Phospholipid-Cholesterol Bilayers under Osmotic Stress

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ABSTRACT Isothermal (27°C) phase behavior of dimyristoyl phosphatidyl choline-cholesterol mixtures at various osmotic pressures and cholesterol contents was investigated by means of isothermal sorption microcalorimetry and ^{2}H-nuclear magnetic resonance. The calorimetric method allows for simultaneous measurement of the partial molar enthalpy and the chemical potential (the osmotic pressure) of water, thus providing an almost complete thermodynamic description of the sorption process. From the experimental results, the $\Pi_{osm} - X_{chol}$ and the ternary composition phase diagrams are constructed. We note that there are strong similarities between the $\Pi_{osm} - X_{chol}$ phase diagram and the previously reported $T - X_{chol}$ phase diagram at excess water. At high cholesterol contents a single liquid ordered ($L_\alpha(o)$) phase is present over the whole range of water contents, implying that this phase has a remarkable stability not only at decreasing temperature but also at increasing osmotic pressure. At low cholesterol contents, the microcalorimetric experiments confirm the extraordinary property of cholesterol not to cause any substantial melting point depression. One important conclusion in the present study is that the P_β phase can dissolve cholesterol more readily than the L_β phase and that the addition of cholesterol induces the P_β phase. Finally, the putative $P_\beta - L_\alpha(o)$ periodic modulated structure is discussed.

INTRODUCTION

Cholesterol is a major constituent of many biological membranes. It comprises a lipid fraction of ~30% in, for example, the plasma membrane of eukaryotic cells (Yeagle, 1985) and the lipid matrix of stratum corneum, the outer part of human skin (Wertz et al., 1992). There is an intriguing interplay between the biological function of cholesterol and the physical-chemical properties of the membrane. This is reflected by the fact that cholesterol has a profound effect on the thermodynamic and mechanical properties of the lipid bilayers, thus influencing the membrane stability and the barrier properties (Yeagle, 1985; Bloom et al., 1991; Demel and de Kruyff, 1976). Cholesterol is also associated with some specific biological functions. Recently, the so-called lipid raft model has been proposed. It describes small size cholesterol-sphingolipid domains as membrane lipid rafts, which can serve as platforms for lipid and protein transport or as relay stations in intracellular signaling (Simons and Ikonen, 1997). The lipid rafts are closely related to the lipid domains around the protein caveolin, referred to as caveloae (Marx, 2001). It has further been demonstrated that the chemical potential of cholesterol in phospholipid bilayers depends on the lipid composition in a strongly nonideal way (Radhakrishnan et al., 2000). This is a consequence of the complex cholesterol-phospholipid intermolecular interactions, and it can be seen as a regulating mechanism, allowing for large variations in cholesterol content in adjacent compartments of the eukaryotic cells even though there is an equilibrium with respect to transfer of cholesterol. In other words, a small concentration difference in another lipid or protein component can result in a large difference in cholesterol content.

The effect of cholesterol on lipid bilayers has been extensively studied (Yeagle, 1985; Bloom et al., 1991). The majority of the studies undertaken to elucidate the effects of cholesterol on lipid morphology in bulk have been performed on systems containing phospholipids due to their frequent occurrence in biological membranes. Cholesterol has been referred to as a "crystal breaker" as it disturbs the translational order of the phospholipid molecules in the crystalline (gel) state (Vist and Davis, 1990; Ipsen et al., 1987). Cholesterol also causes a straightening of the disordered phospholipid acyl chains in liquid-like phases and reduces the mean headgroup area (Vist and Davis, 1990; Lafleur et al., 1990). This property is often referred to as the stabilizing effect of cholesterol.

The phase behavior in model systems of saturated phospholipids and cholesterol has been widely studied (Vist and Davis, 1990; Shimshick and McConnell, 1973; Ipsen et al., 1987; Anderson and McConnell, 2000; Nielsen et al., 1999). It has been demonstrated that phosphatidyle choline (PC)-cholesterol phase diagrams have a universal form with the main difference of translations along the temperature axis when varying the acyl-chain lengths (Thewalt and Bloom, 1992). Fig. 1 shows the $T - X_{chol}$ phase diagram of dimyristoyl phosphatidyl choline (DMPC) and cholesterol in excess water (Almeida et al., 1992). The phase diagram shows several remarkable features, indicating very specific PC-cholesterol interactions (Ipsen et al., 1987). In excess water pure DMPC goes through a phase transition from gel to liquid crystalline state at $T_m = 23.5$°C. Cholesterol is to some extent soluble in phospholipids in the gel state. This is an unusual property for a solid, which is normally not a

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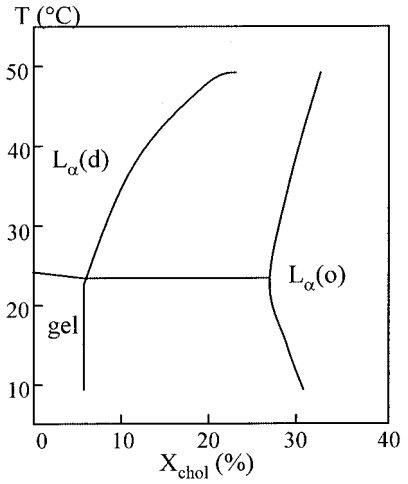

FIGURE 1 $T - X_{chol}$ phase diagram for DMPC-cholesterol in excess water. Phase diagram adapted from Almeida et al. (1992). The gel phase has been characterized as a P_β gel phase (Mortensen et al., 1988).

good solvent. In fact, the solubility of cholesterol is almost as high in the gel phase as in the liquid crystalline ($L_\alpha(d)$) phase, resulting in only very marginal melting-point depression. The notation, $L_\alpha(d)$, liquid disordered, refers to the common lamellar liquid crystalline phase observed above the chain melting temperature, which is generally called the L_α phase. The gel has been characterized as a rippled P_β gel phase (Mortensen et al., 1988).

At high cholesterol contents the system behaves like a liquid over the whole range of temperatures, and in contrast to the low concentration behavior, the cholesterol strongly favors the liquid phase over the solid phase at these compositions. The liquid phase at high cholesterol concentrations has been denoted a liquid ordered phase ($L_\alpha(o)$), motivated by the fact that several independent studies have demonstrated a high degree of the acyl-chain order in this lamellar liquid crystalline phase. Two two-phase regions are also present in the phase diagram in Fig. 1. Above T_m, two liquid phases, $L_\alpha(o)$ and $L_\alpha(d)$, coexist, and below T_m the $L_\alpha(o)$ and gel phases coexist. Furthermore, a narrow coexistence region ending in a eutectic point separating the gel and $L_\alpha(d)$ phases is expected on thermodynamic grounds.

In the majority of experimental work, phospholipid-cholesterol phase equilibria is studied in relation to variations in temperature. However, for many biological applications the chemical potential of water (osmotic pressure) is an equally relevant intensive variable as temperature for studying the relation between molecular interactions and phase behavior. The osmotic forces are important in regulating a number of biological membrane processes. Membrane fusion processes can be induced by creating a local osmotic stress (Hui et al., 1999). The effect of osmotic pressure on lipid phase behavior is also of uttermost importance in the case of human skin, due to the large difference in water chemical potential across the skin (Sparr and Wennerström, 2001).

Phase behavior of pure phospholipid-water binary systems have previously been studied both experimentally and theoretically (Guldbrand et al., 1982; Ulmius et al., 1977; Gabriella-Madelmont and Perron, 1983). In a few cases, $T - \Pi_{osm}$ phase diagrams for phospholipid-water systems have been established (Smith et al., 1990; Markova et al., 2000). One of the outcomes of these studies is that a first order phase transformations from a gel to a liquid crystalline phase can be induced by a decrease in the osmotic pressure analogous to the transition induced by an increase of temperature in excess water. The response in phospholipid-cholesterol equilibria to changes in osmotic pressure has received much less attention, and no experimental phase diagrams of this kind can be found in the literature. Faure et al. (1997) presented a partial phase diagram for DMPC-cholesterol mixtures at varying water contents, but the authors made no connections to the intensive variable of the osmotic pressure.

In this paper, we investigate the phase behavior of DMPC-cholesterol mixtures at various osmotic pressures and cholesterol contents. An isothermal sorption microcalorimeter was used to monitor lipid hydration. This calorimeter allows for simultaneous measurement of the partial molar enthalpy and the chemical potential (the partial molar free energy or osmotic pressure) of the water (Wadsö and Markova, 2000). As a support to the calorimetric results the ^{2}H-nuclear magnetic resonance (NMR) quadrupolar splitting of heavy water is studied at various water contents. From the experimental results the $\Pi_{osm} - X_{chol}$ phase diagram is constructed. A ternary phase diagram based on molar compositions is also outlined.

MATERIALS AND METHODS

DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphatadylcholine (98% pure, molecular weight = 678 g/mol) and cholesterol (molecular weight = 386.66 g/mol) were obtained from Larodan Fine Chemicals (Malmö, Sweden). DSC measurements showed no evidences of impurities. The Millipore water used was deionized, distilled, and filtrated through Millipore Q Purification System (Millipore Corporation, Bedford, MA). Samples of cholesterol and DMPC at different compositions were dissolved in 2:1 chloroform:methanol. The mixtures were heated for 10 min at 40°C, vortexed for 2 min, and then dried in vacuum at room temperature. The samples were thereafter dried in a vacuum pistol with a few drops of water to remove traces of the solvent. All samples were used directly after drying or annealed by prehydration and subsequent drying. According to Nilsson et al. (1991) and McIntosh et al. (1987) such a careful drying procedure is sufficient to remove all water from the lipid sample. The fact that the lipids were stored at −4°C before drying further insures the dry state of the lipids (Cevc and Marsh, 1987). However, other authors claim that one or maximal two water molecules per lipid are so strongly associated to the lipid headgroups that they are very difficult to remove in practice (Small, 1986). We report compositions based on the assumption that there is no water in the maximally dried sample (see below).

A novel double twin isothermal microcalorimeter was used to study the water vapor sorption of the phospholipids. A detailed description of the instrument is presented elsewhere (Wadsö and Markova, 2000). The calorimetric cell consists of two vessels connected by a steel tube. At the start of the measurements the bottom vessel contains 40 to 100 mg of a dry