Lipid dynamics in nanoparticles formed by maleic acid-containing copolymers: EPR spectroscopy and molecular dynamics simulations

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ARTICLE INFO

Keywords:
Nitroxide spin label
Coarse-grained molecular dynamics
SMALP
DIBMA
Phospholipid bilayer
Liposome

ABSTRACT

Amphiphilic maleic acid-containing copolymers account for a recent methodical breakthrough in the study of membrane proteins. Their application enables a detergent-free extraction of membrane proteins from lipid bilayers, yielding stable water-soluble, discoidal lipid bilayer particles with incorporated proteins, which are wrapped with copolymers. Although many studies confirm the potential of this approach for membrane protein research, the interactions between the maleic acid-containing copolymers and extracted lipids, as well as possible effects of the copolymers on lipid-embedded proteins deserve further scrutinization. Here, we combine electron paramagnetic resonance spectroscopy and coarse-grain molecular dynamics simulations to compare the distribution and dynamics of lipids in lipid particles of phospholipid bilayers encased either by an aliphatic diisobutylene/maleic acid copolymer (DIBMALPs) or by an aromatic styrene/maleic acid copolymer (SMALPs). Nitroxides located at the 5th, 12th or 16th carbon atom positions in phosphatidylcholine-based spin labels experience restrictions of their reorientational motion depending on the type of encasing copolymer. The dynamics of the lipids was less constrained in DIBMALPs than in SMALPs with the affinity of spin labeled lipids to the polymeric rim being more pronounced in SMALPs.

1. Introduction

The preparation of single membrane proteins or membrane protein complexes in a native-like but water-soluble environment, as required for the spectroscopic investigation, e.g., is challenging. Amphiphilic maleic acid-containing copolymers provided a significant methodical breakthrough in this field. Styrene maleic acid (SMA) copolymers with styrene-to-maleic acid ratios of 2:1 and 3:1 were found to directly solubilize phospholipids and membrane proteins, both from artificial and natural bilayers, yielding discoidal SMA/lipid particles (SMALPs), (see [1–4] for the pioneering publications on their application in biology and [5–9] for recent reviews). Quite recently, one more amphiphilic alternating copolymer of diisobutylene and maleic acid (DIBMA), has been shown to extract phospholipids and membrane proteins and form discoidal particles (DIBMALPs) [10,11]. SMA- and DIBMA-wrapped lipid particles are comprised of lipid or lipid/protein cores surrounded by a polymer belt, and have, depending on the preparation routine, typical diameters of 10–30 nm. Within a particular preparation, the size of particles is uniform, which renders them suitable for different kinds of spectroscopy, as well as other techniques that require relatively small, homogenous water-soluble particles or single membrane proteins in their native lipid environment. In the last few years, SMALPs were also shown to be suitable protein carriers for cryo-electron microscopy [12,13].

Up to recently, the most common method of membrane protein purification for the above applications was their extraction from the membrane into detergent micelles. However, detergents can affect the stability and functionality of membrane proteins, see e.g. [14,15]. In
contrast, maleic acid-containing copolymers extract integral membrane proteins from the lipid bilayer in the absence of detergents. This is their major advantage, particularly in comparison to the well-studied nanodiscs, where amphipathic helical scaffold proteins stabilize discoidal lipid patches after removal of detergents [16,17]. Membrane proteins encapsulated in lipid particles stabilized by copolymers could be studied by different biophysical methods without loss of their structural and functional properties (see [9,18] for a recent survey). In the case of SMALPs, the proteins remained functional, but possessed somewhat higher thermal stability, which might reflect their constrained dynamics [18,19]. In DIBMALPs, the dynamics of lipids were less constrained, whereas the ability to extract proteins from membranes was somewhat compromised [10,11].

Although many empirical studies indicate the great potential of maleic acid-containing copolymers for membrane protein research, the molecular mechanisms of lipid membrane solubilization by these copolymers, the interactions between the copolymer belts and solubilized lipids and their possible effect on lipid-embedded proteins remained unclear. Recent coarse-grained (CG) molecular dynamics (MD) simulations examined the interaction of SMA copolymers, which varied in their component and electric charge, with lipid membranes, as well as the behavior of lipodiscs encased by distinct maleic acid-containing copolymers [20–22]. It was found that SMA copolymers tend to aggregate in solution into clusters, which could account for the uniform size of SMALPs, and that these clusters directly interact with the lipid bilayer [22]. Consequently, clusters of SMA copolymers pull lipid patches out of the lipid bilayer forming SMALP-like structures [22].

The introduction of lipid spin probes is an established method to investigate the dynamics of various parts of fatty acid chains in phospholipid bilayers using EPR spectroscopy [23–25]. In particular, nitroxides attached to carbon atoms at different positions along the hydrocarbon chain allow in-depth probing the lipid bilayer in order to characterize the segmental chain mobility [25]. Recently, EPR spectroscopy yielded insight into the properties of the lipid bilayer and characterizes the segmental chain mobility (25). The introduction of lipid spin probes is an established method to probe the dynamics of various parts of fatty acid chains in phospholipid bilayers using EPR spectroscopy [23–25]. In particular, nitroxides attached to carbon atoms at different positions along the hydrocarbon chain allow in-depth probing the lipid bilayer in order to characterize the segmental chain mobility [25].

Our study, we apply EPR spectroscopy and CG MD simulations to compare the dynamics of lipid spin labels inserted in lipid bilayer containing nanoparticles depending on different polymer and lipid composition. We used a series of three phosphatidylcholine (PC)-based spin labels with the nitroxides attached to the carbon atoms at the 5th, 12th and 16th positions in the hydrocarbon chain of the host phospholipid. We tested two types of commercially available maleic acid-containing copolymers, namely an aliphatic DIBMA copolymer and aromatic SMA copolymers with 3:1 styrene-to-maleic acid ratios (hydrolyzed version of SMA3000 and XIRAN SL25010 S25). We probed two types of PC lipids, saturated 2-dimyristoyl-sn-glycerol-3-phosphocholine (DMPC) and monounsaturated palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC). Liposomes prepared from these lipids with addition of PC-based nitroxide spin labels served as a reference model for comparison with the lipid nanoparticles. EPR spectroscopy and CG MD simulations show that the dynamics of lipids in DIBMALPs appears to be less constrained than in SMALPs. Furthermore, the MD simulations provide evidence and explanation for the significant interaction of the nitroxides with the copolymer belt of SMALPs rendering the spin labels very sensitive for lipid-polymer interaction.

2. Materials and methods

2.1. Chemicals

2-Dimyristoyl-sn-glycerol-3-phosphocholine (DMPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-stearoyl-(5-doxyl)-sn-glycero-3-phosphocholine (16:0-5 doxyl PC), 1-palmitoyl-2-stearoyl-(12-doxyl)-sn-glycero-3-phosphocholine (16:0–12 doxyl PC), 1-palmitoyl-2-stearoyl-(16-doxyl)-sn-glycero-3-phosphocholine (16:0–16 doxyl PC) were purchased from Sigma-Aldrich (St. Louis, MO). SMA copolymer with styrene-to-maleic acid molar ratio of 3:1 (MW 9500 Da; supplied as an aqueous sodium salt solution SMA 3000 HNa) was kindly provided as a gift by Grafix Valley (Exton; PA; USA). Polysciences (Pennsylvania) kindly provided an SMA product under the trade name XIRAN SL25010 S25 as a gift. DIBMA copolymer commercially available under the trade name Sokalan CP9 was kindly provided as a gift by BASF, Germany. All other reagents were of analytical grade.

2.2. Liposome preparations

Powdered DMPC and POPC were dissolved in chloroform and mixed with 1 mol% of lipid spin label. Chloroform was evaporated under a stream of nitrogen gas. The resulting lipid film was dried under vacuum for at least 2 h. The dried lipids were suspended in 10 mM Tris (pH 8.0), 150 mM NaCl buffer (Buffer A) and vortexed. Subsequently, the multilamellar suspension underwent ten freeze–thaw cycles, and, if not used directly, was stored in aliquots at −80 °C. Before reconstitution, the suspension of liposomes was extruded at least 11× through polycarbonate membranes (Whatman) of 400 nm or 200 nm pore size using a Mini-Extruder Set (Avanti Polar Lipids, Alabaster, AL, USA).

2.3. Preparation of lipid bilayer containing nanoparticles

To form SMALPs or XIRAN/lipid particles (XIRAN-LPs), 1 ml of a 2.5% (w/v) solution of SMA or XIRAN, which were extensively dialyzed against Buffer A before, was added dropwise to 1 ml of the lipid suspension to get a final lipid-to-copolymer ratio of 1:2.5 (w/w). Similarly, 1 ml of a 5% (w/v) solution of DIBMA was added dropwise to 1 ml of the liposome suspension to get a final lipid-to-copolymer ratio of 1:5 (w/w). The assembly mixture was allowed to equilibrate for 1 h at room temperature and afterwards for 16 h at 4 °C. The resulting samples were centrifuged (126,000 g; 30 min; 4 °C) to remove aggregates.

2.4. Dynamic light scattering

Dynamic light scattering (DLS) measurements were performed on a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK) at 550 nm and 25 °C. Data represent the average of three sets of 14 runs of 10 s each. The particle size distribution was obtained by using the ZETASIZER software package Ver. 7.02, under the assumption that nanoparticles were spherically shaped.

2.5. Transmission electron microscopy

Copper grids (300 mesh formvar/carbon-coated) (Ted Pella, USA) were hydrophilized by glow discharge (~20 mA; 45 s) with a Pelco EasyGlow apparatus (Ted Pella, USA). Diluted samples of the nanoparticle preparations (3 μL) were placed onto the grid and incubated at RT for 30 s. The excess of the sample was removed with filter paper. Grids were stained twice with a 1% aqueous uranyl acetate solution for 30 s at RT and air-dried.

Micrographs were acquired using an analytical transmission electron microscope Jem-2100 (Jeol, Japan) equipped with a 2 K × 2 K CCD camera Ultracell 1000XP (Gatan, USA). The microscope was operated at 200 kV in a low dose mode, with a magnification of x40000 (2.5 Å/pix) and a defocus of 0.5–1.9 μm.

The images of individual particles were picked from the micrographs automatically using the cYLO neural network [33]. For each polymer-lipid composition, a few hundred of micrographs were acquired and processed using the EMAN2.3 suite [34,35]. For XIRAN-
POPC 17000 particles, for DIBMA-DMPC 2500 particles, and for all other samples 7000 particle images were collected. Particles were windowed to a box size of 100 × 100 pixels for SMA- and XIRAN-derived samples, and 200 × 200 px for DIBMA-derived samples. The set of particle images of each sample was subjected to reference-free 2-D classification that includes iterative centering, rotational and translational alignment and multivariate statistical analysis (MSA) of images. This classification allows combining similar particles together and calculating the class average - normalized sum of particle images with increased contrast. Particles were distributed in classes according to their structural features, the number of particles in different classes varies.

2.6. Electron paramagnetic resonance spectroscopy

EPR measurements were performed as described previously [36]. Briefly, room temperature EPR spectra were recorded on a home-built EPR spectrometer equipped with a dielectric resonator (Bruker Biospin, Germany). Glass capillaries of 0.9 mm inner diameter were filled with sample volumes of ~20 μL at ~250 μM final spin label concentration. The magnetic field modulation amplitude was set to 0.2 mT and the MW power leveled to 2 mW. The sample temperature was stabilized by a room temperature nitrogen gas stream through the resonator and checked with a thermocouple wired to the inside of the resonator thermo jacket, with measured values of 292.5 ± 1.5 K.

2.7. EPR spectra simulations

As an approximation for the reorientational motion of the lipid bound spin labels we assumed a model of anisotropic Brownian rotational diffusion with axial symmetry and orienting potential. The order parameter, S, and the rotational correlation time constant, τc, of the reorientational motion of the lipid bound nitroxides were determined from fittings of the experimental EPR spectra using the software Multicomponent [37]. This program is based on the numerical solution of the stochastic Liouville equation [38,39]. A single spectral component was found to be sufficient to reasonably fit the experimental spectra. During the fittings, the hyperfine tensor components Axx and Ayy were held fixed at 4.65 and 5.25 G, respectively, the g-tensor components were taken from [38] or [40]. The value of the hyperfine tensor component Azz was allowed to vary during fittings to account for different environmental polarities and to approximate partial motional averaging due to possible fast (ps) small scale wobbling of the nitroxide as pointed out by Timofeev et al. [41]. Values for Azz in the range of 33.2 ± 0.8 G were found to provide the best fittings. The rotational diffusion tensor of axial symmetry defined by R∥ and R⟂ with its average R1 =( R∥ R⟂)1/3, R∥ = Rzz, is related to the molecular frame of the nitroxide by the angle βD, which was determined by preliminary best-fit results for spectra of all present preparations and ultimately set fixed to an average value of 37.8°. The value of βD for a doxyl nitroxide spin label bound to the lipid tail was reported to be 34° [42] and thus the value resulting from our simulations reasonably agrees. The effective reorientational correlation time, τc, is given by 1/6R1.

Other variables, which were allowed to vary during the fittings, were the coefficient for the orienting (restoring) potential, C20, and the linewidth tensor, W. The values of the order parameter S were calculated from C20 according to the algorithm provided by Multicomponent. Error limits were determined by repeating simulations using different starting values of the fitting parameters and changing the value of βD by ± 15°.

Fig. 1. The structural formulae and the acronyms of SMA and DIBMA copolymers (A), spin-labeled lipids (B) and synthetic lipids (C) used in this work. Periodically repeating chemical components of polymers are enclosed in breakers and are marked with n, m and x letters. Mapping of all-atom structures to the coarse-grained MARTINI models are shown with the dotted contours.
2.8. Molecular dynamics simulations

Coarse-grained models of nanoparticles containing doxyl-PCs were developed within the framework of the MARTINI force field [43], which has gained broad popularity for simulations of biological membranes and polymers [44] despite certain limitations [45]. The standard MARTINI library contains the CG force field parameters for POPC and DMPC, while parameters for DIBMA and doxyl PCs were derived based on all-atom (AA) simulations according to the iterative scheme we used before [46] until satisfactory agreement between the AA and CG models was achieved (Fig. S1-S5, see Supplementary Information). The AA DIBMA copolymer model consisting of 52 monomers with the 1:1 diisobutylene:maleic acid ratio was built using the OPLS AA force field [47]. The reference AA simulation was run in NPT ensemble for 100 ns (T = 303.15 K maintained by the V-rescale algorithm, P = 1 bar controlled by Parrinello-Rahman barostat; the integration time step was set to 2 fs; the Verlet cutoff scheme and particle mesh Ewald (PME) were used for the nonbonded interactions with the cutoff value set to 1.2 nm). The set of CG parameters for SMA was taken from [22]. The mapping scheme between all-atom and CG models is shown in Fig. 1. The selection of MARTINI CG particle types was guided by the automated parametrization toolkit for small organic molecules [48] and in analogy with the existing topologies (Fig. S6). All of the CG parameters and structure files are available at https://hpc.mipt.ru/zhmurov/lab/.

The AA simulations of doxyl PCs were carried out using the set of parameters and the starting conformations from [49]. These simulations were run for 100 ns each and the simulation protocol suggested by the authors was utilized.

We used Gromacs 2018.1 for all MD simulations [50]. For each system, energy minimization using the steepest descent algorithm was run prior to equilibration simulation in the NVT ensemble (simulation time equaled 1 μs) maintained by means of the V-rescale thermostat (T = 320 K, ττ = 1.0 ps) during which the phosphate groups of lipids were constrained in the XY plane perpendicular to the normal of the lipid patch using a harmonic potential with kspring = 1000 kJ/mol/nm2. The production simulations were run for 1 μs in the NPT ensemble using the same thermostat and the Parrinello-Rahman barostat (time constant = 12.0 ps, compressibility = 3 × 10^{-4} bar^{-1} as recommended in [51] applied isotropically). All CG simulations were performed with the standard water model and the reaction field approach was used to treat the long-range electrostatics with the relative permittivity of 15. A time step of 20 fs was used for all simulations.

![Fig. 2. Size distribution based on DLS data for DMPC (left) and POPC (right) containing particles: SMALPs (blue), XIRAN-LPs (green), DIBMALPs (black) and liposomes (red).](image)

![Fig. 3. Structural characterization of nanodiscs containing lipid fragments of DMPC (left panel) and POPC (right panel) and stabilized by amphiphilic synthetic polymer molecules: top row – SMA; middle row – XIRAN; bottom row – DIBMA. Left in each panel – images of representative classes of particles, extracted from TEM images of individual particles; right in each panel – plot of size distributions, based on TEM data. The bar size is 10 nm.](image)
Verlet pair-lists cutoff scheme was used and the neighbor list was updated every 20 steps. Periodic boundary conditions were applied in all simulations.

For analysis, the nanodiscs were aligned such that their center-of-mass was moved to the origin of the coordinate axes and their principal axes were aligned with the coordinate axes at every trajectory step using a custom TCL script. In-house Python scripts exploiting MDAnalysis [52] were further used for calculations of the nanodiscs thickness, densities of the nanoparticles components, and the order parameters of lipid acyl chains. We were not able to accurately estimate the order parameters for doxyl-PCs because of their relocation close to the polymeric rim during the simulations and away from the planar central region of nanodiscs.

3. Results

3.1. Size and shape of the lipid bilayer containing nanoparticles

Particle average sizes and shapes of the lipid nanodiscs were determined for compositions of three variants of maleic acid-containing copolymers (SMA, XIRAN and DBMA) and two variants of lipids (DMPC and POPC) (Fig. 1) using dynamic light scattering (DLS) and transmission electron microscopy (TEM). The DLS measurements of nanodisc preparations showed unimodal intensity weighted size distributions with particle average sizes in the range of about 8–10 nm for SMALPs and XIRAN-LPs, whereas the size distribution for DIBMALPs was in the range of about 10–35 nm (see exemplary graphs shown in Figs. 2, 3). The DLS data also revealed a monodisperse character for all types of assembled nanodiscs. Visualization of individual nanodiscs deposited on a solid support was performed by TEM imaging of negatively stained nanodisc preparations. Micrographs showed the individual nanodiscs of all compositions studied as monodisperse well-defined disc-shaped particles. Individual particles were clearly visible on the micrographs but did not reveal sufficient contrast for automatic size calculations based on image binarization. To overcome this issue we used an approach from single particle analysis workflow (for details, see Section 2.5).

Fig. 3 shows the particle diameter distribution of each sample, which is consistent with the DLS data. The SMALPs and XIRAN-LPs diameters are between 5 and 10 nm with maxima around 7–8 nm for both DMPC and POPC. The DBMA-LPs size distributions are wider: 10–25 nm for DMPC with maximum around 14 nm, and 10–50 nm for POPC with a maximum at 25 nm. In the case of POPC, the major nanoparticle population additionally included larger structures of 30–50 nm diameter.

3.2. Dynamics of spin-labeled lipids in lipid nanoparticles

The mobility of spin labeled lipids was determined for the same set of maleic acid-containing copolymer compositions (SMA, XIRAN and DBMA) and lipids (DMPC and POPC), and compared to the results obtained from liposomes prepared from the same lipids. To monitor the dynamics of spin-labeled lipids, EPR measurements were performed for each composition, with nitroxides bound either to the 5th, 12th or 16th carbon atoms of the host phosphatidylcholine chain, 5-, 12-, and 16- doxyl PC (in the following abbreviated 5-PC, 12-PC and 16-PC) (Fig. 1B). The line shape of EPR spectra reflects the dynamics of the spin-label. Unrestricted reorientational motion of a nitroxide results in three equally spaced sharp lines of similar amplitude if the reorientational correlation time is in the ps time range. Increase of the correlation time and/or spatial restriction of the reorientational motion leads to an increase of both the linewidth and the apparent hyperfine splitting. Fig. 4 shows the room temperature EPR spectra of the three different spin-labeled lipids in DMPC and POPC in liposomes or encased in the different nanodiscs with corresponding simulations. For all combinations of lipids and copolymers, the spectra could be simulated by a single spectral component using the model of anisotropic Brownian motion with axial symmetry and orienting potential. In liposomes and in all types of prepared nanodiscs, the EPR spectra of 5-PC and 12-PC show broadening of the lines with an increase of the splitting between the outer hyperfine peaks suggesting restricted motion of the spin labels if compared to 16-PC. The apparent hyperfine splitting is largest for 5-PC. As expected, the nitroxides located closer to the lipid head groups show the most restricted reorientational behavior compared to the samples with spin labels located deeper in the bilayer. Furthermore, the reorientational motion of the spin labeled lipids encased in nanodiscs seems to be more restricted than that of their counterparts in liposomes as obvious from the increased hyperfine splitting for 5-PC or the increased linewidths for 16-PC. The case of 12-PCs is more complex. These labels appear to be immobilized in SMALPs and XIRAN-LPs, but to be much less constrained in case of liposomes. The spectra of 12-PCs in DIBMALPs resemble the previously reported spectra of partly immobilized nitroxide labels, cf. e.g. to ref. [53].

To describe our results quantitatively, we performed spectra simulations based on a model of axial symmetric Brownian reorientation diffusion [38,39,42,54]. This model is justified by the shape of the lipids and their interaction within the membrane, which favors a less restricted reorientational motion around their long axis compared to the reorientation perpendicular to that. The inclination of the nitroxide axes with respect to the main lipid diffusion axes is accounted for by a diffusion tilt angle, $\beta_0$. The rotational correlation time constant, $\tau_c$, is thus a measure of the motion of nitroxides around the two symmetry axes. The segmental order parameter, $S$, describes the motional restriction of the bound nitroxides, which results from the restoring potential acting on the lipids in the membrane.

The bar plots in Fig. 5 depict values of the order parameter, $S$, and the rotational correlation time constant, $\tau_c$, for the three acyl chain positions in preparations of nanodiscs from both DMPC and POPC in comparison to results obtained for liposomes. The values are given in Table 1.

A rigid crystal structure of the membrane is described by the maximum value of the order parameter, $S = 1$, whereas membranes in a state of total dynamic disorder are characterized by the lowest possible value, $S = 0$. For DMPC preparations, the largest values of $S$, between 0.62 and 0.76, are found for 5-PC in all types of particles. Thus, the corresponding nitroxide position represents the most ordered region of the bilayer in liposomes with only minor impact of the polymer on this parameter in the nanodiscs. The values of $S$ for 12-PC range from 0.1 for liposomes to 0.5 for SMALPs and thus represent intermediate values for the set of spin label positions studied. The observed difference between the order parameter values for nanodiscs and liposomes could be explained by the interaction of the spin labeled lipids with the encasing polymers. Noticeably, DIBMALPs exhibit smaller $S$ values than SMALPs and XIRAN-LPs indicating a weaker interaction between the polymer and the spin labeled lipid. 16-PC and thus the nitroxide at position 16 shows small order parameter values ranging from 0.02 to 0.15. These data do not reveal any significant differences between nanodiscs and liposomes. Located close to the end of the lipid tail and in the middle of the bilayer, the 16th position is less restricted compared to the 5th and 12th positions. Similar results were obtained for POPC preparations, confirming that in all preparations the lipid bilayer is in the liquid-crystalline phase, despite the higher phase transition temperature of pure DMPC ($T_{\text{m}} \sim 296 \text{ K}$) compared to POPC ($T_{\text{m}} \sim 271 \text{ K}$).

The inspection of the reorientational correlation times, $\tau_c$, reveals the slowest motion for 5-PC with values of about 6 ns for SMALP and XIRAN-LP preparations, about 3 ns for DIBMALPs and 2 ns for liposomes. SMALPs and XIRAN-LPs exhibit slower motion of spin labels compared to liposomes while DIBMALPs have values in-between. Deeper in the bilayer, at position 12, we observe the same tendency for a decreased spin-label mobility for preparations of nanodiscs compared to liposomes. Both SMALPs and XIRAN-LPs show reorientational correlation times of about 4 ns, whereas DIBMALPs reveal values of
approximately 3 ns. Liposomes, in comparison to the other preparations, show slightly faster motion of the nitroxides with time constant values of about 2.75 ns being closer to those of DIBMALPs. The motion of nitroxides at position 16 is the fastest with rotational correlation times of about 1.5 ns for all combinations of maleic acid-containing copolymers and lipids. As expected, the results obtained for SMALPs and XIRAN-LPs are almost identical in all preparations. Similar to the behavior of the order parameter, the correlation time values show only small differences between DMPC and POPC preparations.

3.3. Molecular dynamics simulations of lipid nanoparticles

3.3.1. Assembly of coarse-grained models of lipid nanoparticles

In order to provide detailed insights into organization and dynamics
of the lipid nanoparticles at the molecular level and to allow for direct comparison with EPR data, we have built CG models of SMALPs and DIBMALPs containing doxyl PCs. The results of the present electron microscopy and dynamic light scattering experiments indicate that both DIBMA and SMA form a substantial fraction of 10 nm sized polymer-lipid nanoparticles (see Figs. 2, 3). Thus, we have modeled SMALPs and DIBMALPs of similar size in order to allow their direct comparison. The parametrization of the CG models, including the development of CG topologies for three types of doxyl PCs with nitroxides attached at different positions along the acyl chain of the lipid, is described in the Section 2.8. The CG models were based on all-atom simulations of the doxyl PCs labeled at positions 5 (proximal to the head group), 12 (middle), and 14 (distal to the head group), which correspond to 5, 12, and 16 doxyl PCs used experimentally. The SMALPs were assembled using periodic SMA polymers with a 3:1 styrene to maleic acid ratio and uniform lengths ([SSSM]13, MW = 5.8 kDa). In our previous simulations [22], this type of periodic SMA polymer showed a behavior similar to that of statistical polymers. For DIBMALPs, periodic DIBMA polymers consisting of S2 units ([DM]26, MW = 5.9 kDa) were used. In the present simulations each of the maleic acid (MA) groups in both SMA and DBMA polymers carried a total charge of −1 (i.e., with only one carboxyl group deprotonated per unit) according to [55].

We assembled nanodiscs starting from preformed disc-shaped patches of POPC or DMPC bilayers containing 142 lipids and 8-doxyl PCs labeled at different positions (see Fig. 6A–B). This corresponds to a disc with a diameter of approximately 10 nm and it is consistent with the typical size of nanodiscs observed in this study as well as in the previous works [1,3,4,56–61]. These preformed lipid patches were surrounded by twelve polymer molecules of either SMA or DIBMA and placed in the periodic water box. This amount of polymers was chosen because it is sufficient to form a single layer of polymers around the cylindrical lipid patch of ca. 10 nm in diameter, which is able to stabilize SMA- and poly(methacrylate) (PMAQA)-containing nanodiscs according to previous computational studies [20–22]. Twelve different types of nanodiscs were assembled: with DMPC or POPC lipids, stabilized by SMA or DIBMA, and containing doxyl PCs labeled at position 5, 12 or 14 (see Table S1 for the detailed description of the simulated systems). During the assembly simulations, the lipid head groups were constrained at their original positions while the polymer molecules could move without restriction for 1 μs. During these simulations, the polymers

Fig. 5. Order parameter, S (stroke-colored bars), and rotational correlation times, τc (full color filled bars), for 5-PC (left column, blue), 12-PC (middle column, green), and 16-PC (right column, orange) in nanodiscs and liposomes consisting of DMPC and POPC lipids.
Table 1
Order parameter, S, (upper part) and reorientational correlation time, \( \tau_c \), (lower part) obtained for doxyl PCs spin labeled at three different positions in SMALPs, XIRAN-LPs, DIBMALPs, and liposomes with POPC and DMPC.

<table>
<thead>
<tr>
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<th>5-doxyl PC</th>
<th>12-doxyl PC</th>
<th>16-doxyl PC</th>
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<tr>
<td><strong>Order parameter S</strong></td>
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<tr>
<td>SMA</td>
<td>0.76 ± 0.02</td>
<td>0.61 ± 0.07</td>
<td>0.10 ± 0.10</td>
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<td>XIRAN</td>
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<td>0.38 ± 0.02</td>
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<td>Liposomes</td>
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<td><strong>Reorientational correlation time, ( \tau_c / \text{ns} )</strong></td>
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<td>6.0 ± 0.2</td>
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<tr>
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<tr>
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<tr>
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<tr>
<td>XIRAN</td>
<td>6.0 ± 0.5</td>
<td>3.7 ± 0.2</td>
<td>1.6 ± 0.1</td>
</tr>
<tr>
<td>DIBM</td>
<td>3.3 ± 1.0</td>
<td>3.0 ± 1.0</td>
<td>1.5 ± 0.2</td>
</tr>
<tr>
<td>Liposomes</td>
<td>2.1 ± 0.3</td>
<td>2.1 ± 0.5</td>
<td>1.1 ± 0.1</td>
</tr>
</tbody>
</table>

rapidly absorbed at the preformed lipid discs adhering mainly to the region of hydrophobic lipid tails exposed to the solvent in the all of the investigated systems (see Fig. S8).

The assembly simulations were followed by the production runs during which all of the constraints were released and each system was simulated for additional 1 \( \mu \)s. In order to estimate the dimensions of the nanodiscs to calculate the radial distribution functions for components of nanodiscs and the order parameters of acyl chains, the nanodiscs in every MD frame were aligned with their principal axes of inertia such that their centers of masses were placed into the axes origin and the X, Y and Z directions matched the principal axes.

All simulated nanodiscs remained stable and retained their flat round shape during the whole simulation time with the aspect ratio along the first two principal axes in a range of 1.0–1.1 and with the average thickness ranging from 5.5 nm to 5.8 nm. The fluctuations of the nanodiscs dimensions along the first two principal axes appeared to be somewhat higher for DIBMALPs compared to SMALPs (0.4–0.5 nm and 0.3–0.4 nm, respectively, see Fig. S7). This might imply a relative destabilization of DIBMALPs by electrostatic repulsion of the DIBMA polymers, which bear a larger charge for a single polymer due to the larger fraction of maleic acid residues compared to SMA.

3.3.2. Distribution of polymers and doxyl PCs in lipid nanoparticles

The SMA polymers in SMALPs arrange into two distinct belts surrounding the lipids as indicated by two peaks for MA units in the density plots (see Fig. 6C). In contrast, in DIBMALPs the polymers form a single broader belt as indicated by the density plots for maleic acid units of DIBMA polymers (Fig. 6C). Similar density plots for the phosphate groups of POPC show that lipids inside both SMALPs and DIBMALPs are oriented similar to those in the native membrane and appear mainly as in a flat bilayer.

In both SMALPs and DIBMALPs, spin-labeled doxyl PCs tend to relocate towards the polymer belt and adsorb at the lipid-polymer interface (see Fig. 7). However, this tendency is more pronounced for SMALPs. Also, for the 12-PCs but not for the 5-PCs or 14-PCs in SMALPs, we observed a shift of the corresponding peak of the radial distribution function towards the average position of the MA units of SMA (Fig. 7A, red curve). This finding implies that the labeled lipids in this case form tighter contact with the polymer belt and that the nitroxide moieties intercalate between the aromatic rings of SMA. In contrast, in DIBMALPs the nitroxides of all the three types of spin-labeled lipids do not penetrate into the polymer rim but remain largely concentrated at the interface between DIBMA and the lipid tails (Fig. 7B).

3.3.3. Order parameters of lipids

The order parameters calculated for the acyl chains of POPC and DMPC lipids decrease along the acyl chains both in DIBMALPs or SMALPs and in planar bilayers, the latter used as a reference. Positions, which are closer to the polar head group, exhibit more restricted mobility compared to positions, which are more distant from the head group (see Fig. 8). At the same time, both types of lipids exhibit increased mobility in planar bilayers compared to lipids encapsated in nanodiscs. The results revealed a slight decrease of the order parameters for both POPC and DMPC in DIBMALPs compared to SMALPs with the most pronounced difference observed for the middle position in the acyl chain.

![Fig. 6](https://example.com/fig6.png)

Fig. 6. Coarse-grained models of DIBMA- (A) and SMA-stabilized (B) lipid particles. Phosphate, choline and maleic acid (MA) moieties are shown as orange, blue, and red spheres, respectively, styrene rings in SMA/diisobutylene in DIBMA as yellow triangles/spheres, backbones of SMA copolymers and lipids as gray and white sticks, respectively. C. Mass densities along the normal to the plane of SMALP/DIBMALP calculated for the phosphate groups of POPC lipids, MA of polymers and the spin label of 12 doxyl lipids.
chains of POPC (see Fig. 8A).

4. Discussion

In this work, using a combined experimental and in silico approach, we compared the mobility of lipid acyl chains in DIBMALPs and SMALPs containing DMPC or POPC. We applied EPR spectroscopy to study the behavior of spin labeled lipids with nitroxides attached to carbon atoms at different positions along the hydrocarbon chain. Furthermore, we performed CG MD simulation of SMALPs and DIBMALPs containing spin labeled PCs to address the lipid distribution within these LPs.

The DLS measurements yield identical average particle sizes of 8–10 nm for all assembled SMALPs and XIRAN-LPs, which corresponds to the results obtained by other authors [1,3,4,56–59]. For DMPC/DIBMALPs and POPC/DIBMALPs, the DLS data reveals average sizes of 14–16 nm and 10–40 nm, which are also in agreement with published data [10,11]. The TEM images show the individual nanoparticles of all studied compositions as well-defined disc-shaped particles consistent with previously reported TEM data [1,11,18,56,58,59]. Based on particle class analysis, the lateral sizes of SMALPs and XIRAN-LPs for both saturated DMPC and monounsaturated POPC have maxima around 7–8 nm. Thus, the unsaturation of lipids seems not to influence the size of SMALPs. Interestingly, DIBMALPs prepared under comparable conditions had maxima around 14 nm for DMPC and 25 nm for POPC being larger than SMALPs. It appears that the size of DIBMALPs increased with the extent of lipid unsaturation, which is in line with previous data obtained by Keller and colleagues [11].

The X-band (9 GHz, 0.3 T) EPR spectroscopy of nitroxides is very sensitive to molecular motions in the nanosecond time range and resolves the reorientation dynamics of spin labels bound to different parts of the fatty acid chains [23,42,53]. Our EPR experiments revealed the influence of the immediate environment on the degree of motional constraints of the spin labels in all preparations of nanodiscs. The line shapes of the EPR spectra vary between broad asymmetric lines, indicating immobilized nitroxides and three narrow lines in the case of fast isotropically moving nitroxides. For all types of nanodiscs, we observed broadening of the lines for all three nitroxide positions compared to liposomes. The nitroxides at the 5th carbon position are located close to the lipid head groups and, accordingly, 5-PCs reveal the mostly ordered nitroxides compared to 12-PCs or 16-PCs in nanodiscs and in liposomes. Interestingly, SMALPs and XIRAN-LPs show a higher degree of spin label immobilization than DIBMALPs and liposomes, most obvious in the spectra of 12-PCs.

The two parameters extracted from the spectra, the reorientation correlation time, $\tau_c$, and the order parameter, $S$, allow quantitative comparison of the dynamical restrictions of the encased lipids (Table 1, Fig. 5) with those in liposomes. In the following, values for $S$ reported...
previously for liposomes will be briefly discussed in relation to our data. (Numerical values of data recorded at similar temperatures (297 ± 3 K) to ours were determined from published figures, thus their approximated values are marked by ~). Our S value for 5-PC in DMPC liposomes, 0.62 ± 0.09, agrees with reported values which range from ~0.52 [62] to ~0.64 [31]. For POPC liposomes, reported S values range from ~0.60 [31] to ~0.68 [63], which again correlates with our finding of 0.66 ± 0.04. These values mark the most restricted motion of the nitroxide due to their location close to the densely packed lipid headgroups. For 12-PC in DMPC liposomes, the order parameter values reveal a less restricted motion of the nitroxide compared to 5-PC. Our value, S = 0.29 ± 0.13, agrees with reported values of between ~0.23 [62] and ~0.31 (for 10-PC) [31]. Similar values were found for POPC liposomes, S = 0.34 ± 0.10 (our work), which agree with literature values ranging from ~0.24 [30] to ~0.54 [31]. Close to the center of the liposome bilayer, the nitroxides of 16-PCs undergo the least restricted reorientational motion with order parameter values close to 0. The S values of 0.12 ± 0.15 (DMPC) and 0.02 ± 0.04 (POPC) found in this work are again in agreement with reported values ranging from ~0.09 [31] to ~0.12 (for 14-PC) [62] for DMPC, and from ~0.12 [31] to ~0.18 for POPC [63]. This overall agreement between our data determined for liposomes and the corresponding literature values prove the present approach of data evaluation reasonable and thus applicable for the following characterization of doxyl PC dynamics in nanodiscs.

For doxyl PCs in nanodiscs, we observe increased motional restrictions. This behavior is most prominent for 12-PCs in SMALPs and XIRAN-LPs, as concluded from the increased order parameter and correlation time values in comparison to liposomes. Similar behavior was reported before in SMALPs for 10-PC [31], 11-PC [4] and for 12-PC [32], and the generally increased motional restriction was concluded to support the idea of a tightly packed bilayer system creating a stable structure [31]. Within error margins, order parameter and correlation time values for SMALPs and XIRAN-LPs are identical for the respective doxyl PCs (Table 1). In contrast, a smaller impact of DIBMA on the doxyl PC and lipid dynamics is observed (Figs. 5, 8). Experimental order parameter and reorientational correlation time values of doxyl-PCs in DIBMALPs more closely resemble those of doxyl PCs in liposomes than those found for SMALPs. Due to the larger average size of DIBMALPs compared to SMALPs (cf. Figs. 2, 3), in DIBMALPs a smaller fraction of lipid molecules is in close proximity to the polymer and thus affected directly in its dynamics. The results of our MD simulations discussed below provide an additional explanation for the different impact on the lipid and doxyl PC dynamics observed for the two copolymers.

The CG MD simulations performed here are related to our previous study where we used the same approach to follow the pulling of lipid patches from DMPC bilayers by SMA copolymer, which resulted in formation of SMA-encased DMPC [22]. The morphology of these discs, with two belts of SMA copolymers stabilizing the rim of the lipid patch, was similar to the arrangements proposed earlier [5,6] and was also observed in earlier MD simulations by Xue et al. [20] and Sahoo et al. [21]. We observed that the process was driven mainly by hydrophobic interactions, as it had been suggested earlier for the formation of SMALPs [64]. The MD simulations showed that a fraction of the styrene groups exhibits hydrophobic interactions with the lipid tails upon formation of SMALPs [22]. The results of these MD simulations indicated the influence of the delicate balance of hydrophobic and hydrophilic units in SMA copolymers on their interaction with membranes. Hydrophilic groups of lipids first interact with maleic acid groups while hydrophobic tails tend to form hydrophobic interactions with disobutylene and styrene.

In the present work, we showed that in contrast to SMA, the interaction of DIBMA with lipids leads to the formation of single-belt disc structures. Both types of modeled phosphatidylcholine lipids, POPC and DMPC, were less constrained in DIBMALPs than in SMALPs as confirmed by the difference in the calculated order parameters of lipids in the two types of nanodiscs (Fig. 8). In line with this observation, the simulations indicate that the spatial dimensions of DIBMALPs fluctuate to a larger extent as compared to SMALPs (Fig. 57), which might be due to a larger negative charge of a single DIBMA copolymer (built of [DM"] units) as compared to a single SMA copolymer (built of [SSSM"] units).

Furthermore, the MD simulations revealed that doxyl PCs show the tendency to relocate closer to the polymeric rim of nanodiscs (Fig. 7). This tendency was more pronounced in SMALPs than in DIBMALPs. Only the 12-PCs, in contrast to the other types of spin-labeled lipids, tend to penetrate deeper into the polymeric rim of SMALPs, with their spin labels specifically intercalating within the aromatic rings of styrene groups of SMA (Fig. 7A). The observed interactions of spin labels with polymers may be due to the formation of intermolecular hydrogen bonds, electrostatic interactions and stereochemical interactions [65]. However, it could be difficult to precisely delineate the main driving force of the observed affinity of the spin labels to the polymers due to limitations of the utilized CG model [45], which does not account explicitly for hydrogen bonds and proper distribution of partial charges (approximated by integer charges localized only on the charged CG beads), and intrinsically leads to smoother stereochemical interactions [66]. These contacts become favorable most likely due to the match between the position of the 12-PC nitroxide and the SMA belts (Fig. 6C). In contrast, DIBMA copolymers contain no aromatic rings complementary to the ring of the spin label. According to the results of our MD simulations, the experimentally observed significant immobilization of the 12-PCs in SMALPs could thus be explained by dwelling of the 12-PC lipids predominantly at the polymer-lipid interface with the lipids being tethered by interactions with the styrene groups of the copolymers. In contrast, the EPR spectra of 12-PCs in DIBMALPs show only partially immobilized spin labels, presumably due to the weaker interaction of the spin label with the polymer and the different structure and position of the copolymer belt, as revealed by MD simulations.

We cannot fully exclude that the experimentally measured higher lipid mobility in DIBMALPs (Table 1, Figs. 4, 5) could correlate with their larger size as compared to SMALPs (Fig. 3). Indeed, the relative fraction of lipid molecules that are dynamically constrained by their proximity to the copolymer ring should be smaller in larger particles. Still, we believe that the major cause of hampered mobility of spin-labeled lipids was their strong interaction with the polymer both in DIBMALPs and SMALPs as uncovered by MD simulations (Fig. 7). This interaction leads to concentration of the majority of spin-labeled lipids at the lipid-polymer interface (i.e., in the polymer rim of the nanodiscs, see Fig. 7) which, in principle, should reduce the effect of particle size on the dynamics of spin-labeled lipids. In addition, the unlabeled lipids appear to experience certain immobilization not only in SMALPs, but also in DIBMALPs (see Fig. 8). Still, the interplay between the size of lipodiscs, the lipid-polymer interactions, and the physico-chemical properties of lipodisc-stabilizing polymers deserves further investigations.

In conclusion, our EPR experiments and MD simulations reveal that the dynamics of spin labeled and unlabeled lipids is less constrained in DIBMALPs compared to SMALPs, rendering DIBMALPs a versatile tool for obtaining nanodiscs with more "native" lipid bilayer dynamics. Furthermore, our results evidence that the spatial distribution of lipophilic compounds, which may be present in the nanodiscs, could be modulated by tailoring the composition or functionalization of the maleic acid-containing copolymers.

Declaration of competing interest

The authors of the manuscript “Lipid Dynamics in Nanoparticles Formed by Maleic Acid-containing Copolymers: EPR Spectroscopy and Molecular Dynamics Simulations“ declare that they have no conflict of interests.
Acknowledgements

This work was supported by German Research Foundation (DFG, STE640/15) to H.J.S., the O斯塔partnerechaftenprogramm of DAAD and RFBR grant no. 18-504-12045 to K.V.S. and O.S.S. were supported in part by the RFBR grant no. 18-504-12045 to H.J.S., the Ostpartnerschaftenprogramm of DAAD and Lomonosov Moscow State University. K.V.S. and O.S.S. were supported in part by the Program of Leading Scientific Schools ‘Depository of the Living Systems’ in the framework of the Lomonosov Moscow State University Development Program. The samples for TEM imaging were prepared in the electron microscopy (EM) unit of Integrated Bioimaging Facility (iBiOs) at Center of Cellular Nanoanalytics (CellNanoOs), University of Osnabrück. We are very thankful to Dr. Katerina Psathaki for the support. Molecular dynamics simulations were performed using facilities of FRCCP RAS (state task AAAA-A19-119012990175-9).

Author contributions

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Data and materials availability

All data needed to evaluate the conclusions in the paper are present in the main text and/or the Supplementary Information. Additional data related to this paper may be requested from the authors.

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