CHAPTER II

LITERATURE REVIEW

Psoriasis

Psoriasis is a common, chronic, inflammatory skin disorder, characterized by the appearance of whitish or silvery laminated scales on slightly raised, well marginated, reddish patches (Barker, 1991). If the fine scale is removed, a salmon-pink lesion is exposed, perhaps with punctuate bleeding from prominent dermal capillaries (Diprio et al., 2002). Prevalence is between 0.1 and 3.0% of world's population (Linden and Weinstein, 1999).

Psoriasis can start at any age and psoriatic patients may be segregated into two groups based on age of onset, clinical course and genetic background. The first group occurring at 16-22 years of age (before the age of 15 years) tends to be more severe, resistant to therapy, associated with a positive family history, and to have a poorer prognosis. The second group occurring at 57-60 years of age is usually less severe and without family history (de Jong, 1997).

The exact cause of psoriasis is unknown. There are several hypothesis regarding the pathophysiology of psoriasis such as defects in epidermal cells; disruption in arachidonic acid metabolism; genetics; exogeneous trigger factors: climate, stress, infection, trauma, and drugs; and immunologic mechanism (Diprio et al., 2002).

PTU in the Treatment of Psoriasis

Propylthiouracil (PTU) is a thioureylene antithyroid drug which impairs thyroid hormone synthesis by binding to the T3 receptor. This receptor belongs to the family of

steroid receptors and includes retinoid and vitamin D receptors. PTU exhibits immunomodulatory activities on T-cell lymphocytes and the mechanism of its action is not known. Intercellular adhesion molecule-1 (ICAM-1), a marker of early T-cell activation, and interleukin-2 receptor levels do not decline in patients with psoriasis who are treated with orally administered thioureylenes (Elias et al., 1993b; Elias et al., 1993c). Elias et al. (1995) concluded that PTU inhibits keratinocyte growth enhancers such as proliferating cell nuclear antigen (PCNA) and enhances production of potential keratinocyte-growth-inhibitory factor (P53). Thus, it is likely that PTU may be acting as suppressing agents beyond the level of early lymphocyte activation. In patients with psoriasis treated orally with PTU alone and combination with methimazole, a significant improvement in the Psoriasis Area and Severity Index (PASI) was observed (Elias et al., 1993a; Elias et al., 1993b; Elias et al., 1993c; Elias et al., 1995; Chowdhury and Mark, 2001; Kose et al., 2001). Possible side effects of PTU treatment are subclinical hypothyroidism and leucopenia (Elias et al., 1993b; Elias et al., 1993c; Chowdhury and Mark, 2001; Kose et al., 2001). In a different study, topical application of 5% PTU lotion (PTU in propylene glycol and hydrophilic petrolatum) to patients with plaque psoriasis resulted in a significant improvement of the lesion (Elias et al., 1994). None of the patients receiving topical PTU developed clinical hypothyroidism or elevation of serum thyroid-stimulating hormone levels. Furthermore, Laifong Mona Asavisanu (1997) studied the effect of 10% PTU lotion in patients with psoriasis. She found a significant improvement of the lesion and the side effect in 11% of patients is contact dermatitis. Although PTU may be an effective agent in the treatment of psoriasis, further clinical trials are required to evaluate the efficacy and safety of PTU for the treatment of psoriasis. Development of a suitable topical formulation that is more efficient in delivery the drug to its target sites may increase efficacy and safety of the drug.

Niosomes (Uchegbu and Vyas, 1998; Uchegbu and Florence, 1995; Florence, 1993)

Niosomes which is a nonionic surfactant-based vesicles are formed from the self-assembly of nonionic surfactants in aqueous media resulting in closed bilayer structures. The assembly into closed bilayers is rarely spontaneous and usually involves some input

of energy such as physical agitation or heat. These structures are analogous to phospholipid-based vesicles (liposomes) and are able to encapsulate both hydrophilic and hydrophobic solutes, either in the bilayer, in the entrapped aqueous volume or at the bilayer interface, to serve as drug carriers. The low cost, greater stability, ease of storage and preparation, niosomes have to the exploitation of these vesicles as alternatives to liposomes. Niosomes are classified by their size and number of bilayers into multilamellar vesicles (MLVs: several bilayers, size 0.1-20 μm), small unilamellar vesicles (SUVs: single bilayer, size 0.01-0.1 μm), and large unilamellar vesicles (LUVs: single bilayer, size 0.1-1 μm).

Materials Used in the Preparation of Niosomes

As stated earlier, niosomes are formed from the self assembly of non-ionic surfactants in aqueous media resulting in close bilayer structures. In brief, the common components of niosomes are as follows (Roson, 1989; Kibbe, 2000):

1. Non-ionic surfactants

Non-ionic surfactants with a wide variety of structures, usually in the presence of CHO, form both multilamellar and unilamellar vesicles. Polyoxyethylene alkyl ethers (PAE) and long chain carboxylic acid esters are non-ionic surfactants most commonly used in niosomal preparations. Other non-ionic surfactants that form vesicles are the following: alkyl polyglycosides, alkyl polyglycerol ethers, alkyl sucrose esters, alkyl polyethyleneglycol polyglycerol ethers, steroidal oxyethylene ethers, dialkyl polyglycerol ethers, dialkyl polyoxyethylene ethers, dialkyl oxyethylene glycerol ethers, and hexadecyl glycerol (Florence, 1993). The properties of some commonly used surfactants are as follows:

1.1 Polyoxyethylene alkyl ethers (Kibbe, 2000)

Polyoxyethylene alkyl ethers (PAE) are non-ionic surfactants widely used in topical pharmaceutical formulations and cosmetics. PAE are a series of polyoxyethylene glycol ethers of n-alcohols (lauryl, myristyl, cetyl, and stearyl). It can be produced by the polyethoxylation of linear fatty alcohols. These products tend to be mixtures of polymers of slightly varying molecular weights, and the numbers used to describe polymer lengths are average values. The most common trade names of this group are Brij® and Steareth®. PAE are chemically stable in strong acidic or alkaline conditions. Brij® can only form vesicles in the presence of CHO. Brij® surfactants that have been reported to be vesicle forming agents include Brij® 30 (Manosroi et al., 2003; Manconi et al., 2002; Manconi et al., 2003; Manconi et al., 2006), Brij® 52 (Hofland et al., 1993; Guenin and Zatz, 1995; Arunothayanun et al., 1999; Manosroi et al., 2003), Brij® 72 (Hofland et al., 1993; Arunothayanun et al., 1999; Manosroi et al., 2003), Brij® 76 (Hofland et al., 1993; Niemiec et al., 1995; Jayaraman et al., 1996).

1.2 Long chain carboxylic acid esters

In this group, polyoxyethylene sorbitan fatty acid esters and sorbitan fatty acid esters are usually used in niosome preparation.

1.2.1 Polyoxyethylene sorbitan fatty acid esters

Polyoxyethylene sorbitan fatty acid esters are series of fatty acid esters of sorbitol, its anhydrides copolymerized with approximately 20 moles of ethylene oxide for each mole of sorbitol and its anhydrides. They are also known under the names of Tween® and polysorbate. These compounds are hydrophilic surfactants and widely used in food, drug, and cosmetic products. Polysorbate is stable to weak acids and bases. Tween® used in niosomal formulations is Tween® 20 (Carafa et al., 1998; Ruckmani, Jayaker, and Ghosal, 2000; Carafa et al., 2002; Shahiwala and Misra, 2002), Tween® 40 (Udupa et al., 1993), Tween® 60 (Udupa et al., 1993; Pillai and Salim, 1999), Tween® 61

(Manosroi et al., 2003; Manosri, et al., 2005), Tween® 80 (Udupa et al., 1993; Ruckmani et al., 2000), Tween® 85 (Naresh et al., 1994).

1.2.2 Sorbitan fatty acid esters

Sorbitan fatty acid esters are series of mixtures of partial esters of sorbitol and its mono- and di-anhydrides with fatty acids. Sorbitan esters are widely used in food, cosmetic and pharmaceutical products as lipophilic surfactants. Their proprietary name is Span®. Many niosomal formulations contain Span®, including Span® 20 (Udupa et al., 1993; Yoshioka, Sternberg, and Florence, 1994; Namdeo and Jain, 1999; Hao et al., 2002; Shahiwala and Misra, 2002), Span® 40 (Udupa et al., 1993; Yoshioka et al., 1994; Namdeo and Jain, 1999; Hao et al., 2002; Manconi et al., 2002), Span® 60 (Udupa et al; 1993; Reddy and Udupa, 1993; Yoshioka et al., 1994; Uchegbu et al., 1995; Naresh et al., 1996; Namdeo and Jain, 1999; Ruckmani et al., 2000; Agarwal et al., 2001; Hao et al., 2002; Shahiwala and Misra, 2002; Manconi et al., 2002; Manosroi et al., 2003), Span® 80 (Udupa et al; 1993; Yoshioka et al., 1994; Dimitrijevic et al., 1997; Namdeo and Jain, 1999; Ruckmani et al., 2000; Hao et al., 2002; Shahiwala and Misra, 2002), and Span® 85 (Yoshioka et al., 1994; Shahiwala and Misra, 2002).

1.3 Glycerol diesters

Glycerol diesters are diesters of glycerol and lipophilic non-ionic surfactants with HLB of 4-6. They are used in foodstuff, medicine and cosmetic industries, an emulsifier, stabilizer, defoamer, and thickener. Glycerol diesters used in niosomes are glyceryl distearate (GDS) and glyceryl dilaurate (GDL). GDS and GDL are used as vesicle forming agent for many drugs (Margalit, et al., 1992; Dowton et al., 1993; Niemiec et al., 1995; Fleisher, et al., 1995; Ohta et al., 1996; Jayaraman et al., 1996; Waranuch et al., 1997; Waranuch et al., 1998).

1.4 Sucrose fatty acid esters

Sucrose fatty acid esters are non-ionic surfactants with a sucrose substituent as the polar head group. They are nontoxic and biodegradable surfactants approved by FAO/WHO as food additives. Since they are nonirritant to the skin, they are also suitable for therapeutic and cosmetic applications. Sucrose fatty acid esters used in dermatological preparations in the forms of liquid crystals and microemulsions are sucrose laurate and sucrose ricinoleate (Ayala-Bravo et al., 2003). Sucrose laurate ester (L-595) contains 30.3% monoester, 39.3% diester, and 30.4% triester (Kunieda et al., 1993). L-595 used in noisome preparation is usually used with octaoxyethyleneglycol-8-laurate ester (PEG-8-L) (van den Bergh, Vroom et al., 1999; van den Bergh, Bouwstra et al., 1999; Li, Danhof, and Bouwstra, 2001; van den Bergh et al., 2001; Honeywell-Nguyen, Frederik, et al., 2002; Honeywell-Nguyen, de Graaff et al., 2002; Honeywell-Nguyen, Arenja et al., 2003; Honeywell-Nguyen and Bouwstra, 2003; Honeywell-Nguyen, Groenink et al., 2003).

2. Membrane additives

2.1 Cholesterol (CHO)

Various additives must be included in the formulation in order to prepare stable vesicles. The most common additive found in vesicular systems is CHO. CHO does not by itself form bilayer structures, but it can be incorporated into the bilayer membrane. Since CHO is an amphipathic molecule, it inserts itself into the bilayer with its hydroxyl group oriented towards the aqueous surface and the aliphatic chain aligned parallel to the alkyl chains in the center of the bilayer. Inclusion of CHO is essential to some non-ionic surfactants that can not form vesicles by themselves such as Tween®, Brij®, and polyglycerol ethers, etc. These surfactants can form stable vesicles in the presence of CHO because CHO modulates CPP values and abolishes the phase transition of the total lipids (New, 1997; Uchegbu and Vyas, 1998). Devaraj et al. (2002) used fatty alcohols instead of CHO in ketorolac tromethamine niosomes containing polyglyceryl-3-

di-isostearate and polysorbate-80 prepared by ether injection method. They reported that niosomes containing fatty alcohol showed similar properties to those containing CHO with slower release rate.

2.2 Stabilizers

2.2.1 Dicetylphosphate (DCP)

Dicetylphosphate (DCP) is a negatively charged lipid and it prevents vesicle aggregation by electrostatic repulsion. It has been widely used in both liposomes and niosomes (Reddy and Udupa, 1993; Udupa et al., 1993; Naresh et al., 1994; Yoshioka et al., 1994; Carafa et al., 1998; Uchegbu and Vyas, 1998; Namdeo and Jain, 1999; Carafa et al., 2002). Inclusion of DCP in niosomal formulations affects drug entrapment and stability.

2.2.2 Solulan® C24

Solulan® C24 (poly-24-oxyethylene cholesteryl ether) is one of additive stabilizers useful in vesicle preparation for antiaggregation by steric hindrance. Solulan® C24 has been used as a stabilizer in many niosomal formulations (Uchegbu et al., 1995; Uchegbu and Florence, 1995; Uchegbu et al., 1997; Dimitrijevic et al., 1997; Arunothayanun et al., 1999; Arunothayanun et al., 2000). Solulan® C24 has been used at a concentration of 5% by weight without toxicity (Dimitrijevic et al., 1997). Addition of Solulan® C24 in niosomes influences niosomal properties such as entrapment efficiency, viscosity, stability, and toxicity.

2.3 Micelle-forming agents

Niosomes prepared from L-595 form rigid vesicles. Incorporation of a micelle-forming agent surfactant, PEG-8-L, into the vesicle bilayers would result in partial solubilization of the bilayer and thereby increasing the elasticity of the vesicular

system. Therefore, a series of vesicles can be obtained, ranging from very rigid to very elastic by changing the ratio of the vesicle forming and the micelle forming agents. van den Bergh et al. (2001) studied the elasticity of elastic vesicles containing various ratios of L-595 and PEG-8-L by using electron spin resonance, electron microscopy, and extrusion measurement. They concluded that when molar content of PEG-8-L was increased the elasticity was increased. Similarly, Honeywell-Nguyen, Frederik et al. (2002) investigated the effect of addition of drug molecules, pergolide, on the physicochemical characteristics as well as the morphology of the vesicular system using Cryo-TEM. They found that increasing the PEG-8-L content increased vesicle elasticity, vesicle stability, and drug solubility but a further increase in the PEG-8-L content resulted in a decrease in drug solubility and vesicle stability. The elastic vesicles consisting of L-595, PEG-8-L and sodium sulfosuccinate in the molar ratio of 50:50:5 gave the most elastic and stable formulation in their study.

There are some researchers who investigated the effect of elastic vesicles containing PEG-8-L on skin delivery of drugs, such as pergolide and rotigotine, compared with rigid vesicles (Honeywell-Nguyen, Frederik et al., 2002; Honeywell-Nguyen, Arenja et al., 2003). In addition, some researchers studied the effect of elastic vesicles on ultrastructure of skin using various techniques and found that elastic vesicles induced ultrastructure changes of the deeper layers of the skin (van den Bergh, Vroom et al., 1999; van den Bergh, Bouwstra et al., 1999; Honeywell-Nguyen, de Graaff et al., 2002).

Niosome Preparation Methods (Uchegbu and Vyas, 1998)

There are many methods available for niosome preparation. Most methods used for liposomes may be applied to niosomes. In addition, methods devoid of organic solvents are also possible because non-ionic surfactants are heat stable. In brief, most methods consist of hydration of a mixture of the surfactants/lipids at the elevated temperature, which is above the phase transition temperature of the systems, followed by optimal size reduction. In case of hydrophilic drugs, separation of the free drug

molecules from the entrapped molecules is usually required. This can be done by various methods such as exhaustive dialysis, centrifugation, ultracentrifugation, and gel filtration. For hydrophobic molecules, the amount of drug in the formulation is usually predetermined, without free drug left in the final preparation. Some of the preparation methods commonly used for niosomes are ether injection (Baillie et al, 1985), film hydration method (Baillie et al, 1985; New, 1997), reverse phase evaporation (New, 1997), mixing of melted surfactants/lipids with the aqueous phase (Wallach and Philippot, 1993; Niemiec et al., 1995; Handjani-vila et al., 1979), enzymatic conversion (Chopineau, Lesieur, and Ollivon, 1994), and the bubble method (Talsma et al, 1994). The most commonly used method is film hydration method. The mixing of melted surfactants/lipids with the aqueous phase method does not require the use of organic solvents and formation of vesicles can be facilitated by sonication. Another method where the use of organic solvents is avoided is the bubble method.

Niosomes prepared as described above are usually in the micron size range although some of the methods produce niosomes in the sub-micron size range. Reduction of vesicle size is often desirable since vesicle size is known to have an impact on biodistribution. Reduction of vesicle size may be achieved by a number of methods such as probe sonication, extrusion through polycarbonate membrane with pore size of 100 nm, combination of sonication and filtration, use of microfluidizer, and high-pressure homogenization (Uchegbu and Vyas, 1998).

Factors Governing Niosome Formation

1. Nonionic surfactant structure

Vesicle formation can be predicted by critical packing parameter (CPP) proposed by Israelachvili (Uchegbu and Vyas, 1998). CPP is a dimensionless parameter described as follows:

$$CPP = v/(l_c a_0)$$

where v = hydrophobic group volume

l_c = the critical hydrophobic group length

a_0 = the area of the hydrophilic head group

Nonionic surfactant structure is related to critical packing parameter (CPP). CPP is the value which indicated the arrangement of surfactant monomer in the aqueous medium. A calculated value of CPP below 0.5 indicates the spherical micelle formation, value between 0.5 and 1 indicates that the surfactant monomers assemble preferentially into vesicles while a CPP of above 1 would predispose a compound to form inverted micelles. The true CPP depends on the molecular geometry of the surfactant monomer which is not a fixed parameter. Factors such as ionic strength, temperature, degree of hydration of hydrophilic head groups and the physicochemical property of the drugs will affect monomer conformation and the vesicle formation.

Thus, theoretically niosome formation requires the presence of a particular class of non-ionic surfactant and aqueous medium. Non-ionic surfactant molecules may consist of one or two alkyl or perfluoroalkyl groups or a single steroidal group. The alkyl chain length usually contains 12 to 18 carbon atoms. Perfluoroalkyl surfactants that can form vesicles may have chain length as short as 10 carbon atoms. The two portions of the molecule may be linked via ether, amide, or ester bonds. Crown ether amphiphiles with a steroidal or C_{16} alkyl chain have been shown to form vesicles. On the other hand, there is a wide variety of the hydrophilic head group in vesicle-forming non-ionic surfactants. Surfactants with two alkyl chains normally form vesicles while surfactants with one alkyl chain form micelles in diluted aqueous solutions. Surfactants with single alkyl chain will form vesicles in the presence of CHO. However, Manosroi et al. (2003) found that some single alkyl chain surfactants can form vesicles prepared by film hydration without CHO such as Span® 60, Brij® 72, and glyceryl monostearate. Although some surfactants have optimal CPP values, not all of them can form stable vesicles. For example, $C_{16}G_3$, $C_{16}C_{12}G_7$, and $C_{16}G_2$ have CPP values of 0.64, 0.63 and 0.7, respectively, indicating that these compounds would all form stable vesicles. However, while $C_{16}G_3$ forms stable vesicles without CHO inclusion and $C_{16}G_2$ forms less stable aggregates with or without

CHO, $C_{16}C_{12}G_7$ can not form vesicles without the inclusion of CHO. The CPP thus serves as a guide only to the ability for vesicle formation of any surfactants.

Bouwstra and Hofland (1994) showed the effects of polar head groups and alkyl chain length of alkylpolyoxyethylene surfactants (C_nEO_m) on the vesicle formation. They concluded that these surfactants can form vesicles only in the presence of CHO. The similar alkyl chain length compounds with smaller ethylene oxide group form vesicles more readily and a series of C_{16} with m varied from 7-21 form micelles in dilute solution. For surfactants with similar EO (m = 3), increase in carbon chain length (C_{10}-C_{18}) decreases stability of vesicles.

Bouwstra et al. (1997) concluded that $C_{12}EO_3$ is the only surfactant that is able to form stable vesicles without CHO using hand shaking method since it has optimal CPP. In the absence of CHO, vesicles can be obtained from $C_{12}EO_3$, $C_{14}EO_3$, $C_{16}EO_3$, $C_{18}EO_3$, $C_{14}EO_5$ and $C_{16}EO_5$ using sonication method. When CHO is added, vesicles can be prepared from each of all surfactants in the series of polyoxyethylene alkyl ethers because CHO improves CPP value to optimal value. For sugar ester surfactants, no vesicles can be formed without the addition of CHO.

In addition, Hydrophilic Lipophilic Balance (HLB) is a good indicator of the vesicle forming ability of surfactant. With Span® surfactants, an HLB number of 4-8 was found to be compatible with vesicle formation. On the other hand, water soluble surfactants with high HLB values like Tween® surfactants will not form stable vesicles. However, some Tween® such as Tween® 40, 60, and 80 (Udupa et al., 1993), Tween® 20 and 80 (Ruckmani et al., 2000), Tween® 20 (Carafa et al., 1998; Carafa et al., 2002), Tween® 60 (Pillai and Salim, 1999), Tween® 85 (Naresh et al., 1994) can form vesicles in the presence of CHO. In addition, Bouwstra et al. (1997) found that vesicle formation from polyoxyethylene alkyl ethers is possible with an HLB between 7.5 and 10.5 depending on the preparation method.

2. Membrane additives

The most common additive found in vesicular system is CHO particularly in the system that the surfactant itself can not form a vesicle. Thus in cases where a mixture of surfactants or CHO is used to prepared vesicles, the operational CPP values will be those of the entire components. CHO is known to abolish the gel to liquid phase transition of vesicular systems (New, 1997). The inclusion of CHO influences the properties of the vesicles such as membrane permeability, entrapment efficiency, bilayer rigidity, and toxicity, etc. Charge stabilizer, such as DCP or stearylamine, is often added to the bilayer of the vesicles to prevent aggregation. The quantity of these non-vesicle-forming additives must be carefully adjusted. Otherwise, structures other than liposomes or niosomes may result (Uchegbu and Vyas, 1998).

3. Nature of the entrapped drug

The nature of the entrapped drug may affect vesicle formation ability of non-ionic surfactants. Hydrophobic or amphiphilic drugs can affect the CPP value of the system since these molecules will be incorporated into the vesicle membrane. Span® 60 niosomes containing DCP form homogeneous dispersion when the entrapped compound is a water-soluble dye, carboxyfluorescein. However, the same niosomal system forms an aggregated dispersion with an amphipathic drug, doxorubicin. Doxorubicin is thought to be incorporated in the vesicle membrane since it has been shown to alter the electrophoretic mobility of hexadecyl diglycerol ether niosomes in a pH dependent manner (Uchegbu and Vyas, 1998). Similar result was observed from the studies of Yoshioka et al. (1994) and Manconi et al. (2002). Span® 80 niosomes containing Span® 80:CHO:DCP in the molar ratio of 47.5:47.5:5 form stable vesicles when the entrapped compound is a water-soluble dye, carboxyfluorescein (Yoshioka et al.,1994). If the drug is a hydrophobic drug, tretinoin, Span® 80 niosomes are not stable and show phase separation. The cause of this result is intercalation of tretinoin into the vesicle bilayers (Manconi et al., 2002).

4. Surfactant and lipid levels (Uchegbu and Vyas, 1998;)

The level of surfactant/lipid used to make niosomes is generally 10-30 mM (1-2.5 % w/w). Altering the surfactant to water ratio during the hydration step may affect microstructure and hence properties of the system (Cevc, 2004). However, increasing the surfactant/lipid level may increase or decrease the total of drug encapsulation depending on the nature of the drug. If the total surfactant/lipid concentration is too high the system is highly viscous.

5. Temperature of hydration

As a rule, the temperature used to make liposomes or niosomes should be above the gel to liquid phase transition temperature of the system.

6. Method of preparation

The assembly of non-ionic surfactants into closed bilayers is rarely spontaneous and usually involves some input of energy such as physical agitation or heat. The different method gives different energy input. Thus, vesicle formation depends on method of preparation. Parthasarathi, Udupa, and Pillai (1993) found that vesicles of vincristine sulfate prepared from Brij® 35 and Brij® 78 could not form by film hydration and transmembrane pH gradient methods, whereas they could form by reverse phase evaporation technique. Tween® 20 and Tween® 80 could not form vesicles by reverse phase evaporation and transmembrane pH gradient methods but they could form by film hydration method. Bouwstra et al. (1997) found that using hand shaking method, $C_{12}EO_3$ could form stable vesicles without CHO while $C_{14}EO_3$, $C_{16}EO_3$, $C_{18}EO_3$, $C_{14}EO_5$ and $C_{16}EO_5$ could form vesicles by sonication method.

Characterization of Niosomes

1. Morphology

Electron microscopy is used to verify vesicle formation and to examine lamellarity and morphology of vesicles. This technique includes Freeze fracture electron microscopy (FFEM) (Hofland et al., 1993; Yoshioka et al., 1994; Bouwstra et al., 1997; van den Bergh et al 2001; Carafa et al., 2002; Carafa et al., 2004; Harvey et al 2005), transmission electron microscopy (TEM) (Arunothayanum, 2000; Manconi et al., 2002; Manconi et al., 2003; Guinedi et al., 2005; Manconi et al., 2006), scanning electron microscopy (SEM) (Touitou et al. 2000; Manconi et al 2006), and cryo-TEM (Honeywell-Nguyen, Frederik et al., 2002). Some researchers used various types of optical microscopy to examine morphology of the vesicles and completion of vesicle formation (Kiwada et al., 1985; Fleisher et al., 1995; Jayaraman et al., 1996; Agarwal et al., 2001; Devaraj et al., 2002; Manosroi et al., 2003; Manosroi et al., 2005).

2. Size and size distribution

The most commonly used method to measure the particle size and size distribution of the vesicles in the nanometer size range is photon correlation spectroscopy. Some researches use other methods, such as, laser diffraction method (Arunothayanun et al., 2000; Agarwal et al., 2001), electron microscopic method (Ruckmani et al., 2000), and microscopic method (Parthasarathi et al., 1993) to measure niosomal size.

3. Entrapment efficiency

Entrapment efficiency (EE) describes drug loading in niosomes and is thus crucial in application of niosomes as delivery systems. There are several ways of describing drug loading in niosomes. For example, drug loading can be described as the percentage of drug entrapped. In this case, the initial drug to surfactant/lipid ratio must be

specified. Another way to describe drug loading is the volume of drug solution, usually in liter, entrapped per mole of surfactant/lipid. This method assumes no change in the concentration of the drug in the aqueous phase throughout the process of preparation and analysis steps. The ratio of drug to surfactant/lipid by weight (g/g) or on molar basis (mol/mol) can also be used to describe drug loading. The lather gives adequate information on the level of excipient that must be concomitantly administered at each dose level of the drug. It is propably the most useful description to formulators.

In determining EE, unentrapped drug must be removed from the vesicles by various methods. The methods that have been used for removing unentrapped solute include:

- Exhaustive dialysis (Baillie et al., 1985; Kiwada et al., 1985; Namdeo and Jain, 1999; Ruckmani et al., 2000; Hao et al., 2002; Devaraj et al., 2002; Honeywell-Nguyen, Arenja et al., 2003; Manosroi et al., 2003; Manosroi et al., 2005;)
- Gel filtration (Dowton et al., 1993; Parthasarathi et al., 1993; Yoshioka et al., 1994; Yoshioka and Florence, 1994; Waranuch et al., 1997; Manconi et al., 2002; Carafa et al., 2002; Manconi et al., 2003; Carafa et al., 2004; Manconi et al., 2006)
- Ultracentrifugation (Arunothayanun et al., 2000; El Maghraby et al., 2000a; Fang, Hong et al., 2001; Agarwal et al., 2001; Guinedi et al., 2005)

4. Phase transition

Differential scanning calorimeter (DSC) is used to evaluate phase transition temperature of the vesicle systems and effect of other compounds (such as CHO, other additives, and drugs, etc) on membrane fluidity. The phase transition temperature of the vesicle systems indicates the thermodynamic state of the bilayer. If the phase transition temperature of the vesicle systems is higher than ambient temperature, the bilayer is in gel state. If the phase transition temperature of the vesicle system is lower than ambient temperature, the bilayer is in liquid crystalline state (Arunothayanun et al., 1999; Arunothayanun et al., 2000). If the phase transition temperature changes when drugs or

CHO or other compounds are included, it means that interaction between other compounds and the membrane occurs. Trotta et al. (2004) used DSC to evaluate the interaction between dipotassium glycyrrhizinate (KG, a surfactant) and liposomes. They found that phase transition of liposome with KG was lower than that of liposome itself. It indicates that KG perturbs the packing characteristics and thus fluidizes the bilayer.

5. Physical stability

The stability of any pharmaceutical products is usually defined as the capacity of the formulation to remain within defined limits for a predetermined period of times (shelf life of the product). The stability of the vesicular products should preferably meet the standard of conventional pharmaceutical products. Both chemical and physical stability aspects are involved. Possible stability problems include loss of entrapped drug, change in the vesicular structure (including particle size distribution and aggregation and fusion of the vesicles), and chemical instability of entrapped drug (Weiner, Martin, and Raiz, 1989).

Factors Influencing Niosome Characteristics

The vesicular formulations of various drugs can be optimized in terms of entrapment efficiency, size and size distribution, stability, and the rate of drug release by altering their physicochemical parameters. The effects of these parameters on vesicular characteristics are as follows:

1. Effect of surfactant structure

Surfactant structure may affect the properties of the vesicles such as drug entrapment, vesicle size, and rate of drug release, etc. There are many studies on the effect of surfactant structure on these parameters. The effects of surfactant structure on properties of vesicles are concluded as follows:

1.1 Effect of surfactant structure on entrapment efficiency (EE)

The chemical nature of the niosome membrane may be manipulated to increase drug entrapment by altering the nature of the hydrophilic part and/or hydrophobic part. When a series of cetyl glycosides is examined for the encapsulation of [^{14}C]-sucrose follows the rank order of glucose > mannose > galactose > lactose. These differences may be due to the different levels of hydration of these sugars (Kiwada et al., 1985). Some researchers found that for Span® niosomes, the drug loading directly depended on the hydrophobic alkyl chain length. When a series of Span® were examined, the drug loading of 5-fluorouracil and colchicines decreased with decreasing alkyl chain length (Hao et al., 2002). Similar result was observed by Manconi et al. (2002) who reported that tretinoin niosomes prepared from Span® 60 by film hydration gave higher entrapment than that of Span® 40 niosomes.

Some researchers have concluded that the less fluid the membrane bilayer is, the higher the drug encapsulation is achieved. The surfactant with higher phase transition temperature usually forms less leaky vesicles, and thus resulting in higher drug entrapment. Similarly, unsaturation in the alkyl side chain can lead to a more leaky membrane and lower drug loading. Yoshioka et al. (1994) reported that the entrapment of carboxyfluorescein in niosomes prepared by hand shaking method varied with vesicle membrane composition. They reported that Span® 60 and Span® 40 gave the higher entrapment than Span® 20 and Span® 80 and that Span® 60 were the least leaky due to the highest phase transition temperature. Similarly, niosomes prepared from Tween® 80, Tween® 20, or Span® 60 gave comparable cytarabine entrapment, whereas a much lower entrapment was seen with Span® 80 (Ruckmani et al., 2000). In another study, acetazolamide entrapment in niosomes prepared from Span® 60 by both film hydration and reverse phase evaporation methods was higher than that of niosomes prepared from Span® 40 (Guinedi et al., 2005).

However, Parthasarathi et al. (1993) reported that Span® 40 niosomes gave the highest vincristine entrapment as compared with Span® 20 and Span® 60

niosomes. Similar result was observed by Namdeo and Jain (1999) who prepared 5-fluorouracil niosomes from a Span® series and they reported that Span® 40 niosomes gave the highest entrapment as compared with other Span® niosomes. In another study, [^{14}C]-sucrose entrapment in C_{16} glucoside niosomes was the highest when compared to those of C_{18} and C_{14} compounds (Kiwada et al, 1985). Therefore, other factors rather than the fluidity of the bilayers must also influence the entrapment efficiency.

1.2 Effect of surfactant structure on drug release

In general, in vitro drug release is used to evaluate drug delivery from topical products. The result from such study can predict the behavior of drug release to the skin in vivo. There are a number of previous studies in which the effect of surfactant structure on drug release from niosomes is evident. Yoshioka et al. (1994) reported that the release of carboxyfluorescein from Span® 60 and Span® 40 niosomes was slower than that from Span® 20, Span® 80, and Span® 85 niosomes. This result agrees well with those of Namdeo and Jain (1999) who studied the release of 5-fluorouracil from niosomes prepared from a Span® series. They found that Span® 60 and Span® 40 niosomes gave a slower drug release rate than those of Span® 20 and Span® 80 niosomes. Span® 60 and Span® 40 have higher phase transition temperatures and form rigid less permeable, bilayers than those of Span® 20, Span® 80, and Span® 85. In general, short chain non-ionic surfactants produce more fluid membranes than membranes of long chain surfactants and unsaturation in the alkyl chain can lead to a more permeable membrane. Ruckmani et al. (2000) also reported that the release of cytarabine hydrochloride from niosomes prepared from Span® 60 and Span® 80 by film hydration method depended on niosomal composition. They found that drug release from Span® 60 niosomes was slower than that from Span® 80 niosomes. In another study, niosomes prepared from Span® 60 gave slower release than those prepared from Span® 40 (Guinedi et al., (2005).

However, Yoshioka and Florence (1994) reported that the release rates of carboxyfluorescein from Span® 20, Span® 40, Span® 60 and Span® 80 niosomes prepared by hand shaking method were almost the same. Similarly, niosomes of tretinoin

prepared from Span® 60 and Span® 40 by film hydration method gave the comparable release rates (Manconi et al., 2002).

1.3 Effect of surfactant structure on vesicle size

The vesicle size also depends on alkyl chain length of surfactants. Increasing hydrocarbon chain length of surfactant monomers lead to smaller vesicles (Barlow, Lawrence, and Timmins, 2000). Increasing hydrophobicity of surfactants increase hydrophobic interaction, thereby the vesicle size was decreased. This effect was observed from the study of Yoshioka et al. (1994) who reported that the size of the vesicles prepared from the Span® series by hand shaking method was dependent on the HLB of Span® used. The lower HLB gives the smaller size of the vesicles. On the other hand, Manconi et al. (2002) reported that tretinoin niosomes prepared from Span® 60 by film hydration method was larger than Span® 40 niosomes though the difference was very small.

1.4 Effect of surfactant structure on stability

The choice of membrane surfactant determines the properties of the bilayer membrane and usually affects the stability of the niosomal system. Thus, in making a choice of surfactant, the higher phase transition surfactants appear to yield more desirable stability. The rank order of the leakiness of carboxyfluorescein from niosomes prepared from a series of Span® surfactants was Span® 80 > Span® 20 > Span® 40 > Span® 60 (Yoshioka et al., 1994).

1.5 Effect of surfactant structure on toxicity

Non-ionic surfactants are weak irritants (Endo, Yamamoto, and Ijuin, 1996; Dimitrijevic et al., 1997). In the study of skin toxicity using the cell proliferation of human keratinocytes in vitro, neither the length of alkyl chain nor the polyoxyethylene had any influence on the skin toxicity of alkyl polyoxyethylene (C_nEO_m) niosomes.

However, surfactants with ester bonds were less toxic than those with ether bonds because of their chemical instability. The cholesterol content did not appear to have an effect on the proliferation of the keratinocytes (Hofland et al, 1991; Hofland et al, 1992).

Dimitrijevic et al. (1997) studied the toxicity of Solulan® C24 or Solulan® C16 in two niosomal systems on Caco-2 cell monolayers using MTT test, measurement of transepithelial electrical resistance, and [^{14}C]-metformin transport compared with free solution and micellar form of Solulan® C24 or Solulan® C16. These two niosomal systems were Span®80:CHO:Solulan® C24 or Solulan® C16 and hexadecyl diglyceryl ether ($C_{16}G_2$):CHO:Solulan® C24 prepared by film hydration method. They concluded that the toxic effect of niosomes is principally a result of the amount of free Solulan® present in the niosomal suspensions and the niosome-forming agent is non toxic. With rabbit skin test, polyoxyethylene alkyl ethers cause the greatest irritation and polysorbates cause more irritation than sorbitans do (Mezei et al., 1966).

2. Effect of surfactant/lipid concentration

Variation of the surfactant/lipid concentration affects the drug entrapment of the vesicles. Increasing surfactant/lipid concentration results in increase drug loading since the number of vesicles formed is increased. Kiwada et al. (1985) reported that encapsulation capacity for [^{14}C]-sucrose of cetyl glucoside noisomes depended on total lipid concentration. High encapsulation efficiency was observed when the vesicles were prepared with a high total surfactant or lipid concentration. This result is in accordance with those of Yoshioka et al. (1994) who concluded that Span® 80 niosomes of carboxyfluorescein prepared from higher total lipid concentration gave higher drug entrapment than those prepared from lower total lipid concentration. Similarly, entrapment efficiency of 5-fluorouracil in Span® niosomes prepared by various methods was linearly increased when the total lipid was increased (Namdeo and Jain, 1999).

However, the effect of total concentration is not always in linear relationship with drug entrapment. Hao et al. (2002) studied the effect of total lipid

concentration on entrapment of colchicines in Span® 60 niosomes prepared by evaporation-sonication method. They reported that the concentration of surfactant from 8×10^{-5} to 3.2×10^{-4} mol/L was optimal for drug entrapment and lower or higher concentrations from this range gave lower drug entrapment.

3. Effect of membrane additives

Membrane additives used in niosomes for stabilization are CHO and stabilizers such as DCP and Solulan® C24. The effects of these additives on niosomal properties are as follows:

3.1 Effect of membrane additives on entrapment efficiency

Inclusion of CHO abolishes phase transition of the system and CHO affects fluidity of the membrane. Addition of CHO may alter the physical structure of niosomes as well as the drug entrapment (Arunothayanun et al., 2000). Polyhedral niosomes were formed from a system containing $C_{16}G_2$ and Solulan® C24. When CHO was added to the system, spherical vesicles mixed with tubular structures resulted. The polyhedral systems were reported to have a larger particle size and high entrapment. Inclusion CHO reduces the leaky space in the bilayer, which allows enhanced drug loading of water-soluble drugs in vesicles. However, Namdeo and Jain (1999) reported that inclusion of CHO in 5-fluorouracil Span® 40 niosomes reduces 5-fluorouracil loading in the vesicles since the vesicles size is decreased.

However, entrapment efficiency for Span® 60 and Span® 40 niosomes of carboxyfluorescein prepared by hand shaking method increases with increasing CHO contents (Yoshioka et al., 1994). The increased drug entrapment is most likely to be the result of increased vesicle size. If no relationship between vesicle volume and drug entrapment exists, increased bilayer thickness is usually the cause of increased drug loading.

The quantity of CHO must be carefully adjusted to optimum concentration. Otherwise, structures other than liposomes or niosomes may result (Uchegbu and Vyas, 1998). Dithranol entrapment in niosomes prepared from Span® 60 by film hydration method was increased when CHO content was increased. If CHO content is increased more than this, the entrapment is reduced. CHO content beyond a certain content starts disrupting the bilayer structure leading to loss of drug entrapment (Agarwal et al., 2001). This result agrees well with those of Hao et al. (2002) and Guinedi et al. (2005). Hao et al. (2002) reported that for colchicine niosomes prepared from Span® 60 by evaporation-sonication method, the quantity of drug entrapped was increased with increasing CHO concentration. The surfactant:CHO molar ratio of 1:1 gave the highest drug entrapment and with CHO more than this level, drug entrapment was reduced. Similarly, the encapsulation of acetazolamide in Span® 60 and Span® 40 niosomes prepared by film hydration and reverse phase evaporation methods was highest at Span®:CHO molar ratio of 7:6 (Guinedi et al., 2005).

Furthermore, inclusion of a positively charged lipid, stearylamine, gives higher entrapment efficiency for the anionic drugs such as all-trans retinoic acid (Desai and Finlay, 2002). Similar result was observed in the study of Manconi et al. (2006) who prepared tretinoin niosomes from octyl-decyl polyglucoside and decyl polyglucoside without CHO by film hydration method. They found that niosomes with stearylamine had higher drug entrapment than those with DCP and also had a larger size.

3.2 Effect of membrane additives on vesicle size

CHO and DCP may affect the vesicle size. The effect of CHO and DCP on vesicle size depends on surfactant type. Bouwstra et al. (1997) reported that addition of CHO and DCP in C_nEO_m niosomes prepared by sonication method reduced the vesicles size. The reason for this result is the effect of CHO in decreasing tendency of the surfactants to aggregate at elevated temperatures, while DCP might increase the curvature of the bilayer and thus increasing hydrophilic surface area. However, they also found that for vesicles from sugar ester surfactants, CHO content did not influence the

vesicle size because this system was stable to temperature but addition of DCP largely reduced vesicle size since the vesicles showed a strong tendency to aggregate due to the increased surface area. In another study, inclusion of CHO and DCP reduced vesicles size of 5-fluorouracil-containing Span® 40 niosomes (Namdeo and Jain, 1999). On the other hand, Agarwal et al. (2001) concluded that no effect of DCP on vesicle size was observed in Span® 60 niosomes of dithranol prepared by film hydration method.

3.3 Effect of membrane additives on drug release

Since CHO and DCP affect membrane fluidity, they may affect drug release from the vesicles. If CHO decreases membrane fluidity, the drug release will be decreased when CHO content is increased. This is especially true for water-soluble drugs. Baillie et al. (1985) studied carboxyfluorescein release from niosomes prepared from non-ionic surfactants by ether injection method. They concluded that addition 50% CHO reduced drug release by a factor of 10 because of membrane stabilizing effect of CHO. This result agrees with those of Namdeo and Jain (1999) and Guinedi et al. (2005). Namdeo and Jain (1999) reported that addition of CHO and DCP in Span® 40 niosomes of 5-fluorouracil reduced drug release. Guinedi et al. (2005) found that increasing CHO content in Span® 60 and Span® 40 niosomes of acetazolamide reduced drug release. In addition, Devaraj et al. (2002) investigated drug (ketorolac tromethamine) release from niosomes containing various fatty alcohols as bilayer stabilizers instead of CHO. These niosomes consisted of polyglyceryl-3-di-isostearate and polysorbate-80 and were prepared by ether injection method. They reported that niosomes containing fatty alcohols showed a slower release rate than those containing CHO and the rank order of release rate was cetostearyl > lauryl > cetyl > myristyl.

3.4 Effect of membrane additives on stability

Other additives in the final formulation may also affect stability of niosomes. High concentrations of detergents (soluble surfactants) are incompatible with niosomal systems. Eventually, they can cause solubilization of the vesicles to form mixed

micelles. The destruction of $C_{16}G_2$ niosomes by octyl glucoside appears to proceed via the build up of a localized concentration of the detergent within the niosome membrane followed by micellization of the system (Saras et al., 1994). Similarly, $C_{16}G_2$ niosomes are solubilized by Solulan® C24 and converted into mixed micelles (Uchegbu, Bouwstra, and Florence, 1992).

4. Effect of method of preparation

The method of drug loading can also alter entrapment efficiency of vesicular drug carriers. Thus, niosomes prepared from different methods have different properties. Entrapment efficiency of water soluble drugs such as carboxyfluorescein, vincristine sulfate, 5-fluouracil are followed the rank order of ether injection > reverse phase evaporation > film hydration methods (Baillie et al., 1985; Parthasarathi et al., 1993; Namdeo and Jain, 1999). The difference may be due to greater encapsulated volume in unilamellar/oligolamellar vesicles resulted from ether injection and reverse phase evaporation methods than that of the multilamellar structure from film hydration method. Water soluble drugs do not associate with bilayers, hence they will be entrapped within the aqueous compartment and this depends on the encapsulation volume.

On the other hand, for water insoluble drug, acetazolamide, the drug entrapments of Span® 60 and Span® 40 niosomes prepared by film hydration method were higher than those of niosomes prepared by reverse phase evaporation method (Guinedi et al., 2005). Similarly, the rank order of tretinoin entrapment in Span® 40 niosomes was from film hydration (MLVs) > extrusion (LUVs) > sonication (SUVs) methods (Manconi et al., 2002).

Various techniques may be used to optimize drug loading. Transmembrane pH gradient drug uptake process (remote loading method) dramatically increases the entrapment of vincristine sulfate in Span® 60 and Span® 40 niosomes (Parthasarathi et al., 1993). So do the dehydration-rehydration method which dramatically increases the entrapment efficiency of PK1 in $C_{16}G_2$ niosomes (Uchegbu and Duncan, 1997). Other

methods that have been reported to increase drug entrapment in the vesicles include the use of pH gradients and ammonium sulfate to trap the drugs inside the vesicles (Haran et al., 1993). This intravesicular trap has been used successfully with doxorubicin in Span® 60 niosomes (Uchegbu and Florence, 1995). Luteinizing hormone releasing hormone displays high entrapment in polyhedral niosomes when the remote-loading methods using pH or ammonium sulfate gradients are applied. This is in contrast with the other methods, including the direct hydration at pH 7.4 or pH 4.3, dehydration-rehydration, and reverse phase evaporation (Arunothayanun et al., 1998).

5. Effect of the physicochemical properties of the drug

The nature of the drug to be entrapped also governs the apparent entrapment efficiency in niosomes. It is challenging to predict drug loading in niosomal preparations since interaction between the drug and the membrane can exist in various ways. For hydrophobic drugs, lipid packing seems to be a major determinant. On the contrary, ionic interaction seems to play a major role for ionizable drugs (Philippot and Schuber, 1995). The extent of drug entrapment varies between drugs and membrane compositions as well as methods of preparation. All of these factors have to be simultaneous considered. When pentoxifylline niosomes were prepared by film hydration method, the drug entrapment was only 9.4% (Gaikwad et al., 2000). On the other hand, rifampicin niosomes prepared by the same technique had an entrapment efficiency of 33% (Kamath et al., 2000). Therefore, it is often difficult to compare results from different laboratories, especially when percent EE are reported.

For ionizable drugs, chemical form of the drug (acidic, basic, or salt form), vesicle composition, and environment pH conditions all seem to be major determinants. This has been shown with lidocaine niosomes. Lidocaine has a pKa of 7.8. At pH 5.5, most of lidocaine is positively charged. At pH 8.6, most of the drug is uncharged. In niosomes prepared from Tween® 20 and CHO, amount of lidocaine entrapped at pH 8.6 was negligible for all formulations, whereas the best drug entrapment was obtained at pH 5.5. However, for charged vesicles containing DCP or N-cetylpyridinium chloride,

remarkably low entrapment was obtained in all cases, even at pH 5.5 regardless of the chemical form of lidocaine (Carafa et al 2002).

Hao et al. (2002) compared drug entrapment between 5-fluorouracil and colchicine in Span® 60 niosomes prepared by evaporation-sonication method. They reported that the encapsulation efficiencies of 5-fluorouracil and colchicines were similar. The authors concluded that this finding could be due to the difference in the interaction between drug and membrane. Although 5-fluorouracil has a small molecular weight, it possesses two amides while large molecular weight colchicine has only one amido group. Therefore, interaction between 5-fluorouracil and membrane is stronger than that of colchicine. Considering the molecular weight and interaction between drugs and membrane, the entrapment efficiency of 5-fluorouracil and colchicines were similar.

The encapsulated drug can be the major determinant of niosome stability in terms of drug leakage. A drug polymer conjugate, PK1, remained entrapped within the vesicles for a relatively long period of time. The entrapped polymer was thought to lead a more stable system since the membrane was sufficiency impermeable to the macromolecule (Gianasi et al., 1997). The physical nature of the entrapped molecules also affects stability of the system. Doxorubucin loaded vesicles using an ammonium sulfate gradient method led to gel formation within the vesicles, resulting in less leaky niosomes (Haran et al., 1993).

6. Effect of temperature

The temperatures affecting the properties of niosomes are both temperature of preparation and temperature of storage. As mentioned earlier, the temperature of preparation must be over the phase transition temperature of the system. Hao et al. (2002) found that the colchicine content loaded in Span® 60 niosomes prepared at room temperature was less that that at 60 °C. The temperature of storage may affect noisomes stability. Thus, storage temperature must be controlled. Changes in the temperature of the

system often lead to a change in fundamental nature of the system, including drug leakage.

Niosomes as a Topical Drug Delivery System

As with liposomes, niosomes have been studied as potential carriers for topical skin delivery. Both hydrophilic and hydrophobic drugs/compounds can be incorporated in niosomal vesicles. The advantages of niosomes over liposomes are as follows: good chemical stability, relatively low cost, ease of preparation and storage, preparation without organic solvent possible, and a large number of non-ionic surfactant available, etc. There are some reports on comparison of drug permeation between liposomes and niosomes. Drug penetration from niosomes is evident to be superior to that from liposomes. These drugs are cyclosporine-A (Dowton et al., 1993; Waranuch et al., 1997; Waranuch et al., 1998), α-interferon (Waranuch et al., 1998), enoxacin (Fang, Hong et al., 2001), lidocaine and lidocaine hydrochloride (Carafa et al., 2002), and cimetidine (Lieb et al., 1994).

Niosomes have been reported to decrease side effect, give sustained release and enhance penetration of many drugs through skin. Niemiec et al. (1995) reported that niosomes enhanced topical delivery of peptide drugs into pilosebaceous units in the hamster ear model. Once the drug gets into the pilosebaceous units, it can freely diffuse to the viable epidermis, depending on the partition coefficient of the drug. There are a numbers of previous reports on investigation of enhancement of skin delivery of both hydrophilic and hydrophobic drugs including mechanism of action of this enhancement.

Many hydrophobic drugs have been successfully encapsulated in niosomes for topical applications such as estradiol (Hofland et al., 1994; Megrab, Williams, and Barry, 1995), cyclosporine-A (Dowton et al., 1993; Niemiec et al., 1995; Waranuch et al., 1997; Waranuch et al., 1998), erythromycin (Jayaraman et al., 1996), cimetidine (Lieb et al., 1994), dithranol (Agarwal et al., 2001), lidocaine (Carafa et al., 2002), growth hormone releasing peptide (Fleisher et al., 1995), enoxacin (Fang, Hong et al., 2001), pergolide

(Honeywell-Nguyen, Frederik et al., 2002; Honeywell-Nguyen, Bouwstra et al., 2003; Honeywell-Nguyen, Groenink et al., 2003), rotigotine (Honeywell-Nguyen, Arenja et al., 2003), retinoic acid (Montenegro et al., 1996), tretinoin (Manconi et al., 2002; Manconi et al., 2003; Sinico et al., 2005; Manconi et al., 2006), flurbiprofen and piroxicam (Reddy and Udupa, 1993), all-trans retinoic acid (Desai and Finlay, 2002), etc. Some hydrophilic drugs also have been encapsulated in niosomes such as alpha-interferon (Niemiec et al, 1995), diclofenac sodium (Naresh et al, 1994), lidocaine hydrochloride (Crafa et al., 2002), glycolic acid and glycerol (Ohta et al., 2002). However, niosomes of lyophobic drugs such as PTU have not been studied. In conclusion, the effects of niosomes on drug transport through the skin can be either impairment or enhancement, depending on the drug and the vesicle composition. In addition, niosomes appear to have potential as a drug carrier system for both dermal and transdermal delivery of drugs (Hofland et al., 1994).

Factors Affecting Drug Permeation into/through the Skin

Although it has been accepted that the use of vesicles with proper composition should increase skin drug delivery, many questions arise about factors that can affect skin delivery of these formulations. If one knows about these factors, one can formulate the proper formulations that increase maximum or optimum drug permeation. Therefore, there are many researchers who investigated, both in vivo and in vitro, factors affecting drug permeation across the skin from both liposomes and niosomes. Some factors that have been studied are the thermodynamic state, size and lamellarity, melting point of lipid components and the existence of vesicular structure.

1. Thermodynamic state

After the introduction of liposomes as drug delivery systems for transdermal drug delivery, Knepp et al. (1990) studied release and permeation of progesterone from various liposomes suspended in agarose gel across hairless mouse skin compared to those from agarose gel alone. Two types of liposome studied were composed of egg

phosphatidylcholine (EPC) and dipalmitoyl phosphatidylcholine (DPPC). They reported that progesterone released from an agarose gel alone was very fast compared to that from liposomes embedded in the agarose gel and that liposomes, compared to agarose gel, reduced progesterone permeation rate. They also found that the gel state liposomes (DPPC) resulted in a lower skin permeation rate than that of the liquid crystalline liposomes (EPC). In addition, they also reported that transdermal delivery of progesterone from vesicles containing cis-unsaturated phospholipids (i.e., EPC and dioleoyl phosphatidylcholine (DOPC)), which were in the liquid crystalline state, was faster than that from vesicles consisting of saturated acyl chain phospholipids (i.e., DPPC and dimyristoyl phosphatidylcholine (DMPC)), which were in the gel state.

However, Yu and Liao (1996) reported that permeation of triamcinolone acetonide from liposomes across rat skin was higher than that from commercial ointment. They also reported that the vesicle size, charge and CHO did not affect drug permeation. Permeation of triamcinolone from PC liposomes was significant higher than that from DPPC liposomes. Furthermore, liposomes consisting of skin lipid composition in gel state significantly enhanced drug permeation better than other types of liposomes due to its optimum miscibility with skin lipid. Similar results were observed in the study of Fresta and Puglisi (1997) who reported that the blanching effect of corticosteroid from skin lipid liposomes was more pronounced than that from phospholipids vesicles and skin lipid liposomes gave the highest drug deposition within the deeper skin layers. In another study, unfractionated heparin penetration into epidermis was only detected for Phospholipon® 80 (PL80) liposomes. The extent of low molecular weight heparin penetration was independent of the formulations. However, PL80 liposomes accumulated in deeper epidermal layers as compared to the aqueous formulation (Betz, Nowbakht et al., 2001).

From the above in vitro studies, drug permeation from gel state liposomes seems to be lower than that from liquid crystalline state liposomes. This finding has been confirmed by in vivo studies. Ogiso et al. (1995) investigated betahistine permeation across rat skin in vivo from various gel formulations consisting of EPC-lipid disperse

systems (LDS) and hydrogenated soya PC-LDS (HSPC-LDS). They reported that the plasma concentrations of betahistine from gel containing EPC-LDS and D-limonene were higher than those from HSPC-LDS and D-limonene. They also found that the fluidity of the stratum corneum lipids was dramatically increased following the treatment with the fluid EPC-LDS, whereas the fluidity was significantly decreased by the solid HSPC-LDS. Similar result was reported by Perez-Cullell et al. (2000) who investigated the penetration of fluorescein and its sodium salt in Caucasian volunteers from gel state liposome (HPC) and liquid crystalline state liposomes (PC) using stripping method.

Hofland et al. (1994) studied the in vitro permeation of estradiol from niosomes in various thermodynamic states through human strtum corneum. The gel state niosomes were composed of $C_{18}EO_3$ and the liquid crystalline vesicles consisted of $C_{12}EO_3$ and Brij® 96 ($C_{9=9}EO_{10}$). All formulations were saturated with estradiol making the thermodynamic activity equal among these formulations. They reported that the fluxes of the two liquid crystalline niosomes were not significantly different and the gel state niosomes showed no effect on estradiol penetration compared to the control.

On the other hand, El Maghraby et al. (2001) studied the permeation of 5-fluorouracil from an ultradeformable (PC:sodium cholate) and two standard (PC and DPPC) liposome formulations across human epidermis. They found that 5-fluorouracil penetration from ultradeformable liposomes was the highest and drug permeation from PC liposomes was higher than the permeation from DPPC liposomes at 12 hours but permeation of 5-fluorouracil from both liposomes was not different at 36 hours. This result is in accordance with other studies on some hydrophilic drugs, for example, low molecular weight heparin sodium (Betz, Nowbakht et al., 2001), methotrexate (Trotta et al., 2004), and sodium ascorbyl phosphate (Foco et al., 2005).

Trotta et al. (2004) studied the permeation of methotrexate from deformable liposomes containing PC and hydrogenated PC (HPC) using dipotassium glycyrrhizinate (KG) as a surfactant. They reported that the methotrexate amount permeated across pig skin from liposomes containing KG was 3 to 4 fold higher than that from solution or

normal liposomes and no significant differences were observed between KG containing PC liposomes and KG containing HPC liposomes. In another study, permeation of sodium ascorbyl phosphate through pig ear epidermis from non-hydrogenated soybean lecithin and hydrogenated soybean lecithin was not significantly different (Foco et al., 2005).

From these previous studies of liposomes, it may be concluded that for hydrophobic drugs, permeation from liquid crystalline state is higher than that from gel state vesicles across both animal and human skins, whereas no difference in permeation of hydrophilic drugs between gel state and liquid crystalline state vesicles is evident.

Cevc and Blume (1992) claim an intact vesicle penetration through the skin with the Transfersomes®. Transfersomes® are composed of phospholipids and edge activator. An edge activator is a single chain surfactant, such as sodium cholate or sodium deoxycholate, that destabilizes the lipid bilayer of the vesicles and increases the deformability of the bilayers. Transfersomes® have been successfully used as drug carriers for many drugs, for example, insulin (Cevc et al., 1995; Cevc et al., 1998) and corticosteroid (Cevc et al., 1997). El Maghraby et al. (2000b) studied the transport of estradiol across human skin in vitro from ultradeformable liposomes containing PC with Tween® 80 and Span® 80 as the edge activators compared to those with sodium cholate. They reported that ultradeformable liposomes increased estradiol fluxes more than those of traditional liposomes. However, the effect was less dramatic than that reported by the group of Cevc. Furthermore, other investigators have confirmed that deformable liposomes were more effective compared to standard liposomes for skin drug delivery. In addition, deformable liposomes deliver estradiol across human skin better than the traditional liposomes (El Maghraby et al., 1999; El Maghraby et al., 2000a). In another study, 5-fluorouracil permeation through human skin from deformable liposomes was also higher than that from rigid liposomes (El Maghraby et al., 2001).

van den Bergh and co-workers (2001) prepared elastic vesicles with sucrose laurate (L-595) and octaoxyethyleneglycol laurate ester (PEG-8-L). They studied the

effect of PEG-8-L content on vesicle elasticity without drug and concluded that increasing PEG-8-L content from 10 to 90 mol% increased elasticity of vesicle bilayers. In addition, Honeywell-Nguyen, Frederik et al. (2002) studied the effect of PEG-8-L content on vesicle morphology, stability and elasticity in the presence of the drug pergolide and reported that increase in PEG-8-L content from 0 to 50 mol% increased vesicle elasticity, changed vesicle shape and decreased stability of the vesicles.

Some researchers investigated drug permeation from elastic vesicles containing L-595 in combination with PEG-8-L compared to that of rigid vesicles and micelles. Honeywell-Nguyen, Frederik et al. (2002) investigated the effect of elastic and rigid vesicles on permeation of pergolide across human skin under non-occlusive condition. A series of L-595:PEG-8-L vesicles, ranging from very rigid (at 100:0 molar ratio) to very elastic (at 50:50 molar ratio), were prepared at pH 5.0. They found that elastic vesicles enhanced drug transport compared to the buffer control, whereas rigid vesicles decreased drug transport. This result was in accordance with the study of Honeywell-Nguyen, Arenja et al. (2003) who investigated the permeation of rotigotine across human skin from the same series of elastic vesicles as in the previous study (Honeywell-Nguyen, Frederik et al., 2002). Rotigotine is a lipophilic drug with a pKa of 7.9. Thus, they prepared a series of vesicles using pH 5.0 and 9.0. At pH 9.0 rotigotine was expected to be highly associated to the vesicle bilayers. In contrast, rotigotine was expected to be mainly in the aqueous phase of the vesicles at pH 5.0. They reported that the very elastic vesicles (L-595:PEG-8-L at 50:50 molar ratio) was the most effective formulation. However, they found that the rigid vesicles also increased drug transport compared to the buffer control. Moreover, they reported that the vesicles prepared at pH 9.0 increased drug permeation by the factor of 80 as compared to the buffer solution while the vesicles prepared at pH 5.0 were not significantly different from solution. Therefore, they concluded that it is essential that drug molecules are highly associated to the vesicle bilayers in order to penetrate into the skin. Similar results on the effect of pH were confirmed by Honeywell-Nguyen and Bouwstra (2003). They studied the effect of pH at pH values of 5.0, 6.0 and 7.0 on permeation of pergolide (pKa 5-6) from L-595:PEG-8-L:sodium sulfosuccinate (50:50:5) niosomes across human skin. They found that pH 5.0

gave the highest drug incorporation and highest drug transport since pergolide is mostly positively charged at pH 5.0.

Besides in vitro and in vivo studies regarding the effect of thermodynamic state on drug permeation through the skin, there are many studies investigating further the vesicles-skin interaction using various methods such as electron microscopy (van den Bergh, Vroom et al., 1999), two-photon excitation microscopy (van den Bergh, Vroom et al., 1999), TEM (van den Bergh, Bouwstra et al., 1999), FFEM (van den Bergh, de Vries, and Bouwstra, 1998; Honeywell-Nguyen, de Graaff et al., 2002), CLSM (Zellmer, Pfeil, and Lasch, 1995; Kirjavainen et al., 1996; van Kuijk, Janssen et al., 1998 van Kuijk, Junginger et al., 1998; van Kuijk, Mougin et al., 1998; Betz, Imboden, and Imanidis, 2001), and double label CLSM (Cevc, Schatzelein, and Richardsen, 2002), microdutoradiographic images (Waranuch et al., 1998). One of the first studies in which vesicles-skin interaction was performed with isolated human stratum corneum (Junginger, Hofland and Bouwstra, 1991). The stratum corneum was incubated for 48 hours with vesicles-A mostly consisting of PC up to 80% and vesicles-B mostly consisting of 28% PC. Vesicles-A had a very strong effect on the microstructure of human stratum corneum, whereas there was no interaction between the vesicles-B and the skin lipids and no vesicular structures could be detected within the stratum corneum.

In another in vitro study, the effect of lipid composition of various liposomes labeled with fluorescent lipid bilayer markers on stratum coeneum organization using confocal scanning electron microscope (CLSM) was studied under occluded condition (Kirjavainen et al., 1996). They concluded that dioleyl phosphatidylcholine-liposomes were able to penetrate deeper into the stratum than other liposomes and negative charge, CHO inclusion and chain length of phospholipids had no effect on the stratum coeneum structure.

After the above study, some researchers studied this effect using CLSM under occlusive conditions and reported similar results. Gel state vesicles aggregate, fuse and adhere on the stratum corneum surface, thereby depositing stacks of lamellar sheets and

forming lipid bilayer networks and can not induced ultrastructural changes in the skin while liquid crystalline state vesicles might act not only in the stratum corneum surface, but may also induced ultrastructural changes in the deeper layers of the stratum corneum. For example, Betz, Imboden et al. (2001) found the distribution of phopholipid in the lipid matrix of the stratum coeneum surrounding the corneocytes from the liquid crystalline state Phospholipon® liposomes but not from the gel state liposomes containing sphingomyelin.

In addition, van den Bergh et al. (1998) showed by FFEM that gel state liposomes (DSPC and ceramide liposomes) aggregated, fused and adhered on the stratum corneum surface, thereby depositing stacks of lamellar sheets and forming lipid bilayer networks. On the contrary, liquid crystalline vesicles (DLPC) did not aggreagate nor fuse on the surface but induced interaction with intercellular lipids in deeper layers of the stratum corneum.

Similar results were also observed under non-occlusive conditions from the study of Zellmer et al. (1995) who found that after 18 hours of application, the fluorescent label of dimyristoyl phosphatidylcholine (DMPC) and distearyl phosphatidylcholine (DSPC) vesicles applied on human cadaver skin remained on the top of the skin but DMPC vesicles showed interaction with stratum corneum as evident by the results of DSC method. On the other hand, for dilauryl phosphatidylcholine (DLPC):$C_{12}EO_7$ vesicles, fluorescent label was detected mainly in the stratum coeneum after 1 and 3 hours of application and in the dermis after 6 hours (van Kuijk, Janssen et al., 1998). A similar result seen with DLPC:$C_{12}EO_7$ vesicles was observed in the in vitro study of van Kuijk, Junginger et al. (1998) using human skin and in the in vivo study of van Kuijk, Mougin et al. (1998) using rat skin. In addition, these authors also reported that the label applied in micelles and in gel state vesicles (DSPC) did not penetrate as deep as that applied in liquid crystalline state. The liquid crystalline state vesicles with flexible bilayer (DLPC:$C_{12}EO_7$) showed the highest fluorescence intensity in the dermis. Moreover, Kirjavainen et al. (1999) evaluated the interaction of vesicles-skin using CLSM and reported that EPC liposomes prepared from aqueous solution did

not penetrate into the human skin while EPC prepared from ethanolic solutions penetrated deeply into the stratum corneum.

The above studies suggest that components of liquid crystalline state vesicles can enter the deeper layers of the stratum corneum and can modify the intercellular lipid lamellae. Vesicles-skin interaction is the most possible explanation for their more effectiveness of vesicles in the liquid crystalline state in enhancing drug transportation into and through the skin.

The effect of elasticity of vesicles prepared from L-595:PEG-8-L on either penetration pathway or ultrastructure of the skin was investigated in several studies. The vesicles were applied onto human and animal skin both in vitro and in vivo (van den Bergh, Vroom et al., 1999; van den Bergh, Bouwstra et al., 1999; Honeywell-Nguyen, de Graaff et al., 2002). van den Bergh, Vroom et al. (1999) used TEM, FFEM and two-photon fluorescence microscopy (TPE) to study the interaction of the vesicles and human skin in vitro. They found that treatment skin with gel state vesicle (Wasag-7:CHO) revealed no ultrastructural changes in the stratum corneum while pretreatment with rigid vesicles (L-595:CHO) showed the presence of vesicle materials on the skin surface and lipid materials with electron dense spot between the upper 2-3 cell layers of the skin were detected. Four types of interactions were observed after treatment with elastic vesicles (L-595:PEG-8-L): 1) the presence of spherical lipid structures containing or surrounded by electron dense spots, indicating for the presence of vesicle materials both on the skin surface and between the upper 2-3 cell layers was evident, 2) oligolamellar vesicles and bilayer stacks were detected between the upper 3-4 cell layers, 3) large areas containing lipids, surfactants, electron spots and vesicle bilayers were observed deeper down into the stratum corneum, but no intact vesicles were observed, and 4) small stacks of bilayers in intercellular space of the skin were found. Furthermore, TPE showed tread-like channels within the entire stratum corneum. The similar results, especially the ultrastructural changes, formation of lamellar stacks and tread-like channels within the entire stratum corneum, were observed in hairless mouse skin treated with elastic vesicles (van den Bergh, Bouwstra et al., 1999).

In another study, the in vivo and in vitro interactions between elastic-, rigid vesicles and micelles with human skin were studied using tape stripping in combination with FFEM (Honeywell-Nguyen, de Graaff et al., 2002). A fast penetration of intact elastic vesicles into the deeper layer of stratum corneum, where these vesicles accumulated in channel-like regions was evident. No ultrastructural change was found in skin treated with rigid vesicles. Treatment with micelles resulted in rough, irregular fracture planes. No differences were found in the structure of the stratum corneum between treatment in vivo and in vitro. Also, there was no evidence to suggest that elastic material could penetrate beyond the stratum corneum in to the viable epidermis in large quantity.

In a recent study, tape stripping in combination with freeze-fracture electron microscopy method was used to investigated the in vivo interaction of L-595:PEG-8-L elastic vesicles with human skin by Honeywell-Nguyen, Groenink et al. (2003). They found a fast penetration of intact elastic vesicles into the stratum corneum via channel-like regions after 1 hour non-occlusive treatment and found intact vesicles in the ninth tape-strip and in the 15th tape-strip after 4 hours. A higher volume of application increased the presence of vesicle materials found in the deeper layers of the stratum corneum.

From the above mentioned studies, it seems very clear that the thermodynamic state of the bilayers of the vesicle plays an important role in the effect of vesicles on drug permeation across the skin both in vivo and in vitro.

2. Vesicle size

It was expected that smaller vesicle size would increase drug deposition if the intact vesicles cross the stratum coeneum. du Plessis et al. (1994) showed that smaller size (60 nm) liposomes prepared from PC did not result in higher levels of radiolabelled cyclosporine-A and cholesteryl sulfate in the deeper skin layer of hairless mouse, hamster and pig skin. However, they found that intermediate size (300 nm) liposomes

gave both the highest reservoir in the deeper skin layer of hairless mouse and hamster skin and the highest drug concentration in the receiver in hairless mouse and hamster skin and concluded that the follicular route played an important role in drug transfer. Similarly, Hofland et al. (1994) also found that MLVs (> 1 μm) prepared from $C_{9=9}EO_{10}$ gave higher fluxes of estradiol across human skin than SUVs did (100-200 nm).

On the other hand, Yu and Liao (1996) reported that the size of MLVs liposomes prepared from DPPC or PC did not show significant difference in permeation and retention of triamcinolone acetonide. In another study, the influence of liposome and niosome size on the penetration of hydrophilic substances across pig ear skin using electron spin resonance method was investigated (Sentjurc et al., 1999). Liposomes composed of DPPC or soya lecithin or hydrogenated soya lecithin and niosomes consisting of glyceryl distearate or PEG stearate were used. They reported that transport into the skin did not depend on the vesicle size and the transport was significantly decreased for small vesicles (< 200 nm) since they disintegrated immediately upon contact with other surface.

However, Verma et al. (2003) showed the effect of vesicle size on the permeation of hydrophilic and lipophilic fluorescent compounds from deformable vesicles consisting of PC and sodium cholate across human skin using stripping and CLSM method. The results revealed that penetration of hydrophilic and lipophilic fluorescent compounds was inversely related to the size of liposomes.

From the previous findings, the effect of size seems to depend on lipid composition of the vesicles and physiocchemical properties of the drug. Since intact vesicles do not penetrate across the skin, the physical parameters such as size and lamellarity of the vesicles are less important than the thermodynamic state of the bilayers.

3. Melting point of lipid compositions

Some researchers investigated various drug permeation from nonionic liposomes prepared from GDS:CHO:Brij® 76 and GDL:CHO:Brij® 76 and they found that GDL liposomes gave higher drug permeation into the skin than GDS liposomes. These drugs are cyclosporin-A (Dowton et al., 1993; Niemiec et al., 1995), alpha-interferon (Niemiec et al., 1995), growth hormone releasing peptide (Fleisher et al., 1995), and glycolic acid and glycerol (Ohta et al., 1996). Penetration of cimetidine and erythromycin from GDL vesicles seem to be superior to other dosage forms both in vivo and in vitro (Margalit et al., 1992; Jayaraman et al., 1996).

Weiner et al. (1994) purposed a hypothesis that after topical application under non-occlusive condition, GDL and GDS liposomes undergo gradual dehydration. When the temperature of the dehydrating formulation exceeds the melting point of GDL (30 °C), the lipid component melts and releases of both free GDL and Brij® 76 which are known skin penetration enhancers. On the other hand, GDS has a melting point of 54 °C, thus GDS does not melt and release of GDS and Brij® 76 does not occur. Thus, permeation from GDL liposomes is better than that from GDS liposomes. Therefore, the factors affecting dehydration process are phase transition temperature of lipid and the presence of components that affect bilayer packing (CHO) or humectants. Ohta et al. (1996) investigated the effect of glycerol on deposition of glycolic acid from GDS and GDL liposomes in hairless mouse skin both in vivo and in vitro. They found that the deposition of glycolic acid was decreased when glycerol was included in GDL liposomes while the deposition from GDS liposomes was unaffected. The addition of a humectant such as glycerol to GDL liposomes delays melting of the lipid components by slowing down dehydration of the formulation. In addition, Waranuch et al. (1997) studied the effect of GDL:Brij® 76 ratios (CHO being fixed at 15% w/w) on topical delivery of cyclosporine-A using hairless mouse skin. They reported that increasing GDL content increased the rate and extent of cyclosporine-A and the rate and extent of the drug was highest between GDL and Brij® 76 ratio of about 1 to 1.5. They also proposed the mechanism of action of GDL liposomes that after dehydration, lipid melts and the melt

containing the dissolved cyclosporine-A fills the follicles and is transported into the deeper skin layer. Thus, the rate and extent of cyclosporine-A would be inversely proportional to the melting point of the lipid mixture.

Moreover, Waranuch et al. (1998) investigated the proposed mechanism of action of GDL liposomes mentioned in the previous study (Waranuch et al., 1997). They showed that cyclosporine-A uptake rate depended on GDL:Brij® 76 ratios and decreased as the melting point of lipid mixtures increased. Cyclosporine-A uptake was maximum at the ratio of 45:40 w/w which was corresponding to the lowest melting point (23 °C). They also showed that the kinetic profile for cyclosporine-A transport into and across living skin layer of hairless mouse from liposomes was similar to lipid melts. Thus, vesicles consisted of lipid components with lower melting point than the skin temperature would provide higher drug permeation than those with higher melting point than the skin temperature.

4. The existence of vesicular structure

Several studies have shown that the presence of vesicular structure is important for liposomes and niosomes to enhance skin delivery. Ganesan et al. (1984) found that permeation of hydrocortisone from liposomes was higher than that from solution of free drug, whereas addition of empty liposomes to free hydrocortisone in normal saline had no effect on the permeation of the drug. It indicates that entrapment of the drug by liposomes is an important factor. In addition, the investigation on the effect of skin pretreatment with empty vesicles on skin drug delivery showed that the enhancement ratio was less than that obtained from direct application of the same vesicles (Hofland et al., 1994; El Maghraby et al., 1999; Honeywell-Nguyen, Arenja et al., 2003; Honeywell-Nguyen and Bouwstra, 2003). Therefore, the effect of the existence of vesicular structure on drug permeation was investigated. El Maghraby et al. (2000a) investigated the importance of liposome structure in permeation of oestradiol across the human skin by comparing drug permeation from lipid solution in 90% w/w propylene glycol with that from corresponding liposomes. They studied four formulas of

liposomes: PC, PC with sodium cholate, PC with Span® 80, and PC with oleic acid. They found that the vesicular forms of all four formulas tested gave higher relative fluxes than the corresponding lipid solutions. Similarly, Fang, Hong et al. (2001) studied the transport of enoxacin across the nude mouse skin from Soybean PC liposomes and Span® 60 niosomes. They reported that Soybean PC liposomes did not gave higher permeation than Soybean PC physical mixtures but Span® 60 niosomes gave higher permeation than Span® 60 physical mixtures. A similar result was observed in the study of Carafa et al. (2002). They showed that the permeation rates of lidocaine and lidocaine hydrochloride from the drug dispersions in the presence of surfactant (Tween® 20:CHO) or PC in micellar form were quite lower than those from vesicles with similar compositions.

Mechanism of Action of the Vesicles

There are many approaches to maximize permeation of drugs into and across the skin, for example, modification of vehicle-drug interaction, to use of the vesicles and other particulate systems, modification of the stratum corneum, to bypass or removal the stratum corneum, and to use of the electricity (Barry, 2001). One of the most controversial methods is the use of the vesicles and particulate systems. There are a number of articles in which liposomes have increased permeation of both hydrophilic and hydrophobic drugs (Lieb et al., 1994; Niemeic et al., 1995; Waranuch et al., 1997; Betz, Nowbakht et al., 2001; El Maghraby et al., 2000a; Agarwal et al., 2001; Fang, Hong et al., 2001; Carafa et al., 2002). Similar to liposomes, niosomes improve transport of many drugs (Reddy and Udupa, 1993; Hofland et al., 1994; Lieb et al., 1994; Niemeic et al., 1995; Ohta et al., 1996; Jayaraman et al., 1996; Waranuch et al., 1997; Agarwal et al., 2001; Fang, Hong et al., 2001; Carafa et al., 2002). Although it has been accepted that vesicles increase skin drug delivery, mechanism of action is not clear. There are many studies focusing on elucidation of mechanism of action of vesicles and many possible mechanisms of action have been proposed.

Ganesan et al. (1984) and Ho et al. (1985) showed that neither liposomes nor phospholipid molecules diffused through intact skin of hairless mouse. They suggested

three probable mechanisms (Figure 1): 1) release of the drug from liposomes and percutaneous absorption of the free drug; 2) release of the drug from the vesicles in combination with skin permeation of free drug and also direct liposome/skin drug transfer; and 3) skin permeation involving liposome/skin drug transfer. They also concluded that the first mechanism is applied to glucose, a hydrophilic drug entrapped in the aqueous phase of the liposomes, since permeation of glucose depended on release rate. For progesterone, a lipophilic drug associated in lipid bilayers, the in vitro release rate was very slow so they suggested a direct liposome/skin transfer mechanism. Release rate of hydrocortisone was higher than that of progesterone while their permeation was comparable. Thus, they suggested that both free drug and liposome/skin transfer mechanisms operated on hydrocortisone permeation.

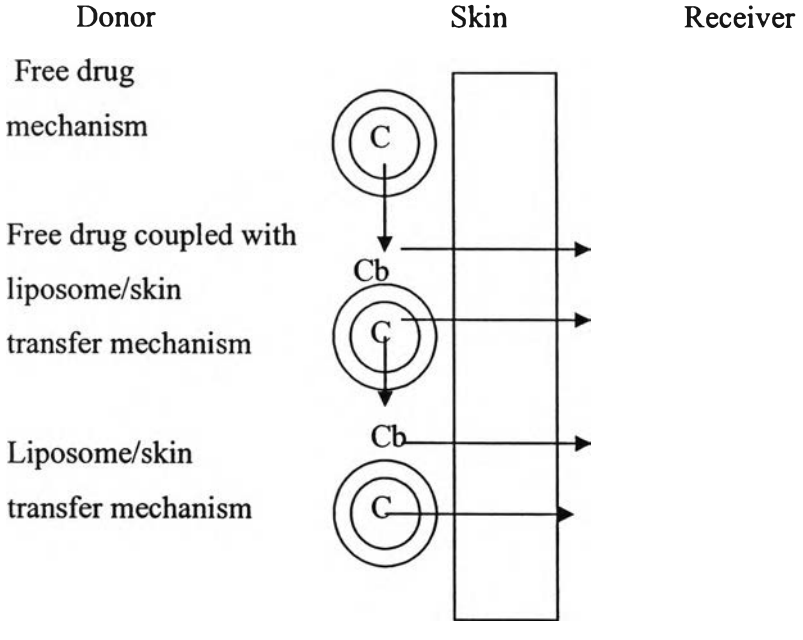

Figure1 Schematic description of various mechanisms in the skin drug permeation of liposomes (From Ganesan et al., 1984)

If the mechanism of action of the vesicles is solely the free drug mechanism whereby the drug is released from the vesicles and then freely permeates the skin, the rate limiting step of skin transport is the release rate. However, there are some previous reports on the lack of relationship between drug release and permeation. For example,

Montenegro et al. (1996) compared the release of retinoic acid from DPPC liposomes with permeation across human skin. They reported that retinoic acid release was not significantly affected by the inclusion of a positively charged compound, stearylamine, in liposomes, whereas positively charged liposomes provided greater drug permeation compared to other liposomes. Similarly, release of enoxacin (Fang, Hong et al., 2001) and lidocaine and lidocaine hydrochloride (Carafa et al., 2002) did not correlate with drug permeation. Furthermore, El Maghraby et al. (1999) found that the peak flux of estradiol from deformable and traditional liposomes through human skin occurred at a time during which drug release was negligible and they concluded that the free drug mechanism did not operate for all liposomal formulations.

One of the rationales for the use of vesicles as topical drug carries is that they may serve as organic solvent for the solubilization of poorly soluble drugs, resulting in higher local drug concentrations. Thus, vesicles with high entrapment efficiency would provide higher drug permeation than vesicles with low drug entrapment. Hofland et al. (1999) studied estradiol permeation from niosomes containing $C_{9=9}EO_{10}$ and found that MLV and SUV niosomes had similar drug entrapment but drug permeation across the human skin from MLV niosomes was higher than that from SUV niosomes. Similar results were also observed by El Maghraby et al. (1999) who investigated mechanism of skin delivery of estradiol from deformable and traditional liposomes using human stratum corneum. They also found no correlation between entrapment efficiency and relative flux in all deformable and standard liposomal formulas. In addition, Fang, Hong et al. (2001) also reported that transport of enoxacin from both niosomes prepared from Span® 40 and Span® 60 and liposomes prepared from PC and DMPC across nude mouse skin did not directly relate with drug encapsulation. Similarly, liposomes containing the same drug loading gave different permeation rate of tretinoin across newborn pig skin and drug permeation depended on phase transition temperature of the main liposomal components rather thandrug entrapment efficiency (Sinico et al., 2005). These lines of evidence do not support the solubilization mechanism.

Another rationale for the use of vesicles as topical drug carries is that they may serve as penetration enhancer by penetration of individual phospholipid or non-ionic surfactant molecules into the lipid bilayers of the skin and thus facilitating drug delivery. Many researchers investigated this mechanism by comparing drug permeation from saturation solution after pretreatment of the skin with empty vesicles with that from the drug loaded vesicles. If drug permeation from both conditions is not different, the mechanism of action of the vesicles is consistent with penetration enhancement of the lipid components.

Pretreatment of human strtum corneum with empty niosomes provides much lower permeation of estradiol than with direct application of the drug loaded vesicles (Hofland et al., 1994). Besides, direct contact between estradiol niosomes and the skin is imperative to exert the highest effect on drug transport. In addition, permeation of estradiol from traditional and deformable liposomes through human skin is superior to pretreatment of the skin with empty vesicles (El Maghraby et al., 1999). Similary, when L595:PEG-8-L elastic vesicles were used for enhancing permeation of rotigotine and pergolide, direct application of L595:PEG-8-L vesicles enhanced drug permeation across human skin higher than pretreatment with empty vesicles (Honeywell-Nguyen, Arenja et al., 2003; Honeywell-Nguyen and Bouwstra (2003). These findings indicate that, for maximum penetration, drugs should be encapsulated in the vesicles and that penetration enhancement from vesicle components might not be the major mechanism for liposomes and niosomes.

El Maghraby et al. (1999) also investigated other mechanisms such as the mechanism in which liposomes improve drug uptake in skin and the possibility that intact vesicles penetrate through the skin. They studied improvement of estradiol uptake in the skin by determining estradiol uptake in the human stratum corneum after dipping the skin into different deformable and traditional liposome formulations and aqueous solution for 10 mintues. They reported that uptake ratios for vesicles and solution were no significant different among individual formulations. They also concluded that one possible mechanism was a process of adhesion of liposomes on to the skin, and fusion or

mixing of liposomes with the skin lipid. The possibility that intact vesicles penetrated through the skin was also studied, assuming that this permeation depended on the vesicle size, by comparing drug permeation from large MLVs (at least 557 nm in diameter) with that from SUVs (mean size 136 nm). No significant differences between MLVs and SUVs were found, suggesting that intact vesicles did not penetrate across the human skin in vitro.

For deformable liposomes and elastic vesicles, Cevc and Bloom (1992) suggested that water gradient was an important driving force for drug diffusion from these vesicles. They have shown that transdermal lipid transport may occur spontaneously provided that the deformable vesicles, Transfersomes®, are exposed to a dehydration force resulting from an osmotic gradient between the skin surface and the deeper skin tissue. Therefore, it has been suggested that deformable and elastic vesicles are more efficient under non-occlusive condition than under occlusive condition.

There are several previous studies on drug permeation enhancement effect of deformable liposomes (El Maghraby et al., 1999; El Maghraby et al., 2000a; El Maghraby et al., 2000b; El Maghraby et al., 2001), and elastic vesicles (Honeywell-Nguyen, Frederik et al., 2002; Honeywell-Nguyen and Bouwstra, 2003; Honeywell-Nguyen, Arenja et al., 2003). These vesicles are superior to traditional vesicles under the non-occlusive condition. In addition, some researchers used non-occlusive condition in investigation of the effect of these elastic vesicles on ultrastructure of the skin (van den Bergh, Vroom et al., 1999; van den Bergh, Bouwstram et al., 1999; Honeywell-Nguyen, de Graaff et al., 2002; Honeywell-Nguyen, Groenink et al (2003). They concluded that elastic vesicles improved skin drug delivery or induced ultrastructural changes of skin under non-occlusive condition.

However, the exact mechanism of elastic vesicles to enhance drug transport is not fully understood. It is not clear that under which conditions elastic vesicles provide the best enhancement of skin drug delivery. Some investigators have studied drug permeation from elastic vesicles under non-occlusive and occlusive conditions. For

deformable liposomes, El Maghraby et al. (2001) investigated human skin delivery of estradiol from ultradeformable liposomes containing soybean PC and edge activators such as sodium cholate, Tween® 80 and Span® 80 and compared with traditional liposomes under occlusive and non-occlusive conditions. They reported that under non-occlusive condition both ultradeformable and traditional liposomes improved estradiol skin delivery but ultradeformable liposomes were superior. Occlusive condition reduced skin delivery of both types of vesicles.

Honeywell-Nguyen and Bouwstra (2003) compared pergolide skin penetration from L-595:PEG-8-L elastic vesicles under occlusive and non-occlusive conditions across human skin. They concluded that non-occlusive condition improved skin delivery of pergolide compared to the buffer control. Occlusion increased drug transport from both vesicles and buffer solution due to the fact that water is an excellent penetration enhancer. However, the action of elastic vesicles themselves was diminished under occlusion. In addition, Honeywell-Nguyen, Groenink et al. (2003) investigated the in vivo interaction of L-595:PEG-8-L elastic vesicles with human skin using the tape stripping technique in combination with freeze-fracture electron microscopy method. They found a fast penetration of intact elastic vesicles into the stratum corneum via channel-like regions after non-occlusive treatment. Although micrographs showed very few intact vesicles in the deeper layers of the stratum corneum, the presence of lipid plaques was frequently observed after occlusive condition.

From the results of the previous reports above, non-occlusion is a suitable condition for the elastic vesicles to enhance drug permeation through the skin. Thus, the possible mechanism for elastic vesicles is that the vesicles improve skin drug delivery by penetrating through the stratum corneum by transepidermal osmotic gradient induced by non-occlusive condition.

In Vitro Permeation Study

1. Theory of drug diffusion (Martin, 1993)

A frequently employed technique to test the relative permeability of topical drug involves the *in vitro* use of excised skin mounted in diffusion chambers. In this case, the release of drug is controlled by membrane and the Fick's first law is applicable because the absorption mechanism is usually a passive diffusion. An equation for an amount (M) of material flowing through a unit cross-section (A) of a barrier in unit time (t) is known as the flux (J). Therefore, the Fick's first law of diffusion is derived as follows:

$$J = \frac{dM}{A.\,dt} \qquad (1)$$

If a membrane separates the two compartments of a diffusion cell with cross-sectional area (A) and thickness (h), and if the concentrations in the membrane on the donor and receptor chamber are C_1 and C_2, respectively, equation (1) may be written as:

$$J = \frac{D(C_1 - C_2)}{h} \qquad (2)$$

in which $(C_1 - C_2)/h$ approximates dC/dx. The gradient $(C_1 - C_2)/h$ within the membrane must be assumed to be constant for a quasi-stationary state to exist. Equation (2) presumes that the aqueous boundary layers on both sides of the membrane do not significantly affect the total transport process.

The concentration C_1 and C_2 within the membrane ordinarly are not known but can be replaced by the partition coefficient (K) multiplied by the concentration in the donor side (C_d) or on the receiver side (C_r). The partition coefficient (K) is given by

$$K = \frac{C_1}{C_d} = \frac{C_2}{C_r} \qquad (3)$$

Therefore, equation (2) can be written as:

$$\frac{dM}{dt} = \frac{DAK(C_d - C_r)}{h} \quad (4)$$

and, if sink conditions hold in the receptor compartment or $C_r = 0$,

$$\frac{dM}{dt} = \frac{DAKC_d}{h} = PAC_d \quad (5)$$

in which

$$P = \frac{DK}{h} \quad (6)$$

where P is a permeability coefficient (cm/sec). Eventually, the amount of drug permeating into a sink bears the following relationship to time:

$$M = PAC_dt \quad (7)$$

Permeation profile (Figure 2) is constructed by plotting the cumulative amount of drug permeating per diffusional area against time. The steady-state flux (Jss) of drug permeation is determined from the slope of the permeation profile. The permeability coefficient (P) can be then obtained from the steady-state flux dividing by C_d, providing that C_d remains relatively constant throughout the time of study.

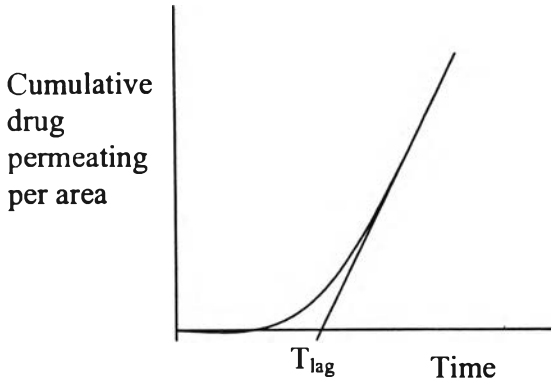

Figure 2 Typical permeation profile for a molecule diffusing across the skin

2. Diffusion cell design (Brain, Walters, and Watkinson, 2002)

Most common methods for evaluation of in vitro permeation use diffusion cells. The major advantage is that the experimental condition can be controlled precisely. The disadvantage is that little information on the metabolism, distribution and effects of blood flow on permeation can be obtained.

Static diffusion cell used is usually of the upright ("Franz"), or side-by-side, with receptor chamber with a side arm sampling port volumes of about 2-10 mL and surface area of exposed membranes of nearly 0.2-2 cm^2. The jacket is positioned around the receiver chamber and heated with an external circulating bath. Excised skin is mounted as a barrier between donor and receptor chambers. The amount of drug permeating from the donor to the receptor side is determined as a function of time. The small volumes are withdrawn from the stirred receiver solution for analysis at predetermined times and the receiver chamber is refilled with receiver solution to keep the volume of solution in the receptor side constant during the experiment. A modified Franz diffusion cell is shown in Figure 3

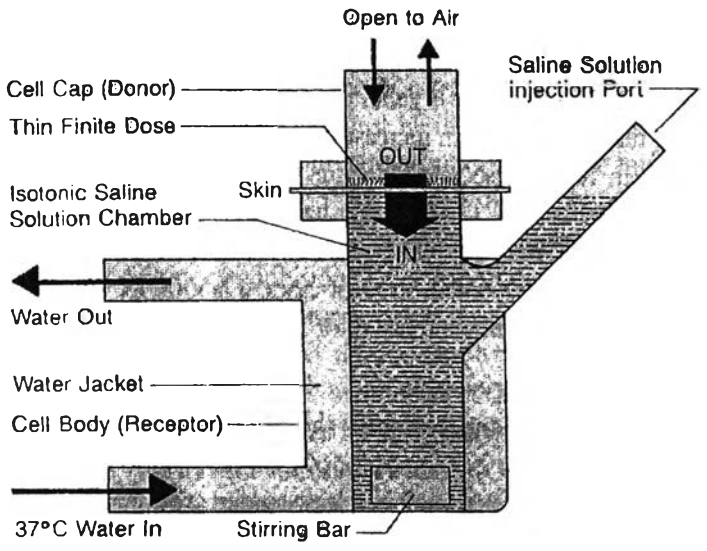

Figure 3 A modified Franz diffusion cell

3. Receptor chamber and medium

The receptor phase can act as a sink, while ensuring that sample dilution does not preclude analysis. As a general rule the drug concentration in the receptor side should not exceed approximately 10% of saturation solution. The most commonly used receptor medium is phosphate buffered saline, pH 7.4. It has been postulated that if drug has a water solubility of less than 10 μg/mL, then the addition of solubilizers becomes necessary.

4. Selection of skin membrane

Animal skin is widely used as a substitute for human skin owing to difficulties in obtaining human skin. The thickness of stratum corneum tends to increase with animal size, for example, the thickness of the stratum coeneum of rats is about 20 μm, whereas that of pig and human is about 30 μm. In addition, the lipid content decreases with size.

From in vivo data on the penetration of several unionized solutes through the skin of a variety of species, the rank order of skin permeability is rabbit > rat > pig > monkey > human. Although the monkey skin seems to be close in skin permeability to human skin, it is not suitable ethically to use as model skin. Therefore, the pig skin should be a suitable skin membrane for in vitro test. The pig skin is frequently used as model membrane for human skin showing a similar penetration for topically applied drugs such as capsaicin and it derivatives (Fang, Wu et al., 1995), captopril (Wu, Huang, and Tsai, 1997), salicylic acid, theophylline, 2,4-dimethylamine, diethyl hexyl phthalic acid, and p-aminobenzoic acid (Wester et al., 1998), terbinafine, clotrimazole, hydrocortisone, and salicylic acid (Schmook, Meingassner and Billich, 2001), and flufenamic acid (Jacobi et al., 2005), etc. In addition, pig skin can be used as a model membrane in iontophoresis studies (Marro, Guy, and Delgado-Charro, 2001).