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Experimental determination and computational interpretation of biophysical properties of lipid bilayers enriched by cholesteryl hemisuccinate

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ABSTRACT

Cholesteryl hemisuccinate (CHS) is one of the cholesterol-mimicking detergents not observed in nature. It is, however, widely used in protein crystallography, in biochemical studies of proteins, and in pharmacology. Here, we performed an extensive experimental and theoretical study on the behavior of CHS in lipid membranes rich in unsaturated phospholipids. We found that the deprotonated form of CHS (that is the predominant form under physiological conditions) does not mimic cholesterol very well. The protonated form of CHS does better in this regard, but also its ability to mimic the physical effects of cholesterol on lipid membranes is limited. Overall, although ordering and condensing effects characteristic to cholesterol are present in systems containing any form of CHS, their strength is appreciably weaker compared to cholesterol. Based on the considerable amount of experimental and atomistic simulation data, we conclude that these differences originate from the fact that the ester group of CHS does not anchor it in an optimal position at the water–membrane interface. The implications of these findings for considerations of protein–cholesterol interactions are briefly discussed.
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1. Introduction

Biological membranes are essential parts of living cells. They provide a highly selective permeable barrier for cells, in which the interactions between lipids and proteins allow fundamental processes such as photosynthesis, signal transduction, perspiration, and transport of ions and nonpolar molecules to take place. In essence, biological membranes are comprised of a lipid bilayer that acts as a host to numerous membrane proteins embedded in the bilayer. The specific and usually strong interactions between lipids and proteins play an important role in maintaining the structure and function of membrane proteins; protein crystal structures thus often contain lipids or their fragments co-crystallized together with the protein. In many cases [1–6], cholesterol (CHOL) has been resolved as a part of the protein crystal structure. Such proteins are thereby known as cholesterol-binding and it is assumed that cholesterol has an important effect on their function, such as an ability to promote thermal stability or to induce conformational changes in protein structure. In many cases, however, the cholesterol-type molecules involved in crystal structures are actually not cholesterol but cholesteryl hemisuccinate [7–9]. Cholesteryl hemisuccinate (CHS) is one of the cholesterol-mimicking detergents that is not present in nature, but is widely used in protein crystallography, in biochemical studies of proteins, and in pharmacology. CHS is more soluble in water than cholesterol and, therefore, easier to use in biochemical studies of proteins. It is commercially available and shares an identical structure with CHOL in the nonpolar part of the membrane where many of the key effects of CHOL originate. Therefore, it is understandable that CHS is commonly employed to replace CHOL in studies of the effects of membrane composition on structure and stability of various proteins (especially G-protein coupled receptors) [7,10–14].

CHOL is known to increase the mechanical strength of lipid membranes and decrease membrane permeability to water, small molecules, and ions [15]. Due to these properties CHOL has applications in drug delivery where it is widely employed in the production of liposomes [16]. For this reason, CHS has been tested as a potential component of drug delivery systems. CHS can form pH-sensitive liposomes [17,18] designed to undergo rapid destabilization in an acidic environment [19]. Such a condition occurs after cellular uptake of liposomes in endocytic vesicles. pH-sensitive liposomes have been shown to be more efficient in delivering their contents to the cells than traditional

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ones [20]. CHS may also be used in combination with polymers to coat liposomes to form so-called stealth-pH sensitive liposomes, and it is used in gene delivery where together with other lipids it coats the so-called polyplex-complex of DNA with polymers [21].

For reasons listed above, it is important to know how well CHS can mimic the behavior of CHOL in lipid bilayers. CHOL and ergosterol are the most common sterols in nature, yet various other naturally occurring and synthetic sterols exist, too. The key effects that CHOL has on lipids are the so-called ordering effect (increase of acyl chain order in bilayers with CHOL) and the condensing effect (decrease of surface area per lipid) [22,23]. Numerous studies have shown that even small modifications in CHOL structure modify both effects, typically decreasing their magnitude [24–28]. This also concerns modifications of the polar part of cholesterol. For instance, the change of conformation of the hydroxyl group from beta to alpha [29] or esterification [30] with sulfuric acid has been shown to decrease cholesterol's effects on membrane properties. What is more, even seemingly tiny changes in cholesterol structure, such as a change of an individual double bond to a single one, have been shown to change the function of cholesterol, e.g., replacing cholesterol with desmosterol in raft membranes was reported to impair the function of the insulin receptor [31].

Biophysical studies on the effects of CHS are rather limited. CHS has been shown to act as a membrane stabilizer in the preparation of liposomes [32,33] and it has been found to alter the motion of acyl chains and the fluidity of cell membranes [34,35]. Experimental studies comparing the properties of CHS- and CHOL-containing bilayers have shown that CHS affects their properties less than CHOL [36]. Our recent atomistic molecular dynamics (MD) simulations on the behavior of CHS in saturated lipid bilayers [37] indicated that the protonated version of cholesteryl hemisuccinate (CHS_{prot}) mimics many of the membrane properties of cholesterol quite well, while the deprotonated version (CHS_{deprot}) is less appropriate for this purpose.

Here, we apply time-resolved fluorescence spectroscopy and dynamic light scattering experiments, assisted by atomistic molecular dynamics simulations to study the effects of CHS vs. CHOL on unsaturated lipid bilayers. Our main objective is to clarify how well CHS is able to mimic the physical behavior of cholesterol in membranes rich in unsaturated phospholipids. In this context, it is important to note that in unsaturated lipid membranes under physiological pH, about 98% of cholesteryl hemisuccinate is in its deprotonated state (CHS_{deprot}), since its pKa is about 5.8 [17]. Therefore, given that the experiments reported in this work were carried out at pH equal to 7.4, their results correspond to the deprotonated CHS (CHS_{deprot}). In MD simulations, we yet considered both states (CHS_{prot}, CHS_{deprot}) in order to explore the dependence of CHS behavior on protonation. Building on our previous computational evidence from saturated bilayers [37], we can expect properties of membranes with cholesteryl hemisuccinate (in both protonated and deprotonated forms) to differ from those containing cholesterol. Indeed, the present experiments and simulations demonstrate that, compared to cholesterol, cholesterol hemisuccinate has a smaller condensing effect on the lipid bilayer, making the bilayer less ordered and restricting less the lipid mobility at the glycerol level. This may alter the strength and dynamics of protein–lipid interactions, indicating that cholesteryl hemisuccinate may not be an ideal cholesterol-mimicking detergent for sterol–protein co-crystallization.

2. Materials and methods

2.1. Chemicals and liposome preparation

POPC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine) and CHOL (ovine wool cholesterol) were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). CHS (cholesteryl hemisuccinate), POPOP (2,2′-(1,4-phenylene)bis[5-phenyl-oxazole]), NaCl, and NaOH were obtained from Sigma-Aldrich (St. Louis, MO, USA). Fluorescent probes: DPH (1,6-diphenyl-1,3,5-hexatriene), and Laurdan (6-lauroyl-2-dimethylaminonaphthalene) were purchased from Invitrogen (Eugene, OR, USA). Hepes (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer (Fluka, Buchs, Switzerland) was dissolved in Mili Q water (Milipore, USA). Organic solvents of spectroscopic grade were supplied by Merck (Darmstadt, Germany). All chemicals were used without further purification.

Extruded large unilamellar vesicles (LUVs) were prepared as follows: The appropriate volumes of chloroform solutions of POPC, and either CHS or CHOL, were mixed with methanol solution of a fluorescent probe. Final molar ratio of lipids to probe was 100:1. The organic solvents were evaporated under a stream of nitrogen and then under vacuum overnight. The dried lipid film was suspended in Hepes buffer: 10 mM, pH 7.4 (NaOH), 150 mM NaCl. After 4 min of continuous vortexing the suspension of multilamellar vesicles was extruded through polycarbonate membranes with a nominal pore diameter of 100 nm (Avestin, Ottawa, Canada).

2.2. Dynamic light scattering

The samples were transferred to UV grade poly(methyl methacrylate) cuvettes (Kartell, Noviglio, Italy), and equilibrated at 298 K for 3 min before each measurement. The light scattering setup of Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, UK) consisted of a He–Ne laser (532 nm) and an avalanche photodiode detector (APD). The scattering intensity was collected at the angle of 173°. Intensity-weighted size distributions were obtained using regularized fitting implemented in Zetasizer Software 6.2 (Malvern Instruments Ltd., Worcestershire, UK).

2.3. Fluorescence instrumentation

All fluorescence measurements were performed in 1 cm quartz cuvettes. Temperature was maintained at 310 ± 0.5 K using a water-circulating thermostat. Samples were equilibrated for 10 min before each measurement. Steady-state fluorescence spectra were collected using a Fluorolog-3 spectrofluorimeter (model FL3-11, JobinYvon Inc., Edison, NJ, USA). Fluorescence decays were recorded on a time-correlated single-photon counting (TCSPC) spectrometer: model 5000 U SPC equipped with a NanoLED 11 laser diode (370 nm peak wavelength, 80 ps pulse width, 1 MHz repetition rate) and a cooled Hamamatsu R3809U-50 microchannel plate photomultiplier (IBH, Glasgow, UK). The emission wavelengths were chosen using monochromators. 399 nm cutoff filter was used to eliminate scattered light. The signal level was kept below 2% of the light source repetition rate (1 MHz). Data was collected in 8192 channels (0.014 ns channel width) until the peak value reached 5000 counts. The full width at half maximum (FWHM) of the instrument response function was 85 ps.

2.4. Laurdan fluorescence (GP and TDFS)

Laurdan-labeled LUVs dispersion with 1 mM total lipid concentration was investigated. Steady-state emission spectrum (EX = 373 nm) and two excitation spectra (EM = 440, and 490 nm) were recorded. The excitation spectra were used to calculate excitation generalized polarization spectra (GP_{EX}) [38]:

$$GP_{EX}(\lambda_{EX}) = \frac{I_{440} - I_{490}}{I_{440} + I_{490}} \quad (1)$$

where I_{440} and I_{490} represent fluorescence intensities emitted at 440 nm and 490 nm, respectively (excited at excitation wavelength λ_{EX}).

For time-dependent fluorescence shift method (TDFS), fluorescence emission decays were measured at a series of emission wavelengths (400–540 nm with a 10 nm step). The decays were fitted in DAS6 software (IBH, Glasgow, UK) with multi-exponential function using reconvolution method. The fitted fluorescence decays together with

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