the vector DC-Chol (3b-[N-(N',N' dimethylaminoethyl) carbamoyl]cholesterol) with cholesterol as the hydrophobic

portion was subsequently reported(Figure 1.5). It is noteworthy that the transfection activity of cationic lipids (especially those which are incapable of forming bilayers alone) can be increased by their formulation as stable liposomes with the neutral colipid DOPE (dioleoyl phosphatidylethanolamine (Figure 1.5).

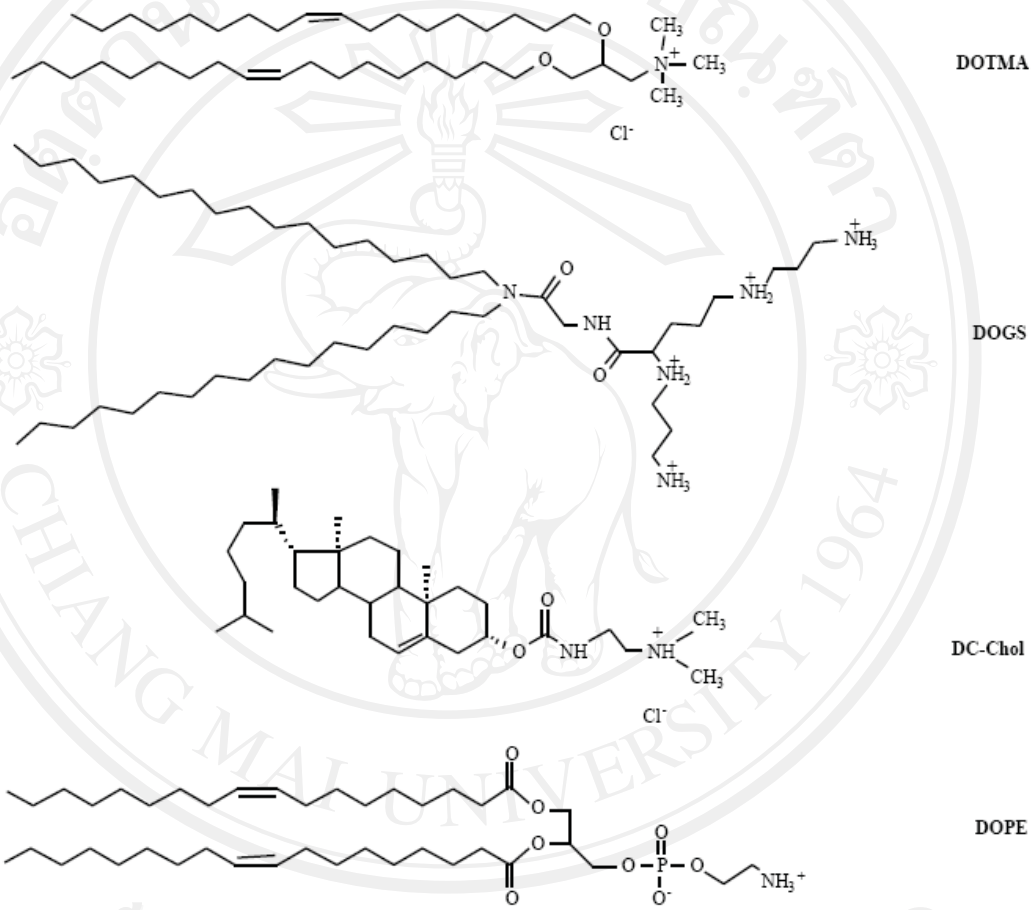


Figure 1.5 Structure of the early cationic lipids and colipid DOPE

Source: Martin et al., 2005

1.4.2.2. Types of cationic lipids

All cationic lipids are therefore positively charged amphiphile systems. They can be classified into various subgroups according to their basic structural characteristics (Figure 1.6) (Morille et al., 2008):

1. **Monovalent aliphatic lipids** They are characterized by a single amine function in their head group, e.g. DOTMA, 1,2-dioleyl-3-trimethyl-ammoniumpropane (DOTAP), *N*-(2-hydroxyethyl)-*N,N*-dimethyl-2,3-bis (tetra-decyloxy \-1-propanaminiumbromide)(DMRIE).

2. **Multivalent aliphatic lipids** Their polar head groups contain several amine functions such as the spermine group, e.g. DOGS.

3. **Cationic cholesterol derivatives**, e.g. DC-Chol, bis-guanidiumtren-cholesterol (BGTC).

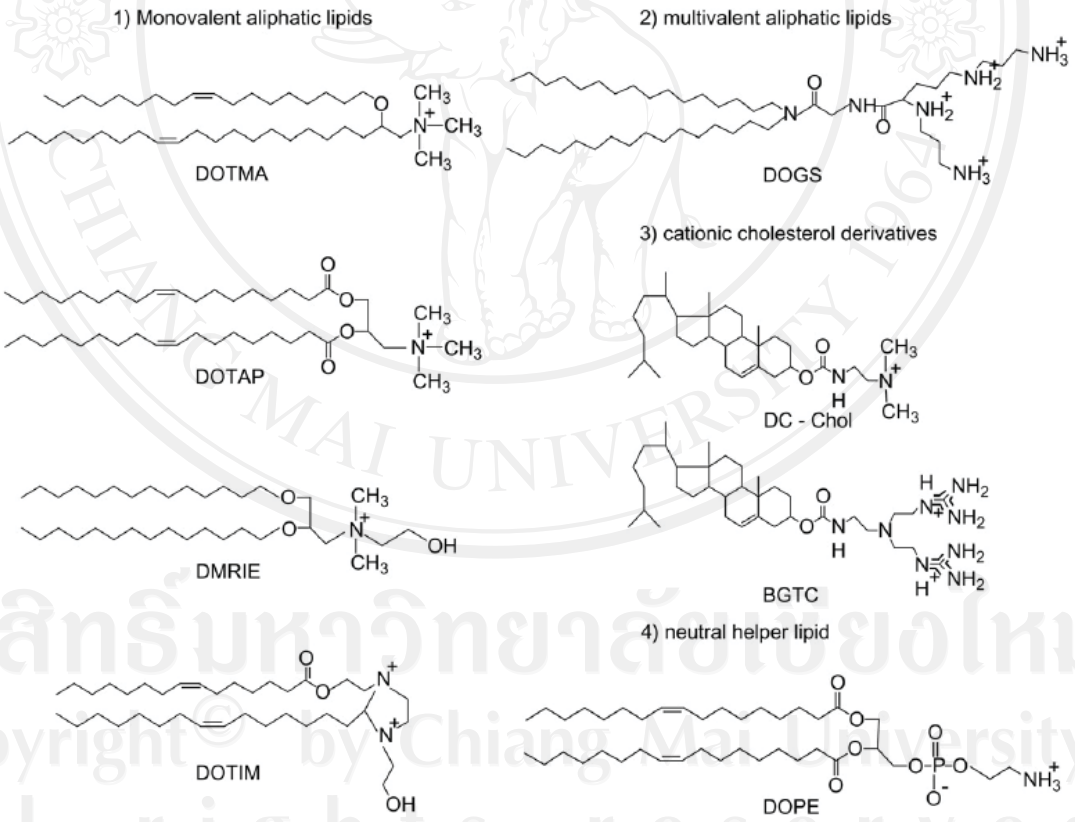


Figure 1.6 Structure of current cationic lipids used in gene therapy and the helper lipids

Source: Morille et al., 2008

In the majority of report studies, the cationic liposomes function most efficiently when the cationic lipid is combined with a second lipid known as a helper. The role of these helper lipids is to stabilize the liposome/DNA complex or to assist membrane fusion events within the cell, thus leading to the escape of the DNA from the endosomes. Helper lipids include unsaturated phosphatidylethanolamine (DOPE). The effectiveness of this agent is generally believed to rest on its property to form non-layer structure skin to membrane fusion intermediates. The use of cholesterol as a helper lipid had been reported to be effective in animal studies of transgene expression. The reason for the enhancement *in vivo* has been attributed to the ability of cholesterol-containing complexs to maintain the ability to interact with cell membranes in the presence of serum, which may not be the case with DOPE-containing liposomes (Sullivan, et al., 2003).

1.4.2.3. Applications of cationic lipids

Gene therapy is a promising approach for the treatment of cancer and inherited diseases. The most important factor in gene therapy is the vector which efficiently packages the DNA, carries it through the membranes and delivers the gene to the nucleus for expression. An ideal vector should be highly efficient in delivering the gene in a target-specific manner, being stable *in vitro* as well as *in vivo*, protect the gene from nuclease degradation, being non toxic, being non immunogenic and easily prepared in large scale.

Liposomes comprised of natural lipids (e.g. neutral and anionic lipids) have low plasmid entrapment efficiency because the negatively charge of the plasmid DNA, which repels the anionic lipids, and the relatively high molecular weight of the

plasmid. Cationic liposomes employ specific types of lipids as the function component to transport DNA into the cell.

Cationic liposomes are the most widely non-viral vector, owing to their capacity in packing well the DNA fragments with their positive charge. They have many important qualities such as less or non immunogenic and non toxic, no limitation in the size of DNA, can be custom-synthesized for targeting and easy expanded for large scale production.

DC-Chol (3β–[N-(N',N'-dimethylaminoethyl)carbamoyl] cholesterol)) is one of the cationic lipids which is used more in liposome preparation by combination with DOPE to deliver nucleic acid fragments in gene therapy. DC-Chol has a tertiary amino group and a spacer that offer a good transfection activity, a stable carbamoyl bond that provides excellent stability and transfection efficiency as well as potential biodegradability and hence reduce toxicity in comparing to other cationic lipids which have an ether bond.

A partial list of varieties of commercially available cationic agents is shown in Table 1.1. At present, the best way to select an appropriate cationic liposome is often through trial and error. Each cell line can demonstrate individual response in terms of both effectiveness and toxicity for various cationic lipids. Researchers can also produce their own cationic liposomal formulations from several readily purchased cationic lipids.

Table 1.1 Some commercially available cationic lipids (Sullivan et al., 2003)

Cationic lipid	Chemical name	Commercial source
Lipofect ACE	-	Life Tecnologies, USA
Lipofection	-	Life Tecnologies, USA
LipofectAMINE	-	Life Tecnologies, USA
CellFECTIN	-	Life Tecnologies, USA
DMRIE-C	-	Life Tecnologies, USA
DDAB	Dioctadecyldimethylammonium bromide	Sigma, USA
DC-Chol	3β–[N-(N',N'-dimethylamino-ethyl)carbamoyl) cholesterol)	Sigma, USA
DOTAP	1,2-dioleoyloxy-3-[trimethyl-ammonio]propane	Boehringer Mannheim, Germany Avanti Polar Lipids, USA Biontex, USA
ARX-230 and MRX-220	-	Avanti Polar Lipids, USA
Transfectam	-	Promega, USA
TransFast™	-	Promega, USA
Tfx™-10, Tfx™-20 and Tfx™-50	-	Promega, USA
ProFection-DEAE-$CaPO_4$	-	Promega, USA
ProFection-DEAE-Dextran	-	Promega, USA
GeneSHUTTLE-40	-	Quantum Biotechnologies, South Africa
CLONfectin™	-	Clontech, USA
METAFECTENE™	-	Biontex, USA
INSECTOGENE	-	Biontex, USA
Effectene	-	Qiagen, Germany

Table 1.1 Some commercially available cationic lipids (Sullivan et al., 2003) (continue)

Cationic lipid	Chemical name	Commercial source
FuGENE6	-	Roche Molecular Biochemicals, USA
GENESEAL™	-	MTTI, USA

Source: Sullivan et al., 2003

1.4.2.4.Toxicity of cationic liposomes

Most biology systems are negatively charged. One can expect two different toxicities originated from cationic liposomes and lipids. Germicidal action of cationic surfactants against viruses, bacteria, fungi, spored, protozoa, and invertebrates is well known. On the colloidal level, positively charged particles may induce aggregation, flocculation, thrombosis, or platelet aggregation. On the molecular level, these lipids can act as surfactants, causing membrane solubilization, poration, hemolysis, as well as changing the properties of the membranes and membrane proteins. Kinase C inhibition by cationic lipid is the best known example (Bottega and Epand, 1992). While colloidal toxicity seems to be unavoidable, biodegradation can mostly reduce the toxicity on the molecular level. Colloidal toxicity depends on the interactions of cationic particles in plasma, which may differ for liposome and genosomes containing different lipids. For instances, two liposomes (DOIC(1- [2-(oleoyloxy)ethyl]-2-oleyl-3-(2-hydroxyethyl)imidazo-linium chloride) /Chol and DMEPC(dimyristoylglycero-3-ethylphosphocho line)/Chol at the molar ratio1:1), as well as genosomes prepared from them (charge ratio -/+, $\rho = 0.5$), differ significantly in *in vitro* plasma stability tests. Liposomes show faster precipitation rate

than genosomes, which can be understood by their lower surface charges. The differences between various cationic lipids may come from the kinetics of adsorption versus aggregation with oppositely charged particles. In systemic administration, adsorption of albumin and some other macromolecules is quicker than colloidal aggregation. Thus, this system may show prolonged stability and reduce colloidal toxicity.

Conventional liposomes are normally tested for toxicity by performing hemolysis, thrombosis, and cytotoxicity assays. Their effects on phagocytic activity of the reticuloendothelial system and pyrogenicity are tested as well. To date such studies have been performed with cationic liposomes.

The interaction of three different liposomal systems with blood was tested, and rather dissimilar behavior was observed. Liposomes containing stearylamine provoked a strong increase in plasma turbidity while DOTMA an BisHOP (2, 3-dihexadecyloxyl–proply - *N,N,N* -trimethylammoniumchloride) provoked a strong clotting response. BisHOP liposomes did not cause homolysis while the other two did. Parameters measured, including turbidity, clot weight, and percent hemolysis, depended on the mole percent of cationic lipid in liposomes as well as on liposomal concentration (Senior et al., 1991). Macroscopic effects were not observed below a threshold concentration of 0.25 μM/ml. It was speculated that an increase uptake *in vivo* may be due to the high negatively charged proteins. Interactions were reduced when more-rigid bilayers were used.

1.4.2.5. Cost

Cationic lipids for cationic liposomal preparation are very expensive because it is difficult to produce. Table 1.2 showed the price of the commercially available cationic lipids.

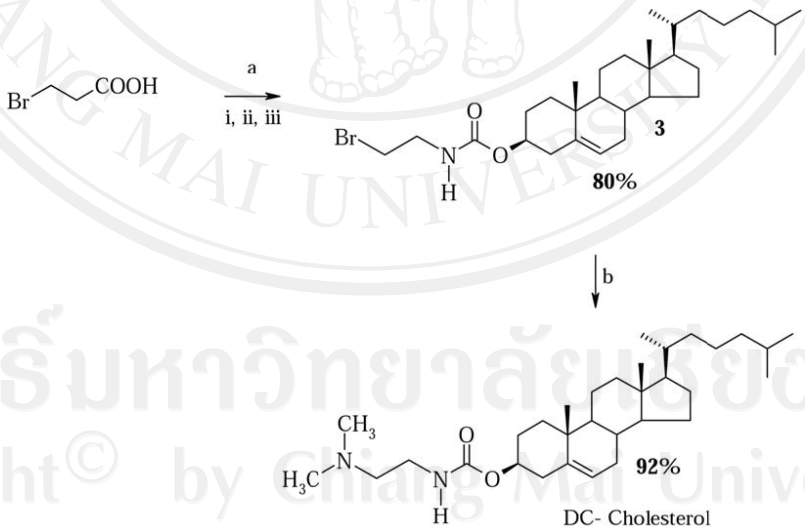
Table 1.2 Prices of some cationic lipids for liposome preparation

Cationic lipid	Commercial source	Price ($)	Price (baht)
DC-Chol (3β–[N-(N',N'-dimethylamino-ethyl)carbamoyl) cholesterol)	Sigma, USA	25 mg = 31.50 100 mg = 80.50	1,260 3,220
DDAB (Dioctadecyldimethylammonium bromide)	Fluka, USA	5 g = 160.00 25 g = 632.00	6,400 25,280
	Aldrich, USA	1 g = 43.90 5 g = 145.00	1,756 5,800
DDAB (Dioctadecyldimethylammonium bromide)	Avanti Polar Lipids, USA	25 mg = 58.00 200 mg = 110.00 500 mg = 175.00 1 g = 285.00	2,320 4,400 7,000 11,400
DOPE (dioleoylphosphatidylethanolamine)	Sigma, USA	25 mg = 71.10 100 mg = 218.50 500 mg = 696.00	2,844 8,740 27,840
	Avanti Polar Lipids, USA	25 mg = 45.00 100 mg = 85.00 500 mg = 240.00	1,800 3,400 9,600
DOTAB (1,2-dioleoyloxy-3-[trimethyl-ammonio]propane)	Avanti Polar Lipids, USA	25 mg = 85.00 200 mg = 175.00 500 mg = 370.00 1 g = 660.00	3,400 7,000 14,800 26,400

Source: http://www.sigmaaldrich.com, http://www.avantilipids.com/

1.4.2.6. Synthesis of cationic lipids for liposome preparation

DC-Chol is widely used for liposome preparation in gene therapy. With DOPE, it forms stable liposomal formulation which can be stored for months at 4 ^{0}C without any change in size or lipid degradation (Sorgi et al., 1996). DC-Chol/DOPE liposomes show a better transfection activity than other cationic lipid formulations *in vivo* (Iwanuma Y. et al., 1996). The industrial synthesis of DC-Chol is based on the reaction of cholesterol with phosgene derivatives. The obtained cholesteryl chloroformiate, is treated with *N,N*-dimethylethylene diamine to give the final product with a 21% overall yield. Improvement of Curtius reaction (reaction of changing carboxylic acid to primary amine) for the synthesis of DC-Chol gives the product with 92 % (Figure 1.7). The phosgene derivative in the industrial synthesis of DC-Chol should be avoid because the phosgene is very toxic to environments.



a)i) $SOCl_2$, 1,2 dichloroetane, 60°C, 1h; ii) NaN_3 , Benziltriethylammonium chloride, toluene, 80°C, 1h; iii Cholesterol, r. t.; b) Dimethylamine solution 2M in THF, THF, r.t, overnight.

Figure 1.7 Synthesis of DC-Chol from the improvement of Curtius reaction

Source: Armandodoriano B., 2003

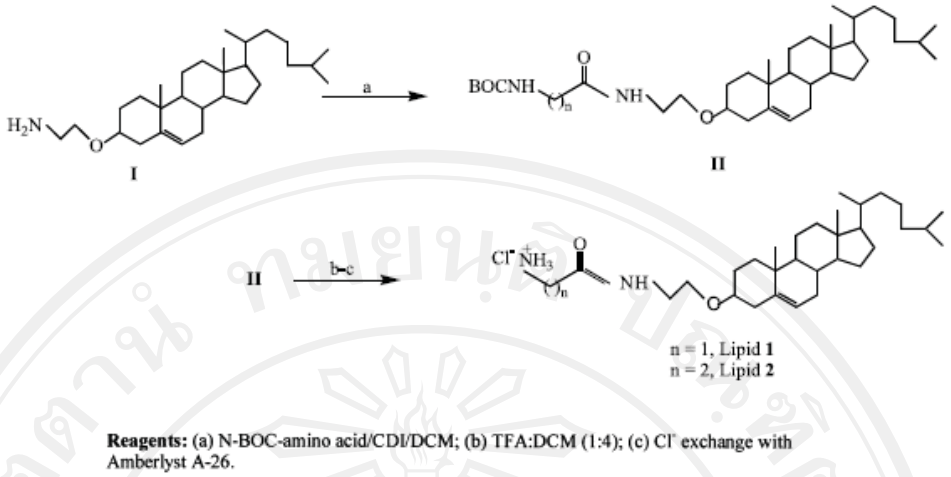


Figure 1.8 Synthesis of 2-(*N*-glycinyl) aminoethyl-cholesteryl ether (lipid I) and 2-(*N*-β-alaninyl) aminoethyl-cholesteryl ether (lipid II)

Source: Rajkumar S. et al., 2004

Cholesterol-based cationic amphiphile is synthesized from an amino acid which the amine group was blocked before reacted with cholesterol such as the synthesis of 2-(*N*-glycinyl) aminoethyl-cholesteryl ether (lipid I) and 2-(*N*- β-alaninyl) aminoethyl-cholesteryl ether (lipid II) (Figure 1.8) and cholesterol β-alanine-diethanolamine-cholesterol(A_2D-Chol) and lysinediethanolamine-cholesterol (K_2D-Chol) (Figure 1.9).

The synthesis of steroidal cationic lipids has been focused on the combination of cationic amine groups with limited variations of the steroidal backbone (Figure 1.9). The study of cationic lipid-based gene delivery systems and cell targeting (Ren T., 1999) were interested in knowing how the geometry of the hydrophobic tail in cholesterol-based cationic lipids affects the outcome of transfection.

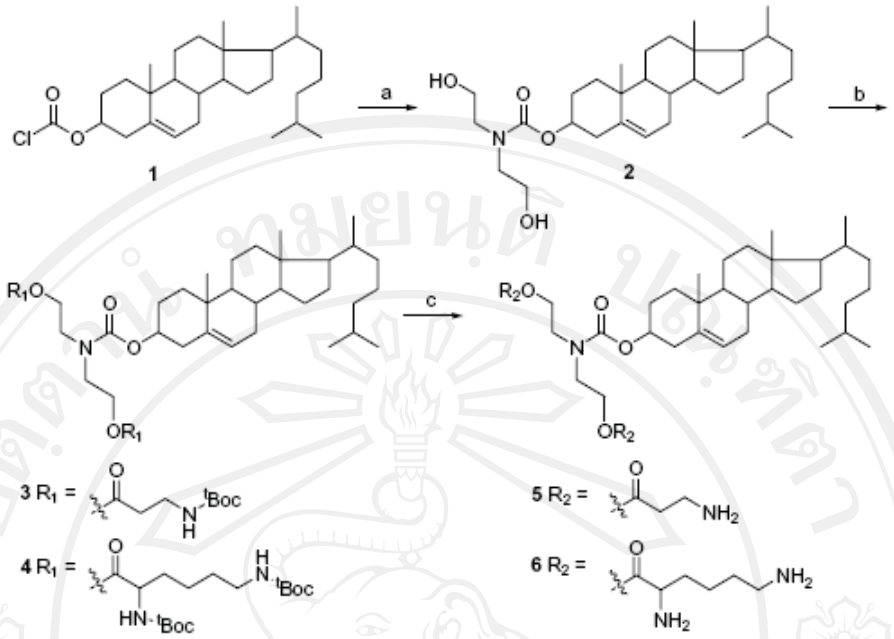


Figure 1.9 Syntheses of A2D-Chol and K2D-Chol.

Source: Yan L et al., 2004

Vitamin D analogues are secosteroids with a 9, 10 carbon-carbon bond breakage of B ring on the steroidal backbone, which would serve as a hydrophobic domain in the cationic lipid- mediated gene transfection. The transfection activity of the synthetic vitamin D-based cationic lipids (1d, 2d) (Figure 1.10) formulated with DOPE as a co-lipid is comparable to that of DC-Chol. These synthetic lipids are effective in transfecting a variety of cell lines. These results suggest that vitamin D-based cationic lipids are useful transfection reagents for *in vitro* gene transfer studies.

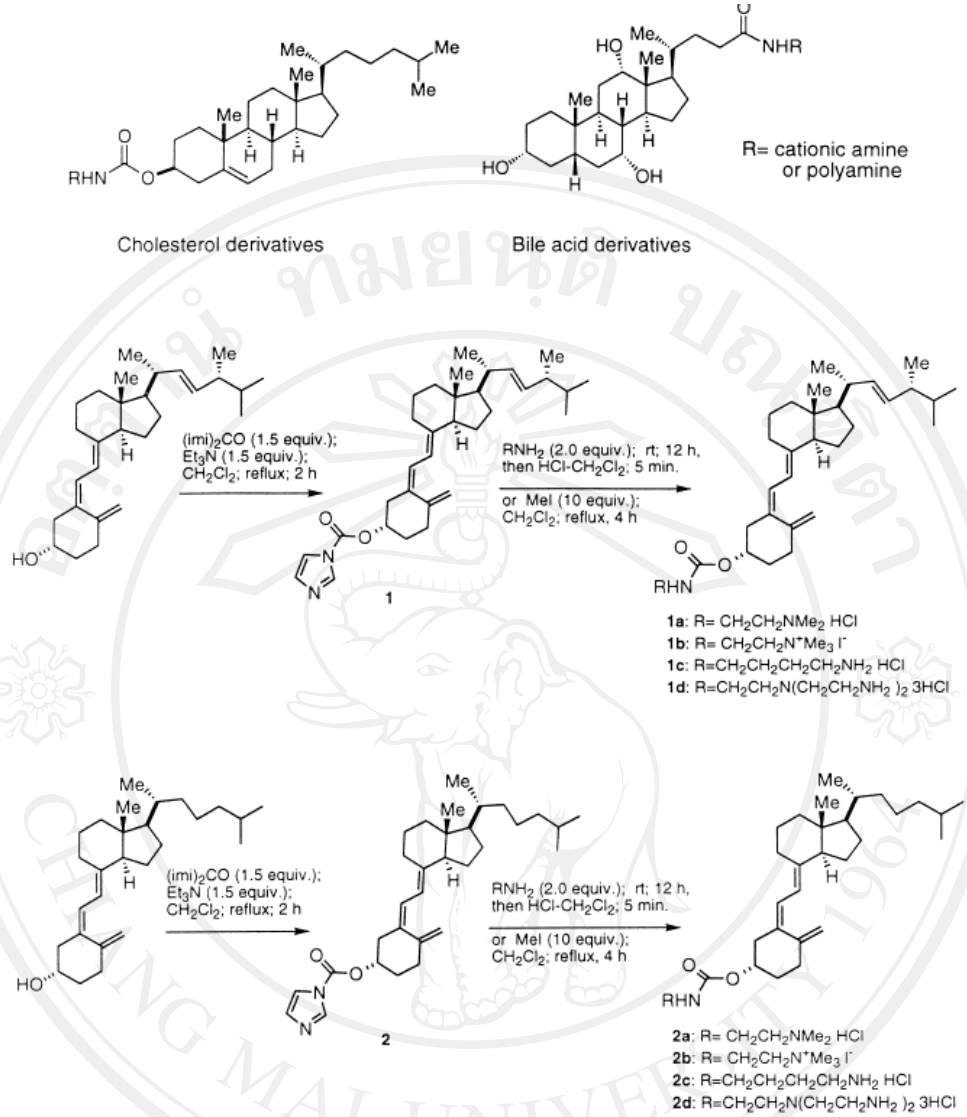


Figure 1.10 Synthesis of vitamin D-base cationic lipids

Source: Tan R et al., 2000

1.4.3 Esterification reaction for cationic lipid preparation

Esterification is the general name of a chemical reaction in which two reactants (typically an alcohol and carboxylic acid) form ester as the product, which is a reversible reaction (Figure 1.11). Hydrolysis-literally "water splitting" involves adding water and a catalyst (commonly NaOH) to an ester to get sodium salt of the

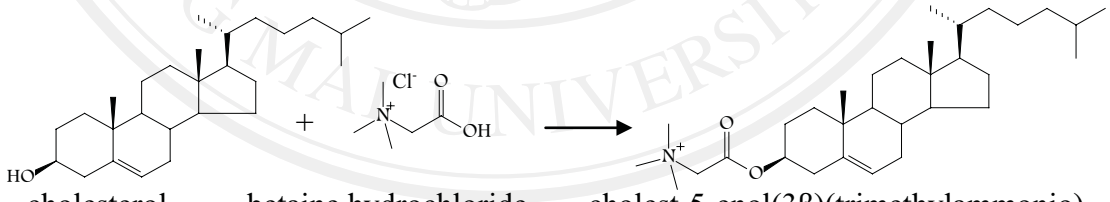
carboxylic acid and alcohol. As a result of this reversibility, many esterification reaction are equilibrium reactions. Therefore, it needs to be driven to completion according to Le Chatelier's principle.

$$CH_3COOH + CH_3CH_2OH \rightleftharpoons[H_2SO_4\ (conc)] CH_3COOCH_2CH_3 + H_2O$$

Figure 1.11 Esterification reaction of ethanolic acid reacts with ethanol in the presence of concentrated sulfuric acid as a catalyst to produce the ester, ethyl ethanoate

Source: http://www.chemguide.co.uk/physical/catalysis/esterify.html

This present study has focused on the synthesis of cationic lipids from cholesterol and other steroids with betaine hydrochloride by Steglich esterification (Figure 1.12).



cholesterol betaine hydrochloride cholest-5-enol(3β)(trimethylammonio) acetate chloride

Figure 1.12 Esterification reaction of betaine hydrochloride reacts with cholesterol to produce the cationic lipid [cholest-5-enol (3β) (trimethylammonio) acetate chloride]

Steglich Esterification is a mild reaction, which allows the conversion of sterically demanding and acid labile substrates. It is one of the convenient methods for the formation of *tert*-butyl esters because *t*-BuOH tends to form carbocations and

isobutene (Figure 1.13) after a subsequent elimination under the conditions employed in the Fischer Esterification.

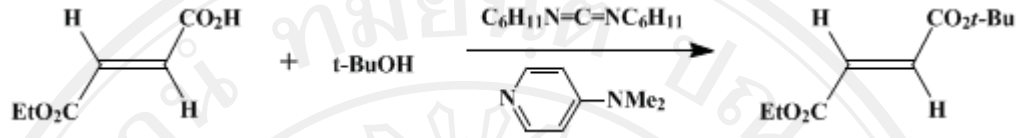


Figure 1.13 Steglich esterification of carboxylic acids with dicyclohexyl-carbodiimide(DCC)/4-dimethylaminopyridine(DMAP) to produce (*E*)-2-Butenedioic acid, ethyl 1,1-dimethylethyl ester

Source: http://www.orgsyn.org/orgsyn/prep.asp

The mechanism of this reaction is suggested (Figure 1.14). First, DCC and carboxylic acid are able to form an *O*-acylisourea intermediate, which offers reactivity similar to the corresponding carboxylic acid anhydride. After that, the alcohol is added to the activated carboxylic acid to form the stable dicyclihexylurea (DHU) and ester.

In practice, the reaction of carboxylic acids, DCC and amines leading to amides has no problems, while the addition of approximately 5 mol-percent DMAP is crucial for the efficient formation of ester. *N*-Acylureas, which may be quantitatively isolated in the absence of any nucleophile, are the side products of an acyl migration that takes place slowly. Strong nucleophiles such as amines react readily with the *O*-acylisoures and therefore need no additives. A common explanation of the DMAP acceleration suggests that DMAP, as a strong nucleophile than the alcohol, reacts with the *O*-acylisoures leading to a reactive amide. This intermediate can not form intramolecular

side products, but reacts rapidly with alcohols. DMAP acts as an acyl transfer reagent, and subsequent reaction with the alcohol gives the ester.

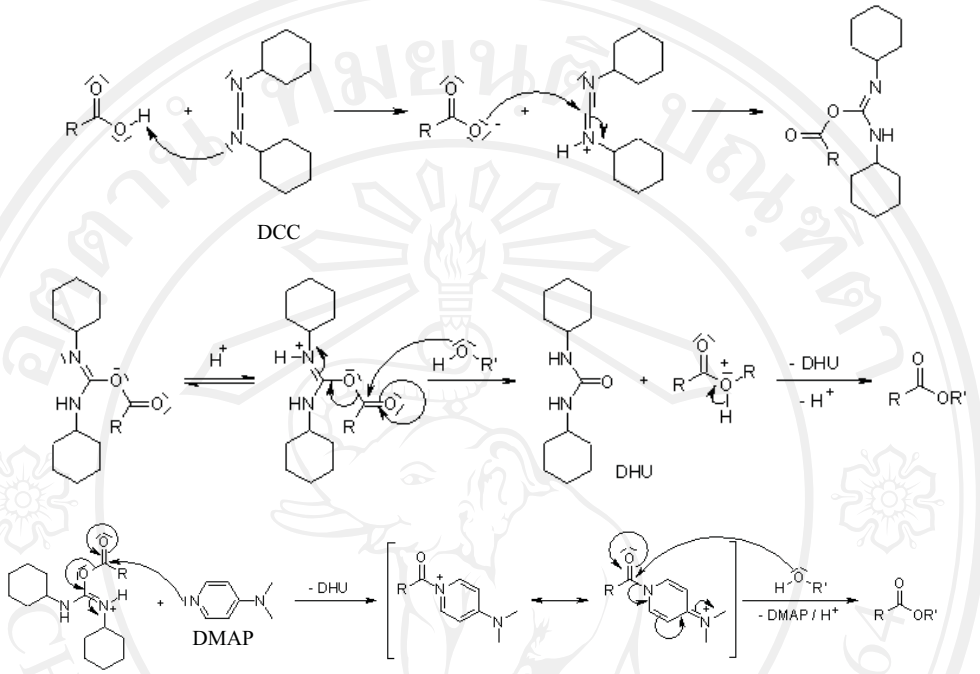


Figure 1.14 Mechanism of Steglich esterification with DCC/DMAP

Source: Neises B. et al., 1978

1.4.4 Precursor for cationic lipid preparation

1.4.4.1. Betaine hydrochloride

Betaine hydrochloride (*N,N,N*-trimethylmethanaminium chloride) is an organic compound with a quaternary ammonium as a cation and chloride counterion (Figure 1.15). It is usually the first crystallized form obtained after extraction from beets. The molecular structure is $(CH_3)_3N^+CH_2COOH{\cdot}Cl^-$ and the molecular weight is 153.61g/mol. It is stable under normal temperatures, soluble in water and ethanol, and insoluble in ether and trichloromethane.

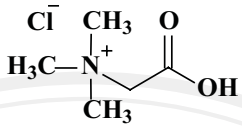


Figure 1.15 Chemical structure of betaine hydrochloride

Source: http://www.sigmaaldrich.com/catalog/ProductDetail

Betaine is a vitamin-type compound found in grains and other foods. Betaine hydrochloride, which is the acidic form of betaine, is primarily prescribed as a supplemental source of hydrochloric acid for people who have difficulty in digestion due to the lack of stomach acid.

1.4.4.2. Cholesterol

Cholesterol is a lipid, waxy alcohol found in cell membranes and transported in blood plasma of animals. Chemically, cholesterol is an organic compound belonging to the steroid family. The molecular formula is C_{27} H_{46} O (Figure 1.16), which is a white, crystalline substance, odorless, tasteless, insoluble in water, and stable under ordinary conditions during use and storage.

Cholesterol is essential for animal life. It is a primary component of the membrane surrounded each cell. It is the starting material or an intermediate compound when the body synthesizes bile acids, steroidal hormones, and vitamin D. Cholesterol is synthesized by the liver and several other organs. It circulates in the bloodstream. Human beings also ingest considerable amounts of cholesterol in the course of a normal diet. A compensatory system regulates the amount of cholesterol

synthesized by the liver with the increased dietary intake of cholesterol resulting in the decreased synthesis of the compound in the liver.

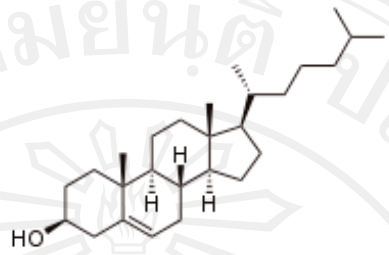


Figure 1.16 Chemical structure of cholesterol

Source: http://www.sigmaaldrich.com/catalog/ProductDetail

1.4.4.3. Cholestanol

Cholestanol (coprostanol or dihydrocholesterol) is a cholesterol derivative found in human feces, gallstones, eggs, and other biological matters. The molecular formula is C_{27} H_{48} O (Figure 1.17), practically insoluble in water, and stable under ordinary conditions during use and storage. This compound has frequently been used as a biomarker for the presence of human faecal matter in the environments.

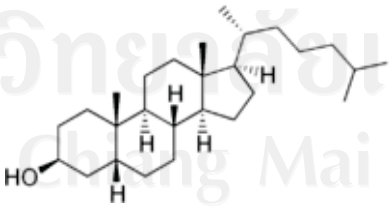


Figure 1.17 Chemical structure of cholestanol

Source: http://www.sigmaaldrich.com/catalog/ProductDetail

1.4.4.4. Solasodine

Solasodine is a steroidal alkaloid from plants of the Solanaceae family. The molecular formula is $C_{27}H_{43}NO_2$ (Figure 1.18). Solasodine and its glycosides are of considerable interest clinically. They are widely used as starting products for the synthesis of various steroidal drugs. Solasodine is a precursor for the synthetic cortisone and cyproterone acetate (Manosroi A et al., 2003).

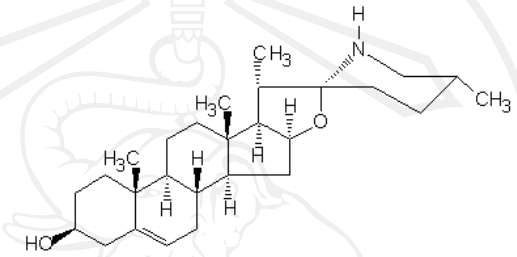


Figure 1.18 Chemical structure of solasodine

Source: http://www.sigmaaldrich.com/catalog/ProductDetail

1.4.5 Insulin

The word “Insulin” is from Latin *insula*, "island", as it is produced in the Islets of Langerhans in the pancreas. Insulin is an anabolic polypeptide hormone that regulates carbohydrate metabolism. Apart from being the primary agent in carbohydrate homeostasis, it has the effects on fat metabolism and changes the liver activity in storing or releasing glucose, processing blood lipids, and other tissues such as fat and muscle. The amount of insulin in blood circulation has extremely widespread effects throughout the body.

Insulin is used medically to treat some forms of diabetes mellitus. Patients with type 1 diabetes mellitus, who have no the hormone, depend on external insulin

(most commonly injected subcutaneously) for their survival. Patients with type 2 diabetes mellitus have insulin resistance, relatively low insulin production, or both. Some type 2 diabetics eventually require insulin when other medications become insufficient in controlling blood glucose levels.

Insulin is a small peptide composed of 51 amino acids in two chains connected by disulfide linkages. Human insulin has the chemical formula of $C_{257}H_{383}N_{65}O_{77}S_6$ and a molecular weight of 5808 (Figure 1.19). The amino acid sequence of the insulin molecule varies with the animal sources. Having a pI of 5.6, insulin is soluble at both acidic and physiologic pHs.

Within vertebrates, the similarity of insulins is extremely close. Bovine insulin differs from human in only three amino acid residues, and porcine insulin in one. Even insulin from some species of fish is similar enough to human to be clinically effective in humans. Insulin in some invertebrates (eg, the *Caenorhabditis elegans* nematode) is quite close to human insulin, has similar effects inside cells, and is produced very similarly.

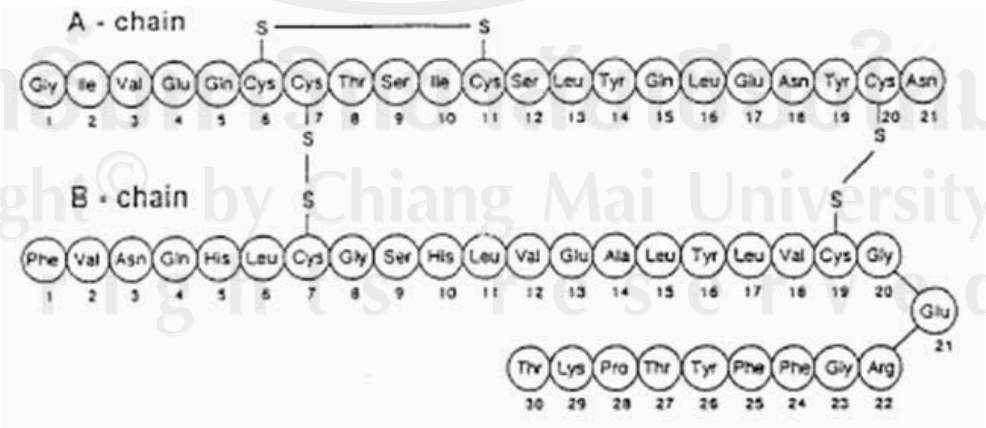


Figure 1.19 Structure of human insulin

Source: http://dailymed.nlm.nih.gov/dailymed/drugInfo.cfm

Insulin, like other peptide and protein drugs, possesses a large number of functional groups and, therefore, may undergo a variety of chemical or physical alterations with times, depending on the storage conditions. The changes that occur may not affect the safety and efficacy of the protein as a drug. It may be degraded non-enzymatically by various chemical reaction. The side chain amide groups of glutamine and aspargine are hydrolyzed to form free carboxylic acids. The rate of de-admidation is dependent upon temperature, pH, ionic strength, primary, secondary, and special intermolecular interaction. Physical alternations such as conformational changes and aggregation may also affect stability and bioactivity of the protein. Insulin may exist as monomers, dimmers, hexamers, and higher oligomers, all at equibrium in a neutral pH solution. Aggregation leads to the reduction in biological activity and obstruction of delivery routes. In contrast, larger aggregates (hexamers) of insulin have lower degradation rates than smaller aggregates (dimmers) when exposed to proteolytic enzymes. This arises from the fact that smaller species offer a correspondingly larger number of the exposed peptide linkages susceptible to enzyme attack.

Insulin or other peptides drugs are ineffective due to the low stability and poor absorption. In order to improve their oral bioavailability, many strategies have been tested, such as a co-administration of absorption enhancers which increase the permeability of peptides through the intestinal membrane (Marishita et al., 1993), synthesis of their stable derivative (Bruce et al., 1995) and the development of special dosage forms by utilizing the micro or nano-particle as a drug carrier (Michel et al.,

1991; Jenkin et al., 1994; Damge et al., 1995; Huang et al., 2006). Liposomes are one of the most potent candidates for such an insulin carrier system.

Liposomes have been applied for insulin therapy as a means to selectively target insulin to the liver, enhance oral absorption of insulin, and prolong insulin action. Liposomes are an effective means of delivering insulin specifically to hepatocytes. The usefulness of hepatically targeted liposomes in the treatment of diabetes is restricted due to the requirement that they must be given intravenously, the dilute concentration of insulin present in liposomal preparations, and the cost associated with liposome production. Encapsulating insulin in liposomes results in an enhanced oral absorption of insulin. The high doses of liposome entrapped with insulin required perorally, coupled with extreme variability in the glycemic response to peroral liposomes, limit the value of peroral liposomal insulin as a viable diabetic therapy. Insulin action can be sustained via encapsulation of insulin in liposomes given subcutaneously. Most insulin appears to remain at the injection site, and the presence of a lipid matrix for subcutaneous insulin delivery raises concerns over enhanced antigenicity of liposomal insulin and given subcutaneously. Viewed in the light of the limitations outlined above, the contribution of liposomal insulin to understanding and treatment of diabetes mellitus will probably be via use of hepatically targeted liposomes as a pharmacological probe to decipher the role of the liver in the metabolic complications associated with diabetes mellitus (Spangler et al., 1990).

It was reported that in *in vivo* oral administration study, insulin entrapped in cationic liposomes composed of DPPC (dipalmitoylphosphatidylcholine)/cholesterol/ SA (stearylamine) at the molar ration of 10:10:1 significantly decreased the plasma

glucose level, whereas insulin entrapped in neutral liposomes composed of DPPC (dipalmitoylphosphatidylcholine)/chol at the molar ratio of 10:10 and insulin solution showed no pharmacological effect. It is obvious that insulin administered in solution was rapidly degraded in the GI fluid before absorption. However, the reason for the difference in the effect of neutral liposome and cationic liposome is not clear from the *in vitro* experiment only. One of the most plausible explanations is that, because the surface of the biomembrane was negatively charged, the adhesive the ability of the cationic liposome to the intestinal epithelium is considered to be stronger than that of the neutral liposomes. In vitro release experiments showed the fast initial release of insulin from the cationic liposomes due to the burst of liposomes by the interaction with the bile salts. Therefore, the rapid effect of cationic liposomes would correspond to the fast release of insulin from liposomes adhered to the epithelium which temporarily raise the insulin concentration at the surface of the cell membrane (Iwanaga et al., 1997).

In this study, human insulin at pH less than 5.6(pI) was used as the model of an anionic drug representing the anionic characteristic of DNA. The characteristic as well as the entrapment efficiency of insulin entrapped in various cationic liposome containing several cationic lipids were investigated and evaluated.