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REVIEW ARTICLE

STABILITY AND USES OF LIPOSOMES

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ABSTRACT:

The present article reviews information about the stability and uses of liposomes.

Liposomes are vesicles having concentric bilayers of lipids. They are spontaneously formed when a dried film of phosphatidylcholine (lecithin) or sphingomyelin is hydrated by an aqueous buffer. Other phospholipids are added to impart a charge on liposomes. For example, phosphatidylinositol, phosphatidylglycerol, phosphatidic acid, phosphatidylserine, dicetylphosphate are included to impart negative charge and stearylamine is added to give charge. Cholesterol (a sterol) is included to increase physical stability of liposomes particularly in the presence of biological fluids such as plasma (Yatvin and Lelkes, 1982; Juliano, 1987). Synthetic phospholipids (generally saturated) and polymerizable synthetic phospholipids with diacetyelenic group(s) are also used in place of natural phospholipids for the formation of liposomes. Liposomes from polymerizable phospholipids on exposure to UV light lead to the formation of polymerized liposomes (Ringsdorf et al., 1988). Now it has been shown that many other nonphospholipid amphiphillic compounds such as synthetic amino acids (Neumann et al., 1987), oleic acid (long chain fatty acid) (Gebicki and Hicks, 1973), dodecyl sulfate (Hargreaves and Deamer, 1978), dialkyl sufactant compounds such as dioctadecyldimethyl ammonium chloride and dihexyldecylphosphate (Fendler, 1980) are also capable of forming vesicles. Both the terms liposomes and vesicles are present in literature. The term liposomes is used when vesicles are composed of phospholipids.

A number of reviews have been published on liposomes in general (Riaz et al, 1989) and about various aspect of liposomes--- about methods of preparation (Hausr, 1982; Deamer and Uster, 1983), mechanism of liposomes fusion (Rand and Parsegiam, 1986), therapeutic applications of liposomes (Nayhew and Papahadjopoulos, 1983; Storm et al., 1991), as drug carrier (Stuhne-Sekalec and Stanancev, 1991), liposome-surfactant interaction study by microcalorimetry (Rozycka-Roszak, 1990), liposome mediated DNA

transfection (Akao and Osaki, 1991) and liposome immobilized in gel beads as a stationary phase for column chromatography (Lundahl and Yang, 1991). From above the importance of liposomes is clear. In the present article information about the stability and uses of liposomes have been reviewed.

Multilamellar liposomes (MLV) usually range from 500 to 10,000 um. Unilamellar liposomes have been classed as small (SUV) or as large (LUV); SUV are usually smaller than 50 um and LUV are larger than 50 nm. The liposomes of very large size are called giant liposomes (10,000 - 1,00,000 um). The liposomes containing encapsulated vesicles are called multivesicular liposomes. These have size ranging from 2,000 - 40,000 nm (See Fig.l) (Yatvin and Lelkes, 1982; Kim et al., 1983; Talsma et al., 1987). In asymmetric liposomes, the phospholipid distribution in the inner and outer sides of the bilyer is different (Culls et al., 1987). Two important parameters are used to describe the entrapment of drugs within loposomes. The capture volume (also called the internal volume) is the volume enclosed by a given amount of lipid. The encapsulation efficiency describes the % of the aqueous phase that becomes entraped during liposome preparation. These two parameters can easily be determined (Digiulo et al., 1991).
Aqueous Compartment

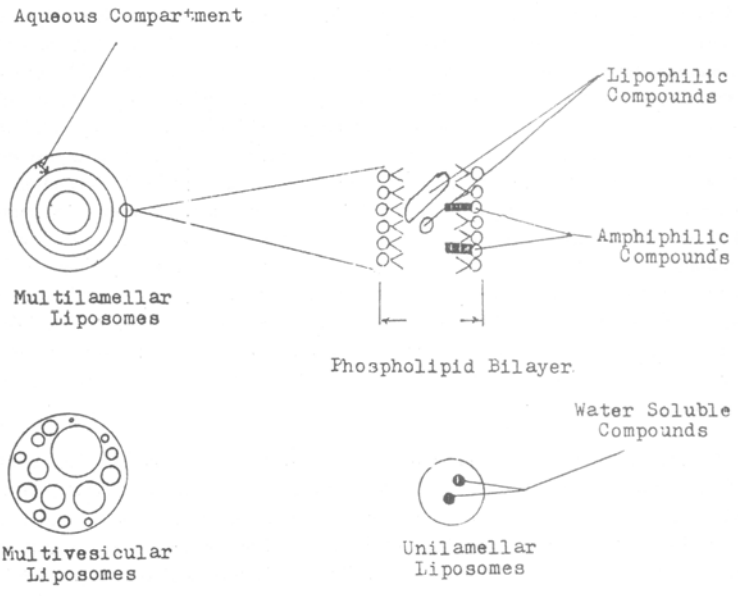

Fig. l: Schematic representation of liposomes.

(A) **Physical and Chemical Stability**

Cholesterol and phospholipids containing unsaturated fatty acids undergo oxidation. One solution to this problem is to use phospholipids which contain saturated fatty acids. Synthetic saturated lecithins provide a good alternative to egg or soybean lecithin (Eibl, 1981). The amount of lipid peroxidation can be monitored by noting the ultraviolet absorbance ratio (Absorbance at 233 nm/absorbance at 215 nm) which is a measure of diene conjugation produced by oxidative damage (Klein, 1970). The peroxidation of lipids can be minimized by use of antioxidants such as Vitamin E and butylated hydroxytoluene (BHT) (Matson and Martin, 1987). Ultraviolet light and divalent metal ions particularly manganese, iron and cobalt ions also accelerate the rate of oxidation (Klein, 1970).

Lecithin undergoes hydrolysis to give lyso-lecithin and other degradation products. The presence of lyso-lecithin in lipid bilayers greatly enhance the permeability of liposomes. Therefore, it is important to start with phospholipids which are free of lyso-lecithin (also of any phospholipases). The rate of hydrolysis of distearoyl-phosphatidylcholine in aqueous solution at 70°C was found to be dependent on pH 6.5 (Frokjaer et al., 1984).

The formation of ice crystals in liposomes (for example when liposomes *are par*-tially frozen and thawed), the subsequent instability of bilayers leads to the leakage of entraped material. When liposomes are subjected to a freeze thaw cycle and freeze drying in the absence of a cryoprotectant such as lactose, they lose the entraped compounds on reconstitution (Crommelin and Bommel, 1984). In another study it was found that addition of a cryoprotectant such as 20% dimethylsulphoxide to frozen liposomes may help liposomes against the leakage (Machy and Leserman, 1984).

Freeze drying (lyophilizalion) can be useful in some cases to solve long term stability problems of liposomes. On reconstitution (rehydration) most of drug remains within liposomes. It has been shown that liposomes when freeze dried in the presence of trehalose (a sugar) retained as such as a 100% of their contents (Crowe et al., 1986, 1987; Madden et al., 1985). Another way to increase stability of liposomes is to use synthetic phospholipids which polymerize on exposure to UV light (Hayward et al., 1936). To solve long term stability problems, recently'Proliposomes" were prepared using sorbitol and methanolic solution of phospholipids. These are dry powders containing *water* soluble compound/drug coated by phospholipids. They give liposomes on hydration above the gel-liquid crystalline phase transition temperature (Payne et al., 1987).

The physical stability of liposomes on storage can be studied by monitoring the amount of leaked material from liposomes and by the size of liposomes. The liposome

size increases due to aggregation and finally decreases due to fusion. Egg lecithin liposomes have more tendency to form aggregates than liposomes containing egg lecithin and acidic phospholipids. Rand and Parsegian (1986) have described video enhanced light microscopy to study various stages during the fusion of liposomes. Connor et al. (1984) found that SUV containing phosphatidylethanolamine and palmitoylhomocystein fuse rapidly when pH of the medium was rapidly lowered from 7 to 5. During the fusion almost all of the encapsulated calcein was released.

An increase in physical stability of liposomes can be achieved by increasing amount of charge on liposomes. For example, Frokjaer et al. (1982) reported an increase in physical stability against aggregation and fusion by decreasing the ionic strength and increasing surface charge density of liposomes consisting of phosphatidylcholine and phospholidylserine. In vivo, the surface large density has been found to influence the distribution of liposomes (Rahman et al., 1980).

The presence of divalent metal ions such as Ca^{t+} and Mg^{t+} causes the aggregation of liposomes particularly those with negative charge. Therefore, aqueous buffer used for the preparation of liposomes should be free of divalent metal ions. Some workers like to treat water with EDTA to remove any divalent metal ions present (Wang et al., 1984).

Hunt and Tsang (1981) have shown that in MLV composed of egg lecithin, there was no detectable chemical degradation and less than 5% leakage of entraped sucrose was observed after six months at pH 7.4 and 22°C in the absence of light and oxygen. In the presence of light and oxygen, autooxidation of lipids can resulted in the complete loss of entraped solute within 48 hours. The incorporation of a-tocopherol retards the autooxidation of liposomes. Crommelin and Bommel (1984) showed that the liposomes composed of disteroy phosphate dylcholine, cholesterol and dipal-mitoylphosphatidylglycerol were stable for at least six months when stored at 4-6°C.

The stability of doxorubicin-HCL (DXR) liposomes containing phosphatidylcholine, phosphadylserine, cholesterol (10:1:4) was studied by Janssen et al., (1985). At pH 4, no significant decomposition of DXR was observed when liposomes dispersions in Tris as well as phosphate buffer were stored for 40 days protected from light at 4°C and pH 7.4, DXR decomposition rates were larger than at pH 4. The stability of anthralin containing liposomes was studied by Mahre et al. (1991). Anthralin is oxidized within days in liposomal phospholipids. The oxidation process is retarded by using liposomes containing mostly negative charged phospholipids. Alamelu and Rao (1991) prepared stable liposomes *containing* carboxymethylchitosan. In an attempt to see the successful application of liposomes as topical opthalmic delivery device, the stability of liposomes composed of egg lecithin, stearlylamine and cholesterol in the presence of rabbit tear

fluid was studied. The rate of release of carboxyfluorescein was significantly increased in the presence of tear fluid at all cholesterol levels (Barber and Shek, 1986).

(B) **Stability In Biological Fluids**

The stability of liposomes in the circulation is of great interest when they are to be applied as intravenous drug carriers. This is a well established fact that the liposomes are generally unable to retain their entraped substances when incubated with blood or plasma. Generally MLV are most stable since only a portion of the phospholipid is exposed to the attach and SUV are the least stable because of the stress imposed by their curvature. The incorporation of cholesterol some times increases the stability of liposomes in the presence of plasma. Senior and Gregoriadis (1982) studied the stability of SUV in mouse serum at 37°C. Liposomes composed of egg lecithin, dioleylphosphatidylcholine or sphingomyelin became rapidly permeable to entraped carboxyfluorescein (CF) but the incorporation of cholesterol in the liposomes reduced CF leakage. Further it was found that the stability of liposomes in plasma can be in-creased by using saturated phospholipids but this stability was dependent on the gel-liquid crystalline phase transition temperature of the phospholipid(s) present in the liposomes.

It has been found that proteins particularly high density lipoprotein (HDL) are mainly responsible for the increase in permeability of liposomes. The lipid from liposomes are transferred to the protein which in turn results in an enhanced leakage of the entraped solute probable due to the formation of pores in the bilayer(s) (Kirby and Gregoriadis, 1981). Further transfer of lipid occurs from liposomes to plasma membranes and vice versa is also true (Ostro, 1987). Agarwal et al. (1986) reported that the transfer of phospholipid from liposomes to HDL could be prevented by using a phosphatidylcholine analogue.

The permeability studies were made on liposomes made from polymerizable diacetylenic phospholipids in the presence of plasma. The incubation of liposomes containing Cu identical chain PC showed high permeability of monomeric vesicles to both carboxyfluorescein and ^{3}H-inulin in plasma. While permeability of polymerized Cu identical chain PC liposomes was unaffected in the presence of plasma, with vesicles retaining most of their entraped 3H-inulin after 50 hours. These findings suggest that the polymeric liposomes have resistance against the destructive actions of plasma components particularly HDL (Freeman et al., 1987).

The stability of liposomes in gastrointestinal tract is very important if they are to be used as drug carrier by the oral route. A lot research has been done to study the stability of liposomes to maintain their integrity against enzymes found in the GIT, bile salts and gastric acidity. The pancreatic lipase was capable of degrading naturally occurring

phospholipids. It has been found that liposomes containing short chain fatty acids were more stable against destructive action of lipase (Dapergolas and Gregoriadis, 1977). Phospholipase A_1 and A_2 cause the formation of lyso-phosphatidylcholine which then induced lysis of the liposomes when these were incubated at or near transition temperature of the phospholipid (Horlos et al., 1977). The addition of cholesterol some times increase the stability of liposomes. For example only 16% of ^{125}1-PVP was released from distearoylphosphatidylcholine liposomes in 10mM bile salts at 37°C in one hour when cholesterol was incorporated in the liposomes (Rowland and Woodley, 1980). It was found that lipsomes containing phosphatidylcholine (PC), cholesterol and stearylamine were stable when they were incubated for 3 hours at 37°C in solution of pepsin, trypsin and pancreatin since a considerable amount of entraped insulin was still associated with these liposomes (Hasqimoto and Kawada, 1979).

Whitmore and Wheeler (1979) reported a complete loss of entraped marker when the liposomes were incubated with 1% v/v bile acids solution. Chiang and Weiner (1987) studied the stability of distearoylphosphatidylcholine / cholesterol liposomes at pH 2, in the presence of bile salts and in the presence of pancreatic lipase. A complete loss of entraped carboxyfluorescein was observed after 1 hour in the presence of a mixture of 10mM bile salts and 10,000 U/mmole of lipase (a simulation of conditions encountered in gastrointestinal tract) and about 10% entraped glucose was released at pH 2 after 120 minutes.

A novel quantitation method for the evaluation of the stability of liposomes in blood circulation based on the clearance concept for liposomal degradation was observed. The method is simple and useful in pharmacokinetic study of liposomes (Kume et al., 1991).

(C) **Use of Liposomes**

a) **Treatment of Diseases**

Drug containing liposomes have been shown to be effective against diseases in test animals and in some cases in human beings (See Table 1).

b) **As Vaccine Carriers**

Liposomes carrying antigens (derived from infectious organisms) particularly viral antigens, malaria antigens and bacterial toxins have been successfully used to produce humoral or cellular immunity in test animals. The indicates that liposomes have potential as vaccine carriers but still more research is needed to produce vaccines based on liposomes for human use (Alving, 1987).

c) In Cell Physiology

Liposomes have been useful in the field of cell physiology to understand the functions of proteins in a natural membranes. Liposomes can be constituted so that cell membranes are functionally expressed (working like those in natural membranes) on the surface of liposomes, thereby providing a model system for studying receptor-membrane interactions. Such studies have indicated that functional membrane proteins (enzymes) have requirements not only for a lipid milieu but in some cases may require specific lipids. For example, the membrane bound (Na^+, K^+) -AtPase enzyme requires anionic lipids such as phosphatidylserine (Malathi, 1983).

d) As Diagnostic Agents

The intravenous administration of liposomes containing contrast agents such as ^{99m}Tc have been used to visualize certain malignant tissues such as cancerous tissues of breast. Another liposome formulation is available for assaying rheumatoid factor based on agglutination technology. Leap RF rheumatoid factor test kit is available from Cooper Biomedical, Inc., Pennsylvania, USA.

e) As Model Membranes

i) Liposome have been used successfully to investigate the mechanism involved in the ototoxicky of amonglycoside antibiotics. On the basis of these studies, the following multistep mechanism was proposed:

(1) Interaction of aminoglycoside with phospholipids in the outer plasma membranes.
(2) Aminoglycoside translocation into cell and
(3) Binding of the drug to phosphatidylinositol 4, 5 diphosphate (PIP_2).

Further these studies have shown that the binding of PIP_2 containing liposomes to aminoglycosides is in accordance with the established toxicity of aminoglycosides: neomycin > gentamicin > amikacin > neamine (Au et al., 1987).

ii) Liposomes have been used to understand the mode of action of local anesthetics. These studies have indicated that the action of a local anesthetic is mediated by the specific interaction with phosphatidylserine. These interactions probably cause significant membrane fluidization and displacement of Ca^{2+} from membranes. These two effects are also associated with anesthetic action of excitable membranes (Ohki, 1984).

iii) Liposomes appear to be convenient in vitro models for studying the phototoxic effects mediated through active oxygen species, membrane damage and altered Ca fluxes (Bhatia et al., 1991).

f) **In Cosmetics**

To investigate the use of liposomes in cosmetics, studies have been made to study interaction between skin and liposomes (Carson et al., 1990; Junginger et al., 1991).

g) **In Textiles**

A new method of wool chlorination at pH < 7 using liposomes as vehicles for oxidizing agents is suitable for inhibiting or modulating the formation of cystic acid in wool fibers. The chlorination using liposomes inhibits the formation of cystic acid (De La Man et al, 1991).

Table 1
Therapeutic uses of liposomes in test animals and humans

Disease	Drug Encapsulated	References
Protozoal		
(I) Leishmaniasis (hamster)	Antimonial	Chapman et al., 1984
(II) Malaria (mice)	Primaquine sulphate	Prison et al., 1982
Hepatitis (mice)	Interferon (immunomodulator)	LaBonnardiere, 1978
Systemic fungal infection (human)	Amphotericin B	Lopez-Berestein, 1987
Rheumatoid arthritis (human)	Cortisol palmitate	De Silva et al., 1979
Cancer		
(i) human	Doxorubicin	Pharm. Journal, 1987
(ii) mice	Cortifen	Kaledin and Kurunov,
(iii) Ovarian cell (human)	Cisplatin, Valinomycin	Daoud and Forde, 1991
Oral Ulcers (hamster)	Triamcinolone acetonide palmitate	Harsanyi et al., 1986
Pneumonia (mice)	Pentamidine	Debs et al., 1987
Hemophilia A (human)	Blood clotting factor VIII	Hamker et al., 1980
Endocarditis	α_1-antitrypsin	Schaff et al., 1991
Genital herpes (guinea pigs)	Genetically engineered interferon (Reaferon)	Kobrinski and Melinikov, 1991

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