Self-Assembly of X-Shaped Bolapolyphiles in Lipid Membranes: Solid-State NMR Investigations

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ABSTRACT: A novel class of rigid-rod bolapolyphlic molecules with three philicities (rigid aromatic core, mobile aliphatic side chains, polar end groups) has recently been demonstrated to incorporate into and span lipid membranes, and to exhibit a rich variety of self-organization modes, including macroscopically ordered snowflake structures with 6-fold symmetry. In order to support a structural model and to better understand the self-organization on a molecular scale, we here report on proton and carbon-13 high-resolution magic-angle spinning solid-state NMR investigations of two different bolapolyphiles (BPs) in model membranes of two different phospholipids (DPPC, DOPC). We elucidate the changes in molecular dynamics associated with three new phase transitions detected by calorimetry in composite membranes of different composition, namely, a change in \( \pi-\pi \)-packing, the melting of lipid tails associated with the superstructure, and the dissolution and onset of free rotation of the BPs. We derive dynamic order parameters associated with different H–H and C–H bond directions of the BPs, demonstrating that the aromatic cores are well packed below the final phase transition, showing only 180° flips of the phenyl ring, and that they perform free rotations with additional oscillations of the long axis when dissolved in the fluid membrane. Our data suggests that BPs not only form ordered superstructures, but also rather homogeneously dispersed \( \pi \)-packed filaments within the lipid gel phase, thus reducing the corrugation of large vesicles.

INTRODUCTION

Lipid molecules constitute the main building blocks of cell membranes, the latter being complex entities that contain many additional components such as proteins, carbohydrates, and cholesterol. The organization of all these components within the membrane determines biological function, and its molecular-scale study is a major research goal. The self-organization of synthetic amphiphilic molecules within lipid membranes to form entities with controlled functionality such as, e.g., synthetic ion channels, has found much interest in recent years.\(^1\)–\(^9\) Also, facial amphiphilic molecules were shown to be useful for drug delivery.\(^10\) The interactions of different parts of such synthetic molecules with the lipids within the membrane, as well as their influence on structure and dynamics of the lipid molecules, have not been investigated systematically. It is thus of interest in which way the properties of the membrane as a whole or that of the individual lipid molecules are changed in the presence of the synthetic molecules. In addition, the self-assembly of the guest molecules and the formation of a composite structure with lipids within the bilayer may lead to membrane domains where the lipids show similar behavior as previously found for the so-called liquid-ordered phase in phospholipid-cholesterol systems.\(^11\),\(^12\)

In two recent publications\(^13\),\(^14\) we have reported on novel bolapolyphlic molecules (BPs), which can be incorporated into model lipid bilayers (see Figure 1a). The BPs consist of a rigid hydrophobic phenylene-ethinylene backbone with two hydrophilic glycerol headgroups attached at both ends. In addition, two hydrophobic, flexible alkyl chains are connected to the central phenyl ring, giving the molecules an X-shaped structure. One of those molecules is the BP B12, which was hypothesized to self-organize into a honeycomb lattice, where the honeycomb walls are formed by the rod-like \( \pi-\pi \)-stacked backbone and its cells accommodate the alkyl side chains as well as confined lipids.\(^13\) The glycerol headgroups are aligned along the upper and lower edges of the honeycombs. This structural model was based upon an extensive multimethod approach,\(^13\) and included the assignment of phase transitions to different molecular processes on the basis of integrals from \(^1\)H high-resolution magic-angle spinning solid-state NMR spectra.

In the present paper, we provide a detailed description and discussion of these results and report on a variety of advanced, site-resolved solid-state NMR experiments aimed at fully characterizing structural and dynamic properties of the membrane components. Probes of dynamics include the mentioned simple \(^1\)H magic-angle spinning (MAS) NMR line widths and intensities as well as residual \(^13\)C–\(^1\)H dipolar couplings from dipolar-shift correlation (DIPSHIFT) dephasing curves\(^15\),\(^16\) and residual \(^1\)H–\(^1\)H dipolar couplings from double-quantum (DQ) sideband patterns\(^17\)–\(^19\). These were
measured for pure B12 and its mixtures with DPPC (molar ratios 1:10 and 1:4) and DOPC (molar ratio 1:10). A headgroup-modified BP (E12/7) was also investigated in a mixture with DPPC. With these sources of information we could assign the three additional new phase transitions in the differential scanning calorimetry (DSC) thermograms of the B12/DPPC mixtures to one specific molecular process each. Furthermore, we obtained a coherent picture of the molecular dynamics and thus also structural aspects of the BP molecules within the lipid bilayer as well as in the bulk.

■ EXPERIMENTAL SECTION

$^1$H and $^{13}$C−$^1$H MAS NMR. MAS NMR investigations were carried out on multilamellar vesicle (MLV) preparations made by codissolving lipid and the respective BP in methanol/chloroform, drying, and subsequent rehydration by adding 50 wt % of H$_2$O/D$_2$O to the powder. All data was acquired on a Bruker Avance III instrument with 400 MHz $^1$H Larmor frequency using a Bruker 4 mm MAS WVT double-resonance probe head at 5–10 kHz spinning frequency, relying on a flow of heated air for temperature regulation. Typical 90° pulse lengths were 3 and 3.2 μs for $^1$H and $^{13}$C, respectively. $^{13}$C spectra were usually taken with a 5 s recycle delay using either direct polarization (DP) by a 90° pulse or cross-polarization (CP), with 1.5 or 5 ms contact time using spin-lock nutation frequencies of 83 kHz and 71.5 kHz for $^{13}$C and $^1$H, respectively. SPINAL64$^{20}$ was used for $^1$H dipolar decoupling at a nutation frequency of 83 kHz. Heating effects due to bearing air friction under MAS and long rf irradiation were taken into account by a calibration and by sufficiently long recycle delays, respectively, as tested on the phase transition temperatures of hydrated pure lipids.

Quantitative $^1$H MAS Spectra. Weight-controlled samples were prepared in 4 mm MAS rotors using Teflon spacers for reproducible sample positioning. The recycle delay was at each temperature adjusted to a value much larger than $T_1$ relaxation time, and the spectral intensities were corrected for the Curie temperature dependence, i.e., multiplying them by $T/323$ K (T in K), allowing for a quantitative evaluation of the integrals. The aliphatic, aromatic, and glycerol resonances of immobilized BPs and DPPC in the gel phase (20 °C) at the given spinning frequency of only 5 kHz are subject to dipolar line broadening down to the baseline level due to the well-defined packing and low mobility, precluding their observation in sufficiently narrow integration ranges. Just above the main lipid phase transition, all resonances of pure hydrated DPPC become well-resolved and quantitatively detectable, while their intensity remains lower for the mixtures. The resolved glycerol resonance of the pure DPPC preparation at 323 K (50 °C) served as an external intensity standard, defining 100% of the signal for each group of resonances. Significant intensity is found in sharp spinning sidebands, which are also integrated. Deviations of experimental high-temperature data from 100% mobilization demonstrate that the measurements are subject to systematic errors on the order of 20%, which can be explained by baseline problems upon integration, and the external calibration, where a change of sample in a MAS rotor can lead to setup variations and thus deviations in the absolute spectral intensities.

DIPSHIFT and Recoupled DIPSHIFT. Dipolar modulation curves were acquired using the pulse sequences described in our previous publications$^{14,21,22}$ using Lee-Goldburg homodecoupling at an effective nutation frequency of 85 kHz (absolute nutation frequency of 62.5 kHz at 49.1 kHz offset), relying on $^{13}$C DP or CP, depending on which of the two gave a better signal-to-noise ratio for the BP resonances of interest. It is stressed that we can exclude a bias due to subensembles of different mobilities, as the $^1$H integrals demonstrate that the respective BP is either fully immobilized or fully mobile at experimental temperatures well above or below relevant phase transitions. Recoupled DIPSHIFT data, necessary to quantify rather weak couplings, was taken with $N=4$ rotor periods of recoupling.$^{14}$ In order to obtain residual $^{13}$C−$^1$H dipole−dipole coupling constants from the measured modulation curves, the data was fitted to analytical formulas for the powder-averaged signal according to published procedures, taking into account the specificities of CH, CH$_2$, and CH$_3$ groups (see, e.g., the Supporting Information of ref 21). Note that $^{13}$C-centered DIPSHIFT techniques, as opposed to so-called proton-detected local field techniques$^{25}$ provide only one effective order parameter for CH$_3$ groups, even in cases where the two protons are magnetically and dynamically inequivalent.

$^1$H−$^{13}$C DQ Spinning-Sideband Analysis. For the neighboring aromatic protons, the corresponding dipole−dipole coupling constants were determined by DQ$^{26}$ spinning-sideband analysis$^{22,27}$ using the BaBa-xy$^{25,26}$ at 10 kHz MAS and 4 rotor periods of recoupling. The pattern was fitted to a numerically calculated and Fourier-transformed powder average of the theoretical time domain signal, assuming a Gaussian distribution of residual dipole−dipole couplings $D_{	ext{res}}$. Its standard deviation was usually around 10% of the average, indicating weak apparent distribution effects.$^{22}$ Such effects do not necessarily arise from an actual coupling distribution, but can also be assigned to changes in the pattern shape arising from couplings to remote protons$^{27}$ or from a bias in the assumed isotropic powder average$^{22}$ as a consequence of an anisotropic transverse relaxation time $T_2$.

■ SUMMARY OF PREVIOUS RESULTS

Fluorescence imaging and spectroscopy experiments on giant unilamellar vesicles (GUVs) prepared from DPPC and B12 at a 10:1 molar ratio revealed a self-assembly of B12 within the membrane exhibiting a dendritic structure of a snowflake-like appearance with 6-fold symmetry, as seen in Figure 1b. From orientation-dependent (polarized) fluorescence measurements an incorporation of B12 in transmembrane orientation could be deduced. Furthermore, in DSC thermograms of such mixtures, three new phase transitions appeared in addition to the main phase transition of the pure lipid at about 42 °C, see Figure 2a and Figure 3 of ref 13. This was also interpreted as an indication of phase separation between pure phospholipid and B12-rich domains, the latter with variable B12 and significant lipid content. For high temperatures ($T \geq 75$ °C), a homogeneous mixture was assumed and proven with different methods.$^{14}$
Further structural investigations included X-ray diffraction measurements. Above the main phase transition temperature of the lipid, intense WAXS peaks were still visible, indicating the sustained presence of ordered lipid domains. These were interpreted as a modified gel phase with different packing of the alkyl chains as compared to a pure lipid gel phase. In addition, the coexistence of lipid domains with tilted and nontilted chains with respect to the membrane normal was suggested, in which the nontilted chains are assigned mainly to the B12/DPPC complexes. Two coexisting structural domains were also evidenced by $^{31}$P NMR measurements. Analysis of the $^{31}$P line shape and $T_2$ relaxation time, probing headgroup dynamics, revealed that the two structural fractions exhibit different motional amplitudes: one as in the pure DPPC and another one with notably larger amplitude despite alkyl chain order persisting up to higher temperatures.

Based on these measurements, a structural model for B12 in DPPC in the temperature range of phase separation was derived, including an explanation for the hexagonal structure. This honeycomb model includes stacked B12 backbones arranged along honeycomb walls, while the resulting hexagonal cells are filled by the lateral alkyl chains of B12 as well as those of DPPC. The DPPC alkyl groups are thus more ordered and immobilized due to the confinement caused by the B12 molecules surrounding them, raising their main phase transition. See Figure 12 further below for a sketch.

For a different lipid, the unsaturated DOPC, a different situation is suggested by fluorescence imaging (see Figure 1c). The B12 molecules appear to be homogeneously distributed within the bilayer, showing no macroscopic phase separation. In addition, structural variation of the hydrophilic headgroups of the BP leads to different interactions with the lipid DPPC. E12/7 with its oligo(ethylene oxide) (EO) headgroup (see Figure 1a) is also incorporated in a transmembrane fashion, but again a nearly uniform distribution of the BP in the bilayer is suggested by GUV imaging (see Figure 1d). Here, we either have freely mobile, individual E12/7 within the bilayer, or only small complexes of E12/7 and possibly lipid. This difference was attributed to the ability of a BP headgroup to either form hydrogen bonds or not, which appears to be a key factor for macroscopic phase separation within the membrane. The question of whether we indeed have full solubility or possibly nanometer-scale phase separation in the latter two cases will be addressed below.

## RESULTS AND DISCUSSION

### DPPC+B12. The $^1$H and $^{13}$C NMR spectra in Figure 2 of the DPPC-B12 mixture are well-resolved, enabling site-resolved measurements. In particular, all B12 core signals are accessible, which can thus be analyzed in detail. As described above and in our recent paper, temperature-dependent quantitative $^1$H NMR spectra were acquired to investigate the phase behavior between 20 and 75 °C qualitatively. We here discuss these published results in more detail than was possible in our first account.

**Mobility from $^1$H Signal Intensities.** As the measured $^1$H spectral integrals (including spinning sidebands) are proportional to the number of mobile molecules in the sample, the values for the alkyl chain, the glycerol-2 proton ($\text{g}_2$) as well as for the B12 aromatic rings were analyzed (see Figure 3a). For the pure DPPC below its main phase transition temperature, only very low signal intensities are detectable, indicating mostly immobile lipids. At the main transition (∼40 °C), the integral increases to the expected maximum, meaning that all lipid molecules become mobile at this temperature.

For the B12-DPPC mixture, a different picture is observed. Below the main lipid transition, all lipids as well as the B12 aromatic cores show negligible signal, indicating immobile, thus well-packed molecules. At the main lipid transition, only a fraction of the lipid molecules becomes mobile for the different mixtures, as the alkyl and $\text{g}_2$ intensities do not reach the expected maximum value. Upon increasing the temperature beyond the main transition, the intensity of the lipid signals...
increases further. This supports the assumption of phase separation and partitioning: some of the lipid molecules are confined and therefore stay immobilized at the main lipid phase transition. The comparably wide temperature range over which the spectral changes take place is attributed to sample inhomogeneity, as is also apparent from the DSC traces.

For the B12 aromatic cores, the intensity rise is even delayed by 5–10 K as compared to the immobilized part of the lipid, which indicates that the phase-separated B12 molecules are part of a distinct structure. Above all phase transitions (T > 75 °C), all integrals reach the expected maximum, indicating fully mobilized molecules and absence of phase separation. These findings agree well with the IR and fluorescence anisotropy results presented before, where only partial fluidization of the DPPC chains was observed above the first DSC peak and fluidization was only complete at high temperature. Fluorescence anisotropy data for B12 showed that it becomes only mobile at higher temperature at which the additional DSC peaks were seen.

Closer inspection reveals that the inflection points of the sigmoidal temperature-dependent signal increases for the mixtures roughly (within the accuracy of the temperature calibration of the order of 1–2 K) coincide with the second (lipid) and third (B12 core) additional phase transition seen in the DSC thermogram. Thus, two out of the three additional phase transitions can be assigned on the basis of the 1H integrals: transition II is accompanied by the mobilization of lipid molecules that are presumably confined and immobilized within honeycomb cells, and transition III is related to the dissolution of the supramolecular structure and the formation of a homogeneous mixture. The assignment of the first additional phase transition is not possible on the basis of these data.

**Mobility and Packing from 13C Spectra.** To solve this question, and now turning to new and unpublished data, a closer look at temperature-dependent 13C spectra is instructive. Spectra for the 1:4 mixture of B12 and DPPC were taken with two excitation methods, DP and CP (see Figure 4). With such spectra we are able to distinguish different mobilities, namely, the more mobile carbons being mainly excited by DP and the immobile ones being excited more efficiently by CP. The acquired spectra indeed look different for different temperatures and the respective excitation methods. For the lipid signals (not shown), phase separation and coexistence is evidenced by the appearance of an additional set of signals for a given temperature above the main lipid transition, as well as by the gradual in- and decrease of signal intensities on heating in the DP and CP spectra, respectively. Above 70 °C, only one set of signals is observed, in agreement with the notion that all molecules are now mobile, as evidenced from the 1H integrals.

Turning to B12, note first that not all core resonances could be uniquely assigned. The numbering scheme in Figure 2 simply follows the resonance position; CH (ar1,7–10) and quaternary Cq (ar1,2,5,6) resonances could be distinguished by CP contact time variation in bulk B12. Relative peak intensities and comparisons to solution-state spectra allowed us to assign ar8 and ar10 to the CH resonances of the central ring and of the outermost ring-CH, respectively, while ar1,4,7,9 represent the remaining ring-CH resonances. Also, the glycerol headgroup resonances of B12 (G1–4) were not assigned further. The multiplicity of the resonances, being higher than expected by molecular symmetry, suggests a symmetry break as a result of molecular packing.

An interesting observation is made for the aromatic core of B12. For the signal arπ, a temperature-dependent change in chemical shift is evidenced (see Figure 4). Whereas at 50 °C only one signal at approximately 134.4 ppm is visible, two signals can be clearly distinguished at 60 °C. The first signal decreases and finally disappears, while the second one at approximately 133.7 ppm increases. Therefore, it is suggested that the two signals reflect the same carbon atom but with different molecular packing. The change in chemical shift is very likely due to a different position of the respective carbon in the vicinity of another aromatic ring, i.e., a packing-related current effect. As the spectra also indicate different mobilities, the change in packing obviously provides increasing motional freedom. By referring again to the DSC thermogram of the corresponding 1:4 mixture of B12 and DPPC (see Figure 2a), the temperature of the packing alteration indeed fits to the first additional phase transition. We thus assign transition I to a change in π–π packing as well as mobility of the B12 cores.

**Dynamic Order Parameters.** In order to elucidate the molecular dynamics in the different phases in more detail, 13C–1H DIPSHIFT experiments were applied for the determination of dynamic order parameters related to different CH bond directions. In general, the DIPSHIFT method provides the magnitude of 13C–1H dipolar coupling constants DCH. Dipolar couplings are sensitive to both distance and internuclear orientation of the involved nuclei. At fixed distance, they thus probe the extent of averaging by motions of the respective moiety with a rate much higher than 100 kHz. This dynamic range is related to the magnitude of the static-limit coupling constant DCH,stat in units of rad/s (DCH,stat/2π ≈ 21 kHz). Since the angle dependence of dipolar coupling follows the second Legendre polynomial (P2(cos θ)), a dynamic order parameter

\[
S_{CH} = D_{CH,exp}/D_{CH,stat}
\]

is defined. Its value can be used to distinguish between different motional geometries. Note that, from our data (modulation curves reaching again maximum intensity at maximum modulation time), we can exclude the presence of significant “intermediate” motions with rates in a range between 1 and 100 kHz. Slower motions cannot be detected with this method.

We first focus on the pure B12 substance, for which DIPSHIFT measurements were performed at two different temperatures, T = 30 and 90 °C (data for the lower

**Figure 4.** 13C MAS spectra of the aromatic spectral region for the 1:4 B12-DPPC mixture at different temperatures, using two excitation schemes (DP: upper, CP: lower spectra). Two peaks with chemical shifts differing by about 0.7 ppm are observed for resonance arπ, one converting into the other upon increasing the temperature.
temperature not shown). From the dephasing curves (for an example, see Figure 5a), different residual couplings were determined that indicate distinct motional amplitudes within the B12 molecule. From the corresponding dynamic order parameters \( S_{CH} \approx 0.6 \) for all aromatic ring carbons with directly bonded protons, and \( S_{CH} \approx 1 \) for quaternary ring carbons (see Figure 5b), relevant conclusions can be drawn. The CH dipolar tensors for the two carbon types (see the inset) differ in their sensitivity to different motional models. Comparing the \( S_{CH} \) values to predictions according to \( n \)-site jumps, diffusion on/within a cone, etc.,\(^{28,31} \) we conclude on fast two-site 180° jumps around the molecular long axis. This is a well-known motif for para-substituted and well-packed phenyl rings.\(^{28,33} \)

In the 1:4 mixture of B12 and DPPC, the B12 resonances were sufficiently intense to also investigate the dynamics of B12 in the mixture, whereas this was not possible for the 1:10 mixture due to low signal-to-noise ratios. We discuss data for two temperatures, one in the region of phase separation (\( T = 45 \) °C), and one above all phase transitions (\( T = 75 \) °C). Again, order parameters were determined from the DIPSHIFT experiments, the results for which are also shown in Figure 5b. As can be seen for the lower temperature, there is no significant difference in the order parameters of the B12 carbons in the mixture as compared to pure B12. This means that all aromatic carbons show the same motional behavior in the mixture as in the B12 crystal, i.e., fast 180° flips of the aromatic rings. Also, the order parameters of the alkyl chains as well as the glycerol carbons did not change in the mixture for the low temperature as compared to the bulk substances. This, along with the fact that all \(^{13}\)C resonances are rather narrow, indicates that the B12 molecules are well packed and ordered within the separated supramolecular structure.

At the high temperature, all carbon atoms exhibit strongly reduced order parameters. All lipid molecules are then in the mobile liquid-crystalline phase, and also the B12 molecules show increased mobility. On the basis of suitable motional models (diffusion on or within a cone), we find that our data is compatible with uniaxial rotation of the aromatic rings about the molecular long axis, with additional wobbling motions that reduce the order parameter further. Here, also the alkyl chains and glycerol carbons show increased molecular amplitudes (lower \( S_{CH} \)) due to the much increased motional freedom. These observations support the above interpretation of free dissolution of the B12 in the fluid membrane.

In addition to the \( S_{CH} \) values characterizing the dynamics of the B12 cores via C–H coupling directions, we also measured homonuclear residual dipolar couplings by \(^{1}H–^{1}H\) DQ spinning sideband analysis.\(^{17–19} \) These provide dynamic order parameters \( S_{HH} \) characterizing the dynamics of the fairly isolated aromatic \(^{1}H\) pairs of the para-substituted phenyl rings. Notably, \( S_{HH} \) is not sensitive to axial rotation, but only to the additional fluctuations (wobbling) of the molecular long axis (see again the inset of Figure 5b).

These measurements were performed for high temperatures between \( T = 65 \) and 80 °C for both mixtures of DPPC and B12, i.e., the range within which a fraction of the fast-rotating B12 molecules are observed. Note that \( \pi \)-packed B12 cores exhibiting only the fast yet restricted 180° flips are not observable under the given experimental conditions.

From the measured sideband patterns (for an example, see the inset of Figure 6), the residual dipolar coupling was determined and hence \( S_{HH} \) calculated. The “static-limit” (rather: perfect-rotation) reference coupling of 8.3 kHz was estimated from the average distance of two aromatic ring protons of about 2.44 Å. In this way, \( S \approx 0.6–0.8 \) were found, as shown in Figure 6. It can be seen that the values are nearly constant for the different temperatures, thus the motional amplitude is not significantly altered upon heating.

Figure 5. (a) \(^{13}\)C–\(^{1}H\) DIPSHIFT (left) and recoupled DIPSHIFT (right) dephasing curves for aromatic resonance \( \alpha _{n} \) of B12 in the 1:4 mixture with DPPC; (b) dynamic order parameters \( S_{CH} = D_{CH}/D_{HH} \) for all resolved B12 resonances as well as expected values for specific motional models for B12 (dashed lines). Error intervals are estimated from the experimental signal-to-noise ratio and the standard deviation over repeated experiments. See Figures 2c and 9 and the main text for the peak assignments. The inset shows schematically the different dipolar tensors probed.

Figure 6. \(^{1}H–^{1}H\) order parameters \( S_{HH} \) for two B12 aromatic resonances in 1:4 and 1:10 mixtures with DPPC as a function of temperature. The error bars indicate the dipolar distribution width \( \sigma \) from the fit; the actual experimental error is smaller and apparent from the deviations from the trend lines. Inset: DQ spinning sideband pattern for the 1:4 mixture taken at 65 °C including a fit.
Interestingly, the order parameter for the 1:10 mixture is systematically lower than that for the 1:4 mixture. This points to a larger motional amplitude for the B12 molecules in the 1:10 mixture as compared to the 1:4 mixture of B12 and DPPC. As the orientation of relevant dipolar coupling tensor is parallel to the molecular long axis, the orientation of the tensor represents molecular orientation with respect to the membrane normal. Depending on the assumed motional model (rotation on or within a cone), the orientation angle of the molecules with respect to the membrane normal can be estimated to about 20°−30° and 35°−45° for the 1:4 and 1:10 mixtures, respectively.30,31 The notably smaller angle for the 1:4 mixture reflects a reduced motional freedom of the B12 molecules, suggesting a crowding effect of the dissolved B12 on its own dynamics.

Finally, we address the question of whether the B12 molecules only influence their own dynamics or also that of the lipid molecules. We thus used the DIPSHIFT methods to also determine the $S_{C^1}$ of the lipid carbons in the pure hydrated lipid and in the mixtures (Figure 7). It is obvious that the order parameters of the lipid molecules are not altered in the mixture. This indicates no significant change of amplitude of the local lipid dynamics, meaning that the lipid molecules in the liquid-crystalline phase are not detectably restricted by the presence of the B12 molecules.

This finding is notable in relation to the effect of, e.g., cholesterol in model membranes. Because of its small headgroup and primarily hydrophobic nature, cholesterol is thought to be inserted preferably below the lipid head groups (umbrella model), leading to the well-known condensing and thought to be inserted preferably below the lipid head groups. Because of its small headgroup and primarily hydrophobic nature, cholesterol is thought to be inserted preferably below the lipid head groups (umbrella model), leading to the well-known condensing and thought to be inserted preferably below the lipid head groups. Because of its small headgroup and primarily hydrophobic nature, cholesterol is thought to be inserted preferably below the lipid head groups (umbrella model), leading to the well-known condensing and thought to be inserted preferably below the lipid head groups. Because of its small headgroup and primarily hydrophobic nature, cholesterol is thought to be inserted preferably below the lipid head groups (umbrella model), leading to the well-known condensing and thought to be inserted preferably below the lipid head groups (umbrella model), leading to the well-known condensing and thought to be inserted preferably below the lipid head groups (umbrella model), leading to the well-known condensing and thought to be inserted preferably below the lipid head groups (umbrella model), leading to the well-known condensing and thought to be inserted preferably below the lipid head groups (umbrella model), leading to the well-known condensing and thought to be inserted preferably below the lipid head groups (umbrella model), leading to the well-known condensing and thought to be inserted preferably below the lipid head groups (umbrella model), leading to the well-known condensing and thought to be inserted preferably below the lipid head groups.

DOPC+B12. B12 was also studied in membranes of an unsaturated model lipid, namely DOPC, but, for substance availability reasons, only in a 1:10 molar ratio. The fluorescence images (Figure 1c) suggest a homogeneous distribution of the B12, therefore, we have no indication of macroscopic phase separation. Hence, it is interesting to compare the dynamic properties of this mixture with the phase-separated case.

Dynamic Order Parameters. Order parameters $S_{C^1}$ for the lipid resonances from DIPSHIFT experiments (not shown) actually averaged virtually the same “no-result” as discussed above for the case of B12 in DPPC membranes (Figure 7), suggesting again weak specific interactions. As $^{13}$C detection of B12 is unsuitable for sensitivity reasons, we only show results based upon temperature-dependent $^1$H−$^1$H DQ, sideband patterns, thus discussing order parameters $S_{C^1}$ from the measured homonuclear residual dipolar couplings in the temperature range between 5 and 65 °C. The results are shown in Figure 8, where the inset also shows a typical measured sideband pattern.

We observe a somewhat reduced order parameter for all measured temperatures, with a trend toward decreasing values upon heating. As the mixture of DOPC and B12 is homogeneous, the lipid molecules are expected to be in the mobile liquid-crystalline phase typical for the pure membrane. Therefore, uniaxial rotation of the B12 can be taken for granted, and reduced $S_{C^1}$ values are again explained by additional wobbling motions, i.e., a molecular tilt accompanied by diffusion on or within a cone. The reorientation angle is thus estimated to be in the range of 35°−45° with respect to the membrane normal. The angle (motional amplitude) obviously increases somewhat upon heating, reducing $S_{C^1}$.

It is further notable that the order parameters of the B12 protons in the 1:10 mixture with DOPC are comparable with those of the mixture with DPPC at the same molar ratio, i.e., the geometry of motion of B12 molecules is rather similar. Again, we take this as an indication of rather comparably weak interactions.

Figure 7. Order parameters $S_{C^1}$ of the DPPC carbons in the pure lipid and in the 4:1-mixture with B12 at 75 °C.
interactions with the different parts of the lipid, considering that the B12 concentration effect in the DPPC mixture (Figure 6) is more significant.

**DPPC+E12/7.** For the headgroup-modified E12/7 molecules in mixture with DPPC, a homogeneous distribution was indicated in the fluorescence images (see Figure 1d). Also in DSC measurements, no high-temperature phase transition was found, suggesting again an absence of macroscopic phase separation in a 1:4 mixture. However, in our $^{13}$C NMR spectra, the E12/7 resonances, notably also the aliphatic side chains as well as the EO terminal chains, could only be detected by CP excitation (see Figure 9). This indicates rather strong dipolar couplings and a lack of substantial mobility in the mixture.

**Mobility from $^1H$ Signal intensities.** $^1H$ spectral integrals expectedly showed mobile lipid molecules (i.e., narrow signals) above the lipid main phase transition, with a rather sharp and quantitative discontinuous change at the transition temperature (see Figure 10). However, despite the quantitative mobilization of the lipid, the aromatic resonances of E12/7 indicate a mobile fraction of only about 10% in the 1:4 mixture. This suggests that the two components are largely independent of each other, in contrast to the mixture of B12 with the same lipid.

**Dynamic Order Parameters.** Turning to $S_{\text{CH}}$ at the measured temperatures most E12/7 resonances showed rather restricted mobility, thus most residues could be characterized by normal rather than recoupled DIPSHIFT (Figure 11a). Only one of the methyl and one of the EO residues required recoupling due to low order parameters at the chains’ termini.

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**Figure 8.** Order parameters $S_{\text{HI}}$ for the main B12 aromatic resonance in a 1:10 mixture with DOPC as a function of temperature. The error bars indicate the dipolar distribution width $\sigma$ from the fit. Inset: DQ spinning sideband pattern taken at 65 °C including a fit.

**Figure 9.** (a) Aliphatic and (b) headgroup regions of the $^{13}$C MAS NMR spectrum of the 1:4 mixture of E12/7 and DPPC taken at 50 °C, including resonance assignment. Note that different excitation schemes were used (DP: upper, CP: lower spectra). The label "EO$_n$" refers to specific carbon resonances of the (EO)$_n$OCH$_3$ chain.

**Figure 10.** Temperature-dependent proton integrals for different spectral regions for the 1:4 mixture of E12/7 and DPPC. The DSC thermogram is also included in the graph.

**Figure 11.** (a) $^{13}$C–$^1H$ DIPSHIFT (left) and recoupled DIPSHIFT (right) dephasing curves for different resonances of E12/7 in the 1:4 mixture with DPPC, (b) dynamic order parameters $S_{\text{CH}} = D_{\text{eq}} / D_{\text{eq}}$ for all resolved E12/7 resonances as well as an expected value for the aromatic cores according to a $\pi$-flip model (dashed line). See Figures 2c and 9 for the carbon resonance assignments; the label "EO$_n$" refers to specific signals of the (EO)$_n$OCH$_3$ chain.
Inspection of the results shown in Figure 11b reveals lower order parameters, thus somewhat higher motional amplitude, of the aromatic core of E12/7 as compared to B12 molecules in both the crystalline state and in the phase-separated mixture (see also Figure 5b). The values are, however, not compatible with free rotation, so we conclude on a somewhat less defined \( \pi-\pi \) packing structure.

So despite of the lack of evidence for macroscopic phase separation in fluorescence imaging and DSC experiments, the NMR results unambiguously demonstrate that a high fraction of E12/7 molecules is still immobilized in the mixture with DPPC even at the highest studied temperatures. This points to small micro- or even nanodomains, which we imagine to be \( \pi-\pi \) packed fibrils that are too small to be visible in microscope images and do not melt/disintegrate within the studied DSC temperature range. The fibril hypothesis is motivated by the lack of corrugation that is typical for GUVs made of pure DPPC in the gel phase (Figure 1), suggesting that the fibrils separate small patches of DPPC along line defects. See Figure 12b for a sketch of the structural model.

As to the EO headgroups, the order parameters reveal an increasing flexibility toward the chain ends. Thus, larger parts of these terminal chains are not subject to motional restrictions to the same extent as the glycerol headgroups of B12 (see also Figure 5b). This difference is likely due to different ability to form hydrogen bonds and therefore due to a different mode of interaction of the E12/7 headgroups with each other as well as with the lipid as compared to B12. The observed higher thermal stability of the E12/7 superstructure is in tune with the known high-temperature insolubility of poly-EO in water and the related peculiarities in the phase behavior of oligo-EO-based surfactants.

Finally, we note that in Figure 9a two methyl signals of the alkyl side chains of E12/7 (denoted \( \text{al}_{12} \)) are visible. Therefore, we also report two order parameters for these groups (see Figure 11b). A possible explanation is that there are two different packing modes for the two lateral alkyl chains of the E12/7 in the fibrillar structure (realized, e.g., in a double strand). As another alternative that we cannot ultimately exclude, the signal could of course also arise from a more ordered minority fraction of the lipid in interaction with the E12/7.

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**Figure 12.** Sketch of (a) the structural model of the BPs forming either (b) \( \pi \)-packed fibrils (E12/7) or (c) hexagonal honeycomb structures (B12); in panels b and c, the view is along the \( \pi \)-conjugated rods, i.e., parallel to the membrane normal.

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**SUMMARY**

In this work, the dynamics, structural aspects, and thus interactions of bolapolyphilic molecules in two lipid membrane model systems, DPPC and DOPC, were investigated by means of solid-state NMR and complementary techniques such as fluorescence microscopy and DSC. For the unsaturated lipid DOPC in the 10:1 mixture with the bolapolyphil E12/7, a homogeneous mixture was found, in which the local dynamics of lipid molecules in the mobile liquid-crystalline phase was virtually unaffected. This was also found for DPPC lipid in interaction with B12 at high temperatures. In both cases, measurements of dynamic order parameters indicated free rotation of the B12 molecules within the fluid bilayer. Focusing on fluctuations of the B12 molecular long axis and considering diffusion on- or within-a-cone models, our data suggests an opening angle in the range of 20–45° that is mainly dependent on the B12 concentration in the membrane. This lead us to the conclusion that the B12 molecules interact comparably weakly with the fluid membrane components.

For the B12/DPPC mixtures, three new transitions appeared in the DSC traces above the main lipid transition, accompanied by macroscopic phase separation with snowflake-like appearance as seen in fluorescence images. We have previously explained this by a honeycomb superstructure with 6-fold symmetry, see Figure 12c. On the basis of the finding that the B12 cores in this structure undergo 180° flips in the same way as in the bulk crystal, the B12-rich mixed phase was shown to feature well-packed aromatic B12 cores that are presumably stabilized by \( \pi-\pi \) interactions. We could assign the additional phase transitions of this phase on the basis of our NMR results to one molecular process each. The first one corresponds to a change in aromatic \( \pi-\pi \) packing of the B12 molecules accompanied by an increase in their mobility. The second additional phase transition is assigned to a mobilization of the still ordered lipid tails and B12 side chains that are presumably confined by B12-rich filaments within a hexagonal honeycomb arrangement. During the third transition, all molecules in the mixture become mobile, leading to a homogeneous distribution of B12 within the mobile liquid-crystalline lipid matrix.

For another bolapolyphil (E12/7) with oligo(ethylene oxide) rather than glycerol headgroup in mixtures with DPPC, fluorescence microscopy also suggested a homogeneous distribution within the membrane. However, the NMR results demonstrated E12/7 to be organized in a rather rigid self-assembled structure not involving lipid molecules. These findings are compatible with a nanometer-scale filamentous structure (Figure 12b), which can explain the intriguing finding that GUVs seen in fluorescence microscopy are regular rather than corrugated spheres, the latter being the common finding for DPPC vesicles in the gel phase. Nanofilmaments can reside in defect lines and thus reduce local corrugation. The same phenomenon can also explain the spherical appearance of DPPC vesicles with B12, i.e., the macroscopic snowflake-like aggregates likely coexist with filaments.

The present work served to elucidate the molecular interactions and processes underlying the self-organization of two synthetic bolapolyphilic molecules in lipid membranes. Based upon these insights, future research will be devoted to further varying the lateral and headgroup substituents, and studying mixtures, in order to explore whether other new, preferably well-defined supramolecular structures can be obtained. For example, controlled and dispersed fibrillar...
structures can be used to fine-tune membrane properties such as their elasticity. The design of possibly guest-selective synthetic membrane channels is another attractive long-term goal.

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**Notes**

The authors declare no competing financial interest.

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