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The Effects of Liposome Composition and Temperature on the Stability of Liposomes and the Interaction of Liposomes with Human Neutrophils

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Abstract: The effects of temperature and lipid composition on the liposome stability (fusion) *in vitro* were studied. It has been obscure that temperature affects phosphatidylcholine (PC) liposomes stability and the optical densities of these liposomes were increased with time (days), while incorporation of cholesterol 25 mol % to the liposomes (PC: cholesterol) reduce the temperature's effects and the optical densities show a very slight change with time (days) because cholesterol in high concentrations prevent the phospholipids packing and induce orientation and more rigidity to those phospholipids and therefore prevent liposome aggregation, even if the oxidation of these lipids takes place (diene production and formation of lyso compounds). Interaction of these liposomes with human neutrophils was also studied. The liposomes containing cholesterol is more efficient in enhancing the activity of these cells than PC liposomes.

Key words: Liposomes, phospholipids, cholesterol, fusion, neutrophils, radicals

Introduction

Liposomes are microscopic spherical vesicles that form when phospholipids are hydrated. When mixed in water under low shear conditions, the phospholipids arrange themselves in sheets, the molecules aligning side by side in orientation, "head" up and "tail'' down. These sheets then join tail-to-tail to form a bilayer membrane, which encloses some of the water in a phospholipid sphere (Hafez *et al*., 2000; Sandip *et al*., 2000).

Liposomes have a long history in the study of biological membranes. More recently, liposomes have been evaluated as delivery systems for drugs, vitamins and cosmetic materials. Liposome can be designed for almost any need by varying the lipid contents size, surface charge and method of preparation, also liposomes with covalently attached proteins on their surface, coated with antibodies or their fragments for immunotargeting of entrapped drugs to cells or coated with protein antigens, analyses of coupling efficiency preservation of antibody activity and liposomes (Reinhard *et al*., 1986; Devine *et al*., 1994) and influence of phosphate asymmetry on fusion of large unilamellar vesicles (Eastman *et al*., 1992), polyethylene induced fusion (Fenske *et al*., 2001; Parr *et al*., 1994), Ca^{2+} -induced fusion (Wilschut *et al*., 1992; Wilschut *et al*., 1985) and Liposome stability in blood (Gabizon and Papahadjopoulos, 1988; Allen *et al*., 1991; Liu *et al*., 1991). Phospholipids (PLs) are the important constituents of biological membranes and their physico-chemical properties in membrane arrangements have been the subject of extensive studies (Davis and Keough, 1991). The molecular packing, structure and distribution, in mixed bilayers of PLs is determined by several factors among which are (a) the composition and degree of similarity of the PL acyl chains, particularly the degree of acyl chain unsaturation; (b) the nature, orientation, interaction and extent of hydration of the polar head groups; ©the ambient temperature relative to the thermotropic phase transition of the component PLs; (d) the extent of peroxidative degradation of the polyunsaturated membrane phospholipids (Cevec *et al*., 1987). Also the asymmetric trans-bilayer distributions of lipids commonly observed in biological membranes may be expected to play a role in regulating membrane fusion *in vivo* (Eastman *et al*., 1992). Upon hydration, most naturally occurring phospholipids adopt either the bilayer organization or the hexagonal H11 phase (Eastman *et al*., 1991).

Cholesterol is considered as a major constituent of biological membranes, its proportion being especially high in the surface membranes of mammalian cells (Dini *et al*., 1991), making up as much as 50 % (w/w) of the lipid fraction in case of the human erythrocyte membrane (Kariel *et al*., 1991). Studies of the influence of cholesterol on the physicochemical properties of the membranes have indicated that cholesterol modifies the packing of phospholipids in membranes (Vist and Davis, 1990).

Previous studies have indicated that the quantitative effect of cholesterol on physicochemical parameters in model membranes depends upon the structure of the phospholipid that is association with the sterol (Meyer *et al*., 1997).

However, very few studies have investigated the effects of liposome composition and temperature on liposome stability *in vitro*.

The aim of this work is to study the stability of different liposomes at different temperatures (for one month) by measuring the optical densities of the liposomal suspensions at different wavelengths, determination of lipid peroxides by the oxidative index and also to indicate the efficiency of the empty liposomes to activate the human neutrophils *in vitro*.

Materials and Methods

Phosphatidylcholine (Lecithin Soybean, BDH. England), cholesterol JANSEEN CHIMICA, Belgium), spectrophotometer (Milton Roy spectronic and PYE UNICAM Pu 8600 UV/Vis-Philips), chloroform (BDH Laboratory Supply Pool, England), ethanol (HAYMAN, Limited, England), Rotary evaporator (RV o5-ST Janke and Kunkel, IKA, Germany), nitroblue tetrazolium (Sigma), ammonium chloride (GCC, Gainland Chemical Company, UK.), di-sodium hydrogen phosphate anhydrous (Fluka, Chemika), potassium dihydro-ortho phosphate (Laboratory Rasagan, purified LR), Percol (Sigma. lot 98H10621).

Preparation of empty liposomes: Lipid mixture of phosphatidylcholine (PC) and cholesterol in ratio of 25 mol% in chloroform solution was dried to leave a thin film on the wall of round-bottom flask by removing the organic solvent with a rotary evaporator. The lipids film was hydrated with water and agitated. For PC liposomes, PC (5 mg/ml) in chloroform solution was dried and evaporated as mentioned above. Unilamellar liposomes were obtained by sonication for 10 min, at 4°C of lipid dispersions (5 mg/ml) in 0.1 M K, Na-phosphate buffer (pH 7.4) until the suspension became clear (Jizomoto *et al*., 1989; Kagan *et al*., 1990).

Optical density: The optical densities of the liposome suspensions were measured at the desired time (days) at visible light (range from 350–600 nm) and the size was calculated according to Klenine method (Trofimove *et al*., 1990).

Oxidative index (OI): To each 0.1 ml of sample (4.5 mg of phospholipid) added 3 ml of absolute ethanol, then the absorbance was measured at 233 nm. The molar extinction coefficient was assumed to be 30,000 . The OI was calculated according to the

formula of Halliwell and John (1991).

Cell isolation: Anticoagulated blood samples (EDTA) were obtained from volunteers. Granulocytes were isolated by a one-step Percol technique (Nilsson and Palmblad, 1988), followed by lysis of residual erythrocytes with 0.155 M ammonium chloride. With this technique, platelets were removed by an initial centrifugation step and neutrophils comprised > 95% of the cells, as were determined from stained smears. Cells were suspended in Hanks' balanced salt solution (HBSS) at pH 7.45, followed by 15 min incubation at 37°C.

Superoxide ion production: Superoxide ion production was analyzed by the nitroblue tetrazolium method (Tonetti *et al.*, 1991; Colepicolo *et al.*, 1990). In brief, cells (2 x 10^6) was incubated with liposomes followed by addition of NBT solution. After incubation for 15-20 min at 37°C, the reaction was stopped by addition of cold 0.5 N HCl, then centrifuged at 1000 g for 20 min. To the pellet was added dioxane solution, followed by incubation for 15 min at boiling water bath, then cooled and read at 520 nm.

Statistical Analysis: Results were analyzed using student's t-test. Any p value less than 0.05 was considered statistically significant.

Results and Discussion

Analysis of the lipids content of liposomes indicated that little oxidation or lytic degradation occurred during liposome preparation (Table 1).

Incubation of these liposomes at room temperature (RT) and 4°C for 28 days suggests that with time lipid peroxidation occurred and this oxidation depends on the composition of liposomes and temperature of incubation. PC liposomes have slight increase in the oxidation index at RT when compared to the same liposomes which were incubated at 4°C (Table 1).

Also with time the optical densities of these liposomes were increased when compared to the optical densities of these liposomes at zero days (Fig. 1 and 2) especially after two weeks for PC liposomes which incubated at RT and this increase may be due to liposome fusion and aggregation. The presence of the reactive hydroxyl radical species (diene production) are capable of inducing peroxidation of unsaturated lipids (Marsh, 1991) and the resulting damage to PLs could impart intrinsic membrane disturbances, thus, affecting the packing and distribution of PLs. The generation of these oxidized lipids create disturbance among PL acyl chains, increase membrane rigidity and promote PL flip-flop (Lentz *et al.*, 1992) and the oxidation of aqueous dispersion of PLs has been found to increase the gel to liquid-crystalline transition temperature creating multiphasic structural arrangements (Madden *et al.*, 1988). This packing irregularities created by peroxidation of membrane lipids may be envisioned to facilitate the membrane fusion.

Liposomes composed of lipid mixture PC: cholesterol (25 mol%), although have an increased oxidation index (Table 1) but their optical densities slightly changed (Fig. 3 and 4) and this may be due to the presence of cholesterol which inserts its hydroxyl group oriented towards the aqueous surface and the aliphatic chain aligned parallel to the acyl chains in the center of the bilayer. Therefore, reducing the freedom of motion of these carbons; while at the same time creating space for a wide range of movement for the remaining carbons towards the terminal end of the chain. Thus, cholesterol causes dramatic changes in membrane molecular order and dynamics in the fluid and gel phases as well as introducing a new thermodynamic phase at high cholesterol. The change in hydrocarbon chain order within the gel phase, as cholesterol is added, is small because in the gel phase the chains are already largely all-trans.

Present findings for the incorporation of cholesterol to liposomes indicate that the liposomes become more stable (Fig. 3 and 4) due to the ordered phospholipids chains and satisfied what has been given in literature, that the bilayer has a lateral diffusion rate; the axial molecular reorientation is rapid; the phospholipid chains are highly ordered; and the bilayer becomes more thick (Meyer *et al.*, 1997).

Table 1: The oxidation index of different types of liposomes at different temperatures

	Type of liposome			
Days	Phosphotidyl-choline*	Phosphotidyl-choline#	Cholesterol*	Cholesterol#
0	0.20	0.20	0.25	0.25
7	0.21	0.20	0.33	0.30
14	0.273	0.30	0.431	0.35
21	0.35	0.36	0.44	0.38
28	0.60	0.504	0.45	0.42

* At room temperature, # At 4°C. n = 4. P < 0.05

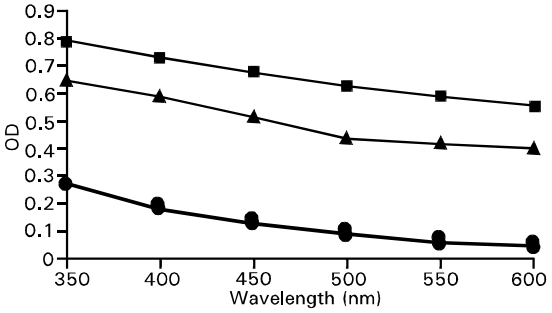

Fig. 1: The optical densities (OD) of PC liposomes incubated at RT

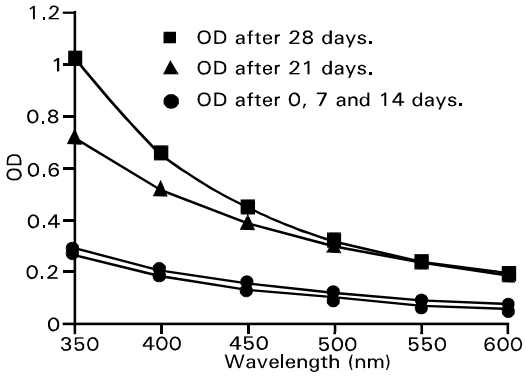

Fig. 2: The optical densities (OD) of PC liposomes incubated at 4°C

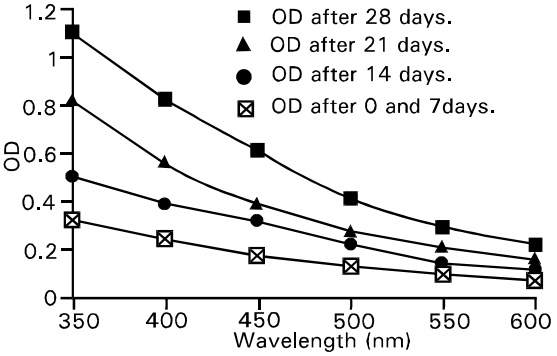

Fig. 3: The optical densities (OD) of (PC- cholesterol) liposomes incubated at RT

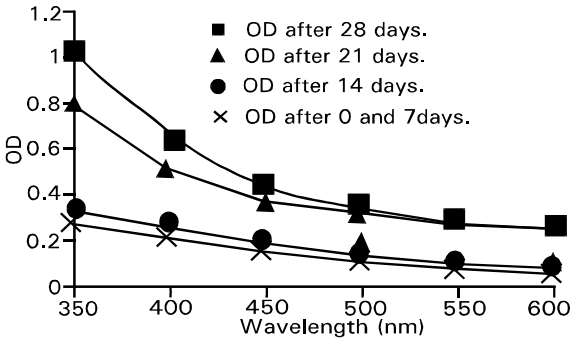

Fig. 4: The optical densities (OD) of (PC- cholesterol) liposomes incubated at 4°C

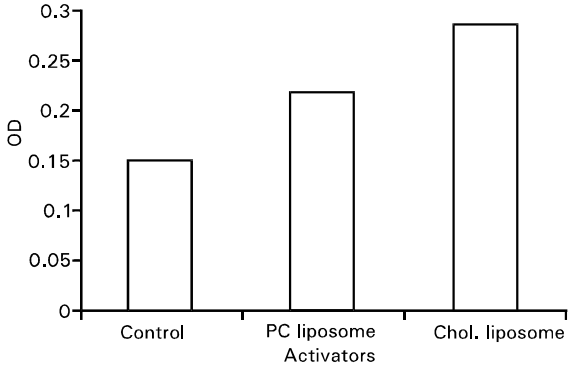

Fig. 5: The optical densities (OD) of nitroblue tetrazolium solution of the incubated liposomes with human neutrophils at 520 nm. N = 8, p< 0.05

Other experiments illustrated that interaction of these liposomes with human neutrophils shows that liposomes containing cholesterol have high activity in enhancing the production and release of reactive oxygen species than liposomes containing only PC as measured by the reduction of nitroblue tetrazolium salt (Fig. 5) . This increase in the activity may be due to the presence of cholesterol, which enhance the interaction between liposomes and cells .

In conclusion, the effects of incorporation of cholesterol in phospholipid bilayers are able to increase the thickness and "strength" of the bilayer while maintaining the "fluid" environment conducive to high lateral mobility. This modification of the physical characteristics of the bilayer may be one of its major functional roles in the membrane. Cholesterol causes a dramatic increase in orientation order of the phospholipid hydrocarbon chains and it decouples the position and conformational degrees of freedom of the physiological molecules.

Incorporation of cholesterol to the PC liposomes not only prevents their fusion, but also increases their activity for stimulation of human neutrophils and therefore, enhances the release of the reactive oxygen species, which has been considered to be indicative of the killing capacity of the phagocytic cells.

More studies are needed to evaluate the exact role of cholesterol in liposome stability and which concentration is more efficient.

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