CHAPTER 1
INTRODUCTION

1.1 Statement and significance of the problem

The evaluation of liposomes started in 1965 has been initially used as models for studying the biological membranes and later considered more frequently as drug carriers. The application of liposomes in skin are interesting and the dermal liposome products have since 1987 been exploited by the cosmetic industries because of the properties of moisturization and the enhancement of penetration through the skin. The principle is that liposome can not only enhance the penetration of the drug into the skin with the slow release of the drug from liposomes but also decrease the clearance of the drug by minimizing the absorption into the bloodstream. There are many successful application of liposomes for topical use. Pharmacodynamic and clinical studies of hydrocortisone and local anesthetic agents entrapped in liposomes showed higher efficacy than the commercially available drug not entrapped in liposomes. The first liposomal drug product named Pevaryl® Lipogel has been proved superior to Pevaryl® Cream in clinical practice.

Amphotericin B is the antifungal drug. It is the drug of choice for systemic fungal infection. Liposomal amphotericin B is now available in parenteral dosage form. There are also the liposomal amphotericin B products for eye or lung application, however, there is none for topical application on skin. Because of the bulky structure of amphotericin B, it can not be absorbed through the skin. Since liposomes can enhance skin absorption, the entrapment of amphotericin B in liposomes should be advantageous. Amphotericin B will be incorporated in the bilayer because of its lipophilic property. This will make the percentage of the entrapment of the drug in liposomes maximum. Thus, amphotericin B entrapped in liposomes should be a reasonable approach for skin application. The liposomal amphotericin B was expected to be advantageous over other topical antifungal drug for the treatment of candida infection because of both the outstanding fungicidal drug activity of AmB as well as the enhancing absorption property of liposomes.

1.2 Objective

The objective of this study was to compare the physical/chemical stability and the absorption through the rat skin of various liposomal amphotericin B formulation (neutral, positive and negative surface charged modified liposomes) with free AmB in solution and powder forms.

1.3 Scope of study

This study is divided into 7 steps which were the followings :

1. Preparation of eight liposome formulations : 1:1, 7:2, 7:2:1(+), 7:2:1(-), 1:1AmB, 7:2AmB, 7:2:1(+)AmB and 7:2:1(-)AmB
2. Characterization of liposome formulations such as the size and size distribution by SEM, the lamellarity by TEM, the charges by Zeta-Meter and the transition temperature and the enthalpy of transition by DSC
3. Qualitative and quantitative analysis of amphotericin B and the drug in Fungizone® and liposome formulations by HPLC
4. Determination of the percentages of the entrapment of amphotericin B in all liposome formulations by HPLC
5. Physical and chemical stability study of amphotericin B entrapped in liposome formulations comparing with the drug in solution and powder form when kept in amber glasses at 4±1°C, 30±1°C and 45±1°C sampling at 0, 5, 20, 40 and 90 days and the degradation rates and shelf life of the drug were determined.
6. Transdermal absorption of amphotericin B entrapped in liposome formulations comparing with AmB in solution and dispersion by the vertical Franz diffusion cells using the full-thickness abdominal skin from the male Wistar rats, 50:50 v/v of ethanol/water as the receiver medium, at 37°C for 24 hrs.
7. The results from this study were collected, assessed, evaluated and discussed.

1.4 Literature reviews

The contents of the literature review were divided to three parts of liposome, amphotericin B and percutaneous absorption.

First, the topic of liposomes which was the reviews of

- Definition
- The formation of lipid bilayers
- The phase transition
- The materials used in liposome preparation
- Types
- The preparation techniques
- Characterization
- Quality control advantages
- Applications

Second, the topic of amphotericin B which was the reviews of

- Physical and chemical properties
- Pharmacology
- The spectrum
- The pharmacokinetic
- Indications
- Preparations
- Stability
- The lipid formulations of amphotericin B

Third, the topic of percutaneous absorption which was the reviews of

- Skin
- Skin permeation techniques *in vitro*
- The mechanisms of liposomal topical drug delivery systems.

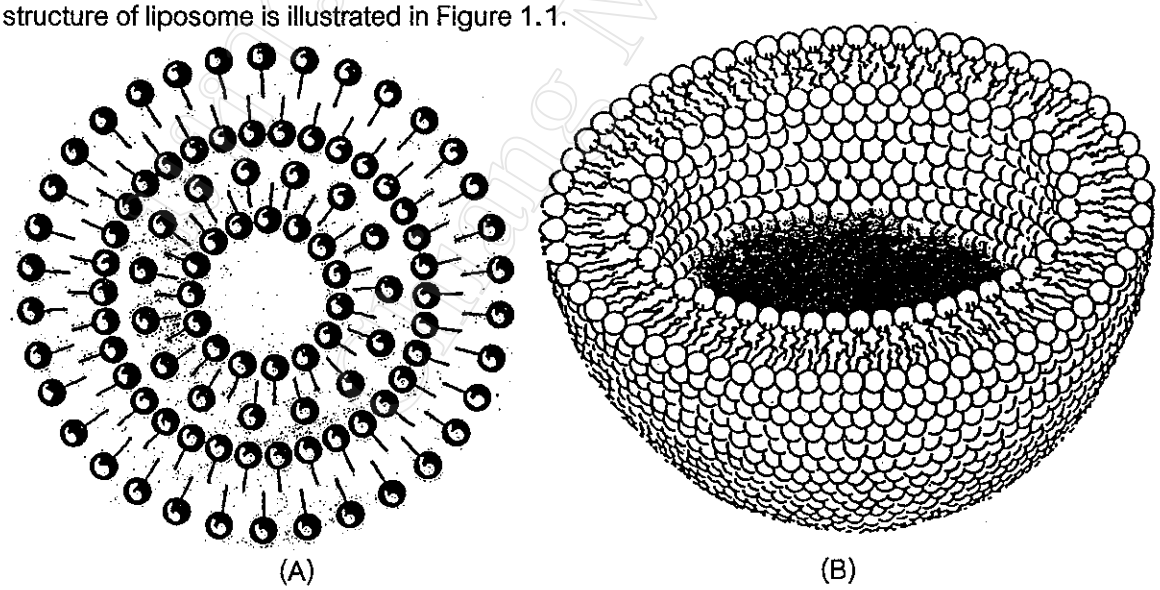
These contents were described as follows.

1.4.1 Liposomes

The application of liposomes as therapeutic devices started in the mid-1950s with the work of Friedman and co-workers. They administered phosphatidyl choline dispersed in the aqueous phase by prolonged ultrasonic radiation (small unilamellar vesicle) intravenously to rabbits. It was found that the treatment was very effective for artherosclerosis. Liposome research started in the mid-1960s by Bangham and co-workers at Babraham in England (Swarbrick and Boylan, 1994). They found that "hand-shaken aqueous phospholipid dispersions" in aqueous phases can spontaneously form microscopic closed vesicles. These vesicles consisted of water surrounded by bilayered phospholipid membranes. Bangham called these tiny fat bubbles "smectic mesophases". They were later named "liposomes" by his colleague Gerald Weissman (Hiemenz and Walsh, 1996).

1. Definition

Liposomes are sealed sacs in the micron or submicron range dispersed in an aqueous environment. The wall of the sacs consists of bilayers composed of suitable amphiphiles. The nature of the bilayers ensures the formation of internal aqueous compartments which can differ from the outside medium (Swarbrick and Boylan, 1994). The structure of liposome is illustrated in Figure 1.1.



(A) (B)

Figure 1.1 : Structures of Liposome (A) and sectional view of liposome (B)
(Lucas Meyer GmbH, 1991)

2. Formation of lipid bilayer

The amphiphiles are composed of defined polar and apolar regions. When such amphiphiles are present in an aqueous phase, the exposure of their hydrophobic moiety is thermodynamically unfavorable. This is the driving force behind the self-aggregation process. Thus, above a critical concentration, which depends on the exact structure of the amphiphile as well as on the composition and temperature of the aqueous medium, most amphiphiles self aggregate. All types of aggregates share a common feature, that is, their hydrophobic moiety forms an apolar core, whereas their hydrophilic moiety faces the aqueous phase. However, the exact type of assembly is dependent on the molecular structure of the amphiphiles. Therefore, the amphiphile is defined by a packing parameter, n (Swarbrick and Boylan, 1994). The equation of packing parameter (p) is defined by

$$p = v / a_0 l_c$$

where v is the molecular volume of the hydrophobic part, a_0 is the optimum surface area per molecule at the hydrocarbon-water interface, and l_c is the critical half-thickness for the hydrocarbon region, which must be less than the maximum length of the extended lipid chains (Crommelin and Schreier, 1994).

Amphiphiles in which the hydrophobic cone occupies a smaller lateral area than the polar region ($n < 1.0$), the molecule in total assumes a cone shape. When packing cones together the resultant structural order is that of a spherical micelle. However, this cone shape is also possible in inverted form of the hydrophobic regions are widened to dimensional areas longer than that of the polar head group ($n > 1.0$), the molecule in total assumes a inverted cone shape. When packing cones together the resultant structural order is that of an inverted micelle and the stacked inverse micelle produces the hexagonal phase by a secondary ordering. The amphiphiles with the polar and hydrophobic region approach similar lateral diameters ($n \sim 1.0$), the molecules as a whole appear as cylinders which can therefore pack together tightly into lamellar sheet that have the potential to form liposomes (Swarbrick and Boylan, 1993). The assembly conformations of amphiphiles are illustrated as Figure 1.2.

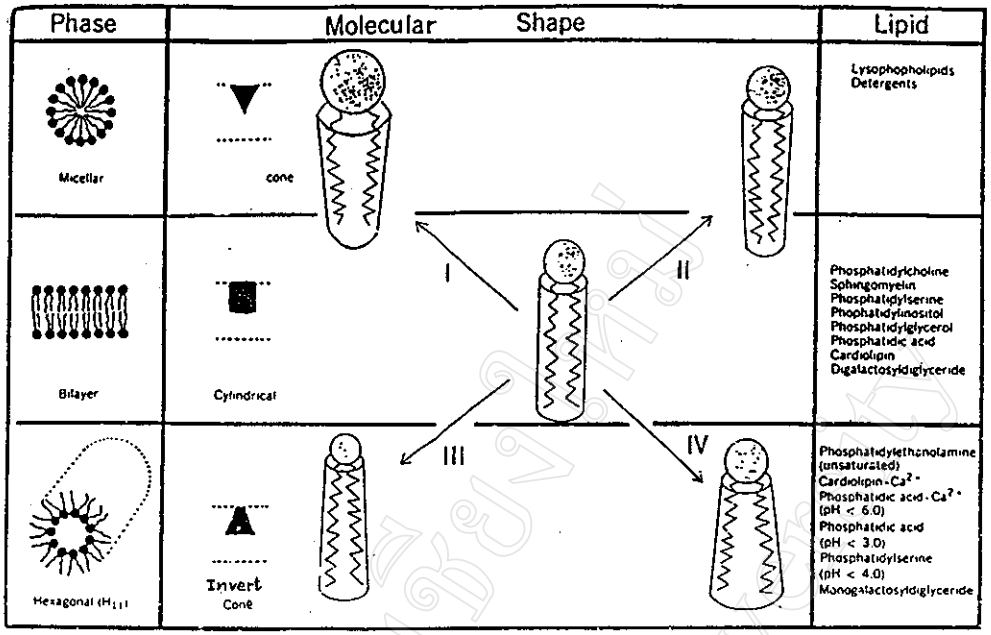


Figure 1.2 : The assembly conformations of amphiphiles (Swarbrick and Boylan, 1994)

3. Phase transition

An understanding of phase transitions and fluidity of phospholipid membranes is important both in the manufacture and exploitation of liposomes, since the phase behaviour of a liposome membrane determines such properties as permeability, fusion, aggregation, and protein binding, all of which can markedly affect the stability of liposomes, and their behaviour in biological systems (New, 1990). The simplest distinction between two lipid phases is in terms of chain order : common grouping is into crystalline, ordered (gel-like), and disordered (fluid or liquid-crystalline) membrane phases. These phases are described as follows and illustrated as Figure 1.3.

3.1 Crystalline phase and lipid subtransition

Hydrated lipids at relatively low temperature typically form densely packed crystalline structure. With increasing temperature, these hydrated lipid crystals become energetically unfavorable, owing to the thermal, initially chiefly rotational, chain excitations. In consequence, at subtransition temperature, $T = T_s$, the two-dimensional lipid crystals revert into a more expand lipid-gel (β or β') phase of untilted chains (β-) or, more frequently, tilted chains (β'-).

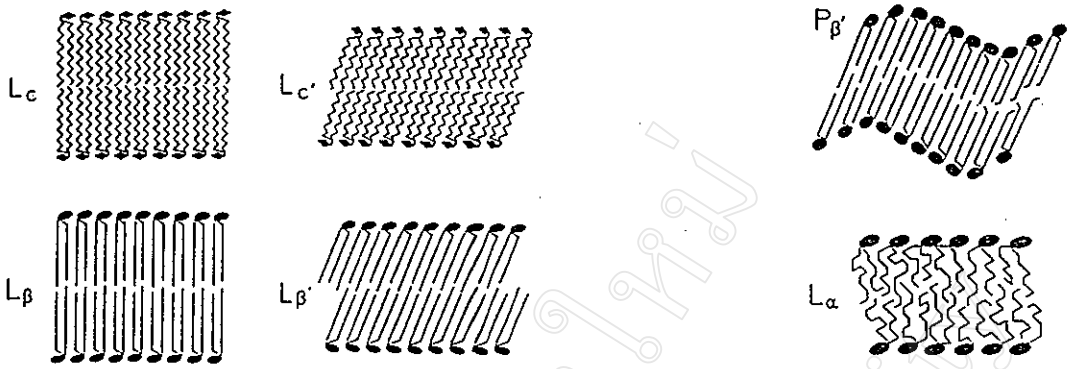


Figure 1.3 : Schematic representation of the lipid polymorphism, dashed symbols correspond to the phases with titled chains ; L_c, crystalline lamellar phase ; L_β, ordered or gel lamellar phase ; P_β ,periodical gel phase ; L_α , disordered or fluid lamellar phase (Cevc, 1993)

3.2 Gel phases and lipid pretransition

Heating lipids in the gel, L_β or L_β' (gel lamellar with untilted or tilted chains, respectively) phase speeds up oscillations of the hydrocarbon chains. This may, but need not, terminate in an essentially unhindered, long-axis chain rotation, often in a cooperative manner at the pretransition temperature, $T = T_p$. Lipid head-group mobility, most notably the rotation of the lipid headgroups around the P-O bond to the glycerol backbone.

It is very probable that the lipid pretransition individual chains shift mutually along their long axes to stay in close contacts. This may be one of the reasons why the bilayer surface at a pretransition breaks up in a series at periodic, asymmetric, quasilamellar bilayer segments which in the electron microscopy pictures resemble undulations or ripples. Regular surface ripples are always diagnostic of the P_β or P_β' (periodical gel phase and gel with untilted or tilted chains, respectively) phase, however, less uniform surface undulations may also be found in the mixed lipid systems.

Lipid pretransition is observed solely if the polar lipid headgroups are sufficiently hydrated and if the packing between fully saturated chains is sufficiently week. Phospholipids with unsaturated chains do not form rippled bilayers, possibly owing to the restricted ability of such chains to slide mutually along their long axes (Cevc, 1993).

3.3 Fluid bilayers and lipid "main transition"

In the low-temperature gel phases, the hydrocarbon chains are in an orientationally well-ordered state in which the hydrophobic molecular segments are nearly completely in an all-trans configuration. As the temperature increases, the fatty acid chains tend to adopt conformations often than the all-trans straight chain configuration, such as the gauche conformation state illustrated as Figure 1.4., and this tends to expand the area occupied by the chains, and hence the membrane, at the same time as it reduces the overall length of the hydrocarbon chains, giving rise to a decrease in a bilayer thickness (New, 1990). The disordered and melting of hydrocarbon chains results in a cooperative, gel-liquid crystalline transition at the main transition temperature, $T = T_m$ (Cevc, 1993, Swarbrick and Boylan, 1992).

Figure 1.4 : Disposition of phospholipid diacyl chains by rotation about one C-C single bond from a trans- to a guache- comformation (New, 1990)

3.4 Non lamellar fluid phases

When the repulsion between hydrocarbon chains is greater than that of the oppositely directed interfacial repulsion, lamellar lipid bilayers are no longer stable. Strong chain disorder ($T \geq T_m$), chain unsaturation, addition of long-chain fat-soluble substance, and/or the tendency for tight headgroup packing all lead to such a situation and, consequently, induce bilayer-to-nonbilayer phase transitions (Cevc, 1993).

4. Materials used in the preparation of liposomes

The formation of lipid bilayers in liposomes require the amphiphiles that have a structure with a packing parameter of n~1.0. The phospholipids are the major structural components of biological membranes and mostly used for preparing liposomes for pharmaceutical applications. The alternative amphiphiles are the modified natural phospholipids and nonionic surfactants. Others compounds do not form bilayer structures by themselves but they are incorporated in liposome membrane for appropiate properties of liposomes (Swarbrick and Boylan, 1994).

4.1 Components in membrane formation.

4.1.1 Natural phospholipids

These lipids have the general structure in which two hydrocarbon chains are liked to a phosphate-containing polar headgroup. Two type of phospholipids exist, there are glycerophospholipid and sphigophospholipid. In glycerophospholipid, the linkage of fatty acids to headgroup is via a bridge region consisting of the three-carbon glycerol. In sphingolipids, the lipid sphingosine forms one of the hydrocarbon chains and is linked directly to the phosphate. Phospholipids can possess fatty acids of different chain length and unsaturation and may have different hydrophilic species linked to the phosphate, according to which individual members of the phospholipid category are classified (New, 1995) and illustrated in Figure 1.5 (New, 1990).

- Phosphatidyl choline (PC)

PC is amphiphatic molecules in which a glycerol bridge links a pair of hydrophobic acyl hydrocarbon chains, with a hydrophilic polar headgroup, phosphocholine. PC, also known as lecithin, is the predominant phospholipid found in natural membranes. They are often used as the principal phospholipid in liposomes for a wide range of applications, because of their low cost relative to other phospholipids, and because of their neutral charge and chemical inertness. Lecithin from natural sources is in fact, a mixture of phosphatidyl cholines, each with chains of different lengths and varying degrees of unsaturation. Lecithin from plant sources has a high level of polyunsaturation in the fatty acyl chains, while that from mammalian sources contains a higher proportion of fully saturated chains (New, 1990).

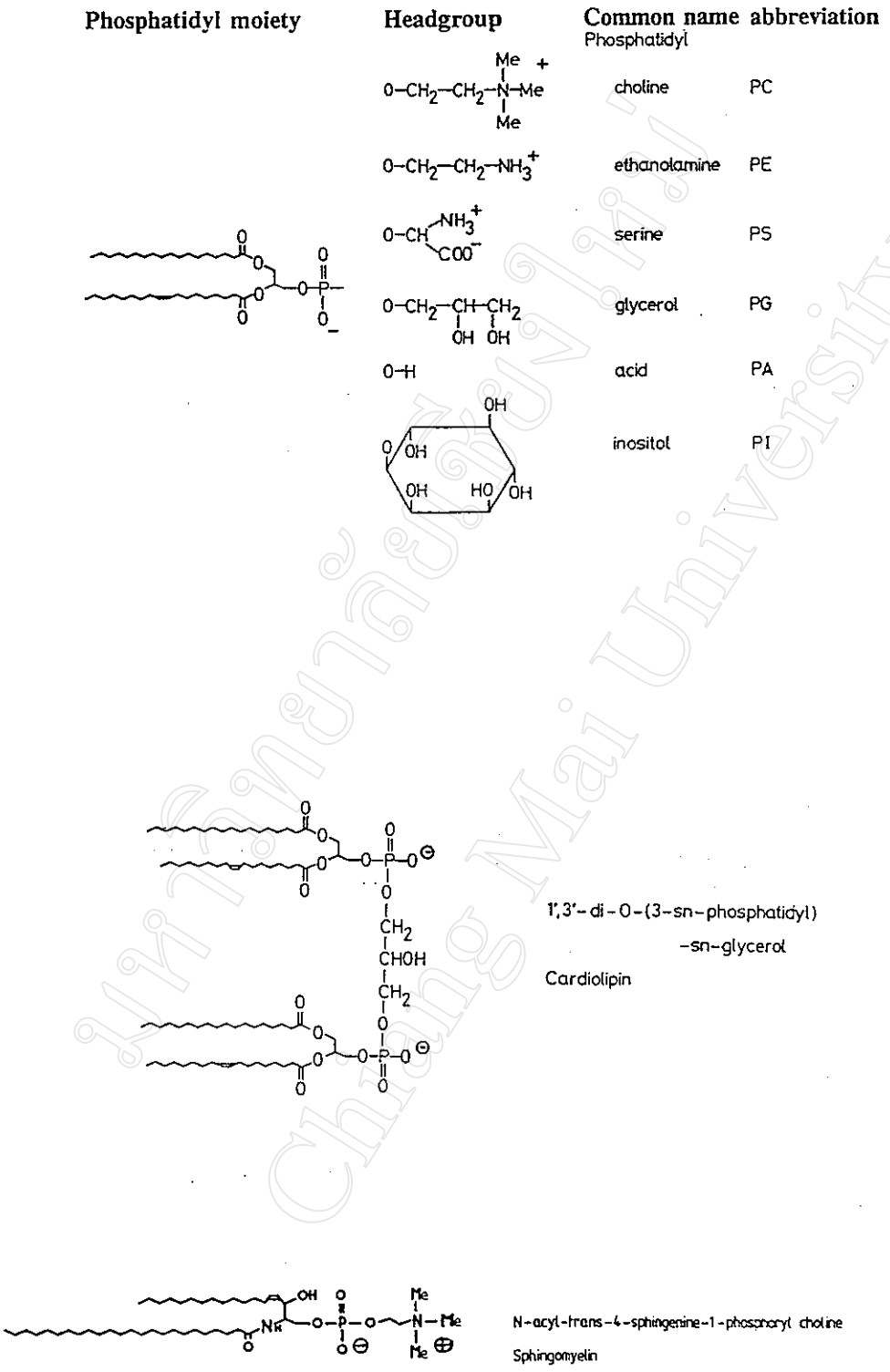


Figure 1.5 : Structures of phospholipids used in liposome formation (New, 1990)

- Phosphatidyl ethanolamine (PE)

PE has a smaller headgroup than PC, and the presence of hydrogens directly attached to the nitrogen of ethanolamine permits interactions of adjacent molecules in the membrane by hydrogen bonding. At low or neutral pH, the amino group is protonated, giving a neutral molecule, which prefers to form hexagonal II phase inverted micelles to lamellar structures when above the main phase transition temperature. The saturated PEs are in the bilayer gel phase at room temperature, while unsaturated PEs are in the hexagonal (nonliposomal phase). The presence of other lipids can stabilize the membranes so that this is prevented, and the ratio of lipids can be carefully arranged if so desired, such that the membrane converts from stable lamellar to non-lamellar with change of pH. Natural PEs tend to be more highly unsaturated than average and have fatty acids of longer and more assymetric chain lengths.

- Phosphatidyl glycerol (PG)

PG possess a permanent negative charge over the normal physiological pH range. In addition to isolation direct from natural sources, it may be readily prepared semi-synthetically from other lipids by the action of phospholipase D in the presence of glycerol.

- Cardiolipin (CL)

Identical to PG, except that a glycerophosphatide moiety is linked to both ends of the headgroup glycerol, to give a molecule with two negatively charged phosphates and four fatty acid chains. CL is found in high proportion in mitochondria of heart tissue. The alternating phosphate-glycerol structure produces an entity that is similar antigenically to the sugar-phosphate backbone of DNA, and, for this reason, CL liposomes can be used diagnostically for detection of conditions such as SLE.

- Phosphatidyl serine (PS)

Serine is linked to the phosphate via its hydroxyl group, leaving the carboxyl and amino functions both free and ionized to form a neutral zwitter ion. The net charge of the PS headgroup is therefore negative, as a result of the charge on the phosphate. Membranes containing PS show a marked sensitivity to calcium, which interacts directly with the carboxyl functions on the headgroups, causing PS molecules to aggregate within the membrane, resulting in a condensed phase separate from that of the bluk lipids. Together with this phase separation goes the appearance of packing irregularities at phase boundaries. Calcium also

causes bridging interactions between PS on membranes of different liposomes, so that aggregation of these liposomes, in which packing defects have been introduced, often results in fusion. However, it has been reported that the presence of PS in membranes helps to stabilize them during freeze-drying in the presence of sugars.

- Phosphatidic acid (PA)

Absence of any subsititution on the phosphate in PA confers a very strong negative charge to the molecule. Dispersions of PA alone in water have a pH of between two and three, and rapid neutralization with acid can cause membrane reorganization, under the influence of electrostatic effects, to produce unilamellar vesicles. In a similar way to PS, addition of calcium can lead to aggregation and fusion, although higher concentrations of the divalent cation are usually required.

- Sphingomyelin (SM)

SM is found to varying extents in the erythrocyte plasma membranes of a number of mammalian species and completely replaces PC in sheep red cells. It is also readily extracted from nervous tissue. It is a neutral molecule with the same phosphocholine headgroup as PC. SMs have hydrocarbon chains often markedly different in length and with a degree of unsaturation giving rise to T_cs between 20°C and 40°C. Membrane packing is tighter than for PC, by virtue of the extra hydrogen bonding made possible in the bridge region by the presence of the amide hydrogen, which participates in interaction between adjacent sphingomyelin molecules, and probably also with cholesterol (New, 1995).

4.1.2 Modified natural phospholipids

Modified natural phospholipids that are natural phospholipids chemically modified by processes such as partial or complete hydrogenation in order to reduce the degree of unsaturation to different extents, as defined by the iodine value supplied by the manufacturer. This improves the appearance and the resistance to peroxidation (Swarbrick and Boylan, 1994) such as hydrogenated phosphatidyl choline. The head group can also be modified with the help of enzymes such as phospholipase D. This enzyme make it possible to convert PC of any acyl chain composition to PG, PE, PS and other phospholipids.

4.1.3 Nonionic surfactants

Nonionic surfactants with a wide variety of structures, usually in the presence of cholesterol, form both multilamellar and unilamellar vesicle. The first reports, circa 1972, from L'Oreal 's laboratories, related to alkyl and dialkyl polyglycerol ethers, although early L'Oreal patents cite a range of vesicle-forming nonionic surfactant compounds. Since then, a number of groups have discovered the vesicle-forming properties of a wide range of nonionic surfactants. The examples of the nonionic surfactants which can forms vesicle such as polysorbate 80, alkyl glycosides and alkyl galactosides, sorbitan monostearate (Florence, 1993), some polyoxyethylene alkyl ethers with a single alkyl chain and the saccharose diesters with two hydrocarbon chains, latter example is illustrated each compounds's structure in Figure 1.6 (Swarbrick and Boylan, 1994).

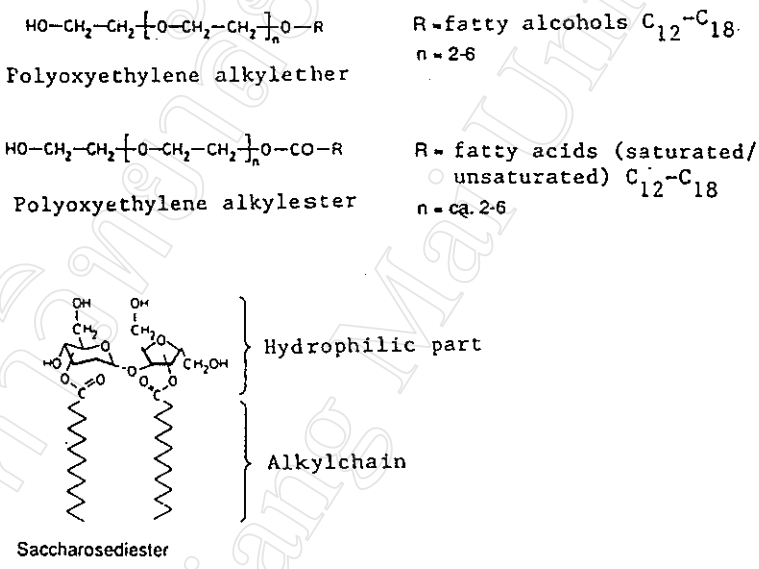


Figure 1.6 : Structural formula of some synthetic, for the formation of nonionic surfactant vesicles (Swarbrick and Boylan, 1994)

4.2 Other components

Others components that incorporate into bilayer membrane for modified the properties of liposomes are the followings :

- Cholesterol

Sterols are important components of most natural membranes, and incorporation of sterols into liposome bilayers can bring about major changes in the properties of these membranes. Cholesterol does not by itself form bilayer structures, but it can be incorporate into phospholipid membranes in very high concentrations-up to 1:1 or even 2 :1 molar ratios of cholesterol to PC. In natural membranes, the molar ratio varies from 0.1-1, depending upon the anatomical and cellular location. Being an amphipathic molecule, cholesterol inserts into the membrane with its hydroxyl group oriented towards the aqueous surface, and the aliphatic chain aligned pararallel to the acyl chains in the center of the bilayer. The 3β-hydroxyl group is positioned level with the carboxyl residues of the ester linkages in the phospholipids, with very little vertical freedom of movement. The presence of the rigid steroid nucleus alongside the first ten or so carbons of the phospholipid chain has the effect of reducing the freedom of motion of these carbons, while at the same time creating space for a wide range of movement for the remaining carbon towards the terminal end of the chain (New, 1990). The structure of cholesterol and the position of cholesterol in the membrane bilayer are illustrated in Figure 1.7.

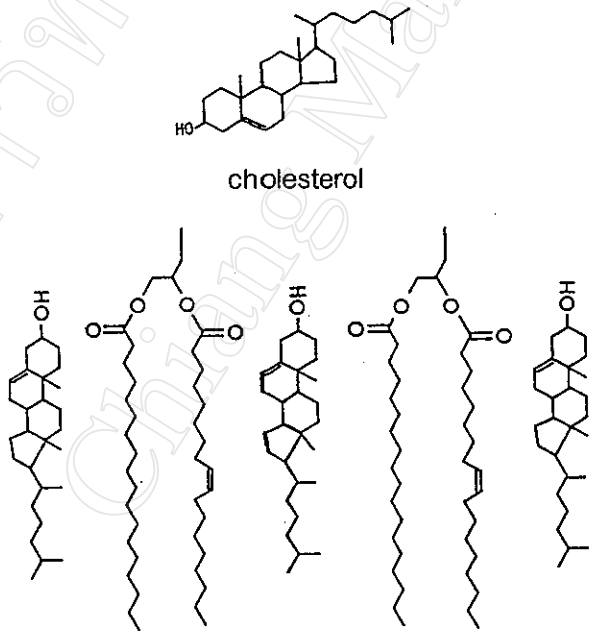


Figure 1.7 : The structure of cholesterol and position occupied by cholesterol in the membrane bilayer. (New, 1990)

Cholesterol reduces the net fluidity of membranes in the fluid phase above the main phase transition temperature but increases it in gel phase membranes below the T_c. Permeability to water-soluble solutes is affected accordingly. In addition, although cholesterol has virtually no effect on the temperature as the phase transition occurs, at high levels it reduces the enthalpy of the transition, which results in the discontinuities that occur in this region also being eliminated—further increasing the stability of the membrane as the temperature changes (New, 1995).

- Charge species

Surface charge of liposome can modified by inclusion the phospholipids which have the charge into liposomal membrane but in natural, some type of phospholipids have negatively charge and no natural positively charged phospholipid exist (New, 1995). Other charged compounds such as stearylamine and dicetyl phosphate, refer to positively charge and negatively charge respectively. The usefulness of stearylamine when included in the liposomal membrane, for examples, increase the stability of drug such as ibuprofen (Sivakumar *et al.*, 1994), increase the efficiency of entrapment of drugs such as factor IX (Ueno, 1982), heparin (Ueno, 1987) and cromolyn sodium (Taylor, 1990), increase the absorption of drug via skin such as retinoic acid (Montenegro, 1996). Inclusion of dicetyl phosphate in the liposomal membrane give the advantages, for examples, increase the proportion of the partitioning of albutarol in the liposomes (Farr, 1989), associate the conjugation between modified mouse monoclonal antibodies and liposomes (Betagari, 1993) and reduces the size of liposomes (Talsma, 1994). Because of the surface charge modification can enhance the properties of liposomes and the method of inclusion the charge does not complicate thus the charge modification is the interest way to manipulate.

- Polyethyleneglycol (PEG)

The liposomes contain PEG-PE, a phosphatidylethanolamine to which a polyethylene glycol group with a chain length ranging from MW 1900 up to 6000 has been attached covalently. These lipids (PEG-PE) has the ability to extend the circulation half-life (Swarbrick and Boylan, 1994). The incorporation of natural hydrophilic components such as gangliosides or phosphatidylinositol, essentially mimicking the outer surface of red blood cells, or of synthetic hydrophilic polymers, specifically PEG will develop the sterically stabilized liposomes

and this new type of liposome is sometimes referred to as a Stealth® liposome (Crommelin and Schreier, 1994).

4.3 Impurity

- Lyso-phospholipids

When the phospholipids lose a fatty acid chain, by either chemical or enzymatic hydrolysis, to give single chain amphiphiles. While they do not form membranes themselves, they are often present in membranes as impurities, either of the starting components, or as a result of degradation during storage. In high concentrations, lysophospholipids can disrupt membranes, and, indeed, they can be highly toxic for cells and whole organism. Membrane disruption with lysophosphatidylcholine (l-PC) only occurs when there is an imbalance in chains in the membrane relative to the headgroups. The action of phospholipase A, converting PC to l-PC and fatty acid does not lead to perturbations until the fatty acid has been removed from the membrane (New, 1995).

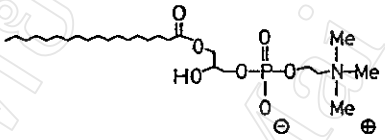


Figure 1.8 : The structure of lysolecithin (New, 1990)

5. Types of liposomes

Liposomes are classified according to their structural parameter or based on their method of preparation. These two classification methods are independent from each other. For example, vesicles made by reverse evaporation can be large or small and single-, oligo-, or multilamellar. Although the classification based on structural parameters is mainly based on size and number of lamellae, the exact properties are also dependent on the method of preparation. Therefore, liposome users have to be specific in difining the liposome preparation by describing the structural properties (size, number of lamellae) and also the method by which the liposomes have been prepared (Swarbrick and Boylan, 1994).

Liposomes when classified by structural properties are divided into three classes. The liposomes when composed of one bilayer are called unilamellar vesicles. The unilamellar

vesicles with the size of under 100 nm are small unilamellar vesicles (SUVs). Those larger size are classified as large unilamellar vesicles (LUVs). Liposomes with lamellae more than one bilayer are classified as multilamellar vesicles (MLVs). Their sizes range from 100 nm to 1 μm (Betageri *et al.*, 1993). The details of the above classification are show in Table 1.1. The structural of lipid bilayer(s) is demonstrated in Figure 1.9.

Table 1.1 : Liposome classification (Swarbrick and Boylan, 1994)

Classification	Abbreviation
Based on Structural Parameters	
Multilamellar large vesicles, > 0.5 μm	MLV
Oligolamellar vesicles, 0.1 -1 μm	OLV
Unilamellar vesicles (all size range)	UV
Small unilamellar vesicles, 20 - 100 nm	SUV
Medium sized unilamellar vesicles	MUV
Large unilamellar vesicles, > 100 nm	LUV
Giant unilamellar vesicles (cell size vesicles with diameters >1 μm)	GUV
Multivesicular vesicles (usually large > 1 μm)	MVV
Based on Method of Preparation	
Single or oligolamellar vesicles made by reverse-phase evaporation	REV
Multilamellar vesicles made by the reverse-phase method	MLV-REV
Stable plurilamellar vesicles	SPLV
Frozen and thawed MLV	FAT-MLV
Vesicles prepared by extrusion methods	VET
Vesicles prepared by French press	FPV
Vesicles prepared by fusion	FUV
Dehydration-rehydration vesicles	DRV

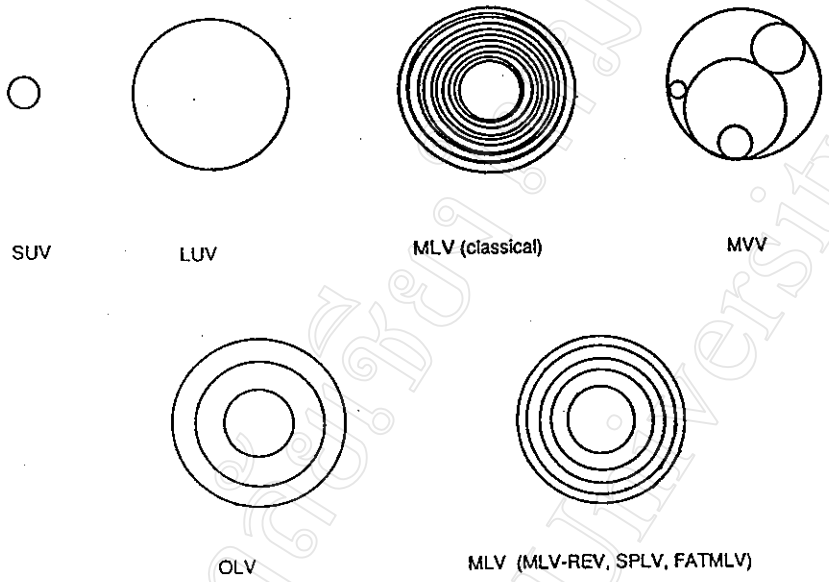


Figure 1.9 : Schematic two-dimensional representation of types of liposomes (Swarbrick and Boylan, 1994)

6. Liposome preparation (Crommelin and Schreier, 1994)

Generally, liposome preparation is divided into 3 steps. Preparation procedures of liposomes are concern a general pattern : First, the lipid must be hydrated. Second, liposomes have to be sized. Third, the nonencapsulated drug has to be removed. These steps are shown in Figure 1.10. and the details of each step are described as follows.

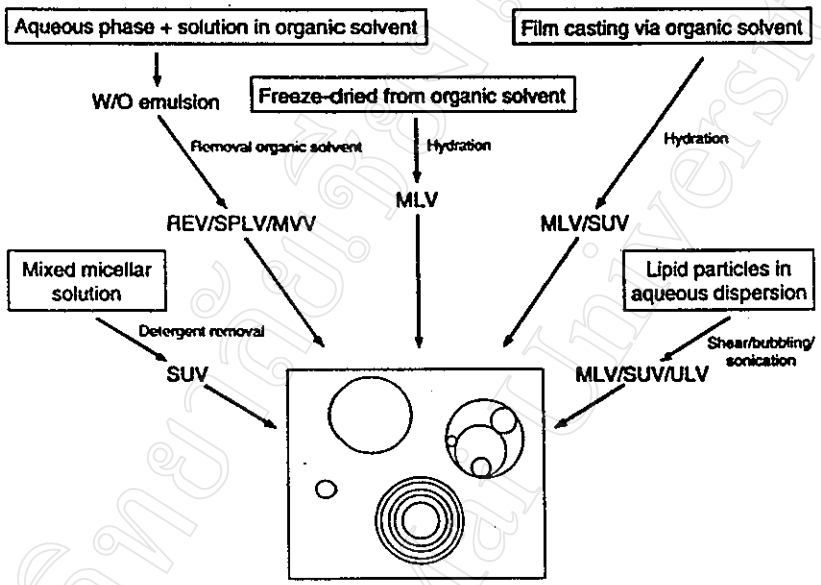


Figure 1.10 : Schematic diagram of regularly used methods for liposome preparation. (Crommelin and Schreier, 1994)

6.1 Hydration step

Many methods have been used for this step.

6.1.1 Mechanical method

The hydration of the thin lipid films deposited from the evaporation of the lipid-organic solvent solution on a glass wall can be obtained by shaking the film with aqueous solution at temperatures above the phase transition temperature of the lipid. The MLVs obtained with wide size distributions are usually further narrowed down by pressure extrusion or ultrasonication procedure.

6.1.2 Replacement of organic solvents by aqueous media method

- Immiscible solvents with excess aqueous phase

Solvent injection or solvent infusion techniques were described (Deamer and Bangham, 1976). Ether or ether/methanol solutions of lipids are slowly injected into a water phase. The organic solvents are continuously removed by evaporation, either at elevated temperatures and/or under reduced pressure. Then, the LUV are formed. Care must be taken to avoid impurities in solvents, in particular peroxides in those solvents liable to oxidative damage. Chlorofluorocarbons have been used as organic solvents as well.

- Immiscible solvents with excess organic solvent phase in the initial stage

The lipid is dissolved in water-immiscible organic solvents. The organic solvent is initially present in excess can vary widely : from cell-size, large unilamellar liposomes down to stable plurilamellar liposomes (SPLV) and REV. The structure of the liposomes strongly depends on bilayer constituents, ratio organic phase/lipid/water and application of shear. The cell-size unilamellar liposomes are prepared by forming the double emulsions (Kim and Martin, 1981). Water in oil (w/o) emulsions are made with water drops emulsified inside the organic phase. This is followed by emulsifying the w/o emulsion in a water phase creating w/o/w emulsions and evaporation of the organic phase. After centrifugation, the cell-size liposomes are found in the pellet and the yield is low. By manipulating the water droplet size in the w/o emulsion, either unilamellar or multivesicular, large (>> 1 μm) vesicles could be obtained.

REV phase evaporation vesicles (REV) are unilamellar or oligolamellar liposomes. They are formed after emulsifying a lipid-containing water-immiscible solvent (chloroform or ether) with a water phase by sonication and subsequent controlled removal of the organic phase by evaporation. Essential for reproducible formation of REV is the proper emulsion formation by sonication. A critical step in the preparation procedure of REV occurs when most of the organic solvent has been removed. In order to avoid gel forming, a vigorous vortexing is needed to converted into a viscous fluid state with the liposomes. The collapse of the gel supposedly coincides with the conversion of the w/o emulsion into the liposomal form.

Unilamellar REV can be only generated when the ratio of the organic solvent/water/ (phospho)lipid is chosen within certain limits. In the initial stage a typical formulation contains 3 ml of ether, 1 ml of aqueous buffer, and 60μmol lipid. When the water content drops

considerably and/or higher lipid concentrations are selected, so-called MLV-REV are formed. With MLV-REV, a large aqueous core is surrounded by many lipid bilayer. The encapsulation efficiency of these MLV-REV is relatively high compared to that of MLV. The generation of SPLV and MLV-REV requires similar relative amounts of organic solvent, water, and lipid. The difference between MLV-REV and SPLV preparation procedures is that for the SPLV the removal of the organic solvent is performed under sonication. Structurally, MLV-REV 's differ from SPLV because SPLV lack the large aqueous core. Moreover, the encapsulation efficiency of the interbilayer spaces is more important for SPLV than for MLV, as the bilayer repetition distance in SPLV is wider than in MLV (with neutral lipids).

- Solvents miscible with water

For the ethanol injection method, the liposomes is prepared by a solution of lipid(s) in ethanol is injected in the aqueous phase. As the ethanol is diluted, the precipitating lipids form liposomes. Liposomes with diameters as small as 25 nm may obtain when injected rapidly and larger when injected slower.

6.1.3 Detergent removal method

Lipids, lipophilic compounds and amphipathic proteins can be solubilized by detergents forming mixed micelles. Upon removal of the detergent by dilution, gel filtration, dialysis, or the addition of polymeric adsorbents (e.g. Biobeads) small vesicle formation can occur when the detergent is removed rapidly. Detergent removal techniques are the first choice for incorporating integral membrane (glyco)proteins into liposomes as well as to be used for the preparation of immunoliposomes.

6.1.4 Size transformation and fusion method

- Freezing/Thawing

When MLV are exposed to series of freezing/thawing cycles, structural changes of liposomes can occur. Multivesicular structures are frequently found whereas stacked bilayer structures are rare. The frozen/thawed MLV are called FATMLV. An increase in the encapsulation efficiency can be obtained by the improved swelling and the elimination of bilayer stacks. Importantcy, with this technique an equilibrium distribution of solute is observed. This technique allows the loading of proteins and other compounds that are

sensitive to organic solvents or detergents to be incorporate into liposomes, providing that freezing (with the accompanying solute concentration effects) is not destructive. The freezing-thawing cycle can be repeated if required. The trapped volume initially increases with the number of freezing-thawing cycles but reachs a plateau upon continuing cycling. The presence of cryoprotectants may interfere with efficient loading of the vesicles in a freezing-thawing cycle. The freezing/thawing procedure can be modified to prepare giant unilamellar vesicles or oligolamellar vesicles with diameters of over 10 μm. These giant vesicles are formed upon freezing/thawing SUV in the presence of a high concentration of electrolytes and subsequent dialysis against a low electrolyte concentration.

- Dehydration/Rehydration vesicles

Kirby and Gragoriadis described a preparation method based on hydration of freeze-dried liposomes (Kirby and Gragoriadis, 1984). Upon hydration of freeze-dried liposomes in a small volume of water, large liposomes were generated again, and high encapsulation efficiencies were encountered (dehydration/rehydration vesicles, DRV). In the preparation, the material to be encapsulated can be added either before freeze drying or in the rehydration medium. As cryoprotectants tend to protect cells and liposome structures, their presence may interfere with the formation of the desired liposome structure during the freeze-drying/rehydration precedure.

6.1.5 pH adjustment Method

Upon transient exposure to alkaline pH values, PA dispersions spontaneously form small unilamellar vesicles. Apart from the small unilamellar vesicles in the 20-60 nm range, larger vesicles are formed as well. The extent of vesiculation depends on the experimental conditions such as pH and the ratio PC/PA. Phospholipid structure (PA, PG), phospholipid mixture (PC/PG), cholesterol content, ionic strength, and timing of the pH adjustment procedure substantially influenced the particle size of the resulting dispersion. The pH gradient over the bilayer (generated by the pH jump) acts as the energy source driving the spontaneous formation of the small vesicles with high curvature. Until now this preparation method has not been used frequently for pharmaceutical preparations, probably because of the rather low encapsulation efficiency, size heterogeneity, and the relatively high costs of negatively charged lipids such as PA and PG.

6.2 Sizing step

Different approaches are available for the size reduction of liposomes. In the 1960s, sonication was introduced to reduce liposome particle size (Saunders *et al.*, 1962). Because of the higher energy power densities that can be reached, probe sonicators are more effective than bath sonicators. However, probe sonicators have a number of potential drawbacks such as the exclusion of oxygen during sonication is difficult and the peroxydation reactions may be induced in unsaturated acyl chains during the sonication process, titanium probes tend to shed metal particles and generate the aerosols. The bath sonicators these drawbacks can be avoid. However, the experimental conditions should be kept constant for reproducible results. The development of high-power cup horn sonifiers has substantially enhanced the efficiency of bath sonication.

In late 1970s, the French press was used for the liposomal size reducing purpose. For examples, the exposure to high shear force (Lelkes, 1984). The high-shear homogenizers have been introduced to narrow down the size range and average diameter of the liposomes by some workers (Talsma *et al.*, 1989 ; Brandl *et al.*, 1990).

A "low pressure" extrusion of the liposome dispersion under pressures up to about 1 Mpa through polycarbonate membranes has also been used (Olson *et al.*, 1979 ; Szoka *et al.*, 1980). Two stacked polycarbonate filters with 0.1 μm pores and pressures up to 4Mpa (high pressure extrusion) were used to convert highly concentrated MLV dispersions into unilamellar liposomes in the 60 - 100 nm range (Hope *et al.*, 1985). For the extrusion of large volumes equipment with a built in stirrer may be advantage (Amselem *et al.*, 1993). Alternatively, ceramic extrusion systems may be used. The advantage is the relative ease of unclogging the system through back flushing and the possibility of cleaning the filters (Martin and Morano, 1988).

6.3 Removal of nonencapsulated materials

Many lipophilic drugs exhibit a high affinity to the bilayer and are completely liposome associated. However, for other compounds, the encapsulation efficiency is less than 100%. The nonencapsulated fraction of the active compound can cause unacceptable side effect or physical instability. For removal of the nonencapsulated material, the techniques of dialysis

and ultrafiltration, ultracentrifugation, gel permeation chromatography, and ion exchange reactions have been used. The details of these methods are as follows.

6.3.1 Dialysis and ultrafiltration

Conventional dialysis membranes can be used with molecular weight cutoff characteristics dependent on the molecular weight of the "free" compound to be removed from the liposome dispersion. Typically, membranes with a molecular weight cutoff between 10 and 100 kDa are used. In ultraflitraition, the dispersion is stirred or circulated by a pump. This convection process must not induce leakage of encapsulated material.

6.3.2 Ultracentrifugation

Ultracentrifugation can be used for the removal of dissolved nonliposome associated materials, the separation of liposomes with different sizes and/or subfractions with different densities, and the concentration of dilute liposome dispersion (Barenholz *et al.*, 1977). Discontinuous or continuous density gradient ultracentrifugation techniques are powerful tools for separating different colloidal structures. Centrifugation techniques can be extremely helpful for purifying liposome dispersion on a lab scale. However, they are difficult to integrate into large-scale liposome production schemes.

6.3.3 Gel permeation chromatography

The gel permeation uses the size exclusion concept for separate the nonencapsulated materials. Sephadex®, Sepharose®, or Bio-Gel® columns are regularly used to separate liposome-associated material from nonencapsulated materials. The liposomes are not retained by the gel bed and eluted in the void volume ; the free materials elute in later fractions. This technique is frequently used for assessment of the encapsulation efficiency. Major drawbacks limit the use of gel permeation chromatography are that the dilution occurs during the elution process and the technology is difficult to scale up, in particular for the production of parenteral products.

6.3.4 Ion exchange reactions

The exchange of ion between resin and sample is useful for separate the nonencapsulated materials. Dowex 50W-X4®, a cation exchange resin, is used regularly. Dowex® removed free doxorubicin, vincristine, cisplatin, cytarabine, and methotrexate from liposome dispersions even under (pH) conditions where the drug was not present in the cationic form. This led to the conclusion that the interaction with Dowex® may not be simply electrostatic in nature but may also include a hydrophobic component (Strom *et al.*, 1985). In general, ion exchange resins can be utilized for efficient and fast removal of nonliposome-associated materials without dilution of the dispersion, if they have a high affinity for the nonliposome-associated compound.

7. Liposome characterization and quality control (Swarbrick and Boylan, 1994)

Chemical and physical characterizations are very important for a meaningful comparison of different liposome preparations or different batches prepared according to the same protocol. The chemical characterization focuses on the quality of liposome constituents (raw material) and the prepared liposomes. Different techniques are used based on chemical, enzymatic or chromatographic principles. As a rule, quality and stability assays have to be based on the descriptions of both the lipids and the entrapped materials.

The following parameters are determined in physical characterization assays. Liposome dispersions consist of a population of particles and the properties should therefore be expressed as a distribution function, for example, of size or charge.

1. Mean size and size distribution : by electron microscopy, dynamic light scattering, coulter counter, and polarization intensity different scattering (PIDS).
2. Number of lamellae : by small-angle x-ray scattering (SAXS), electron microscopy, colored or fluorescent probes, and NMR.
3. Entrapped volume and osmotic behavior : by colored or fluorescent probes.
4. Distribution of the agents between liposome membranes and its internal aqueous compartments : by comparison with encapsulated volume, fluorescence, or zeta potential.
5. Structural and motional behavior of the lipids in the liposomal assembly : by fluorescing probes, NMR, ESR, and DSC.

6. Electrical surface potential and surface pH : by microelectrophoresis or zeta potential probes (fluorescence, ESR, etc.)
7. Distribution of lipid components between the two faces of the lipid bilayer with special emphasis on the components exposed to the external medium : by NMR or chemical labeling.

There is a difference between quality control (QC) strategies to characterize vesicles during the development of a liposomal dosage form and QC assays used to routinely characterize batches for clinical applications. During the development of invasive methods, labels or agents irrelevent for the formulation itself can be used. This cannot be done with batches employed in the clinical or in final stages of animal toxicity or efficacy testing.

8. The advantages and applications of liposomes

The imagination of liposome properties are possible to transfer to real liposomes because of the fact that liposome properties can be vary substantially with lipid compositions, sizes, surface charges and methods of preparation (Betageri *et al*, 1993). Thus, many properties of liposomes can be prepared for various fields of sciences, especially in pharmaceuticals. The profits and applications of liposomes are described and examples of drugs used are demonstrated as follows.

8.1 Enhancement of activity and reduction of toxicity of drugs

Liposome formulations can enhance the activity of drugs encapsuled in liposomes, such as tetracaine entrapped in liposomes showed the deeper anesthesia effect with shorter onset in the volunteers than the unentrapped drug (Foldvari, 1994) pilocarpine hydrochloride entrapped in liposomes showed longer period of miotic response than the drug in solution (Khalil *et al*., 1992). Besides, cyclosporine entrapped in liposomes gave lesser nephrotoxicity than oily cyclosporine preparation (Akbarieh *et al*., 1993). Streptokinase entrapped in liposomes showed stronger effects on the fibrinolytic enzyme in test animals (Schmidt and Michaelis, 1992). It has been also showed that netilmicin entrapped in liposomes has substantial reduction of acute toxicity as well as the prolongation of half-life circulation time (Mimoso *et al*., 1997). However, chloramphenicol when encapsulated in liposomes, the

antibacterial activity (*in vitro*) was slightly lower than that commonly used chloramphenicol ophthalmic solutions (Szulc *et al.*, 1990).

8.2 Various possible routes of administration

Liposomes can be prepared in various formulations such as gel, aerosol, solutions for injection and from these formulations can ease to administer in vary routes.

Parenteral administration, for examples, the liposomal bovine serum albumin can be administered by subcutaneous (s.c.) injection to study about antibody response (Phillips *et al.*, 1996), methotrexate liposome administered by intramuscular (i.m.) injection was markedly localized in the lymph nodes (Kim and Han, 1995), paclitaxel liposome for intraperitoneal (i.p.) injection appears to be promissing therapy for malignancies confined to the peritoneal cavity (Sharma *et al.*, 1996) and amphotericin B liposome administered by intraveneous (i.v.) injection for the treatment of cutaneous leismaniasis (Yardley and Croft, 1997).

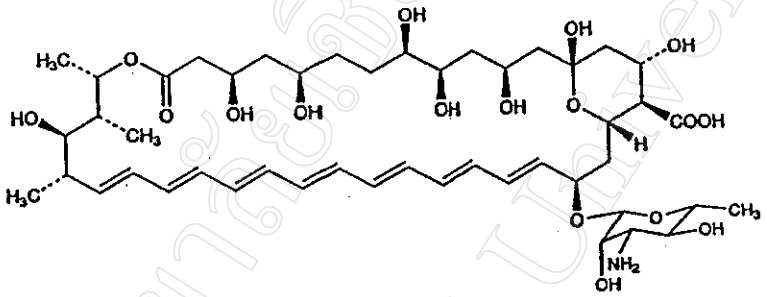
Topical administration of liposomes includes application to skin, eyes, lungs or body cavities, for examples, triamcinolone acetonide liposome for skin application produced significantly lower blood levels in systemic circulation (Mezei and Gulasekharam, 1982) and when administered by ophthalmic instillation, they can produce significantly higher drug levels in ocular tissue (Singh and Mezei, 1983). Fentanyl liposome administered to lung by inhalation provide sustained release (Hung *et al.*, 1995). DNA-liposome complexes administered to mucosal membrane for immunization (Klavinskis *et al.*, 1997) and albendazole liposome via oral route (Wen *et al.*, 1996) can increase the uptake of drug.

8.3 Targeting of liposomes

The advance application of liposomes in drug targeting is the use of drugs that affect disease tissue while not harming the healthy tissue. For examples, ofloxacin liposome was able to deliver ofloxacin into human synovial fibroblasts (McCoy cells) in a greater amount than the free drug (Fresta *et al.*, 1995). The tumor-selective delivery of boron liposome that is useful for the treatment of cancer by boron neutron capture therapy (Hawthorne and Shelly, 1997). The long-circulating liposomes coated with PEG and conjugation to monoclonal antibodies (Mabs) as a drug-targeting device were directed against erbB-2 oncoprotein, a functional surface antigen (Goren *et al.*, 1996).

1.4.2 Amphotericin B

Amphotericin B, a potent antifungal substance with a polyene structure was isolated in 1956 by Gold *et al.* from an actinomycete, *Streptomyces nodosus*, found in a soil sample from Venezuela. The antibiotic material was a mixture of two closely related compounds designated amphotericin A and B. The B compound was more active and is consequently the one employed therapeutically. Its structure and absolute stereochemistry have been determined. Its structure and molecular weight are shown in Figure 1.11(Delgado and Remers, 1991).



$C_{47}H_{73}NO_{17}$, molecular weight 924.09

Figure 1.11 : The structure of amphotericin B (Budavari, 1996)

The rigid heptaene chain elongates the macrocycle, so that one side (polyene) is hydrophobic while the other side (aliphatic) is hydrophilic due to the presence of seven hydroxyl groups and an ester carbonyl group. This may account for its ability to act as an ion-channel in membranes. A mycosamine residue is attached to one end providing a free amino group (Asher *et al.*, 1977).

1. Physical properties

Amphotericin B is bright yellow powder. Microscopic examination reveals prisms or needles for samples freshly recrystallized from dimethylformamide. Amphotericin B has no evidence of the melting up to 250°C (Asher *et al.*, 1977) but decomposes gradually above 170°C (Budavari, 1996).

Amphotericin B is insoluble in water at pH 6 – 7 but soluble in water at pH 2 or pH 11 of about 0.1 mg/ml. Water solubility of amphotericin B can be greatly increased by adding sodium lauryl sulfate or sodium desoxycholate (as in a commercial injectable product "Fungizone®"). Amphotericin B also dissloves in lecithin-cholesterol vesicles and sterol-containing natural membrane. Solubility in methanol is 1.60 mg/ml, in anhydrous methanol is 0.2 - 0.4 mg/ml (Asher *et al.*, 1977), in dimethyl formamide is 2 – 4 mg/ml, in dimethyl formamide + HCl is 60 – 80 mg/ml and in dimethyl sulfoxide is 30 – 40 mg/ml (Budavari, 1996). Amphotericin B sodium desoxycholate complex gives high solubility of 50 mg per ml in water.

The partition coefficient (octanol/water) of amphotericin B is high and the two pKa 's (COOH, NH_2) of amphotericin B are 5.7 and 10 (Dollery, 1991). Ultraviolet absorption spectrum of amphotericin B are 406, 382, 363 and 345 nm. It is distinguished from amphotericin A because amphotericin A's ultraviolet absorption spectrum are 318, 304, 291, 280 and 228 nm (Asher *et al.*, 1977).

2. Pharmacology

Amphotericin B is usually used as a fungistatic in action with the obtained clinically concentrations. But, it may be fungicidal in high concentrations or against with very susceptible organisms (McEvoy, 1996). Its mode of action is at least partly due to the binding to ergosterol in the fungal cell membrane. As a result of this binding, amphotericin B appears to open pores in the fungal cell wall thus increasing permeability to allows amino acids and other small molecular weight nutrients to escape from the cells. The maintenance of potassium pool is also disrupted. In addition, low concentrations of amphotericin B have stimulatory effects on cell proliferation of both fungal and human cells. Amphotericin B at low concentrations also stimulates macrophages and has a potent humoral immunostimulant effect as well as causing an increase in the cell-mediated tissue response (Dollery, 1991).

Amphotericin B is not active against organisms (e.g., bacteria) that do not contain sterols in their cell membranes. Since amphotericin B has selective binding to sterols, it will account for several aspects of its toxicity. Some mammalian cells (such as certain kidney cells and erythrocytes) contain sterols that can bind amphotericin B and thus are subject to alterations in cellular permeability. Thus, amphotericin B shows toxicity to kidney and heart.

3. Spectrum

3.1 Fungi

In vitro, amphotericin B with the concentrations of 0.03-1.0 μg/ml usually inhibit *Aspergillus fumigatus*, *Paracoccidioides brasiliensis*, *Coccidioides immitis*, *Cryptococcus neoformans*, *Histoplasma capsulatum*, *Mucor mucedo*, *Rhodotorula* spp. and *Sporothix schenckii*. *Blastomyces dermatitidis* may require slightly higher drug concentrations for inhibition. The minimum inhibitory concentration (MIC) of amphotericin B for *Candida* varies with species and strains. The MIC for *C. albican* and *C. guilliermondi* is 0.2 – 1.9 μg/ml, and ranging from 0.2 – 25 μg/ml for strains of *C. tropicalis*. It has been suggested that to treat a systemic fungal infection successfully, serum amphotericin B concentrations should be maintained at twice the *in vitro* MIC.

3.2 Protozoa

Amphotericin B is active *in vitro* and *in vivo* against *Leishmania braziliensis*. The drug is also active *in vitro* and *in vivo* against *L. mexicana* and *L. donovani*, including antimony-resistant strains of the organisms. In *in vitro*, amphotericin B concentrations of 1 μg/ml result in complete elimination of *L. donovani* amastigotes in human monocyte-derived macrophages and *L. donovani* promastigotes in cell-free media. The drug is also active *in vitro* against *L. tropica*.

Amphotericin B is active *in vitro* and apparently *in vivo* against *Naegleria* spp., particularly *N. fowleri*. The drug has variable and limited activity *in vitro* against *Acanthamoeba castellanii* and *A. polyphaga*.

3.3 Other Organisms

Amphotericin B is not active against bacteria, rickettsiae, or viruses (McEvoy, 1996).

4. Pharmacokinetics

Amphotericin B is not absorbed through the skin or mucous membrane when applied topically. Little or no absorption occurs from either the normal or ulcerated gastrointestinal tract (Dollery, 1991). It must be given parenterally to treat systemic fungal infections.

Distribution is apparently multicompartmental, however more information on the distribution of amphotericin B is limited. The volume of distribution of the drug has been reported to be 4 L/kg (McEvoy, 1996). Amphotericin B is highly protein bound (>90%) and is poorly dialyzable (Drug Fact, 1995). The routes of elimination and metabolic pathways for amphotericin B are not known.

5. Indications

5.1 Candidosis of the mouth, oropharynx, oesophagus and gastrointestinal tract

Amphotericin B is not absorbed following oral administration but remaining in an active form in the mouth and gut. In oral oropharyngeal and oesophageal candidosis, the yeast is primarily on and in the mucosal surface, although in some cases they may penetrate into deeper layers. Amphotericin B lozenges and suspension are indicated in candidosis of the mouth, oropharynx, and oesophagus. The success of the treatment depends on maintaining an adequate concentration of the drug on the infected mucosal surface for a sufficient length of time to affect the yeast cells. Oral tablets are indicated in patients with candidal overgrowth in the faeces associated with broad-spectrum antibiotics. The reduction or eradication of faecal *Candida* is also part of the recognized treatment of anal candidosis and recalcitrant vaginal candidosis.

5.2 Denture stomatitis

Amphotericin B lozenges and suspension can significantly reduce *Candida* on the oral mucosa but do not affect the yeasts on the upper fitting surface of the denture.

5.3 *Candida* prophylaxis

In patients who are prone to develop vaginal candidosis following broad-spectrum antibacterial therapy, amphotericin B tablets can be given with the antibacterial compounds. The yeast population in the lower gastrointestinal tract will be reduced. Amphotericin B lozenges can be similarly used in patients undergoing open heart surgery. They should be used in conjunction with amphotericin or nystatin tablets and an anticandidal vaginal pessary.

5.4 Vaginal candidosis

Amphotericin B pessaries can be used in the management of women with acute vaginal candidosis.

5.5 Cutaneous and mucocutaneous candidosis

The amphotericin B can be used for the majority of superficial *Candida* infections arise in moist areas of the skin, especially in areas of folds and creases, and in the nail bed and nails and in babies *Candida* infection resulting from nappy rashes as well.

5.6 Systemic mycoses

Amphotericin B is the most effective antifungal agent in the management of patients with systemic mycoses. As drug resistance is not a problem with amphotericin B, laboratory susceptibility testing is not necessary and treatment should commence as soon as the diagnosis of a systemic mycosis is made. It is not possible to indicate in percentage terms the efficacy of amphotericin in systemic mycoses. It remains the mainstay of antifungal drugs for systemic mycoses but it is frequently only given in the late stage of infection either because of delay in diagnosis or fear of toxicity (Dollery, 1991).

6. Preparations

Amphotericin B is available in oral, topical and parenteral forms. Oral preparations include tablet (100 mg), lozenges (10 mg) and suspension (100 mg/ml). Topical preparations include cream (3%), lotion (3%), ointment (3%), pessaries(50 mg), eye drop (5 mg/ml) and amphotericin B in orabase (2%, Fungilin®) (Dollery, 1991).

The parenteral preparation includes amphotericin B for injection (Fungizone®) that is the lyophilized yellowish powder which contains amphotericin B 50 mg, sodium desoxycholate 41 mg and sodium phosphates 20.2 mg (McEvoy, 1996).

7. Stability

Dry powder of amphotericin B appears to be very stable at room temperature, it is stable for days when dissolved in solution of isopropanol and water (1:1) at pH 6-8. It is apparently stable when dissolved in phosphate citrate buffer at pH 5-7 and stable for 14 days

when dissolved in sodium dodecyl sulfate (1%) at room temperature (Bach, 1984). It is stable up to eighteen months at -20°C when dissolved in the solutions of DMSO and methanol (1:1 v/v) (Wang *et al.*, 1992).

In biological matrices, amphotericin B in the environment liquid of plasma or liver is stable when stored at -20°C (Wang *et al.*, 1992). It shows initial loss of about 5% in serum and no further decrease when frozen at -70°C (Bach, 1984).

The degradation of amphotericin B can accelerated by greater than 60% within six months in 100% DMSO (Wang *et al.*, 1992), in liquid medium of L-asparagine or aqueous dextrose solution at pH 7.4, 37°C, amphotericin B gives half-life of 4-5 hours. In 0.25% sodium dodecyl sulfate, it degrades slightly after three days at room temperature but none degradation is found at 4°C (Bach, 1984).

Amphotericin B in plasma or liver homogenates has the degradation half-life at 37, 25 and 4°C of 10 hours, 1 day and 7 days respectively (Wang *et al.*, 1992). Amphotericin B in serum gives the half-life at room temperature and 4°C of 21 hours and 20 days respectively. When amphotericin B in serum mixed with guanidine, the half-life at room temperature about 1.5 hours. This shows more rapid degradation of the drug than in serum alone (Bach, 1984).

Amphotericin B for injection (Fungizone®) when reconstituted with sterile water for injection and protected from light are stable for 24 hours at room temperature or one weeks when refrigerated at 2-8°C. Precipitation occurs when solution containning sodium chloride or bacteriostatic agent (eg., benzyl alcohol) is used for reconstitution. Coagulation of colloidal partical is observed when pH of the injection is less than 5 (McEvoy, 1996).

8. Lipid formulations of amphotericin B

Lipid formulations of amphotericin B have the promising to improve the therapeutic index of amphotericin B. In 1990, amphotericin B in liposomes under the brand name of AmBisome by Vestar and in other lipid base by Liposome technology (Amphocil ; amphotericin B colloidal dispersion) and Liposome Company (Abelcet ; amphotericin B lipid complex) (Gulati *et al.*, 1998) was introduced into the market. Many lipid formulations of amphotericin B can be prepared in the lab scale, such as amphotericin B in lipid emulsion. The details of lipid formulations of amphotericin B are described in the following topics.

8.1 Amphotericin B lipid complex (ABLC ; Abelcet®)

ABLC is a suspension product of amphotericin B complexed with the lipids L-α-dimyristoylphosphatidylcholine (DMPC) and L-α-dimyristoylphosphatidylglycerol (DMPG). It appears as ribbons by freeze-fracture electron microscopy (Bhamra *et al.*, 1997). ABLC shows reduced toxicity relative to that of amphotericin B deoxycholate while maintaining antifungal activity (Hiemenz and Walsh, 1996 ; Bhamra *et al.*, 1997).

8.2 Amphotericin B colloidal dispersion (ABCD ; Amphocil®)

ABCD is a stable complex of amphotericin B and cholesteryl sulfate in a 1:1 molar ratio (Sanders *et al.*, 1991). This lipid formulation of amphotericin B forms disklike structure of about 115 nm in diameter. ABCD shows less toxicity effect than amphotericin B deoxycholate while the antifungal activity is similarly (Sanders *et al.*, 1991 ; Hiemenz and Walsh, 1996).

8.3 Amphotericin B in lipid emulsion

Amphotericin B in lipid emulsion is prepared by mixing amphotericin B with 10% or 20% of Intralipid. These preparations show good antifungal activity with lower the side effects in mucocutanous candidosis (Chavanet *et al.*, 1992) and visceral leishmaniasis (Herbrceht *et al.*, 1996). However, the evidence of incompatibility of these formulations were frequently reported especially in case of sedimentation (Trissel, 1995 ; Lopez *et al.*, 1996) and creaming (Cleary, 1996). An amount of 0.2 mg/ml of amphotericin B in 20% intralipid shaked for 18 hours by an orbit environmental shaker showed the longest stability time of at least 1 month (Shadkhan *et al.*, 1997).

Many investigators suggested additional studies of efficacy (Cleary, 1996), stability and compatibility (Sievers *et al.*, 1996) for these formulations although they show the advantanges of side effects reduction.

8.4 Liposomal amphotericin B (L-AmB ; AmBisome®)

Liposomal amphotericin B consists of hydrogenated soy phosphatidylcholine, distearolyphosphatidylglycerol, cholesterol and amphotericin B in a molar ratio of 2 : 0.8 : 1 : 0.4. There are the small unilamellar vesicles with spherical sizes of 60-70 nm in diameter (Hiemenz and Walsh, 1996).

There are several reports about the improving of efficacy and therapeutic index of L-AmB. The higher efficacy with less toxicity of L-AmB than amphotericin B deoxycholate was demonstrated when given in patients with systemic mycose (Lopez *et al.*, 1989), persisting fungemia (Sculier *et al.*, 1989), hepatic candidosis (Hudson *et al.*, 1991), visceral leishmaniasis or those who are the new born infants with systemic candidosis (Vincent *et al.*, 1992) and those who are very low birth weight infants with disseminated fungal infections (Lackner *et al.*, 1992).

The toxicity especially in renal toxicity was decreased when given L-AmB since L-AmB which have the affinity to high density lipoprotein (HDL) and can not transfer drug from HDL to low density lipoprotein (LDL). Therefore, renal cells express low affinity to HDL receptors. Thus, L-AmB distributes less to kidney and the toxicity to kidney are then decreased(Wasan *et al.*, 1994). Some datas have reported that L-AmB has low volume of distribution (Alak *et al.*, 1996) causing higher distribution to liver and spleen than to kidney and lung (Tollemar *et al.*, 1992). The anaphylactic reaction when treated by L-AmB are not shown in 187 transplanted recipients (Ringden *et al.*, 1994) but an anaphylactic reaction in a 10 years old girl with candidosis was observed after following intraveneous injection of L-AmB (Torre *et al.*, 1996)

L-AmB is indicated to be administered only by i.v. injection. Further efforts to improve clinical application of L-AmB, such as aerosolized L-AmB administered to the lung with aspergillosis (Purcell and Corris, 1995) and cryptococcosis have been performed (Lambros *et al.*, 1997). L-AmB was applied to rabbit eyes with ocular mycosis (Pleyer *et al.*, 1995), L-AmB with polyethylene glycol (PEG-L-AmB) has been developed to prolong residence time of L-AmB in blood (van-Etten *et al.*, 1995). The conjugate of PEG-L-AmB with monoclonal antibody 34A (34A-PEG-L-AmB) has been administered to i.v. injection in BALB/c mice for lung targeting (Kohno *et al.*, 1997).

1.4.3 Percutaneous absorption

1. Skin

The skin is the body's largest organ accounting for more than 10% of body mass. The skin enables the body to interact most intimately with its environment. Figure 1.12 shows the structure of the skin. The skin consists of four layers which are the stratum corneum (SC) (nonviable epidermis), the remaining layers of the epidermis (viable epidermis), dermis, and subcutaneous tissue. There are also a number of associated appendages such as hair follicles, sweat ducts, apocrine glands, and nails.

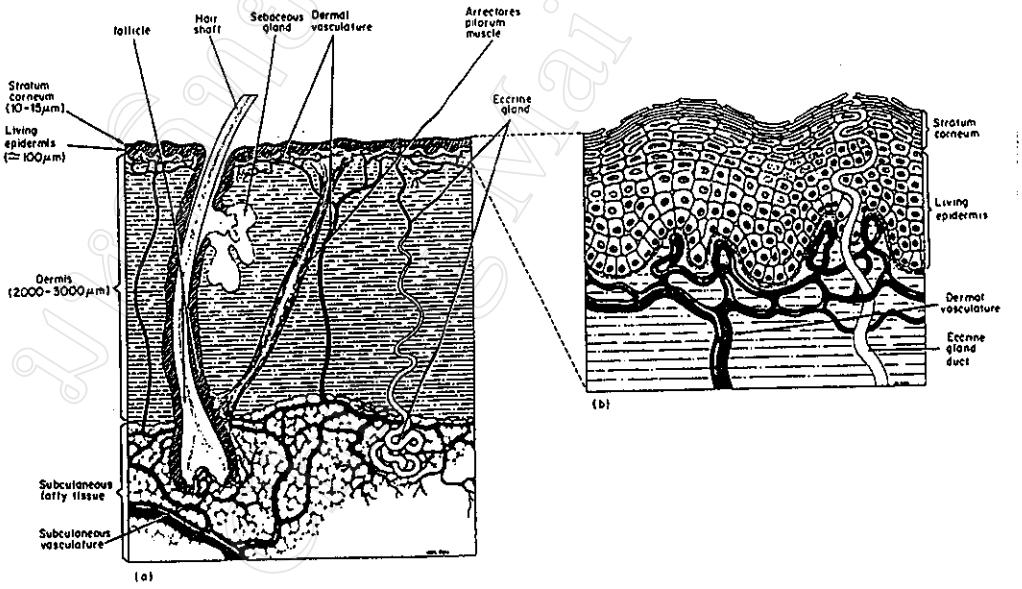


Figure 1.12 : The structure of the skin. (Potts *et al.*,1992)

1.1 The epidermis (Eckert, 1992)

1.1.1 The keratinocyte

The major cell type of the epidermis is the keratinocyte. It comprises more than 90% of the cells of the epidermis layer. As such, it is the cell type responsible for formation of the protective shealth (epidermis) that repels pathogens, guards against fluid loss, and is abrasion resistant. To accomplish this, keratinocytes undergo a programmed process of differentiation in which proliferative, undifferentiated cells are converted to highly differentiated, nondividing cells. The epidermis can be divided into several layers based on the state of keratinocyte differentiation (Figure 1.13), which are basal layer, spinous layer, granular layer, transition zone and cornified layer.

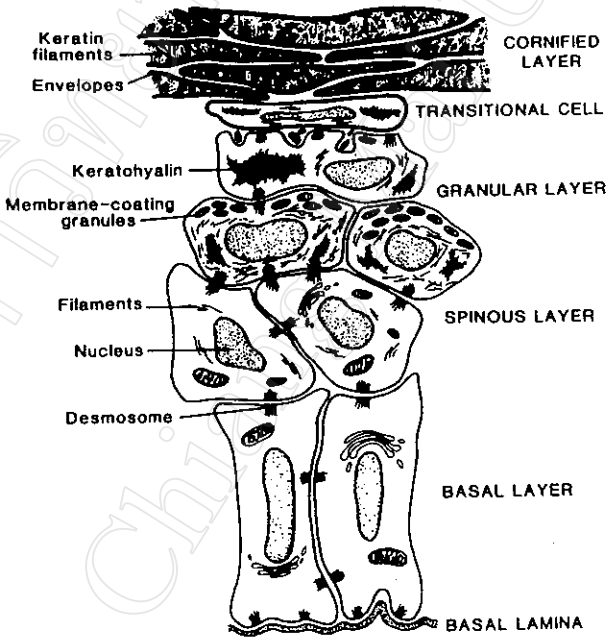


Figure 1.13 : The structure of the epidermis (Eckert, 1992)

A. Basal cell layer

The basal layer consists of a continuous carpet of stem cells (basal cells) that reside along the basal lamina (the border between the epidermis and dermis). These cells are relatively undifferentiated, columnar epithelial cells that are linked to the basal lamina by hemidesmosomes and to each other by desmosomes. Basal cells are relatively undifferentiated cells that lack the biochemical markers that are typical of the more differentiated cells of the upper layers. Basal cells proliferation is regulated by a variety of intrinsic and extrinsic factors and is not completely understood. The composition of the dermis also influences the differentiation of this layer. Grafting experiments indicate that the dermis can directly influence the morphology and differentiation of the epidermis.

B. Spinous layer

The spinous layer is situated directly above the basal layer. It appears spine-like (thus spinous) due to the presence of numerous desmosomes joining adjacent cells. The cells are also biochemically distinct in that they synthesize a different set of macromolecules.

C. Granular layer

The granular layer contains electron-dense keratohyalin granules that contain profilaggrin. These granules pepper this layer and are responsible for its name (granular layer). Granular layer cells are living, as evidenced by intact cellular metabolic function.

D. Transition zone

Between the granular and cornified layer is a "transition zone" between living and dead epidermis. This is a zone of extensive cellular remodeling. Most of the existing cellular organelles, DNA and RNA, are destroyed by the activity of proteases and nucleases in this layer. In addition, the lipid contents of the membrane coating granules are released into the extracellular matrix, the keratin filaments are restructured to a more stable form, and the cornified envelope is formed in this layer.

E. Cornified layer

The terminal stage of keratinocyte differentiation is its metamorphosis into the corneocyte. The corneocyte is a flattened polyhedron that is held to adjacent corneocytes by modified desmosomes and an interdigitating system of ridges and grooves. *In vivo*, the cornified layer is a remarkably highly ordered array of interlocked corneocytes. The major protein component of the corneocyte is keratin that has been structured into macrofibrillar bundles. The second major corneocyte component is the cornified envelope which consists of a covalently crosslinked sheet of protein. Several precursors have been identified that become incorporated into the envelope during crosslinking.

1.1.2 Other cells of the epidermal layer

A. Langerhans cell

The other cell types found in the epidermal basal layer is the Langerhans cell. Although it has been proposed that these cells play a role in the control of the proliferation of keratinocytes, they have since become recognized as the prominent antigen-presenting cells of the skin immune system. As such, their main function appears to be to pick up contact allergens in the skin and present these agents to T-lymphocytes in the skin-draining lymph nodes (Roberts and Walters, 1998).

B. Melanocytes

The melanocytes are pigment-producing cells that originate in the neural crest and are distributed among the basal keratinocytes along the basal lamina. The most distinctive feature of the melanocyte is the melanosome, which is the site of melanin synthesis. Melanocytes transfer pigment to nearby keratinocytes by transfer of the complete melanosome to the keratinocyte. Once within the keratinocyte, single melanosomes or melanosome aggregates exist within a membranous structure. In the upper epidermal layers, the melanosome and pigment is destroyed along with other organelles during terminal keratinocyte differentiation. Thus, most of the skin coloration is the result of pigment present in the lower epidermal layers (Eckert, 1992).

C. The Merkel cell

These cells which are found in the basal layer of SC, can be distinguished from the keratinocytes by their clear cytoplasm and lack of tonofilaments. The cells are closely associated with nerve endings, present on the other side of the basement membrane, which suggest that they function as sensory receptors of the nervous system. Although histochemical evidence demonstrating the presence of acetylcholinesterase suggests a sensory role for Merkel cells, there has been no direct evidence for the release of neurotransmitters. Indeed, acetylcholinesterases have been found in keratinocytes. Despite this lack of confirmation, most researchers in the field agree that Merkel cells play a role in the mechanosensory system, trophic action on peripheral nerve fibers, in stimulating and maintaining proliferation of keratinocytes and release of bioactive substances to subepidermal structures (Roberts and Walters, 1998).

1.1.3 Differentiation in the epidermis (Roberts and Walters, 1998)

The development of the SC from the keratinocytes of the basal layer involves several steps of cell differentiation which has resulted in a structure-based classification of the layers above the basal layer (the stratum basale). Thus, the cells progress through the stratum spinosum, the stratum granulosum, and the stratum lucidum to the SC. Cell turnover, from stratum basale to SC, is estimated to be on the order of 21 days. The stratum spinosum (prickle cell layer), which lies immediately above the basal layer, consists of several layers of cells which are connected by desmosomes and contain prominent keratin tonofilaments. It is clear that the α-keratins of the stratum spinosum are somewhat different from those found in the stratum basale, indicating an initiation in the differentiation process. In the outer cell layers of the stratum spinosum, membrane-coating granules appear. This reflects the border between this stratum and the overlying stratum granulosum. Further keratin differentiation occurs in the stratum granulosum. But, the most characteristic feature of this layer is the presence of many intracellular membrane-coating granules. The assembly of these granules appears to take place in the endoplasmic reticulum and Golgi regions. Within these granules, lamellar subunits arranged in parallel stacks are observed. These are believed to be the precursors of the intercellular lipid lamellae of the SC.

In the outermost layers of the stratum granulosum, the lamellar granules migrate to the apical cell surface, where they fuse and eventually extrude their contents into the intercellular space. At this stage in the differentiation process, the keratinocytes lose their nuclei and other cytoplasmic organelles, become flattened, and are compacted to form the stratum lucidum which eventually forms the SC. The extrusion of the contents of lamellar granules is a fundamental requirement for the formation of the epidermal permeability barrier. The disturbances in this process have been implicated in various dermatological disorders.

1.2 The dermis

The dermis comprises the largest fraction of the skin and is responsible for providing its structural strength. It is comprised mainly of connective tissue. Dermis also provides an environment for nerve and vascular networks and appendages requires to support the epidermis. The main cell types of the dermis are fibroblasts, macrophages and mast cells. Other cells such as lymphocytes and plasma cells frequently populate the dermis in response to injury and other stimuli. The dermis consists of two main layers which are papillary and reticular.

1.2.1 The papillary dermis

It begins below the basal lamina which directly underlies the epidermis. The major cell type of the papillary dermis is the fibroblast. Fibroblasts of the papillary dermis possess a relatively high synthetic and proliferative capability. A major synthetic product in papillary dermis is type III collagen. Some type I collagen is also present. It is organized into small fiber bundles that contrast with the larger type I collagen fiber bundles found in the reticular dermis. Collagenase activity is also localized principally in the papillary layer.

1.2.2 The reticular dermis

This layer underlies the papillary dermis and is superficial to the hypodermis. It is composed primarily of type I collagen organized in large fibrillar bundles. It contains large, fully mature elastic bundles that extend between the collagen fiber bundles. The elastic fibers and collagen bundles progressively increase in size toward the hypodermis.

1.3 The hypodermis

The hypodermis is a layer of mesenchymally derived adipose cells that abut the connective tissue layer of the reticular dermis. It is the innermost layer of skin and its functions to provide a cushion between the external skin layers and the internal structures such as bone and muscle. It also provides an energy reserve, allows for skin mobility, structures of body contours, and insulates the body.

1.4 Skin appendages (Roberts and Walters, 1998)

There are four skin appendages which are the hair follicles with their associated sebaceous glands, eccrine sweat glands, apocrine sweat glands and the nails. Each appendage has different functions.

1.4.1 The hair follicles

They are distributed across the entire skin surface with the exception of the soles of the feet, the palms of the hand and the lips. Each follicle is associated with sebaceous glands which vary in size from 200 to 2000 μm in diameter. The subum secreted by these glands, consisting of triglycerides, free fatty acids and waxes. These excretions protect and lubricate the skin as well as maintaining pH of about 5. The fractional area for these is slightly more than 1/1000 of the total skin surface.

1.4.2 The eccrine and apocrine glands

The eccrine and apocrine glands are about two third and one third of all glands, respectively. The ecrine glands are epidermal structures which are simple, coiled tubes arising from a coiled ball of approximately 100 μm in diameter located in the lower dermis. It secretes dilute salt solution with the pH of about 5. The secretion is stimulated by temperature such as when exercise or in high environmental temperature as well as emotional stress through the autonomic (sympathetic) nervous system. These glands have a total surface area of about 1/10,000 of the total body surface. The apocrine glands are limited to specify body regions such as axillae, nipples and anogenital. The size of these glands is about 10 times of the eccrines. They extend as low as the subcutaneous tissues, and are paired with hair follicles.

1.4.3 The nails

In many respect the nail may be considered as vestigial in man. However, some manipulative and protective function can be ascribed. Certainly, nail plate compositions and layers of flattened keratinized cells fused into a dense but somewhat elastic mass will afford some protection to the highly sensitive terminal phalanx. The cells of the nail plate originate in the nail matrix and grow distally at a rate of about 0.1 mm/day. The chemical composition of the nail plate is not remarkable and is similar to that of the hair. Thus, the major components are keratin proteins with small amounts (0.1% to 1.0%) of lipid, the latter presumably located in the intercellular spaces. The principal plasticizer of the nail plate is water which is normally present at a concentration of 7% to 12%.

1.5 Function of the skin

Skin has the protection, homeostatic and sensing functions with four reasons. First, the protective and homeostasis role of the skin is from its barrier properties in the survival of humans in an environment of variable temperature, water content (humidity and bathing), and presence of environmental dangers such as chemicals, bacteria, allergens, fungi, sharp and blunt objects, radiation such as sun rays. Second, the skin is also a major organ for the maintenance of homeostasis of the body, especially in terms of its composition, heat regulation, blood pressure control and excretory roles. Third, the skin is also a major sensory organ in terms of sensing environmental influences such as heat, pressure, pain, and allergen and microorganism entry. Fourth, the skin is in a continual state of regeneration and repair (Roberts and Walters, 1998).

For barrier function of skin, the SC presents the main barrier to permeability. The other parts of the epidermis and dermis also contribute to the overall resistance and in certain circumstances, can be rate limiting. The SC is the matrix of keratinized cells (corneocytes) surrounded by lipids. The lipids are unique in a number of ways. They consist primarily of cholesterol, free fatty acid and ceramides, and no phospholipids are present. The lipids of the SC are most abundant in the intercellular spaces. The SC lipids exist in multilamellar arrays which form the only continuous medium from top to bottom of this tissue (Potts *et al.*,1992).

1.6 Mechanism of percutaneous absorption

Percutaneous absorption involves the following sequence of events as shown in Figure 1.14.

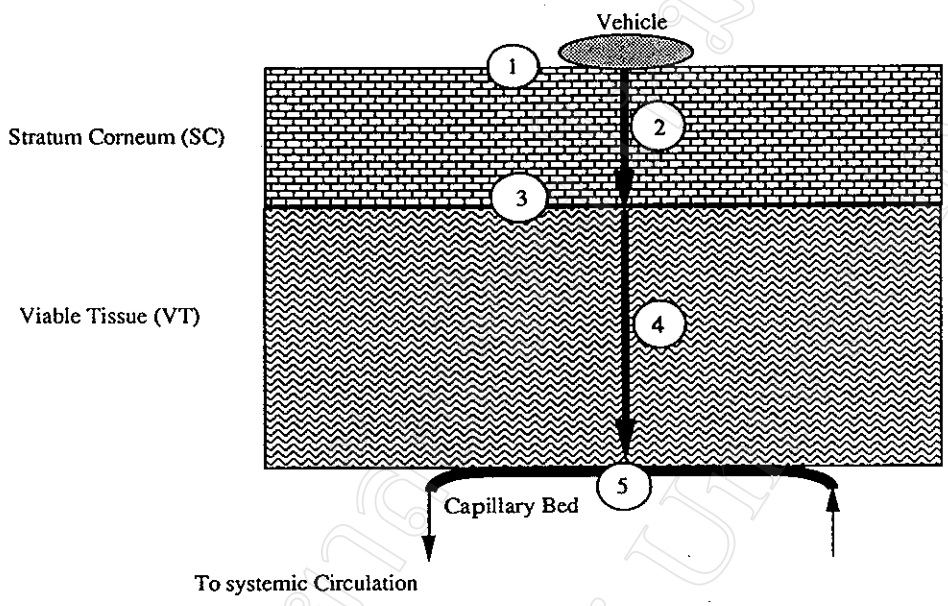


Figure 1.14 : The sequential steps involved in percutaneous absorption. (Potts *et al.*,1992)

1. Partitioning of the molecule into the SC from the applied vehicle phase
2. Diffusion through the SC
3. Partitioning from the SC into the viable epidermis
4. Diffusion through the epidermis and upper dermis
5. Capillary uptake

The route of penetration across the SC can take one or more routes of the routes presented in Figure 1.15. The SC has been likened to a brick wall with the keratinocytes corresponding to the bricks and the intercellular lipids analogous to the mortar(Potts *et al.*,1992). However, it is clear that the major route of penetration across the SC is via the tortuous but continuous intercellular lipid (Roberts and Walters, 1998).

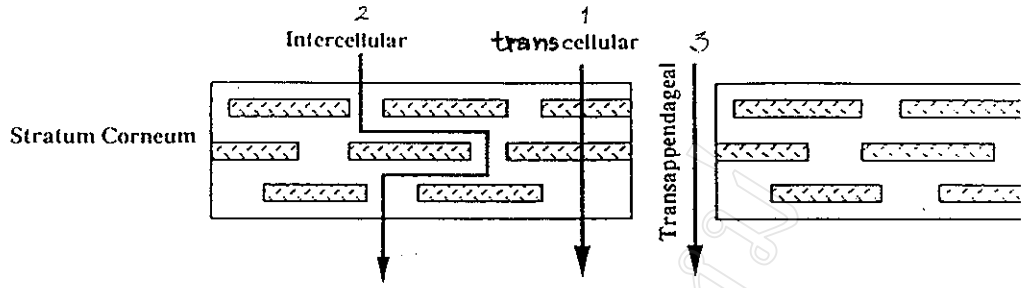


Figure 1.15 : The putative pathways of penetration across the SC (Potts *et al.*,1992)

1. The Transcellular path, indicating that the molecules transfer sequentially and repeatedly through the "bricks" and "mortar"
2. The intercellular path - via the tortuous but continuous intercellular lipids
3. The transappendageal path via hair follicles, etc.

In general, for polar solutes, the diffusional resistance of SC is large compared to that presented by the viable epidermis and dermis. For more lipophilic molecules, the resistance of SC is smaller, due to the larger K (partition coefficient). However, the SC maintains a rate-controlling role since a lipophilic molecule does not favorably partition out of the SC into the more aqueous viable epidermis. In addition, it appears that the permeability of molecules is related inversely proportional to molecular size (Potts *et al.*,1992).

2. *In vitro* skin permeation techniques (Brain *et al.*, 1998)

2.1 *In vitro* skin diffusion cells

The most common method for the evaluation of *in vitro* percutaneous penetration is the use of diffusion cells. The major advantage of *in vitro* investigations is that the experimental conditions can be controlled precisely. The only variables are the skin and the test materials. However, the disadvantage is that little information on the metabolism, distribution, and effects of blood flow on permeation can be obtained.

It is essential to consider the ultimate use of resulting data when developing of experimental protocols. Routinely, simple mathematical models which are based on certain

assumptions or boundary conditions are applied to experimental data. The common used assumptions for diffusion in *in vitro* situation are the following :

1. The receptor phase is a perfect sink.
2. Depletion of the donor phase is negligible.
3. The membrane is a homogeneous slab.

None of these assumptions are wholly true in practice. The potential significance of these imperfections must not be overlooked. Careful experimental desige can be used to achieve a close approximation to reality.

2.1.1 Diffusion cell design

A. Factor affecting diffusion experiment

In vitro systems range in complexity from a simple two-compartment "static" diffusion cell to multijacketed flow-through cells. The construction materials for the cells must be inert. Glass is most common, although teflon and stainless steel are also used. In all cases, excised skin is mounted as a barrier between a donor chamber and a receptor chamber. The amount of the compound permeating from the donor to the receptor side is determined as a function of time. Efficient mixing of the receptor phase and sometimes at the donor phase is essential. The sample removal procedure should be simple. Neither of these processes should interfere with diffusion of the permeant. Continuous agitation of the receptor medium, sampling from the bulk liquid rather than the side arm and accurate replenishment after sampling are important practical considerations. It is essential that air bubbles should not be introduced below the membrane during sampling.

B. Type of diffusion cells

Static diffusion cells are usually of the upright ("Franz") or side-by-side type, with receptor chamber volumes of ~2 to 10 ml and surface areas of exposed membranes of ~0.2 to 2 cm^2. The main difference in the application of these two static cell types is that side-by-side cells can be used for the measurement of permeation from one stirred solution, through the membrane, and into another stirred solution. This is of particular advantage when examining flux from saturated solutions in the presence of excess solid where accumulation of solid on

the membrane surface must be prevented. This type of cell can also be modified to allow the absorption of permeants in the vapor phase. Upright cells are particularly useful for studying absorption from semisolid formulations spread on the membrane surface and are optimal for simulating *in vivo* performance. The donor compartments can be capped to provide occlusive conditions or left open according to the objectives of the particular study. Upright static diffusion cells offer a simple, low-cost and very versatile system which can be employed on a large scale and adapted to meet the particular requirements of a wide range of studies.

C. Characteristics of diffusion cells

A well-designed skin diffusion cell should be as follows :

1. Be inert
2. Be robust and easy to handle
3. Allow the use of membranes of different thicknesses
4. Provide thorough mixing of the receptor chamber contents
5. Ensure intimate contact between membrane and the receptor phase
6. Be maintainable at constant temperature
7. Have precisely calibrated volumes and diffusional areas
8. Maintain membrane integrity
9. Provide easy sampling and replenishment of the receptor phase
10. Be available at reasonable cost

2.1.2 Receptor chamber and medium

The ideal receptor phase provides an accurate simulation of the conditions pertaining to *in vivo* permeation of the test compound. As a general rule, the concentration of the permeant in the receptor fluid should not be allowed to exceed ~10% of saturation solubility. Excessive receptor phase concentration can lead to a decrease in the rate of absorption, which may result in an underestimate of bioavailability. The most commonly used receptor fluid is pH 7.4 phosphate-buffered saline (PBS), although this is not always the most appropiate material. It has been postulated that if a compound has a water solubility of less than 10 μg/ml, then a wholly aqueous receptor phase is unsuitable and the addition of solubilizers becomes necessary. Receptor fluids described in the literature range from water

alone to isotonic phosphate buffers containing albumin and preservatives. One particularly useful fluid is 25% (v/v) aqueous ethanol, which provides a reasonable "sink" for many permeants, whilst removing the need for other antimicrobial constituents.

2.1.3 Preparation of skin membranes

A major potential variant in the design of *in vitro* skin diffusion experiments is the nature of the skin membrane. Animal skins are widely used as substitutes for human skin primarily due to difficulties in obtaining human tissue.

Differently methods can be used to prepare human skin. The membrane can be the following ;

1. Full-thickness skin, including the SC, viable epidermis and dermis
2. Dermatomed skin, in which the lower dermis has been removed
3. Epidermal membranes, comprising the viable epidermis and the SC (prepare by heat separation)
4. SC alone (prepared from 3. by enzyme treatment)

The preparation of epidermal membranes and SC is time-consuming, and the necessary processing increases the possibility of damage to the skin membrane. Careful consideration of the most appropriate type of skin preparation is required. This should address the physicochemical nature of the penetrating species, the data required, tissue availability and the time scales involved. With animal skin, full-thickness membranes are usually used because it is difficult to isolate intact epidermis or SC due to the presence of numerous hair follicles, which may also compromise dermatomed tissue.

Dermatomed skin is usually prepared as follows :

1. The majority of the subcutaneous fat is removed leaving only a small quantity in place
2. The skin is placed dermal side down onto a metal plate
3. The residual fat adheres to the metal and a thin sheet of plastic is placed over the SC to protect it before a second metal plate is applied
4. The two plates are clamped together and cooled to -20 °C until completely frozen
5. The upper plate is gently warmed to ease removal from the SC

6. The plastic sheet is removed and a dermatome is used to remove strips of skin of the desired thickness (usually ranging from 200 to 600 μm). Care must be taken in the preparation of dermatomed skin to ensure that the damage to hair follicles is minimized if these damages are severed, erroneously high penetration may result.

For human skin, the separation technique of the dermis from the epidermis is relatively simple. First, the subcutaneous fat is removed by blunt dissection. The full-thickness skin membrane is then totally immersed in water at 60 °C for 45 sec. Following removal from the water, the skin is pinned with dermal side down, on a dissecting board and the epidermis is gently peeled back using a pair of blunt curve forceps. The epidermal membrane is then be floated onto warm and placed onto a membrane support (membrane or paper filter). The skin is then ready to be mounted in a diffusion cell. To isolate SC from the epidermal membranes, the membrane are placed in trysin solution (0.0001%) and incubated at 37°C for 12 hours. The treated membrane is rubbed (with a cotton bud) to remove the epidermal cells, rinsed in distilled water and air-dried.

2.1.4 Applying of the permeant on the skin

There are two basic approaches on applying samples on the skin. First, the infinite dose techniques which involve the application of sufficient permeants that can make up any changes in donor concentration due to diffusion (i.e., the concentraion is effectively infinite). This condition is desirable if the calculation of diffusional parameters, such as permeability coefficients and the investigation of mechanisms of penetration enhancement are required. Second, the finite dose techniques which is designed to model "in-use" conditions involving application of a dose that may show marked depletion during an experiment. This occurs when the proportion of permeants entering the membrane is large relative to the amount applied. Alternatively, the permeant may be removed from the skin surface during the absorption process, for example, the simulation of a rinsing or washing procedure. The permeation profile of this technique may exhibit the characteristic plateauing effect that accompanies donor depletion. The finite dose technique may also involve the application of permeants and /or enhancers in small volumes of volatile solvents (e.g., ethanol). This allows assessment of the gross effects of enhancers, but results are always more difficult to interpret mechanistically.

2.2 Other *in vitro* skin permeation technique

2.2.1 Skin stripping techniques

Skin stripping using adhesive tape is commonly used *in vivo* and *in vitro*. Tape-stripping experiments are performed as follows :

1. A permeant is applied to the skin surface for a fixed period of time
2. Permeants remaining on the skin surface is removed (where possible)
3. A succession of SC layers are removed by sequential tape strips
4. The permeant contents of the tape strips are determined.

This experiment can be evaluated how the concentration of a permeant applied to the skin surface changes with depth of the skin especially the SC and other epidermal layers. The shape of this concentration VS depth profile is varied with time and type of permeants since the rate and degree of uptake by the SC are dependent on these factors.

2.2.2 Artificial skin techniques

The technology behind the construction of human skin equivalents (i.e. artificial skin) is derived predominantly from research for the treatment of burns. A classification and evaluation of numerous different types of human skin equivalents have concluded that the technique is limited to the reconstitution of the epidermis with SC. Human skin equivalents have been used to investigate both cutaneous metabolic events and dermal irritation with varying degrees of success. At present, the use of artificially cultured skin in permeation experiments has been found to be both expensive and not clearly predictive of *in vivo* results. *In vitro* skin equivalents per se are approximately 10 times more permeable than human skin. However, the reconstructed epidermis transplanted onto a nude athymic mouse had a similar permeability to normal human skin after 1 month.

2.2.3 Autoradiography techniques

Autoradiographic techniques have been used extensively to visualize and quantify penetration through and distribution within animal skin. The general procedure is as follows :

1. A radiolabeled permeant is applied to the skin surface

2. After a suitable time, a skin sample is excised and microtomed perpendicular or parallel to the surface to produce a section of the skin
3. The section is placed in contact with a photographic emulsion
4. Exposure to radiation produces an image showing the pattern of distribution of the radiolabeled within the sample
5. Evaluation of the intensity of the image allows qualitative and quantitative evaluations of routes and degrees of permeation.

The technique is particularly useful in the assessment of attempts to target drugs to specific regions of the skin. For example, compounds that act against acne can be targeted to the pilosebaceous unit.

3. Mechanisms of the liposomal topical drug delivery system (Mezei, 1994)

Most researchers have investigated the potential of liposomes for systemic drug delivery. Only a few studies have been reported on the use of liposomes for drug topical application of drugs. Liposomes have been found to be suitable for localization of topically applied drugs at or near the site of application. The localizing effect is mainly due to the fact that liposomes, especially the MLV types, may act as slow release vehicles.

Results of biodisposition studies in rabbits and guinea pigs indicate that most liposomal formulations in multiple-dose topical treatment provide higher drug concentrations of triamcinolone, progesterone, econazole, minoxidil, retinoids and local anesthetic agents in rat skin than conventional (ointment, cream, gel, or lotion) dosage form. Certain liposomal formulations greatly enhance both dermal and transdermal drug delivery. With appropriate formulations, the drug could be targeted even within the skin.

For previous *in vivo* experimental results and other literature data on the basis of autoradiographic and electron microscope couples, Mezei (1994) has proposed the possible pathways for liposome-skin interactions as illustrated in Figure 1.16. Multi- and unilamellar liposomes adsorbed on the skin surface can intact with the skin before their penetration through the skin. Some liposomes can be ruptured just on the surface of the skin. The penetration of smaller vesicles is more probable, however, it is possible that the intradermally localized uni- or oligolamellar vesicles are derived from multilamellar liposomes that lost their outer bilayers during penetration.

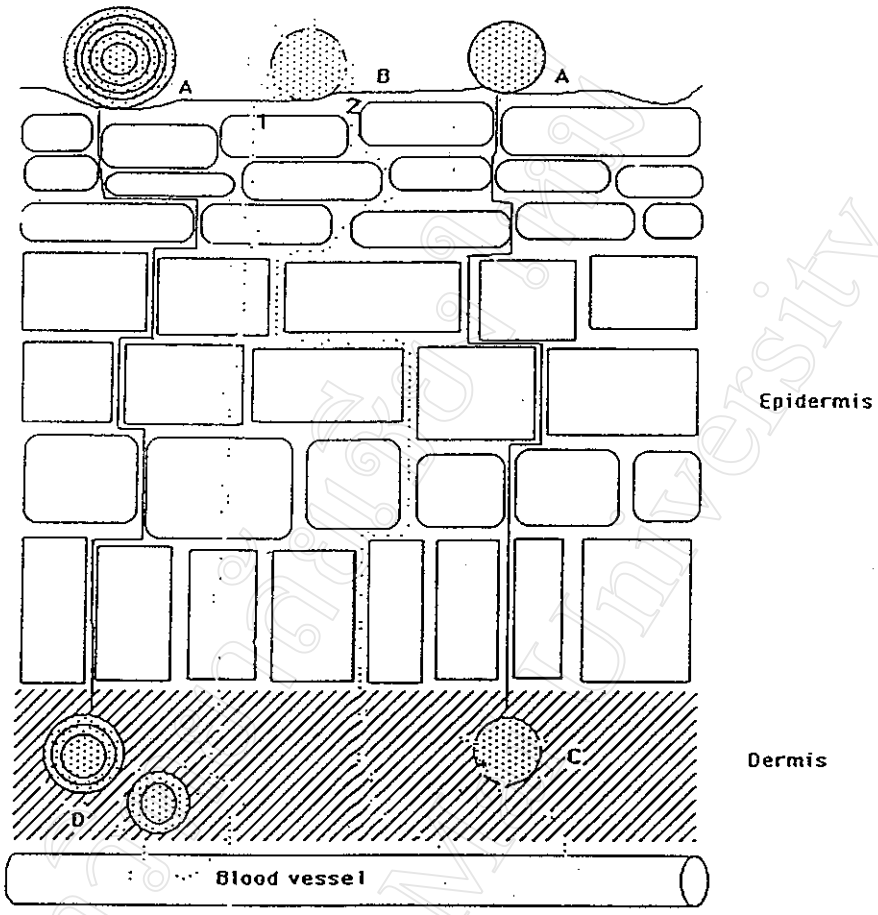


Figure 1.16 : Proposed mechanisms for the interaction of liposomes with the skin (Mezei, 1994)

(A), Adsorption of liposomes to the skin surface ; drug transfer from liposomes to skin.

(B), Rupture of vesicles, release of content, and penetration of free molecules into the skin via (1) intracellular or (2) intercellular route.

(C), Penetration of unilamellar vesicles via the liposome-rich channels to the dermis, where they slowly release their content due to disruption or degradation of liposomal membranes.

(D), Penetration of multilamellar vesicles via the lipid-rich channels.