CHAPTER II

LITERATURE REVIEWS
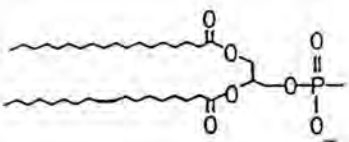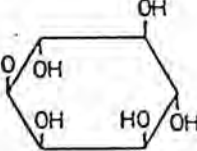
1. Formation of liposomes (Bangham et al., 1965; Mezei, 1993; Kulkarni et al., 1995)

Liposomes are closed microscopic vesicles composed of membrane like lipid layers surrounding aqueous compartments. The lipid layers are consisted mainly of phospholipids. Phospholipids are amphipathic molecules; they have a hydrophobic tail and a hydrophilic or polar head. The hydrophobic tail is composed of two fatty acid chains that contain 10-24 carbon atoms and 0-6 double bonds in each chain (Figure1). The polar end of the molecule is composed of phosphoric acid bound to a water-soluble molecule such as choline, ethanolamine, serine, glycerol, acid and inositol. The amphipathic nature of these molecules causes phospholipids to form closed bilayers in the presence of water. When phospholipids are placed in the aqueous medium, the fatty acid tails align towards each other, excluding water from this hydrophobic domain in that process. Conversely, the polar head groups orient themselves towards the bulk aqueous phase, leading to a bilayer configulation. The large free-energy difference between the aqueous and the hydrophobic environments promotes the formation of bilayer structures in order to achieve the lowest free energy level. The vesicles consist of simple lipid bilayers that resemble biological membranes, so they have also been used as a tool to study the structure and function of biological membranes.

Depending on the number of bilayers formed and diameter of the vesicles, liposomes are broadly classified into small unilamellar vesicles (SUVs; single bilayer, size 10-100 nm); large unilamellar vesicles (LUVs; single bilayer, size

100-1000 nm); multilamellar vesicles (MLVs; several bilayers, size 100 nm-20μm); and multivesicular vesicles (MVVs; size 100nm-20μm) (Figure 2).

Liposome components (Phospholipids)

Phosphatidyl moiety	Headgroup	Common name	abbreviation Phosphatidyl-
	$O-CH_2-CH_2-\overset{Me}{\underset{Me}{\overset{+}{N}}}-Me$	choline	PC
	$O-CH_2-CH_2-NH_3^+$	ethanolamine	PE
	$O-CH\overset{NH_3^+}{\underset{COO^-}{}}$	serine	PS
	$O-CH_2-\underset{OH}{CH}-\underset{OH}{CH_2}$	glycerol	PG
	O-H	acid	PA
		inositol	PI

Figure 1. Chemical structure of naturally occurring phospholipids (From Kulkarni, 1995).
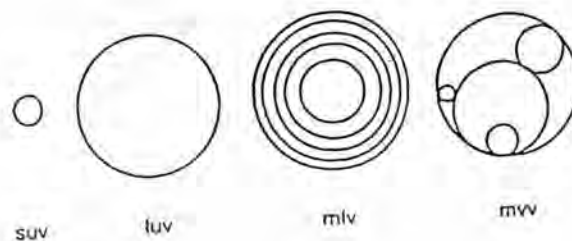
Figure 2. Types of liposomes: SUV=small unilamellar vesicle; LUV=large unilamellar vesicle; MLV=multilamellar vesicle; MVV=multivesicular vesicle (From Kulkarni, 1995).

2. <u>Materials used in the preparation of liposomes (Betageri et al., 1993; Sriram and Rhodes, 1995)</u>

Phospholipids

Liposomes can be prepared from a variety of lipids and lipid mixtures. Phospholipids are most often used especially phosphatidylcholines which are amphipathic molecules in which a glycerol bridge links a pair of hydrophobic acyl hydrocarbon chains with a hydrophilic polar head group (Figure 1). Phosphatidylcholines contrast markedly with other amphipathic molecules (detergents, lysolecithins) in that bilayer sheets are formed in preference to micellar structures because the double fatty acid chains give the molecule an overall tubular shape, more suitable for aggregation in planar sheets than in other aggregate structures (New, 1990).

Phosphatidylcholines, also known as lecithin, can be derived from both natural and synthetic sources. They are readily extracted from egg yolk and soya bean but less readily from bovine heart and spinal cord. 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, their neutral charge, and their chemical inertness. Lecithin from natural sources is, in fact, a mixture of phosphatidylcholines, 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.

In addition to phosphatidylcholine, neutral lipid bilayers may be composed of sphingomyelin, or alkyl ether lecithin analogues which substitute entirely for lecithin in the membranes. Replacement of ester groupings by ether linkages increases the resistance of such lipids to hydrolysis, while apparently not greatly affecting the physical properties of the membranes. In sphingomyelin, the presence of

the amide linkage and hydroxyl groups (in the region corresponding to the glycerol backbone of lecithin), gives rise to hydrogen bond interactions which may explain the more highly ordered gel phase relative to phosphatidylcholine.

The other neutral phospholipid found commonly in natural membrane is phosphatidylethanolamine (PE). Possessing an unsubstituted quaternary ammonium group, which is protonated at neutral pH, this lipid differs from lecithin in two respects: first, its headgroup is smaller than the bulky phosphocholine of lecithin; second, it is able to take part in hydrogen bonding interactions with its neighbors in the membrane.

In negatively charged (acidic) phospholipids (e.g. cardiolipin, phosphatidylserine (PS), phosphatidylglycerol (PG) , all three posible forces regulating headgroup interaction of bilayer membranes come into play, namely steric hindrance, hydrogen bonding, and electrostatic charge. Membranes composed of acid phospholipids can bind strongly to cations particularly divalent cations such as calcium and magnesium. The reduction in electrostatic charge of the headgroups as a result of binding causes the bilayer to condense, increasing the packing density in the gel phase, and as expected, raising the transition temperature. Thus, at the appropriate ambient temperature, addition of cations can induce a phase change from liquid-crystalline to gel phase.

Cholesterol

Cholesterol is a major component of natural membranes, and its incorporation into liposome bilayers causes major changes in the properties of vesicles. Cholesterol does not, by itself, form bilayer structures, but it can be incorporated into phospholipid membranes at high concentrations. In natural membranes, the molar ratio varies from 0.1-1, depending on the anatomical and cellular location. Being an amphipathic molecule, cholesterol inserts into the membrane with its hydroxyl group oriented towards the aqueous surface and with the
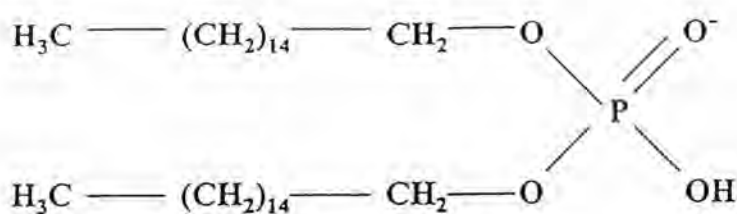
aliphatic chain aligned parallel to the acyl chains in the center of the bilayer (Figure 3). Cholesterol has been used in the preparation of liposomes to improve the bilayer characteristics of liposomes. It improves fluidity of the bilayer membrane, reduces permeability of water soluble molecules through the membrane, and improves stability of bilayer membranes in the presence of biological fluids such as blood or plasma. Liposomes without cholesterol tend to react with blood protein components such as albumin, m-transferrin, and macroglobulin. These components tend to destabilize the liposomes and reduced the utility of liposomes as drug delivery systems. Cholesterol appears to reduce this type of interaction with blood proteins. Although the presence of a large quantity of cholesterol in a vesicle will effectively protect it against plasma-induced solute release, the loss of liposomal phospholipid cannot be entirely prevented (Damen et al., 1981; Kirby et al., 1980).
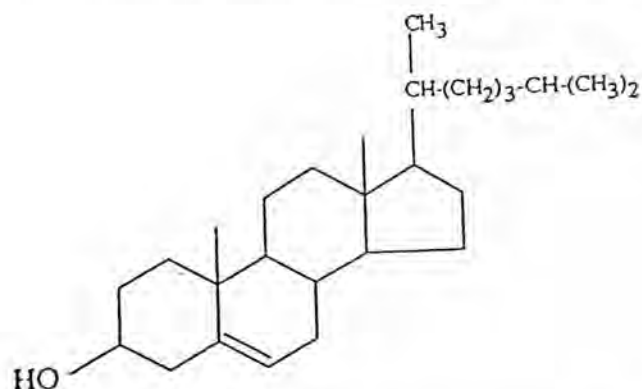
Figure 3. Structural formula of cholesterol (From Kulkarni et al., 1995).

Other substances

Diacylglycerol, stearylamine and dicetylphosphate have been incorporated into liposomes in order to impart either a negative or positive surface charge to these structures (Figure 4). In liposomal preparation, a charged species may be added (normally at 5-20%w/w) to prevent aggregation, and small amounts of antioxidants such as α-tocopherol or BHT are also included when polyunsaturated natural lipids are used.

$$H_3C \longrightarrow (CH_2)_{14} \longrightarrow CH_2 \longrightarrow O$$

Dicetylphosphate

$$H_3C \longrightarrow (CH_2)_{16} \longrightarrow CH_2 \longrightarrow \overset{+}{N}H_2$$
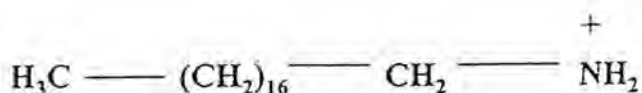
Stearylamine

Figure 4. Chemical structures of dicetylphosphate (DCP) and stearylamine (SA).

3. Method of preparation by reverse phase evaporation technique

Liposomes made by reverse phase evaporation method were developed by Szoka and Papahadjopoulos in 1978. They can be prepared by forming water in oil emulsions of phospholipids in an excess organic phase followed by removal of the organic phase under reduced pressure. The two phase are usually emulsified by sonication. Removal of the organic solvent under vaccuum causes the phospholipid coated droplets of water to coalesce and eventually form a viscous gel. Removal of the final traces of solvent under high vacuum or mechanical disruption (such as vortexing) results in the collapse of the gel into a smooth suspension of LUVs. A schematic diagram of the processes that might be occuring during formation of REVs is shown in Figure 5.
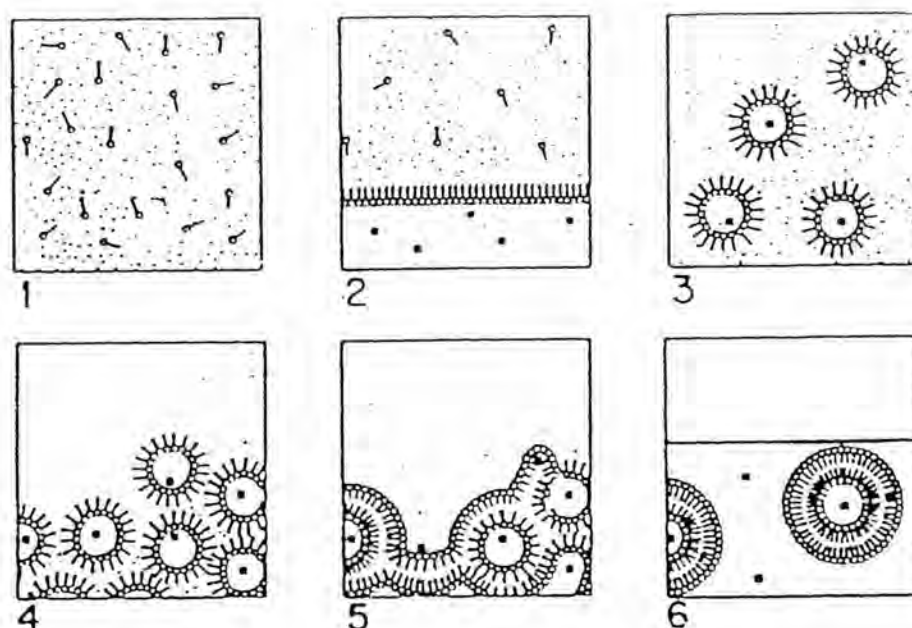
Figure 5. Diagram of the formation of REVs. First, bilayer-forming phospholipids are dissolved in appropiate solvents to form a lipid-in-solvent solution. The aqueous phase containing the drug (squares) is added to form a two-phase system that is subsequently emulsified by sonication to form a water-in-oil emulsion. As the organic solvent is removed, the preparation becomes viscous and usually forms an intermediate gel. As the last traces of solvent are removed, the gel collapses into a smooth suspension of single-layered REV liposomes (From Szoka and Papahadjopoulos, 1978).

Liposomes formed by the reverse phase evaporation method are known to have high encapsulation efficiency. Size ranges, internal volumes, and encapsulation effciencies for different liposome preparations are reported in Table 1.

Table 1. Summary of size, encapsulation efficiency, and internal volume of different liposome preparations (From Szoka and Papahadjopoulos, 1978).

Liposomes	Encapsulation efficiency	Captured volume, ml/mg %	Diameter nm (range)
REV (PG/PC/ Chol, 1:4:5)	35-65	13.7	200-1000
REV (PG/PC 1:4)	30-45	8.1	100-300
MLV (PG/PC/ Chol, 1:4:5)	5-15	4.1	400-3500
SUV (PG/PC/ Chol, 1:4:5	0.5-1	0.5	20-50
LUV (PS)	0.5-1	9.1	200-1000

4.Encapsulation of drugs in liposomes

For a manufacturer, the physical and chemical stability and the drug encapsulation capacity are the major points of concern. In order to achieve optimum efficacy and cost effectiveness for a drug delivery system, it is necessary to encapsulate the maximum possible quantity of the drug. For a water-soluble (non-bilayer interacting) drug, encapsulation means the entrapment within the aqueous compartments; this depends on the encapsulated aqueous volume (Figure 6). The internal or trapped or capture volume is expressed as aqueous entrapped volume per unit quantity of lipid (µl/µmol or µl/mg) (Table 2). It is determined by entrapping a water-soluble marker such as 6-carboxyfluorescein, ^{14}C or ^{3}H-glucose or sucrose in liposomes and then the amount of the marker in the vesicle is determined after lysing the liposomes by the use of a detergent such as Triton X-100. Determination of the amount of marker that is trapped enables one to back-calculate the volume of entrapped water. The encapsulation efficiency describes the percent of the aqueous phase (and hence the percent of water-soluble drug) that becomes entrapped during liposome preparation. The remaining drug remains outside of the liposome and is therefore 'wasted'. Encapsulation efficiency is usually expressed as % entrapment/mg lipid. The internal or trapped volume and encapsulation efficiency greatly depends on liposomal composition, lipid concentration, method of preparation and drug used (Weiner et al., 1989). For a hydrophobic drug, the encapsulation means entrapment within the lipid bilayers (Stamp and Juliano, 1979), also called ' incorporation into the bilayers' (Figure 6). Encapsulation efficiency or loading efficiency is usually defined as the percent fraction of the total input drug encapsulated in lipid bilayers in the liposomes at a particular phospholipid concentration (Szoka and Papahadjopoulos, 1980).

When comparing encapsulation efficiencies for different drugs, it is necessary to measure at a constant lipid concentration. Therefore, another term that needs to be defined is 'encapsulation capacity' (EC). It is the maximum amount of drug that can be encapsulated at a particular phospholipid concentration and it is
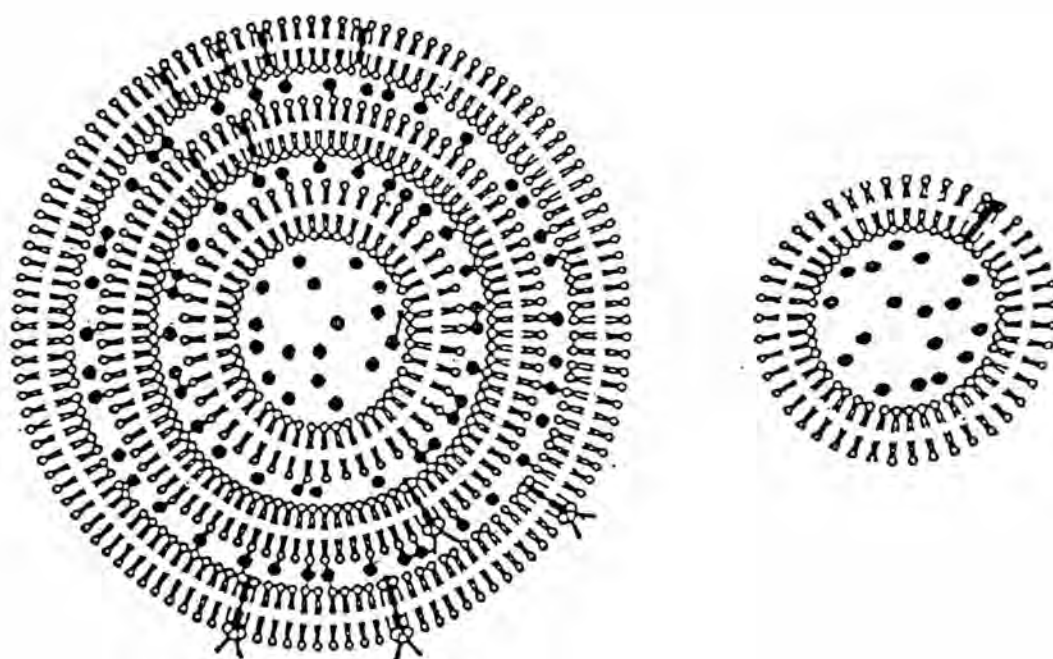
Figure 6. Schematic representation of a unilamellar and a multilamellar vesicular structure.

• hydrophilic active or drug molecule

_ lipophilic active or drug molecule

>o- amphiphilic active or drug molecule, the hydrophobic part is immobilized

in   the lipophilic domain of the bilayer (From Simonnet, 1994)

Table 2. Typical values of different types of liposomes (Weiner et al., 1989).

Liposome Type	Internal Volume μl / μmol lipid	Entrapment Efficiency %/mg lipid
SUV	< 0.5	<1
MLV	> 4	5-15
REV	> 10	35-65

usually expressed as moles of drug per mole of phospholipid. Benita et al. (1984) suggested an equation for the calculation of EC:

$$EC = C_e \ / \ (\ C_i C_L \ )$$

where, C_e is the concentraion of the encapsulated drug, C_i is the input drug concentration, and C_L is the concentration of the lipid. They have further suggested that the encapsulation capacity can also be determined by calculating the molar fraction of drug entrapped per mole of lipid.

5. Factors affecting drug entrapment and release characteristics

To obtain optimum encapsulation of a drug into a liposomal formulation, parameters influencing both the liposome and the drug need to be carefully considered during the preformulation stage. In addition, some factors can also affect the amount of drug released *in vitro* and *in vivo*. Most of the factors studied so far are as follows:

5.1 Type of vesicles

MLVs are suitable for the encapsulation of bilayer-interacting hydrophobic drugs, and less appropriate for hydrophilic drugs because they have a low aqueous encapsulation volume. LUVs are more suitable for hydrophilic drugs due to their large entrapped aqueous volume, while SUVs have very low encapsulation efficiency for these drugs because of the low encapsulation volume (Stamp and Juliano, 1979; Szoka and Papahadjopoulos, 1980). However, entrapment of highly hydrophobic drugs is affected to a lesser extent by vesicle type, since they remain entrapped within the phospholipid bilayers. Thus, for hydrophilic drugs, encapsulation appears to increase in the order of MLVs < SUVs < LUVs. In the case of hydrophobic drugs, size and type of liposomes may not play a major role.

5.2 Phospholipid selection

Although there is a variety of phospholipids for the preparation of liposomes, the choice of phospholipid is often limited to the family of the phosphatidylcholine (PC) and phosphatidylglycerol (PG), mainly from toxicological considerations, the availability of pure compounds and the cost. Phospholipids undergo a characteristic gel-liquid crystalline phase transition at a temperature range (known as phase transition temperature, T_c), which is a function of the acyl chains, branched chains, or chains carrying bulky side groups. Vesicles that are at temperature < T_c are considered solids (gels), whereas above this temperature they are in fluid state. Length of alkyl chains and degree due to the high surface density of the bilayer of saturation affect the encapsulation of lipid soluble drugs and stability of liposomes. Increasing the alkyl chain length of phospholipid increases partitioning of hydrophobic drugs into the bilayers. The partitioning is also influenced by the fluidity of the membrane structures; at low temperatures, very small amounts of drug are found to be entrapped due to the high surface density of the bilayer. An increase in temperature improves fluidity of the bilayers an thus improves drug encapsulation (Ma et al., 1991).

Encapsulation of a hydrophilic drug, dideoxyionosine triphosphate, is higher in DPPC liposomes than in DMPC liposomes(Betageri, 1993). Another hydrophilic drug, 5-fluorouracil, has higher encapsulation efficiency in DMPC liposomes than in sphingomyelin liposomes (Elorza et al.,1993). This difference was ascribed to their difference in fluidity state of the bilayer. Fluidity of bilayers is also affected by addition of detergents or surface active agents. Downing et al. (1993) reported that incorporation of sodium dodecyl sulfate changes the fluidity of liposome bilayers and the permeability of the liposomes is increased.

Incorporation of glycolipids in phospholipid bilayers affects encapsulation of some hydrophilic drugs into MLVs or LUVs. The hydrophilic head

functional groups exposed outside. The drug molecules may bind to the galactose head groups by hydrogen bonding. This leads to an increase in the encapsulation efficiency, usually in proportion to the concentration of glycolipid. For example, the higher encapsulation of mitoxantrone was observed when galactocerebroside was incorporated in liposomes and this increase in encapsulation was proportional to the concentration of galactocerebroside (Law et al., 1991).

5.3 Presence of charge

The presence of negatively charged lipids such as phosphatidylserine, phosphatidic acid, phosphatidylinositol and phosphatidylglycerol or positively charged detergents such as stearylamine tends to increase the interlamellar distance between successive bilayers due to charge repulsion in the MLVs structure and thus lead to a greater overall entrapped volume. This is particularly true in low ionic strength buffers or in solutions of nonelectrolytes (such as sucrose) since the electrostatic repulsive forces which give rise to the effect are greater under these conditions. However, there is no uniform rule with respect to the effect of charge on the encapsulation efficiency. It is dependent on the characteristics of the drug being entrapped. Encapsulation of mitoxantrone was found to be higher in negatively charged liposomes than in positively charged and in neutral liposomes (Laws et al., 1991). Conversely, Arien et al. (1993) reported higher encapsulation of calcitonin in positively charged liposomes than in neutral liposomes. Generally, about 10-20 mole percent of a charged species is used in liposomal formulations. The presence of charge may also prevent aggregation of liposomes (Puisieux and Benita, 1982).

5.4 Effect of bilayer rigidity

Most of liposomes are permeable and not sufficiently rigid; thus, they often leak encapsulated drugs during storage. Frequently, cholesterol is incorporated into the lipid bilayers to impart rigidity. Another important compound which is often added to the liposomal preparation is α-tocopherol (Figure 7). Similar to cholesterol,

tocopherol improves membrane rigidity and makes it more hydrophobic and less permeable; in addition to this, it also acts as an antioxidant (Kulkarni, 1995).
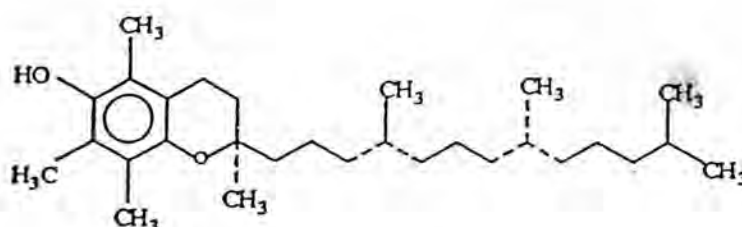
Figure 7. Chemical structure of α- tocopherol (From Kulkarni et al., 1995).

Addition of cholesterol reduces the permeability of liposomes, provides more physical stability against ultrasonication, and increases encapsulation efficiency (Benita et al., 1984). Encapsulation of citicoline is increased linearly with addition of cholesterol up to 1:1 (m/m) to DPPC (Puglisi et al.,1992). This increase was attributed to the reduction in the membrane permeability. Incorporation of cholesterol also improves the physical stability of the liposomes during sonication, centrifugation and other mechanical operations. In addition, it appears that cholesterol improves encapsulation of hydrophobic drugs only if the drug input is less than the encapsulation capacity for that drug. Kulkani (1993) reported a slight increase in encapsulation of hydrophobic steroids as the concentration of cholesterol was increased when the drug input was below the encapsulation capacity, but it had an opposite effect when the drug input was equal to or higher than the encapsulation capacity. For hydrophilic drugs, cholesterol improves encapsulation by decreasing permeability of bilayers.

All liposomal preparations are prone to autooxidation (except those made with fully saturated phospholipids), which is usually accelerated by elevated temperature, light, metal ions and some solutes. When it is difficult to avoid exposure to oxygen, it is advisable to use tocopherol to reduce the autooxidation and prolong the shelf lives of liposomes. Herandez-Caselles et al. (1990) reported higher retention

of a water-soluble dye (carboxyfluorescein), a reduction in the lipid peroxidation, and a decrease in the formation of lysophospholipid when tocopherol was added.

5.5 Method of preparation

Method of preparation of liposomes can also affect drug location and overall entrapment efficiency (Weiner et al., 1989). Several different methods are available for liposomal preparation . The cast film method is simple but the major drawback with MLVs prepared is relatively low encapsulation in terms of aqueous space per mole of lipid, and with solvent injection method dilute preparation of liposome with a low encapsulation efficiency is obtained (Jaggi and Khar, 1989). Szoka and Papahadjopoulos (1978) developed the reverse phase evaporation method for the encapsulation of water-soluble drugs. Using this method, a maximum of 65% encapsulation may be obtained, but the major drawback of this method is that macromolecules such as proteins may get denatured by organic solvent. In this procedure, a water-in-oil emulsion is prepared by sonication, and the emulsion is dried down to a semisolid gel using a rotary evaporator. The gel is then subjected to vigorous mechanical shaking, which converts it into a LUVs suspension. Shew and Deamor (1985) have reported a novel method for encapsulation of macromolecules. They encapsulated haemoglobin and alkaline phosphatase in phosphatidylcholine liposomes using a dehydration-rehydration cycle for liposome formulation. In this method, a suspension of empty liposomes is lyophilized. Since such lipid has a highly organized structure, addition of water with a desired solute can rehydrate, fuse and reseal vesicles with a high capture effciency.

5.6 Solubility and partition coefficient of the drug

The total amount of liposomal lipid used and the internal volume of the liposome will affect the total amount of loading of non-polar and polar molecules into liposomes. Efficient capture will depend on the use of drugs at concentrations which

do not exceed the saturation limit of the drug in the aqueous compartment (for polar drugs) or the lipid bilayers (for non polar drugs) (Weiner et al., 1989).

The location of a drug within liposomes is based on the partition coefficient of the drug between the aqueous compartment and the lipid bilayer, and the maximum amount of drug that can be entrapped within a liposome is dependent on its total solubility in each phase. In incorporation of drugs that have intermediate partition coefficients (significant solubility in both the aqueous phase and the bilayer), encapsulation efficiency may be improved by changing the pH of the aqueous medium. Any change in pH causes a change in the aqueous solubility of these drugs and thus affects their partitioning. Encapsulation of tin-mesomorphin was found to be higher (about 90%) at pH 5 than that at pH 7 (<10%) (Cannon et al., 1993). For bilayer-associated drugs, the encapsulation of the drug depends on the mechanism of interaction. If the drug-liposome interaction is based on electrostatic forces, then encapsulation is affected by the density of the charge-inducing bilayer constituent and the ionic strength of the aqueous medium.

Generally, drugs which are water soluble accommodate in the aqueous layer lattice of liposomes. Their encapsulation is low, and, on storage, leakage of drug is the problem especially in the step of removal of free drug from liposomal preparation. These factors do not encounter if the drugs are lipid soluble as they remain lipid membrane bound, and such preparation can be stored in a dry form to be rehydrated prior to use. Several efforts have been done to avoid the problems of water soluble drugs, the most promising one is making them lipid soluble. The approaches include the following:

a) Addition of ion pairing and complexing agents

Encapsulation of polar drugs can also be increased by addition of ion pairing or complex-forming agents to make those polar drugs more hydrophobic. Lee et al. (1988) improved the encapsulation of isopropamide iodide by using an ion

pairing agent, sodium taurodeoxycholate. The ion pair complex of isopropamide with sodium taurodeoxycholate was highly hydrophobic, and a three times increase in encapsulation was observed.

b) Chemical modification of the drug

Chemical modification of drugs has been employed to improve the yield of liposomal encapsulation of several drugs. A usual way to improve the encapsulation of a less hydrophobic drug is to make it more hydrophobic by derivatizing it into a fatty acid ester. This method is quite useful for improving encapsulation of steroidal drugs. Shaw et al. (1976) prepared various fatty acid derivatives of hydrocortisone and found that hydrocortisone 21-palmitate had maximum encapsulation efficiency in MLVs. Similar results were reported by Goundalkar and Mezei (1984); triamcinolone acetonide 21-palmitate showed 85% encapsulation compared with 5% encapsulation of triamcinolone acetonide in MLVs.

6. Stability of liposomes

Possible stability problems

Possible stability problems include loss of entrapped drug; changes in liposome structure, including particle size distribution and aggregation/fusion of liposomes; sedimentation of liposomes; and chemical instability of the liposomes and entrapped drug (Fildes, 1981).

If the entrapped drug is lipophilic, stability problems are reduced because the liposomes can be stored dry (possibly freeze-dried). The liposomes can be rehydrated (possibly with gentle heat and agitation) immediately prior to use, and the lipophilic drug will naturally associate with the liposomal membrane up to the "solubility" of the drug in the membrane. This would solve many of the problems listed above and also eliminate the need for stabilizers for the phospholipids and/or the

drug. The drug entrapped is often less stable than the phospholipids. Thus, only drug stabilization is often required to extend the shelf-life of the liposome product to the point of commercial feasibility.

There are many additional potential stability problems when hydrophilic drugs are entrapped in liposomes. Because only a fraction of the total hydrophilic drug is entrapped during liposome formation, it is probably not feasible to prepare the liposomes immediately prior to use. Also, the necessary removal of unentrapped (free) drug after liposome formation is currently beyond the capabilities of the average pharmacy. In some cases, it may be possible to overcome these problems through the use of a lipid soluble prodrug, and the prodrug is readily transformed to the parent compound *in vivo* (Knight, 1981).

Stability of phospholipid and cholesterol

For maximum stability it is preferable to store phospholipids in hexane, chloroform or ethanol at -20°C under nitrogen. The most common mechanism of phospholipid degradation are oxidation of fatty acids and hydrolysis of the ester bonds linking the fatty acids to the glycerol backbone (Martin, 1990).

In the absence of specific oxidants, the oxidation of fatty acids is initiated by abstraction of hydrogen from the lipid chain, and free radicals are formed. Double bonds present in acyl chains of polyunsaturated lipids render these chains particularly susceptible to oxidation (New, 1990). Phospholipids from common sources, such as egg and soy are relatively inexpensive but unfortunately have a significant number of double bonds. This is especially true for soy phospholipids. Stability of these phospholipids can be achieved by complete or partial hydrogenation, which is cost-effective. Phospholipids with I_2 values of 30-40 are ideal since they are resistant to both oxidation and thermal phase transition at lower temperatures. Oxidation of phospholipids can also be minimized by the addition of an antioxidant (butylated hydroxytoluene, butylated hydroxyanisole, α-tocopherol or its more stable

hemisuccinate derivative), protection of the phospholipids from light, storage under an inert gas such as argon or nitrogen, and the addition of EDTA to chelate any trace heavy metal ions (Martin, 1990). The order of effectiveness among lipophilic antioxidants is butylated hydroxyanisole > catechin > butylated hydroxytoluene > α-tocopherol > chlorogenic acid.

The hydrolysis of ester bonds that link fatty acids to the glycerol moiety normally occurs before oxidation and follows first-order kinetics. These reactions become significant above $40^{\circ}C$ and are strongly pH-dependent with a pH of maximum stability around 6.5 (Martin, 1990).

The loss of phospholipids may be monitored through extraction followed by thin layer chromatography (silica gel with chloroform) if it is necessary to separate the various lipid components. Densitometry may be performed after staining the plate with molybdenum blue to expose the phospholipids or with 50% sulfuric acid to expose cholesterol or α-tocopherol. Total phosphorus may be assayed spectrophotometrically by the Bartlett assay (Bartlett, 1959). A better method may be the Stewart spectrophotometric assay since inorganic phosphates in the buffer do not interfere. However, this test is unresponsive to phosphatidylglycerol (New, 1990).

There is no single test to determine the extent of oxidation of phospholipids. The simplest method to detect oxidation is to monitor for dienes by scanning for a peak at 233 nm (the molar extinction coefficient is 30,000 for dienes) and comparing the scan to the original concentration of phospholipids. Extensive degradation yields an additional peak at 270 nm. The formation of lyso-compounds (lysolecithin) through hydrolysis of ester bonds is detected through extraction and thin layer chromatography or, preferably, by high-performance liquid chromatography (HPLC). However, in HPLC it is difficult to relate these peak heights to quantities. Lyso-compounds cause changes in the properties of bilayers, including an increase in permeability. Thus, it is vital to minimize their formation during storage. The oxidation of cholesterol can be monitored through gas liquid chromatography using a

silica capillary column to separate cholesterol from its oxidative products (New, 1990). Unfortunately, there is little information available on the effect of entrapped drug on the stability of phospholipids.

Stability of liposomes on shelf (Sriram and Rhodes, 1995)

A stability study program for a pharmaceutical product must include a section for product characterization and another section to study the product stability during storage. As a first step of product stability program, one must identify various parameters of the product that characterize the drug as well as the dosage form. Each of the product parameters of interest should be developed and validated prior to their use in the stability study. All liposome preparations reported in the literature are heterogeneous in size with potential physical and chemical stability problems. Average size distribution of liposomes determined at the time of their preparation changes on their storage. Liposomes tend to fuse and grow into bigger vesicles, which is a thermodynamically more favorable state. Fusion and breakage of liposomes on storage also poses a more important problem of drug leakage form the vesicles. Interactions of the drug with the phospholipid also lead to the chemical stability problems. Chemically, phospholipids are susceptible to oxidation and hydrolysis reactions. Majority of therapeutic liposome formulations are parenteral products (either injectable or inhalation dosage forms), and thus they must be sterilized to remove the microbial contamination from the product.

Other factors to be taken into consideration prior to starting a stability study are the formulation factors and environment conditions which may influence the stability of liposomes. Knowledge upon formulation factors such as pH, buffer species, ionic strength, and solvent system and their influence on the liposome play a major role in stabilizing a liposome formation. Influence of environmental factors such as temperature, light, oxygen, and heavy metal ions may initiate chemical or physical reactions. These reactions may include changes in size distribution of vesicles and oxidation and hydrolysis of lipid.

a) Physical stability

Liposomes are susceptible to aggregation or fusion, forming larger liposomes or aggregates, and leakage of the encapsulated drug upon storage. Therefore, it is important that a liposomal product remains stable on the shelf, under ambient conditions of storage for a reasonable period. It was reported that the increase in size and leakage of encapsulated drug of small unilamellar liposomes depends on the nature of the lipid composition employed and the encapsulated drug (Frokjaer et al.,1984). Szoka and Papahadjopoulos reported the retention behavior of several lipid compositions under various conditions of storage (Szoka and Papahadjopoulos, 1980). SUVs are more sensitive to size changes than are MLVs.

b) Chemical Stability

The chemical stability of the encapsulated drug and the lipids used to prepare liposome affects the stability of liposomal products. Phospholipids containing unsaturated fatty acids are known to undergo oxidative reactions. The reaction products can cause permeability changes in the liposome bilayers. Furthermore, the reaction products can also alter the shelf-life of liposomes. It has been identified that the lipid peroxidation and the lipid hydrolysis are the two major routes of lipid degradation. Oxidative degradation of the lipids in general can be minimized by protecting the lipid preparations from light by adding antioxidants such as α-tocopherol or BHT, by adding EDTA to lipid formulations to remove heavy metals, and by producing the product under nitrogen or argon environment. Hydrolysis of lipids leads to the formation of lyso-phosphatidylcholine. Both unsaturated and saturated phospholipids are susceptible to hydrolysis in an aqueous phase leading to the formation of lyso-phospholipid and fatty acid. The rate of hydrolysis is dependent on the pH of the medium. The presence of lyso-phosphatidylcholine enhances the permeability of liposomes.

It is possible to formulate liposomal products for storage either as a suspension or in a form to be reconstituted prior to administration. If the liposomal preparation is stable with respect to leakage of the encapsulated drug, then it is advantageous to supply it in a ready-to-use suspension form. However, a reconstitutable form avoids both physical and chemical stability problems associated with storage. Lyophilization (freeze- drying) has great potential as a method to solve long term stability problems with respect to liposomal stability. Liposomes containing drug entrapped in their bilayers would be better candidates for lyophilization than liposomes containing drug entrapped in their aqueous compartments since the lyophilization procedure would cause some disruption and subsequent leakage (Weiner et al., 1989). This technique can also be used to increase the shelf-life of liposomal products.

7. Liposomes for dermal drug delivery

Topical application of a drug may have two objectives: either to induce local effects or to produce systemic activities. In dermal drug delivery, due to the intention that the drug penetrates into the skin and localizes at its site of action without being taken up by the blood circulation and distributed to systemic sites, we need a vehicle or a drug carrier that enhances penetration of the active ingredient into the skin, localizes the drug at the site of action, and reduces percutaneous absorption. On the other hand, in transdermal drug delivery the drug has to penetrate through the skin and should have adequate uptake by the blood circulation to provide therapeutic concentrations at the site of action within an internal organ, so we need a vehicle or a drug carrier that enhances percutaneous absorption, or we need a drug molecule that freely penetrates the skin (Mezei, 1993).

To achieve these goals, liposomes have attracted considerable interest and have generated many speculative claims concerning their potential utility, both as a drug carrier and a reservoir for controlled release of drug within various layers of the skin (Moghimi and Patel, 1993). An appropriately prepared drug containing liposomal

formulation should enable a prolonged and sustained release of the drug to the skin at the site of application (Egbaria and Weiner, 1990). The drug is released when the bilayer of the liposomes becomes permeable to the drug or when the bilayer is decomposed. If the drug is hydrophobic it will be released when the phospholipid molecules of the outer bilayer are hydrolyzed or oxidized. If the drug is hydrophilic, it is released when the outermost aqueous layer breaks down or the membrane becomes more permeable. While a drug is entrapped in the liposomes, it cannot be metabolized since it is not exposed to the metabolizing enzymes. Consequently, liposomes can act as depots for a drug (Mezei, 1991). In summary, the major advantages of topical liposomal formulations include the potentials to reduce serious side effects and incompatibilities that may arise from undesirably high systemic absorption of the drug, to enhance significantly the accumulation of the drug at the site of administration as a result of the high substantivity of liposomes with the stratum corneum, and to incorporate readily both lipophilic and hydrophilic drugs because liposomes have both hydrophilic and hydrophobic compartments (Weiner et al. 1994).

7.1 Structure and function of the skin (Mezei, 1991, 1993; Moghimi and Patel, 1993)

The skin is the largest and most heterogeneous organ of the body. The primary function of the skin is to protect the underlying tissues from mechanical, chemical and radiant injuries and from invasion by pathogenic organisms. The skin is composed of tissue that grows, differentiates and renews itself constantly. It is divided into three major layers: epidermis, dermis, and subcutaneous tissue.

The epidermis

The epidermis is composed of four strata; the stratum germinativum (or basal layer), the stratum granulosum (the malpighian layer), the stratum lucidum (the granular layer) and the stratum corneum (the horny layer). The basal layer has a high proportion of phospholipids (e.g., phosphatidylcholine, phosphatidylethanolamine,

and sphingomyelin). Within the viable epidermis is the granular layer, which has a transient composition between the basal layer and the stratum corneum. It consists of a relatively small phospholipid and high ceramide content. The outermost layer of the skin, the stratum corneum, has been identified as the principal barrier for penetration of most drugs. In the non-hydrated state, the stratum corneum is about 10 μm thick and contain 10-15 layers of dead keratinized cells, corneocytes, embedded in lamellar lipid domains. Lipid composition of the intercellular space is rather non-polar in nature and consists of ceramides, sterols (predominantly cholesterol), sterol esters (such as cholesteryl sulfate), and fatty acids, which are organized in multilaminated sheets.

The dermis

The dermis interfaces with the epidermis at the epidermal-dermal junction. It is ten to forty times thicker than the epidermis, depending on the area of the body. It is a matrix of loose connective tissue composed of polysaccharides and proteins (collagen and elastin) containing nerves, blood vessels, hair follicles, sebaceous and sweat glands. Additionally, mast cells, macrophages, melanocytes, leukocytes, and endothelial cells of the blood vessels are also located in the dermis.

The subcutaneous tissue

The subcutaneous tissue serves as a receptacle for the formation and storage of fat, having functions as both heat regulator and shock absorber. It is a place for dynamic lipid metabolism; it supports the nerve and blood vessels that pass to the dermis. The deeper hair follicles and sweat glands originate in this layer.

7.2 Mechanism of liposomal dermal drug delivery (Mezei, 1993; Moghimi et al., 1993)

There are two assumed pathways for permeation through the stratum corneum; these are the transepidermal route either through the transcellular or intercellular route and the transappendageal route through the hair follicles, sweat or sebaceous glands (Schafer et al., 1979; Gray and Elias, 1982). The pathway of penetration depends on the physicochemical characteristics of the drug, although more than one pathways may be used at the same time (Figure 8).

To explain the mechanism by which liposomes achieve selective dermal drug delivery, the ultrastructural electron microscopic evaluation of guinea pig skin treated with liposomes containing an electron dense material, colloidal iron was studied. Foldvari et al. (1990) encapsulated colloidal iron into large multilamellar vesicles and studied their fate following topical application using electron microscopy. Their electron micrographs indicated that intact liposomes can penetrate into the skin of guinea pigs through the intercellular pathway . Moreover, in some instances, the liposomal structure was highly distorted in the intercellular regions. These investigators reasoned that the distortion of liposomes could be due to the narrow channels in the skin, or partial degradation of the vesicles. In addition, electron microscopic observations also demonstrated the presence of a number of intact liposomes in the dermis. The ability of intact liposomes to reach the dermis, however, is rather confusing since liposomes must penetrate through the basement membrane which lies between the epidermis and the dermis. However, the transappendageal pathway may provide a possible route for penetration of topically applied vesicles into dermis. Beacause of their relatively small area (0.1 percent of the total surface), the appendageal route along hair follicles, sebaceous and sweat glands may be of minor importance to account for the reported significant increase in disposition of liposomally entrapped drugs in the dermis (Mezei and Gulasekharam, 1980,1981; Gesztes and Mezei, 1988; Mezei 1988). It is possible that the increase in drug concentration in the dermis, as mediated by liposomes, is more likely to be the result of passive diffusion of the drug molecules from destabilized vesicles at basal epidermis.
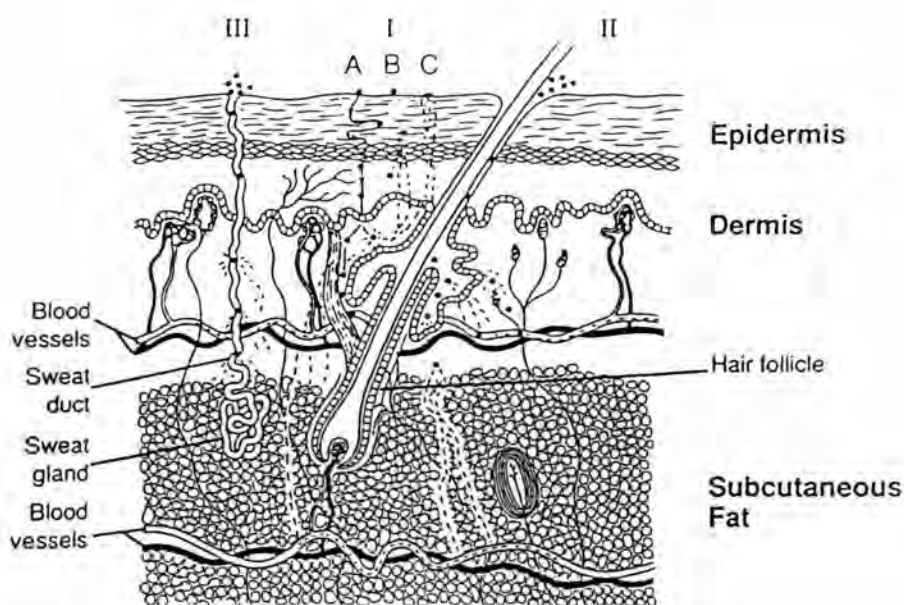
Figure 8. Diagram of skin structure and possible routes of liposome (: :) penetration. Route I. Across the contineous stratum corneum membrane: (A) Intercellular route, (B) Transcellular route, and (C) Fusion of MLV. Route II. Through the hair follicles with their associated sebaceous glands. Route III Via the sweat ducts. (From Patel and Moghimi, 1993).

On the basis of autoradiographic and electron microscopic findings coupled with previous *in vivo* experimental results and other literature data, one can propose the following pathways for liposome-skin interactions. Multilamellar and unilamellar liposomes can be adsorbed onto the skin surface where they are miscible with the surface lipids, which may act as a barrier for some polar drugs. Some lipid vesicles might rupture on the surface of the skin , due to interaction with surface lipids and possibly on the action of bacterial flora present. Consequently some encapsulated drug becomes 'free' and penetrates as was indicated by the presence of the colloidal iron grains scattered within the epidermis and the dermis. The penetration of smaller liposomes is more likely possible via the lipid rich channels (lipid bilayers) present in the stratum corneum and viable epidermis. It is possible that larger oligolamellar or multilamellar lipid vesicles lose their outer bilayers during penetration and carry some

drug encapsulated in the intact vesicles down to the dermis. The intact liposomes, as identified within the dermis with the aid of colloidal iron, definitely indicate this possibility. Another possibility is that some liposomes break up either on the skin surface or during penetration through the epidermis due to metabolic or other reactions. However, if the drug is lipophilic, it can be carried into the skin by the lipid fragments derived from the liposomal bilayers. The encapsulated or the lipid-bound drug has less chance of penetrating the blood vessels and being cleared from the skin. The slow clearance would be due to less uptake by the blood circulation and lack of metabolism of the entrapped drug. This obviously can lead to drug accumulation within the skin during a multiple-dose therapy (Meizei, 1993).

Hofland et al. (1995) studied the interaction between liposomes and human stratum corneum *in vitro* using freeze fracture electron microscopy and small angle x-ray scattering techniques. Their results suggested that liposomes do not penetrate intact into the skin, but that their constituents are able to penetrate molecularly dispersed into the stratum corneum, mix with the stratum corneum lipids, and induce a penetration-enhancing effect by producing ultrastructural changes in the intercellular lamellae.

In conclusion, following application of liposomes to the skin at least two types of interactions seem possible: 1) some intact vesicles can penetrate through the stratum corneum, perhaps through the intercellular and appendageal routes; hence they can act as reservoirs for drugs in the skin; and 2) vesicles may fuse with lipid bilayers and/or corneocytes and, as a result, they may act as penetration enhancers.

8. Properties of lactic acid (Fahad et al., 1993)
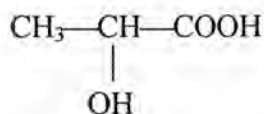
Generic name

Lactic acid

Chemical names

2-Hydroxypropanoic acid

2-Hydroxypropionic acid

α-Hydroxy propionic acid

Propanoic acid-2-hydroxy

Empirical formula   **Molecular weight**

$C_3H_6O_3$ 90.08

Structural formula

$$CH_3—CH—COOH$$
$$|$$
$$OH$$

Functional category

Acidify agent and acidulant

Applications in pharmaceutical formulation or technology

Lactic acid is used in beverages, foods, cosmetics and pharmaceuticals as an acidifying agent. In topical formulations, particularly in cosmetics, it is used for its softening and conditioning effects on the skin. It is also used as a food preservative; in injections, in the form of lactate, as a source of bicarbonate for the treatment of metabolic acidosis; as a spermicidal agent; in pessaries for the treatment of leucorrhea; in infant feeds; and in topical formulations for the treatment of wart.

Use	Concentration (%)
Injection	0.012-1.16
Topical preparation	0.015- 6.6

Description

Lactic acid consists of a mixture of 2-hydroxypropionic acid, its condensation products, such as lactoyl-lactic acid and other poly lactic acids, and water. It is usually in the form of the racemate, (RS)-lactic acid, but in some cases the (S)-(+)-isomer is predominant.

Lactic acid is a practically odorless, colorless or slightly yellow-colored, viscous, hygroscopic, nonvolatile liquid.

Typical properties

Boiling point: $122^{\circ}C$ at 2 kPa (15mmHg)

Dissociation constant: $pK_a = 3.9$ ($25^{\circ}C$)

Melting point: $17^{\circ}C$

Osmolarity: A 2.3% w/v aqueous solution is iso-osmotic with serum.

Solubility: miscible with ethanol, ether, and water; practically insoluble in chloroform.

Stability and storage conditions

Lactic acid is hygroscopic and will form condensation products, such as polylactics on contact with water; the equilibrium between the polylactic acids and lactic acid is dependent on concentration and temperature. At elevated temperatures lactic acid will form lactide which is readily hydrolyzed back to lactic acid.

Lactic acid should be stored in a well-closed container in a cool, dry place.

Incompatibilities

Lactic acid is incompatible with oxidizing agents, iodides, and, albumin. It reacts violently with hydrofluoric acid and nitric acid.

Method of manufacture

Lactic acid is prepared by the fermemtation of carbohydrates, such as glucose, sucrose and lactose, with *Bacillus acidi lacti* or related microorganisms. Wheat, corn starch, potatoes, or molasses are used as a source of carbohydrate on a commercial scale . Lactic acid may also be prepared synthetically by the reaction between acetaldehyde and carbon monoxide at $130-200°C$ and high pressure, or by the hydrolysis of hexoses with sodium hydroxide.

Lactic acid prepared by the fermentation of sugars is levorotatory; that prepared synthetically is racemic. However, lactic acid prepared by fermentation becomes dextrorotatory on dilution with water due to the hydrolysis of (R)-lactic acid to (S)-lactic acid.

9. Alpha hydroxy acids as anti-aging agents

Alpha Hydroxy Acids (AHAs), including hydroxy fatty acids, have been found notable for their wide range of positive dermal effects. The range of acids so far considered for this purpose includes those shown in Figure 9. Use of AHAs and other acids as skin peels and emollients is well known. Such benefits result from keratolytic action of the acids which facilitates the removal of dead surface skin cells from the outer skin. These hydroxy acids are thought to stimulate protein utilization by living epidermis cells, promoting a faster renewal of the latter; such an action results in an increase in the new cell turnover.

Used topically, AHAs reduce the thickness of hyperkeratotic stratum corneum by decreasing corneocyte cohesion. After treatment, the stratum corneum is not strongly attached to the lower part of the epidermis (Hermite, 1992). Scott and Yu (1984) have advanced the hypothesis that AHAs interfere with ionic bonding of the cells of the stratum corneum. In addition, they suggested that a topical AHA might turn on the biosynthesis of dermal glycoaminoglycans and other intercellular ground substances that could be responsible for the eradication of fine wrinkles.
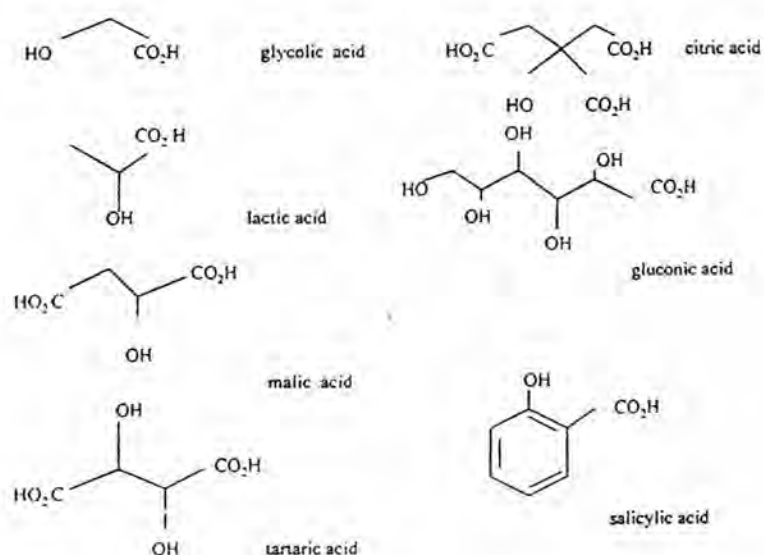
Figure 9. Structures of some α-hydroxy acids.

The role of pH values in relation to exfoliation and anti-aging effects of AHAs has been studied. Smith (1991) found that when pH increased, the ability of AHAs to stimulate cell renewal decreased. Additionally, for all the AHAs tested, a maximal stimulation of renewal was observed at a pH of about 3. Since the normal skin surface pH is in the range of 4.2-5.6 (Scott and Yu, 1994), products exhibiting a pH significantly lower than that of the skin are likely to have greater potential for irritation or the potential to elicit a stinging sensation than would the same formulation partially buffered or neutralized to a pH closer skin pH. The pH of finished product in the range of 3.5-4.5 is acceptable for balance between minimizing potential irritation and optimizing effectiveness of AHAs.

However, long term studies are still necessary as to the effects of AHAs when applied to human skin. Although it has been stated that concentrations of up to 10% are safe, the acids have been associated with injuries of the skin. Possible itching, redness and stinging have been mentioned, especially on sensitive skin.