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EPR-GHOST CHARACTERIZATION OF SPIN LABELED ALKYLPHOSPHOLIPID LIPOSOMES WITH DIFFERENT CONCENTRATION OF CHOLESTEROL

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Characterization of biological membranes is important to understand physiological aspects of cellular functions. Liposomal bilayers can be used as model system, in which the composition can be varied easily in order to follow the effect of a particular lipid component on the membrane domain structure and modes of molecular motions within these domains. Electron paramagnetic resonance (EPR) together with spectral simulation is one of the methods by which it can be obtained. The number of spectral parameters needed for the characterization of EPR spectra increases substantially due to the membrane heterogeneity. The problem of accurate determination of EPR spectral parameters, together with personal influence of the spectroscopist, therefore implies the usage of an automatic optimization procedure. In this work a newly developed characterization procedure (hybrid evolutionary optimization HEO and GHOST condensation method) was applied to the EPR spectra of alkylphospholipid liposomes, with different concentration of cholesterol (CH). The results were compared to those obtained previously for the same system, as well as to the theoretical predictions of possible membrane structure in presence of cholesterol and the measurements obtained by other authors. This is the first time that the HEO-GHOST method was applied to characterize the membrane properties of liposomes in more detail. For this purpose a four-spectral-component model was used to simulate the EPR spectra and HEO procedure was applied 200 times. The solutions are presented on GHOST diagrams as the basis for determination of spectral parameters and relative proportion of spin probe motional and polarity modes within the domains. It was found that OPP liposomes with less than 45% of CH can be described with four modes of spin probe motions. An increase in CH resulted in a shift of solutions to higher ordering of the membrane domains and increased proportion of the most ordered domain with complementary disappearance of one of the disordered domains. Based on these results we assume that domains with random lipid distribution coexist with domains with regular lipid distribution in OPP liposomes with CH concentration below 0.45 mol%.

INTRODUCTION

Cell membranes represent a selective barrier for transport of ions and molecules into and out of cells. Besides that most of the biochemical and biophysical events happen in cell membranes (Shinitzky, 1984). In the Singer-Nicolson fluid mosaic model a membrane is an oriented, two dimensional, viscous solution of amphiphatic proteins and lipids in a thermodynamic equilibrium (Singer & Nicolson, 1972). In this model proteins and lipids are randomly distributed and are dynamically rearranging via Brownian motion. In the recently proposed "dynamically structured mosaic model", emphasis is shifted from fluidity to mosaicism (Vereb, Szollosi, Matko, Nagy, Farkas, Vigh, Matyus, Waldmann & Damjanovich, 2003). This model takes into account that specific kinds of membrane proteins form small-scale clusters at the molecular level and large-scale groups of clusters at submicrometer scale (domains). It is now being thought that the fluid properties of the membrane permit a dynamic restructuring of molecular clusters according to the needs of the cell, dependent on the environment. Free diffusion of membrane components proposed by the Singer Nicolson model still holds but only within domains. Rafts are an example of such membrane domains (Simons & Ikonen, 1997). In general lipid and protein mobility is far from being unrestricted.

Lateral lipid domain structure is a possibility how mosaicism can restrict free diffusion of molecules in the membrane. Recently it was proposed that lipids tend to adopt regular distributions in fluid mixed bilayers, where distribution of lipid components depends on the shape of molecules and the charge of lipid headgroups (Somerharju, Virtanen & Cheng, 1999; Virtanen, Cheng & Somerharju, 1998; Chong & Sugar 2002). These regular structures are in dynamic equilibrium with areas in which lipids are distributed randomly

Table 1: Composition of OPP liposomes

Name	$R_{CH/OPP}$[1]	Molar ratios OPP:CH:DCP	Percent of CH
N15	1.5	10:15:2	29
N12	1.2	10:12:2	38
N10	1	10:10:2	43
N9	0.9	10:9:2	45
N7.5	0.75	10:7:2	50
N5	0.5	10:5:2	56

Abbreviation used: [1] Molar ratio of CH to OPP.

(Wang, Sugar, Chong, 1998; Parker, Miles, Cheng & Huang, 2004).

One of the important aspects of phase behavior within the two-dimensional membrane is the gel-liquid crystal phase transition. Depending on the lipid composition and temperature typical arrangement of molecules is liquid crystal state in coexistence with some regions, which are in gel states. Both states can have many different molecular arrangements and mobility characteristics. The phase transition is a first order phase transition with a discontinuity – a sharp change in measurable quantity such as the order parameter or the mobility of molecules or lipid chains, which changes with composition of lipids in the membranes (Lasic, 1993). For example, calorimetric studies showed only one phase transition in lipid bilayers of pure palmitoylsphingomyelin (Bruzik, 1987) while by ^{31}P-NMR spectroscopy four distinct gel phases were identified. This indicates that phase behavior of a simple system consisting of only one kind of lipids can be already very complex (Bruzik, Sobon & Salamonczyk, 1990).

Typical time in which lipid molecule exchanges its place with one of its neighbors in the membrane is around 10^{-7} s. Since the sensitivity of fluorescence method is on a nanosecond time scale it was possible to experimentally detect the existence of lateral domains with regular distributions of lipid and cholesterol molecules by this method (Liu, Sugar & Chong, 1997; Cannon, Heath, Huang, Somerharju, Virtanen & Cheng, 2003). Electron paramagnetic resonance (EPR) is sensitive on a similar time scale. However, by this method it is possible to detect not only different types of domains and the distribution of the probe within these types of domains, but also different modes of molecular motions of the corresponding spin probe within the same domain. In latter case different modes of spin probe motions originate from different environments of the probe within the particular domain.

In this article we present results obtained with the newly developed characterization based on EPR spectra analysis (Štrancar, Koklic & Arsov, 2003; Štrancar, Koklic, Arsov, Filipič, Stopar & Hemminga, 2004). It provides a deeper insight into the membrane domain structure and motional modes of spin-label molecules on the nanosecond time scale and more valuable description as could be obtained by other optimization procedures (Simplex Downhill, simple genetic algorithm, or their combination; Koklic, Šentjurc & Zeisig, 2002). The example will be presented how increasing cholesterol concentration influences the properties of liposome membranes composed of alkylphospholipids, cholesterol and dicetylphosphate. Liposomes were used in this study because they represent a good model for investigation of bilayer propeties. It is also easy to change the composition of liposomes and follow the changes in lateral lipid organization of the membrane. OPP liposomes were already studied by spectral decomposition method, with combined genetic algorithm and simplex method optimization procedure (Koklic *et al.*, 2002). Therefore it would be possible to compare the data obtained by the two optimization procedures and the information obtained with newly developed procedure.

MATERIALS AND METHODS

Materials

Octadecyl-(1,1-dimethyl-piperidino-4-yl)-phosphate (OPP) was a generous gift from Dr. Hilgard (ASTA Medica, Frankfurt, Germany). Dicetylphosphate (DCP) and cholesterol (CH) were obtained from Serva (Heidelberg, Germany). Dichloromethane and methanol from Merck AG (Darmstadt, Germany) were used in Lichrosolv® gradient grade quality. The spin probe 5-doxylpalmitoyl methyl ester (MeFASL(10,3)) was synthesized by Dr. Pečar (Faculty of Pharmacy, University of Ljubljana, Ljubljana, Slovenia).

Liposome preparation

Liposomes used in this study consist of OPP, CH and a charged component – dicetyphosphate (DCP). The composition of liposomes prepared is described in the Table 1.

Multilamellar liposomes (MLV) with OPP concentration of 10 mM were prepared by the thin film/hydration method from appropriate mixtures of stock solutions of the components in CH_2Cl_2/MeOH, (7:3, v/v). Rotary evaporation of the solvent resulted in a thin lipid film, which was hydrated with phosphate buffer saline (PBS) at

40°C. The resulting MLV suspension was further shaken for 12 hours at room temperature. Finally, large unilamellar liposomes (LUVET) were prepared from these MLV as described previously (Zeisig, Eue, Kosch, Fichtner & Arndt, 1996) by repeated extrusion through polycarbonate filters (diameter of pores: 100 nm) using a LiposoFast^{tm} Basic System (Avestin, Inc. Ottawa, Canada) until the suspensions were unimodal (19-29 times). Composition of liposomes was determined with high performance thin layer chromatography (HPTLC) method according to the procedure described elsewhere (Zeisig, Arndt, Stahn & Fichtner, 1998). Each LUVET liposomal formulation was prepared and measured at least three times.

EPR measurements

For EPR measurements liposomes were spin labeled with the lipophilic spin probe MeFASL(10,3) in a molar ratio of MeFASL(10,3) to all other liposome components in the membrane of 1/600 (mol/mol) in the following way: a thin film of MeFASL(10,3) was prepared on the wall of a glass tube by rotary evaporation of an ethanol solution of the spin probe. The liposome solution was then added to the tube and vortexed at room temperature for 2 minutes. After that the samples were transferred into the glass capillary for EPR measurements, which were performed on an X-band EPR spectrometer Bruker ESP 300 at room temperature.

Computer simulation of EPR spectra

Generally, to describe the EPR spectra of spin labels, the stochastic Liouville equation is used (Budil, Lee, Saxena & Freed, 1996; Robinson, Thomann, Beth, Fayer & Dalton, 1985; Schneider & Freed, 1989). However, in a membrane system labeled with fatty acid spin probes, measured at room temperature, local rotational motions are fast with respect to the EPR time scale. Modeling of the spectra taken at physiological temperature is therefore simplified by restricting the motions to the fast motional regime. Since the basic approach was already discussed elsewhere (Štrancar, Šentjurc & Schara, 2000; Schindler & Seelig, 1973) it is only summarized here. The model takes into account that the spectrum is composed of several spectral components reflecting different modes of restricted rotational motion of spin probe molecules in different environments of the membrane, described with different sets of spectral parameters: order parameter (S), rotational correlation time (τ_c), polarity correction factors of hyperfine and g tensors (p_A and p_g) and broadening constant (W). S is related to time averaged amplitude of rotational motion of nitroxide group relative to its average direction. ($S = 1$ for perfectly oriented

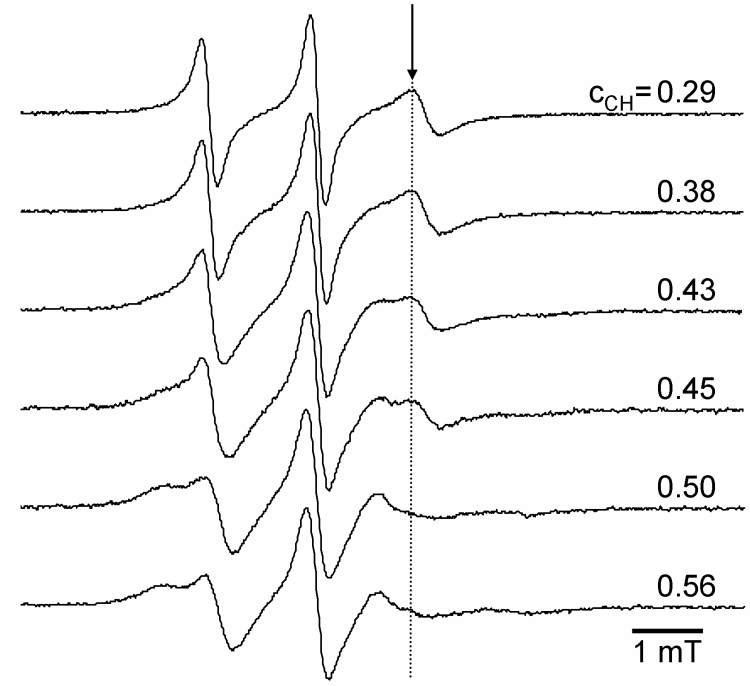

Fig. 1: EPR spectra of lipophilic spin-probe methyl ester of 5-doxylpalmitate (MeFASL(10,3)) in the membrane of OPP liposomes with different molar ratios of cholesterol to OPP (the amount of cholesterol is indicated in mol percent, at the right side of each spectrum). The arrow is pointing to a peak that is vanishing with an increasing amount of cholesterol in the liposome membrane.

molecules and $S = 0$ for isotropic motion of molecules), τ_c describes the rate of motion, polarity corrections are due to the effect of neighboring electric fields, which influence the electron density distribution of the spin probe and W arises primarily from unresolved hydrogen super-hyperfine interactions and contributions from other paramagnetic impurities (e.g., oxygen, which is usually present), external magnetic field inhomogeneities, and field modulation effects, as well as from spin-spin interaction. It is important to note, that the line broadening can differ among the domains due to different partitioning of spin probes and/or oxygen in different regions of the membrane. Besides, the relative proportion of a particular spectral component d is determined. It describes the relative amount of the spin probes with particular motional mode and depends on the distribution of the spin probe between the domain types as well as on distribution and position of the spin probe within the domain. It should be stressed that the lateral motion of the spin probe is slow on the time scale of EPR spectra (Träuble & Sackmann, 1972, Johnson, Berk, Blankschtein, Golan, Jain & Langer, 1996). Therefore an EPR spectrum describes only the properties of a spin label's nearest surrounding. The computer simulation procedure is implemented by means of the software package EPRSIM (http://www.ijs.si/ijs/dept/epr/).

GHOST condansation procedure

To obtain best fit of calculated to experimental spectra the deterministic and robust optimization method like Simplex Downhill can be applied. It provides good results only if starting points are close to the solutions (Štrancar *et al.*, 2000). This inherently leads to the convergence into a local rather than in global minimum. To eliminate these problems the stochastic and population-based genetic algorithm is used. This requires no special starting points and no user intervention. It is good at finding promising regions in complex search space. When combined with Simplex Downhill and knowledge-based operators into evolutionary optimization method (HEO) (Filipič & Štrancar, 2001) it is also capable of fine-tuning.

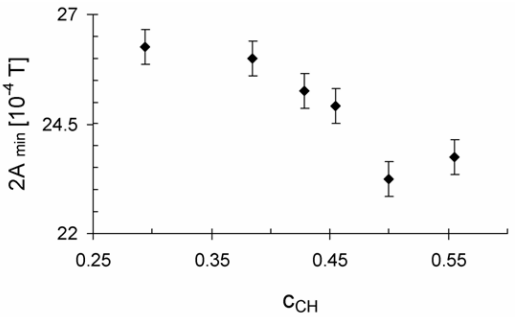

Fig. 2: Minimum hyperfine splitting 2Amin of EPR spectra of lipophilic spin-probe methyl ester of 5-doxylpalmitate (MeFASL(10,3)) in membranes of OPP liposomes with different concentration of cholesterol.

In order to get a reasonable characterization one still has to define the number of spectral components before applying the optimization. To resolve this problem multi-run HEO optimization is used together with a newly developed GHOST condensation procedure. According to this method 200 independent HEO simulation runs for each EPR spectrum were applied, taking into account 4 different motional modes of spin probe (23 spectral parameters), which is around the resolution limit of EPR nitroxide experiments. From these runs only the set of parameters, which correspond to the best fits were used. All the best fit sets of parameters obtained by 200 optimizations were evaluated according to the goodness of the fit (χ^2 filter) and according to the similarity of the parameter values of best fits (density filter). The parameters of the best fits were presented by three two-dimensional cross-section plots using four spectral parameters: order parameter S, rotational correlation time τ_c, line broadening W, and polarity correction factor p_A (S-τ_c, S-W, and S-p_A) (Štrancar *et al.*, 2004). Groups of solutions, which represent the motional modes of spin probes in particular surrounding and which could correspond to different types of membrane domains, can be resolved either graphically on GHOST diagrams or numerically within GHOST condensation. Even if the number of different motional modes is more than four, some information about the molecular arrangement in the membrane can be obtained (Štrancar *et al.*, 2003; 2004). From these plots, information about the membrane domain types, dynamics of motion and ordering within the domain types as well as about the polarity of spin probe surrounding can be obtained. Therefore the changes in the domain types due to the interaction of membrane with biologically active compound, due to temperature and inclusion of other molecules into the membrane can be studied. Starting values of parameters of spectral components were finally defined using the average parameters taken from the GHOST diagrams. Due to the discrete nature of the groups of solutions found within GHOST diagrams, the relative proportions of groups were found with EPR spectra simulations within 4-spectral-component model using Simplex Downhill optimization.

RESULTS

Information obtained directly from the EPR spectra

The amount of cholesterol in the membrane has a pronounced effect on the line-shape of an EPR spectrum (Fig. 1). From the spectra we can see that with increasing concentration of CH the spectral lines become broader.

Quantities that can be directly measured from the spectra are maximum $2A_{max}$ and minimum $2A_{min}$ hyperfine splitting, which give information about the changes in average ordering of lipids in liposome membranes (Marsh, 1981). The minimum hyperfine splitting $2A_{min}$ (Fig. 2) decreases with increasing amount of cholesterol in the liposome membrane, showing that with increasing concentration of cholesterol ordering of lipid molecules in the liposome membranes increases.

At cholesterol concentration above $c_{CH} = 0.5$ $2A_{min}$ remains constant, within the range of experimental error. This can also be seen directly from the EPR spectrum at the largest concentration of CH at $c_{CH} = 0.55$ which has almost the same shape as the EPR spectrum at $c_{CH} = 0.5$ (Fig. 1).

Some other changes can also be seen from the spectra, which can not be quantitatively described in a simple way. The arrow is pointing to the peak that is present in the EPR spectra of liposomes with a small amount of cholesterol, but is vanishing with an increasing amount of cholesterol in the liposome membrane (vanishes at cholesterol concentration $c_{CH} = 0.50$) (Fig. 1). To get a more detailed description about the changes in liposome membrane with increasing concentration of cholesterol, computer simulation of the EPR spectra was performed with the newly developed characterization procedure (hybrid evolutionary optimization HEO and GHOST condensation method).

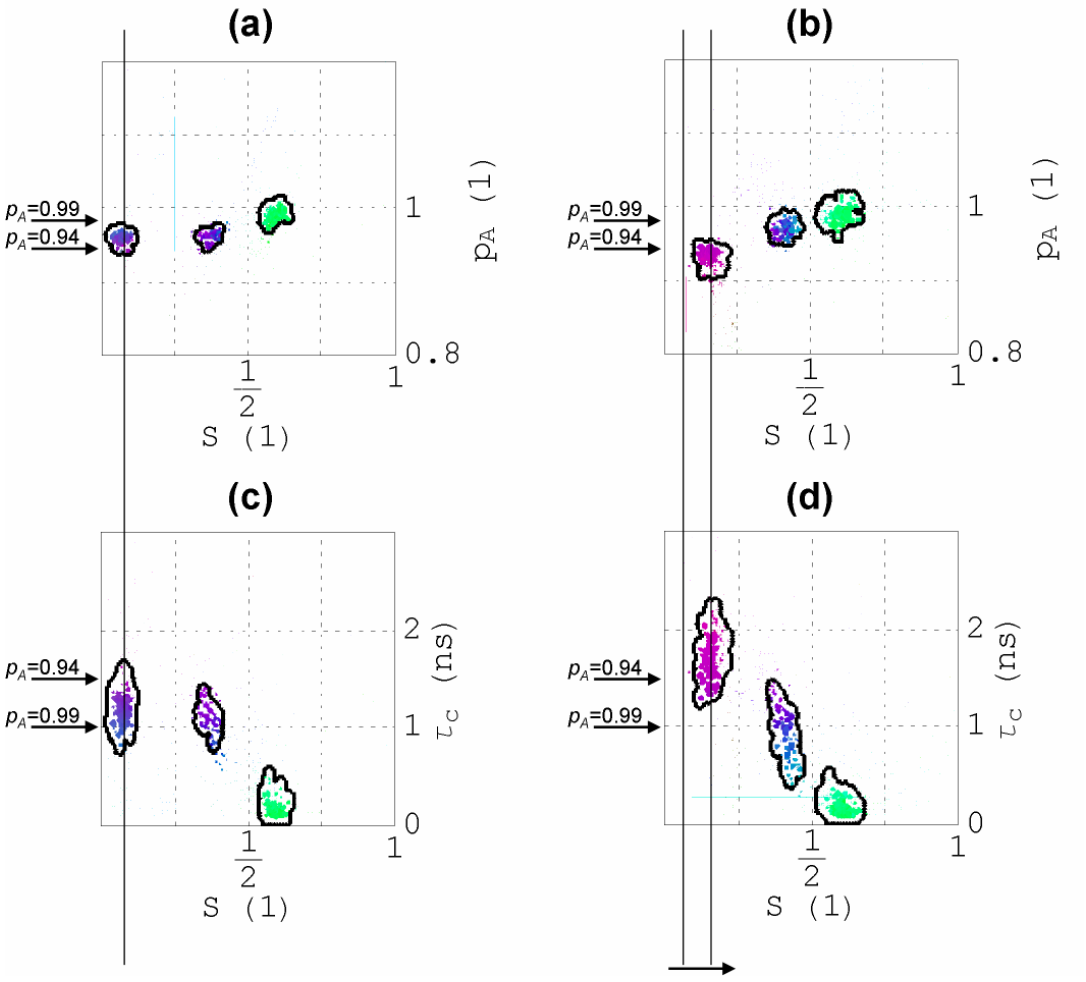

Fig. 3: GHOST diagrams of EPR spectral parameters of spin probe motional modes in OPP liposomes. (a) and (c) for liposomes with 0.45 mol% of cholesterol, (b) and (d) for liposomes with 0.5 mol% of cholesterol: S-p_A (order parameter – polarity correction) diagram: S-τ_c (order parameter – rotational correlation time) diagram. Arrows indicate the shift in order parameter and polarity corrections p_A, respectively of the most disordered mode of spin probe motions.

Results obtained by the GHOST method

One can see from the GHOST diagrams of EPR spectral parameters that in the case of OPP liposomes with c_{CH} = 0.45 EPR spectrum is composed of at least three modes of motion as indicated by three groups of solutions in the diagrams (Fig. 3 (a) and (c)), which could correspond to three types of different lateral membrane domains. When comparing diagrams of liposomes with c_{CH} = 0.45 and c_{CH} = 0.5 (Fig. 3 (b) and (d)), one can see that there are two different groups of solutions that define the most disordered mode of spin label motion with order parameter S around 0.1. There is a part of solutions in liposomes with c_{CH} = 0.45, at p_A close to 0.99 which is absent at c_{CH} = 0.5, while the other part of solutions, shifts to lower values of p_A, to higher values of rotational correlation time τ_c, and to higher values of order parameter. The value of additional broadening parameter, for this group of solutions, also shifts to higher values (data not shown). This indicates that the portion of spin probe molecules in the part of liposome membrane with the most disordered motion of molecules becomes more ordered after the addition of cholesterol, with probably decreased lateral diffusion and is shifted to a less polar region of the membrane.

Spectral decomposition

To quantify the relative proportion of each group of solutions we have decomposed the EPR spectra to four spectral components each representing one group of solutions found by the GHOST method. Since the group of solutions with the lowest value of order parameter seems to be composed of two groups of solutions with different polarity according to GHOST diagrams (Fig. 3 (a) and (c)) we used two spectral components with fixed values of p_A at 0.99 and 0.94 to characterize the modes of motion represented by these groups of solutions. The other parameters and proportions of different types of spin probe motional modes were obtained after applying Simplex Downhill optimization procedure with the starting values of parameters which were chosen as weighted averages of the groups of solutions.

Each spectral component represents a mode of motion of a portion of the spin probes partitioned in a part of the membrane with particular fluidity properties. One of the most important parameters that define a property of spin label motion is order parameter S. The changes of relative proportions and order parameters of all spectral components with cholesterol concentration are shown in Fig. 4. It is important to emphasize that one of the spectral components disappears at the concentration of cholesterol c_{CH}=0.5, as was also indicated in the GHOST diagrams. This concentration coincides with the solubility limit of cholesterol for similar mixed systems (Huang & Feigenson, 1999) and indicates that at certain concentration of cholesterol the entire lipid membrane becomes ordered.

DISCUSSION

In the case of OPP liposomes GHOST method proved to be successful in the automatic characterization of liposome membrane properties. To analyze spin-probe properties in liposome mem-

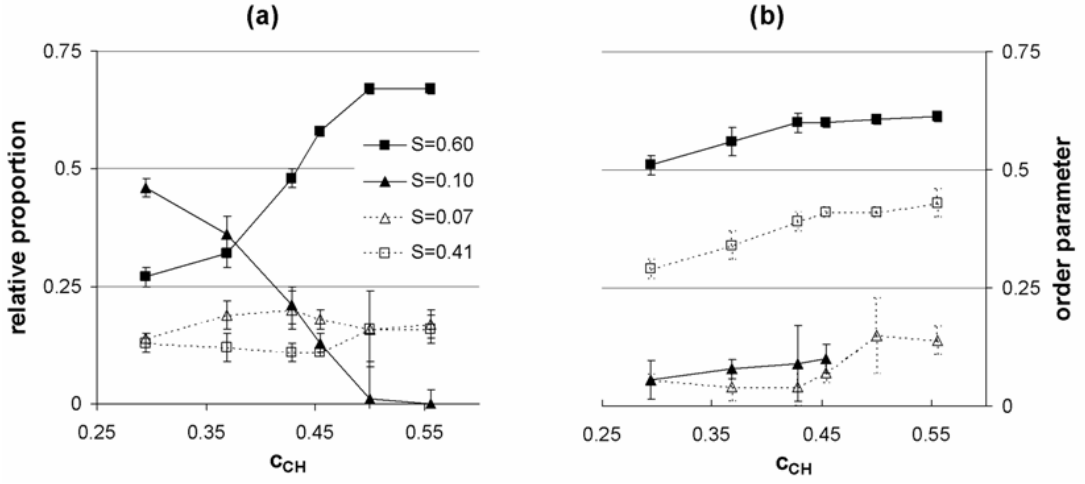

Fig. 4: Dependence of (a) relative proportions and (b) order parameters of spectral components on cholesterol concentration c_{CH} in the membranes of OPP liposomes as derived by the tuning of the calculated to the experimental spectra. Each spectral component is denoted by the value of its order parameter at c_{CH}=0.45. The spectral component with S=0.60±0.01 at c_{CH}=0.45 corresponds to the most ordered mode of spin probe motion, similarly the spectral component with the order parameter S = 0.10 ± 0.01 at c_{CH}=0.45 corresponds to the disordered mode.

brane we defined the starting values of EPR parameters with the help of the GHOST diagrams. These starting values were then used in the simulation of EPR spectra with four spectral components. It was shown that at low cholesterol concentration four spectral components exist, while at cholesterol concentration greater than 0.45 one of the spectral components disappears. If we compare the obtained results with previously published results, obtained with the use of HEO procedure only, the portion of spin probes with the least ordered mode of motion which is found in the polar region characterized with p_A around 0.99, was not detected (Koklic *et al*., 2002). This proves that with the newly developed optimization procedure more precise information about the domain types and their motional characteristics in OPP liposome membrane could be obtained.

Our results also agree well with the results and models predicted by other authors. When the concentration of cholesterol is high enough to exceed the maximum solubility of cholesterol in a lipid bilayer, excess cholesterol precipitates in the form of cholesterol monohydrate crystals (Huang & Feigenson, 1999). Monte Carlo simulations showed that at the cholesterol solubility limits, highly regular lipid distributions form in the bilayer (Huang & Feigenson, 1999). Magnitude of interaction energies in the order of several *kT*s are required to obtain any large stable regular distribution of lipids with a perfect long-range order. Simulations demonstrated that small dynamic domains with regular distribution of lipids exist even if the magnitude of interactions is smaller then *kT* (Parker *et al*., 2004). It was estimated that, at 50% cholesterol in PC membrane, the area of regular distribution domains can be as high as 80% of the total membrane surface area (Wang *et al.*, 1998). Our results support the possibility of growing domains with regular distributions of lipids after the addition of cholesterol and give us accurate prediction of relative proportions of domains with random lipid distributions. Based on the results shown in Fig. 4 we can predict that domains with random lipid distribution coexist with cholesterol rich domains, with regular lipid distribution, in liposomes made of alkilphospholipids, dicetylphosphate and less then approximately 50% cholesterol.

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