Chemistry and Physics of Lipids 199 (2016) 3–10

Contents lists available at ScienceDirect

Chemistry and Physics of Lipids

journal homepage: www.elsevier.com/locate/chemphyslip

In vitro determination of the solubility limit of cholesterol in phospholipid bilayers

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ARTICLE INFO

Article history:
Available online 21 July 2016

Keywords:
Cholesterol
Cholesterol crystals

ABSTRACT

Cholesterol has limited solubility in phospholipid bilayers. The solubility limit is strongly dependent on the nature of the lipid with which the cholesterol is mixed while properties of the crystals formed can be modified by phospholipid-cholesterol interactions. In this review we summarize the various methods that have been developed to prepare hydrated mixtures of cholesterol and phospholipid. We point out some of the factors that determine the form adopted when cholesterol crystallizes in such mixtures, i.e. two- or three-dimensional, monohydrate or anhydrous. These differences can greatly affect the ability to experimentally detect the presence of these crystals in a membrane. Several methods for detecting cholesterol crystals are discussed and compared including DSC, X-ray and GIXRD diffraction methods, NMR and EPR spectroscopy. The importance of the history of the sample in determining the amount and nature of the cholesterol crystals formed is emphasized.

1. Biological relevance of cholesterol crystals

Biological membranes are comprised of a large number of molecular species of lipids and proteins, as well as covalently-linked carbohydrates, asymmetrically distributed across the bilayer. In order to understand the behavior of individual membrane components, it is helpful to study *in vitro* model systems that are comprised of a limited number of molecular species. A lipid component of particular interest for mammalian plasma membranes is cholesterol. Cholesterol is one of the most abundant lipid species in this membrane. It has been associated with domain formation required for signal transduction as well as for modulating the physical properties of the membrane. In addition to its role as a major lipid component of the bilayer, cholesterol has a marked tendency to form crystallites. It has only limited solubility in membranes and at higher molar fractions cholesterol will form crystals.

Cholesterol crystals are generally found *in vivo* in pathological conditions. The presence of cholesterol crystallites in atherosclerotic plaques is well known to pathologists (Nasiri et al., 2015; Kataoka et al., 2015; Varsano et al., 2015). In addition to being present in atherosclerotic plaques, cholesterol crystals activate innate immunity (Kiyotake et al., 2015) and play an important role in inflammation (Samstad et al., 2014; Grebe and Latz, 2013) that is also associated with heart disease. Although earlier studies suggested the involvement of cholesterol crystals in the formation of cataracts in the human lens, more recent studies suggest that the opposite is true and that a lens with cataracts has less cholesterol (Mainali et al., 2015). However, cholesterol crystals within the eye have been associated with severe pathologies (Khan and Parulekar, 2014). There is also evidence that cholesterol crystals may have a role in age-related macular degeneration (Pang et al., 2015). The formation of gall stones is another pathology that is triggered by the formation of cholesterol crystals that occurs when the concentration of cholesterol exceeds its limit of solubility in bile (deBari et al., 2015; Van Erpecum, 2011; Venneman and Van Erpecum, 2010).

The conditions required for the formation of cholesterol crystals in disease processes are of course related to the question of how much cholesterol can dissolve in a membrane with a particular lipid composition. Additionally, interaction of cholesterol with phospholipids will affect the activity of cholesterol and determine

Abbreviations: CP, cross polarization; DMPS, dimyristoyl phosphatidylserine; DSC, differential scanning calorimetry; EPR, electron paramagnetic resonance; GIXRD, grazing incidence X-ray diffraction; ULVs, unilamellar vesicles; MAS, magic angle spinning; POPC, 1-palmitoyl-2-oleoyl phosphatidylcholine; POPS, 1-palmitoyl-2-oleoyl phosphatidylserine; SAXS, small angle X-ray scattering; SOPS, 1-stearoyl-2-oleoyl phosphatidylserine; T1, spin-lattice relaxation time.

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http://dx.doi.org/10.1016/j.chemphyslip.2016.06.006
0009-3084/

its partitioning between the plasma membrane and intracellular membranes as well as the availability of cholesterol to interact with membrane proteins. This information can be most readily obtained by investigating the behavior of model membrane systems. However, since lipids are insoluble in water, it is important to consider how two or more lipids can be mixed to increase the likelihood that the mixture will be close to its equilibrium state when hydrated. We will first review the methods of sample preparation to achieve this goal.

2. Sample preparation

2.1. Cholesterol/phospholipid multilamellar vesicles: deposition from organic solvents followed by hydration

Cholesterol and phospholipids are insoluble in water. In order to obtain mixtures of these lipids for *in vitro* studies, the most common procedure has been as follows. The compounds are first dissolved in organic solvents and mixed at the appropriate ratios; the solvents are evaporated with a stream of dry nitrogen and the resulting lipid film is then hydrated with water or buffered salt solution. The question of which solvents to use and how to hydrate the lipids without obtaining either artifactually high or low cholesterol miscibility, was already discussed by Freeman and Finean 40 years ago (Freeman and Finean, 1975). These authors prepared egg phosphatidylcholine–cholesterol mixtures dissolved in ethanol or in chloroform, and then studied either the dry or hydrated forms. Phase separated cholesterol, if present as crystallites, could be detected by small angle X-ray diffraction. Depending on: (i) the ratio of cholesterol to phosphatidylcholine; (ii) the solvent used for lipid solubilization; (iii) the hydration state of the sample; and (iv) the time of incubation, either a single phase or a biphasic mixture containing cholesterol crystallites was obtained. Their results are summarized in Table 1 (Freeman and Finean, 1975). The authors suggested that in samples prepared from chloroform, metastable, cholesterol/phospholipid aggregates are present, resulting in an overestimation of cholesterol solubility in the phospholipid investigated. These metastable aggregates disassemble with time and/or hydration, accompanied by the appearance of cholesterol crystallites. Ladbrooke et al. (1968) prepared mixtures of dipalmitoyl phosphatidylcholine-cholesterol in chloroform, but eliminated the long lived, metastable phases with extensive hydration. The true solubility limit of cholesterol in egg phosphatidylcholine and dipalmitoyl phosphatidylcholine was concluded to be molar ratio 1:1 (Freeman and Finean, 1975; Ladbrooke et al., 1968). Since that early work, researchers have used various organic solvents and protocols for preparing phospholipid:cholesterol mixtures. For example, in their investigation of mixtures of phosphatidylcholine with cholesterol or cholesterol analogs (McMullen et al., 1994) solubilized the lipids in either chloroform, a mixture of chloroform:methanol 2:1 v/v, choloroform:methanol 1:2 v/v, or methanol. They did not find significant differences in the thermotropic or spectroscopic properties of the mixtures. The same authors noted that in order to obtain homogeneous samples of saturated phosphatidylserine/cholesterol mixtures, it was necessary to heat to 40–50 °C during evaporation of the solvents with a stream of dry nitrogen, followed by 18 h under vacuum (McMullen et al., 2000). The reason for heating during evaporation was that at ambient temperature, cholesterol crystals formed, which could not be redissolved upon hydration with heating. The lipids were then hydrated in NaCl solution buffered with either Tris or phosphate buffer during heating to 10–20 °C above the phase transition with vigorous vortexing to produce multi-lamellar samples. Historically, the most commonly used solvent mixture has been chloroform: methanol 2:1 v/v, although there are examples of the use of benzene as well as mixtures with chloroform and 2,2,2,-trifluoroethanol (Barrett et al., 2013). In our own research on the solubility limit of cholesterol in phospholipid multilamellar vesicles (beginning with (Bach, 1984)), we generally dissolve the lipids in chloroform:methanol 2:1 v/v, mix at the appropriate ratios, evaporate the solvents with dry nitrogen at ambient temperature, hydrate the solid with NaCl in buffer while incubating for 0.5 h to temperatures >60 °C with frequent vortexing. Although in some phospholipid mixtures (e.g. DMPS or POPS) with mol fraction cholesterol below the solubility limit, there is evidence for the presence of cholesterol crystals in the dry state, they then disappear upon proper hydration of the mixture (Fig. 1). The cholesterol solubility limits that we have measured for material prepared in this way have been consistent and reproducible over many years, displaying marked dependence on the particular phospholipid used. For example, mixtures with POPS display different solubility of cholesterol as compared with SOPS (Bach et al., 1992), even though there is little structural difference between these two phospholipids and both have similar phase transition temperatures (14 °C, 18 °C). At cholesterol molar fraction 0.5 in POPC, no cholesterol crystals are formed, while with POPS, 80% of the cholesterol is in the form of crystals (Epand et al., 2002). Such a dramatic difference would not be expected if the formation of cholesterol crystals were a result of solvent evaporation. Nevertheless, alternative protocols, which did not include a dry state, have been considered (see below).

Table 1
Occurrence of a cholesterol phase in dry and hydrated samples of cholesterol/lecithin mixtures prepared from either chloroform or ethanol solutions at 1 day and at 14 days. C indicates cholesterol observable by diffraction. Lecithin is the name formally used for phosphatidylcholine. Taken from (Freeman and Finean, 1975) with permission.

Solvent		Chloroform			Ethanol		
Cholesterol/Lecithin Ratio		1:1	3:2	2:1	1:1	3:2	2:1
1 day	Dry				C	C	C
	Hydrated			C	C	C	C
14 days	Dry	C	C	C	C	C	C
	Hydrated	C	C	C	C	C	C

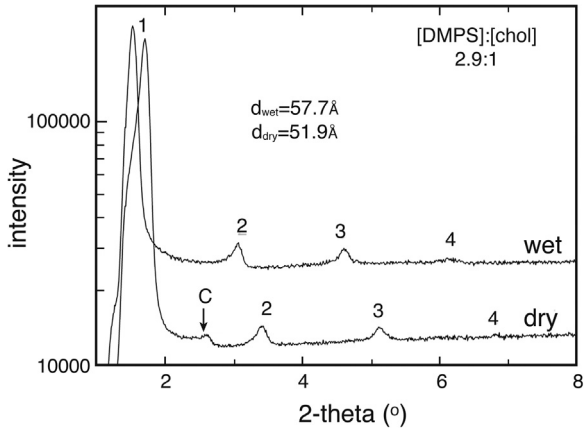

Fig. 1. SAXS patterns of mixtures of DMPS with cholesterol at molar ratio 2.9:1, dried from chloroform:methanol 2:1 v/v (lower trace), and following hydration with NaCl in buffer while incubating for 0.5 h to temperature >60 °C with frequent vortexing (upper trace). The numbers indicate the diffraction orders of the phospholipid and C indicates the 34 Å peak of cholesterol crystals. 2-theta is the scattering angle: $d^{-1} = 2\sin(\text{theta})/\lambda$, where λ is the X-ray wavelength..