THE INFLUENCE OF DOPC ON THE PERMEABILITY OF LIPID MEMBRANE: DETERGENT SOLUBILIZATION AND NMR STUDY

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Abstract. There is an efficient, systemic transgene expression in many cell lines (*in vitro*) by using anionic liposomes, composed of equimolar amounts of 1, 2-dilauroyl-sn-glycero-3-phosphoethanolamine (DLPE), 1, 2-dioleoyl-sn-glycero-3-phospho-L-serine (DOPS) and cholesterol (CHOL). 1, 2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) is added to this liposomal formulation to increase the stability of small unilamellar vesicles in the presence of fetal calf serum (FCS). The liposomal stability was studied by monitoring the retention of a trapped marker, carboxyfluorescein (CF), in the internal liposome compartment. The liposomes containing DOPC had a lower stability in serum compared to the DOPC free liposomes. The extremely low retention in the DOPC liposome was ascribed to a formation of the inverted hexagonal phase of the liposomal membrane. A higher sodium deoxycholate detergent concentration is needed to solubilize DLPE/DOPS/CHOL (1:1:1) liposomes than DLPE/DOPC/DOPS/CHOL (1:1:1:1) liposomes. Phosphorus-31 nuclear magnetic resonance (^{31}P-NMR) is used to investigate the lamellar (L_α) → hexagonal (H_{II}) phase transition of unilamellar lipid vesicles containing DOPC. It was found that unilamellar lipid vesicles containing DOPC are in the bilayer state at pH 4 and undergo a lamellar (L_α) → hexagonal (H_{II}) phase transition at pH 8.

Key words: NMR, DOPC, liposome, permeability, solubilization, hexagonal phase.

INTRODUCTION

Following decades of extensive research, lipid-based delivery systems have entered the mainstream as carriers of nucleic acids, either for plasmid delivery or as agents for carrying short antisense single strand oligonucleotides (ON) to express or to down-regulate target genes in therapeutic applications. With many of the early problems related to carrier toxicity, biodegradability and stability recently ameliorated, a notorious drawback of the lipid based vehicles remains poor efficiency [3, 4, 8, 16, 20, 21, 24].

However, the potential use of non viral vectors as gene carriers by intravenous injection is limited by their low stability in bloodstream. Therefore,

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most *in vitro* transfection protocols avoid serum, which makes the extrapolation to *in vivo* situations difficult. The stability of liposomes in the presence of serum can be thought as a first approximation to gain insight into the behavior of liposomes in biological fluids.

There is an efficient, systemic transgene expression in many cell lines (*in vitro*) by using anionic liposomes, composed of equimolar amounts of 1, 2-dilauroyl-sn-glycero-3-phosphoethanolamine (DLPE), 1, 2-dioleoyl-sn-glycero-3-phospho-L-serine (DOPS) and cholesterol (CHOL). The efficiency and safety of the *in vitro* use of these liposomes have been demonstrated in many cell lines [11, 15, 17, 26].

DOPC is added to this liposomal formulation to increase its stability in the presence of fetal calf serum (FCS). The choice of the highly unsaturated DOPC lipid, already used as a reliable constituent of model membranes and able to form liposomes with a stable and well characterized bilayer structure [19], has been mainly driven by its well-known NMR spectrum [12, 29]. On the contrary, the liposomes containing DOPC had a lower stability in serum compared to the DOPC free liposomes. The extremely low retention of CF in the DOPC liposome was ascribed to a formation of the inverted hexagonal phase of the liposomal membrane.

Non-bilayer arrangements of phospholipids, such as the inverted hexagonal (H_{II}) phase and the inverted cubic (I_{II}) phase, can be formed under a variety of conditions. They are believed to play an important role as transient structures in complex biological processes such as membrane fusion and are also implied as structural features in tight junctions. Depending on the experimental conditions and the membrane composition the interplay of different geometries leads to the formation of micelles, bilayers, or hexagonal phases, respectively.

The present work deals with the influence of DOPC on these anionic liposomes properties. Stability measurements in the presence of serum, solubilization process by sodium deoxycholate detergent and ^{31}P-NMR spectroscopy provide a suitable experimental system to investigate the formation of the inverted hexagonal phase in DOPC liposome bilayer.

MATERIALS AND METHODS

1, 2-dilauroyl-sn-glycero-3-phosphoethanolamine (DLPE); 1, 2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1, 2-dioleoyl-sn-glycero-3-phospho-L-serine (DOPS) were obtained from Avanti Polar-Lipids Inc. (Ottawa, Canada). Tris ultra pure buffer and sodium deoxycholate were obtained from ICN Biomedicals Inc. (Ohio, USA). 4(5)-carboxyfluorescein (CF) was purchased from Fluka (Germany). All reagents were of analytical reagent grade. Cholesterol (CHOL) was obtained from Calbiochem (La Jolla, CA, USA).

Anionic liposomes are composed of equimolar amounts of DLPE, DOPS and CHOL [26]. Liposomes were formulated according to the well-established method of extrusion [18]. In short, the appropriate phospholipid composition was mixed in organic solvent (chloroform) in a 50 ml round flask. The organic solvent was evaporated to dryness by a Mini-Rotavapor (Büchi, Switzerland). The resulting thin lipid film was hydrated with tris buffered saline (10 mM tris, 140 mM NaCl) containing 100 mM CF at pH 7.4. The resulting lipid suspension was extruded through 200 nm nucleopore membranes using a commercially available extruder device (Liposofast, Avestin Inc., Canada). Free CF was removed by dialysis (Mini Lipoprep, Dianorm GmbH) in Tris buffered saline containing 10 mM Tris and 140 mM NaCl at pH 7.4 after extrusion [28].

Liposome stability is defined as their capability to retain the structural integrity of the lipid bilayer and to prevent leakage of their aqueous contents [13]. The release experiments were run immediately after the separation of free CF from encapsulated CF in liposomes. The liposomes were incubated with 100% FCS at 37°C. At indicated time points, 10 µl of the suspension were diluted 200 times with Tris buffered saline and the fluorescence intensity (F; excitation at 470 nm, emission at 520 nm) was measured. To lyse liposomes completely, 20 µl of 10% sodium deoxycholate was added and the total fluorescence F_{total} (corresponding to 100% release) was measured. The percentage of CF release was calculated by dividing F by F_{total}. A plot of % CF release *versus* time is used to compare the stability of different liposomal formulations in the presence of FCS [2].

The solubilization of uni-lamellar liposomes composed of DLPE/DOPS/ CHOL(1:1:1) liposomes and DLPE/DOPC/DOPS/CHOL (1:1:1:1), containing 100 mM CF, of a certain lipid concentration was followed by continuous addition of sodium deoxycholate detergent and monitored by measuring their fluorescence intensity. The sodium deoxycholate solution was continuously added at a constant rate to the cuvette, equipped with a paddle stirrer and containing 2 ml of liposomes solution. During sodium deoxycholate injection, fluorescence intensity of the mixed solution was measured. Consequently, % CF release profiles of solubilized liposomes were plotted as a function of total detergent concentration. The initial concentration of lipid was 0.33 nmole/ml.

NMR spectroscopy is one of the principal techniques used to obtain physical, chemical, electronic and structural information about molecules. It is a powerful technique that can provide detailed information on the topology, dynamics and three-dimensional structure of molecules in solution and the solid state. The phase behavior of unilamellar lipid vesicles composed of DLPE/DOPS/CHOL (1:1:1) and DLPE/DOPC/DOPS/CHOL (1:1:1:1) liposomes is monitored with ^{31}P-NMR spectroscopy (Varian Mercury VX 300 NMR spectrometer, 300 MHz) as a

function of pH. Lipid concentration was 5 μmole/ml. The ^{31}P chemical shielding anisotropy ($\Delta\sigma$) was evaluated from the separation between the low-field and the high-field shoulder at half-height of the signal intensity [27].

RESULTS AND DISCUSSIONS

Because of the fast progress of nucleic acid-based technologies in the treatment of diseases, the call for appropriate delivery vehicles becomes increasingly important. One of the problems confronting researchers who wish to use liposomes in the treatment of disease is the rapid removal of liposomes *in vivo*. The ideal vehicle should avoid immediate uptake by the mononuclear phagocyte system and have prolonged circulation in the blood, thus increasing the probability of reaching the desired targets. In addition, the vehicle should be able to deliver its contents efficiently into the cell cytoplasm, avoiding lysosomal degradation [22].

Recently, an anionic liposomal formulation, composed of equimolar amounts of DLPE, DOPS and CHOL, has been used successfully to deliver oligonucleotides (ON) [26] and DNA [17] into a variety of cells *in vitro*. The present experiments were designed to look at leakage of contents from a variety of these vesicles in the presence of undiluted FCS at 37°C. To optimize the potential of these anionic liposomes as gene carriers, it is important to characterize their stability in terms of controlled release of their contents *in vitro*, simulating physiological conditions *in vivo* [14]. DOPC is added to this liposomal formulation to increase its stability in the presence of fetal calf serum (FCS).

Comparison between DLPE/DOPS/CHOL liposomes and DLPE/DOPC/ DOPS/ CHOL liposomes incubated with 100% FCS at 37°C showed that serum promotes CF release from DLPE/DOPC/DOPS/CHOL (1:1:1:1) liposomes faster than DLPE/DOPS/CHOL (1:1:1) liposomes (Fig. 1). A number of reports describe the transfer of lipids from liposomes to high-density lipoproteins (HDL), which represents the major factor responsible for the disruptive effect of serum on the integrity of liposome bilayer [1]. The process of this lipid transfer seems to be mediated by apolipoproteins. Insertion of apolipoproteins into the membrane of small unilamellar vesicles at conditions near the phase-transition temperature of the lipids may cause an accelerated leakage of the entrapped contents [10, 25].

It is known that cholesterol markedly increases the negative curvature of DOPC [7]. However, sufficient unsaturation in the lipid acyl chains is required for mixtures of cholesterol and PC to spontaneously convert to the hexagonal phase.

An additional interesting feature of the formation of a hexagonal phase in DOPC is that it appears to promote the phase separation of cholesterol crystals. If the H_{II} phase may form in the DOPC, the low retention of CF in the DOPC

liposomes would be expected because the formation of such a non-bilayer structure can lead to the leakage of the marker from the inner aqueous phase of vesicles [6]. The phase behavior of mixtures of phospholipids and detergents in aqueous solutions is an issue of basic importance for understanding the solubilization and reconstitution of biological membranes.

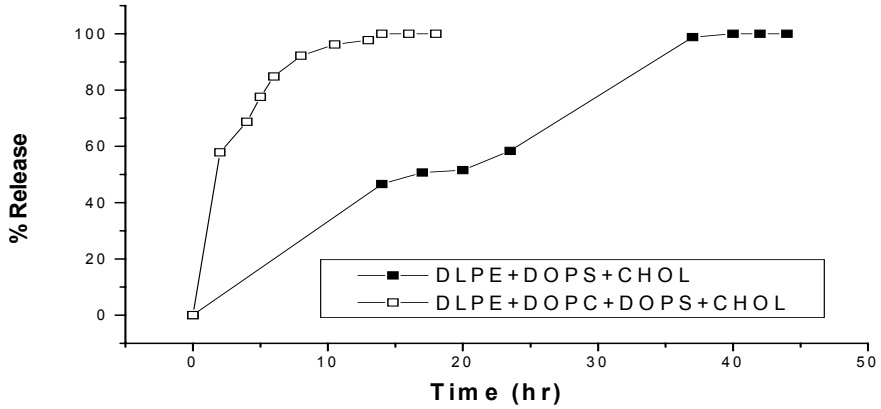

Fig. 1. % CF release from DLPE/DOPS/CHOL (1:1:1) and DLPE/DOPC/DOPS/ CHOL (1:1:1:1) liposomes measured at 37 °C in 100% FCS.

Figure 2 illustrates the variation of the % CF release of the two liposomal formulations as a function of the detergent concentration. A higher sodium deoxycholate detergent concentration is needed to complete solubilization of DLPE/DOPS/CHOL (1:1:1) liposomes than of DLPE/DOPC/DOPS/CHOL (1:1:1:1) liposomes. The coexistence phase, vesicular/mixed micellar, was shifted towards higher detergent concentrations for liposomes free from DOPC, indicating increasing membrane resistance to the detergent and hence changes in the natural membrane permeation properties. For example, the detergent concentration at which 50% of CF is still encapsulated was 0.527 μM and 0.48 μM for DLPE/DOPS/CHOL (1:1:1) liposomes and DLPE/DOPC/DOPS/CHOL (1:1:1:1), respectively. The data indicate that the membrane of DLPE/DOPS/CHOL liposomes is rigid and less soluble by the detergent compared to the same formulation containing DOPC.

Nuclear magnetic resonance (NMR) is a physical phenomenon based upon the quantum mechanical magnetic properties of an atom's nucleus. Characteristic ^{31}P-NMR spectral profiles are indicative of phopholipids in bilayer, hexagonal H_{II}, or isotropic phases, or in mixed phases. This technique provides information on

molecular structure and can be used to confirm the identity of a particular compound or help identify an unknown.

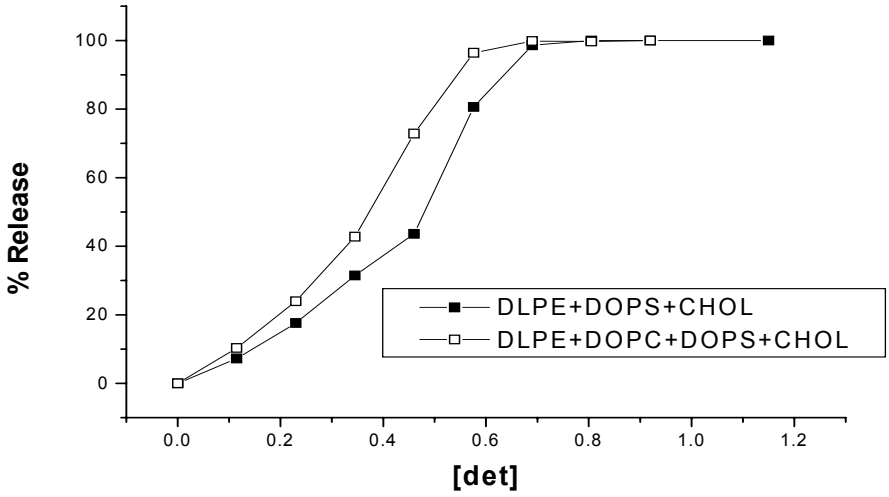

Fig. 2. The variation of % CF release from DLPE/DOPS/CHOL (1:1:1) and DLPE/DOPC/DOPS/ CHOL (1:1:1:1) liposomes as a function of the Na deoxycholate detergent concentration (μM). Initial lipid concentration was 3 nmole/ml.

Figures 3A & 3B show a ^{31}P-NMR spectrum of lipid vesicles composed of DLPE/DOPS/CHOL (1:1:1) and DLPE/DOPC/DOPS/CHOL (1:1:1:1) respectively, at pH 4 and pH 8. The data show that unilamellar lipid vesicles containing DOPC are in the bilayer state at pH 4 and undergo a lamellar (L_α) → hexagonal (H_{II}) phase transition at pH 8. The ^{31}P chemical anisotropy $\Delta\sigma$ shielding was –25 ppm. The asymmetric spectrum (Fig. 3B) is typical for a hexagonal arrangement of the lipid molecules [5, 6, 9]. Fig. 3 B provides evidence for a proton-induced lipid phase transition from the lamellar (L_α) to a hexagonal phase, most probably the H_{II} phase, at pH 8 and a constant temperature of $T = 25$°C.

It has been shown that cholesterol enhances the negative curvature tendency of phospholipids and will facilitate the formation of the H_{II} phase [7]. However, sufficient unsaturation in the lipid acyl chains is required for mixtures of cholesterol and PC to spontaneously convert to the hexagonal phase. An additional interesting feature of the formation of a hexagonal phase in DOPC is that it appears to promote the phase separation of cholesterol crystals. These findings therefore agree with those of Chen and Rand that at sufficiently high cholesterol concentrations, the curvature properties of DOPC become similar to those of DOPE [7]. Crowe *et al*. [9] clearly demonstrated that liposomal fusion and/or

aggregation could be one of the factors inducing the leakage of contents entrapped in the inner aqueous compartment of liposomes. In general, if lipids are approximately cone shaped (the area of the head group is smaller than the area of the acyl chain region), their membranes easily make a transformation from the lamellar to inverted hexagonal phase [5].

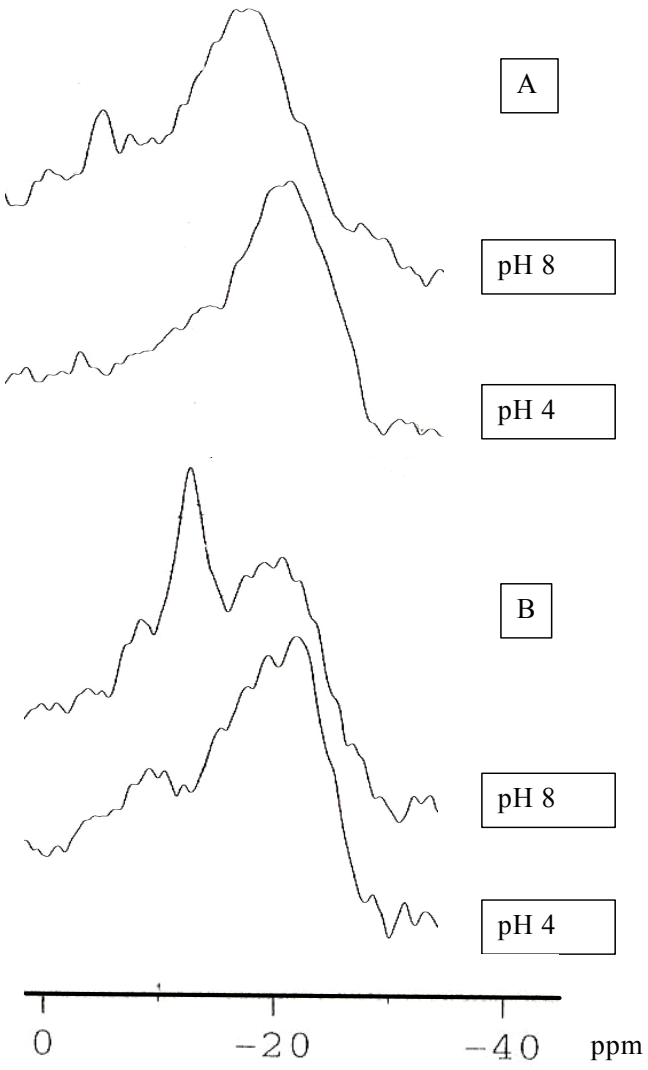

Fig. 3. ^{31}P-NMR spectroscopy of unilamellar lipid vesicles composed of A) DLPE/DOPS/CHOL (1:1:1) and B) DLPE/DOPC/DOPS/CHOL (1:1:1:1) liposomes at varying pH, (25 ^{o}C). Lipid concentration was 5 μmole/ml.

The canonical 'lipid raft' mixture utilized in many studies of lipid model membranes is made up of an equimolar mixture of PC (usually DOPC) and cholesterol [23]. These discrete lipid raft domains were postulated to exist in a relatively ordered lamellar liquid-crystalline phase (the L_o phase) while the continuous non-raft regions of the membrane exist in a relatively disordered lamellar liquid-crystalline phase (the L_α or L_d phase). The results suggest that DOPC has a preference to be located in a disordered phase, where most of the DOPC is squeezed out of L_o phase.

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