CHAPTER II

LITERATURE REVIEW

A. Liposomes

1. Definition of liposomes

Liposomes are microscopic spherical lipid vesicles consisting of bilayer membranes covering the aqueous part within. They, first, were described by Bangham, Standish, and Watkins (1965) and initially used as models for studying the biological membranes.

Many substances can be incorporated in either part of liposomes depending on their properties. Hydrophobic substances are entrapped in the bilayer membranes but hydrophillic ones are entrapped in the aqueous parts while amphiphilic ones can be incorporated in both parts as shown in Figure 1 (Strauss, 1989)

2. Classification of liposomes

Liposomes are classified by several persons based on their properties. New (1990b) classified liposomes into four classes on the basis of size and number of lamellae.

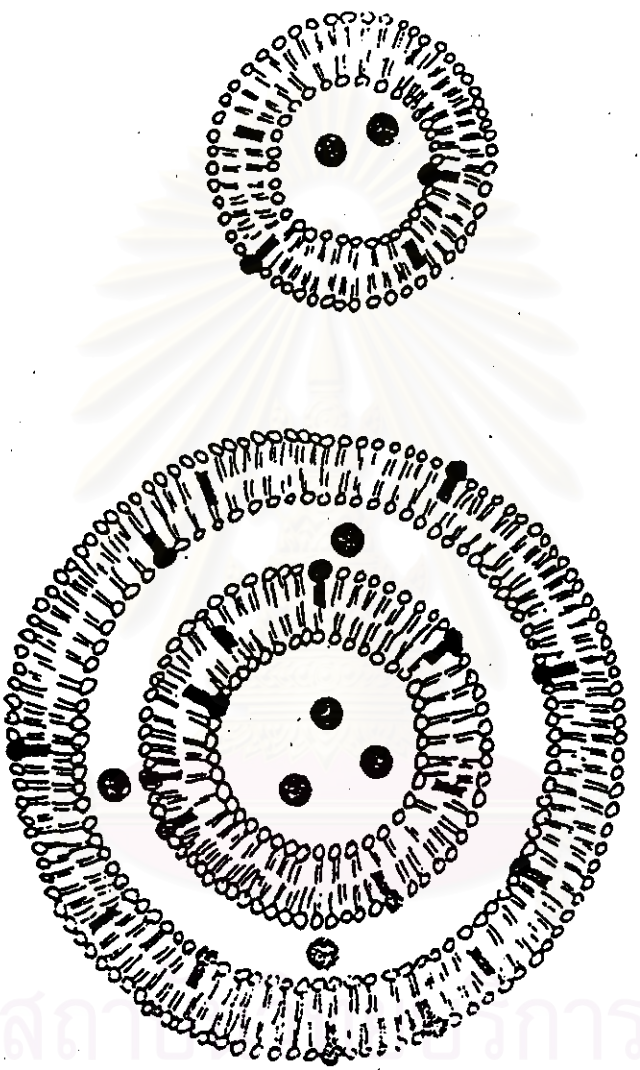


Figure 1 Schematic representations of liposomes and location of hydrophilic, hydrophobic and amphiphilic solutes encapsulated in vesicles as shown.

A : unilamellar liposomes

B : multilamellar liposomes

First, multilamellar vesicles (MLV) are the vesicles of which various number of bilayer membranes, generally consisting of five or more lamellae. Oligo-lamellar vesicles (OLV) or paucilamellar vesicles are the names of vesicles composed of a few concentric lamellae. The major drawback of MLV is low encapsulation capacity for hydrophilic substances expressed in terms of liters of aqueous space per mole lipid (1-4 l/mole). So, hydrophobic substances are more suitable incorporated in MLV. They were entrapped in the bilayer membranes. The sizes of MLV range from 100 nm to 1,000 nm.

Small unilamellar vesicles (SUV) are small spherical shape vesicles with small radius of about 20 nm. Their population are homogeneous and prepared from MLV by simple techniques such as sonication of MLV. However, there are many disadvantages of SUV such as limitation of low encapsulation efficiency, the possibility of asymmetric distribution of various lipids between the inner and outer monolayer and the low ratio of captured volume per mole of lipid.

The third class, large unilamellar vesicles (SUV) are vesicles of which consisting of single bilayer membranes. They were selected for hydrophilic substances by means of the high internal capture volumes. The diameters of these liposomes range up to 1,000 nm.

Intermediate-size unilamellar vesicles (IUV) are the last class classified on the basis of size and number of lamellae. They have diameters in the order of magnitude of 100 nm.

Vemuri and Rhodes (1995) classified liposomes either by the method of their preparations or by the number of bilayers presence in the vesicle or by their sizes.

By the method of preparation, they classified liposomes into three classes, reverse phase evaporation vesicles (REV), french press vesicles (FPV), and ether injection vesicles (EIV) with approximate sizes of 0.5, 0.05 and 0.02 μm, respectively.

Unilamellar vesicles (ULV) and multilamellar vesicles (MLV) are two kinds of liposomes classified on the basis of number of bilayers presence in vesicles. Their approximate sizes are 0.025-0.1 and 0.05-10 μm, respectively.

They classified liposomes into two classes by approximate sizes of 0.025-0.05 μm as small unilamellar vesicles (SUV) and large unilamellar vesicles (LUV) called at approximate size of 0.1 μm.

Recently, Sharma and Sharma (1997) have classified liposomes in terms of compositions and mechanism of intracellular delivery into five types as (1) conventional liposomes; (2) pH-sensitive liposomes; (3) cationic liposomes; (4) immunoliposomes; and (5) long-circulating liposomes as shown in Table 1.

However, the descriptions of liposomes by lamellarity and size are more common than others.

Table 1 Liposomes classification based on composition and mode of drug delivery

Type	Composition	Characteristics
Conventional liposomes	Neutral and/or negatively charged phospholipids plus cholesterol	Subject to coated-pit endocytosis; contents ultimately delivered to lysosomes, if they do not diffuse from the endosome; useful for RES targeting; rapid and saturable uptake by RES; short circulation half-life; dose-dependent pharmacokinetics.
pH - sensitive liposomes	Phospholipid such as PE or DOPE with either CHEMS or OA	Subject to coated-pit endocytosis; at low pH , fuse with cell or endosome membranes and release their contents in cytoplasm; suitable for intracellular delivery of weak bases and macromolecules; biodistribution and pharmacokinetics similar to conventional liposomes.
Cationic liposomes	Cationic lipids: DDAB, DOGS, DOSPA, DOTAP, DOTMA, DMRIE, and DORIE with DOPE	Possibly fuse with cell or endosome membranes; suitable for delivery of negatively charged macromolecules (DNA, RNA, oligos); ease of formulation; structurally unstable; transfection activity decreases with time; toxic at high doses; mainly restricted to local administration.
Long-circulating liposomes	Neutral high T_C lipids, cholesterol, plus 5-10% of PEG-DSPE, GM1 or HPI; ≤ 0.1 μm in size	Hydrophilic surface coating; low opsonization and thus low rate of uptake by RES; long circulation half-life (about 40 hours); dose-independent pharmacokinetics upto 10 μmol/mouse lipid dose.
Immuno-liposomes	Conventional liposomes or long-circulating liposomes with attached antibody or recognition sequence	Subject to receptor-mediated endocytosis; cell-specific binding (targeting); can release contents extracellularly near the target tissue and drugs may diffuse through plasma membranes to produce their effects.

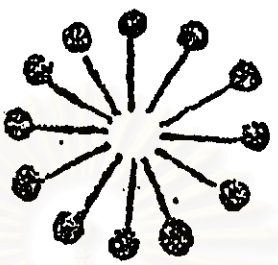
3. Composition of liposomes

The important materials used for preparing liposomes are phospholipids. Phospholipids are one of the major lipid components of all living organism (Neidleman, 1993). They fulfill two functions: the emulsification of water insoluble substances such as in blood and in the digestive tract; the formation of compartments as the major component of biological membranes. They probably affect the oxidative, radical-induced destruction of unsaturated fatty acids in biological membranes. They are one of the most commonly used in many ways, for examples, emulsifiers in foodstuffs, membrane substitutes or surfactant including solution promotors in pharmaceutical products.

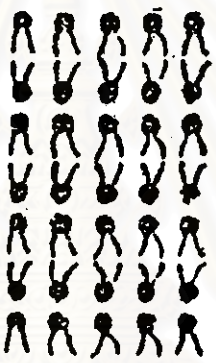
Phospholipids are amphiphilic molecules containing of a highly polar head group, the phosphate group, and a hydrophobic tail. The tail lengths depends on the types of fatty acids present, from 14 to 24 carbon atoms. One of these acids usually contain one or more double bonds, unsaturated, while the others are saturated, no double bounds. Phospholipids were found that they are only soluble in organic solvents such as chloroform, methanol or mixtures of chloroform/methanol. They spontaneously aggregate in water due to their amphiphilic nature (Blume, 1992).

According to the type of phospholipid present, the types of fatty acids bonded, the lipid/water ratio, and the temperature, this process can form one of two very different structures, micelles (as A) or bilayers (as B and C) as shown in Figure 2 (Citernesi and Sciacchitano, 1995).

In the presence of water, phospholipid tends to associate spontaneously into double layers. In each layer, the molecules are closely packed together and



A



B

C

Figure 2 Possible structures formed by phospholipids dispersed in aqueous medium.

A : micelles

B : flat biomolecular layered structure

C : global biomolecular bilayered structure (liposome)

identically aligned so that the polar headgroups are directed towards the surrounding aqueous medium or the internal aqueous space and the hydrophobic fatty acid chains compose the interior of the bilayers. However, the phospholipid tends to form as closely sealed vesicles called 'liposome' in order to minimize the unfavorable interaction between the bulk aqueous phase and the long hydrocarbon fatty acid chains.

Two sorts of phospholipids exist : phosphodiglycerides and sphingolipids, together with their corresponding hydrolysis products. Phosphatidylcholine molecules (PC) are the most common phospholipids known as ' lecithin'. Lecithin can be derived from both natural, animals like egg yolk or plants like soybean, and synthetic sources (New, 1990c).

Phospholipids of animal (hen egg) or vegetable (soybean) origin can be used to prepare phospholipid liposomes. While animal phospholipids contain mainly unsaturated fatty acid, phospholipid from soybean primarily contain polyunsaturated fatty acid. The chemical structure and composition of phosphatidylcholine from soybean was shown in Figure 3 and Table 2, respectively. This showed that it contained linoleic acid greater than 70% (Nattermann Phospholipid GmbH, 1995a).

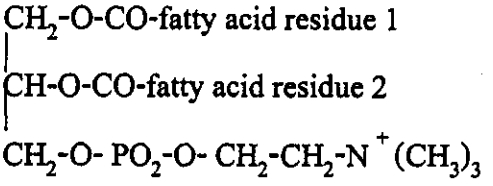


Figure 3 Chemical structure of soybean lecithin

Table 2 Composition of phosphatidylcholine from soybean

Fatty acid	Fatty acid 1 (%)	Fatty acid 2 (%)	Total (%)
C 16:0 palmitic acid	24.0	1.7	12.9
C 18:0 stearic acid	7.9	1.0	4.4
C 18:1 oleic acid	10.9	10.0	10.5
C 18:2 linoleic acid	52.4	80.6	66.5
C 18:3 linoleic acid	4.7	6.7	5.7

The transportation of linoleic acid to skin in large amounts by means of soybean-derived phosphatidylcholine created new aspects for the treatment of, for example, skin inflammation, since this essential fatty acid exhibits strongly suppressive effects on the oxygen metabolism and the phagocytosis activity of neutrophils (Ghyczy and Gareiss, 1994).

The purity of phosphatidylcholine is very important for the formation of liposomes, loading capacity and stability structure. It was found that there was only 4% difference in loading capacity between liposomes prepared from 99% phosphatidylcholine and ones prepared from 90% of phosphatidylcholine. Besides this, the difference of liposomes in term of stability was no significant (Memoli et al, 1993).

Cholesterol is added in liposomes in order to improve the fluidity of bilayer membranes by reducing the permeability of water soluble molecules through the membranes.

Cholesterol could not form liposomes by itself. Its molecules attach among the phospholipid molecules as it turns its hydroxyl group facing towards the water phase, the tricyclic ring sandwiched between the first few carbons of the fatty acyl chains, into the hydrocarbon core of the bilayer. Incorporation of cholesterol increased the rigidity of fluid state liposome bilayers (New, 1990d).

Cholesterol reduced the interactions between some substances in biological fluids such as blood and plasma (Kirby, Clarke, and Gregoriadis, 1980).

Addition of charged lipids affected the preparation of liposomes. The negatively charged lipids such as phosphatidyl serine, phosphatidyl inositol, or phosphatidyl glycerol or positively charged adjuvants such as stearylamine had a propensity to increase the interlamellar distance between successive bilayer and thus provide greater encapsulation ability. In addition, the presence of charged moiety in the lipid formulation also reduced the probability of vesicular aggregation following the formation of MLV. Generally, as low as 10-20 mole% of charged species was used to provide the charge effect in the preparation of MLV (Weiner, Martin, and Raiz, 1989; Vemuri and Rhodes, 1995)

Stearylamine improves the affinity of liposomes to attach with the negatively charged sites on the bacterial surface. Recently, Sanderson and Jones (1995) have reported the strong affinity of stearylamine-containing liposomes for *Staphylococcus epidermidis* bacteria. *Staphylococcus epidermidis* bacteria originate and spread within hospital themselves. The skin around the insertion site for the implant is the most common source of the infecting bacteria. The interaction between positively charged liposomes and the bacterial biofilm seemed to be an electrostatic nature and involved negative charges associated with the bacterial biofilm. Such these

vesicles could be used to deliver agents such as vancomycin, an antibiotic which is used to treat the patient infected by bacteria with hopefully minimizing the undesirable side effects of the free drug.

α-Tocopherol added in the preparation of liposomes acted as an antioxidant. It protected phospholipids from oxidative degradation reaction. It improved membrane rigidity and more hydrophobic. It was found that 0.1 mole% of α-tocopherol doubled the shelf-life of multilamellar liposomes containing no cholesterol that were stored in air and light at 22°C (Anthony and Tsang, 1981). However, it was found that the efficiency of inhibition of oxidation by α-tocopherol in liposomal membranes was lower than in homogeneous solution. It might be attributed to lower mobility of α-tocopherol and, more importantly, to higher chance of propagation in the tightly packed structure of liposomal membranes (Niki, Takahashi, and Komuro, 1986).

On widespread regulatory acceptability, α-tocopherol is of value in oil or fat-based pharmaceutical products and were normally used in the concentration range of 0.001-0.05% (Wade and Weller, 1994a).

4. Preparation of liposomes

There are various methods for preparing liposomes. Briefly, all methods of preparing liposomes involved three or four basic stages: drying down of lipids from organic solvents, dispersion of the lipids in aqueous media, purification of the resultant liposomes, and analysis of the final product (New, 1990a).

4.1 Mechanical dispersion

Basically, the lipids are dried down onto the solid support and shaken after adding an aqueous medium. After that the lipids generally form multilamellar liposomes. The major drawback of this group of method is the low entrapment efficiency, only about 5-10%, for hydrophilic substances in the aqueous part of liposomes but up to 100% entrapment efficiency for lipophilic substances.

4.1.1 Hand-shaken multilamellar vesicles (MLV)

This method is the simplest and most widely used for preparation of multilamellar vesicles. Briefly, the lipid mixture is dissolved in the organic solvent in a round bottom flask. The thin film is deposited on the wall of the flask after evaporating of organic solvent by rotary evaporator under reduced pressure. An aqueous buffer is added to the system and then it is hydrated above the transition temperature of the lipids. A homogenous liposomes suspension is formed after shaking vigorously by hand or vortex. The final liposomes have a wide range of size, number of lamellae and low entrapment efficiency.

4.1.2 Non-shaken vesicles

This method was developed by Reeves and Dowben (1969). The thin film was dispersed in distilled water or an aqueous solution of non-electrolyte. Introducing of ions with a net charge between the bilayer membranes increased the intermembrane attraction and prevented their separation from each other during swelling. In this method, the hydration and swelling processes were carried out in two separate steps. The hydration was initiated by exposing the dry thin film to

the stream of water saturated with nitrogen and followed by swelling process in the aqueous medium without shaking. The resulting liposomes are large unilamellar or oligolamellar liposomes with size about 0.5 to 10 μm in diameter.

Lasic, Belic, and Valentincic (1988) improved a method for the instant preparation of large unilamellar vesicles. They suggested that this method was extremely simple, rapid and avoidance of all potentially harmful treatments. In this method, the finely-etched silicon wafer was used as the supporting of the thin film. Because of its irregular surface with many different angles and planes, the lipid was broken up into small sheets upon hydrating. This may help to define the size of the final liposomes.

4.1.3 Pro-liposomes

Proliposomes were first prepared by Payne et al (1986). They described a procedure for preparing proliposomes with approximate size of 0.1 μm in diameter. The final product obtained was a free flowing granular product which on addition of water produced isotonic multilamellar liposomal suspension. Briefly, lipids and drug were coated on the inert carrier materials, like sorbitol and sodium chloride, which should have good water solubility and poor solubility in organic solvent with two advantages, ease of liposome hydration and ease of processing, respectively. Sorbitol was found that it was the best inert carrier material for this preparation. When the liposomes were hydrated with water, sorbitol would be dissolved and the isotonic solution was formed. This method may be suitable for preparing liposomes in the large scales and pro-liposomes obtained can be stored in dried form in sealed vials before using by reconstitution with water.

A new method for preparing multilamellar vesicles from the initial pro-liposomes preparation was developed by Perrett, Golding, and Williams (1991). This method initiated with dissolving of lipid mixture in warmed alcohol and aqueous buffer. This mixture was then warmed again on the water bath and allowed to cool to the room temperature. Finally, the proliposomes mixture was converted to stable liposomes at approximate size of 0.5 μm in diameter after diluting with excess aqueous phase. This method is simple and avoids the pharmaceutical unacceptable solvents and the sonication.

4.1.4 Processing of lipids hydrated by physical means

Due to the properties of MLV like size and heterogeneity in population, processing of method to prepare MLV is modified to reduce their size.

4.1.4.1 Micro-emulsification liposomes (MEL)

Brandl et al (1990) have described the use of a high pressure homogenizer, Gaulin Micron, to prepare small unilamellar or small multilamellar vesicles which have narrow particle size distribution. This is a single step liposomes preparation method. Lipid composition in the form of lipid dispersion was introduced into the homogenizer. The fluid collected can be recycled through the pump and interaction chamber until vesicles with the required dimensions are obtained.

A microfluidizer used to produce liposomes in the large scale was developed by Vemuri et al (1990). The liposomes obtained was the small size multilamellar vesicles. After three times passing through the microfluidizer, the

mean size of the liposomes reduced drastically from 0.64 μm to 0.16 μm. They could produce the liposomes from 100 ml to 4,000 ml (Vemuri et al, 1990). Although this method is easy to scale up the liposomal production, but there is the potential problem, the contamination of the dispersion with small amounts of metal particles after production (Talsma et al, 1989).

4.1.4.2 Sonicated vesicles

This method is most widely used for preparing small vesicles by the exposing of MLV to ultrasonic irradiation. Either a probe or a bath-type sonifier can be used as the machine to prepare liposomes (Yotsuyanagi, Nonomura, and Ikeda, 1981).

Probe sonication is used for suspensions requiring high energy in a small volume and it is a good method for reducing the size of large liposomes. However, there are some disadvantages in a risk of lipid degradation due to the high intensity of the input energy and the contamination of metal particles from the probe.

Bath sonication is more suitable for large volumes of diluted lipids and much milder in the intensity of input energy than probe sonification. The advantages for this method are the maintenance of sterility of the suspension, free from metal fragments and lower risk of lipid degradation. There are some disadvantages in the time-consuming and a low yield of liposomes.

However, there was a simpler method to prepare small vesicles described by Barenholz et al (1977). This method is based on the differential

high-speed ultracentrifugation. Its advantage is a higher yield of vesicles without dilution and the rapidity of preparation.

4.1.4.3 French pressure cell liposomes

This method was developed to solve the problem of sonication. This method involved the introducing of aqueous suspension of liposomes passing through a french pressure cell by rapid extrusion at high pressure. The resulting liposomes are rather homogenous unilamellar or oligo-lamellar vesicles of intermediate sizes (30-80 nm in diameter, depending on the pressure used). Its advantages are simple, rapid, high entrapment efficiency resulting from using of higher lipid concentration (Barenholzt, Amselem, and Lichtenberg, 1979; Robert et al, 1980).

4.1.4.4 Liposomes produced by membrane extrusion

This method required lower pressure than the french pressure cell. It is a more gentle method to reduce the size of liposomes by passing them through a membrane, usually polycarbonate membrane, filter of defined pore size which limit the upper size of the resulting liposomes. This method can be used to prepare LUV as well as MLV. Diameters of liposomes are decreased and more homogeneous size distribution is received (Olson et al, 1979).

4.1.4.5 Dried-reconstituted vesicles (DRV)

This procedure involves to disperse the solid lipid in finely divided form before contact with the aqueous fluid. A suspension of empty

SUV is frozen and lypophilized. This is rather like the proliposomes without the support, which, on addition of water , can rehydrate, fuse and reseal to form vesicles with a high capture efficiency. Liposomes suspensions of several hundred milligrams per millitre can be obtained routinely in this way (New, 1990e).

4.1.4.6 Freeze-thaw sonication (FTS) method

A freezing and thawing process is used to rupture and refuse SUV (Oku and MacDonald, 1983; Mayer et al, 1985). During the process, the solute is equilibrated between the inside and outside of liposomal membrane and the liposomes themselves fuse and increase in size which resulting in enhancement of entrapment efficiency due to the rising of their entrapment volume to 30% of total volume of the suspension. Several disadvantages compared with DRV method were found that it is impossible to prepare neutral liposomes because of the requiring of charge exist to aid in the rupture/fusion process. However, the method is still very simple, rapid and mild for entrapped solutes and results in a high proportion of large unilamellar vesicles.

4.1.4.7 pH-induced vesiculation

Hauser and Gains (1982) described the method to produce unilamellar vesicles without using sonication of high pressure. It is a simple method with changing the pH. This is an electrostatic phenomenon. The transient change in pH increases the surface charge density of the lipid bilayer ; provided this exceeds a threshold value, spontaneous vesiculation will occur. The liposome suspensions obtained are small unilamellar vesicles with diameters of 20 - 60 nm.

4.1.4.8 Calcium-induced fusion to produce large unilamellar vesicles

A potential method for creating very large unilamellar vesicles has been suggested by Papahadjopoulos et al (1975). In this method, calcium is added to small unilamellar vesicles in order to induce the aggregation and fusion. It causes the formation of large, cylindrical, and folded multilamellar structures in a cochelate cylinders, a spiral configuration. The incubation with EDTA and the removal of calcium, the large and closed spherical unilamellar vesicles are formed. The disadvantage of this method is the requirement for acidic phospholipid and the inevitable presence of calcium inside the liposomes.

4.2 Solvent dispersion

Basically, the lipids are dissolved in an organic solution, which is then brought into contact with the aqueous phase. At the interface between these phases , the lipids align themselves into a monolayer which forms the basis for half the bilayer the liposomes.

4.2.1 Ethanol injection

This method first described by Batzri and Korn (1973). The final preparation was single bilayer liposomes with approximate 25 μm in diameter. The process initiated with the rapid injection of an ethanolic solution of phospholipid into an aqueous medium through a fine needle. The advantages of this method are fast, highly reproducible, unlimited amounts of liposomes used in the preparation, and less risk in chemical degradation of lipid. However, its major drawbacks are the

low entrapment efficiency of drug and the difficulty of the removal of ethanol from the liposomal preparations.

4.2.2 Ether injection

This method basically involves the injection of a solution lipid dissolved in ether into an aqueous phase containing material required to be encapsulated through a narrow bore needle at a temperature which is higher than the boiling point of ether. At this temperature, ether is removed by rapid vaporization during the process (Deamer and Bangham, 1973). It yields unilamellar liposomes with average size of 150-1,200 nm in diameter. This method is very gentle for lipids with very little risk of causing oxidation. Its disadvantages are that it takes long time to produce a batch of liposomes and the careful control needed for introduction of the lipid solution.

4.2.3 Water in organic phase

This group of liposomes is prepared with a basic principle of two steps. First, the inner monolayer is assembled enclosing the internal aqueous compartment, then the outer monolayer is enveloped around the droplet and the intervening solvent is removed.

4.2.3.1 Double emulsion vesicles

In the process of this method, the outer leaflet of the liposome membrane is created at a second interface between two phases by emulsification of an organic solution in water. When the organic solution which

already contains water droplets is introduced into excess aqueous medium, followed by mechanical dispersion, a multi- compartment vesicle (water-in-oil-in-water system) is obtained. Removal of the organic solvent results in intermediate-sized unilammellar vesicles. The theoretical entrapment yield is 100%, and can approach this value on occasions, depending on the nature and concentration of the material to be entrapped (New, 1990f).

4.2.3.2 Multivesicular liposomes (MVL)

This method was described by Kim et al, (1983). The resulting product was called multivesicular liposomes (MVL) with high encapsulation efficiency. They can be produced by the evaporation of chloroform-ether solution, containing with the combination of amphiphilic lipids and a small amount of triglycerides, which suspended in water by a stream of nitrogen. After that, MVL gradually formed and the resulting size range from 5.6 to 29 μm with the high percentage of entrapment, up to 89%.

4.2.3.3 Reverse-phase- evaporation vesicles (REV)

This method was developed for the preparation of LUV and OLV with the size and number of lamellae being dependent upon the nature of lipid mixture (Szoka and Papahadjopoulous, 1978; Szoka et al, 1980). The various drugs and biological active material can be loaded in liposomes prepared with this method. The encapsulation efficiency of drug is up to around 50%. There are many advantages such as the high entrapment efficiency for hydrophilic compounds, the ease of scale up and the almost homogenous size of liposomes. The disadvantage of this method is the difficulty for reproducing on a large scale production due to the

need for sonication of the mixture of lipid solution and aqueous drug solution to form a suitable emulsion. The other one is that it is difficult to entirely remove the organic solvent from the preparation and the requirement of high temperature for evaporation organic solvent under reduced pressure which is not suitable for some labile drugs.

4.2.3.4 Stable plurilamellar vesicles (SPLV)

The process of this method initials with the preparation of a water in oil dispersion with the excess lipid. After that, the drying down process, carried out using a stream of nitrogen, is continued by bath sonication, during which redistribution and equilibration of aqueous solvent and solute occur between the various bilayer in each plurilamellar vesicles (Gruner, 1985). This is the suitable method for hydrophilic substances because of the internal structure with large aqueous core, the located part of aqueous molecules between adjacent lamellae. The percentage entrapments are normally around 30% which is higher than MLV of about 60% and SPLV without leakage after 15 months while MLV leaked in a few days.

4.2.4 The polyol dilution method

Yamauchi et al (1994) have described a new method for preparing liposomes. It was considered for the industrial scale production of liposomes with good reproducibility. The method makes up with mixing membrane components with a water-soluble, non-volatile organic solvent (e. g. glycerin, propylene glycol), and then, dispersing this mixture in an aqueous medium and results in liposomal preparation. They prepared liposomes containing adriamycin with size of 100 nm and 100% of the encapsulation efficiency before lyophilization.

4.3 Detergent solubilization

In this method, detergent is used as an intermediate substance for phospholipid and aqueous phase. The detergent molecules are associated with phospholipid molecules in form of micelles.

In general, membrane-solubilizing detergents have a higher affinity for phospholipid membranes than for the pure detergent micelles. So, when detergent is added in increasing amounts to the membrane preparation, more and more detergent will be incorporated into the bilayer, until a transition from the lamellae to the spherical micellar phase configuration occurs. Techniques which cannot remove other small water soluble materials such as dialysis and column chromatography, are used for the removal of detergent Triton-X, bile salt, and alkyl glycoside which are the most common detergents used (Brunner, Skrabal, and Hauser, 1976; Rhoden and Goldin, 1979). The advantages of this method are; the ability to incorporate lipophilic proteins into mixed micelles up to 100% with non-denaturation; and ability to vary the size of liposomes by controlling the removal of detergent (Zumbuehl and Weder, 1981).

5. Liposomes and skin

5.1 Drug distribution in the skin

Skin is generally described in term of three tissue layers: the epidermis, the dermis, and the subcutaneous fat tissue as shown in Figure 4 (Chien, 1992).

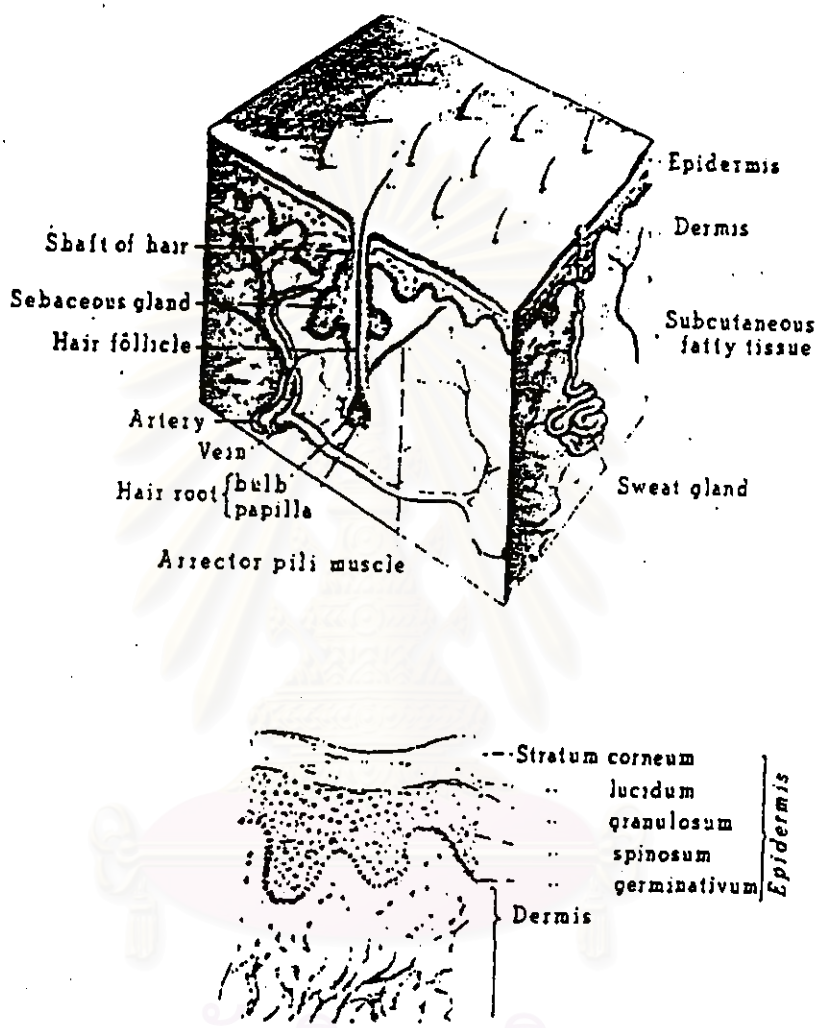


Figure 4 Three dimensional view of the skin showing various skin tissue layers and appendages and cross-sectional view of various epidermal layers and the dermis.

Epidermis is the outer layer of the skin composed of stratified squamous epithelial cells. The two main parts of the epidermis are the stratum corneum and the stratum germinativum. The stratum corneum acts as primary barrier to percutaneous absorption. It is poorly permeable to water and aqueous solutions and also an efficient permeability barrier for lipophilic substances.

Dermis is 10-20 times thicker than the epidermis. The structure of dermis is made up of a network of robust collagen fibers of fairly uniform thickness with regularly spaced cross-striations. The network structure is responsible for the elastic properties of the skin. Dermis is often a preferred site for the dermatological drug delivery while the stratum corneum is seldom a goal of therapeutic drug applications, except when certain fungal and bacterial infections are to be eradicated. The subcutaneous tissue is a sheet of fat-containing areolar tissue attaching the dermis to the underlying structure.

Mechanisms of various kinds of substances molecules after applied on the skin are different (Ceve, 1992). The diffusion of amphiphilic agents is only little hindered by the intermediate aqueous or fatty compartments. Amphiphilic molecules have a responsible high propensity for a rapid and homogeneous redistribution in composite lipid-water systems over distances in the order of several micrometers.

Lipophilic substances are less favorable. Their molecules can only diffuse relatively freely through, or accumulate in the lipidic sub-compartments. It is very difficult to penetrate passing any water-filled region, a high permeability barrier.

In contrast to lipophilic substances, hydrophilic ones can diffuse nearly freely in the water-filled region but have a strong resistance to permeate through the lipid region, stratum corneum.

5.2 Liposomes as carriers into skin

Liposomes were lipid vesicles selected using in the field of topical dermal applications (Mezei and Gulasekharam, 1980; 1982; Wohlrab and Lasch, 1987; Gesztes and Mezei, 1988). They entrapped either lipophilic drugs or hydrophilic drugs in the bilayer membranes and aqueous parts, respectively. While amphiphilic ones can be incorporated in the both parts of liposomes.

Mechanisms of liposomes as carrier into skin were discussed by many investigators. Some investigators (Foldvari, Gesztes, and Mezei, 1990) explained that there were more than one mechanism occurred in the liposomes-skin interaction process and they proposed a cascade of events as shown in Figure 5.

1) Multi- and unilamellar liposomes could be absorbed to the skin surface intact before their penetration into the skin and the release of the free drug by diffusion (as A) or rupture of the vesicles (as B) resulting in direct drug transfer from liposomes to the skin. The free drug molecules penetrated the skin via either intracellular (1) or intercellular (2) pathway.

2) Penetration of unilamellar liposomes were described in two ways. First, they could rupture on the surface of the skin and they slowly released their contents drugs via intracellular and intercellular pathway due to disruption or degradation of liposomal membranes (as B). The other one, they penetrated through

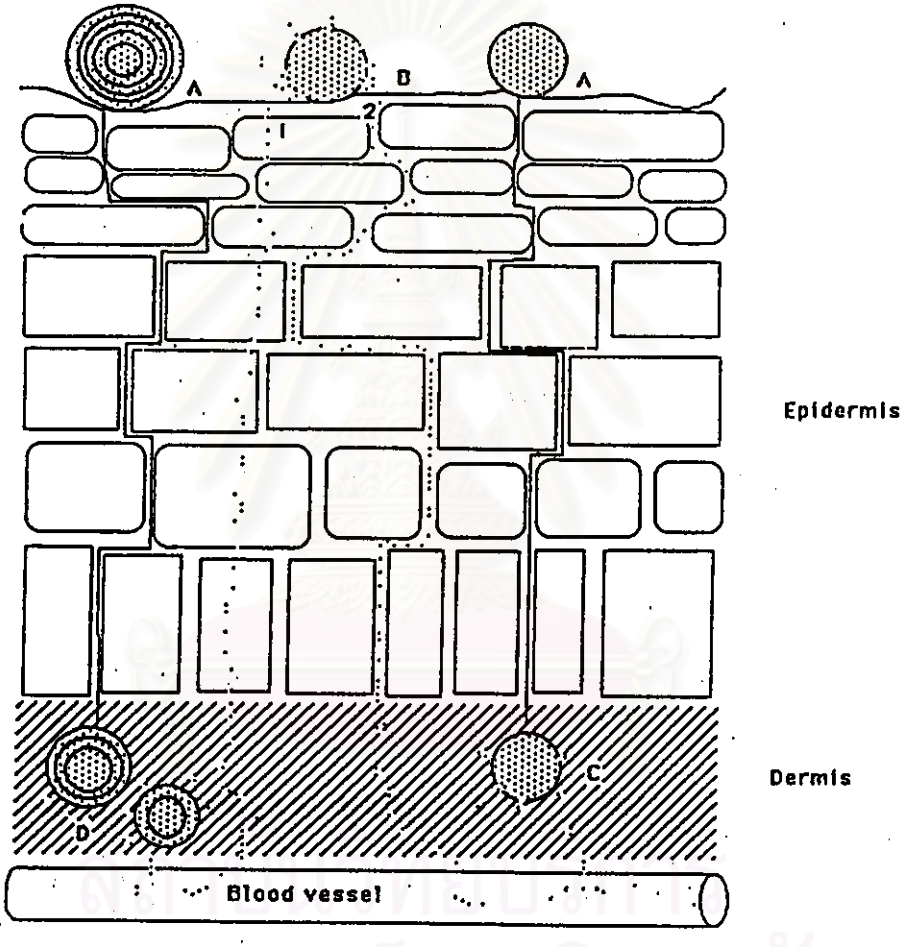


Figure 5 Proposed mechanisms for the interaction of liposomes with the skin.

pore of epidermis cells and acted as a depot of drug in the dermis (as C).

3) Multilamellar liposomes can be absorbed to the skin surface intact before their penetration into the skin through the intercellular of epidermis cell (as A). They have the elasticity for penetration through the smaller distance of pore of skin but the lamellae were decreased and they would be new liposomes like oligolamellar or unilamellar liposomes (as D). Lipophilic drugs encapsulated in liposomes were also released in this pathway.

The other interesting mechanism of liposomes penetrating into skin described by Bouwstra et al (1992). They explained that the interaction between lipid membranes of liposomes and stratum corneum changed the stratum corneum. They also found that only liposomes with high amount of phosphatidylcholine could interact with the corneocyte of stratum corneum. The higher amount of phosphatidylcholine prepared liposomes, the higher change of the skin.

In 1992, however, Ceve and Blume described that lipid vesicles could penetrate through intact skin by a dehydration force resulting from an osmotic gradient between the skin surface and the deeper skin tissues.

Recently, transfersomes were described (Ceve, Schatzlein, and Blume, 1995). The movement of transfersomes through the pore was so high. They with an average diameter of 500 nm could be transported through the pores 5 times smaller, nearly as rapidly and efficiently as pure water. They penetrated the skin permeability barrier spontaneously.

5.3 The penetration of liposomes into skin

Discussing about the penetration of liposomes into skin were referred. Some investigators (Artmann et al, 1990a; 1990b) observed that when applied liposomes-encapsulated antibodies with molecular weights between 20,000 and 50,000 Da, they distributed rapidly into deep cutaneous region, whereas antibodies did not penetrate into skin while applied as aqueous solution. They confirmed with using $[^{14}S]$ heparin and ^{99m} technetium as marker.

Some investigators (Lasch, Laub, and Wohlrab, 1991) found that liposomes with complete structures could only penetrate into the stratum corneum, they could not penetrate into the deeper skin.

In contrast to this, transfersomes could carry a hydrophilic fluorescent marker normally got into the skin in large quantities when applied in the form of a liposomal suspension, rhodamine 123, transporting extensively and reached a depth of at least 30 μm of the skin tissues (Ceve, Schatzlein, and Blume, 1995).

5.4 Factors affecting the penetration of liposomes into the skin

1) Type and size of liposomes

It was found that unilamellar liposomes could carry drug into the skin much more than multilamellar liposomes while there was no significant difference in the depth of liposomes in the skin tissue (Michel et al, 1992). So, it was possible that unilamellar liposomes, small size liposomes, would be the suitable liposomes for penetrating with their contents into the skin.

In the contrast to this, Du Plessis et al (1994) found that smaller size liposomes did not penetrate in the large quantities more than the bigger ones. Only suitable size of liposomes could penetrate into the skin. They found that liposomes with size of 0.3 μm brought Cyclosporin-A through into the deeper skin strata more than liposomes with size of 0.06 μm.

2) Type and charge of selected phospholipids

It was found that the interaction between phospholipid and stratum corneum depending on the saturation of phospholipid selected. Because of their fluidization effect, liposomes prepared from unsaturated soybean phosphatidylcholine interact with stratum corneum in more rapid rate and depth of the skin than saturated soybean phosphatidylcholine ones (Nattermann Phospholipid GmbH., 1996).

Positive charged liposomes could be suitable for carrying drug into the skin because they could interact with stratum corneum more than neutral liposomes or negative charged liposomes. For example, there was a large quantities of retinoic acid released from liposomes with the composition of positive charge lipid, stearylamine, more than from ones with the composition of negative charge lipid, dipalmitoylphosphatidylserine, and from neutral liposomes (Montenegro et al, 1996).

3) Type of substances encapsulated in liposomes

Substances like to attach with the bilayer membranes, lipophilic substances, can penetrate into skin in a small volume because there are more water in the deeper skin than in the stratum corneum. So, it looked like that the increasing of

quantities of lipophilic drug encapsulated in liposomes found in the deeper skin was smaller than ones of hydrophilic drug (Du Plessis, Weiner, and Muller, 1994).

4) Additives (Nattermann Phospholipid GmbH, 1996)

Some additives added in liposomal formula such as oleic acid and propylene glycol are penetration enhancers of substances or drugs into the skin. The oleic acid/phosphatidylcholine-complex is delivered to the skin and the oleic acid is integrated into the structure of the lamellar lipid barrier. Due to the sterical orientation of its molecule, oleic acid has got beside its emulsifying properties also the function of a spacer. The stratum corneum lipid structure is thereby loosened in an efficient way.

Propylene glycol sustains the interaction of liposomes with the lipid barrier part of the stratum corneum by minimizing electrostatic interaction between charged liposomal formulations and keratinocytes remnants in the stratum corneum.

6. Stability of liposomes (Weiner, Martin, and Riaz, 1989; New, 1990g)

Liposomes could change upon storage. Chemical and physical degradation were the reactions found in liposomal preparations.

Chemical degradation of phospholipid such as hydrolysis and oxidation usually occurred upon storage. These reactions could cause dramatic changes in the permeability properties of liposomes.

Due to unsaturated acyl chains as part of the molecular structure, these were vulnerable to oxidative degradation or lipid peroxidation which could occur during preparation, storage or actual use. These could be retarded by the addition of α-tocopherol into the lipid membranes. The antioxidant in most common used at the present time is α-tocopherol because it was a common non-toxic dietary lipid, although it had been suggested that β, γ, and δ-tocopherol might be more effective as long term antioxidant (Anthony and Tsang, 1981).

Lipid hydrolysis of lecithin occurs from the ester bond at the C position of the glycerol moiety resulting in the degradation product called 'lyso-phosphatidylcholine' (lyso-PC). The presence of lyso-PC in bilayer membranes greatly enhanced the permeability of liposomes and caused in the increasing of liposomal sizes. Beside this, the formation of lyso-PC in the micellar form could fuse with liposomal membranes instead of the penetration into membranes. This also decreased the packing of phospholipid membranes and caused in the increasing of liposomal sizes (Inoue and Kitagawa, 1974)

Physical degradation such as leakage and fusion of vesicles could occur as a result of lattice defects in the membrane introduced during their manufacture.

Aggregation and fusion of vesicles of neutral liposomes were brought about by Van der Waals interaction, and tended to be more pronounced in large vesicles. Small unilamellar vesicles were prone to fusion by means of relieving stress arising from the high curvature of the membrane. The presence of charged lipids, negatively charged lipids such as phosphatidyl serine and phosphatidyl inositol, or positively charged lipid such as stearylamine, at the concentration 10 to 20 mole%

was added in order to reduce likelihood of aggregation of multilamellar liposomes (Weiner, Martin, and Riaz, 1989; New, 1990g; Kulkarni, Betageri, and Singh, 1995).

B. Ibuprofen

1. Physicochemical properties (Lund, 1994)

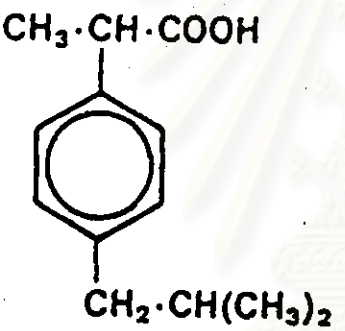


Figure 6 Chemical structure of ibuprofen

Molecular formula : $C_{13}H_{18}O_2$

Relatively molecular mass : 206.3

Chemical name : *p*-Isobutylhydratropic acid;
α-methyl-4-(2-methylpropyl)benzeneacetic acid;
2-(*p*-isobutylphenyl)propionic acid

Description : Colourless crystals or a white, crystalline powder; odour characteristic

Dissociation constant (pK_a) : 5.2

Melting point : range 75 °C to 78 °C

Solubility :

water	practically insoluble
acetone	soluble (1 in 1.5)
chloroform	soluble (1 in 1)
dichloromethane	freely soluble
ethanol	soluble (1 in 1.5)
ether	soluble (1 in 2)
alkaline hydroxide	soluble
carbonate solutions	soluble

Effect of pH

Solubility increased rapidly at pH values higher than the pK_a. The approximate solubilities at pH 4, pH 6 and pH 7 were given as 1 in 35,000, 1 in 1,900, and 1 in 410 respectively.

Effect of temperature

Solubility of ibuprofen in aqueous buffer at pH 2 and in octanol as a function of temperature.

	5°C	25°C	37°C
Aqueous buffer, pH 2.0	3.34×10^{-5} M	4.3×10^{-5} M	5.21×10^{-5} M
Octanol	0.059 M	0.091 M	0.122 M

2. Mechanism of action (Evoy, 1995)

Ibuprofen has pharmacological action similar to those of other non-steroidal antiinflammatory drugs. It has shown antiinflammatory, antipyretic, and analgesic activity in animals and humans. Higher doses are required for anti-inflammatory effects than for analgesia. The action of ibuprofen may be due to inhibition of synthesis and/or release of prostaglandin. Ibuprofen probably produces antipyresis by acting on the hypothalamus with heat dissipation being increased as a result of vasodilatation and increased peripheral blood flow. Animal studies indicate that ibuprofen is a peripherally acting, not a centrally acting, analgesic. The drug does not possess glucocorticoid or adrenocorticoid stimulating properties and has no uricosuric action.

3. Pharmacokinetics (Evoy, 1997)

3.1 Absorption and distribution

Approximately 80% of an oral dose of ibuprofen is absorbed from the gastro-intestinal tract. Absorption rate is slower and plasma concentrations are reduced when ibuprofen tablets or suspension are taken with food. However, the extent of absorption is not affected. It appears that the rate of oral absorption is faster for the suspension compared with that of the tablets. Following oral administration of ibuprofen tablets or suspension, peak serum ibuprofen concentrations are reached within 2 or 1 hour , respectively. Average peak serum ibuprofen concentrations of about 40 μg/ml occur after about 1.5 hours in febrile children receiving a single 10 mg/kg dose of ibuprofen suspension. Following oral administration of ibuprofen

tablets in adults, the area under the serum concentration-time curves(AUCs) of ibuprofen increase proportionally with single ibuprofen dose up to 800 mg: at higher doses, increases in AUCs are less than proportional to the increases in dose. Following oral administration of ibuprofen suspension in febrile children, AUCs increase with increasing single ibuprofen dose up to 10 mg/kg: it appears that absorption of ibuprofen suspension is not affected by age, in children 2 to 11 years of age. In children, the antipyretic effect of ibuprofen suspension begins within 1 hour after oral administration and peaks within 2-4 hours. The antipyretic effect of single ibuprofen dose of 5 or 10 mg/kg may last up to 6 or 8 hours, respectively. Plasma concentrations required for anti-inflammatory effect are not known. A few days to 2 weeks of therapy are required before therapeutic response occurs.

3.2 Distribution

Animal studies indicate that ibuprofen distribution varies according to species: human distribution data have not been published. Approximately 90-99% of a dose is bound to plasma proteins. Ibuprofen and its metabolites cross the placenta in rats and rabbits. In preliminary studies, ibuprofen was not detected in the milk of nursing women.

3.3 Elimination

The plasma half-life of the drug has been reported to be 2-4 hours. Blood concentrations decline as rapidly after multiple doses as after single doses. Ibuprofen is metabolized via oxidation to form 2 inactive metabolites, (+)-2[4′-2-hydroxy-2-methypropryl)phenyl] propionic acid (metabolite A) and (+)-2-[4′(2-carboxypropyl) phenyl]propionic acid (metabolite B), About 50-60% of an oral dose

is excreted in urine as metabolites A and B or their glucuronide conjugates within 24 hours. Less than 10% of the drug is excreted in urine unchanged. Excretion of ibuprofen is essentially completed within 24 hours following oral administration. Some biliary excretion of the drug probably occurs in humans.

4. Ibuprofen in topical dosage form

It was found that there were only two topical dosage form of ibuprofen in the pharmaceutical market for the relief pain in soft tissue inflammation in rheumatics; one was Ibugel® which was a gel formulation containing 5% of ibuprofen and the other was Ibuspray® which was a spray formulation containing 5% of ibuprofen (George, 1996).

It was found that ibuprofen showed significant antifungal activity *in vitro* against dermatophytes at pH 5 (MIC: 5-40 μg/ml). It was more efficient than two well known and medically used antifungal compounds, benzoic acid and salicylic acid. A bacterium, *Staphylococcus aureus* and a pathogenic yeast, *Candida albicans*, which were responsible for skin infections were also susceptible to ibuprofen (MIC below 200 μg/ml). This caused in additional advantage in antifungal treatment of superfacial mycoses by virtue of its anti-inflammatory activity which did not exist in benzoic acid and salicylic acid. The anti-inflammatory property would help in relieve the skin inflammation associated with the infection (Sanyal et al, 1993).

The antibacterial activity of ibuprofen was also investigated by Elvers and Wright (1995). They found that ibuprofen inhibited growth of six gram-positive species, but not affected to two gram-negative species, *Echerichia coli* and *Pseudomonas fluorescens*. Similar to Sanyal et al (1993), the antibacterial activity of

ibuprofen increased potency at lower pH (below pH 7). However, they found that MIC value for *Staphylococcus aureus* at pH 6 was 350 μg/ml whereas at pH 7 exceeded 600 μg/ml.

It was found that ibuprofen in 5% of ibuprofen cream caused the decreasing of thromboxane releasing from the endotoxin-stimulated burn wound *in vitro*. Ibuprofen levels in burn lymph were maintained at 1-2 μg/ml. Thus, it was possibility for the application of ibuprofen in topical dosage form to treat the burn wound (Katz et al, 1985). However, the application of 5% of ibuprofen cream caused the increasing of the wound bacterial content to 10^5-10^7 bacteria/gram of tissue compared to10^2-10^3 for a dry eschar. This could explain that the burn wound applicated with topical cream was softer and hydrated. This would accentuated bacterial growth compared to a dry eschar. It was possible that ibuprofen increased bacterial count by decreasing local immune function. This confirmed by Brown and Collins (1977) who found that ibuprofen could decrease O_2 radical production by neutrophils, a process required for bacterial killing.

5. A step forward in liposomal ibuprofen

It was found that anti-inflammatory activity of ibuprofen in liposomal preparation was shown to be enhanced. Liposomes acted as the drug carrier for distribution of ibuprofen to the inflamed site. It could be attributed to enhance uptake of drug to phagocytic cells at the inflamed site which caused in the increasing concentration of ibuprofen at the inflamed site (Katare, Vyas, and Dixit, 1990).

Sivakamai et al (1994) described the stability of ibuprofen liposomes containing soybean lecithin. They found that the addition of stearylamine in soybean lecithin liposomes caused slightly decreasing in percentage drug encapsulation. They mentioned that it might be caused from the competition of stearylamine in bilayer membranes. However, it was found that the stability of ibuprofen liposomes containing soybean lecithin and stearylamine was improved when carbodiimide, a cross-linking agent, was added. This resulted in retarding the release of ibuprofen from the liposomes when compared with uncrosslinked ibuprofen liposomes.

The addition of carbodiimide formed interlinkages with the carboxyl groups of ibuprofen present in the liposomes and then, the crosslinking of stearylamine with the crosslinked complex appeared. This caused in the rigidity of liposomal membranes and showed in retarding of ibuprofen release from liposomes. This caused the improvement of the stability of ibuprofen liposomes.