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Cholesteryl Hemisuccinate Is Not a Good Replacement for Cholesterol in Lipid Nanodiscs

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Abstract

Nanodiscs are suitable tool for studies of membrane proteins (MPs) due to their ability to mimic native biological membranes and several MP structures are solved in nanodiscs. Among the various cell membrane components, cholesterol (CHL) is known to regulate protein function and their concentration can reach up to 50 mol %. However, studies comprising cholesterol is challenging due to its hydrophobic nature, hence, nanodiscs with only low cholesterol concentration has been studied. To overcome the problem, cholesterol analogs with high solubility in polar solutions are often used and one of them is cholesteryl hemisuccinate (CHS). Nevertheless, in molecular dynamics (MD) simulation, this is not an obstacle. In this study, we performed MD simulations of nanodiscs containing neutral phosphatidylcholine (POPC) lipids, negatively charged phosphatidylglycerol (POPG) lipids, CHL, or negatively charged cholesterol analog, CHS. Our simulations show that CHS increases the order of lipids in nanodiscs, the effect is however weaker than CHL, and even smaller in nanodiscs. Furthermore, CHS gathered around scaffold proteins while cholesterol was uniformly distributed in the nanodiscs. Thus, nanodiscs with CHS are heterogeneous and not equivalent to nanodiscs with CHL. Finally, we also observed the increased concentration of POPG near the scaffold proteins, driven by electrostatic interactions. The MD results are experimentally validated using electron paramagnetic resonance spectroscopy. These results show that nanodiscs are in fact, complex structures not easily comparable with planar lipid bilayers.

Introduction

Nanodiscs are nanoscale macromolecules, composed of lipid bilayer wrapped with two membrane scaffold proteins (MSPs). Nanodiscs are engineered based on apolipoprotein ApoA I, a component of high-density lipoprotein.¹⁻³ Primary reason for the development of nanodiscs are challenges related to studies of membrane proteins (MPs),⁴ which require hydrophobic environment naturally ensured by lipids. Use of nanodiscs as a platform to study MPs seems to be an excellent alternative to commonly used micelles, bicelles, and liposomes,⁵ since all of them have some disadvantages.⁶ Micelles do not have the bilayer structure, bicelles have bilayer like arrangement, but they require high concentration of lipids and detergents. Liposomes are the most suitable systems in structural investigations; however, they are large, unstable, and challenging to prepare.

Nanodisc technology is a flexible method allowing modification of properties of nanodiscs and optimizing them for the desired application. For instance, by changing the length of MSPs, it is possible to obtain nanodiscs of a precisely defined size in the range from 6 nm to 16 nm and well-defined number of lipids.^{2,7,8} Moreover, the lipid composition of nanodisc can be altered to increase the nanodisc stability.⁹ Lipid composition is of particular importance for MPs as they are inseparable in the lipid environment and can affect the structure, activity, and dynamics of MPs. In this respect, cholesterol (CHL) is of particular importance in animal membranes.^{10,11} CHL occurs in large quantities in cell membranes with concentration reaching about 50 mol%, but in intracellular membranes e.g., in endoplasmic reticulum the concentration is only about 10 mol%.^{12,13} CHL regulates not only the physicochemical properties of lipid bilayers like bilayer thickness, rigidity, polarity, and permeability but also known to be a direct allosteric regulator of MPs.¹⁴⁻¹⁸ Nevertheless, due to its highly hydrophobic nature, it is often not possible to use CHL in biochemical studies.¹⁹ For this reason, nanodiscs with only low CHL concentrations have been successfully fabricated.^{20,21} As an alternative, CHL structural analogs with high solubility in polar solutions are often used and one of them is cholesteryl hemisuccinate (CHS).¹⁹

CHS is an ester of cholesterol and succinic acid, and due to the presence of the carboxylic group, it has characteristics of typical detergent (Figure 1). Of late, CHS has been frequently added detergent in the crystallization of MPs, particularly GPCRs.²²⁻²⁴ Thus, the presence of

CHS in 12 MP structures is not surprising.²⁵ The position occupied by CHS in these protein structures is believed to be the same for CHL in natural bilayers. CHS is also used to stabilize MPs structure in biochemical experiments utilizing micelles or bicelles.^{23,26–30} Since CHL and CHS share the same hydrophobic section of the molecule, these sterols are considered to be equivalent in biochemical studies. This assumption is however questionable based on the biophysical studies of sterol induced changes of lipid bilayer properties.¹⁴ The studies showed that even small structural modifications of native cholesterol structure decrease the ability of the sterol to modify lipid bilayer properties.¹⁴ CHS is no exception here, as most sterols it increases the order of the lipid acyl tails and stabilizes the bilayer but these effects are weaker when compared to CHL.^{31,32} The CHS carboxylic group has pKa of 5.8; thus approximately 98% of the CHS present at the physiological pH 7 will be present in the anionic form.³³ Consequently, the presence of CHS causes an effective change of the surface charge of the bilayer.

Figure 1. Figure showing (a) chemical structures of lipid molecules and (b) initial configuration of POPC/POPG/CHL nanodiscs. MSP1D1 is rendered as gray cartoon, CHL as red licorice, phosphorous atoms of POPC and POPG are rendered as VDW spheres and colored pink and

purple, respectively.

The main objective of this study is to compare the effects of CHS and CHL on the properties of lipid bilayer in nanodiscs which we do by combining molecular dynamics (MD) simulations and electron paramagnetic resonance (EPR) spectroscopy. In addition, to compare sterol effects on nanodiscs with more conventional membrane models, lipid bilayer and liposomes were used in MD simulations and EPR measurements, respectively.

MATERIALS AND Methods

Molecular Dynamics Simulations

CHARMM-GUI was used to prepare the initial structures of nanodiscs and planar lipid bilayers^{34–36} for MD simulations. Nanodiscs were composed of MSP1D1 protein (9.7 nm), palmitoyl-oleoyl-phosphatidylcholine (POPC) / palmitoyl-oleoyl-phosphatidylglycerol (POPG) in ratio 3:1, and 25 mol% of sterols (CHL or CHS). Figure 1 shows the structure of molecules used in this work and snapshots of initial structure of nanodiscs and planar lipid bilayers. All systems were solvated using TIP3 water,³⁷ and Na⁺ and Cl⁻ ions were added to ensure physiological salt concentration and neutrality of the systems. CHS was studied in both neutral (CHS0) and deprotonated form (CHS⁻). The exact composition of each system is given in Table 1.

Charmm36 force field^{38,37} was used to describe the protein, lipids and water/ions. Prior to MD simulations, all the systems were energy minimized using steepest-descent algorithm. The equilibration was performed with position restraints applied to the lipid and protein atoms.

Table 1. Numbers of lipids, water, and ions in planar lipid bilayers and nanodiscs used in MD simulations.

	POPC	POPG	Sterol	Water	Na+	Cl-
Planar Lipid Bilayers						
POPC/POPG	96	32	-	6180	44	12
POPC/POPG/CHL	72	24	32	5769	35	11
POPG/POPG/CHS0	72	24	32	6917	36	12
POPG/POPG/CHS ⁻	72	24	32	7050	69	13
Nanodiscs						
POPC/POPG	132	44	-	122565	276	222
POPC/POPG/CHL	126	42	56	95337	225	173
POPC/POPG/CHS0	126	42	56	120501	270	218

POPC/POPG/CHS ⁻	126	42	56	123247	331	223
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For the long-range electrostatic interactions, the Particle-Mesh Ewald (PME) method was applied with a Coulomb cut-off of 1.2 nm. Lennard-Jones interactions were truncated at the distance of 1.2 nm.³⁹ To preserve the covalent bond lengths the linear constraint solver (LINCS) algorithm was employed,⁴⁰ and the time step of simulations was set to 2 fs. The neighbor search was performed using the Verlet cut-off scheme. The neighbor list was updated every 20 steps. Temperature, 310 K, was maintained with Nose-Hoover thermostat, separately for protein, lipids, and water/ions.^{41,42} Pressure, 1 bar, was coupled with Parrinello-Rahman barostat.⁴³ The time constant for thermostat was set to 1 ps and for barostat 5 ps. All restraints were removed for the final production run and simulations were carried out using GROMACS 5 package.⁴⁴ The nanodisc system were simulated for 1 μ s each and planar lipid bilayers for 500 ns each. All analysis were performed using gromacs tools and two dimensional maps were calculated using g_thickness, g_mydensity, g_ordercg scripts⁴⁵. Figures were rendered using VMD⁴⁶ tool. The stability of the nanodiscs structure during MD simulations was monitored via evaluation of the secondary structure of the MSP. Both the secondary structure and the disc shape were preserved during simulations. Besides, we monitored the radius of gyration of nanodiscs, which showed that the size of nanodiscs was not changing during simulations. The radius of gyration was the same for CHL and CHS0 nanodiscs, 4.84 $\pm$ 0.03, 4.83 $\pm$ 0.03 respectively, and 1% larger for CHS- nanodisc, 4.90 $\pm$ 0.03. The shape of nanodiscs was also preserved during MD simulations.

Electron Paramagnetic Spectroscopy and Spectra Simulations

POPC and POPG lipids were purchased from NOF Europe, and the PC spin labels, CHL and CHS from Avanti Polar Lipids. The Plasmids for MSPs expression were purchased from Addgene^{4,47}. All other necessary compounds for protein purification and buffer preparation were purchased from Sigma Aldrich Europe. Both the liposome and nanodisc samples (constisting of POPC/POPG 3:1 or POPC/POPG/CHS 12:3:4) containing 1 mol% of lipid spin label for the EPR measurement were prepared and checked for the quality as described in the previous work.⁴⁸ Three spin labels (5,10 and 16-PC) with a nitroxide free radical moiety attached to different carbon atoms along the lipid alkyl chain were employed. The EPR measurements were conducted using the

Bruker EMX spectrometer equipped with EMX ER 4141 VT temperature control unit. Samples were placed in the gas-permeable methylpentene plastic, TPX capillary (i.d. 0.6 mm) and deoxygenated inside the resonator using nitrogen fumes from the temperature control unit. All samples were measured at 310K.

Nonlinear least squares (NLLS) fitting procedures were performed using the program by Budil et al,⁴⁹ which uses the stochastic Liouville equation by Schneider and Freed.⁵⁰ The magnetic parameters of A-tensor used for the spectra simulations were established by measuring the samples at rigid limit temperatures (120 K). Parameters for g-tensor were taken from previous studies.⁵¹ With fixed magnetic parameters, MOMD (microscopic order and macroscopic disorder) model was employed to perform NLLS fitting to the experimental spectra.⁵² One of the parameters calculated based on EPR spectra simulations was the order parameter S_0 .

RESULTS AND DISCUSSION

CHS and CHL sterols increase order of lipid bilayers

First, we evaluated the effect of sterols on the bulk properties of lipids in nanodiscs and planar lipid bilayers. From MD simulations, we calculated the order parameter S_{CD} for sn-1 acyl tails of POPC and POPG according to the formula:

$$S_{CD} = \left\langle \frac{3}{2} (\cos^2 \theta_i) - \frac{1}{2} \right\rangle$$

where θ_i is the angle between the C-D bond (C-H in simulations) of the i -th carbon atom and the bilayer normal. The S_{CD} profiles (Figure 2) indicate that both sterols increase the order of the acyl chains; however, the effect is weaker in nanodiscs than in planar lipid bilayers. In nanodiscs, CHL and CHS0 induce similar ordering effect on lipid acyl tails, while the effect of CHS⁻ is weaker. Moreover, CHS⁻ in nanodiscs does not increase the order of the sn-1 chain of POPG and a small decrease in the order occurs at the segments located deeper in the membrane (below eight segment). This effect is specific for nanodiscs as in planar lipid bilayers the order of POPG chains increase after addition of sterols to the same level as for POPC acyl chains.

Figure 2. Profiles of the order parameter S_{CD} along sn -1 chain of POPC (A, and C), and POPG (B, and D) in nanodiscs (A, B), and lipid bilayers (C, and D).

Similarly, from EPR spectroscopy we can obtain S_0 parameter which is given by the same formula as S_{CD} parameter where θ_i is the angle between bilayer normal and molecular axis of the label.⁵³ Profiles of S_0 parameter for POPC/POPG liposomes in comparison with POPC/POPG and POPC/POPG/CHS nanodiscs are presented in Figure 3a. Profiles of S_0 parameter are in qualitative agreement with profiles of S_{CD} parameter and show an increase of the order after addition of CHS to nanodiscs. Additionally, we showed profiles of S_0 parameter for POPC/POPG, POPC/POPG/CHL, and POPC/POPG/CHS liposomes (Figure 3b). In agreement with previous studies, the CHS ordering effect is weaker in comparison to CHL.

Figure 3. Profiles of the order parameter S_0 measured in (a) POPC/POPG liposomes, POPC/POPG nanodiscs, and POPC/POPG/CHS nanodiscs; and (b) POPC/POPG liposomes, POPC/POPG/CHL liposomes, and POPC/POPG/CHS liposomes. The approximate locations of the paramagnetic centers in the membrane are marked with arrows.

CHS⁻ and POPG segregate towards scaffold protein

In Figure 4, two-dimensional maps of thickness, order parameter, and density of nanodiscs, and in Figure 5, profiles of the order parameter for four zones of lipids located at different distances from the nanodisc center are shown. Results obtained for POPC/POPG and POPC/POPG/CHL nanodiscs are in agreement with previously published study of nanodiscs with neutral lipids.^{36,54,55} In these nanodiscs the thickness of bilayer is lowest near the MSPs and gradually increases towards the center of nanodisc. Increase in thickness is associated with increase in density and ordering of lipids. For POPC/POPG/CHS0 nanodisc very similar behavior is observed however certain inhomogeneity is seen in the nanodisc. Interestingly for POPC/POPG/CHS⁻ nanodisc we observe increase in thickness near the region neighboring MSPs and decrease in the thickness towards the center of nanodisc. Consequently, there were no increase of the order of the tails and density in the center of nanodisc. Moreover, POPC/POPG/CHS⁻ nanodisc is also heterogeneous in the central portion.

Figure 4. Two-dimensional map showing bilayer thickness (left panel), lipid acyl chain order parameter (central panel), and membrane density map (right panel) for MSP1D1 and nanodiscs at 310 K.

Figure 5. Profiles of the order parameter S_{CD} along hydrocarbon tails of lipids located in four zones: (1) 0-1.5 (black triangles), (2) 1.5-2.5 (red circles), (3) 2.5-3.5 (blue squares), (4) >3.5 (magenta diamonds) nm from the disc center, in POPC/POPG/CHL (a), POPC/POPG/CHS0 (b), POPC/POPG/CHS- (c) nanodiscs. Profiles of the order parameter S_{CD} along hydrocarbon tails for lipids located in first zone (solid lines), and fourth zone (dashed lines), in POPC/POPG/CHL (red line), POPC/POPG/CHS0 (green line), POPC/POPG/CHS- (blue line) nanodiscs (d).

To understand the distinct lipid properties in POPC/POPG/CHS⁻ system, we calculated two-dimensional maps showing sterol density (Figure 6) in nanodiscs, and we visualized final location of sterols in the disc (Figure 7). In CHL and CHS0 systems, the sterols concentrate mainly in the center of nanodisc, whereas in CHS⁻ system the sterols have clear preferences to localize near the MSPs. Figure 6 showing final snapshots of the MSPs and sterols in nanodiscs further confirms the interaction between MSPs and CHS⁻. The difference between neutral and ionic form of CHS indicates that origin of the preference of CHS⁻ towards MSPs is a result of strong electrostatic interactions, salt bridges between carboxylic group of CHS⁻ and

positively charged amino acids of the protein, predominately arginine (Table 2). These interactions most likely slow the mixing of the lipids in the nanodiscs, thus resulting in the heterogeneity of the lipid properties like, e.g., thickness (Figure 4).

Table 2. Numbers of H-bonds between sterols and MSP and positively charged amino acids of the MSP1: lysine (LYS), arginine (ARG) and histidine (HIS) per sterols molecule.

	All bonds	LYS - sterol	ARG - sterol	HIS - sterol
POPC/POPG/CHL	0.05	0.00	0.02	0.00
POPC/POPG/CHS0	0.03	0.00	0.02	0,00
POPC/POPG/CHS ⁻	0.87	0.06	0.63	0,00

POPG lipid is another anionic molecule in our nanodiscs, therefore we also calculated two-dimensional density maps for POPG lipid (Figure 6). Apparently, POPG also accumulates near the MSPs due to strong electrostatic interactions and this is most likely the reason for lack of ordering effect of CHS⁻ on POPG tails.

Figure 6. Two-dimensional map showing density of sterols (A, B, and C) and POPG (D, E, and F) in nanodiscs.

Figure 7. Final snapshots from MD simulations showing locations of sterols in nanodiscs.

CONCLUSIONS

Although lipid nanodiscs seem to be simple structure, mimicking the lipid environment of MPs and the structure of the nanodiscs is complex. Moreover, the effect of MSPs on lipid behavior is significant. Previous studies have shown that lipids in nanodiscs are more ordered than in liposomes and the lipids close to the MSPs are less ordered than in the center of the nanodisc. In the current study, we show that MSPs affect the distribution of anionic lipids in the nanodisc plane. Both CHS⁻ and POPG preferentially locate close to MSPs due to electrostatic interactions with the positively charged residues of MSPs. These interactions affect the properties of lipids in nanodiscs and are very different in comparison with nanodiscs which contain cholesterol. Particularly striking is the inhomogeneity of nanodiscs with CHS⁻ resulting from slow mixing of lipids in the nanodisc. Our results indicate that CHS is not suitable to mimic CHL.

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Supporting Information. Package of input files for simulations in GROMACS used in this study.

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