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INFLUENCE OF CHOLESTEROL ON BILAYER FLUIDITY AND SIZE DISTRIBUTION OF LIPOSOMES

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Abstract

Cholesterol molecules are essential to maintain the integrity and optimal fluidity of the cell membranes. The aim of this work was to study the influence of different cholesterol content (0–50 mol %) on the vesicle size and membrane fluidity of zwitterionic liposomes prepared with l-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine lipids. Temperature dependent anisotropy measurements using the fluorescent probe 1,6-diphenyl-1,3,5-hexatriene confirmed that increasing the cholesterol content in the liposomes decreased the fluidity of liposomal membranes. The dynamic light scattering data have shown that the size of the liposomes increased gradually with the increase in cholesterol concentration. This data provides insight about the optimal proportion of cholesterol to be added during the liposome preparation for diverse biomedical purposes, including drug delivery and in vivo applications.

Key words: fluidity, cholesterol, anisotropy, liposomes

1. Introduction. Cholesterol is a vital component of cell membranes, and its content can vary up to 50 mol % of the total lipid content in cell membranes [1]. It provides structural support, increases rigidity and protects the cell membranes from adverse environmental conditions [2]. Cholesterol is a bulk steroid molecule

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that has four linked hydrocarbon rings (Fig. 1a), and acts as a key molecule in the formation of small membrane domains known as lipid rafts. These cholesterol-rich domains are comparatively thicker and provide a favourable environment for the proper anchorage of membrane proteins [3]. Hence, the maintenance of optimal amounts of cholesterol is essential for the cell membranes to maintain their properties and to perform their functions.

Liposomes are widely used in the pharmaceutical field due to their high efficiency to carry and deliver the drugs to targeted locations in the body. Cholesterol is often added to such liposome preparations to enhance stability, prevent drug leakage, and increase their blood circulation time [4]. It also provides integrity to the membrane and plays an important role in maintaining the essential properties of the membrane such as elasticity, fluidity and permeability of the vesicles. Either enrichment or depletion of cholesterol beyond a specific proportion in the membranes will lead to altered properties. Hence it is important to add the optimal proportion of cholesterol during the liposome preparation. Few reports have predicted that up to 30 mol % of cholesterol is included in liposome-drug formulations to provide beneficial effects in terms of liposome stability and drug delivery [5]. On the contrary, some reports have shown that adding higher concentration of cholesterol did not cause significant changes on the biophysical properties of the liposomal membranes. As the application of liposomes in the biomedical field is increasing, there is an urgent need to clarify the optimal proportion of cholesterol to be added during the liposome formation which will not affect the membrane properties, stability and morphology of the vesicles.

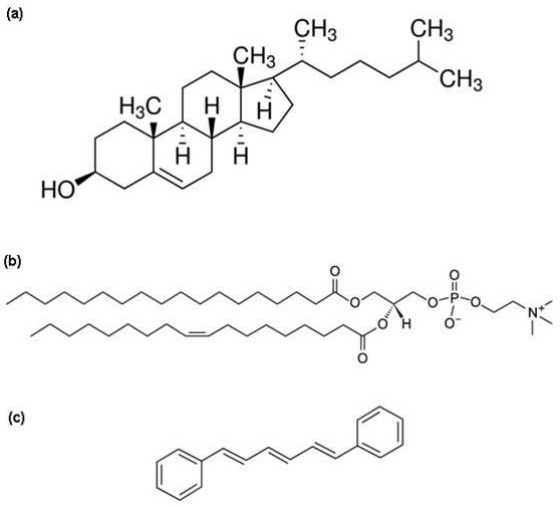

Fig. 1. Structures of (a) cholesterol (b) l-stearoyl-2-oleoyl-sn-glycero-3-phosphocholine (SOPC) and (c) 1,6-diphenyl-1,3,5-hexatriene (DPH)

The present work aims to address this issue and intends to compare the effects of different cholesterol concentrations (0–50 mol %) on membrane fluidity and stability of the liposomes prepared with zwitterionic l-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (SOPC) (Fig. 1b) lipid. Fluidity is an important aspect of cell membranes, and the fluid nature of the plasma membrane enables the free mobility of lipoproteins within the membrane, which is essential for cells to perform various functions, including adhesion, signalling, permeability and responses to external stimuli [6,7]. In the present work, we have employed a widely used fluorescent probe, 1,6-diphenyl-1,3,5-hexatriene (DPH) (Fig. 1c) to study the bilayer fluidity of liposomal membranes. Through this study, we intend to provide more insight for the design and development of novel liposomal formulations incorporated with appropriate cholesterol concentrations for improved stability and applications.

2. Materials and methods. ***2.1. Materials.*** SOPC lipid was purchased from Avanti Polar Lipids Inc. (USA). Cholesterol (5-cholesten-3β-ol) and DPH were obtained from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). All the chemicals obtained have high purity of $> 99\%$ and are used without any further purification.

2.2. Preparation of small unilamellar vesicles. Small unilamellar vesicles (SUVs) were prepared using the thin film method using a rotary evaporator. The stock solution of the lipid was prepared by dissolving 5 mg of SOPC in 5 ml of chloroform. Cholesterol (2 mg) was dissolved in 2 ml of methanol to get a homogenous solution. The control liposomes containing pure lipid without cholesterol were prepared by transferring 1 ml of the stock SOPC-chloroform mixture into a round-bottomed flask. Specific proportion of the cholesterol-methanol solution was added to the SOPC-chloroform mixture in round bottomed flasks in the ratio of 1:9, 2:8, 3:7 and 5:5 to get 3, 6, 10, 15, 20, 30, 40 and 50 mol % of cholesterol, respectively, from a total SOPC-cholesterol content of 1 mg.mL^{-1}. The organic solvent in the flasks was slowly evaporated overnight using the rotary evaporator under reduced pressure (17 mbar), to obtain a thin lipid film inside the flask. The mass of the flask was checked until the samples have reached constant mass to be sure that the solvent was completely removed. The lipid film was subsequently hydrated with double-distilled water at pH 7.0. and the lipid suspension was vortexed vigorously with glass beads for 10 min, to obtain multilamellar vesicles (MLVs). SUVs were then formed by sonicating these MLVs for a total of 30 min, using 10 s on-off cycles at 50% amplitude with a Vibracell Ultrasonic Disintegrator VCX 750 (Sonics and Materials, Newtown, USA). The sample was then centrifuged at 14 000 rpm for 10 min (Eppendorf centrifuge 5415C), to remove the debris from the ultrasonic probe (titanium alloy Ti-6Al-4V). The prepared SUVs containing varying cholesterol contents were used to measure the fluidity and size distribution of the liposomes.

2.3. Fluorescence anisotropy measurements. The fluorescent probe DPH was used to measure membrane fluidity and the anisotropy measurements

were performed in a 10-mm-path-length cuvette using a Cary Eclipse fluorescence spectrophotometer (Varian; Mulgrave, Australia). The 10 µL DPH was added to 2.5 mL 100 µM solutions of SUVs prepared from SOPC lipid to reach a final concentration of 0.5 µM DPH. The anisotropy values of SUVs were measured within the temperature range of 15 °C to 50 °C, by increasing the temperature by 5 °C for every measurement, with a time interval of 7 min with constant mixing. Varian autopolarizers with slit widths with a nominal band-pass of 5 nm were used for both excitation and emission. DPH fluorescence anisotropy values were measured at the excitation wavelength of 358 nm and emission wavelength of 410 nm. The anisotropy values were calculated as shown in Eq. 1 using the built-in software of the instrument:

$$\langle r \rangle = \frac{I_{\parallel} - 2GI_{\perp}}{I_{\parallel} + 2GI_{\perp}} \tag{1}$$

where $I_{\parallel}$ and $I_{\perp}$ are the parallel and perpendicular emission intensities, respectively. The values of the G-factor (ratio of the sensitivities of the detection system for vertically [IHV] and horizontally [IHH] polarized light) were determined separately for each sample. Applying Eq. 2 the lipid-order parameter S was calculated from the anisotropy values using the following expression [8]

$$S = \frac{\left[1 - 2\left(\frac{r}{r_0}\right) + 5\left(\frac{r}{r_0}\right)^2\right]^{\frac{1}{2}} - 1 + \frac{r}{r_0}}{2\left(\frac{r}{r_0}\right)}, \tag{2}$$

where r_0 is the fluorescence anisotropy of DPH in the absence of any rotational motion of the probe. The theoretical value of r_0 for DPH is 0.4, while experimental values of r_0 lie between 0.362 and 0.394 [8]. In our calculation, the experimental value ofr_0 was 0.370 for DPH in SOPC at 5 °C.

2.4. Size distribution and stability measurements. The hydrodynamic diameter of the SOPC SUVs suspended in double-distilled water was measured using a Zetasizer Nano (Malvern, UK) dynamic light scattering (DLS) instrument with a standard 4 mW helium-neon laser (633 nm) at a 173° scattering angle and a digital autocorrelator. These measurements were performed at 25 °C in disposable glass micro cuvettes. Although the addition of cholesterol enhances the stability of liposomes in general, the phase in which the vesicles are stored and the duration of the storage of liposomes are essential criteria in the stability assessments. Since the phase transition temperature of SOPC lipid is 6 °C the prepared vesicles were stored at two different temperatures: below their phase transition temperature (at −20 °C) and above their phase transition temperature (at 20 °C) for a period of 10 days. Freshly prepared liposomes were used as the control and these were analyzed simultaneously using DLS.

3. Results and discussion. ***3.1. Membrane fluidity.*** Membrane fluidity is reciprocal to the viscosity of a real cell membrane or a liposomal membrane [9]. Maintaining the optimal membrane fluidity is essential for a cell, otherwise the variations in fluidity level will affect the permeability which leads to cell deformability. Cholesterol plays a dual role in maintaining the ideal fluidity level of cell membranes [10]. When the environmental temperature increases, cholesterol reduces the bilayer fluidity through the formation of strong hydrophobic interactions with the adjacent phospholipid tails to maintain them intact and thus reduces their mobility [11]. On the other hand, at low temperatures, cholesterol can prevent the cell from freezing [12]. Cholesterol molecules are located between the phospholipid tails in the membrane and act like spacers to segregate them. This sort of segregation is essential to prevent the hydrophobic tails of the membrane lipids from approaching too close to each other and crystallizing [4].

In general, the membrane lipids can exist in different phases such as gel phase, liquid crystalline phase, liquid phase, etc. When the environmental temperature is increased, they can change from the highly ordered gel phase to the less ordered liquid phase and vice-versa. Since, the fluorophores such as DPH and its derivatives are very sensitive to such phase changes and viscosity, they are widely used to study the alterations in membrane fluidity level. The DPH molecules are hydrophobic in nature and tend to be positioned between the hydrophobic tail regions throughout the bilayer. When there is a phase change in the membrane lipids, viscosity of the membrane is also changed which in turn can affect the rotation and free mobility of the probes within a lipid bilayer. Rotational diffusion changes the direction of the transition moments and the light emitted by the fluorophore has unequal intensities along different axes of polarization and this extent of polarization of the emission is measured in terms of anisotropy. The anisotropy values are directly proportional to the order parameter of the bilayer lipids and inversely proportional to membrane fluidity [13].

The order parameter results shown in Fig. 2 indicate that the gradual increase in the cholesterol content of liposomes increases the order parameter values and decreases the bilayer fluidity. For instance, the order parameter values were greater for the liposomes prepared with higher cholesterol concentrations (e.g., 50 mol %) than the control liposomes prepared without cholesterol. The anisotropy for control liposomes at 15 °C was 0.14 ± 0.01, whereas for the samples with 30 mol % and 50 mol % cholesterol the anisotropy was 0.23 ± 0.01 and 0.27 ± 0.01, respectively. The corresponding order parameters for the control and the liposomes prepared with 30 mol % and 50 mol % cholesterol were 0.44 ± 0.01, 0.70 ± 0.01 and 0.79 ± 0.01, respectively.

This data has shown that the increase in the cholesterol content (0–50 mol %) has caused a parallel decrease in the membrane fluidity level. Our result coincides well with the literature data. Numerous reports have shown that enhancing the cholesterol level in the membrane decreases the fluidity level and vice versa [10].

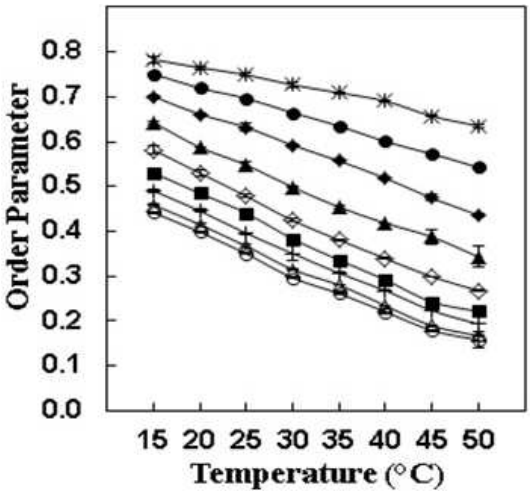

Fig. 2. Lipid order parameter of SOPC SUVs as a function of temperature with varying cholesterol concentrations (∗ 50 mol % cholesterol; • 40 mol % cholesterol; ♦ 30 mol % cholesterol; ▲ 20 mol % cholesterol; ◇ 15 mol % cholesterol; ■ 10 mol % cholesterol; + 6 mol % cholesterol; Δ 3 mol % cholesterol; ∘ Control (SOPC liposomes without cholesterol) measured by DPH fluorescence anisotropy measurements. Data are means ± SD ($n = 2$)

The 3β-hydroxyl group of cholesterol locates near the head groups of the bilayer lipids while the hydrophobic bulky steroid ring lies parallel to the phospholipid tails thereby restricting the free mobility of fatty acid chains in the membrane [14]. This intercalation of cholesterol between the fatty acid tails enhances the degree of membrane packing and acts as a hindrance to the free mobility of lipids in the bilayer, and thus reduces membrane fluidity.

3.2. Size distribution of liposomes. The DLS results showed a gradual increase in the hydrodynamic diameters of the SUVs with the increase in cholesterol content (Fig. 3). Literature reports also have shown that the addition of cholesterol increases the vesicle size [15, 16]. Indeed, the size of the liposomes increased proportionally with the cholesterol concentration, i.e. SUVs with the largest size corresponded to those vesicles which were prepared with the highest cholesterol content (50 mol %). Coldren et al. [17] reported that the size distribution of the liposomes is closely related to the spontaneous membrane curvature, the bending elasticity moduli and stability of the vesicles. When the size of the vesicle increases due to the increment of lumenal contents, the bilayer gets thinner and the area density of the inner lipid head declines much more significantly than that of the outer does. It is also known that the intercalation of cholesterol

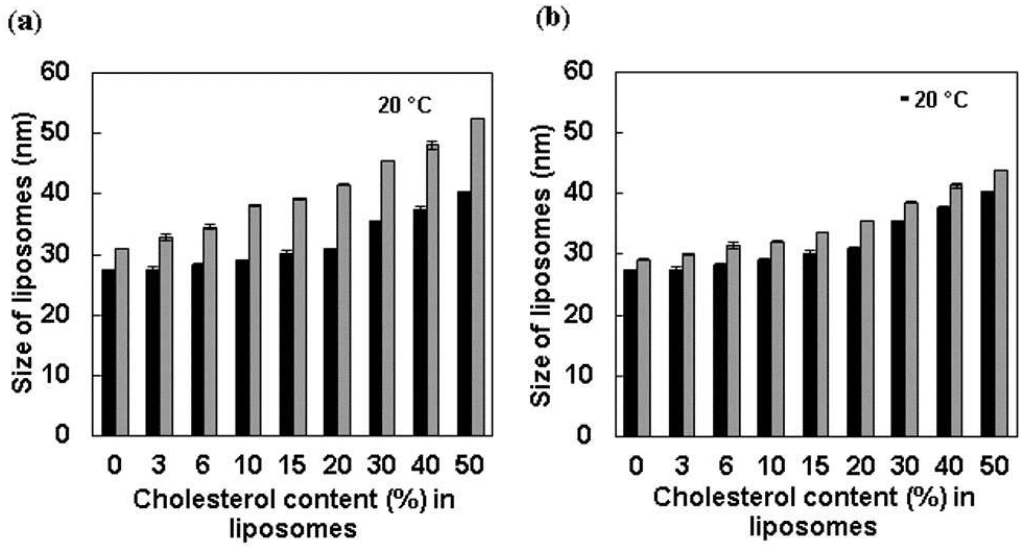

Fig. 3. Size distributions of SOPC SUVs with different mol% of cholesterol stored at (a) 20 °C and (b) −20 °C, as determined by DLS. ■ Size of liposomes measured after 1 h; ■ Size of liposomes measured after 10 days. Data are means ± SD ($n = 2$)

creates more inter-lipid space between the hydrophobic lipid chains and causes lateral membrane expansion which leads to increase in the vesicle size, decrease in the membrane thickness and increase in the bending elasticity.

The liposomes stored at two different temperatures 20 °C and −20 °C showed increase in vesicle size compared to the control liposomes prepared without cholesterol. During the storage period, the vesicles fused to form agglomerates, which led to an increase in the hydrodynamic diameter. Wong and Thompson [16] have reported that small unilamellar dipalmitoylphosphatidylcholine vesicles in the size range of 200 Å increased up to 700 Å and further, below their phase transition temperature due to aggregation and subsequent vesicle fusion. The vesicles stored in the liquid phase at 20 °C (Fig. 3a) were comparatively larger than those stored at the gel phase at −20 °C (Fig. 3b). This is consistent with the study of Saez et al. [15], where the fusion of dipalmitoylphosphatidylcholine SUVs was facilitated at higher temperatures in the high fluid state, which led to increased vesicle size. As the hydrodynamic diameter of the liposomes stored at −20 °C was slightly smaller compared to those stored at 20 °C, the percentage of agglomeration and fusion that occurred between the adjacent liposomes was less at lower temperatures, which indicates that the liposomes stored at −20 °C (gel phase) were more stable compared to the liposomes stored at 20 °C.

The addition of cholesterol also increased the stability of the vesicles, as it provides more rigidity to the membrane and improves the resistance of the vesicles against aggregation [18]. It is also known that the incorporation of cholesterol into

phosphatidylcholine liposomes enhanced the absolute zeta potential values and the electrostatic repulsion between the neighbouring vesicles and thus reduced their fusion. Presti et al. [19] and Genova et al. [20] reported that cholesterol improves the stability by forming hydrogen bonds with its hydroxyl group to the carbonyl groups of the membrane lipids. As a consequence of their interaction, the stiffness and cohesion of the membrane is increased.

4. Conclusions. The effects of varying the cholesterol concentration in small and giant SOPC unilamellar vesicles were analyzed to determine the cholesterol-induced variations in vesicle size, bilayer fluidity, and elastic properties of the liposomal membranes. The fluorescence anisotropy measurements of DPH have shown that increasing the cholesterol content in liposomes increased the anisotropy and order parameters, whereas the bilayer fluidity was decreased. The DLS data have shown that the vesicle size increased steadily with increasing the cholesterol concentration in the liposomes. The size of the liposomes stored at temperatures above their phase transition temperature (at 20 °C) were larger than those stored at temperatures below their phase transition temperature (at −20 °C), which can be attributed to reduced vesicle fusion, indicating that the SOPC liposomes stored in the gel phase are more stable than those stored in the liquid phase.

As cholesterol has been incorporated into various liposome formulations to enhance their stability, especially for pharmaceutical and in vivo applications, it becomes essential to validate the appropriate percentages of cholesterol to be added for the most beneficial effects. The data obtained in the present study provide information about the role of varying cholesterol content on these parameters. Further studies regarding the effects of different cholesterol concentrations on other important parameters, such as membrane permeability and phase behaviour are underway to provide more knowledge about the influence of cholesterol on membranes.

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