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An Electron Paramagnetic Resonance Method for Measuring the Affinity of a Spin-Labeled Analog of Cholesterol for Phospholipids

Justin A. Williams · Cynthia D. Wassall · Marvin D. Kemple · Stephen R. Wassall

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Abstract Cholesterol (chol)–lipid interactions are thought to play an intrinsic role in determining lateral organization within cellular membranes. Steric compatibility of the rigid steroid moiety for ordered saturated chains contributes to the high affinity that holds chol and sphingomyelin together in lipid rafts whereas, conversely, poor affinity of the sterol for highly disordered polyunsaturated fatty acids (PUFAs) is hypothesized to drive the formation of PUFA-containing phospholipid domains depleted in chol. Here, we describe a novel method using electron paramagnetic resonance (EPR) to measure the relative affinity of chol for different phospholipids. We monitor the partitioning of 3β-doxyl-5α-cholestane (chlstn), a spin-labeled analog of chol, between large unilamellar vesicles (LUVs) and cyclodextrin (mβCD) through analysis of EPR spectra. Because the shape of the EPR spectrum for chlstn is sensitive to the very different tumbling rates of the two environments, the ratio of the population of chlstn in LUVs and mβCD can be determined directly from spectra. Partition coefficients (K_B^A) between lipids derived from our results for chlstn agree with values obtained for chol and confirm that decreased affinity for the sterol accompanies increasing acyl chain unsaturation. The virtue of this EPR method is that it provides a measure of chol binding that is quick, employs a commercially available probe and avoids the necessity for physical separation of LUVs and mβCD.

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J. A. Williams · C. D. Wassall · M. D. Kemple · S. R. Wassall (✉)
Department of Physics, Indiana University-Purdue University Indianapolis, Indianapolis, IN 46202-3273, USA
e-mail: swassall@iupui.edu

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Introduction

The concentration of cholesterol (chol) in cellular membranes, which may be as much as 45 mol% of total lipid in mammalian membranes (Yeagle 1993), plays a crucial role in controlling the physical properties and biological function of the membrane (Ohvo-Rekilä et al. 2002; Silvius 2003). Alteration of membrane features and events is primarily a consequence of the sterol's interaction with neighboring lipids and proteins. Unequal affinity of chol for different lipids is potentially a driving factor in promoting the sorting of membrane lipids into patches or domains of specific composition (Lingwood and Simons 2009). The purpose of the domains is to provide a local environment necessary for the function of resident proteins (Levental et al. 2010). Here, we describe a novel electron paramagnetic resonance (EPR) method for measuring the affinity of a spin-labeled analog of chol for lipids and present results obtained on phospholipids with increasing levels of acyl chain unsaturation to illustrate the utility of the method.

Chol is a mostly nonpolar molecule that consists of a tetracyclic ring structure with a hydroxyl group at one end and a short hydrocarbon tail at the other (Fig. 1). It usually resides in membranes so that the hydroxyl group sits just below the aqueous interface while the short chain extends toward the center of the bilayer, the long molecular axis lining up approximately parallel to the fatty acyl chains of lipid molecules (Khelashvilia and Harries 2013). This arrangement places the rigid planar ring system of the steroid moiety at the same depth as the upper portion of

adjacent lipid acyl chains and impedes isomerization between trans and gauche conformations of C–C bonds. The restriction to chain motion leads to an increase in molecular packing, thickness and mechanical rigidity for the membrane (McMullen et al. 2004). Preferential affinity for chol is conferred upon saturated lipids because the extended conformation adopted by saturated chains is compatible with close proximity to the rigid steroid moiety and allows for strong van der Waals interactions (Brown 1998). A tendency for saturated lipids to segregate into domains with chol is a consequence. The tendency is enhanced in the case of sphingolipids by hydrogen bonding between the hydroxyl group on the sterol and the amide on the sphingosine backbone. Lipid rafts, domains 10–200 nm in size enriched in sphingolipids "glued" together by chol (Pike 2006), are purported to serve as the platform for signaling proteins in the outer leaflet of plasma membranes (Simons and Ikonen 1997; Brown and London 2000). Phospholipids containing polyunsaturated fatty acids (PUFAs) represent the opposite extreme to sphingolipids with predominantly saturated chains. The shallow energy barrier to rotation about C–C bonds in the repeating =C–C–C= unit in a PUFA chain produces a multitude of rapidly changing conformations that push chol away and drive the formation of membrane domains rich in PUFA-containing phospholipids but depleted in the sterol (Wassall and Stillwell 2009). These (nonraft) domains are organizationally the antithesis of rafts and, we have hypothesized, are responsible in part for the diverse range of health benefits attributed to dietary consumption of PUFA in fish oils.

There is an immense diversity of natural lipids (Yeagle 1993). The charge, size and ability to hydrogen bond of the head groups vary, while the chains vary in length and degree of unsaturation. These variations produce differing affinities for chol that are potentially significant enough to generate lateral

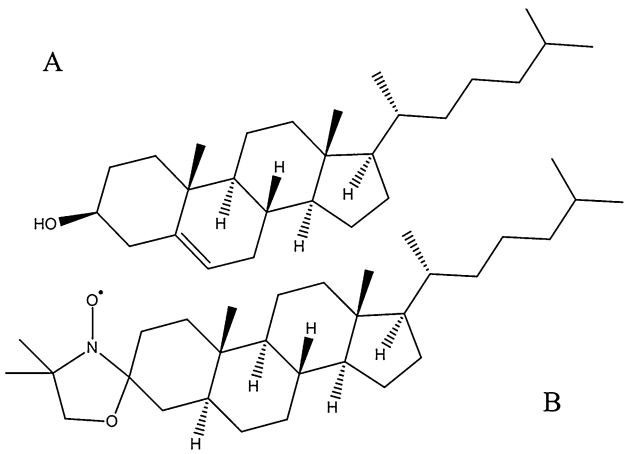

Fig. 1 Molecular structures of cholesterol (chol) (*A*) and 3β-doxyl-5α-cholestane (chlstn) (*B*)

membrane segregation in biological systems (Silvius 2003). Chol's affinity for different membrane compositions has been investigated by a variety of techniques. An early approach was to measure the partitioning of chol between large (LUVs) and small (SUVs) unilamellar vesicles prepared from different lipids (Yeagle and Young 1986). LUVs containing chol were incubated with SUVs or LUVs were incubated with SUVs containing chol and then separated on the basis of size. The relative affinity of the two lipids for the sterol was determined from the equilibrium distribution of chol between the two sizes of vesicle. Subsequent work used methyl-β-cyclodextrin (mβCD) to facilitate the exchange of chol between donor and acceptor LUVs that were separable due to the charge on phosphatidylgylcerol included in the donor vesicles (Leventis and Silvius 2001). mβCD is a ring-shaped molecule consisting of seven glucose units that is soluble in water but possesses a hydrophobic cavity capable of reversibly binding small hydrophobic molecules such as chol. By extracting membrane-bound chol from donor vesicles and forming a water-soluble complex that transfers the sterol to acceptor vesicles, the time taken to reach equilibrium was reduced (López et al. 2011). A later refinement was to measure the partitioning of chol solely between mβCD and LUVs, thereby avoiding the mixing of donor and acceptor lipid species; and the affinity among differing lipids could then be compared indirectly using mβCD as a reference (Nui and Litman 2002; Halling et al. 2008). mβCD (∼1 nm in size) and LUVs (100 nm in diameter) were separated after equilibration by centrifugation across a filter, and the concentration of chol was assayed enzymatically or with a radiolabeled tracer. The necessity to physically separate vesicles and mβCD, which has the potential to introduce artifacts, was eliminated by application of isothermal titration calorimetry (ITC) (Tsamaloukas et al. 2005). In this method an estimate of the partitioning of chol between LUVs and mβCD was obtained from an analysis of power compensation due to the heat of reaction recorded after each of a series of injections of LUVs containing chol into an aqueous solution of mβCD (release protocol) and of LUVs into an aqueous solution containing mβCD–chol complexes (uptake protocol). The same advantage was shared by another method that directly determined the distribution of cholestatrienol (CTL), a fluorescent analog of chol, between mβCD and LUVs from measurements of the steady-state anisotropy (Nyholm et al. 2010). This fluorescence experiment is the inspiration for the current study.

We introduce an EPR method to add to the arsenal of techniques for investigating the partitioning of chol among lipids. EPR spectra for 3β-doxyl-5α-cholestane (chlstn), a spin-labeled analog of chol (Fig. 1) that is commercially available, incorporated into LUVs are distinguished from the EPR spectrum for chlstn complexed with mβCD due to the motional inequivalence of the two environments. Exploiting this property, it is straightforward to calculate

the ratio of the population of chlstn partitioned between LUVs and mβCD at equilibrium by spectral simulation without separating the fractions. Partition coefficients measured for chlstn in this work are compared with results obtained by other methods to demonstrate that the relative affinity of the spin-labeled analog for different lipids matches chol. Measurements made on phosphatidylcholine (PC) LUVs are then reported that support the hypothesis of a decreased sterol affinity with increasing acyl chain unsaturation that could influence lateral organization within a membrane.

Materials and Methods

Materials

Chlstn, chol and mβCD were purchased from Sigma-Aldrich (St. Louis, MO). Fisher Scientific (Pittsburgh, PA) was the source for butylated hydroxyl toluene (BHT). The phospholipids 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (16:0–18:1PC, POPC), 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine (16:0–18:2PC, PLPC), 1-palmitoyl-2-docosahexaenoyl-*sn*-glycero-3-phosphocholine (16:0–22:6PC, PDPC) and 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (18:1–18:1PC, DOPC) were obtained from Avanti Polar Lipids (Alabaster, AL) and used without further purification.

Sample Preparation

LUVs

Stock solutions of PC lipid and chlstn in chloroform were mixed in appropriate volumes. The lipid was dried under a gentle stream of argon, and the film produced was placed under vacuum overnight to evaporate remaining traces of solvent. Sample mass was then checked. After hydration with buffer (100 mM NaCl/10 mM Tris, pH 7.4), four freeze–thaw cycles were completed and LUVs were created by extrusion: 25 passes across a 100-nm nucleopore Whatman track–etched filter performed with an Avanti miniextruder. Samples were protected from oxidation by the addition of BHT (1 BHT: 250 PC lipids), and manipulations were performed in an argon atmosphere.

mβCD–chlstn complex

To produce the mβCD–chlstn complex, chlstn was dried on the bottom of a test tube and mβCD PBS solution was added. The solution was subsequently sonicated at 65 °C until the chlstn film was visibly removed from the glass.

Partitioning Experiments

EPR

We performed partitioning experiments between LUVs and mβCD using both uptake and release protocols. For release experiments, chlstn originates with and partitions from LUVs to mβCD initially free of sterol. For uptake experiments, LUVs free of sterol were introduced into a solution containing mβCD–chlstn complex. Figure 2 shows a cartoon representation of the two types of experiment. The partitioning of chlstn between LUVs and mβCD is characterized by partition coefficients calculated according to the model derived by Tsamaloukas et al. (2005)

$$K_{\mathrm{x}} = \frac{C^{\mathrm{LUV}}_{\mathrm{chlstn}}\left(C_{\mathrm{m}\beta\mathrm{CD}} - 2C^{\mathrm{m}\beta\mathrm{CD}}_{\mathrm{chlstn}}\right)^2}{\left(C_{\mathrm{LUV}} - C^{\mathrm{LUV}}_{\mathrm{chlstn}}\right)C^{\mathrm{m}\beta\mathrm{CD}}_{\mathrm{chlstn}}} \quad (1)$$

In this expression, K_{x} is the hybrid mole partition coefficient, where the molar concentration at equilibrium for chlstn in LUVs, chlstn bound by mβCD, total mβCD and total PC are denoted as $C^{\mathrm{LUV}}_{\mathrm{chlstn}}$, $C^{\mathrm{m}\beta\mathrm{CD}}_{\mathrm{chlstn}}$, $\mathrm{C}_{\mathrm{m}\beta\mathrm{CD}}$ and $\mathrm{C}_{\mathrm{LUV}}$ respectively. The model assumes a stoichiometry of 2:1 for the mβCD–chlstn complex and accounts for the decrease in free mβCD due to binding with chlstn. A maximum uncertainty less than ±20 % applies to the values derived for K_{x}.

Release experiments, in which mβCD was added to a suspension of LUVs containing chlstn, were designed to achieve a partitioning solution composition of 15 mM PC, 0.152 mM chlstn and a variable amount of mβCD. The concentration of chlstn, which corresponds to 1 mol% relative to phospholipid, was chosen to minimize magnetic (dipole–dipole and spin exchange) interactions between spin labels (Sackmann and Traüble 1972). Four trials were performed for each phospholipid with mβCD concentrations of 4, 5, 6 and 7 mM. Partitioning solutions were incubated at 37 °C on a shaker for 1 h to reach an equilibrium distribution. In uptake experiments, where the mβCD–chlstn complex was added to LUVs free of chlstn, the partition solution consisted of 15 mM PC, 0.152 mM chlstn and 5 mM mβCD. This solution was then incubated in the same manner as in the release experiment.

To measure the partitioning of chlstn between LUVs and mβCD by EPR, equilibrated samples were transferred to 25-μl fused silica capillaries that were sealed with Critoseal clay and inserted into the temperature-controlled (37 °C) rectangular TE_{102} cavity of a Bruker (Billerica, MA) X-band ESP 300 EPR spectrometer operating at 9.29 GHz. Experimental EPR parameters were microwave power, 12.6 mW; field center, 3,300 G; sweep width, 100 G; sweep time, 83.89 s; time constant, 655.4 ms; modulation amplitude, 1 G; and data set, 1,000 points. The signal detected was the first derivative of the absorption spectrum

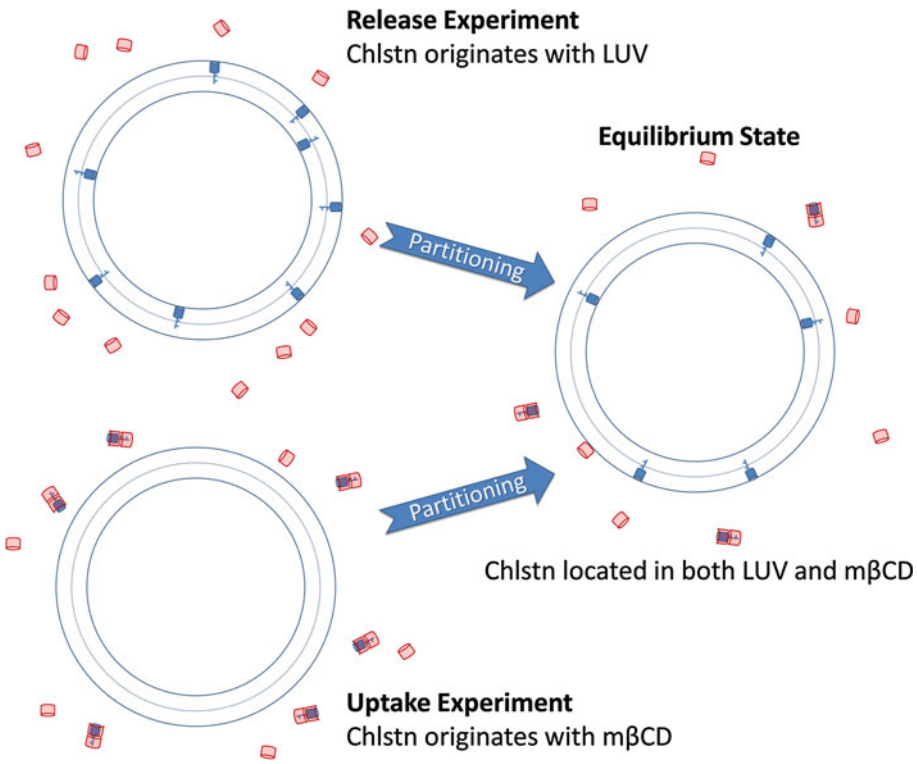

Fig. 2 Cartoon of uptake and release experiments. A single LUV and multiple mβCDs are depicted

composed of three resonances characteristic of the hyperfine coupling between the unpaired electron and ^{14}N nuclear spin of the doxyl label (Marsh 1981). Four scans were taken per sample and averaged. The spectra were normalized according to the double integral, which is proportional to the concentration of chlstn. Reference spectra for each lipid at 15 mM PC with 0.152 mM (1 mol%) chlstn and for 5 mM mβCD with 0.152 mM chlstn were recorded. The percentages of chlstn located in LUVs and mβCD at equilibrium were then determined with a spectral fitting procedure that will be explained in "Results."

ITC

For purposes of comparison, ITC measurements of the partitioning of chol between LUVs and mβCD were performed. A brief description of the experimental method can be found in the Supplementary Material (online resource).

Results

Measurement of K_x by EPR

The basis of our approach to measuring the partition coefficient K_x of chlstn between LUVs and mβCD may be understood by inspecting the EPR spectra presented in Fig. 3. Representative EPR spectra obtained for chlstn incorporated within POPC LUV (Fig. 3A) and bound to mβCD (Fig. 3B) demonstrate that the spectral shape depends upon whether the spin label is located within LUVs or in complex with mβCD. On the timescale to which EPR of a nitroxide group is sensitive (10^{-11}–10^{-7} s), the tumbling of LUVs is too slow to produce motional narrowing so that the spectrum for a spin-labeled lipid in LUVs is a powder pattern comprised of a superposition of spectra from membranes in a spherically symmetric distribution of orientations (Schreier et al. 1978). The resultant spectrum has broad high and low field absorption peaks that are characteristic of the fast anisotropic motion undergone by chlstn within a membrane (Fig. 3A). While bound to mβCD, in contrast, chlstn experiences isotropic motion because the mβCD–chlstn complex is very much smaller than LUVs and tumbles rapidly in solution. The resulting spectrum (Fig. 3B) has high and low field peaks that are substantially narrower compared to the LUV–chlstn spectrum. In a partitioning experiment chlstn resides in both mβCD and LUVs. The spectrum obtained is then a superposition of spectra from both environments that are population-weighted in intensity, as illustrated by the spectrum that was recorded in an uptake experiment when POPC LUVs were equilibrated with mβCD–chlstn complex (Fig. 3C). There the presence of two spectral components is most evident in the appearance of the high field peak that is comprised of a sharp signal due to chlstn bound to mβCD superposed upon a broad component due to chlstn incorporated into LUVs.

Our procedure for determining K_x entails simulating a spectrum that reproduces the EPR spectrum observed in a partitioning experiment. Spectra recorded for chlstn in LUVs prepared from the same lipid as the partitioning experiment and in the mβCD complex separately were combined for the full range of relative intensity between a fractional intensity of 1 for LUVs and 0 for mβCD to 0 for LUVs and 1 for mβCD in 0.0001 increments. The combination that most accurately reproduces the mixed spectrum of the partitioning experiment was next assessed according to a least squares criterion. Specifically, the difference between the simulation and experiment for each data point was squared and summed over the entire spectrum, and the combination with the smallest sum was deemed to be the best fit. The relative intensity of spectra leading to the best fit is equivalent to the ratio of the concentration of chlstn in LUVs and mβCD in the mixed sample, from which a value for K_x was calculated via Eq. (1). Figure S1 in the Supplementary Material (online resource) provides an example of the quality of fit achieved for the spectrum shown in Fig. 3C that was obtained in an uptake experiment where chlstn (0.152 mM) originally bound to mβCD (5 mM) was incubated with POPC LUVs (15 mM). Based on this best fit, 73 % of the total chlstn was found to be taken up into the LUVs, which coincides with a value of $K_x = 4.3$ mM for POPC.

Acyl Chain Unsaturation

To illustrate the utility of our EPR method, we report K_x values measured for chlstn in PC membranes with differing amounts of acyl chain unsaturation. Reference spectra for chlstn in LUVs prepared from all of the lipids under consideration are plotted in Fig. 4, arranged from most unsaturated lipid (PDPC with six double bonds, top) to least (POPC with one double bond, bottom). The spectral shape in DOPC, PLPC and PDPC is very similar to that in POPC. A slight narrowing of resonances at higher levels of unsaturation is apparent on close inspection, which corresponds to a reduction in the degree of anisotropy for the motion of chlstn in a more disordered membrane interior. These LUV reference spectra were used in the same manner as described for POPC to fit spectra recorded in partition experiments conducted on their respective lipid species. The values of K_x determined for chlstn are given in Table 1. They are an average from uptake experiments, where mβCD was the donor and LUVs were the acceptor, and release experiments, where LUVs were the donor and mβCD was the acceptor. Results from the two types of experiment, which agree within experimental uncertainty, can be found in Table S1 in the Supplementary Material (online resource). Comparing POPC, PLPC and PDPC, all having saturated palmitoyl sn-1 chains, a trend toward reduced affinity for chlstn is seen to accompany an increasing level of unsaturation in the sn-2 chain. POPC ($K_x = 4.4 \pm 0.4$ mM) with one carbon–carbon double bond has a K_x value twice as large as PDPC ($K_x = 2.2 \pm 0.4$ mM) with six double bonds. Comparing POPC and DOPC ($K_x = 2.6 \pm 0.4$ mM), furthermore, less favorable interaction with chlstn is seen to follow the introduction of an unsaturation into the sn-1 chain.

Discussion

There has been considerable interest in chol's ability to affect lateral organization within cellular membranes and in the molecule's nonuniform distribution among membranes. One mechanism thought to be influential is variation in the molecular interaction of the sterol with different lipid species. This view has been the motivation behind numerous studies of the affinity of chol for lipids by a range of experimental techniques. The best approach that

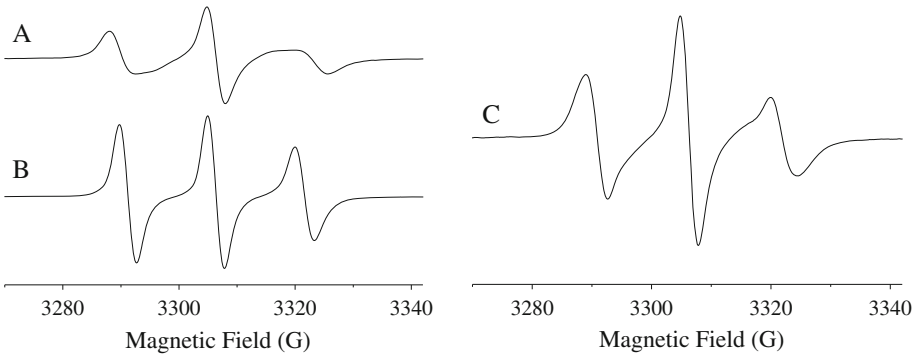

Fig. 3 Representative reference EPR spectra for chlstn within POPC LUV (*A*) and in complex with mβCD (*B*) and an experimental spectrum from a partition (uptake) experiment where chlstn is present both in complex with mβCD and incorporated into POPC LUV (*C*). A fit of spectrum *C* that was obtained from a combination of spectra *A* and *B* scaled in relative intensity is shown in Fig. S1 in the Supplementary Material (online resource). Spectra were recorded at 37 °C. Composition of samples was 15 mM POPC with 0.152 mM (1 mol%) chlstn (*A*), 5 mM mβCD with 0.152 mM chlstn (*B*) and 15 mM POPC and 0.152 mM chlstn with 5 mM mβCD (*C*)

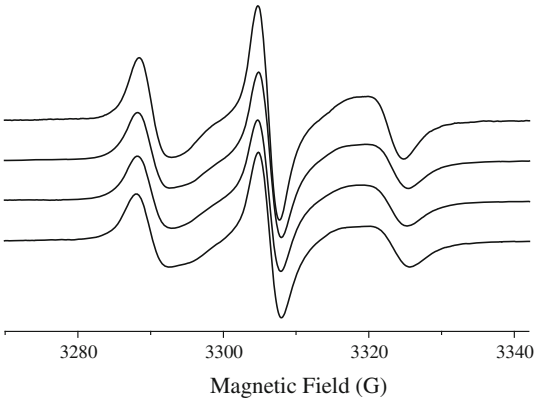

Fig. 4 LUV reference spectra of 15 mM PC with 1 mol% chlstn at 37 °C, arranged from bottom to top: POPC, DOPC, PLPC and PDPC

Table 1 Partition coefficients K_x and K_B^A relative to POPC measured by EPR for chlstn at 37 °C

Lipid	K_x (mM)	K_B^A
POPC	4.4 ± 0.4	1
PLPC	3.4 ± 0.3	0.77 ± 0.14
PDPC	2.2 ± 0.4	0.50 ± 0.14
DOPC	2.6 ± 0.4	0.59 ± 0.14

The K_x values and uncertainties in K_x are an average of the results from release and uptake experiments found in Table S1 of the Supplementary Material (online resource). The error in K_B^A includes the uncertainty in K_x for POPC as well as for PLPC, PDPC or DOPC

has been developed so far is to measure the equilibrium partitioning of chol between LUVs and mβCD, from which a determination of relative affinity between lipids may be made. Various methods, each with a set of pros and cons, have been used to estimate the concentration of sterol in the two environments. A problem with the methods involving physical separation of mβCD from LUVs prior to measurement by enzymatic assay (Nui and Litman 2002) or quantitation with a radiotracer (Halling et al. 2008) is that the equilibrium partitioning may be altered during centrifugation. The ITC method, which monitors the heat of reaction due to the exchange of chol between LUVs and mβCD (Tsamaloukas et al. 2005), eliminates this step and arguably has the potential to set the "gold standard" for measurement. However, care has to be taken in choosing a concentration window where large amounts of phospholipid are not extracted by mβCD and, in our experience, can be extremely time-consuming to achieve. The reliance on a probe that is not commercially available is a drawback of the much quicker technique based upon measurement of the steady-state polarization of CTL (Nyholm et al. 2010) that also avoids the need to centrifuge.

We have devised a new EPR method that measures the equilibrium distribution of chlstn between LUVs and mβCD. This spin-labeled analog of chol has been extensively employed in studies of molecular organization and dynamics in membranes (Raguz et al. 2011). Other analogs should work equally well. 25-doxyl-cholesterol, which better mimics chol (Scheidt et al. 2003), for instance, is an appealing alternative but unavailable commercially. The distinction in the shape of EPR spectra for chlstn incorporated in LUVs (Fig. 3A) versus bound to mβCD (Fig. 3B), which is depicted in Fig. 3 for POPC, is at the heart of our method. Our procedure entails incubating chlstn in mβCD as the donor with LUVs as the acceptor (uptake experiment) or, vice versa, chlstn in LUVs as the donor with mβCD as the acceptor (release experiment). An estimate of the equilibrium distribution of chlstn between donor and acceptor is then ascertained from the resultant spectrum by adding the spectra recorded for chlstn individually in the two environments and scaling their relative intensity to provide a best fit as judged by a least squares analysis. The fit to the spectrum obtained for POPC in an uptake experiment (Fig. 3C) by this process, which identified 0.73 versus 0.27 as the ratio of chlstn in LUVs and mβCD (corresponding to $K_x = 4.3$ mM), may be found in the Supplementary Material, Fig. S1.

Partition coefficients (K_x) measured for POPC by EPR and other methods are compiled in Table 2. It is immediately apparent that the partition coefficient determined for chlstn ($K_x = 4.4 \pm 0.4$ mM) is an order of magnitude less than the range of values ($K_x = 32$–49 mM) obtained for chol. The smaller value of K_x is predominantly ascribed to differential interaction of the spin-labeled molecule with mβCD. Greater affinity for mβCD, together with the more polar nature of the fluorescently labeled analog, was invoked to account for the comparably small partition coefficient that was reported for CTL ($K_x = 5.0 \pm 0.9$ mM) (Ekholm et al. 2011). We suggest that in the case of chlstn, which unlike CTL is less polar than chol, greater exposure to mβCD contributes to the reduction in value for K_x. Specifically, the bulky doxyl group in the spin-labeled analog that replaces the OH group in chol

Table 2 Equilibrium partition coefficients (K_x) measured for chol and analogs of chol between POPC LUVs and mβCD at 37 °C

Method	K_x (mM)
Physical separation, chol (enzyme assay)[a]	49 ± 4
Physical separation, chol (^{3}H radiolabel)[b]	32 ± 2
ITC, chol[c]	37 ± 7
Fluorescence, CTL[d]	5.9 ± 0.9
EPR, chlstn[e]	4.4 ± 0.4

Values taken from [a]Nui and Litman (2002), [b]Halling et al. (2008), [c]Tsamaloukas et al. (2005), [d]Ekholm et al. (2011) and [e]this study (Table 1). The value quoted for Nui and Litman was recalculated from their data assuming a 1:2 ratio for the stoichiometry of the chol/mβCD complex

(Fig. 1) protrudes into the lipid–water interface (Scheidt et al. 2003), making extraction by mβCD at the membrane surface easier.

A relative partition coefficient (K_B^A, lipid A relative to lipid B) that is independent of mβCD can be calculated by taking the ratio

$$K_B^A = \frac{K_x^A}{K_x^B} \tag{2}$$

of partition coefficients K_x^A and K_x^B measured in individual assays with LUVs prepared from the two lipids (A and B, respectively) (Nui and Litman 2002). The associated change in free energy of chol partitioning between the two types of vesicle is then given by

$$\Delta G = -RT \ln K_B^A \tag{3}$$

where R is the universal gas constant and T is temperature. Figure 5 compares partition coefficients derived according to Eq. (2) for chlstn and chol from EPR and ITC experiments, respectively, on DOPC and PDPC relative to POPC. They agree within experimental uncertainty ($K_{POPC}^{DOPC} = 0.59 \pm 0.14$ vs. 0.63 ± 0.25 and $K_{POPC}^{PDPC} = 0.50 \pm 0.14$ vs. 0.38 ± 0.15 for chlstn vs. chol), indicating that chlstn and chol share the same relative affinity for different lipids and validating the EPR method. It would be interesting in subsequent work to compare measurements involving more ordered, saturated lipids such as sphingomyelin as a further test.

The results obtained in experiments performed on phospholipids possessing different levels of acyl chain unsaturation to demonstrate the applicability of the EPR method are consistent with unfavorable interaction between chol's tetracyclic ring and acyl chain double bonds. The partition

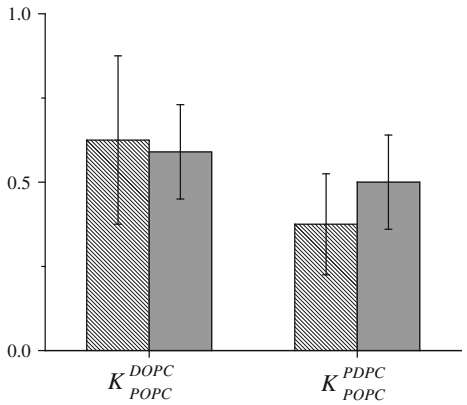

Fig. 5 Comparison of relative partition coefficients (K_B^A) obtained for chlstn ■ by EPR and chol ▨ by ITC. Experiments were conducted at 37 °C. The values for K_B^A for chlstn are taken from Table 1, and those for chol are taken from Table S2 in the Supplementary Material (online resource). The error in K_B^A includes the uncertainty in K_x for POPC as well as for PDPC or DOPC

coefficients [K_x and K_B^A relative to POPC calculated via Eq. (2)] listed in Table 1 confirm that affinity for chlstn is reduced when the number of double bonds in the sn-2 chain increases (POPC > PLPC > PDPC) and when the saturated sn-1 chain is replaced with an unsaturated chain (POPC > DOPC) (Nui and Litman 2002). Invoking Eq. (3), the respective reductions in partition coefficient for PDPC and DOPC correspond to an energy barrier of $\Delta G = 1.8$ kJ/mol for the exchange of chlstn between POPC and PDPC and $\Delta G = 1.4$ kJ/mol for the exchange of chlstn between POPC and DOPC. That the free energy barrier for DOPC with just a single extra double bond in the sn-1 chain is only slightly less than PDPC with an extra five double bonds in the sn-2 chain implies that chol preferentially associates with the saturated sn-1 chain in mixed-chain phospholipids with PUFAs at the sn-2 position. This finding corroborates experimental and computer-modeling studies on saturated-polyunsaturated PC membranes. A tendency to locate next to the saturated stearoyl sn-1 chain was inferred from solid-state ^{2}H NMR spectra for [3α-$^2H_1$]chol revealing that the tilt angle of the sterol is insensitive to the presence of polyunsaturation at the sn-2 position (Brzustowicz et al. 1999), a view supported by a higher rate of chain-to-chol nuclear Overhauser enhancement cross-relaxation that was detected in ^{1}H MAS NMR experiments on 1-stearoyl-2-docosahexaenoylphosphatidylcholine (SDPC)/[25,26,26,26,27,27,27-$^2H_7$]chol membranes (Huster et al. 1998) and by molecular dynamics simulations on an SDPC/cholesterol bilayer in which the sterol prefers solvation by saturated over polyunsaturated chains (Pitman et al. 2004).

In summary, we have established a new method for measuring the relative affinity of chol for different lipids. The method is quick and straightforward. K_x values describing the partitioning of chlstn between LUVs and mβCD are directly determined from EPR spectra without the potentially artifact-inducing step of physically separating LUVs and mβCD fractions. The partitioning coefficient K_B^A between two lipids derived from the ratio of K_x obtained with the spin-labeled analog reliably reflects the relative affinity of chol for the lipids. Measurements made on phospholipids with differing degrees of acyl chain unsaturation demonstrate that chlstn prefers to associate with saturated chains and to avoid increasingly unsaturated chains, which is consistent with a propensity for lipids to be driven into membrane domains enriched or depleted in chol according to the compatibility of their molecular structure. Future work is planned to explore how affinity for chol is affected by other facets of the structure of phospholipid molecules such as the location and type of double bond.

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