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Comparative EPR studies on lipid bilayer properties in nanodiscs and liposomes

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ABSTRACT

Studies of the membrane proteins suggest their close interaction with the lipid surroundings. Membrane proteins and their activities are affected by the composition and structure of the lipid bilayer, therefore adequate surroundings for studied protein are crucial for the model membrane to ensure its biological relevance. In recent years nanodiscs which are small fragments of lipid bilayer stabilised by derivatives of apolipoprotein, called membrane scaffold protein (MSP), have been established as alternative tool in structural and functional studies of membrane proteins. In this study, the influence MSP of different length on structure and dynamics of DMPC and POPC bilayer was investigated and compared to bilayer present in liposomes. EPR spectroscopy technique using different PC-based spin probes was employed to show cholesterol-like organising effect of MSPs on lipid bilayer, thus giving a better insight into the nanodiscs model membrane structure, and its possible implications in the research of membrane protein applications.

1. Introduction

In recent years the introduction of novel biological membrane model based on lipoprotein particles circumnavigating bloodstream called nanodiscs provided a promising system in which insoluble proteins could behave in a way somewhat similar to soluble ones. Nanodiscs are self-assembling systems constituted by lipid bilayer stabilised by homodimers of modified apolipoprotein called membrane scaffold proteins (MSPs) [1]. With specific lipid to MSP ratio homogenous population of discoidal particles can be attained upon removal of detergent from reconstitution solution. The size of these particles, and so the area of lipid bilayer in a single nanodisc, is determined by the length of stabilising MSP proteins, which can force a membrane protein to adopt an oligomeric state of interest. In nanodiscs, the lipid composition can be fully controlled to provide adequate surroundings for a studied protein. MSP proteins are almost exclusively helical and amphiphilic, and stabilise lipid bilayer by forming belt-like antiparallel dimer of proteins [2,3]. Many membrane proteins were successfully reconstituted into nanodiscs and studied both structurally and functionally [1,4–8], taking advantage of the precise control of the lipid environment, protein oligomeric state and access to both intra- and extracellular part of studied protein. As always in case of introduction of a new model experimental system a good understanding of its physical properties is required to evaluate its biological relevance. It is especially important when studying membrane proteins, which are sensitive to their lipid surroundings [8–11].

One of the well-established methods for in-depth investigation of the physical structure of lipid bilayer and changes induced in it by modifying factors is the employment of spin probes in electron paramagnetic resonance (EPR) spectroscopy. Derivatives of phosphatidylcholine with paramagnetic centres attached to different carbon atoms in hydrocarbon chain allow probing of lipid bilayer at different depths [12], and can provide insight into polarity, structure and motion of labelled molecules. This approach was previously successfully used to investigate many factors that modify fluidity of the membrane, such as cholesterol, carotenoids, peptides and membrane proteins [12–14].

In this study we have investigated the effect of the presence of MSP proteins on lipid bilayer stabilised by them, and have tried to evaluate how the introduced changes may affect membrane protein reconstitution. As a reference system liposomes were used, as a well-established and thoroughly studied membrane model [15]. Both semi-unsaturated (POPC) and saturated (DMPC) membrane systems of nanodisc and liposomes were studied. Additionally, nanodiscs assembled using two MSP proteins of different lengths were used to investigate possible structure differences between nanodiscs of 9.7 nm and 12.9 nm sizes based on MSP1D1 and MSP1E3D1, respectively [1]. The aim of the latter comparison was to test possible differences between molecular packing in nanodiscs with different areas of lipid bilayer and different amounts of boundary lipids in contact with scaffold protein.

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2. Materials and methods

2.1. Protein purification

Plasmids for MSPs expression were purchased from Addgene [16,17]. MSPs proteins were expressed and purified using Sligar's protocol [18]. After the initial purification His-tag was removed by incubation with TEV protease for over 24 h in 4 °C in 20 mM Tris buffer (pH 7.8). The proteins were then purified from the cleaved His-tags, TEV protease and some residual impurities by Ni-affinity chromatography. Final purity was verified with SDS-page electrophoresis (Supplementary Fig. 1) and by using size exclusion chromatography on Superdex 200 (GE Healthcare). The concentration of protein was established by measurement of absorbance at 280 nm using calculated extinction coefficients $\varepsilon_{280} = 18{,}200\ M^{-1}\ cm^{-1}$ for MSP1D1 and $\varepsilon_{280} = 26{,}600\ M^{-1}\ cm^{-1}$ for MSP1E3D1 [19]. In the case of insufficient concentration required for effective nanodisc assembly, the protein stock was concentrated using centrifugal concentrators (Sartorius).

2.2. Liposome preparations

POPC and DMPC were purchased from NOF Europe and PC spin labels were purchased from Avanti Polar Lipids. Multilamellar liposomes (consisting of DMPC or POPC) containing 1 mol% of lipid spin label were prepared according to [12]. All compounds were dissolved in chloroform, which was then evaporated under the stream of nitrogen. The formed lipid film was put under vacuum for at least 12 h. The dried lipids were suspended in 20 mM Tris buffer (pH 7.8) and vortexed. Then, the multilamellar liposome suspension underwent five freeze–thaw cycles, after which it was centrifuged at 14 000 *g*, for 15 min at 4 °C, and the resulting pellet was used for EPR measurements. The final lipid concentration was in the range of 60–100 mM to ensure obtaining a good EPR signal to noise ratio and proper EPR spectra under all experimental conditions.

2.3. Nanodisc preparation

Nanodisc samples were prepared using a modified Sligar's protocol [16,20]. Lipid films with addition of spin label were prepared as described in Section 2.2. Protein to lipid ratio in assembly mixture was optimized for purified MSP proteins and lipids with a concentration determined by inorganic phosphorus assay test [21,22]. After the preparation, samples were analysed and fractioned using size exclusion chromatography on Superdex 200 column. This procedure assured that in every preparation only the fractions containing correctly assembled, homogenous nanodiscs were pulled and used for further experiments. Any lipid–protein aggregates which might have been present in the post-assembly solution were therefore discarded. For a typical preparation, depending on its quality, the fractions pulled constituted 30–50% of nanodisc elution peak (Supplementary Figs. 1C, 2 and 3).

The samples after fractioning were concentrated using Sartorius concentrators to yield a final phospholipid concentration of 60–100 mM (the same as in the case of liposomes). Final phospholipid concentration was calculated based on the protein concentration under the assumption that samples contained nearly pure nanodiscs and that the protein to lipid ratio was as given in literature [1].

2.4. EPR measurements

T-PC, 5-PC, 10-PC and 16-PC are lipid spin labels which have a nitroxide free radical moiety responsible for the EPR signal attached to the polar headgroup, 5th, 10th or 16th carbon atom in the alkyl chain, respectively. Therefore, information is obtained from different regions of the membrane: the water–membrane interface, the region close to the polar headgroups and the membrane centre.

For both liposome and nanodisc samples lipid films were prepared with the addition of 1 mol% of spin probe. The spin label to lipid ratio was chosen so that statistically one (or less) spin label molecule per disc will be present, which ensures that EPR spectra will not suffer from homogenous broadening caused by interaction between paramagnetic centres in close proximity.

The EPR measurements were conducted with Bruker EMX spectrometer equipped with a temperature control unit (EMX ER 4141 VT). The suspension of multilamellar liposomes was placed in a gas permeable capillary (i.d. 0.9 mm) made of Teflon and located inside the EPR dewar insert in the resonant cavity of the spectrometer. The sample was thoroughly deoxygenated with nitrogen gas (about 20 min), which was also used for temperature control. During this time any protein–lipid aggregates present in nanodisc samples due to high sample concentration should sediment out of the measurement window. This sedimentation was observed for several samples by slight signal amplitude decrease after the time required for deoxygenation. For polarity measurements, samples were frozen to below −145 °C using liquid nitrogen vapour. For other measurements, the EPR spectra were recorded at 20 °C and at physiologically relevant 37 °C, so that for both nanodisc and liposome samples containing DMPC bilayers were measured below and above the main phase transition temperature (T_M) of 24 °C for DMPC in liposomes and 28 °C in nanodiscs, as established by differential scanning calorimetry (DSC) measurements for MSP1D1 based nanodisc [23]. It was assumed that the T_M phospholipids in MSPE3D1 based nanodiscs would be similar to that in MSP1D1 based ones. Additionally, for POPC samples only temperature dependent effects would be perceived, resulting in the additional insight into both systems. The representative EPR spectra, and parameters directly measured from them are compiled in Figs. 1 and 2.

2.5. DLS measurement

After EPR measurements, the samples were analysed using the dynamic light scattering (DLS) technique in order to check whether the high concentration promotes nanodisc aggregation and/or forming of larger lipid–protein complexes. Also, to consider aggregation caused by freezing samples to −145 °C, samples which were not frozen before were measured. Backscatter angle DLS measurements were performed using Zetasizer Nano (Malvern). Before the measurements, the samples were degassed and diluted to yield a required volume if needed. Samples were then put into a 1 ml 12 mm square polystyrene cuvette or into a 40 μl chamber quartz cuvette. Measurements were performed at 25 °C. The data was analysed using the Malvern Zetasizer software.

3. Results

3.1. Nanodiscs sample aggregation

DLS measurement of concentrated nanodisc samples showed bimodal distribution for samples not frozen prior to the measurement. Besides nanodiscs, a small population of lipid–protein aggregates with a diameter of over 100 nm was present. In samples measured after the freezing sometimes a third larger population (1000 nm) was present. However, the calculated volume partition of aggregates in the sample was negligible (0 up to 0.01% for samples after freezing). Also, the presence of these aggregates did not disrupt the structure and homogeneity of prepared nanodiscs, as nanodisc populations of 10.35 nm in diameter with polydispersity of 0.06 and of 13.5 nm with polydispersity of 0.13 were detected for MSP1D1 and MSP1E3D1 nanodiscs, respectively.

3.2. Effect on lipid mobility in polar headgroup region

To investigate the polar headgroup region of DMPC and POPC model membranes T-PC spin probe was employed. Fig. 3 shows a set of chosen parameters of T-PC EPR spectra, which are a well-established measure

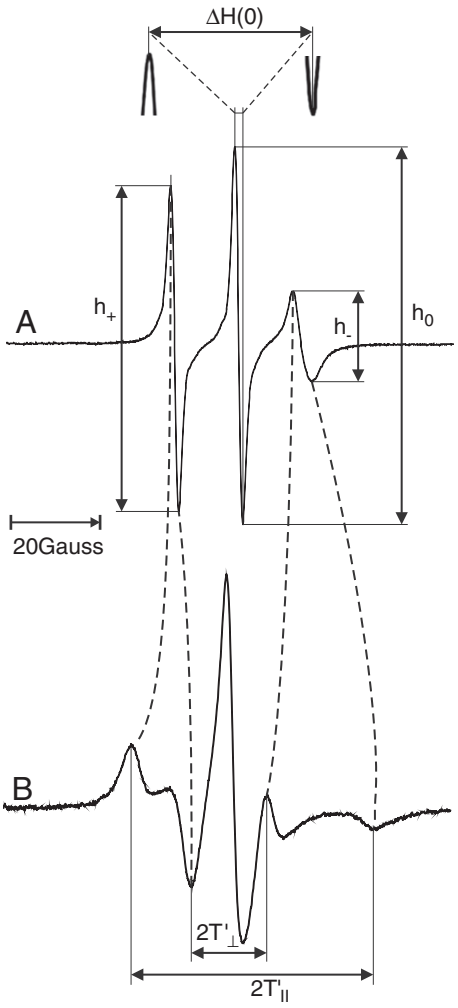

Fig. 1. EPR spectra of 16-PC spin label in MSP1D1/DMPC nanodiscs measured at 37 °C (A) and 5-PC spin label in MSP1E3D1/POPC nanodiscs measured at 20 °C (B). The measured parameters needed for the calculation of order parameter S and rotational correlation times τ_{2B} and τ_{2C} (ref. 3.3) are indicated; for a detailed description please refer to [12]. The dashed lines denote corresponding peaks for different types of spectra.

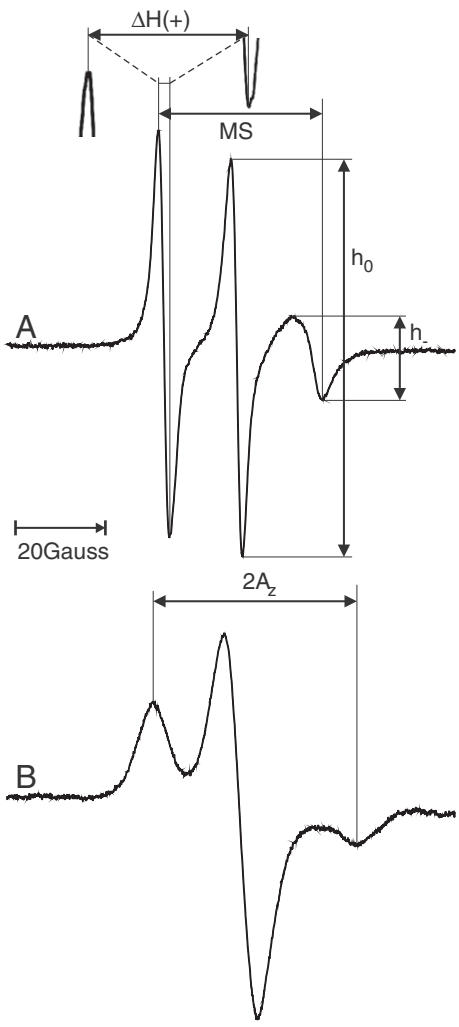

Fig. 2. EPR spectra of T-PC spin label in MSP1E3D1/POPC nanodiscs measured at 37 °C (A) and 10-PC spin label in MSP1D1/POPC nanodiscs measured at −145 °C (B). The measured parameters needed for interpretation of headgroup region mobility (ref. 3.2) and for obtaining polarity profiles (ref 3.4) are indicated; for detailed description please refer to [12,24].

of this probe's motional freedom: peak-to-peak width of the low field line (ΔH (+)), maximum splitting (MS) and ratio of the height of central and high field peaks (h0/h−). It is assumed that the increase in the motional freedom of this spin label molecule results in the decrease of these parameters [24,25]. All of the chosen spectral parameters, excluding h0/h− ratio for POPC samples have smaller values for the nanodiscs than for the liposomes, with no significant difference between MSP1D1 and MSP1D1E3 based nanodiscs. However, the observed differences are significant at room temperature and much smaller at 37 °C. The h0/h− ratio is the least sensitive parameter and MS is the most sensitive one. Generally, the results suggest that in nanodiscs polar headgroups of lipid molecules have more motional freedom than in liposomes.

3.3. Effect on hydrocarbon chains region mobility

To assay the effect of the presence of MSPs on phospholipid bilayer in DMPC and POPC-based nanodiscs, the order parameter S was calculated based on spectral parameters for all doxyl derivative probes according to Marsh [26]. In the case of n-PC, S reflects the segmental order parameter of the hydrocarbon chain segment to which the nitroxide fragment is attached. This parameter is a measure of the semi-cone angle θ_c within which the wobbling motion of this segment is confined: $S = cos\theta_c(1 + cos\theta_c)/2$ [27], however for lipids in the gel phase this cannot be correctly calculated from measured spectral parameters, therefore was not calculated for DMPC samples spectra at 20 °C. For both DMPC and POPC no relevant difference between MSP1E3D1 and MSP1D1 based nanodiscs was observed (Fig. 4). With increase in temperature, a decrease in the order parameter was noted for POPC samples in both nanodiscs and liposomes. In nanodiscs, the order parameter was generally higher than in liposomes, with the most prominent difference for 10-PC label in POPC and all labels in DMPC bilayers (Fig. 4).

Faster motion of 16-PC spin label yields more isotropic spectra and allows employment of another approach to their interpretation [28]. Rotational correlation times for samples with lipids above T_M can be calculated using two terms:

$$\tau_{2B} = 6.51 \cdot 10^{-10} \cdot \Delta H_0 \left[\left(\frac{h_0}{h_-} \right)^{\frac{1}{2}} - \left(\frac{h_0}{h_+} \right)^{\frac{1}{2}} \right] s \quad (1)$$

$$\tau_{2C} = 6.51 \cdot 10^{-10} \cdot \Delta H_0 \left[\left(\frac{h_0}{h_-} \right)^{1/2} - \left(\frac{h_0}{h_+} \right)^{1/2} - 2 \right] s \quad (2)$$

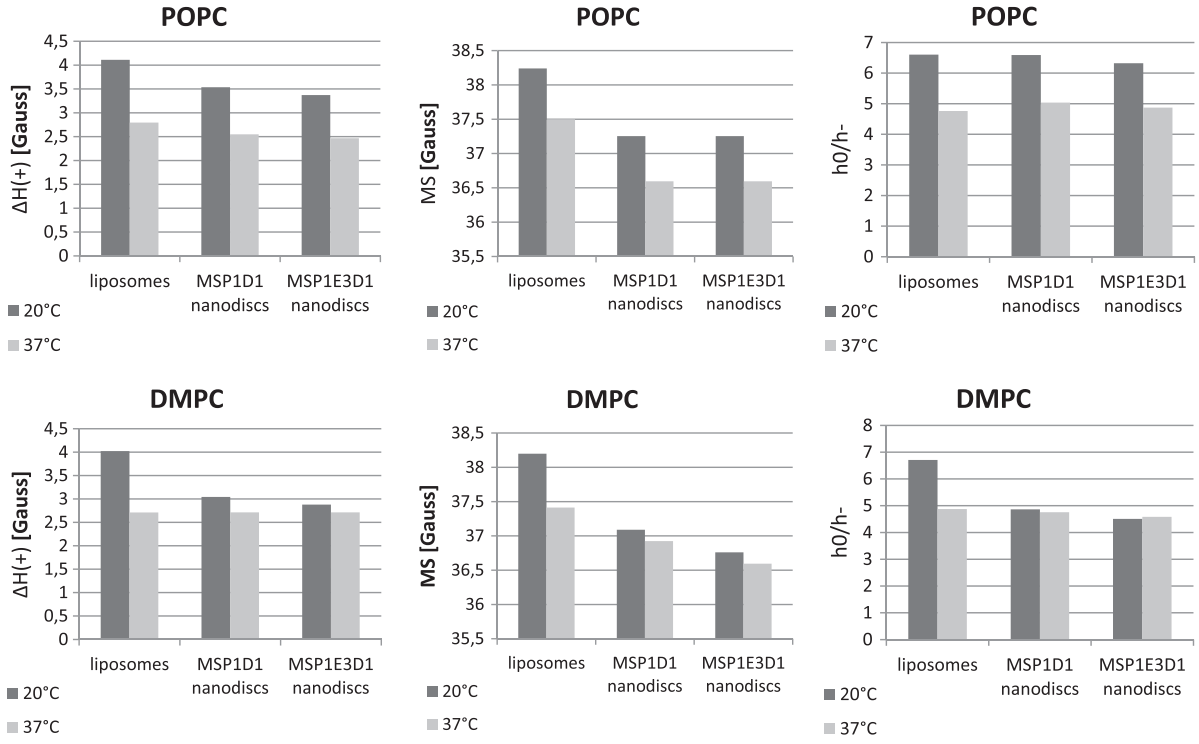

Fig. 3. EPR spectral parameters of T-PC spin probe measured for POPC and DMPC systems at 20 °C and 37 °C. Data for both liposomes and nanodiscs is shown.

where ΔH_0 is the peak-to-peak width of the central line and h_+, h_0 and h_- are amplitudes of respectively low, central and high field peaks (see Fig. 1). Nanodisc samples for both scaffold proteins show significant increase in the correlation times compared to liposomes (Fig. 5). Additionally, it is accepted that the more anisotropic motion of 16-PC spin label, the bigger the difference between correlation times τ_{2B} and τ_{2C} [28]. As shown in Fig. 5, all nanodisc systems exhibit a difference between τ_{2B} and τ_{2C}, whereas for liposomes both correlation times are similar to each other. This means that in the membrane centre the motion of lipid alkyl chains in nanodiscs is not only slower, but also more anisotropic than in liposomes.

3.4. Effect on polarity profiles across a lipid bilayer

Data collected for samples frozen under −145 °C yielded values of $2A_z$ (z-component of the hyperfine interaction tensor, see Fig. 2B) which depend only on polarity of the local environment of the spin probe [12,29–31]. So called polarity profiles (dependence of $2A_z$ on nitroxide group position along the chain) for studied model membranes are shown in Fig. 6. For both POPC and DMPC based systems, 5-PC and 10-PC yielded comparable $2A_z$ values in liposomes and nanodiscs with slightly smaller value (lower polarity) for 10-PC in nanodiscs. Major differences in polarity between nanodiscs and liposomes were noted in the

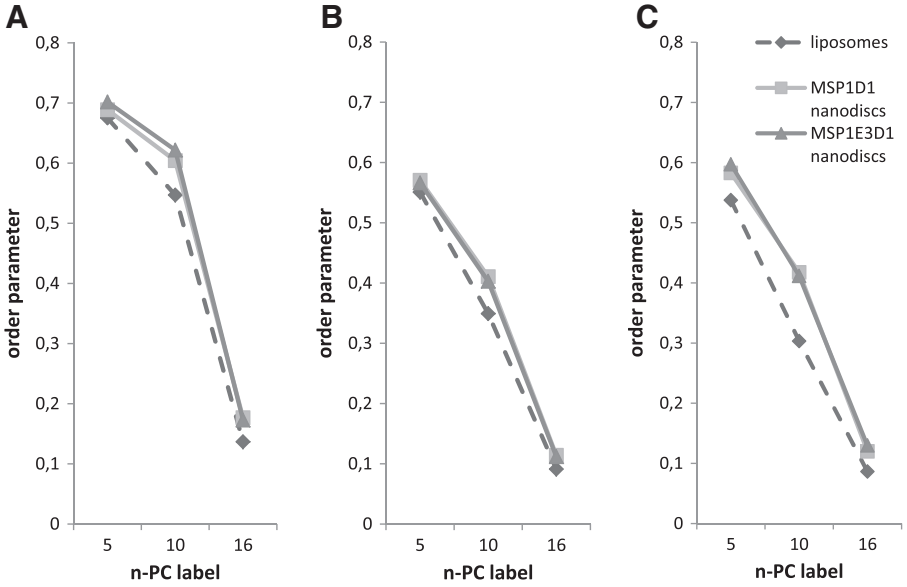

Fig. 4. Order parameter S of n-PC labels calculated for POPC samples in 20 °C (A) and 37 °C (B) and DMPC sample in 20 °C(C). Data for both liposomes and nanodiscs is shown.

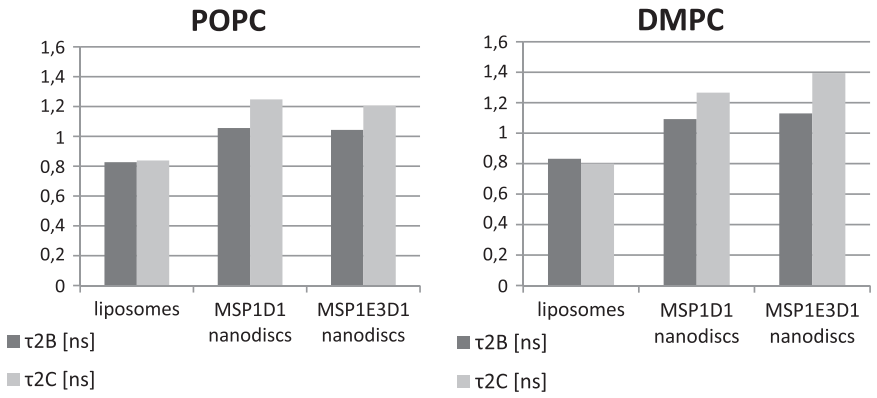

Fig. 5. Rotational correlation times of 16-PC spin probe in POPC and DMPC model membranes at 37 °C. Shorter times observed in liposomes reflect higher membrane fluidity. The difference between τ_B and τ_c calculated for nanodiscs shows a higher degree of anisotropy of spin label motion in these systems as opposed to liposomes where τ_{2B} and τ_{2C} are similar.

polar headgroup region and in the membrane centre. A significant decrease in polarity (lower $2A_z$ values) was observed for 16-PC in nanodiscs compared to liposomes. On the other hand, in the polar headgroup region polarity significantly increased in nanodiscs compared to liposomes, as monitored by T-PC (higher $2A_z$ values). 2Az values measured for the spin labels in nanodiscs stabilised by MSP1D1 and MSP1E3D1 showed no major differences. POPC based system displayed lower overall polarity compared to ones based on DMPC.

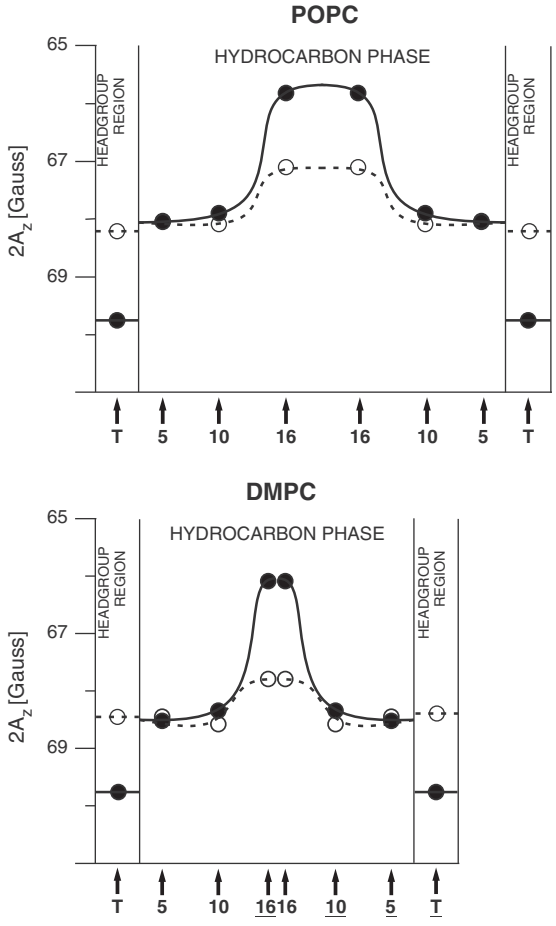

Fig. 6. Polarity profiles across POPC (A) and DMPC (B) bilayers in MSP1D1 nanodiscs (●) and liposomes (○). Upward changes indicate decrease in polarity. Approximate locations of the nitroxide moieties of spin labels are indicated by arrows. The underlined numbers for n-PC in DMPC indicate that these molecules are intercalated mainly in the right half of the bilayer (labelled PCs are longer than the host phospholipid). T stands for T-PC spin label with the nitroxide moiety attached to choline polar headgroup. For more details including the method of calculating membrane thickness and approximating position of nitroxide moieties see refs. [12,32,33].

4. Discussion

Nanodiscs are steadily gaining more recognition as a tool for investigating membrane proteins. Access to both intra- and extracellular parts of membrane protein gives this model great advantage over commonly used liposomes and homogeneity of sample and absence of big quantities of detergent make this model more preferable than another novel membrane model — bicelles. Also, the strict control of lipid quantity content and ability to control nanodisc size by controlling the length of MSP protein make this system a model of choice for structural NMR studies [34], where noise from lipids and detergent can disturb the measurement. It is worth noting that, when considering model of biological membrane, not only its lipid composition, but also physical structure of bilayer is crucial. While the protein part of nanodiscs has been thoroughly structurally studied [35], the bulk of lipids, which constitute surroundings of incorporated membrane protein has been not. This study shows the physical structure changes forced on lipid bilayer by MSP proteins.

Data collected from EPR experiments using 16-PC label consistently shows that in the centre of the lipid bilayer rotational motion of lipid chains is decreased after the introduction of MSPs into the model membrane system. Lower values of rotational correlation times of 16-PC in nanodiscs than in respective liposomes suggest that in nanodiscs lipid chains are packed more tightly because of the pressure applied by proteins stabilising this membrane system. This pressure is expected to be the largest in the centre of the lipid bilayer because at this height MSP dimer is located around the bilayer [2,3,35]. As denoted by the difference in rotational correlation times τ_{2B} and τ_{2C} this tighter packing would not only affect the rate of motion, but also its anisotropy. This kind of interaction introduces mechanical stress into the system, which can be released by loosening of the packing of polar headgroups of nanodiscs. Indeed, we observed a decrease in lipid packing in the polar headgroup region of nanodiscs, compared to liposomes as proved by changes in spectral parameters of T-PC label (Fig. 3). Lower values of T-PC spectral parameters obtained in nanodiscs suggest increased motional freedom of lipid headgroups in this membrane system. This structural change is also reflected in presented polarity profiles (Fig. 6). In nanodiscs, water accessibility to polar headgroup region is significantly increased compared to liposomes, whereas in the centre of the bilayer a hydrophobicity barrier is formed. While the increase in hydrophobicity inside the bilayer is characteristic for introduction of proteins into the liposome membrane as showed for α-helical peptides [36,37], changes in headgroup polarity are not observed in such systems. This supports a notion that the effects observed in nanodiscs do not result only from a simple interaction of lipids with helical MSP proteins. Lipid bilayer in

nanodiscs in terms of polarity profiles, is more similar to that with addition of cholesterol [12]. However, in contrast to cholesterol and transmembrane peptide effect on polarity, no great change is present in the surroundings of 5-PC and 10-PC labels, highlighting the specific interaction of MSPs with the centre of the bilayer. Also, the overall effect of MSP is somewhat smaller than that of cholesterol. The effect of strapping together lipid molecules by MSP proteins is not particularly visible in the distribution of the order parameter S, contrary to cholesterol where the change is significant [25]. Nevertheless, the overall distribution of S does consistently show an increase in the lipid order in nanodiscs compared to liposomes (Fig. 4). It is worth noting, that experiments of introducing cholesterol to lipid membrane are usually conducted with its high concentration (i.e. 50 mol%) [33]. This somewhat cholesterol-like effect is consistent with data form DSC experiments which show the shift of the T_M and phase transition broadening in MPS1D1 nanodiscs [23]. This broadening and the shift in T_M are the result of a smaller amount (compared to liposomes) of lipids in nanodiscs cooperatively undergoing phase transition and overall physical changes in packing and molecular interactions in lipid bilayer structure caused by the presence of protein. Nanodiscs seem to be overall the best solution for reconstitution of membrane proteins mimicking high concentration of proteins in the plasma membrane [23] and to some degree the rigidifying effect of cholesterol. Addition of cholesterol can still be crucial for the stability of the membrane protein [38]. Recent bioinformatic analyses show that in great number of membrane proteins cholesterol binding motifs are present [39], implying its significant role in functioning of integral membrane proteins. However, the overall concentration of the cholesterol employed in nanodiscs can be smaller to ensure native-like membrane confinement in the model system. This feature is also relevant, because preparation of nanodiscs requires homogenous suspension of lipids and detergent in buffer, and cholesterol is known to form detergent resistant fractions with saturated lipids [40,41] thus preparation of nanodisc with high concentration of cholesterol can prove to be unattainable in standard systems. The solution to this problem can be the detergent free systems where up to 10 mol% of cholesterol was successfully incorporated into the DMPC based nanodiscs [4]. The tighter packing introduced into system by MSP protein, is also similar to effect of forcing hexagonal H_{II} phase preferring lipids to form a bilayer. Pressure arising from this effect of "curvature frustration" has been suggested to be an important function of membrane [42,43]. Introduction of hexagonal H_{II} phase preferring lipids into nanodiscs in controlled quantities should be investigated, as a mean of controlling this pressure. In this study we show that changes introduced into lipid bilayer are non-negligible, and should be considered when using this membrane model. Structural studies like the one presented here, while giving the insight into physical structure of chosen model can provide knowledge of how to control and adjust such model to ensure its biological relevancy.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamem.2014.10.004.

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