CHAPTER II

REVIEW OF LITERATURE

Liposomes

Liposomes are microscopic vesicles consisting of phospholipid bilayers enclosing an aqueous compartment. They can also be bound by single bilayer membrane (unilamellar liposomes) or may be composed of multiple concentric membrane (multilamellar liposomes) (Figure 1). Because phospholipids are amphiphilic substances, they form bilayer vesicles spontaneously when dispersed in an excess of aqueous solution, so that liposomes can entrap both hydrophilic and hydrophobic substances in their vesicles (44). Hydrophilic substances are entrapped within the aqueous compartment while hydrophobic substances are bound into the lipid membrane (see Figure 2).

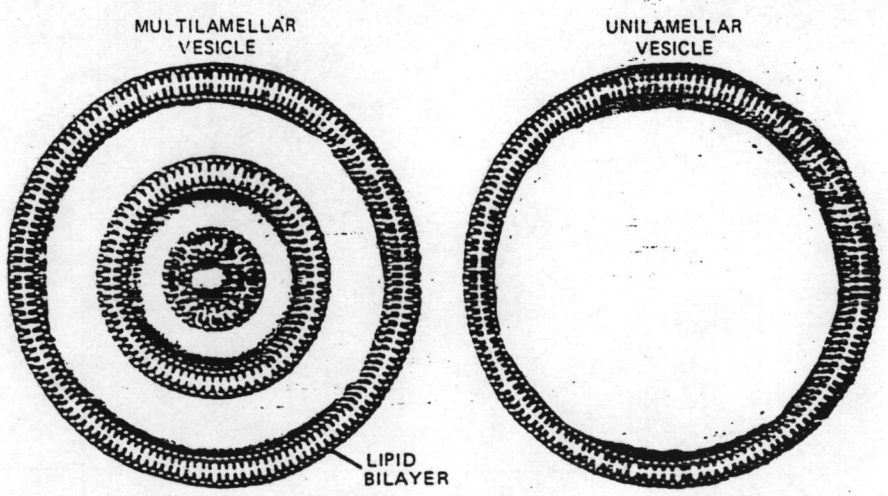


Figure1 Diagrammatic representation of multilamellar and unilamellar vesicles.

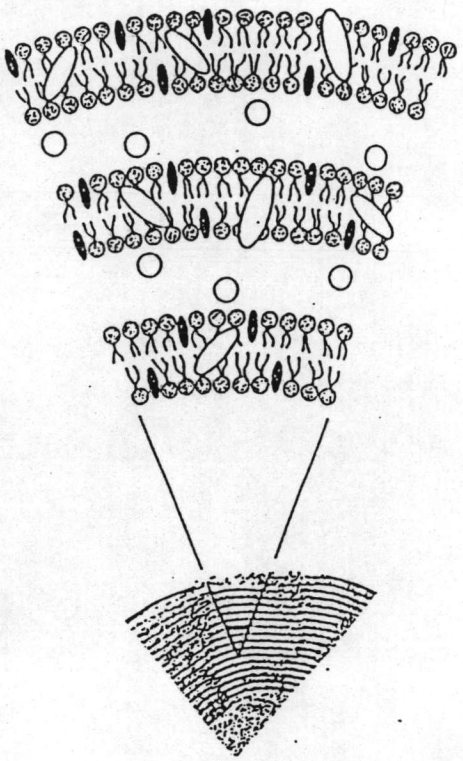


Figure 2. A section of an electron micrograph of multilamellar liposomes shows lipid bilayers alternating with electron opaque aqueous channels. Three of these bilayers are enlarged schematically to illustrate their molecular organization, in which polar heads of phospholipids face the water phase and acyl chains form the hydrophobic regions.

○, open circles denote water soluble drug entrapped in the aqueous channels.

●, filled oblong shapes are cholesterol

0, open oblong shapes are lipid soluble drug entrapped in the membrane.

1. Historical Background

In 1965 , the English scientist Alec D. Bangham made a chance discovery. Introducing water into an erlenmeyer flask containing a thin layer of phospholipid molecules makes these assume an ordinate structure now known as liposomes(6). In the first time, liposomes had been studied as a biological membrane model (44,45). In 1971, liposomes had been extensively studied as drug delivery system (46) ; one aspect of the studies involved the entrapment of enzymes which could then be administered intravenously to patients for the treatment of inherited storage diseases (47). Liposomes have an advantage as a drug delivery system because they were formed from natural molecules which can be metabolized in the body. Because of the versatility of liposomes structure and ability to incorporate almost any drug regardless of solubility , the use of liposomes as carrier vesicles has been extended to many hundred of drugs. They included chelating agent, antibiotics, drugs, which particular emphasis on anti-tumor drugs, peptide hormones, other proteins and genetic materials (49-60).

2. Chemical Constituents.

Chemical constituents of liposomes consist of structural components and non structural components. The structural components are phospholipids and sterols which determine properties such as membrane fluidity ,charge density ,and permeability. The non structural components are not important components for structural construction of liposome. They were incorporated for some special purpose (61).

2.1 Phospholipids.

Phospholipids are the major structural components of liposomes. They are not soluble but dispersible in water (62). Phospholipids are polar lipids, their molecules consist of a hydrophobic "tail" (the pair of fatty acids) and a hydrophilic polar "head"(the phosphate group) (Figure3). The backbone of most common polar lipids is either an alcohol such as glycerol or a sphingosine. Corresponding lipids are thus called glycerides or glycerophosphatides and sphingophosphatides (63).

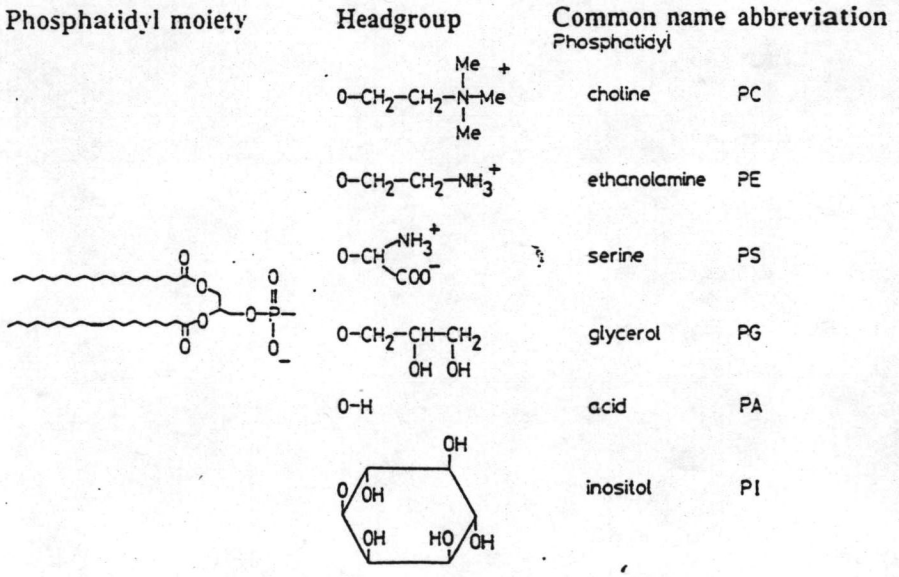


Figure 3. Structure of glycerol containing phospholipids

The most common phospholipids are phosphatidylcholine (PC). They are amphipathic molecules in which a glycerol bridge links a pair of hydrophobic acyl hydrocarbon chains with a hydrophilic polar headgroup. In aqueous media they align themselves closely in planner bilayer sheets in order to minimize the

unfavourable interactions between the bulk aqueous phase and the long hydrocarbon fatty acid chains. Such interactions are completely eliminated when the sheets fold on themselves to form close sealed vesicles. Phosphatidylcholines contrast markedly with other amphipathic molecules (detergents, lysolecithin) in that bilayer sheets are formed in preference to micellar structures. This is thought to be because the double fatty acid chain gives the molecule an overall tubular shape, more suitable for aggregation in planar sheets compared with detergents with a polar head and single chain, whose conical shape fits nicely into a spherical micellar structure (Figure 4). Surfactant molecules, such as dioctadecyl ammonium chloride (DODAC), containing two hydrocarbon chains (as well as single chain fatty acids, e.g. oleic) can, under certain circumstances, also form bilayer membrane vesicles (61).

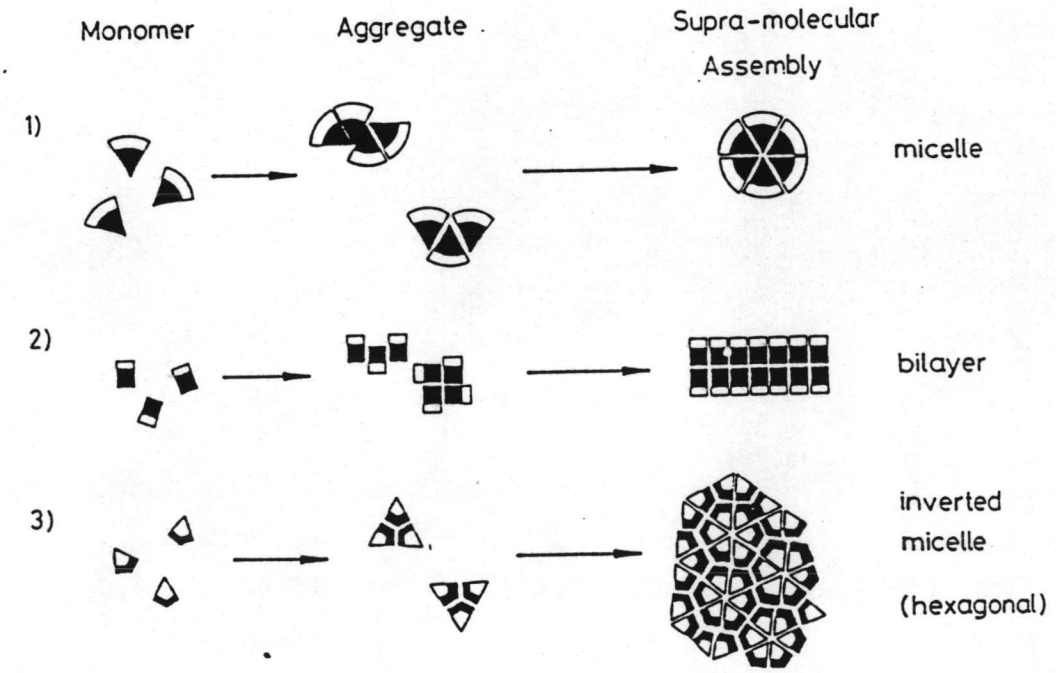


Figure 4. Association patterns of amphiphiles. Relation between molecular shape and structure of aggregates.

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. Forming as they do the major phospholipid component of many cell membranes, they are often used as the principal phopholipid in liposomes for a wide range of applications, both because of their low cost relative to other phospholipids, and their neutral charge and chemical inertness. Lecithin from natural sources is in fact a mixture of phosphatidylcholines, each with chains of different length and varying degrees of unsaturation (Table1). Lecithin from plant sources has a high level of polyunsaturation in the fatty acyl chains, while that from mammalian sources contain a higher proportion of fully saturated chains (64).

At different temperatures, lecithin membranes can exist in different phases. In low temperature(T<Tc) - gel phases, the hydrocarbon chains are in an orientationally well ordered state in which the hydrophobic molecular segments are nearly completely in an all-trans configuration; their extension is thus close to the possible maximum. At higher temperatures (T>Tc),however, such high chain order is lost, owing to the orientational chain excitations. This results in a cooperative, first order chain-melting (order-disorder, gel to fluid) (65,66). Transitions from one phase to another can be detected by physical techniques as the temperature is increased. The most consistently observed of these phase transitions is the one occurring at the highest temperature, in which the membrane passes from a tightly ordered "gel" or "solid" phase, to a liquid-crystal phase at raised temperatures where the freedom of movement of individual molecules is higher. The most widely used method for determining the phase transition temperature (T_c) is differential scanning microcalorimetry (Figure 5).

Table 1. Fatty acids of phospholipid in common use.

Abbreviation	Systematic name	Common name	Relative Abundance in natural source of PC (%)		
			Soya PC	Egg PC	Rat
C12:0	dodecanoic	lauric			
C14:0	tetradecanoic	myristic			
C16:0	hexadecanoic	palmitic	17.2	35.5	28.8
C16:1	cis-9-hexadecenoic	palmitoleic			
C18:0	octadecanoic	stearic	3.8	13.5	18.0
C18:1	*cis*-9-octadecenoic	oleic	22.6	26.8	8.4
C18:2	*cis,cis*-9,12-octadecadienoic	linoleic	47.8		
C18:2	*cis,cis*-6,9-octadecadienoic		5.7	19.4	
C18:3	all *cis*-9,12,15-octadecatrienoic	∝- linoleic	8.6		
C18:3	all *cis*-6,9,12-octadecatrienoic	ϒ- linoleic		0.2	
C20:0	eicosanoic	arachidic			
C20:1	*cis*-9-eicosanoic	gadoleic			
C20:3	all *cis*-8,11,14-eicosatrienoic				1.0
C20:4	all *cis*-5,8,11,14-eicosatetraenoic	arachidonic		1.0	17.0
C20:5	all cis-5,8,11,14,17-eicosapentaenoic		3.6	2.4	
C22:0	docosanoic	behenic			
C22:5	all *cis*-7,10,13,16,19-docosapentaeaoic		1.3	1.5	
C22:6	all *cis*-4,7,10,13,16,19-docosahexaenoic		12.6	3.5	
C24:0	tetracosanoic	lignoceric			

In the numerical abbreviation for each of the fatty acids (left-hand side), the first figure refer to the total number of carbon atoms in the fatty acid chain, and the second to the number of unsaturations (double bonds) which that chain possesses.

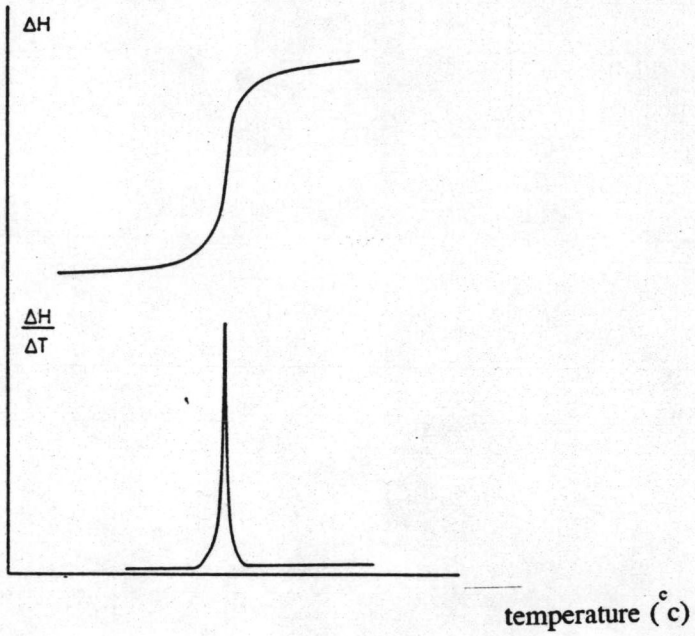


Figure 5. Differential scanning microcalorimeter of phospholipid.

From figure 5 change in heat absorbed by membrane preparation upon increase of temperature, as a result of passing through a transition from one phase to another. The heat required by the sample to maintain a steady upward rise in temperature is plotted as a function of temperature. The lower trace is obtained from the upper by mathematical differentiation, and the area under the peak is the enthalpy of transition.

The influence of hydrocarbon chain length and unsaturation (as well as head group) on the value of T_c for membranes composed of different phospholipids is considerable, and is illustrated in Figure 6. In general, increasing the chain length, or increasing the saturation of the chains increases the transition temperature. Membranes made from egg yolk lecithin have a transition temperature from -15°c to -7°c, compared with membranes from mammalian sources which are usually in the range zero to 40°c.

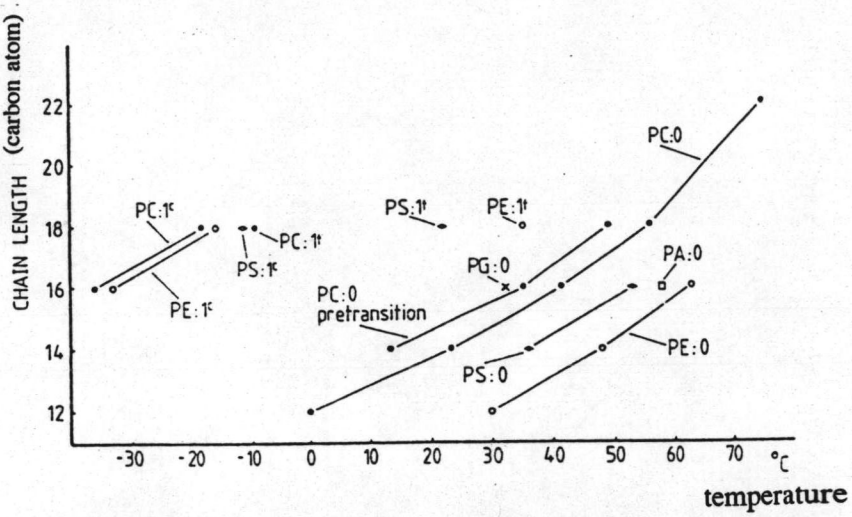


Figure 6. Phase transition temperatures for diacyl phospholipids with different headgroups as a function of chain length.

An understanding of phase transitions and fluidity of phospholipid membranes is important both in the manufacture and exploitation of liposomes, since the phase behaviour of a liposome membrane determines such properties as permeability, fusion, aggregation, and protein binding, all of which can markedly affect the stability of liposomes, and their behaviour in biological systems.

Binary mixtures of synthetic lecithins of different chain lengths give a main transition intermediate in temperature between those of the individual components, unless the chain lengths are very different, in which case two separate transitions are observed; in the temperature region between the transitions solid and fluid phases may co-exist, each enriched in one or the other of the components (Figure 7).

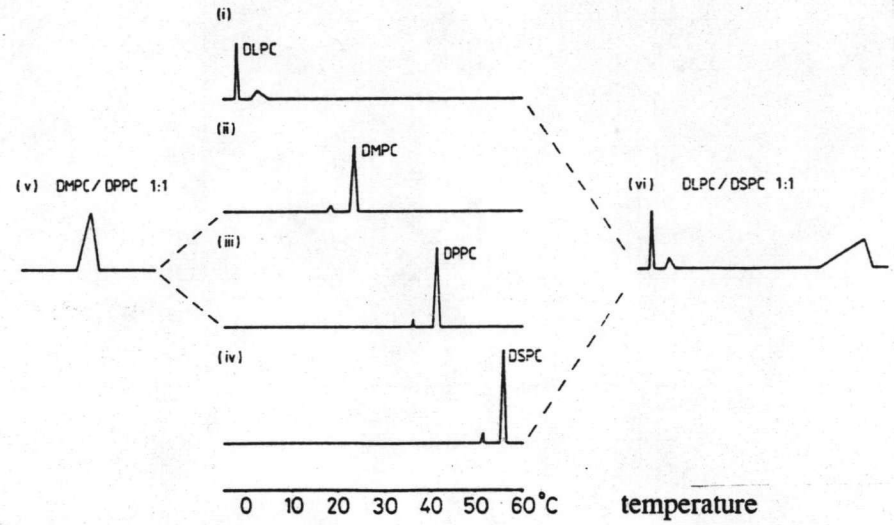


Figure 7. Microcalorimetry curves showing phase transition of membranes containing single components or mixtures of phospholipids.

Membranes composed of mixtures of pure phosholipids can display a single phase transition if the Tc of the individual lipids are close to each other (e.g. DMPC and DPPC). The combined Tc will usually be intermediate between those of the two separate lipids. Lipids with Tc which differ greatly from each other (e.g. DLPC and DSPC) act as isolated components with a degree of mutual immiscibility, undergoing phase transitions independently of each other, and forming membranes composed of two or more separate phases rich in one or other of the individual components. Abbreviations: DLPC, dilauroyl (12:0) phosphatidyl choline; DMPC, dimyristoyl (14:0) phosphatidyl choline; DPPC, dipalmitoyl (16:0) phosphatidylcholine; DSPC, distearoyl (18:0) phosphatidylcholine.

2.2 Sterols

Sterols are apolar lipids (the other apolar lipids, such as paraffins, waxes, fatty alcohols, simple fatty acids, di- and triglyceride, certain steroids,etc.). Normally, they do not interact with water or hydrate only weakly

because of the lack or too small number of hydrophilic residues (61). Sterols are important components of most natural membranes, and incorporation of sterols into liposome bilayers can bring about major changes in the properties of these membranes. In mammals, the predominant sterol is cholesterol , with a significant quantity of 7-dehydro-cholesterol being found in subcellular membranes. Commonly encountered plant sterols are stigmasterol and sitosterol, while ergosterol is an endogenous sterol of yeasts, fungi, and some protozoa. The structure of some sterols were shown in Figure 8. Features common to all of these sterols are a 3β-hydroxyl group, a planar steroid nucleus, and an aliphatic side chain, all of which are essential for the characteristic behaviour of sterols in membranes to be displayed.

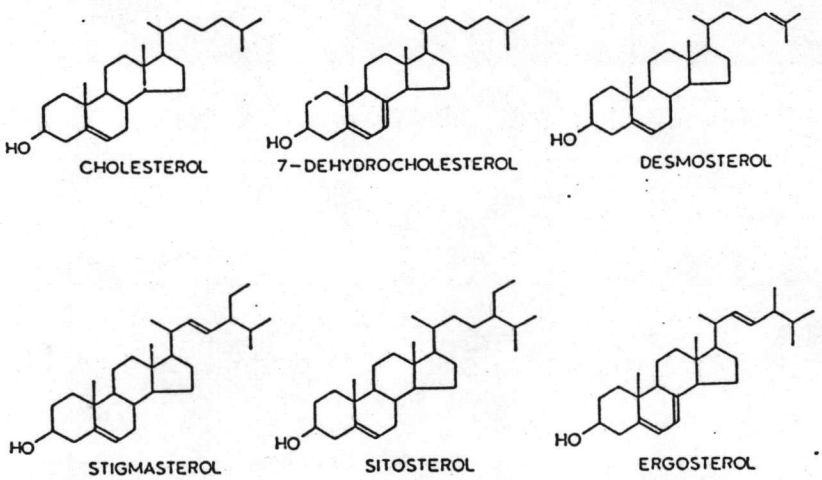


Figure 8. Structure of major sterols found in natural membranes

Cholesterol does not by itself from bilayer structures, but it can be incorporated into phospholipid membranes in very high concentrations-up to 1:1 or even 2:1 molar ratios of cholesterol to PC. In natural membranes, the molar ratio varies from 0.1-1, depending upon the anatomical and cellular location. Being an amphipathic molecule, cholesterol inserts into the membrane with its hydroxyl

group oriented towards the aqueous surface, and the aliphatic chain aligned parallel to the acyl chains in the centre of the bilayer. The 3β-hydroxyl group is positioned level with the carboxyl residues of the ester linkages in the phospholipids, with very little vertical freedom of movement. The presence of the rigid steroid nucleus alongside the first ten of carbons of the phospholipid chain has the effect of reducing the freedom of motion of these carbons, while at the same time creating space for a wide range of movement for the remaining carbon towards the terminal end of the chain (Figure 9).

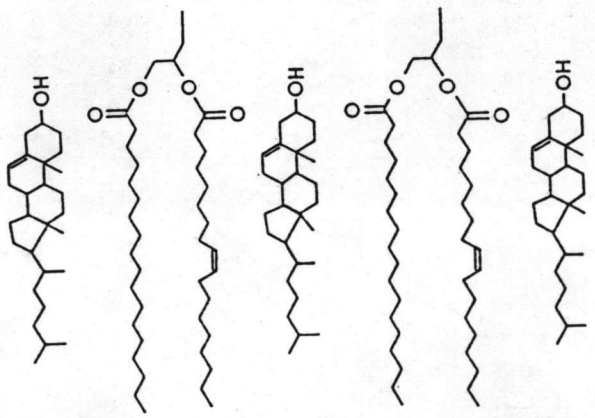


Figure 9. Position occupied by cholesterol in the membrane bilayer.

Cholesterol rests in the membrane with the hydroxyl group on a level with the acyl chain carboxyl groups, and the planar steroid nucleus is thus located parallel with the first nine carbon atoms of the acyl chain, which is the portion of the chain where motion is most severely restricted in the absence of cholesterol. In contrast, motion of the carbons later in the chain is relatively free, and is increased in the presence of cholesterol, as insertion of the steroid nucleus at the head of the chain creates more space for the end carbons in the chain to move.

Above a certain concentration of cholesterol, the membrane area occupied by the acyl chains and sterol combined is greater than or equal to that taken up by the phosphatidylcholine headgroup, so that PC membranes with high levels of cholesterol do not show the chain tilt that is observed in the gel phase of liposomes composed of pure PC .

Addition of cholesterol to PC membranes has a marginal effect on the position of the main transition temperature (T_c): in dipamitoayl phosphatidyl choline (DPPC) the T_c change from 41°c to 44°c with 33 mol% cholesterol. With increasing concentration, however, cholesterol is able to eliminate evidence of a phase transition altogether, reducing the enthalpy of phase change to zero at 50 mol% (1:1 ratio), and in so doing altering the fluidity of the membrane both below and above the phase transition temperature (Figure 10).

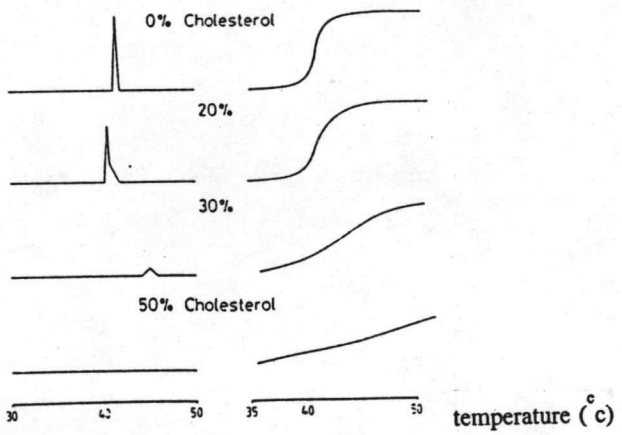


Figure 10. Influence of cholesterol on phase transition.

Cholesterol has relative little effect on the position of the phase transition, but is able to abolish completely the heat of transition. As the concentration of sterol reaches equimolar with phospholipid, the freedom of molecular motion above the phase transition is decreased, while below the phase transition mobility is actually increased.

At temperature higher than transition temperature, the phospholipid are pushed apart, the packing of the headgroups is weakened, and the fluidity is increased. Below at the transition temperature, the reduction in freedom of acyl chains causes the membranes to condense, with a reduction in area, closer packing and a decrease in fluidity. These changes in fluidity are paralleled by changes in permeabiility of the membrane. By high cholesterol, the permeability of membrane decrease at temperatures higher than the T_c but increase at lower temperatures.

2.3 Non-structural components

For structural purposes, no components other than phospholipids and sterols need to be incorporated into the membrane. Because the membrane interior is a very fluid aliphatic medium composed of molecules associated by non-covalent interactions it will readily accept and retain a wide range of lipophilic compounds without the need for any fixed chemical structural specificity. Under normal circumstances, these compounds can probably be accommodated in the membrane to a concentration of about 1-10% by weight without serious disruption of the basis bilayer structure, although the membrane integrity as determined by fluidity or permeability may will be altered. In particular cases, where a specific interaction is known to occur between the compound and other membrane components (e.g. fatty acids, α-tocopherol), concentrations higher than 10% may be achieved. Conversely, relatively low concentrations of certain substances such as some polyene antibiotics, will completely disrupt the membrane, as a result of specific interactions.

3. Physical structure

Physical structure of liposomes, such as vesicle size, shape and lamellarity are an important factors affecting physicochemical stability, encapsulation efficiency, tissue distribution, and in vivo circulation lifetimes (67) . In the study of the interaction of liposomes with cells in culture have shown that the rate and site of uptake in vivo of small and large liposomes may be quite different. For these reasons, many workers have aimed to prepare homogeneous suspensions of small liposomes (68).

Physical structure of liposomes are influenced by chemical components and preparing method. Equilibrium liposome radius is determined by the lipid packing properties(69). Chain length plays a much smaller role than the properties of lipid heads, but chain packing parameters do matter(70,71). Addition of cholesterol, for example, results in greater average vesicle size (72). The classification of liposomes according to size is the most common index of characterization in current use(61).

Liposomes can range in size from the smallest vesicle obtainable on theoretical groups (diameter ~ 25 nm) determined by the maximum possible crowding that headgroups will tolerate as the curvature in the inner leaflet increases with decreasing radius, to liposomes which are visible under a light microscope, with a diameter of 1000 nm (one micron) or greater, equal to the dimensions of living cell. It is usual to define the liposomes on works with as belonging to one of several categories:

(I) Multilamellar vesicles (MLVs). These usually consist of a population of vesicles covering a wide range of sizes (100-1000 nm), each vesicle generally consisting of five or more concentric lamellae. Vesicles composed of just a few concentric lamellae are sometimes called oligo-lamellar liposomes, or paucilamellar vesicles.

(II) Small unilamellar vesicles (SUVs). These are defined here as those liposomes at the lowest limit of size possible for phospholipid vesicles. This limit varies slightly according to the ionic strength of the aqueous medium and the lipid composition of the membrane, but is about 15 nm for pure egg lecithin in normal saline, and 25 nm for dipamitoayl phosphatidyl choline (DPPC) liposomes. Since, according to the definition, these liposomes are at or close to the lower size limit, they will be a relatively homogeneous population in terms of size.

(III) Large unilamellar vesicles (LUVs). These liposomes have diameters of the order of 1000 nm.

(IV) Intermediate-sized unilamellar vesicles (IUVs) . This term is not currently found in the literature, but it is introduced here for convenience and to avoid confusion. These have diameters of the order of magnitude of 100 nm.

Other terms also used in the literature such as REV (reverse-phase evaporation vesicle), DRV (dried-reconstituted vesicle), MVL (multivesicular liposome) relate to the method of manufacture.

4. Physical Properties of Liposomes.

This ability of liposomes to entrap water and therefore solutes within a closed lipid bilayer structure lead to their use as models of biological membranes for permeability studies; phosphatidylcholine vesicles are permeable to

water , ions and non-electrolytes , although the permeabilities depend on the chemical composition of the liposomes. Positively charged liposomes (e.g. phosphatidylcholine plus a positively charged lipid such as stearylamine) are impermeable to cations , while negatively charged liposomes (e.g. containing phosphatidic acid) are permeable to cations; however the permeability of all liposomes to protons is low. Anions diffuse rapidly through negatively and positively charged lipid membrane and also through uncharged lipid membrane. In general it has been found that increasing degree of saturation or the length of the fatty acyl chains produces a decrease in the permeability of the bilayer to all solutes. This is due to the increase in the transition temperature. A decrease in permeability can also be produced by the addition of cholesterol to naturally occurring phospholipid.

The rate which the solutes leak out of the liposomes is dependent on both the nature of the solute and the composition of the liposome. By modification of the composition of the lipid bilayers it is possible to reduce the leakage of particular molecules although any small molecule not having too many hydrogen bonds will leak rapidly from the liposome irrespective of lipid composition, while large molecules such as proteins will not leak out unless the structure of the liposomes is disrupted. The great value of liposomes, as carriers of therapeutic and other materials, is the wide spectrum of materials which they can incorporate in liposomes. The ranging, lipophilic agent located in lipid bilayer, amphipathic compounds located with the phospholipid at the boundary between the aqueous phase and the membrane interior, and the water-soluble molecules entrapped in the aqueous compartment. Because the means of their incorporation is entirely physical, no restrictions are placed on the chemical nature of these agents.

Accordingly, only two classes of compounds are not good candidates for incorporation into liposomes. The first, materials which are insoluble in both aqueous and organic solvents although with certain methods of preparation even these need not be an obstacle; and second , materials whose solubility are high in both media, and for whom a lipid membrane will constitute no barrier to passage from the inside to the outside of liposomes.

5. Methods of Liposome Preparation.

Liposomes have attracted considerable interest in such various fields as the medical, biotechnological and cosmetic ones. A number of different methods have been developed for the preparation of liposomes. Those methods have been classified for convenience into three categories.

5.1 Mechanical dispersion

In this group of methods, essentially the simplest in concept, the lipids are dried down onto a solid support (usually the side of the glass container vessel) and then dispersed by addition of the aqueous medium, followed by shaking. Even before exposure to water, the lipids in the dried-down film are thought to be oriented in such a way as to separate hydrophilic and hydrophobic regions from each other, in a manner not unlike their conformation in the finished membrane preparation. Upon hydration, the lipids are said to 'swell', and peel off the support in sheets, generally to form multilamellar vesicles. In most of the methods described in this section, the aqueous volume enclosed within the lipid membrane is usually only a small proportion of the total volume used for swelling - about 5-10%. This method is, therefore, very wasteful of water-soluble compounds

to be entrapped, although the absolute yield of material may be satisfactory for practical purposes. Lipid-soluble compounds on the other hand, can be encapsulated to 100% efficiency.

5.1.1 Hand shaken multilamellar vesicles (MLVs)

The simplest and most widely used mothod of mechanical dispersion is commonly known as hand shaking, since the lipids are suspended off the sides of a glass vessel into the aqueous medium by gentle manual agitation. In order to increase the entrapment volume,it is advisable to start with a round-sided glass vessel of large volume, so that the lipids will be dried down onto as a large surface area as possible to form a very thin film. Thus, even though the volumes of organic or aqueous starting solutions may be only 1 ml each, it is recommended that one use a 50 - or 100 - ml vessel for drying down. Care and patience is needed in order to resuspend all the dried lipid with minimum loss of fluid. If the materials to be entrapped are lipid-soluble, however, conditions for drying down are not so critical. The temperature for drying down should be regulated so that it is above the phase transition temperature of the lipids in question. At high temperatures, or under conditions where the dried lipid forms a fairly thick film, it may be difficult to remove it from the glass by manual swirling; also lumps of solid lipid may form which do not disperse easily. In this case, the addition of glass beads, either with the aqueous solution, or dried down with the lipid, is a very effective aid in suspending the lipid. Glass beads of any size from 0.5 to 3 mm in diameter are suitable. After addition of the beads, the flask may be re-attached to the rotary evaporator by means of clip or tape, and the lipids suspended by rotation of the flask for a period of half an hour at atmospheric pressure. When large volumes of lipid and aqueous solution are used, the suspension can be carried out by vigorous vibratory motion in a mechanical shaker,

and complete and homogeneous dispersion ensured by continuing the process for several hours.

5.1.2 Processing of lipids hydrated by physical means

After preparation of multilamellar vesicles by hydration of dried lipid it is possible to continue processing the liposomes in order to modify their size and other characteristics. For many purposes, MLVs are too large or too heterogeneous a population to work with, so many of the methods have been devised to reduce their size and in particular to convert liposomes in the large size range into smaller vesicles. These include techniques such as micro-emulsification, extrusion, ultrasonication, and use of a French pressure cell. A second set of methods is designed to increase the entrapment volume of hydrated lipids, and or reduce the lamellarity of the vesicles formed, and employ procedures such as freeze-drying, freeze-thawing, or induction of vesiculation by ions or pH change.

5.1.3 Micro-emulsification liposomes (MEL)

Recenly, the use of a micro-fluidizer to prepare small MLVs from concentrated lipid suspensions has been reported. The micro-fluidizer is a machine which pumps fluid at very high pressure (10, 000 p.s.i.) through a 5 μm filter, after it is forced along defined microchannels which then direct the two streams of fluid to collide together at right-angles at a very high velocity, thus affecting a very efficient transfer of energy. The lipids can be introduced into the fluidizer, either as a suspension of large MLVs or as a slurry of unhydrated lipid in an aqueous medium. The fluid collected can be recycled through the pump and interaction chamber until vesicles of the required dimensions are obtained. After a single pass, the size of vesicles is reduced to between 0.1 and 0.2 μm in diameter, the exact size distribution depending on the nature of the components of the

membrane and of the hydration medium. The presence of negative lipids tends to decrease their size, while increased cholesterol gives larger liposomes. Continuing the cycling time generally bring the size to a steady low value, although in some cases (e.g. liposomes containing doxorubicin) the diameter can increase after prolonged recycling.

5.1.4 Sonicated vesicles (SUVs)

In order to reduce hydrated lipid to vesicles of the smallest size possible, it is necessary to use a method which imparts energy at a high level to the lipid suspension. This was first achieved by exposure of MLVs to ultrasonic irradiation and it is still the method most widely used for producing small vesicles. The starting point is usually a suspension of multilamellar vesicles. Since these vesicles will be completely broken down in the process, it is not necessary be concerned about the initial size of the MLVs, the percentage entrapment or the thickness of the lipid film, so the lipid can be dried down in the same vessel as is used for sonication. There are two methods of sonication, using either a probe or a bath ultrasonic disintegrator. The probe is employed for suspensions which require high energy in a small volume (e.g. high concentrations of lipids, or a viscous aqueous phase) while the bath is more suitable for large volumes of dilute lipids. where it may not be necessary to reach the vesicle size limit.

5.1.5 French pressure cell liposomes

Because of the problems inherent in subjecting biological materials to ultrasonic irradiation (i.e. degradation not only of lipids, but of macromolecules and other sensitive compounds to be entrapped inside liposomes), methods have been developed which are able to cause fragmentation and restructuring of membranes under very mild conditions. One of the first and

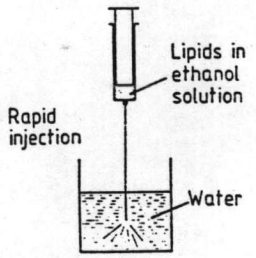
still very useful techniques developed is that of extrusion of pre-formed large liposomes in a French Press under (generally) very high pressure. This technique yields rather homogeneous uni- or oligo-lamellar liposomes preparations of intermediate sizes (30 - 80 nm in diameter, depending on the pressure used). Besides the more gentle preparation conditions, which permit their use as carriers of sensitive macromolecules, these liposomes are more stable than sonicated ones and can be used advantageously as drug delivery systems *in vitro*. In addition, high pressure extrusion of liposomes/protein mixtures appears to be a useful means for reconstitution of solubilized membrane proteins. The French Press, named after one of its inventors, was originally designed and is still being used for the disruption of plant and bacterial cells. The heart of a French Press is the pressure cell. Manufactured in stainless steel, it is designed to resist pressures up to 20,000 or even 40,000 p.s.i. . Two pressure cells are available, of different sizes. The size of the resulting French pressed liposomes (FPL) is variable, depending on the lipid composition used, the temperature, and, most important, on the pressure the average diameter of unilamellar French press liposomes is roughly inversely proportional to the extrusion pressure. However, below a certain critical pressure the shear forces do not seem to suffice to completely disrupt the multilamellar parent liposomes. Lipid mixtures composed of pure phospholipids tend to yield smaller vesicles than mixtures containing cholesterol. Thus inclusion of 30 mol % of cholesterol is found to in crease the average size of the extruded liposomes by around 50 %. Liposomes prepared by this technique, although still small, are somewhat larger than vesicles prepared by sonication, and are less likely to suffer from the structural defects and instabilities known to arise in sonicated vesicles. Leakage of vesicle contents from liposomes prepared using a French Press has been found to be slower than from sonicated liposomes. The French Press has also been used to reduce the

heterogenicity of populations of liposomes obtained by detergent dialysis techniques.

5.2 Solvent dispersion

In this group of methods, the lipids comprising the liposomes membrane are first dissolved in an organic solution, which is then brought into contact with the aqueous phase containing materials to be entrapped within the liposomes. At the interface between the organic and aqueous media, the phospholipids align themselves into a monolayer which forms the basis for half of the bilayer of the liposomes. Methods employing solvent dispersion fall into one of three categories:

5.2.1 Ethanol injection



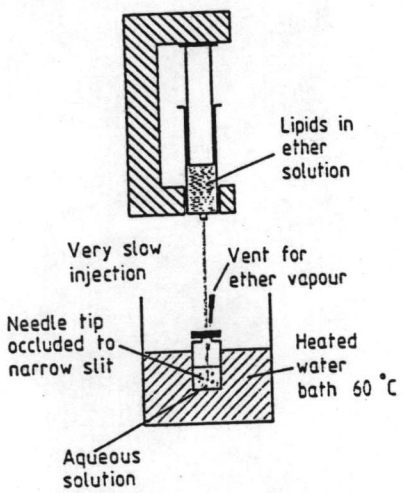
Preparation of SUVs by ethanol injection. This drawing illustrates the salient features of the ethanol injection method for preparation of SUVs. For formation of good vesicles the final ethanol-in-water concentration should not exceed 7.5%.

An ethanol solution of lipids is injected rapidly into an excess of saline or other aqueous medium, through a fine needle. The force of the injection is usually sufficient to achieve complete mixing, so that the ethanol is diluted almost instantaneously in water, and phospholipid molecules are dispersed evenly throughout the medium. This procedure can yield a high proportion of small unilamellar vesicles (diameter 25 nm) although lipids aggregates and large vesicle

may form if the mixing is not through enough. The method has the advantage of extreme simplicity and a very low risk of bringing about degradation changes in sensitive of lipids. It major shortcoming is the limitation of the solubility of lipid in ethanol (40mM for PC), and on the volume of ethanol that can be introduced into the mediurn (7.5 % v/v maximum), which in turn limits the quantity of lipid dispersed, so that the resulting liposomes suspension is usually rather diluted. The percentage encapsulation is thus extremely low if the materials to be entrapped are dissolved in the aqueous phase. Another drawback is the difficulty with which ethanol can be removed from phospholipid membranes.

5.2.2 Ether injection

Although very similar in concept to the method of ether injection contrasts markedly with ethanol injection in many respects. It involves injecting the immiscible organic solution very slowly into an aqueous phase through a narrow bore needle, at such a temperature that the organic solvent is removed by vaporization during the process.



The mechanism whereby large vesicles are formed by this method is not clearly understood, but it may be that the slow vaporization of solvent gives rise to an ether:water gradient extending on both sides of the

interfacial lipid monolayer, resulting in the eventual formation of a bilayer sheet which folds in on itself to form a sealed vesicle. This process can repeat itself many times.

Ether injection is a method which treats sensitive lipids very gently, and runs very little risk of causing oxidative degradation, providing the ether has been carefully redistilled to remove peroxides which arise as a result of spontaneous breakdown. Since the solvent is removed at the same rate as it is introduced, there is no limit to the final concentration of lipid which can be achieved, since the process can be run continuously for a long period of time, giving rise to a high percentage of the aqueous medium encapsulated within vesicles. The disadvantages of the technique are the long time taken to produce a batch of liposomes, and the careful control needed for introduction of the lipid solution, requiring a mechanically operated infusion pump. If working with substances which might be damaged at elevated temperature (60°C), the method may be adapted to use fluorinated hydrocarbons which vaporize at lower temperatures, instead of ether.

The liposomes formed are in the size range 0.1-0.5 μm diameter, with an entrapment volume of 10 - 15 litres mol^{-1} phospholipid. Under the conditions described above, one would expect a total entrapped volume of approximately 50 μl (1 % entrapment).

Parameters which are important in the preparation of liposomes with a good entrapment efficiency are as follows:

(i) The temperature of the medium, which must be between 50-60°C. Injection rates faster than 0.2 ml min^{-1} should not be used, since this can result in cooling of the solution by evaporation.

(ii) The presence of a negatively-charged phospho-lipid, to prevent aggregation. There appears to be no advantage in incorporating PA in proportions greater than 10 mol%.

(iii) The shape of the injection needle orifice. The needle is crimped in order to produce a slight back pressure in the syringe, thus preventing pre-evaporation of ether, which can otherwise cause blockage of the needle or the formation of multilamellar vesicles.

Saturated lipids, such as dipalmitoyl PC, which are poorly soluble in pure ether, can be used with this method provided that the ether contains 20 % methanol by volume. The concentration of lipid in ether may be increased up to 5-fold without deleterious results, to produce liposomes in higher concentration. In the case of materials which are sensitive to high temperatures, the preparation can be carried out by subjecting the vial to reduced pressure (by attaching a water suction tap the gas vent needle) and operating at 37°C.

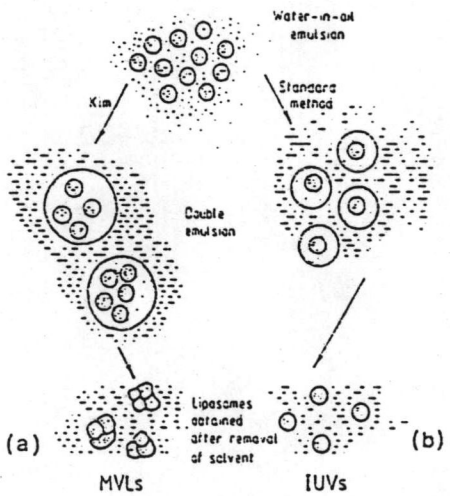
5.2.3 Water in organic phase

In this group of methods, the liposomes is made up in two steps, first the inner of the bilayer, then the outer of bilayer. There are several different variations, the common feature of which is the formation of a water-in-oil emulsion produced by introduction of a small quantity of aqueous medium (containing material to be entrapped) into a large volume af immiscible organic solution of lipids, followed by mechanical agitation to break up the aqueous phase into microscopic water droplets. These droplets are stabilized by the presence of the phospholipid monolayer at the phase interface. The size of the droplets is determined by the intensity of mechanical energy used to form the emulsion, and by the amount of lipid relative to the volume of aqueous phase, since each droplet

requires a complete monolayer of phospholipid covering its surface in order to prevent its coalescing with other droplets, or with the solvent -air interface.

The aqueous compartment, surrounded by the monolayer of phospholipid, forms the central core of the final liposomes. There are several different ways of preparing the water-in-oil droplets, before addition of the outer coating, which under appropriate conditions give droplets of differing sizes. Droplets of diameter 0.1 μm (100 nm) can be formed by probe sonication. The dispersion formed in this way can be converted into a liposomes suspension by forming a double emulsion.

(a) Double emulsion vesicles



In this method, the outer layer of the liposomes membrane is created at a second interface between two phases by emulsification of an organic solution in water. If one uses the organic solution which already contains water droplets, and introduces this into excess aqueous medium, followed by mechanical dispersion, a multi-compartment vesicle is obtained, which may be described as a

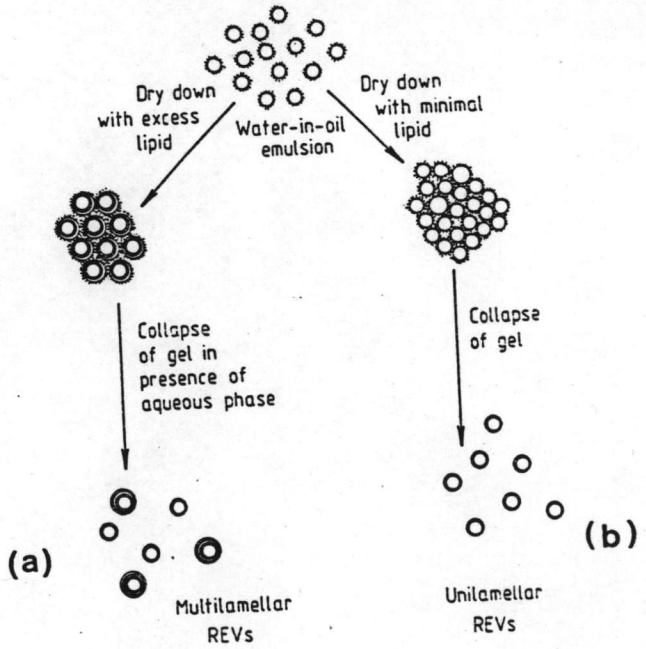
'water-in-oil-in-water' system (i.e. a double emulsion). These vesicles are suspended in aqueous medium, and have an aqueous core, the two aqueous compartments being separated from each other by a pair of phospholipid monolayers whose hydrophobic surfaces face each other across a thin film of organic solvent. Removal of this solvent clearly results in intermediate-sized unilamellar 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. The critical point in this process is probably the evaporation step where the organic phase is removed. At this stage some water droplets may tend to disintegrate releasing the entrapped material or causing a collapse of the double-emulsion structure, giving rise to marked lipid aggregation. In general, the degree of encapsulation of water-soluble material, expressed as a percentage of the total initial starting amount of this material, depends mainly on the nature of material itself and only to a minor extent on the liposomes composition. Optimizing the procedure on the basis of the encapsulation of bovine serum albumin (BSA), an entrapment of 50-80 % can be obtained routinely. Using the same experimental procedure for evaporation as used for BSA, the amount of insulin which is encapsulated is of the same order, whereas the encapsulation efficiency of compounds like inulin and sucrose is much lower, typically 10%. It has been found, however, that simply by using a more gentle evaporation technique than employed in the standard procedure gives an encapsulation percentage of 40-50 % for these compounds.

(b) Multivesicular liposomes (MVLs)

This is a variation of the double emulsion method described above, but which gives rise to liposomes of a type which can be produced by no other method. The novelty lies in the fact that the proportions of lipid, solvent composition and duration of shaking are adjusted in such a way that

instead of just one single water droplet being contained within the 'oil' droplet of the double emulsion, several water droplets are enclosed. Upon removal of the solvent by evaporation, these water droplets remain intact and form multiple compartments within a single liposomes. Like ordinary double emulsion vesicles, these have an entrapment yield of 50 % or greater. Although no applications have yet been reported for MVLs which could not be performed by normal LUVs, it is conceivable that one may want to deliver to cells or organs of the body number of different agents simultaneously which normally are not very stable in each other's presence. Entrapping the materials in separate compartments of the liposomes could overcome this problem. The size of liposomes ranges from 5 to 30 microns in diameter, depending on the duration of shaking employed to produce the second emulsion. Shaking for 10 sec is found to be optimal from the point of view of percentage entrapment, prolonged shaking presumably resulting in rupture and leakage of the internal aqueous compartment to the outside.

(c) Reverse-phase evaporation vesicles



The process involved an emulsion which was the reverse of the standard 'oil-in-water' emulsion, and because the key, novel step in the preparation was the removal of solvent from the emulsion by evaporation. Thus, after the droplets have been formed by bath sonication of a mixture of the two phases, the emulsion is dried down to a semi-solid gel in a rotary evaporator, under reduced pressure. At this stage, the monolayers of phospholipid surrounding each water compartment are closely opposed to each other, and in some cases probably already form part of a bilayer membrane separating adjacent compartments. The next step is to subject the gel to vigorous mechanical shaking with a vortex mixer, in order to bring about the collapse of a certain proportion of the water droplets. In these circumstances, the lipid monolayer which enclosed the collapsed vesicle is contributed to adjacent, intact vesicles, to form the outer leaflet of the bilayer of a large unilamellar liposomes. The aqueous content of the collapsed droplet provides the medium required for suspension of these newly formed liposomes. After conversion of the gel to a homogeneous free-flowing fluid, the suspension is dialyzed in order to remove the last traces of solvent. The vesicles formed are unilamellar, and have a diameter of the order of 0.5 μm. Because it is essential for at least 50 % of the vesicles to collapse, in order to provide the outer monolayer for the surviving 50 %, values for percentage encapsulation greater than 50 % should not be expected. Using the lipid-to-aqueous volume ratio described, the water-in-oil emulsion, in contrast to those described earlier for the double emulsion, has virtually no lipid in free solution after the dispersion has been formed. Almost all the lipid is associated with the monolayer surrounding the water droplets. If, however, the concentration of lipid employed is increased, or the volume of water decreased, then excess lipid will be present which will be dried down with the water droplet, and will coat them with multiple layers of phospholipid membrane. Under these circumstances. it is not necessary for a large number of vesicles to

collapse in orderconvert the gel a liposome suspension, since the outermost lipid monolayer can be transferred from one vesicle to another without destroying the integrity of the donor vesicles.

Liposomes prepared by solvent dispersion methods other than ethanol injection have a higher encapsulation efficiency of aqueous phase than those prepared by method in the other two categories . However, with solvent dispersion methods, all liposomes have at least one of several disadvantages such as low encapsulation efficiency, many fold dilution, heterogeneous size and size distribution, restrictions on lipid composition, and limitations in the solubility of lipid in specific organic solvents.

In general, methanol, ethanol, ether (diethyl, isopropyl, petroleum), hexane, benzene, chloroform, dichloromethane, and other hydrocarbons can be used for the preparation of liposomes by solvent dispersion methods. In particular, the last five organic solvents, which are water immiscible, are useful for liposome preparation using a water in oil (W/O) emulsion, such as the REV method of Szoka and Papahadjopoulos (78), the electroemulsification method of Ishii and Noro (79), and microencapsulation method of Ishii et al (67).

The mean size and size distribution of liposomes are important factors affecting physicochemical stability, encapsulation efficiency, tissue distribution, in vivo circulation lifetimes, and transfer of lipid onto cells.

In a more recent (67), microencapsulation vesicles (MCV) prepared using the emulsification technique were found to be excellent as drug delivery carriers, showing high encapsulation, stability, and reproducibility(see Figure 11).

This method have shown that particle size distribution and mean diameter could be controlled within one order of magnitude by mechanical agitation. The MCV method of in-liquid drying, a microencapsulation technique capable of encapsulating aqueous solutions of materials such as enzymes, hormones, genetic material, and biologically active peptides, is simple, rapid, versatile, and reproducible. Moreover, water-insoluble material can also be encapsulated in a similar manner.

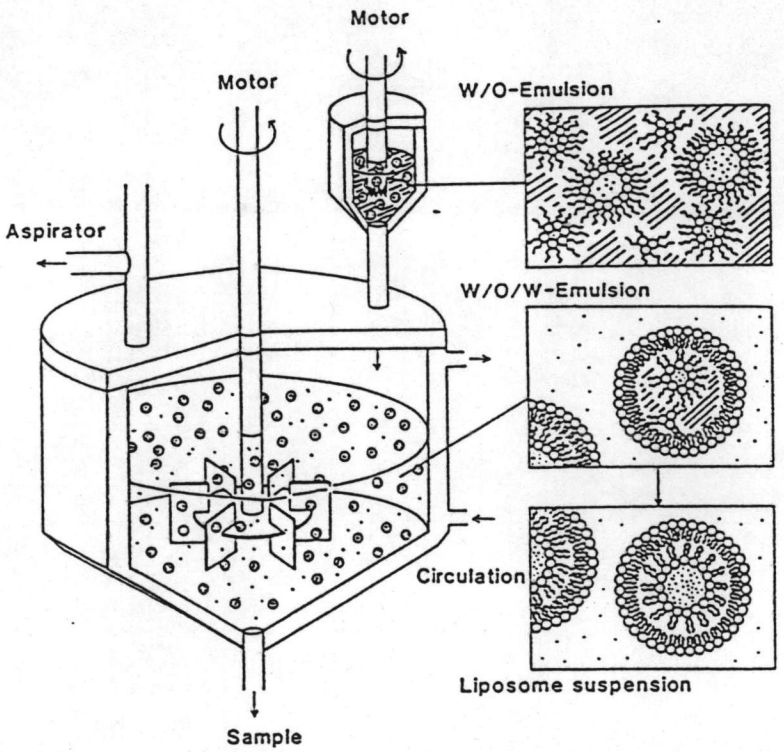


Figure 11. Diagrammatic representation of the preparation of liposomes by emulsification technique.(the mothod of Fumiyoshi Ishii).

As shown in Figure 11, the MCV method is essentially different from the REV vesicle method of Szoka and Papahadjopoulos and lecithin - span 80 mixed vesicle method of Matsumoto etal (81), in the step involving evaporation of the solvent from the oil phase- dissolved lipids. In the latter methods, the lipid solvent is removed from the surface of the oil phase of the W/O emulsion, resulting in poor reproducibility of liposome formation. However, in the MCV method, it is possible to obtain fairly good reproducibility with regard to size distribution, encapsulation efficiency, and physical stability in comparison with the other methods, since removal of the solvent is performed by mechanical agitation during preparation of the W/O/W emulsion. Moreover, an important characteristic of the MCV method is that liposomes can be prepared by emulsification without either an emulsifier or a dispersing agent. That is phospholipid (egg PC) can be used as an emulsifier to prepare the W/O emulsion. Furthermore, the spontaneous swelling of phospholipids that occurs in aqueous solution during preparation of W/O/W emulsion is exploited.

5.3 Detergent solubilization

In this third class of methods for the manufacture of liposomes, the phospholipids are brought into intimate contact with the aqueous phase via the intermediary of detergents, which associate with phospholipid molecules and serve to screen the hydrophobic portions of the molecule from water. The structures which form as a result of this association are known as micelles, and can be composed of several hundred component molecules. their shape and size depending on the chemical nature of the detergent, the concentration, other lipids involved, etc. Detergents can form micelles in the absence of any other lipids providing the concentration is high enough. That concentration of detergent in water at which micelles just start to form is known as the critical micelle concentration

(CMC). Below the CMC, the detergent molecules exist entirely in free solution. As detergent is dissolved in water in concentrations higher than the CMC, micelles form in larger and larger amounts, while the concentration of detergent in the free form remains essentially the same as the CMC. Micelles containg components in addition to the detergent (or composed of two or more detergents) are known as 'mixed micelles'. In contrast to phospholipids, detergents are highly soluble in both aqueous and organic media, and there is an equilibrium between the detergent molecules in the water phase, and in the lipid environment of the micelle. The critical micelle concentration can give an indication of the position of this equilibrium, and from that conclusions can be drawn as to the ease with which molecules of detergent can be removed from the mixed micelle, upon lowering the concentration of detergent in the bulk aqueous phase by dialysis. A high CMC indicates that the equilibrium is strongly shifted towards the bulk solution so that removal from the mixed membrane by dialysis is relatively easy.

As a general rule, membrane-solubilizing detergents have a higher affinity for phospholipid membranes than for the pure detergent micelles. Thus, as detergent is added in increasing amounts to the membrane preparation. more and more detergent will be incorporated into the bilayer, until a point is reached where a transition from the lamellar to the (usually) spherical micellar phase configuration takes place. As the detergent concentration is increased further, the micelles are reduced in size, until they become saturated with detergent, where upon the concentration of free molecules equal to the CMC is attained, and simple detergent micelles are seen to form. Again, it is generally found that a high CMC in advantageous for solubilizing membrane phospholipids, although one might have expected the converse since a high affinity for lipid membranes should be reflected by a low CMC. It may be that kinetic factors are important, and that detergents

with a higher CMC can incorporate more rapidly into the membrane because of the higher concentration of the free entity.

In all methods which employ detergents in the preparation of liposomes, the basic feature is to remove the detergent from pre-formed mixed micelles containing phospholipids, whereupon unilamillar vesicle form spontaneously. Because removal of detergents is carried out using technique which inevitably remove other small water-soluble molecules (e.g. dialysis, column chromatography), the detergent methods are not very efficient in terms of percentage entrapment values attainable; on the other hand, they are certainly the best general methods for preparing liposomes with lipophilic proteins inserted into the membranes, since these proteins can be introduced into the mixed micelles in the presence of mild non-denaturing detergents, to achieve 100 % incorporation without modification of the general method. Another special feature is the ability to vary the size of the liposomes by precise control of the conditions of detergent removal and to obtain vesicles of very high size homogeneity.

Detergents can differ in the way in which the polar and lipophilic moieties are distributed within the molecule, and these differences lead to differences in the structure of the resultant micelles formed. Thus one may draw distinctions between detergents according to whether the boundary between polar and apolar regions is latitudinal, as in the case of alkyl glycosides or longitudinal as for bile salts. The mixed micelles formed are spheroid and discoid respectively.

Detergents fail into three classes: Ionic (anionic, cationic), Amphoteric, Non-ionic. Ionic detergents, such as sulphonated hydrocarbons like sodium dodecyl sulphate (SDS), although possessing ideal characteristics as far as

solubility and CMC are concerned, are usually considered unsuitable for work with biological membranes since they are highly denaturing for proteins. The long flexible aliphatic chains can fit easily into, and disrupt, the hydrophobic clefts of proteins, and the strong electrostatic charges interfere with the hydrogen bonding interactions that maintain the tertiary structure of the protein. One exception to the rule is the class of ionic detergents including bile salts, for example, sodium cholate or deoxycholate, where the charged carboxyl group is not so bulk, and the rigid steroid nucleus has less access to hydrophobic areas in the protein molecule.

The majority of detergents employed for membrane work are non-ionic, of which, Triton-X and alkyl glycosides are very commonly used. Triton-X is a mixture of homologues in which the lipophilic group is an iso-octyl phenol moiety, while the hydrophilic portion is a single long chain consisting of between 8-12 oxyethylene subunits ($-CH_2-CH_2-O$). Brij, Lubrol, Emulphogen, Nonidet, Tween, and others are all non-ionic detergents comprising a polyoxyethylene hydrophilic chain. In contrast, the alkyl glycosides have hydrophobic chains (short chain alkyl groups), and a sugar residue as the hydrophilic headgroup.

Ionic and non-ionic detergents differ in the way in which the critical micelle concentration, and micelle size, are affected by external conditions. Thus, bile salts are very sensitive to pH, precipitating at pH below neutrality, in contrast to Triton or octyl glucoside. Increasing the ionic strength reduces repulsive interaction between charged groups, and leads to formation of micelles at much lower concentrations, In addition. the size of micelle is increased. For non-ionic detergents, increasing the temperature results in a reduction of hydration of polar moieties (e.g. the oxygen atoms in the oxyethylene subunits of Triton-X) leading to

an increase in the size of the micelle. Detergents may be removed either by dialysis, column chromatography, or by dilution.

6. Stability of Liposomes

6.1 Chemical degradation

Many different changes can take place in liposomes with the passage of time. The phospholipids can undergo chemical degradation-oxidation and hydrolysis-leading to a build-up of short-chain phospholipid and lyso-derivatives in the membrane. Either as a result of these changes, or otherwise, liposomes maintained in aqueous suspension may aggregate, fuse, or leak their contents.

Method devised to overcome the problems of liposome instability fall into two categories those designed to minimize the degradation processes which may take place, and secondly, those which contrive to help liposomes survive in the face of conditions which encourage these processes.

Prevention of chemical degradation

The level of oxidation can be kept to a minimum by taking the following precautions : start with freshly purified lipids and freshly distilled solvents; avoid procedures which involve high temperature; carry out the manufacture process in the absence of oxygen; deoxygenate aqueous solutions with nitrogen; store all liposome suspension in an inert atmosphere; include an anti-oxidant as a component of the lipid membrane.

The anti-oxidant in most common use at the present time is α-tocopherol (vitamin E), a common non-toxic dietary lipid, although it has been suggested that β, γ, and δ-tocopherols may be more effective as long-term anti-oxidants since they have a longer lifetime, and are not destroyed so easily in the process of radical neutralization. An alternative approach to the oxidation problem is to reduce the level of oxidizable lipids in the membrane, by using saturated lipids instead of unsaturated ones. Among phospholipids from natural sources, increasing unsaturation is in the order plant, egg yolk, mammalian origin. In the case of egg yolk lecithin, the degree of unsaturation of the phospholipid fatty acids can depend on the diet of the birds, and on the method of purification employed.

Preparations are possible which contain almost wholely 16:0, 18:0 and 18:1 with very little longer chain polyunsaturated material. The mono-unsaturated chains are much less susceptible to oxidation than polyunsaturated ones. Thus sphingomylelins, usually containing only a single double bond, can be expected to deteriorate more slowly than other membrane lipids of mammalian origin. Alternatively, unsaturated lecithins from natural sources may be converted to the saturated homologues by the process of catalytic hydrogenation. Liposomes may also be prepared from entirely synthetic saturated compounds, such as dimyristoyl phosphatidyl choline (DMPC), dipalmitoyl phosphatidye choline (DPPC), and distearoyl phosphatidyl choline (DSPC).

6.2 Physical degradation

Leakage and fusion of vesicles can occur as a result of lattice defects in the membrane introduced during their manufacture. Aggregation (and

sedimentation) of neutral liposomes is brought about by Van der Waals interactions, and tends to be more pronounced in large vesicles, where the increased planarity of the membranes allows greater areas of membrane to come into contact with each other. Although factors such as residual solvent and trace elements can enhance the process, for uncharged membranes it is a natural and unavoidable phenomenon, and the simplest way to overcome it is to include a small quantity of negative charge (e.g. 10% phosphatidic acid (PA) or phosphatidyl glycerol (PG) in the lipid mixture.

Permeability of liposome membrane depends very much on the membrane lipid composition, and on the solute which one has entrapped. Large polar or ionic molecules will be retained much more effectively than low molecular weight lipophilic compounds. In general, for both classes of compound, a rigid, more saturated membrane with a high molar ratio of cholesterol is the most stable with regard to leakage of solutes.

6.2.1 Roles of cholesterol on liposome stability

The addition of cholesterol to the lecithin-water system in the gel phase causes a reduction in the cohesive force between adjacent hydrocarbon chains of the lecithin; this leads to a fluidization of these chains. The cholesterol can exhibit a condensing effect on lipids in the liquid crystalline state and a liquefying effect on lipids in the crystalline state, leading to an intermediate gel state (Figure 12).

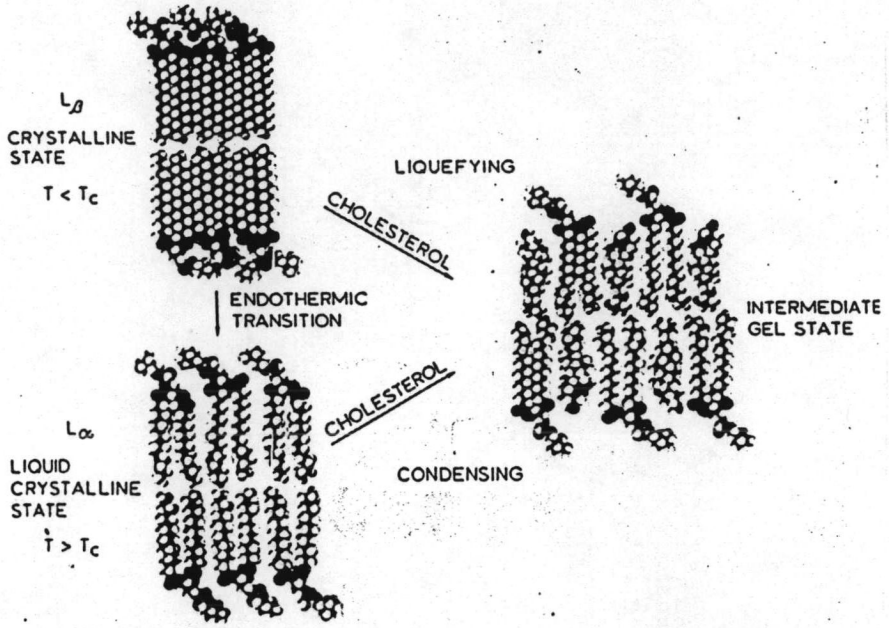


Figure 12. Representation by molecular models of the liquefying and condensing effect of cholesterol.

The additional finding is that cholesterol remove the Tc transition between the crystalline and liquid crystalline states in the presence of an excess of water. The condensing effect of cholesterol on lipids at T > Tc was demonstrated by monolayers, showing a reduction in molecular area; liposomes, black lipid membranes and natural membranes showing a reduced permeability; NMR ESR, X-ray diffraction and fluorescence spectroscopy, showing a reduced mobility of the fatty acyl chains. The liquefying effect of cholesterol on lipids at T < Tc was demonstrated by liposomes showing an increased permeability; NMR, ESR, X-ray diffraction and differential scanning calorimetry, showing an increased chain mobility leading to an elimination of the original phase transition. The intermediate gel phase formed in the presence of cholesterol at temperatures above and below Tc suggest that cholesterol could regulate the permeability of biological membranes by affecting the internal viscosity and molecular motion of

the lipids in the membrane. Organisms like yeast cells and fungi which contain sterols can grow over a wide temperature range(73).

6.2.2 Roles of polymer coating on liposomes stability.

Polymers have also been combined with liposomes in an attempt to stabilize the liposomes. However, only a limited number of papers in the literature refer to coating the surface of liposomes with polymer derivatives (74). Alamelu and Panduranga Rao (1991) used carboxymethyl-chitosan as a coating on liposomes containing dapsone(75). Sunamoto et al. (1984,1987,1988) using liposomes coated with polysaccharides for targeting purposes for example, they developed immunoliposome (an egg phosphatidylcholine liposome) coated with pullulan bearing both a cholesterol moiety and an IgMs fragment (21-23). Several studies utilized chitin derivatives compounds in place of the polysaccharide. Kato and Kondo (1987) used carboxymethyl-chitin to produce their artificial red blood cells, encapsulating hemolysate in liposomes and coating them with this negatively charged polymer (76). All of the mentioned paper, found an increased stability against release of selected markers compared to uncoated liposomes.

The coating efficiency of liposomes by polymer was dependent on the initial concentration of polymer and the coating procedure. Dong and Rogers (1991) reported that when the CM-chitin was added during liposome formation more of the polymer became associated with liposomes than when the polymer was added to preformed liposomes. This is not surprising since the CM-chitin molecules were intimately in contact with all of the bilayers comprising the multilamellar liposome structure when added during liposome formation whereas

only the outer bilayers of the liposomes were available for interaction with the CM-chitin molecules when added to preformed liposomes. In the same study, the former case, the coating efficiency rose only slightly with additional CM-chitin, reached a maximum, then actually decreased when 2% CM-chitin was added. This suggests a capacity-limited interaction process which would occur if the CM-chitin molecules interdigitated the ordered phospholipid molecules in the bilayers. But in the latter case, the coating of liposomes by CM-chitin is proportional to the CM-chitin added which indicates surface binding. Since the coating efficiency increased linearly up to 2% of added CM-chitin, the surface film formed is probably multimolecular(77).

7. Liposomes as Immunological Adjuvants in Vaccines.

Immunological adjuvants are agents can provoke potent immune responses to antigens. They include aluminium hydroxide (alum), saponins complexed to membrane protein antigens (immune stimulating complexes), Pluronic polymers with mineral oil, killed mycobacteria in mineral oil (Freund's complete adjuvant ,FCA), bacterial products such as lipopolysaccharide (LPS) and muramyl dipeptide (MDP) , and liposomes. Adjuvants appear to function by one or both of the following mechanisms. The first mechanism involves creating a depot (as, for example, by alum and oil emulsions) at the site of injection, which prolongs the release and interaction of antigens with antigen-presenting cells (APC). The APC may also invade the depot area in the presence of local inflammation, and adjuvant-antigen complexes may migrate to area in the regional lymph nodes containing T cells. The second mechanism is activation of macrophages (for example LPS, MDP) which release interleukin 1 (IL-1). The combined action of IL-1 and antigen on T cells produces interleukin 2 (IL-2) and other mediators which activate effector T

cells(cell-mediated immunity,CMI) or antibody-forming B cells (humoral immunity,HI). Some of these factors also promote the retention of circulating lymphocytes in the lymphoid tissues adjacent to the site of injection. It has been suggested that strong and persistent immune responses may be elicited by targeting antigens to interdigitating cells and follicular dendritic cells which, unlike macrophages, constitutively express major histocompatibility (MHC) class II antigens. As these specialize in presenting antigen to T and B lymphocytes respectively. CMI and HI could be favoured selectively. Of the adjuvants mentioned, only alum is licensed for use in humans. but it is far from ideal: it is not always effective. it increases CMI only slightly if at all and, as it cannot be lyophilized, it requires refrigerated storage. Many of the other adjuvants are toxic, inducing granulomas, acute and chronic inflammations (CFA), cytolysis (saponins, some Pluronic polymers) and pyrogenicity (LPS and MDP). However with some of the agents, toxicity can be curtailed without loss of adjuvanticity through changes in the adjuvant's structure (LPS, MDP) or through the choice and synthesis of appropriate adjuvant analogs (saponin, Pluronic polymers). Concerted efforts are now in progress to develop safe and effective adjuvants to meet the challenges of subunit and peptide vaccines and new insights into the ways in which immunity is produced. An ideal adjuvant should comply and certain criteria which, in addition to some practical ones (for example inexpensive raw materials, simplicity of manufacture, stability in storage-preferably in a freeze-dried form), would also include a biodegradable, non-toxic, and non-immunogenic nature, the ability to elicit both CMI and HI to antigens administered by a variety of routes, synergistic action with other adjuvants if needed, and a potential for selective interaction with populations of immunocompetent cells. In this review liposomes are discussed as attractive adjuvant candidates.

It is fairly clear that the adjuvant property of liposomes is a physical rather a chemical effect. When injected intravenous liposomes are taken up mainly by the liver and spleen. Interestingly, intravenous administration is a very poor method of immunising mice with material in liposomes intraperitoneal and subcutaneous administration being routes which lead to a greater immune response. It seems likely that liposomes injected by these two routes will remain at the site of injection for a long period. Thus, liposomes may well exert their adjuvant effect by the same method as many other particulate adjuvant that is, by retaining a "depot" of antigen at the site of injection. The phagocytosis of liposomes by cells such as macrophages may also be an important factor. This suggested by the fact that the incorporation of more than 30 mol% cholesterol into liposomes markedly reduces the immune response to the antigen entrapped within them. This level of cholesterol has also been shown to reduce the rate of digestion of liposomes by macrophages. Since Allison and Gregoriadis demonstrated that liposomes could enhance the antibody response to diphtheria toxoid. They have claimed that whilst liposomes enhance the immune response they also prevent any adverse hypersensitivity reaction to the antigen by shielding it from antibodies. They have so far demonstrated for diphtheria toxoid, that liposomal entrapment of antigen prevents Arthus type hypersensitivity reactions when the material is injected into the footpad of a primed mouse, and also prevents the death of mice with high circulating antibody when injected intravenously, the role of liposomes as adjuvants in stimulating an immune response has received a great deal of attention. Since that time liposomes have been shown to be effective adjuvants for a large number of protein antigens(see Table 2).

Table 2 Immunoadjuvant action of liposomes : antigens studied[a]

Diphtheria toxoid	Influenza virus (A/PR/8 strain) reconstituted envelopes
Tetanus toxoid	Influenza virus (X-49 strain) rosettes
Cholera toxin	Polio virus 3VP2 peptide
Mycobacterium leprae antigens	Foot-and-Mouth virus VP1 Peptide
Neisseria gonorrhoeae proteins	Rubella virus (M-33 strain) rosettes
Proteus mirabilis major outer membrane protein and LPS	Adenovirus type 5 hexon
Salmonella typhimurium LPS	Encephalomyocarditis virus (inactivated)
Streptococcus sobrinus ribosomal protein	Semliki Forest virus (inactivated)
Streptococcus pneumonia type 3	*Herpes simplex* virus type 1 antigens
Sterptococcus mutans carbohydrate-protein conjugate	Epstein-Barr virus glycoprotein
Nippostrongylus brasiliensis antigens	Rabies glycoprotein
Leishmania mexicana gp63 and lipophosphoglycan	Gross virus cell surface antigen
Leishmania major soluble antigens (LV39 and J 2 311 promastigotes)	Fibrosarcoma surface antigens
Plasmodium falciparum synthetic peptide	Colon tumour (LS17T) membrane vesicles
Plasmodium falciparum merojoite-enriched antigen	Tumour (L_2C) antigens
Hapetitis B surface antigen	Tuberculins (purified protein derivative and peptide)
Hepatitis B surface antigen polypeptide	Rat colon tumour antigens
Hepatitis B virus envelope pre-S (120-145 sequence) peptide	Spermatozoal polypeptide fraction
Influenza virus (A/PR8/34 strain) glycoproteins	Nigerian *Echis carinatus* venum

[a] Antigens shown are only those of medical or veterinary relevance. Antigens were incorporated by various method into liposomes of different sizes, compositions and other characteristics. In some studies, liposomal antigen formulations also contained other adjuvants

Liposomes are being investigated in the design of subunit vaccines for viral diseases and are able to efficiently present the surface glycoproteins of many enveloped viruses for stimulation of a protective immune response. The viral glycoproteins are anchored in the liposomal bilayer via a transmembrane segment and assume a conformation analogous to their native conformation in the viral envelope. Influenza virus surface proteins have also been shown by Almeida et al. to orientate themselves in liposomes in a manner which, under the electron appears similar to their disposition in the intact influenza virus. Moreover the "virosomes" so formed can be agglutinated by anti-influenza antisera. The production of the virosome was prompted by the knowledge liposomes were adjuvants, and the need for a non-pyrogenic influenza vaccine. Whole influenza virus is a good immunogen, but is also pyrogenic. Influenza proteins are not pyrogenic, but also are not very good immunogens. It is hoped that the combination of non-pyrogenic lipid with the subunits should produce a non-pyrogenic, but immunogenic, preparation.

An important feature of liposomes as adjuvants is their ability to induce CMI. This has been shown by positive DTH reactions the lymph node lymphocyte proliferative response test and the induction of cytotoxic T-lymphocytes. Liposome-induced CMI cannot be explained by the antigen-depot mechanism since adjuvants such as alum and oil emulsions, which act in this way induce only or predominantly HI. Since proteins coupled to lipids are known to induce DTH in proportion to the latter's hydrophobicity, it is likely that the increased internalization of hydrophobic antigen lipids by macrophages ultimately improve antigen presentation to T cells. As with HI, these events may also be favoured by the efficient (and selective) uptake of liposomal antigens into the regional lymph nodes. The adjuvanticity of liposomes is basically a projection of the system's vesicular structure and perhaps of its lipoid nature, rather than of the identity of its lipid

components or other secondary characteristics. The latter, however, are known to effectively control the behaviour of liposomes *in vivo* and may thus be instrumental in the way immunoadjuvant activity is expressed, both qualitatively and quantitatively. Several groups have, therefore, investigated the extent to which bilayer fluidity. Number of lamellae in bilayers, vesicle size and surface charge, lipid to antigen mass ratio and mode of antigen localization within liposomes influence adjuvanticity. All appeared to have an effect but conclusion as to the role of individual parameters have often been contradictory or have not been confirmed.

PASTEURELLA MULTOCIDA

1. Taxonomy

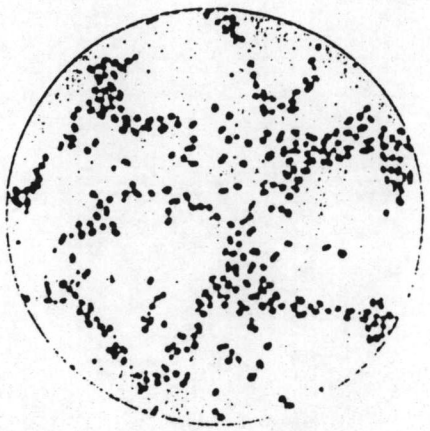
Rosenbach and Merchant 1939 (33) proposed the name Pasteurella in Bergey's Manual of Systemic Bacteriology (83) the genus Pasteurella can be devided among six species with differential characteristics base on the beta hemolysis, growth on MacConkey agar, indole production, urease activity, gas from carbohydrates and acid production from lactose mannitol.

2. Morphology, Growth Characteristic and Biochemical Reactions

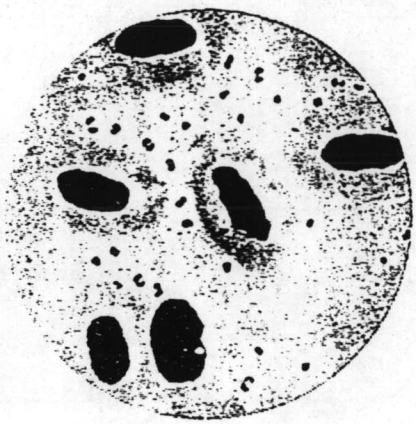
P. multocida is a small coccoid rod gram negative bacteria, their average size are 0.25 to 0.4 by 0.6 to 2.6 microns (Figure 13a). They were stained more distinctly at either pole, giving rise to the term bipolar. Bipolar staining is common especially in preparation made from blood and infected animals tissue

especially in preparation made from blood and infected animals tissue (Figure 13b). They are generally capsulated, but non-capsulated forms have been reported. The majority of the species are non motile and spore forming. After repeated culture on agar, the bacteria tends to form longer rods and to become more pleomorphic, forming chains, filaments and rods of various sizes. When grow in carbohydrate media for prolonged periods, marked pleomorphicity is noted. The organism usually possesses a capsule. The capsules are mostly composed of hyaluronic acid. The bacteria is an aerobe and facultative anaerobe. Its optimum temperature for growth is at 37°C, and the maximum growth is reached in 18 hr in fermentor or shaker condition. The pH growth range is from pH 6-8.8 with an optimum of pH 7.2-7.4 . The use of digested protein media or proteose peptone stimulate the growth of the organism. Bacteria could be grown in beef infusion media, but better growth is obtained when blood or serum is added to the media. Some strain fail to grow in media not containing blood or serum. Three principle colony types are seen on clear screen agar : fluorescent (smooth colonies), intermediate (mucoid colonies) and blue colonies (rough or non-capsulated colonies)(84). The fluorescent colonies are moderate in size , whitish, opaque, generally unstable and pathogenic. The mucoid colonies vary in appearance between fluorescent and the blue form. The blue colonies are smaller, dewdrop-like and of a relatively low virulence (Figure 13c). Strains giving rise to blue colonies are most frequently recovered from chronic infections. While acute infections usually yield strains giving rise to fluorescent colonies. Many group A strains produced mucoid variants on initial isolation on enriched media.

(A)



(B)



(C)

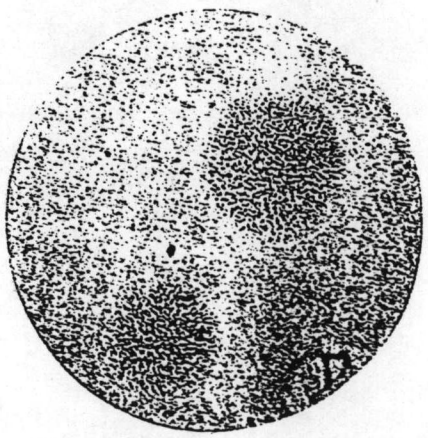


Figure 13. *Pasteurella multocida* colony

(A) smear from agar culture, X 2000

(B) blood smear, X 2000

(C) colony on agar plate, X 70

(From Nowak: Ducumenta Microbiologica, courtesy Gustav Fischer.)

3. Serotyping

The classification that gained widespread acceptance was based on serological properties of the organisms, capsular and cell wall compositions. At the present time, *P. multocida* have been identified as 5 capsular types designated A, B, D, E and F and 16 somatic types (85). The popular method of designating serotypes was combined both capsular and somatic types.

The Namioka-Carter serotyping system is identified by Arabic numerals 1 through 16 representing the somatic types followed by a capital letter representing the capsular type, The important serotypes which are the cause of fowl cholera are 5:A, 8:A and 9:A and of hemorrhagic septicemia are 6:B and 6:E

4. Antigenic Structure (86)

The structure and cellular components of *P. multocida* may contribute to disease or stimulate host immune response. The major antigenic components are capsules, lipopolysaccharides (LPS), toxins, and plasmids.

4.1 Capsules

The antigenic specificity of the capsular determines its serogroup. Capsular type A was composed of hyaluronic acid, which may act to mimic host antigens because hyaluronic acid naturally present in host tissue. Intimate associated with it other polysaccharides, proteins and lipids. The hyaluronic acid not exert antiphagocytic activity, but saline-extractable capsule material contained a factor capable of inhibiting the function of bovine

polymorphonuclear leucocyte. The capsule of avian strains provided production from the action of complement. Removal of the hyaluronic acid surfaces and its susceptibility to phagocytosis. The production of capsular material was affected by subminimal inhibitory concentration of antibiotics. Capsule was considered virulence factor because non encapsulated variants of pathogenic strains were less virulent than encapsulated forms.

4.2 Lipopolysaccharides (LPS)

Examination of strains from a variety of animal sources confirmed that LPS from *P. multocida* was similar to semirough LPS of enterobacteriaceae. The LPS contained lipid, 2-keto-3-deoxyoctanate (KDO), heptose, glucose and polysaccharide. The LPS of rabbit isolates contained either a non-serospeccific antigen (R-LPS), a serospecific antigen (S-LPS) or both, from avian strains found to be R-LPS. The LPS responsible for antigenic specificity of somatic serotype, and when examined electrophoretically the molecular weight of LPS was low.

4.3 Toxins

The production of protein toxin by *P. multocida* has been recognized for some time although it is only in the last decade that some definition has been given to the nature of these products, following the discovery of toxigenic strain. Some strains, particular those of capsular type D produced a factor designated dermonecrotic toxin (DNT). Purified DNT was a protein estimated molecular weight ranging from 112-160 kDa. Crude toxin was inactivated by formaldehyde, heat and proteolytic enzyme suggesting it was a protein. Commercial vaccines therefore incoporate formaldehyde treated whole cell of toxigenic

P. multocida or formaldehyde-detoxified crude bacterial extracts of toxigenic organism. Strains from other hosts also produced the toxin including poultry, claves, cats and dogs, rabbits and human respiratory tract.

4.4 Plasmids

Avian strains contained plasmids and this characteristic together with a degree of complement resistant were correlated to virulence marker. Plasmids confering antibiotic resistance have been isolated from fowl cholera and bovine pneumonia strains.

5. Disease Caused by *P. multocida* (28,30,31)

P. multocida is the most prominant pathogen in domestic animals causing severe disease and major economic losses. The important disease caused by *P. multocida* were fowl cholera in avian species and hemorrhagic septicemia in cattle.

6. Fowl Cholera Vaccine (87)

To control and eliminate pasteurellosis is to develop an effective vaccine. Since Pasteur developed the first fowl cholera vaccine, there have many attempts to produce efficient vaccines against disease.

6.1 Killed Vaccine (Bacterins)

There are many types of killed vaccines used to prevent disease. Vaccines are usually prepared in formalinized saline solution. Bacteria

prepared from tissue of turkeys that died of acute fowl cholera and embryonating turkey egg induced immunity in turkeys against infection with a different immunogenic type. The phenol-killed vaccine was found to be effective in controlling duck cholera. The bacteria are incorporated with adjuvant to improve vaccine efficacy. Immunity induced by this vaccine is type specific. In Thailand, the formalinized vaccine prepared from serotype 8:A local strain is used. The efficacy of this vaccine is 70% dependent on the virulence of an outbreak. Killed vaccines prepared from tissue of infected turkey induce immunity against different immunogenic types, but the bacteria prepared with bacteria grown on conventional agar media does not induce cross-immunogens in vivo than in vitro.

6.2 Live Vaccine (88)

Live vaccine are generally thought to provide higher protection against different serotypes than bacterins. Several live vaccines developed for vaccine efficacy improvement have been reported. The one of live avirulent vaccine was the Clemson University (CU) strain, serotype 3:A, 4:A. The CU strain was found to provide both humoral and cell mediated immunity, and to be protective against the major type that infected turkey. Although the CU strain stimulated an effective immune response in turkey, disadvantage of this was the resulting in mortality following vaccination.

7. Protective Antigens of *P. multocida*

Prevention of disease by using killed and live vaccine can induce protective immunity, but sometimes the results in mortality, morbidity and systemic infection are important problem. For these reasons, many researchers attempt to an

improved vaccine efficacy by the determination of the immunogenic antigens or subcellular materials. There are numerous reports of studies in which vaccine have been prepared by extraction of antigens.

An antigen extracted from *P. multocida* type A by an aqueous solution of 0.5 M KSCN in 0.08 M sodium chloride was found to be immunogenic in chicken against homologous and heterologus challenge(34). Lu et al. 1987 (35) demonstrated that a KSCN antigen extract prepared from a virulent serotype 3:A contained protein, carbohydrate, LPS, DNA and RNA was safe and protected rabbit against homologous challenge. Furthermore, rabbits against homologous challenge, indicating that vaccine stimulated protective antibodies. Subcellular fraction from 2.5% sodium chloride solution contained capsular antigen (39) and complex substance with high molecular weight (40% protein and 15% carbohydrate) was immunogen. Truscott,1988, reported that all of turkey inoculated with this antigen survived from challenge (41).

Lu et al., 1988 (89) identified protein immunogens of OMP by radioimmunoprecipitation (RIP) and Western blot analysis. They demonstrated that rabbits mounted major antibody response against 27, 37.5, 49.5, 58.7 and 64.4 kDa OMP. They further demonstrated that vaccination with OMP protected rabbits against homologous challenge. More specifically, Mab against 37.5 kDa OMP protected both mice and rabbits against challenge. *P. multocida* lipopolysaccharide (LPS) has similar chemical and biological properties to the R-type LPS of the gram negative bacteria. Purified LPS is antigenic, however the level of antibody response following immunization depend on animal species, inoculated dose, LPS type, used, route and method of inoculation. The role of LPS as an immunogen in mammals remains controversial. Mice, cattle and rabbits have been readily protected against infection following immunization with LPS.