Published in final edited form as:

Chem Phys Lipids. 2011 November ; 164(8): 819–829. doi:10.1016/j.chemphyslip.2011.08.001.

Using spin-label electron paramagnetic resonance (EPR) to discriminate and characterize the cholesterol bilayer domain

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Abstract

Electron paramagnetic resonance (EPR) spin-labeling methods make it possible not only to discriminate the cholesterol bilayer domain (CBD) but also to obtain information about the organization and dynamics of cholesterol molecules in the CBD. The abilities of spin-label EPR were demonstrated for Chol/POPC (cholesterol/1-palmitoyl-2-oleoylphosphatidylcholine) membranes, with a Chol/POPC mixing ratio that changed from 0 to 3. Using the saturation-recovery (SR) EPR approach with cholesterol analogue spin labels, ASL and CSL, and oxygen or NiEDDA relaxation agents, it was confirmed that the CBD was present in all membrane suspensions when the mixing ratio exceeded the cholesterol solubility threshold (CST). Conventional EPR spectra of ASL and CSL in the CBD were similar to those in the surrounding POPC bilayer (which is saturated with cholesterol), indicating that in both domains, cholesterol exists in the lipid-bilayer-like structures. The behavior of ASL and CSL (and, thus, the behavior of cholesterol molecules) in the CBD was compared with that in the surrounding POPC-cholesterol domain (PCD). In the CBD, ASL and CSL molecules are better ordered than in the surrounding PCD. This difference is small and can be compared to that induced in the surrounding domain by an ~10°C decrease in temperature. Thus, cholesterol molecules are unexpectedly dynamic in the CBD, which should enhance their interaction with the surrounding domain. The polarity of the water/membrane interface of the CBD is significantly greater than that of the surrounding PCD, which significantly enhances penetration of the water-soluble relaxation agent, NiEDDA, into that region. Hydrophobicity measured in the centers of both domains is similar. The oxygen transport parameter (oxygen diffusion-concentration product) measured in the center of the CBD is about ten times smaller than that measured in the center of the surrounding domain. Thus, the CBD can form a significant barrier to oxygen transport. The results presented here point out similarities between the organization and dynamics of cholesterol molecules in the CBD and in the surrounding PCD, as well as significant differences between CBDs and cholesterol crystals.

Keywords

cholesterol; cholesterol bilayer domain; membrane; spin label; EPR; lipid; fiber cell

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Introduction

In both model and biological membranes with a high cholesterol content, cholesterol structures (often called in the literature, *cholesterol crystalline domains*, *cholesterol crystallites*, or, simply, *cholesterol crystals*) were detected using X-ray and neutron diffraction (Borochoy et al., 1995; Cheetham et al., 1989; Knoll et al., 1985; Preston Mason et al., 2003; Wachtel et al., 1991), differential scanning calorimetry (DSC) (Borochoy et al., 1995; Epand, 2003; Wachtel et al., 1991), and magic angle spinning nuclear magnetic resonance (MAS NMR) (Epand, 2003; Guo and Hamilton, 1996) techniques. In the presence of phospholipids, these structures are formed only when the cholesterol content exceeds the cholesterol solubility threshold (CST) in phospholipid membranes. These structures possess the same properties as those found in one of three previously characterized triclinic crystal structures (formed without phospholipids) depending on the state of hydration and temperature. They show characteristic diffraction peaks (34 Å), either the polymorphic phase transition of the anhydrous form of cholesterol or the dehydration of the monohydrate form, and the characteristic C18 resonance doublet of cholesterol, all which were previously found for crystals (Loomis et al., 1979). Therefore, the consensus in the literature is that the structure of the cholesterol crystalline domain in membranes is the same as the structure of cholesterol crystals (with a pseudo-bilayer structure and a bilayer thickness of 34 Å).

In our previous work (Raguz et al., 2008, 2009), we introduced the EPR spin-labeling approach to characterize lens lipid-membranes extracted from six-month-old pigs and two-year-old cows. In the first case, because the cholesterol/phospholipid (Chol/PL) molar ratio was very low (about 0.7), we induced formation of pure cholesterol structures by adding an excess of cholesterol to an isolated lipid mixture to reach a Chol/PL molar ratio close to 2.0. In the case of lipids extracted from the cow eye-lens nucleus, the Chol/PL molar ratio was already close to 2.0. In both cases, using the discrimination by oxygen transport (DOT) method (Subczynski et al., 2007) with cholesterol analogue spin labels, we confirmed the presence of pure cholesterol structures in lens lipid-membranes. In these studies, we mainly focused on discriminating this new domain from the surrounding phospholipid-cholesterol bilayer and did not provide molecular-level information about the organization and dynamics of the pure cholesterol domain. In reviewing the literature, we first named this domain the “cholesterol crystalline domain (CCD)”. However, results presented in our recent paper (Raguz et al., 2011) indicate significant differences between the organization of cholesterol in domains (i) investigated by the EPR spin-labeling method and (ii) investigated by X-ray diffraction and DSC. Therefore, we have named domains that have been discriminated and characterized by the EPR spin-labeling method “cholesterol bilayer domains” (CBDs) to indicate their (dynamic) bilayer structure and difference from the (rigid) crystalline structure.

There is an explanation for these new results that does not contradict well-documented data from the literature. When the cholesterol content in the phospholipid bilayer exceeds the cholesterol solubility threshold (CST), both the CBD and cholesterol crystals form in the membrane suspension. The CBD is detected by the EPR spin-labeling method; cholesterol crystals are detected by DSC, X-ray diffraction, and MAS NMR. The organization of cholesterol molecules in CBDs is similar to that in phospholipid cholesterol domains surrounding the CBD (the order and dynamics are similar as well), and different from the rigid structure of cholesterol crystals (although the bilayer structure of the CBD and the pseudo-bilayer structure of cholesterol crystals indicate certain structural similarities). It is most probable that when the cholesterol content exceeds the CST, parts of cholesterol molecules form the CBD and parts form cholesterol crystals (see Ref. (Raguz et al., 2011) for further discussion).

In our recent studies (Raguz et al., 2011), we have made the first effort to obtain molecular-level information about the organization and dynamics of cholesterol in the CBD formed in POPS membranes with a very low CST (which take place at a Chol/POPS molar ratio of 0.5 (Bach, 1984; Bach et al., 2004; Bach and Wachtel, 2003; Wachtel et al., 1991). For our studies, we selected two spin-labeled cholesterol analogues: cholestane spin label (CSL) and androstane spin label (ASL) (see their structures in Fig. 1). It must be remembered that experiments carried out with probe molecules necessitate due caution in interpreting results; the labeled molecules cannot be expected to mimic all properties of cholesterol. Nevertheless, the interaction of CSL (or ASL) with phospholipid and/or cholesterol should, to a certain degree, approximate cholesterol-phospholipid and cholesterol-cholesterol interactions in the membrane domain because of the overall similarity in their molecular structures and phase behaviors in phosphatidylcholine-cholesterol membranes (Cadenhead and Muller-Landau, 1979; Muller-Landau and Cadenhead, 1979a, b).

In the work presented here, we applied both conventional and saturation-recovery (SR) EPR techniques with cholesterol analogue spin labels and hydrophobic and water-soluble relaxation agents to discriminate and characterize the CBD in the bilayer formed from a more biologically relevant phospholipid, namely 1-palmitoyl-2-oleoylphosphatidylcholine (POPC). The CST depends on many parameters, including the method of liposome preparation, lipid type, the length and degree of unsaturation of alkyl chains, the presence of charges on the lipid headgroup, and interheadgroup hydrogen bonds (Huang et al., 1999). To avoid traces of solvent (chloroform) in our samples (and due to the uncertainty of chloroform removal efficiency (Buboltz, 2009), we did not use the rapid solvent exchange method to prepare POPC liposomes with the CST at a Chol/POPC molar ratio of 2 (Huang et al., 1999). Instead, we used the film deposition method to prepare POPC liposomes, which showed the CST at a Chol/POPC molar ratio of 1 (Benatti et al., 2008; Borochoy et al., 1995; Bourges et al., 1967). A significant advantage of the film deposition method in the application of EPR spin-labeling to discriminate and characterize the CBD, which was demonstrated and described in detail in our previous paper (Raguz et al., 2011), is that the EPR signal of ASL and CSL from cholesterol crystals is so broad that it cannot be seen in conditions when the signal from CBD (and also PCD) is recorded. It is due to this fact only that EPR spectra were not contaminated by signal from cholesterol crystals. We aim to demonstrate the ability of EPR spin-labeling methods to obtain new information at the molecular level about the dynamics and organization of cholesterol in the CBD. In this sense, this paper focuses on methodology. Most unexpectedly, our results showed the high dynamics of cholesterol molecules in the CBD, which differ from the rigid organization of cholesterol molecules in cholesterol crystals. We believe this new molecular-level data will help us to better understand the physiological functions of cholesterol as well as the functions of CBDs.

2. Materials and methods

2.1. Materials

POPC and cholesterol were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). Cholesterol analogues, androstane spin label (ASL), and cholestane spin label (CSL) were purchased from Molecular Probes (Eugene, OR). Other chemicals, of at least reagent grade, were purchased from Sigma-Aldrich (St. Louis, MO).

2.2. Preparation of lipid bilayer membranes

The membranes used in this work were multilamellar dispersions of POPC and cholesterol (multilamellar liposomes) containing 1 mol% spin label. The membranes were prepared using the film deposition method as described by us earlier (Widomska et al., 2007a).

Chloroform solutions of POPC, cholesterol, and spin label were mixed to attain the desired mixing ratio. Chloroform was evaporated under a stream of nitrogen gas, and the lipid film on the bottom of the test tube was thoroughly dried under reduced pressure (about 0.1 mm Hg) for 12 h. A buffer solution (0.4 mL of 10 mM PIPES and 150 mM NaCl; pH 7.0) was added to the dried lipids at 40°C, vortexed vigorously and centrifuged to increase the signal-to-noise ratio.

In this paper, we use the term “mixing ratio” because the input molar ratio of cholesterol and phospholipids in lipid mixture before preparation of liposomes can be quite different from molar ratios in certain membrane domains after liposome preparation. Multilamellar liposomes are preferred in EPR investigations because the loose pellet after centrifugation contains a high amount of membranes (~20% lipid w/w), which significantly increases the signal-to-noise ratio.

2.3. EPR measurements

The membranes were briefly centrifuged, and the loose pellet was used for EPR measurements. The sample was placed in a 0.6 mm i.d. capillary made of gas-permeable methylpentene polymer, called TPX (Subczynski et al., 2005). The concentration of oxygen in the sample was controlled by equilibration with the same gas that was used for the temperature control (i.e., a controlled mixture of nitrogen and dry air adjusted with flowmeters [Matheson Gas Products, model no. 7631H-604]) (Hyde and Subczynski, 1989; Subczynski and Hyde, 1983).

Conventional EPR spectra were obtained with a Bruker EMX X-band spectrometer with temperature-control accessories. EPR spectra were recorded with a modulation amplitude of 1.0 G and an incident microwave power of 5.0 mW. The z -component of the hyperfine interaction tensor, A_z , for ASL and CSL in the membrane was determined directly from EPR spectra for samples frozen at about -165°C and recorded with a modulation amplitude of 2.0 G and an incident microwave power of 2.0 mW (Subczynski et al., 1994). $2A_z$ values can be measured within the accuracy of ± 0.25 G.

The validity of using $2A_z$ values for hydrophobicity measurements has been confirmed, as cited in Ref. (Subczynski et al., 1994). The strongest supporting evidence has been shown from the simulation of EPR spectra that have been measured for a variety of membranes in the fluid phase using magnetic parameters (including A_z) determined at rigid limit conditions (Earle et al., 1994; Kurad et al., 2003; Swamy et al., 2006). This method was also used earlier for simulation of ASL spectra in phospholipid bilayers (Pasenkiewicz-Gierula et al., 1990).

The spin-lattice relaxation times, T_{1s} , of the spin-labels were determined by analyzing the SR signal of the central line obtained by short-pulse SR EPR at X-band (Kawasaki et al., 2001; Subczynski et al., 1989; Yin and Subczynski, 1996). The SR spectrometer used in these studies was described previously (Subczynski and Hyde, 1983; Yin and Subczynski, 1996). Accumulations of the decay signals were carried out with 2048 data points on each decay. Fits were based on a damped linear least-squares method, which utilizes the Gauss-Newton minimization procedure which includes a scalar factor; this helps to “damp” the process toward the minimum and assures convergence in the iterative steps. The damped least-squares method has proven successful for fitting exponential decay curves (Laiken and Printz, 1970) and EPR spectra (Pasenkiewicz-Gierula et al., 1987). The uncertainties in the measurements of decay time from the fits are usually less than 0.5% whereas the decay times determined from sample to sample are within an accuracy of $\pm 3\%$.

2.4. Theory outline for evaluation of the oxygen transport and NiEDDA accessibility parameters

In dual-probe SR EPR experiments, small paramagnetic molecules, molecular oxygen, or NiEDDA are introduced in the membrane suspension as a relaxation agent. Through bimolecular collisions with nitroxides, these relaxation agents induce spin exchange, which leads to faster spin-lattice relaxation of nitroxides. Molecular oxygen is more soluble in the membrane phase than in water, and NiEDDA is soluble in water. The rate of bimolecular collision between molecular oxygen or NiEDDA and the nitroxide moiety of a spin label placed at a specific location in the membrane is evaluated from the T_1 s of the spin labels.

An oxygen transport parameter, $W(x)$, was introduced as a convenient quantitative measure of the collision rate between the spin label and molecular oxygen (Kusumi et al., 1982; Subczynski et al., 1989):

$$W(x) = T_1^{-1}(\text{Air}, x) - T_1^{-1}(\text{N}_2, x) = AD(x)C(x) \quad (1)$$

$W(x)$ is normalized to the sample equilibrated with air and is proportional to the product of the translational diffusion coefficient, $D(x)$, and the concentration, $C(x)$, of oxygen at a depth “ x ” in the membrane. To obtain the oxygen transport parameter, in principle, two SR measurements could be performed for the sample equilibrated with nitrogen and air (see Eq. (1)). However, to increase accuracy, SR measurements are carried out as a function of oxygen concentration (% air) in the equilibrating gas mixture, and extrapolation to 100% air is performed to obtain the value of the oxygen transport parameter. The oxygen transport parameter is evaluated as a mean value of a minimum of three independent experiments (for samples prepared totally independently) within the range of $\pm 5\%$.

In an analogy to the oxygen transport parameter defined in Eq. (1), we defined the NiEDDA accessibility parameter, $P(x)$, as (Subczynski et al., 1994):

$$P(x) = T_1^{-1}(20 \text{ mM NiEDDA}, x) - T_1^{-1}(\text{No NiEDDA}, x) \quad (2)$$

The NiEDDA accessibility parameter is proportional to the product of the local concentration and the local translational diffusion coefficient of NiEDDA at membrane depth “ x ”, at which the nitroxide moiety is located. Greater $P(x)$ values indicate a greater extent of NiEDDA penetration into the membrane. All accessibility measurements must be performed on deoxygenated samples. The NiEDDA concentration in buffer used for liposome preparation is 20 mM. The NiEDDA accessibility parameter is evaluated as a mean value of a minimum of three independent experiments (for samples prepared totally independently) within the range of $\pm 5\%$.

When located in two different membrane domains, a spin label alone most often cannot differentiate between the two, giving very similar conventional EPR spectra and similar T_1 values. However, even small differences in lipid-packing in these domains can affect partitioning and diffusion of the relaxation agent, which can be detected by observing different T_1 s from spin labels in these locations in the presence of the relaxation agent. Collisions with molecular oxygen (Eq. (1)), which can be quite different in different domains, form the basis of the previously developed DOT method (Subczynski et al., 2007). Here, we introduce a new method in which collisions with the water-soluble, paramagnetic nickel complex NiEDDA (which penetrates differently into different membrane domains) can discriminate these domains. Both methods are used in this study to detect and characterize coexisting PCDs and CBDs.

The major criteria for the goodness of the fit of the SR signals are the residual (the experimental signal minus the fitted curve) and the standard deviation. If substantial improvement in the fitting is not observed when the number of exponentials is increased from one, the SR signal can be analyzed as a single exponential. This is often the case for samples equilibrated with nitrogen. For samples containing membrane domains and in the presence of relaxation agents (oxygen or NiEDDA), fitting the experimental data to a single-exponential mode is often unsatisfactory, while a double-exponential fit can be excellent (good residual and smaller standard deviation; see Sect. 3.2), which is consistent with the existence of two immiscible domains. Additional criteria for the goodness of a fit are the pre-exponential coefficient for the second component (negligible for a single-exponential fit and significant [$\sim 5\%$ or larger] for a double-exponential fit), the standard deviation of T_1 for the second component (when this value is much larger than for the first component, it is a sign that a single-exponential fit is satisfactory; when values are comparable, a double-exponential fit can be satisfactory), and the repetition of the fit for different recording conditions such as number of points and time increment.

2.5. Calculation of the membrane oxygen-permeability coefficient

Knowledge of the profiles of the oxygen transport parameter makes it possible to calculate a significant membrane characteristic—namely, the oxygen permeability coefficient across the membrane. The oxygen permeability coefficient describes the oxygen flux across the lipid bilayer with the difference in oxygen concentration in water on each side of the bilayer. This method is based on the work of Subczynski et al. (Subczynski et al., 1989), and calculations for this method are explained in detail in our earlier papers (Subczynski et al., 1991; Widomska et al., 2007b).

3. Results and discussion

3.1. Does ASL detect the CBD?

Because the molecular structures of ASL and CSL are largely similar to that of cholesterol (Fig. 1), to a certain degree they should approximate the location and orientation of cholesterol molecules in the lipid bilayer. ASL and CSL polar groups should be located in the headgroup region of the bilayer, and their hydrophobic parts should protrude into the hydrocarbon-chain region toward the membrane center. However, the cholesterol analogue, ASL, possesses two polar ends, and, in principle, both can be located in a polar headgroup region. The $-OH$ group is more polar than the nitroxide moiety and is expected to be located in the headgroup region. However, there is a possibility for opposite orientation, especially when the lipid composition of the membrane changes. This presents the question of whether the results described in Refs. (Raguz et al., 2008, 2009) indicate formation of the CBD (Fig. 2A) or a different orientation of ASL (Fig. 2B). In the latter case, the nitroxide moiety of ASL should be located at the membrane surface (similar to the location of the nitroxide moiety of CSL). In both cases, SR signals recorded for ASL in the presence of oxygen should be double-exponential because the oxygen transport parameter is much greater in the membrane center than in the polar headgroup region (Raguz et al., 2008, 2009). To clarify this problem, the local hydrophobicity around the nitroxide moiety of ASL in Chol/POPC membranes (with a Chol/POPC mixing ratio between 0 and 3) was measured and compared with the local hydrophobicity around the nitroxide moiety of CSL (Fig. 2C). Hydrophobicity measured with ASL does not change with an increase of cholesterol content (even above the CST) when the CBD is formed. Measured $2A_z$ values, ~ 64.5 Gauss, are typical of nitroxide moieties located in a membrane center with a high cholesterol content (Subczynski et al., 1994). In contrast, hydrophobicity measured with CSL decreases with an increase in cholesterol content.

These results were expected since cholesterol molecules separate larger phospholipid headgroups, decrease their “umbrella” effect, and increase water penetration into the polar headgroup region where the nitroxide moiety of CSL is located (Subczynski et al., 1994). When the CBD is formed, further increase of cholesterol content above the CST causes further decrease in hydrophobicity. This is understandable because in the CBD the nitroxide moiety of CSL is fully exposed to the water phase. The difference between the values of $2A_z$ detected by ASL (~64.5 Gauss) and CSL (~70.0 Gauss) is so large that the orientation of ASL with the nitroxide moiety in the polar headgroup region (like CSL) should be easily detected at a high cholesterol content. Our results indicate that the nitroxide moiety of ASL is always located in the hydrophobic membrane center (independent of cholesterol content), and that ASL unambiguously detects two coexisting domains but not the distribution of its orientations (see also Sect. 3.2).

Using the procedure described in Sect. 3.5, we obtained rigid-limit EPR spectra for ASL and CSL from the CBD by subtracting the spectrum obtained for the Chol/POPC mixing ratio of 1 (with a weight of 0.33) from that obtained for the Chol/POPC mixing ratio of 3 (data not shown). Calculated $2A_z$ values for ASL and CSL in the CBD were 65.1 and 70.5 Gauss, respectively. These values were added to Fig. 2C to indicate the hydrophobicity of the CBD at interface and in its interior.

3.2. The oxygen transport and the NiEDDA accessibility parameters for cholesterol-analogue spin labels in membranes containing CBDs

Figure 3 shows representative saturation-recovery signals for ASL (Fig. 3A) and CSL (Fig. 3C) in Chol/POPC membranes with a mixing ratio of 3, at 25°C, in the presence and absence of the hydrophobic relaxation agent, oxygen. At this mixing ratio, the cholesterol content exceeds the CST in the POPC membrane threefold (Benatti et al., 2008; Borochov et al., 1995; Bourges et al., 1967), ensuring formation of the CBD. SR signals were fitted using single- and double-exponentials and compared. The single-exponential fit was satisfactory for both ASL and CSL in deoxygenated membranes (top residuals, Figs. 3A and C). For ASL in the presence of oxygen, the single-exponential fit was not satisfactory (middle residual, Fig. 3A), while the double-exponential fit was excellent (bottom residual, Fig. 3A). Spin-lattice relaxation times of the double-exponential curves obtained for ASL in the presence of oxygen were assigned to the bulk PCD (shorter time constant) and the CBD (longer time constant) (see Fig. 4 and Refs. (Raguz et al., 2008, 2009) for more detail). However, all SR signals obtained with CSL for membranes in the presence of oxygen were single exponentials (Fig. 3C). Because CSL should be distributed between the PCD and CBD similarly to cholesterol (as shown in Fig. 2A), we conclude that the collision rate between oxygen and the nitroxide moiety of CSL is the same in the PCD and CBD (see also the discussion in Ref. (Raguz et al., 2008)). Thus, the existence of the CBD can be confirmed using the DOT method and ASL—but not CSL.

To clarify the problem of why the DOT method can discriminate the CBD with the use of ASL but not CSL, we used the water-soluble relaxation agent NiEDDA. NiEDDA strongly affects spin-lattice relaxation of spin labels that have the nitroxide moiety at the membrane-water interface, like CSL. This new approach allows us to develop further the dual-probe SR method for discrimination of membrane domains. For measurements with relaxation agents other than oxygen, samples must be thoroughly deoxygenated.

Figure 3 also shows representative saturation-recovery signals for ASL (Fig. 3B) and CSL (Fig. 3D) in the presence and absence of NiEDDA. Measurements with CSL show that in the presence of NiEDDA, a single-exponential fit was not satisfactory (middle residual, Fig. 3D), while the double-exponential fit was excellent (bottom residual, Fig. 3D). Spin-lattice relaxation times of the double-exponential curves obtained for CSL in the presence of

NiEDDA were assigned to the bulk PCD (longer time constant) and the CBD (shorter time constant) (see also Fig. 4 for more explanation). Thus, the existence of the CBD can also be confirmed with CSL and NiEDDA as a relaxation agent.

All SR signals obtained with ASL for membranes in the presence of NiEDDA were single exponentials, suggesting that ASL in combination with NiEDDA cannot discriminate the CBD. Based on SR curves presented in Fig. 3B, we conclude that in both the PCD and CBD, NiEDDA does not penetrate to the depth at which the nitroxide moiety of ASL is located. These results are in agreement with hydrophobicity measurements for CSL and ASL (data presented in Fig. 2C), showing that hydrophobicity around the nitroxide moiety of CSL decreases at high cholesterol contents and is always very high around the nitroxide moiety of ASL (comparable to that of hexane).

For ASL and CSL in fluid-phase lipid membranes and in the absence of relaxation agents, T_1 typically falls in the range of 3 to 4 μs , but can be as low as 0.1 μs (lower limit for SR measurements) in the presence of air or NiEDDA. The signal-to-noise ratio is typically good enough that changes as small as 10% can be measured. Thus, the upper limit of the time scale of the discrimination method, which is based on the collision of ASL/CSL with oxygen or NiEDDA, is approximately 30 μs . Thus, the upper limit for the exchange rate of spin labels between the CBD and the PCD, which can average EPR results, is $3.3 \times 10^4 \text{ s}^{-1}$.

3.3. Discrimination of the CBD using cholesterol-analogue spin labels and the relaxation agents, oxygen or NiEDDA

The dual-probe SR EPR spin-labeling method was first applied to discriminate the CBD in pig- and cow-lens lipid membranes with a Chol/PL molar ratio of ~ 2 (Raguz et al., 2008, 2009). The DOT method using ASL clearly shows the existence of two domains in these membranes, which were assigned to the bulk PCD and the immiscible CBD. To clarify a few methodological questions (see Sect. 3.1) and further develop the method of discriminating the CBD, we performed experiments on a simple membrane, in which the phospholipid component was known and the cholesterol content was manipulated in a controlled manner. Therefore, we used Chol/POPC membranes with a mixing ratio between 0 and 3 to ensure that measurements would be performed for mixing ratios greater than the CST (even if the CST is not 50 mol% (Benatti et al., 2008; Borochoy et al., 1995; Bourges et al., 1967) but as high as 66 mol% (Huang et al., 1999)). SR measurements were performed as described in Sect. 3.2. The oxygen transport and NiEDDA accessibility parameters were calculated as described in Sect. 2.4. The final results are presented in Fig. 4. We were able to detect two domains when the Chol/POPC mixing ratio was 1.5 or greater, which confirmed that the CST in the POPC bilayer was ~ 50 mol% for our preparations using the film deposition method. Measurements performed for a wide range of cholesterol contents confirmed assignment of SR results presented in Sect. 3.2 to the PCD and CBD. Results presented in Fig. 4A indicate that the oxygen transport parameter in the center of the CBD (at the position of the nitroxide moiety of ASL) is about three to five times smaller than in the center of the PCD. Although CSL data show a single value for the oxygen transport parameter for all cholesterol contents, this does not mean that CSL detects a single homogeneous domain. Data indicate that the collision rate between the nitroxide moiety of CSL and oxygen in both domains is the same. Thus, the value obtained with CSL can be used for both the PCD and CBD to make profiles of the oxygen transport parameter and to calculate permeability coefficients for oxygen across these domains. These statements were confirmed by measurements with NiEDDA, which showed that CSL (1) is located in the PCD and CBD and (2) can discriminate these domains using the NiEDDA accessibility parameter (Fig. 4B). As shown in Fig. 4B, the nitroxide moiety of CSL is more exposed to collisions with NiEDDA when it is located in the CBD and its nitroxide moiety is not protected by the “umbrella” effect of phospholipid headgroups (as in the PCD). These

results are in agreement with hydrophobicity measurements presented in Fig. 2C, which show that the polarity around the nitroxide moiety of CSL increases when the cholesterol content increases beyond the CST.

Both methods (with hydrophobic oxygen and water-soluble NiEDDA relaxation agents) can provide complementary information about the lateral organization and domain properties of the membrane. In a recent paper (Raguz et al., 2011), we first demonstrated that the water-soluble relaxation agent, NiEDDA, could discriminate membrane domains in conditions where the DOT method could not. Thus, the dual-probe SR method of discriminating membrane domains can be significantly broadened and strengthened with the use of hydrophobic and hydrophilic relaxation agents. This new approach can also be used with other spin labels such as n-PCs and n-SASLs.

3.4. Profile of the oxygen transport parameter and evaluation of the oxygen permeability coefficient across the CBD

Figure 5 shows profiles of the oxygen transport parameter across coexisting PCDs and CBDs that were obtained using the DOT method with phospholipid- and cholesterol-analogue spin labels for Chol/POPC membranes. Because the PCD coexists with the CBD, the PCD is always saturated with cholesterol. The profile across the PCD (obtained with the use of phospholipid-analogue spin labels, which are located exclusively in the PCD (Raguz et al., 2008, 2009)) has a characteristically rectangular shape with an abrupt increase in the oxygen transport parameter between C9 and C10. This shape is typical for phospholipid bilayer membranes saturated with cholesterol and is practically independent of phospholipid composition (Raguz et al., 2008, 2009; Widomska et al., 2007a). Values of the oxygen transport parameter obtained with ASL and CSL were also added to the profile across the PCD. Exact locations of these values on the profile confirm assignments made in Sect. 3.2 and also indicate that the location of the nitroxide moiety of ASL in the PCD bilayer is the same as the location of the nitroxide moiety of 10-PC. Another set of values of the oxygen transport parameter obtained with ASL and CSL (see Sect. 3.2) was used to draw a profile of the oxygen transport parameter across the CBD (Fig. 5). It should be noted that the positions of the nitroxide moieties of ASL and CSL in the CBD are shifted toward the membrane center. A “sinking” of cholesterol molecules was evaluated in Ref. (Raguz et al., 2008) as about four C-C bonds deeper compared to cholesterol in the PCD. Values of the oxygen transport parameter in the CBD are significantly lower than values in the PCD and in water.

Based on profiles of the oxygen transport parameter across the CBD and PCD (Fig. 5), permeability coefficients for oxygen across these domains were evaluated as 15.9 and 34.6 cm/s, respectively. Oxygen permeability across the CBD is also significantly lower than across a water layer of the same thickness (67.7 cm/s). Therefore, we can conclude that the CBD can be a barrier to oxygen transport compared to transport across water and the surrounding PCD. The permeability coefficient for oxygen across the CBD is the bulk physical property of this domain. However, it was obtained based on the profile of the oxygen transport parameter describing molecular-level events — namely, collisions between molecular oxygen and the nitroxide moiety of cholesterol analogue spin labels (Widomska et al., 2007b).

3.5. Properties of the CBD: Information derived from conventional EPR spectra

Figure 6 shows a panel of conventional EPR spectra for cholesterol-analogue spin labels ASL and CSL in Chol/POPC membranes with a mixing ratio from 0 to 3. Because of the use of the film deposition method, the CST in the POPC membrane is 1. With a Chol/POPC mixing ratio of 3, about 33% of cholesterol molecules should saturate the POPC bilayer

forming the PCD, and 66% should form the CBD and other pure cholesterol structures (presumably, cholesterol crystals that are not detected by the EPR method with ASL and CSL (Raguz et al., 2011). With this assumption, we can obtain EPR spectra of ASL and CSL from the CBD by subtracting the signal obtained for the Chol/POPC mixing ratio of 1 (with a weight of 0.33) from that obtained for the Chol/POPC mixing ratio of 3 (see Ref. (Raguz et al., 2011) for more detail). These EPR spectra are included in Fig. 6 and are indicated as “CBD(1)”. There are three remarkable features of these spectra: (1) There is no clear indication of the presence of two components in the EPR spectra even at the highest cholesterol content employed in this work. (2) All spectra are characteristic of spin labels in lipid-bilayer-like structures. (3) Changes to the overall shape of ASL and CSL spectra that occur after the addition of cholesterol to the POPC membrane up to the CST indicate a significant increase in molecular order and decrease in fluidity of the lipid bilayer. Further addition of cholesterol (above the CST) affects the overall shape of the spectra much less. Changes in spectra were evaluated through sets of parameters, maximum splitting (Fig. 7A, parameter related to the order parameter that indicates the amplitude of the wobbling motion of the long axes of the ASL and CSL molecules (Kusumi et al., 1986)), and the h_+/h_0 ratio (Fig. 7B, parameter that includes contribution of both the orientation and rotational mobility of the ASL and CSL molecules (Schreier et al., 1978)). Because of the sharpness of the EPR lines maximum splitting values can be measured with the accuracy of ± 0.1 G and h_+/h_0 values with the accuracy of ± 5 %.

The results presented here allow us to conclude that in a wide range of cholesterol contents, ASL and CSL are located in the lipid-bilayer-like environment. ASL and CSL molecules are better ordered in the CBD than in the PCD (Figs. 6 and 7A). However, differences between the maximum splitting values of CBD EPR spectra and appropriate values from PCD spectra are slight (Fig. 7A) and can be compared to the effect of an $\sim 10^\circ\text{C}$ decrease in temperature for the ASL and CSL maximum splitting values in the PCD (data not shown). The maximum splitting values are much smaller than those for rigid limit conditions ($2A_Z$ values for ASL and CSL in CBD, as shown in Fig. 2). Similarly, spectral parameter h_+/h_0 , which describes both the order and dynamics of ASL and CSL molecules (Fig. 7B), indicates an increase in the rate of motion in the CBD compared to that in the PCD. This finding is in agreement with SR measurements presented in Sect. 3.6.

Because the nitroxide moiety of ASL and CSL is firmly connected to the rigid sterol-ring structure of cholesterol, its orientation and rotational motion reflect that of the rigid-ring structure of cholesterol. Thus, the above results demonstrate that cholesterol molecules in the CBD and PCD behave in the same way, showing a similar order and rate of rotational motion. Therefore, these cholesterol molecules cannot be discriminated by conventional EPR spectra or even by SR EPR measurements without a relaxation agent (see Sect. 3.6). Because of the uncertainty about the CST in POPC membranes, we performed a similar procedure to obtain EPR spectra of ASL and CSL in the CBD assuming the CST at a Chol/POPC mixing ratio of 2 (data not shown). In this case, at a Chol/POPC mixing ratio of 3, about 66% of cholesterol molecules should saturate the POPC bilayer forming the PCD, and 33% should form the CBD and cholesterol crystals. EPR spectra and spectral characteristics were similar to those described above, showing that the conclusion will be valid even if the CST in the POPC membrane occurs at a Chol/POPC molar ratio of 2.

3.6. Organization and dynamics of the CBD: Information derived from SR EPR

Our results clearly show that when located in the PCD and CBD, cholesterol analogue spin labels alone cannot differentiate between these domains, giving very similar conventional EPR spectra (Fig. 6) and similar T_1 values (Fig. 3). However, differences in lipid-packing and hydrophobicity in these domains affect partitioning and diffusion of relaxation agents (hydrophobic molecular oxygen and polar NiEDDA), which can be detected by observing

different T_1 s of ASL and CSL in these locations in the presence of relaxation agents (Figs. 3 and 4). Discrimination methods not only allow one to distinguish between different domains but also to obtain values of the oxygen diffusion-concentration product and NiEDDA accessibility parameter in these domains, which are useful physical characteristics of the organization of lipids. These measurements provide a good example to demonstrate the abilities and limitations of dual-probe SR EPR spin-labeling methods. Although the rate of bimolecular collisions between the nitroxide moiety of a lipid-type spin label placed at a specific location in the membrane and small paramagnetic probe molecules is a useful monitor of membrane fluidity that reports on translational diffusion of small molecules, it indicates little about the dynamics of lipid molecules, including the motion of phospholipid alkyl chains and the steroid ring of cholesterol.

Fortunately, the spin lattice relaxation time (measured for samples without relaxation agents) is a spectral parameter that depends primarily on the rate of motion of the nitroxide moiety within the lipid bilayer (Subczynski et al., 2010). Thus, T_1 can be used as a convenient quantitative measure of the rate of spin-label motion (Subczynski et al., 2010), in our case, the rate of motion of ASL and CSL in the POPC lipid bilayer. Figure 7C shows T_1 values for ASL and CSL in POPC membranes as a function of the Chol/POPC mixing ratio. There are two remarkable features of these data: (1) There is no indication of two components in the SR signals, even at the highest cholesterol content, when the coexisting domains, CBD and PCD, are observed. Thus, T_1 values in both domains must be very close, indicating a similar rate of motion for cholesterol analogue spin labels. (2) An increase (up to the CST) in T_1 values that occurs after the addition of cholesterol to the POPC membrane indicates a decreased motion of cholesterol analogues. After further addition of cholesterol (above the CST), T_1 values decrease again, practically to their beginning values, indicating that the apparent rate of motion of ASL and CSL increases even after formation of the CBD. The overall change in T_1 values is small, not greater than 0.4 μ s, which can be compared to a change of ~ 1.0 μ s induced by a 10°C change in temperature. This unexpected result suggests that the rate of motion of cholesterol analogues in the CBD is greater than in the PCD. Because all types of motion can affect T_1 values, we hypothesize that the increase of the rotational motion of ASL and CSL in the CBD about their long axes contributes to decreasing T_1 s, which is also in agreement with data presented in Fig. 7B. Also, here the conclusion is valid independently if the CST in POPC membranes is 50 mol% or 66 mol%.

Conclusions

The results presented here were obtained with EPR spin-labeling methods that allowed us not only to discriminate the CBD in the POPC membrane but also to gather information about the structure and dynamics of the CBD on the molecular level. We conclude that the CBD is formed by cholesterol molecules organized in a bilayer-like structure with order and rotational motion of cholesterol molecules similar to that in surrounding PCD. However, *similar* does not mean the *same*. We indicated higher order and similar (or even higher) rotational motion, but these differences are small. To the best of our knowledge, this is the first report in addition to our recent paper (Raguz et al., 2011), which emphasize the high dynamics of the CBD. The greatest difference between the PCD and CBD is manifested by the different solubility and movement of small hydrophobic (molecular oxygen) and polar (NiEDDA) molecules. The accessibility of water-soluble molecules to the polar headgroup region of the CBD is much greater than to the PCD. In contrast, the solubility and movement of hydrophobic oxygen in the membrane center is greater in the PCD than in the CBD.

We would like to comment on the CST in phospholipid bilayers, following a similar discussion by (Pata and Dan, 2005). They presented schematic phase diagram of phospholipid-cholesterol bilayer and indicated in it that when the cholesterol concentration

in the phospholipid bilayer is increased to its solubility limit (called here the CST), the phospholipid bilayer becomes saturated with cholesterol. Above this concentration, excess cholesterol will precipitate as crystals of pure cholesterol monohydrate (Huang et al., 1999), assuming that nucleation takes place outside the bilayer (Bach and Wachtel, 2003; Huang et al., 1999). Alternatively, excess cholesterol can nucleate into pure cholesterol structures within the phospholipid bilayer and exist there as cholesterol domains that coalesce and precipitate into solution over time as cholesterol crystals (Epand et al., 2003; Troup et al., 2003). It was suggested that the pure cholesterol structures form the immiscible cholesterol domains (called here CBDs) coexisting with the liquid-ordered phase (Troup et al., 2004). In their hypothetical phase diagram, these coexisting phases precede formation of cholesterol crystals, which are formed when the cholesterol concentration exceeds the CST. The location of the phase boundary separating the liquid-ordered phase from the coexisting liquid-ordered phase and CBDs is not clear. (Troup et al., 2004) positioned it at ~33 mol% cholesterol, well below the CST. In our recent paper (Raguz et al., 2011), using EPR spin-labeling approaches and the film deposition method for liposome preparations, we were unable to discriminate between the CST (above which cholesterol crystals are formed) and the phase boundary indicating cholesterol concentration above which CBDs are formed. It is very probable that the CBD is formed first. Only after that, when the cholesterol content exceeds the total amount of cholesterol that can be supported by phospholipids in the form of a bilayer (as cholesterol in the PCD and in the CBD), cholesterol crystallization (presumably outside the membrane) will occur. Based on our results and data from the literature, we cannot yet differentiate between these two possibilities.

In this paper, we directed our attention on the methodology of applying EPR spin-labeling techniques to discriminate the CBD and PCD and to study the organization and dynamics of these domains on a molecular level. We would like to direct readers to our earlier paper where other aspects are discussed (Raguz et al., 2011).

Acknowledgments

This work was supported by grants EY015526, TW008052, EB002052, and EB001980 of the National Institutes of Health.

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Research highlights

EPR spin-labeling methods: discrimination of cholesterol bilayer domains

Molecular level information on cholesterol bilayer domains

Unexpectedly high mobility of cholesterol molecules in cholesterol bilayer domains

Fig. 1. Chemical structures of cholesterol analogue spin labels used in this work: ASL and CSL. Chemical structures of POPC and cholesterol are also included to indicate approximate locations of these molecules across the phospholipid bilayer membrane.

Fig. 2.

(A) Schematic drawing of the distribution and approximate locations of ASL and CSL in domains formed in the POPC membrane when the cholesterol content exceeds the CST. (B) Drawing of the normal and purported “upside-down” orientations of ASL in the PCD that can also explain the appearance of double-exponential SR signals of ASL in the presence of molecular oxygen. The nitroxide moieties of spin labels in (A) and (B) are indicated by black dots. (C) $2A_z$ values, which indicate local hydrophobicity around nitroxide moieties, for ASL and CSL in Chol/POPC membranes plotted as a function of the Chol/POPC mixing ratio (filled symbols). Open symbols indicate $2A_z$ values for ASL and CSL in the CBD. Upward changes indicate an increase in hydrophobicity. The broken line indicates the CST in the POPC membrane.

Fig. 3.

Representative SR signals with fitted curves and residuals (the experimental signal minus the fitted curve) for ASL (A, B) and CSL (C, D) in Chol/POPC membranes with a Chol/POPC mixing ratio of 3. Signals were recorded at 25°C for samples equilibrated with 100% nitrogen (A, B, C, D); with a gas mixture of 50% air and 50% nitrogen (A, C); and in the presence of NiEDDA (B, D). SR signals were satisfactorily fitted to a single-exponential function in the absence of molecular oxygen with time constants of $3.2 \pm 0.01 \mu\text{s}$ (A), $3.2 \pm 0.01 \mu\text{s}$ (B), $3.72 \pm 0.01 \mu\text{s}$ (C), and $3.72 \pm 0.01 \mu\text{s}$ (D) (the upper residuals in A, B, C, and D are for a single-exponential fit). The SR signal in the presence of molecular oxygen can be fitted satisfactorily with a single-exponential function only for CSL with a time constant of $2.34 \pm 0.01 \mu\text{s}$ (C) (lower residual in C is for a single-exponential fit), and with double-exponential curves for ASL with time constants of $2.53 \pm 0.01 \mu\text{s}$ and $0.67 \pm 0.01 \mu\text{s}$ (A) (middle residual in A is for single- and lower residual for double-exponential fits). The SR signal in the presence of NiEDDA can be fitted satisfactorily with a single-exponential function only for ASL with a time constant of $3.18 \pm 0.01 \mu\text{s}$ (B) (lower residual in B is for single-exponential fit) and with double-exponential curves for CSL with time constants of

$3.23 \pm 0.1 \mu\text{s}$ and $1.31 \pm 0.01 \mu\text{s}$ (D) (middle residual in D is for single- and lower residual for double-exponential fits).

Fig. 4.

The oxygen transport parameter (A) and NiEDDA accessibility parameter (B) for ASL and CSL in Chol/POPC membranes plotted as a function of the Chol/POPC mixing ratio. Values of these parameters in coexisting PCD and CBD are indicated. Broken lines indicate the CST in the POPC membrane.

Fig. 5.

Profiles of the oxygen transport parameter (oxygen diffusion-concentration product) at 25°C across certain domains in Chol/POPC membranes with a mixing ratio of 3. Dotted lines indicate the oxygen transport parameter in the aqueous phase. Approximate localizations of nitroxide moieties of spin labels are indicated by arrows. Data obtained with phospholipid-type spin labels (■) were taken from Ref. (Widomska et al., 2007a). Data obtained with ASL and CSL in this work are indicated by open symbols for PCD (□) and CBD (○).

Fig. 6. EPR spectra of CSL and ASL in membranes made of Chol/POPC mixtures. Spectra were recorded at 25°C for different Chol/POPC mixing ratios. “CBD” indicates EPR spectra of CSL and ASL in the CBD after subtraction of the EPR spectra of the PCD (assuming a CST of 1) (see Sect. 3.5 for details of this calculation). Measured values, maximum splitting, and the mobility parameter, h_+ / h_0 , are indicated.

Fig. 7.

Spectral parameters, maximum splitting (A), and the mobility parameter, h_+/h_0 (B), measured for the EPR spectra shown in Fig. 6 are plotted as a function of the Chol/POPC mixing ratio. Filled symbols indicate experimental data for ASL and CSL in Chol/POPC membranes, and open symbols indicate values obtained for CBD spectra. (C) T_1 values for ASL and CSL measured in deoxygenated samples are plotted as a function of the Chol/POPC mixing ratio. Broken lines indicate the CST in the POPC membrane.