CHAPTER II

BACKGROUND

The Greek root of the word "Liposome" means fat body, but a more precise definition may described it as hollow structure made from lipids, likewise membrane of all animal cells [Lasic, 1992]. Because of this similarity to the structure of biological membranes, liposomes are able to fuse with living cells and tissue; however, composition of liposomes can be varied with various interesting substances. The similarity of liposomes to cell membranes and their ability for carrying substances form the basis of their scientific and industrial applications.

McGraw-Hill encyclopedia of chemistry [Parker, 1993] defined liposomes as "aqueous compartments enclosed by lipid bilayer membranes"; and liposomes are also known as lipid vesicles. Major components of liposomes are lipids which the popular one is phospholipid. Its structure consists of an elongated nonpolar (hydrophobic) tail with a polar (hydrophilic) head group as shown in Figure 2.

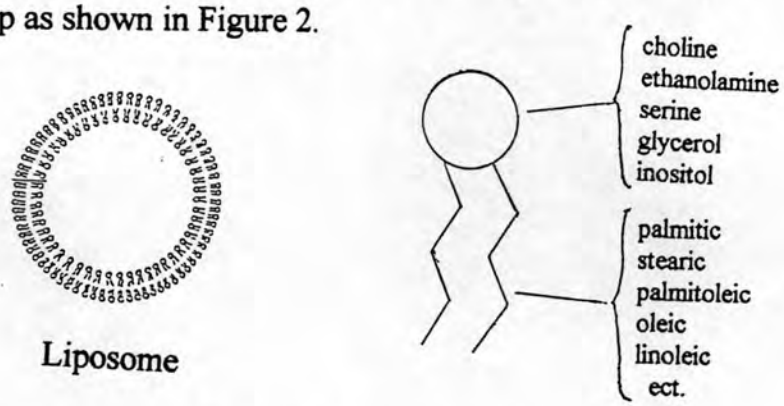


Figure 2. Liposome and chemical diversity in the head group and acyl chain regions of phospholipids.

When lipids are dispersed in water, they spontaneously form bilayer membranes which are also called lamella. The lamella composes of two monolayer sheets of lipid molecules with their nonpolar (hydrophobic) tails facing each other and their polar (hydrophilic) head group facing the aqueous medium. The liposomal membrane encloses a portion of the aqueous phase much like cell membrane which encloses the cell components; in fact, the liposomal membrane is essentially a cell membrane without its protein components.

Liposomes have been widely used in biochemical and biophysical works in the following way.

1) as vehicles for delivery of both water and oil soluble materials to cells

2) as immunological adjuvants

3) as substrate for study of membrane properties such as rotational or translational diffusion in plane of the membrane

4) as intermediates in construction of bilayers for studying electrical properties of membranes

There are two main reasons for the growing interest in liposomes [Houser, 1982]: they are extensively used as models for biological membranes and, more recently, they have become potentially important for drug encapsulation. The latter application is base on their ability to enclose therapeutic molecules such as drugs and enzymes and deliver them to specific sites in the body for the purpose of achieving tissue-specific drug absorption.

Liposome encapsulation can alter drastically the pharmacokinetics and tissue disposition of the encapsulated substances. It can enhance their uptake

into cells by mechanisms that are not normally available for these molecules; thus, it can increased their pharmacological efficacy.

The compatibility of liposomes with both lipophilic and hydrophilic drugs, and their composition which can be varied for achieving a selected release pattern are also great advantage when a drug is administered topically or to mucosal tissue. Another adventage for drug delivery to mucosal tissue is that liposome surface can be modified to provide a degree of bio-adhesion, enhancing the residence time of the liposomes at target site such as eye.

Comparison liposomes with other heterogeneous system

The pharmaceutical industry has successfully produced other heterogeneous systems, such as emulsion and suspensions on a large scale. The most successful example is shown by the phospholipid-stabilized triglyceride emulsions used for hyperalimentation. Products such as intralipid are produced in large quantity requiring carefully control of pharmaceutical quality; since, they are adminstered in fairly large doeses by intraveneous (IV) infusion. Another product such as parenteral emulsion can be substituted by liposomes as well.

Nevertheless, the greatest contrast between the two systems is different in their structures and thermodynamic properties. In emulsions, the oil phase is dispersed into small droplets in excess water by high energy processing methods such as homogenization or high-shear mixing. To prevent coalescence of the oil droplets, an amphipathic emulsifier is included that orients as a monolayer at oil/water interfaces, significantly reducing interfacial tension (Figure 3.). The head groups of the emulsifier molecules are in contact with water (up to 15 water molecules are bound to each headgroup) and the acyl

chains dissolve in triglyceride core. The inclusion of charged species in the emulsifier system lead to the establishment of a surface potential that may also stabilized the suspension.

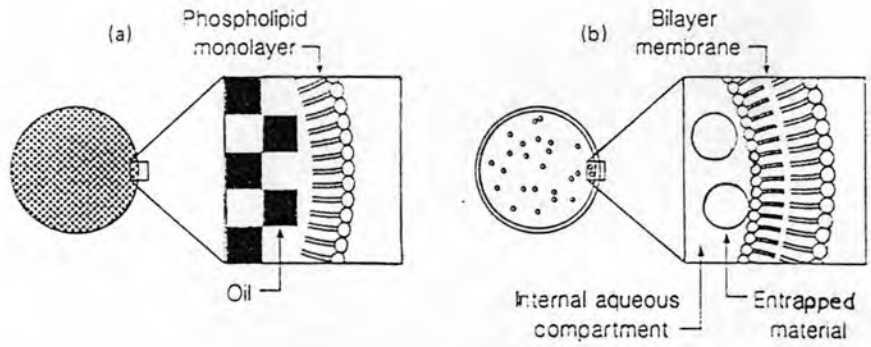


Figure 3. Diagrammatic representations of (a) a phospholipid-stabilized triglyceride emulsion droplet and (b) a phospholipid bilayer vesicle or liposome.

Although the emulsifier in these formulations acts to stave off the inevitable, coalescence of the oil phase will eventually take place as evidently by tendency of such emulsions to oil out with time or under stress conditions such as freezing, centrifugation, filtration, or temperature cycling. In a thermodynamic sense, emulsion systems are not at an energetics; thus, minimum a fraction of the energy used to create the emulsion is stored in the system and there is a pressure to relieve this energy over time.

Liposomes, in contrast, are at true energy minimum. The basis structural element of a liposome resides in its thin but durable membrane, a similar structure to biological membranes. As illustrated in Figure 3 (b), liposomes consist of a lipid bilayer matrix that wrap around an aqueous volume-sealing it from the external medium, much like a cell. The central aqueous core can range in diameter from 20 nm to several micrometers, and liposomes can

contain from one to hundreds of concentric bilayers, each creating its own aqueous compartment likewise the layers of an onion.

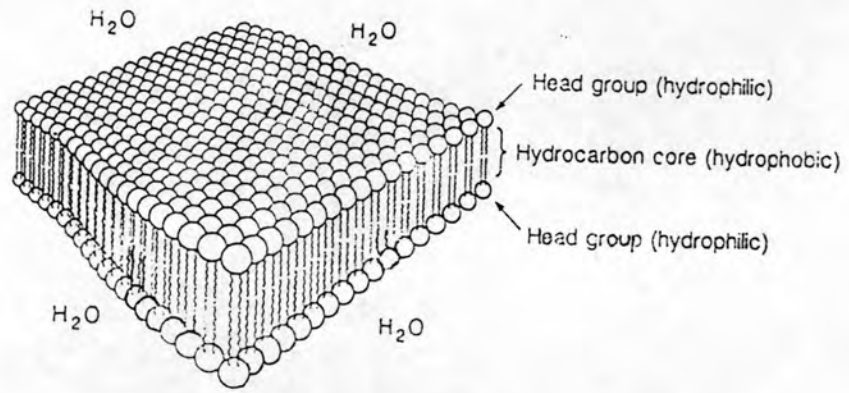


Figure 4. Schematic representation of the lipid bilayer matrix that serves as the basic structural element of liposomes (and cells).

As showed in Figure 4., these bilayer membranes form spontaneously when certain amphipathic molecules (such as phospholipids) are exposed to excess water. The phospholipid molecules align next to one another, forming biomolecular sheets with the polar head groups in close contact with water on either side of the structure and the nonpolar tail groups mixing to form a very thin oil phase sandwiched in between. The bilayer configuration satisfies the dual chemical nature of the lipids, the headgroups fully hydrate and the hydrocarbon tails content mix with each other within the core of the membrane. Although covalent bonds are usually not formed during bilayer assembly, the summation of the nonpolar forces among the many methylene groups, as the hydrophobic effect, explains the remarkable stability of structure.

Liposome-lipoprotein interaction

Scherphof, et al. (1978) reported on the release of sucrose and inulin from phosphatidylcholine liposomes as induced by rat plasma and bovine serum albumin. Although albumin caused similar leakage rate as whole plasma they had reasone to believe that the liposomal phosphatidylcholine, when incubated with whole plasma was not bound to albumin but rather to a component with higher molecular weight, presumably high-density lipoprotein. The massive destruction of the liposomal structure had to occur as a consequence of the loss of the bulk of its phospholipid. The release of phospholipid from the liposomes apparently involved an interaction of liposome and high-density lipoprotein. Albumin, which could bind liposomal phosphatidylcholine in a reversible way, was not required for the transfer to take place.

Nichols, et al. (1978) had reported that incubation of human high-density lipoprotein (HDL) with sonicated unilamellar liposomes of DMPC resulted in uptake of DMPC by the HDL and dissociation of lipid-free apo A-I which formed discs with remaining vesicles. Liposomes were lysed upon their incubation with blood, plasma or serum due to their interaction with HDL [Agawal, 1986]. This lytic effect of HDL was known to be mediated through transfer of lipids from liposomes to the protein, which in turn resulted in a enhanced leakage of the entrapped solutes in the medium. Release of trapping material from liposomes in the absence of serum or serum proteins occurred slowly over a perior of hours and was temperature-dependent [Guo, 1980].

The liposomal phosphatidylcholine is transfered from the liposomes to the whole HDL particle. The HDL releases apolipoprotein A-I which becomes associated with the liposomes and subsequently dissolves these to form a phosphatidylcholine-apolipoprotein A-I complex. The major structural

conversion of liposomes to discoidal particles occur in less than 5 min after mixing unilamellar vesicles and rat apolipoprotein A-I and E at 37 °C [Guo, 1980]. Serum apolipoproteins are the most potent liposome-disrupting agents of serum and free apolipoproteins may exist in native serum; however, the amounts must be too small to account for the observed serum activity.

The transformation of phosphatidylcholine from liposomes to particles of small size by intact HDL is an apolipoprotein-mediated process and dependent on the ratio of liposomes to available apo HDL [Chabanain, 1979]. A possible mechanism for the transformation is that apolipoprotein, loosely associates with HDL, attaches to phosphatidylcholine liposomes subsequent to collision between HDL and a vesicle particle and the resulting apolipoprotein-liposome complex undergoes break down to smaller particles when a sufficient ratio of apolipoprotein to phospholipid is attained. Collision between particles is a diffussion regulated process, dependent upon temperature and the concentration of reactants. The stability of the apolipoprotein-liposome complex and its subsequent break down to smaller particles may depend on temperature and the apolipoprotein:phospholipid ratio. The rate of transformation is depended on the amount of HDL added and is more rapid at 37 °C than at 0 °C. The formation of apolipoprotein-liposome complex necessitates dissociation of apolipoprotein from the HDL particle. Apo A-I is loosely associated with human HDL and may be dissociated in vitro under several mild conditions.

The reports of albumin binding to liposomes may be explained by the present of small amounts of contaminating lipoproteins or apolipoproteins. The observation that $d > 1.25$ g/ml fractions of bovine serum albumin are more potent than the parent albumin in inducing entrapping solute release may be explained by the presence of free apolipoprotein in these fraction. The $d > 1.25$

g/ml fractions from serum known to contain free apolipoproteins also induces marker release, and it is found in various experiments that lipid-free apolipoproteins are the most potent entrapping solute-releasing agents tested [Guo, 1980].

Aspects of lipid-cholesterol interaction

The lipid transfer and the enhanced leakage are considerably reduced by introducing an appropriate structural modification in the phospholipid component of liposome [Agawal, 1986].

The effect of cholesterol on phospholipid membranes has been extensively investigated. Cholesterol, a main constituent of many biomembranes has been the subject of a great number of studies in model systems [Demel, 1976]. Structural parameters change slowly with increasing cholesterol content up to about 32 mol%, and a relatively abrupt structural alteration as revealed by hydrodynamic parameters occurs above this cholesterol content [Chauhan, 1986].

Cholesterol is an ubiquitous structural and functional component of most eukaryotic plasma membranes and occurs in biological interfaces in highly variable concentrations. In the membrane structures of the mitochondria and nuclei, the concentration is low, but in the outer cell membranes, the concentration is normally high and molar ratios of phospholipid:cholesterol are close to 1, although the lack of sterol is found in bacterial membrane. The importance of cholesterol is normally visualized in an association with the other membrane lipids and in theoretical considerations both hydrogen bonding and London-Van der Waals interactions are thought to be of importance [De Gier, 1968]. In discussion on cholesterol's function in these biological interfaces, the

possible molecular interactions between cholesterol and other lipid molecules have been the subject of various theoretical considerations. Furthermore, these interactions have been studied in various authors seen at a first sight to be somewhat contradictory.

By means of a large varieties techniques, it has been demonstrated that the sterol molecules, when introduce into the lipid bilayer, enter into specific interaction with the phospholipid molecules. Spectroscopic studies on liposome systems exhibited a gradual reduction of the rotational freedom of the paraffin chains in the liquid-crystalline state. From calorimetric studies, it appear that the energy content of the gel-to-liquid-crystalline phase transition is reduced and the results favor the conclusion that each cholesterol molecule withdraws two phospholipid molecules from the cooperative lipid phase transition. From these studies, it has been concluded that each cholesterol affects the mobility of the phospholipids so that the lipid bilayer attains an intermediate state in which the hydrophobic interior has a high viscosity [Demel, 1976].

The investigations on mixed monolayer of cholesterol and phospholipid demonstrate reduction in the average area per molecule (the packing of the molecules becomes more condensed) comparing with those in the pure films, although it is found that the extent of this effect is highly depended on the chemical nature of the paraffin chains. A significant condensing effect is observed with phospholipids containing monounsaturated fatty acids; since, molecular interactions increase and lipid expansion of the film is limited in the presence of cholesterol. However, the work of Shah and Schulman showed that introduction of cholesterol into a very closely packed monolayer of dipalmitoylphosphatidylcholine reduced the cohesive forces, as could be seen from the change from a solid to a liquid type of film.

The addition cholesterol to dipalmitoeylphosphatidylcholine in an aqeous system lowers the transition temperature between the gel and liquid crystalline phase; thus, the heat absorbs at the transition. On state decreases the basis of these results, it is assumed that cholesterol controls fluidity of hydrocarbon chains of the phospholipids by disruption of the crystalline chain lactice of the gel phase and by inhibiting the flexibility of the chains in the dispersed liquid crystalline phase. Therefore, the various observations strongly suggest that cholesterol can cause a dual effect on phospholipids, depending on the nature of their fatty acid constituents [De Gier, 1969].

Johnson, (1973) reported enlargement of surface area per phospholipid molecule in sonicated phospholipid liposomes upon adding of cholesterol. This might be due to reorientation of the phosphate head groups in the presence of cholesterol which much more water are required for fully hydration of head group; thus surface expansion of liposomal membrane was obtained. When the proportion of cholesterol was low, decreasing the bilayer thickness was achieved by tilting the rest of the molecule sideways. However, as more cholesterol was add into the bilayer, the head of phospholipid molecules could obtain their water to hydration by overlapping the cholesterol, and their hydrocarbon chain straighten out behind, there by increasing the bilayer thickness. It appeared that the phospholipid:cholesterol ratio was more important in determining membrane thickness than the nature of the phospholipid head group.

The most important constituent of liposome than can control retention of drugs is cholesterol content. Indeed, numerous studies on the use of liposomes as model membrane have shown that cholesterol, by increasing the packing of phospholipid molecule, reduce bilayer permeability to non-electrolyte and electrolyte solutes [Kirby, 1980]. This physical change of the

hydrophobic barrier involves for the permeability properties at the interface. In the studies of the effect on water permeability it can be shown that the change in the rate of osmotic shrinkage after applying a glucose gradient to liposome systems of dipalmitoylphosphatidylcholine decrease with increasing concentration of cholesterol. The results indicated that above the phase-transition temperature (40 °C) there is a gradual but drastic reduction of the permeability, whereas below the transition, some increase in water permeability can be noticed. The same conclusions have been made from studies on the permeability of nonelectrolyte solutes [De Gier, 1969]. The rates of these simple diffusion processes are apparently a direct function of the membrane viscosity [De Gier, 1978].

Kirby, et al. (1980) showed that a high content of cholesterol in such liposomes was essential for the maintainance of their stability under a variety of experimental conditions. Cholesterol-rich liposomes, for instance, remained stable in the blood of intravenously injected animals and *in vitro* in the presence of whole blood, plasma and serum. The stability of cholesterol-rich liposomes *in vivo* was also maintained when their net surface charge was negative or positive. It would thus appeared that interaction of liposomes with plasma proteins believed to occur *in vitro* and *in vivo* or their contact with circulating cell had no detrimental effect on the stability of liposomes when they were rich in cholesterol.

The presence of blood cells, presumably erythrocytes, reduce the rate of marker released seen upon mixing of cholesterol-free or cholesterol-poor liposomes with plasma or serum. Two possible explanations can account for this finding:-

a) erythrocytes are known to interact with lipoproteins in term of phospholipid removal, thus, marker released from liposomes may be reduced due to competition of erythrocyte with liposomes in binding to lipoprotein.

b) erythrocytes donated cholesterol to liposomes and if this take place rapidly enough, cholesterol-free and cholesterol-poor liposomes will be enriched in cholesterol and so less vulnerable to destabilization.

Regardless of either real mechanism, it promotes stability of liposomes.

The mechanism by which cholesterol controls liposome stability within the biological milieu may be related to the evidence that, upon contact with plasma, liposomes with little or no cholesterol lose some of their phospholipid to high-density lipoproteins. This is accompanied with the liberation of entrapped agents. It is conceivable that excess sterol by restricting the mobility of phospholipid, prevents the latter's subsequent loss to lipoproteins [Kirby, 1980].

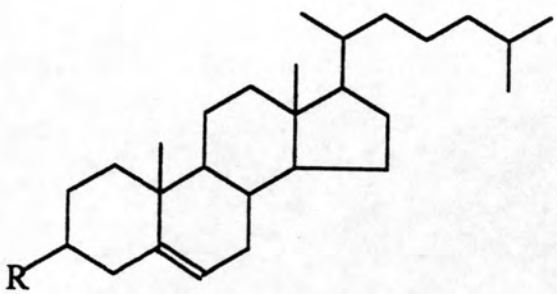
The ability of intact HDL to interact with phospholipid bilayers containing cholesterol may had physiological relevancy. HDL has been involved in the process of cellular cholesterol efflux, and apolipoprotein-phospholipid complexs has been shown to remove cholesterol and phospholipid from cultured cells [Chabanain, 1979]. Cholesterol will exchange rapidly with serum lipoproteins. Cholesterol exchange processes, however, do not impair the protective effect which cholesterol has on egg phosphatidylcholine vesicles incubated in the presence of serum, although phosphatidylcholine exchange processes, on the other hand, appear to destabilize the membrane and render it leaky. A possible explanation is that the presence of cholesterol inhibits the phospholipid exchange process of liposomes with HDL. The defects in the lipid matrix are the sites of insertion of apo A-I. These defects will be most

frequent found at the phase transition, in the absence of cholesterol or in the presence of low levels of cholesterol. The presence of high levels of cholesterol prevents or inhibits the association of apolipoprotein with membrane bilayers; thus, enhancement of liposome bilayer's stability is observed [Allen, 1980].

Inclusion of cholesterol in phosphatidylcholine liposomes (43 mol%), remarkedly reduces disc formation because intact liposomes are the predominant species remaining when cholesterol-containing liposomes are incubated with apoHDL. However, a few discoidal particles are also found, suggesting a heterogeneous distribution of cholesterol between or within liposomes, in contrast with cholesterol-poor liposomes whose bilayers are easier disrupted by the apoproteins.

By using unilamellar liposomes containing increasing amounts of cholesterol, two major apolipoproteins of rat serum, apo E and apo A-I are used to study their interaction with liposomes. As the molar ratio of cholesterol increases, less marker is released by these apoproteins until completely inhibited at 37 mol% cholesterol. This may be due to prevention of conversion of liposome to disc, cause by apoprotein. The more stable cholesterol-containing liposomes are remarkably similar to the abnormal lipoprotein commonly called LP-X, found in the plasma of humans or rats with cholestasis. LP-X is bilayer vesicle composed of equimolar phospholipids and unesterified cholesterol with every small amounts of bound apolipoprotein [Guo, 1980].

Cholesterol analogues



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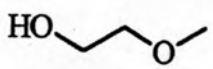
0	
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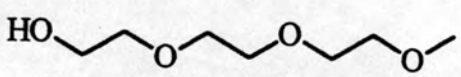
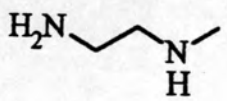
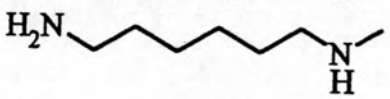
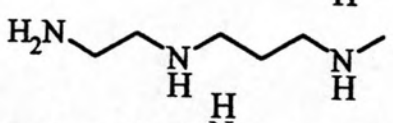
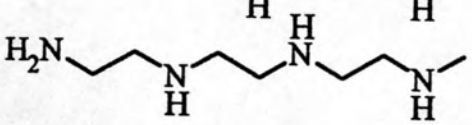
II	
III	
IV	
V	
VI	

Figure 5. Structure of cholesterol analogues. Hydroxyl cholesterol analogues are compound 0, I, II and amino cholesterol analogues are compound III, IV, V, VI.

Analogues 0, I, II have a terminal hydroxyl group and III, IV, V, and VI have terminal primary amino group.

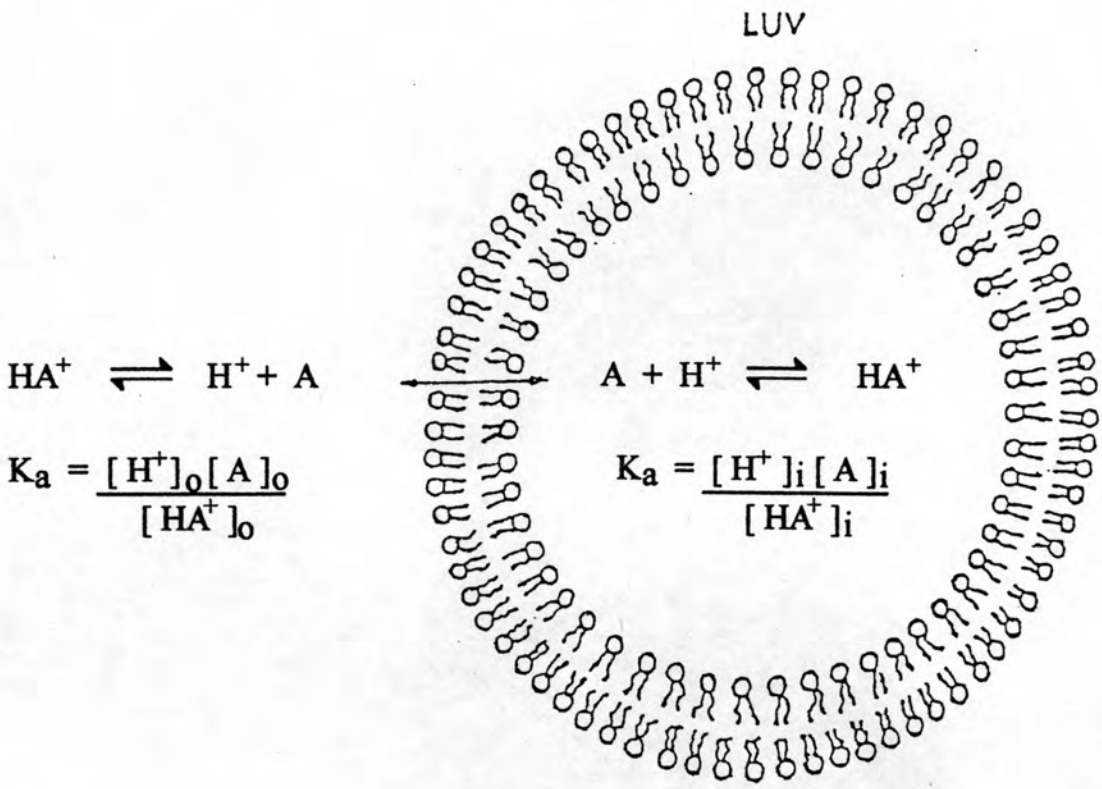
Triethoxycholesterol (compound II), in the absence of any phospholipids, can form stable liposomes capable of encapsulating polar compound [Patel, 1984]. Replacing half of cholesterol with synthetic analogues having a hydroxyl terminal group on the side chain (0 and II) reduce the permeability of DPPC vesicles [Patel, 1985]. However, addition of synthetic analogues with terminal amino group (III, IV, V and VI) increase the permeability of DPPC vesicles. The four amino-containing analogues also cause marked permeability differences. The behavior appears to correlate with the length of the group separating the terminal amino group from the sterol nucleus. Thus, the ten-bond analogues VI in DPPC vesicles is very permeable in both phosphate-buffered saline and serum, while the shortest (four-bonds) derivative III is as permeable as the control vesicles in phosphate-buffered saline , although markedly permeable in serum. The two eight-bond derivatives (IV and V) behave similarly to the ten-bond derivative VI. The poor encapsulation and higher permeability of the longer derivatives may related to either steric effects or charge interactions with the phospholipids or serum proteins.

The advantages of liposomes for drug delivery are primarily based on the reduction of potent toxic effects as exhibited by liposomal formulation of anticancer and other drugs comparing with the free drug, while maintaining or increasing efficacy against the desease state. The development of this potential has been confronted with various problems, including the instability of liposome preparation in blood circulation before reaching target organ and also high efficiently entrapment of drug within liposomes. Mayer (1986) had been reported the passive trapping technique did not allow efficient entrapment of high concentrations of hydrophobic drug such as adriamycin, which could also leaked rapidly from the vesicle carrier. Active trapping technique is the

procedure where drug/lipid ratios of the final preparations are far greater than predicted on the basis of theoretical aqueous trapped volume, base on Henderson-Hasselbach equation. At one extreme is drugs or agent which is virtually insoluble in water and can be incorporated into the lipid bilayer during vesicle formation. The amount of hydrophobic drugs that can be introduced to liposomes are, therefore, highly dependent on packing restrictions in the lipid bilayer and, liposome formulations for drug of this class vary dramatically from one agent to the other. On the opposite side, hydrophilic drug will interact with the polar head group of phospholipids and are sequestered inside to the aqueous content of liposomes. Between these two extremes are amphiphilic drugs which are often the most difficult to retain inside aqueous content of liposomes as they can rapidly permeate through lipid bilayers. Since a large number of commonly used drugs are amphiphilic molecules; therefore, this problem should be resolved.

Bulk accumulation of amphiphilic drug such as antineoplastic agents, local anaesthetics and biogenic amines inside liposomes can be obtained by incubating the drug in the presence of vesicles exhibiting transmembrane ion gradients ($\Delta\psi$) or proton gradients (ΔpH). Although both $\Delta\psi$ and ΔpH systems exhibit efficient entrapment of lipophilic cations, drug uptake in response to ΔpH is preferable; since, exogeneous ionophore is required for $\Delta\psi$-dependent encapsulation whereas ΔpH dependent is not [Mayer, 1984].

Ideally, charged form of the amine is assumed to be unable to penetrate a membrane while the uncharged form can freely penetrate ; thus, its concentration and pK_a on both sides of the membrane are the same. The influence of transmembrane pH gradient on the intravesicular and external drug concentration could be derived as demonstrated in Figure 6.



At equilibrium $[A]_i = [A]_o$, thus $\frac{[HA^+]_i}{[HA^+]_o} = \frac{[H^+]_i}{[H^+]_o}$

Let $[A^T] = [HA^+] + [A]$, then $\frac{[A^T]_i}{[A^T]_o} = \frac{[H^+]_i + K_a}{[H^+]_o + K_a}$

Figure 6. Influence of a pH gradient on the transbilayer distribution of amine drug (HA^+) in LUV.

$$HA^+ \rightleftharpoons H^+ + A$$

with a dissociation constant:

$$K_a = \frac{[H^+][A]}{[HA^+]}$$

As indicated in Figure 6, this corresponds to Henderson-Hasselbach equation.

$$\frac{[A^T]_i}{[A^T]_o} = \frac{[H^+]_i + K_a}{[H^+]_o + K_a} \qquad (1)$$

Where $[A^T]_i$ and $[A^T]_o$ are the total internal and external concentrations of the amine, $[H^+]_i$ and $[H^+]_o$ are internal and external proton concentrations, and K_a was the dissociation constant of the amine. It follows that at pH gradients, acid inside, will drive accumulation of amines into the internal volume. According to classical Henderson-Hasselbach relationships $[A^T]_i/[A^T]_o = [H^+]_i/[H^+]_o$ for situations where $K_a << [H^+]_i , [H^+]_o$. Since the pK_a of amine are more than 9, such as propranolol pK_a is 9.5; thus, this assumption is easily satisfied. For example, for a three unit pH gradient ($\Delta pH = 3$) (inside acidic), this corresponds to an equilibrium concentration of amine drug inside the vesicle system which is 1000 times higher than outside. The pH gradients, thus increases in retention of drugs inside the liposomes once entrapped. Moreover, since this approach does not rely on any specific drug-lipid interaction to enhance entrapment, it can be used for virtually any lipid mixture that forms liposomes capable of maintaining a stable transmembrane pH gradient.