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# Alkane localisation inside a compliant protomembrane model

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## Abstract

Protomembranes at the origin of life were likely composed of short-chain lipids, readily available on the early Earth. On the other hand, membranes formed by such lipids are less stable and more permeable under extreme conditions, so that a novel membrane architecture was suggested to validate the accuracy of this assumption. The model membrane includes the presence of a layer of alkanes in the mid-plane of the protomembrane in between the two monolayer leaflets and lying perpendicular to the lipid acyl chains. Here, we investigated such possibility experimentally for membranes formed by the short-chain phospholipid 1,2-didecanoyl-*sn*-glycero-3-phosphocholine, including or not the alkanes eicosane, squalane or triacontane by means of neutron membrane diffraction and contrast variation. We found strong indications for an incorporation of two of the three alkanes in the membrane mid-plane through the determination of neutron scattering length density profiles and the membrane swelling at various relative humidities indicating a slightly increased bilayer thickness when the alkanes are incorporated into the bilayers. The selectivity of the incorporation points out the role of the length of the n-alkanes with respect to the capacity of the membrane to incorporate them.

## Introduction

The cell membrane is among the most fascinating macromolecular structures that make up all living systems. It is a biological, mainly two-dimensional matrix that separates and protects the interior of the cell from its environment. The basic unit of cell membranes (which generally consist of lipids, proteins and carbohydrates) is a bilayer of polar lipids (phospholipids, glycolipids) whose polar head-groups point outwards and stabilize the bilayer, while the hydrophobic acyl chains constitute the membrane core. Biomembranes promote the compartmentalization of living systems, which is an essential property for the definition of life. This fact probably played a key role in the early stages of the formation of the first membranes and thus in the origin of life on our planet [1-5].

Several structural studies have been performed on lipid membranes to obtain quantitative information on their molecular organization, phase transitions and phase separations, incorporation and effects of host molecules and macromolecules [6], etc. These structural characterizations are often complicated by the fact that the biologically relevant phases are not crystalline, therefore there is no three-dimensional repeat unit that can be resolved through usual crystallographic methods. Instead, lipids typically arrange into liquid-crystalline phases, e.g. gel ( $L_{\beta}$ ,  $P_{\beta}$ ) or fluid ( $L_{\alpha}$ ) lamellar phases, which possess intrinsic static and/or dynamic lateral disorder. X-ray and neutron diffraction techniques have been important tools in this respect, because they allow one-dimensional information of the membrane ordering to

48 be obtained both in the bilayer plane and perpendicular to it, despite the lateral disorder of  
49 the various lamellar phases precisely when in the biologically relevant ( $L_{\alpha}$ ) fluid phase.

50 From seminal studies performed back in the 1970s [7-10], a methodology was developed to  
51 extract the neutron and x-ray scattering length density (SLD) profiles of membranes from  
52 solid-supported stacks of parallel membranes opening the field known today as *membrane*  
53 *diffraction*. The analysis of the SLD profiles gives information on the average positioning and  
54 dimensions of each membrane constituent [11]: hydrocarbon chains, headgroup components  
55 (e.g. the phosphate and the choline groups) [12, 13], water and additional incorporated host  
56 molecules, when present (e.g. cholesterol) [8, 9].

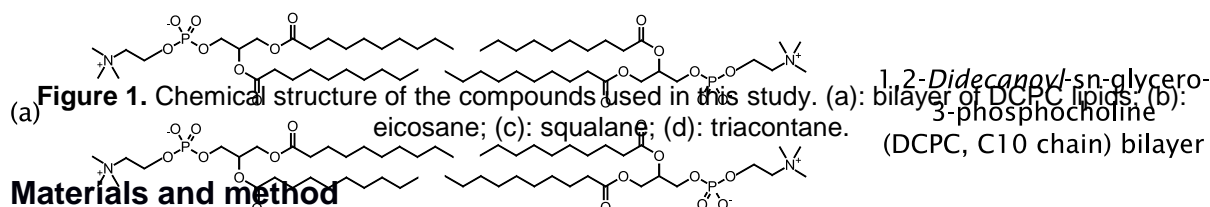
57 With this quantitative information at hand, one can investigate the location of intercalants  
58 within the membrane and correlate it with the observed consequences on the membrane  
59 structure, as well as its properties and functioning. This was done, for example, by using n-  
60 alkanes of varying chain length to investigate the mechanisms of anaesthesia [14, 15]. These  
61 studies proved that a significant amount of n-alkanes (e.g. hexane [16]) entered the bilayer  
62 core, although the resolution was not sufficient to determine whether there was a preferential  
63 positioning (i.e. sitting parallel or perpendicular to the lipid acyl chains). Another study [17]  
64 was performed using high amounts of C6 – C16 n-alkanes (0.3 – 0.9 alkane:lipid ratio) on  
65 egg PC membranes, finding that smaller n-alkane chain lengths contributed to an increase in  
66 bilayer thickness. More recently, the exact positioning of small amounts of squalane  
67 (branched C30 alkane) was determined in model membranes made of DOPC:DOPG [18]  
68 and an archaea-mimicking mixture DOPhPC:DOPhPE [19]. In both cases, squalane was  
69 found to enter the lipid membrane and to position in the mid-plane of the bilayers  
70 perpendicularly to the lipid tails. Such novel membrane architecture was suggested earlier  
71 by Cario et al. [20] and then confirmed experimentally. Moreover, the two studies highlighted  
72 the possibilities for the incorporated molecules to act as proton permeability barrier [18] and  
73 to trigger domain formation [19].

74 In analogy with previous findings, our group has studied the incorporation of n-alkanes in  
75 early life membrane models composed of short single chain amphiphiles (SCAs) with 10-  
76 carbon (C10) chain length [21]. The reason for this lies in the likely presence of a broad size  
77 distribution of n-alkanes (especially linear ones) along with SCAs in the early Earth  
78 environment, as both species are readily formed via Fischer-Tropsch type reactions [22, 23].  
79 If a structural role could be identified for such apolar molecules into the prebiotic  
80 membranes, this would help explain how SCA membranes could have withstood the harsh  
81 environmental conditions of the early planet. In our recent study [21], we found that the  
82 alkanes squalane and eicosane (C20 linear) decrease the temperature induced membrane  
83 swelling of multilamellar vesicles (MLVs) and decrease the membrane sensitivity at high  
84 hydrostatic pressure. These results show that the incorporation of n-alkanes can indeed  
85 confer a higher membrane stability at high temperatures and pressures and therefore could  
86 have played a major role in the formation of the first forms of life.

87 It is now important to determine the actual positioning of such molecules inside short-chain  
88 lipid membranes. First, the existence of a preferential orientation of the alkanes inside the  
89 membrane could help gain insight into the mechanisms by which the bilayers acquire a  
90 higher thermo- and piezo-stability. Second, it might explain the need for more or less  
91 intercalant in order to obtain significant effects in the membrane (for instance, an orientation  
92 of the alkanes perpendicular to the acyl chains might correlate with very small amounts  
93 needed in the membrane). Third, the positioning may affect membrane structural features,  
94 e.g. the bilayer thickness, possibly offering a way to obtain a thicker membrane even in  
95 conditions where only short chain lipids are available.

96 Previous structural studies on model membranes have focused on lipids having chain length  
 97 of 12 carbons or longer [12, 13, 24]. Here, we performed our investigation using the C10 PC  
 98 lipid 1,2-didecanoyl-*sn*-glycero-3-phosphocholine (hereafter DCPC, Figure 1a) as  
 99 phosphocholine C10 analogue of the previously studied SCA membranes [21, 25]. This  
 100 allows to benefit from optimized procedures for successfully producing membrane stacks  
 101 onto silicon substrates [12], which are needed for obtaining structural information by  
 102 membrane diffraction. We combined two approaches to neutron diffraction studies, contrast  
 103 variation and membrane swelling (see Materials and Methods section), to fully characterize  
 104 DCPC bilayers in the absence or presence of small amounts of three alkanes: eicosane  
 105 (Figure 1b), squalane (Figure 1c) and triacontane (C30 linear, Figure 1d).

106 We found that the alkanes eicosane and squalane enter the membrane hydrophobic core  
 107 and lead to an increase of the bilayer thickness compared to the alkane-free sample.  
 108 Conversely, the triacontane does not lead to an increase in membrane thickness, hence  
 109 questioning its incorporation into the membrane and suggesting a role of the n-alkane length  
 110 for a successful inclusion in the bilayer.



116 1. Sample preparation

117 DCPC was purchased from Avanti Polar Lipids (Alabaster, AL, USA). Hydrogenated and  
 118 perdeuterated eicosane, squalane and triacontane were purchased from (c) Sigma  
 119 (Merck, Darmstadt, Germany). All products were used as received from the manufacturer,  
 120 without further purification. 5 mg of DCPC or DCPC with 4% molar of the corresponding  
 121 hydrogenated or deuterated alkane (eicosane, squalane or triacontane) were dissolved in  
 122 chloroform:methanol (2:1), spread on a silicon wafer (Si-Mat., Kaufering, Germany) using the  
 123 "rock and roll" method [26] and dried overnight under vacuum. Next, (b) the samples  
 124 were placed in sealed Falcon tubes containing about 100  $\mu\text{L}$  of  $\text{H}_2\text{O}$  at 40  $^\circ\text{C}$  for 24 hours of  
 125 annealing. Finally, samples were stored at ambient temperature before the experiments.

126 2. Neutron Diffraction experiments

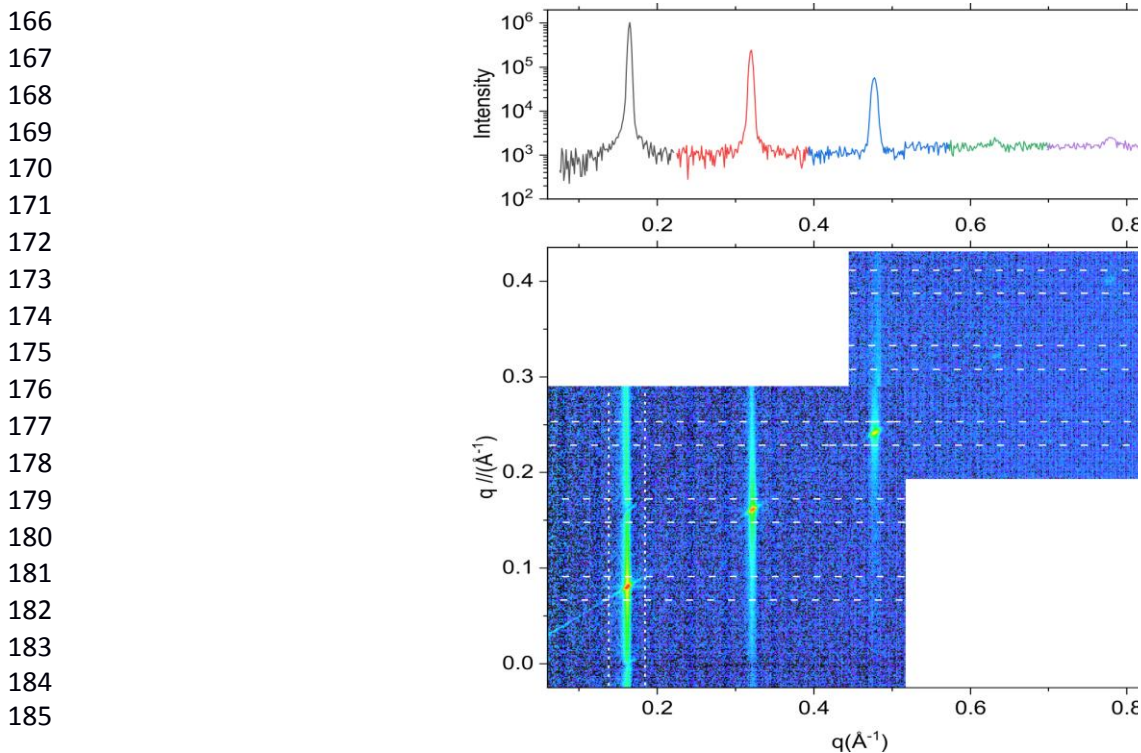
127 Neutron diffraction experiments were performed at the D16 neutron diffractometer [27] at the  
 128 Institut Laue - Langevin (ILL, Grenoble, France). The incident wavelength was 4.46  $\text{\AA}$ . The  
 129 position sensitive Millimeter resolution Large Area Neutron Detector (MiLAND, 320 x 320  
 130  $\text{mm}^2$  with 1 x 1 mm pixel resolution) was set to a sample-to-detector distance of 950 mm.  
 131 Sample rocking scans ( $\Omega$ -scans) were used to collect neutron diffraction data in reflection  
 132 geometry by scanning the sample angle with respect to the incident beam ( $\Omega$ ) using 0.05 $^\circ$   
 133 increments at two detector angles  $\Gamma$  relative to the incident neutron beam,  $\Gamma = 12^\circ$  ( $-1 < \Omega <$   
 134  $12^\circ$ ) and  $\Gamma = 28^\circ$  ( $8 < \Omega < 18^\circ$ ) in order to collect the maximum number of diffraction orders  
 135 (Figure 2). The resulting q-range investigated was  $q = 0.06 \text{ \AA}^{-1} - 0.86 \text{ \AA}^{-1}$ , where q is the  
 136 neutron scattering vector. Diffraction experiments were performed using a set of three  
 137 identical [28] humidity chambers (BerILL) developed at ILL, allowing for precise control of  
 138 the humidity and the temperature. The temperature at the sample position was kept at the  
 139 constant value of  $T = 25^\circ\text{C}$ . The relative humidity (RH) was precisely regulated by the  
 140 temperature difference between the sample and the water reservoir controlled independently

141 using a dedicated controller for the chambers and a Lakeshore 336 (Lake Shore Cryotronics,  
142 Inc., Westerville, OH, USA) for the readout of the high-precision temperature sensors [28].

143 The DCPC samples, pure or containing 4% of either hydrogenated or deuterated alkanes,  
144 were measured following two different procedures. In the first (*contrast variation*  
145 *experiments*), samples were incubated at a constant RH of 97% for 2 hours before starting  
146 each scan. Every sample was incubated three times inside the humidity chamber at different  
147  $D_2O$  contrasts, with the reservoir filled with 20 ml of different mixtures of  $D_2O/H_2O$  (8%, 70%  
148 or 100%). The different contrasts were used to extract the phase of each Bragg reflection  
149 required to determine the membrane neutron scattering length density (NSLD) profile [8].

150 In the second experimental procedure (*swelling experiments*), the  $D_2O/H_2O$  solvent contrast  
151 was set to a zero NSLD obtained using the 8%  $D_2O$  mixture. Here, the RH was varied from  
152 30% up to  $\approx 100\%$  in order to scan the sample lamellar periodicity and Bragg reflection  
153 intensities as a function of humidity from a dehydrated state up to full hydration. Sample  
154 equilibration was gradually increased from 1 hour (used for the lowest humidity steps) up to  
155 2.5 hours for the sample equilibrated at 99.5% RH. For the last measurement aimed at  
156 reaching full hydration, samples were equilibrated offline for 24 hours in the measurement  
157 chamber.

158 An example of reciprocal space map obtained by merging two  $\Omega$ -scans is shown in Figure 2.  
159 The first visible feature is the occurrence of a series of reflections located at the same  
160 periodicity ( $q_z$ ) on top of the line  $q_{||} = (q)/2$  that corresponds to the specular condition. The  
161 periodicity identified by the reflections results from the lamellar ordering of the multilayer,  
162 with a repeat or center-to-center distance given by one bilayer plus one water layer. Each  
163 reflection does not have the shape of a Bragg peak as expected from a perfect crystal, but  
164 that of a Bragg sheet in the vertical ( $q_{||}$ ) direction, due to fluctuations and defects in the liquid  
165 crystal's organization.



186

187 **Figure 2.** Example of reciprocal space map ( $q_{||}$  vs  $q$ ) of DCPC at 8%  $D_2O$  contrast and 30% relative  
188 humidity showing 5 lamellar diffraction orders. Horizontal white dashed lines show the limits of



189 integration for each diffraction order, while the top plot shows the result of the integrations truncated in  
190 the selected  $\Omega$  and  $2\theta$  regions of interest for the peak fitting.

191 These elongated features are a sign of the disorder both static or dynamic of the membrane  
192 stack. Two minima lines can be observed from the 2D map, one that is located at constant  $\Omega$   
193 = 0 and the other tracing the line  $\Omega = 2\theta$ . These minima correspond to the so-called *sample*  
194 *horizons*, and occur at the angles where the scattered beam is attenuated by crossing  
195 longitudinally the lamellar stack on the silicon substrate.

## 196 1. Data reduction

197 Data reduction was performed using the ILL software LAMP [29]. Pixel efficiency as well as  
198 solid angle corrections were performed using a detector calibration obtained from the flat  
199 incoherent scattering signal of a water measurement. The background was obtained by  
200 collecting an empty humidity chamber scan and was subtracted from each measurement.  
201 The background-subtracted data were then exported for further analysis using Origin Pro  
202 (Version 2019, OriginLab Corporation, Northampton, MA, USA). The data were reduced to  
203 1D intensities vs  $2\theta$  plots by integrating for each peak a slice of  $\Omega = 1^\circ$  width around the  
204 peak center (see Figure 2).

205 The integrated intensities of the Bragg peaks were obtained using Gaussian fits, as done  
206 previously [30]. The angle ( $\theta$ ) of a Bragg peak is related to the scattering vector ( $q$ ) by:

$$207 \quad q = \frac{4\pi\sin(\theta)}{\lambda} \quad (1)$$

208 where  $\lambda$  is the wavelength of the incident neutron beam. The periodicity (*d-spacing*) was  
209 derived as in references [31, 32]. For each sample and experimental condition, a weighted  
210 linear fit was performed on a plot of peak positions (in  $q$ ) as a function of the diffraction order  
211 ( $h$ ) (known as *Bragg plot*), the slope ( $s$ ) of which was used to determine the *d-spacing* via  
212 the equation:

$$213 \quad d = \frac{2\pi}{s}. \quad (2)$$

214 The error on the *d-spacing* was obtained by propagating the error of the peak position fitting  
215 and used in the weighted linear fit to the peak positions vs. Bragg order  $h$ .

216 The next step in the data analysis is to convert reciprocal space data into real space 1D  
217 structures. For that, the calculated area of each peak was employed to construct neutron  
218 scattering length density (NSLD) profiles from the sum of the neutron scattering lengths per  
219 unit volume [33]. The way the membrane stack NSLD profiles can be constructed comes  
220 from the definition of the scattering intensity  $I(q)$  as:

$$221 \quad I(q) = P(q) * S(q) \quad (3)$$

222 where  $P(q)$ , the membrane form factor, contains information about the repeat unit structure  
223 (in our case, one bilayer and one water layer) while  $S(q)$ , the structure factor, is related to  
224 the spatial correlation between adjacent membranes that results from their interactions and  
225 leads to the lamellar periodicity and to the Bragg reflections. Therefore, the  $S(q)$  features  
226 (the Bragg peaks) are modulated by the underlying form factor, and this property can be  
227 used to sample the form factor dependence with  $q$  at discrete positions exploiting the  
228 intensity modulation of all Bragg reflections that are observed in a sample scan [7-9]. The  
229 Fourier transform of  $P(q)$  in real space provides the NSLD profiles.

230 In practice, since one only has access to a finite number of  $q$  positions through the Bragg  
231 reflections, approximated NSLD profiles are calculated from the discrete set of Fourier

232 coefficients  $F_h$  (also referred as “structure factors” in the text) using the following equation [7,  
233 8, 10]:

$$234 \quad \rho(z) = \sum_{h=1}^{h_{max}} |F_h| v_h \cos\left(\frac{2\pi h}{d} z\right) \quad (4)$$

235 where  $d$  is the lamellar periodicity of the bilayer stack in the  $z$  direction perpendicular to the  
236 membrane plane,  $|F_h| = \pm\sqrt{I_h h}$  where  $I_h$  is the integrated intensity of the  $h^{\text{th}}$  order Bragg  
237 peak and  $v_h$  is the phase of the structure factor terms that are in general complex quantities  
238 including information on absorption and activation of the sample [34]. However, in the case  
239 of centrosymmetric structures such as bilayers,  $v_h = \pm 1$  and the phase assignment  
240 simplifies into the assignment of a + or - sign for each  $F_h$  term [8].

241 Finally, from the data acquired by scanning the RH (swelling experiments), the osmotic  
242 pressure ( $\Pi$ ) [35, 36] could be calculated from the relative humidity using the following  
243 equation:

$$244 \quad \Pi = \frac{-k_B T}{v_w^0} \ln(RH) \quad (5)$$

245 where RH is defined as  $P/P_0$  (e.g. 0.5 for 50% RH),  $k_B$  is Boltzmann’s constant,  $T$  is the  
246 temperature in Kelvin, and  $v_w^0$  is the molecular volume of water [31].

## 247 2. Scattering Length Density profile determination

248 In order to gather insights about the membrane structural changes, the first step was to  
249 extract the corresponding NSLD profiles of each sample. This was done by exploiting the  
250 modulation in the intensity of each membrane Structure Factor term  $F_h(q)$  as detailed in the  
251 “Data reduction” section. The main challenge relies in the assignment of the phase which, in  
252 the case of centrosymmetric structures such as membrane stacks, simplifies into the  
253 assignment of a positive or negative sign to each term. In this work, we combined three  
254 strategies to obtain an unambiguous phase assignment:

- 255 1. Contrast variation: the use of different  $D_2O / H_2O$  isotopic compositions allows to vary  
256 the water NSLD that is the contrast between bilayers and water layers. By using the  
257 fact that the NSLD of water increases linearly with the  $D_2O$  percentage, one can  
258 detect the occurrence of phase changes.  
259
- 260 2. Swelling: when the RH of the environment increases, the membrane stack  
261 undergoes swelling, as a result of the hydration and the increase of the water layer  
262 thickness. Assuming that the bilayer structure does not change when the water layer  
263 increases, one can perform Relative Humidity (RH) scans and use the displacement  
264 in  $q$  of the Bragg reflections to sample the  $P(q)$  in a finite set of  $q$  positions [8]. With  
265 this technique, one obtains information on the possible sign swapping whenever the  
266 tracing of the  $P(q)$  vs  $q$  highlights the presence of a relative minimum.  
267
- 268 3. Physical considerations from the observed NSLD profiles: as highlighted in the  
269 former points, the contrast variation and swelling techniques both allow detection of  
270 phase changes, but not the absolute phases. However, the features of the NSLD  
271 profiles can be qualitatively judged from our knowledge of the system. For instance,  
272 the acyl chains placed in the middle of the bilayer should give a broad minimum in  
273 the profile because of the high density of low scattering length hydrogen atoms.  
274 Conversely, the phosphate groups of the phosphatidylcholines (the DCPC

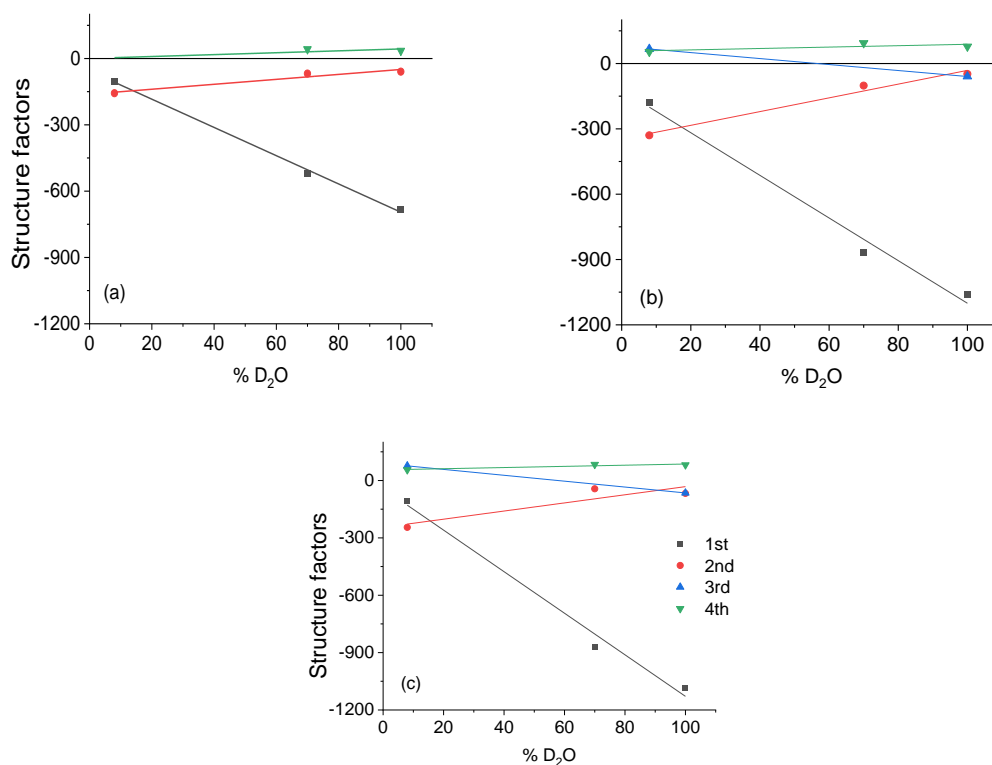
275 headgroups) give local maxima that will be highly visible at the lowest water contrast  
276 of 8% D<sub>2</sub>O. In addition, different D<sub>2</sub>O contrasts at a constant RH do not change the  
277 shape of the NSLD in the hydrocarbon region. Similar considerations were also made  
278 when applying scaling and offset factors to the NSLD profiles to allow for  
279 comparisons between different samples: there, the NSLD profiles of the different  
280 samples were matched with each other in the solvent region, for the measurements  
281 performed at the same D<sub>2</sub>O / H<sub>2</sub>O contrast.

## 282 Results

283 Figure 3 shows examples of the resulting contrast variation plots for the DCPC samples with  
284 and without perdeuterated eicosane or squalane molecules. All numerical values of the  
285 structure factor terms and the measured d-spacing can be found in Table 1 in the Electronic  
286 Supplementary Information (ESI).

287 The choice of the sign for the 1<sup>st</sup> reflection order, the term which is a function of the overall  
288 periodicity of the repeat unit (the d-spacing), is assigned as negative in order to obtain a  
289 local minimum of the NSLD profiles at the z = 0 position (Figure 4). This translates into the  
290 choice of setting the centre of the bilayer repeat unit at the 0 of the x-axis. All other phases  
291 were assigned by making sure that, in the following order:

- 292 1) the linearity in the contrast variation plots was maintained (Figure 3);
- 293 2) combining the shape observation of the NSLD profiles at all 3 contrasts;
- 294 3) applying the phase change when needed (in our case, for the 3<sup>rd</sup> Bragg reflection order  
295 around 70% D<sub>2</sub>O/H<sub>2</sub>O, visible in Figure 3).



296

297

298 **Figure 3.** Examples of contrast variation plots for the DCPC samples with and without added  
299 alkanes. (a) DCPC. (b) DCPC + 4% d-eicosane. (c) DCPC + 4% d-squalane.



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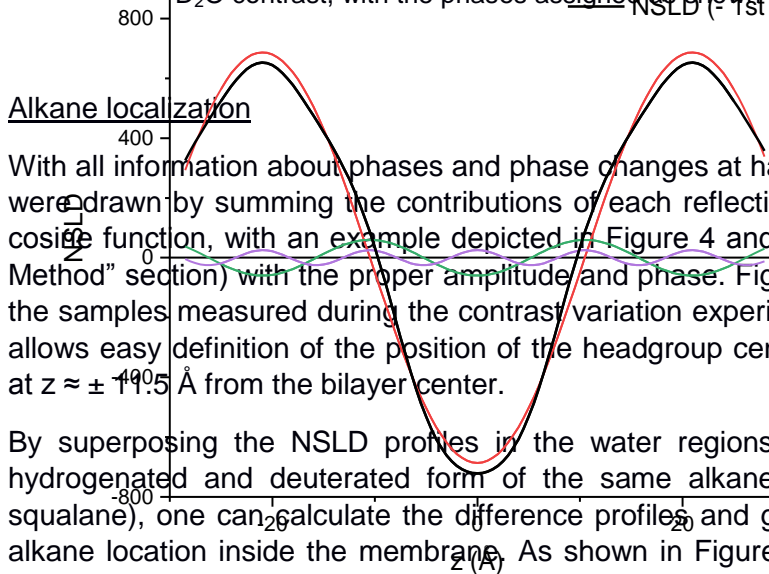
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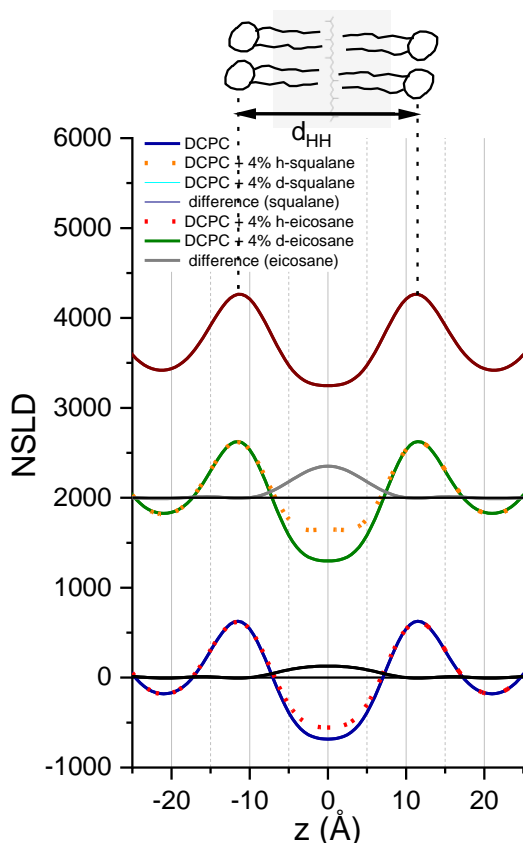
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**Figure 4.** Plot of individual terms that contribute to the NSLD profile of the DCPC sample at 100% D<sub>2</sub>O contrast, with the phases assigned as shown in panel (a) of Figure 3.



With all information about phases and phase changes at hand, the membrane NSLD profiles were drawn by summing the contributions of each reflection order (all having the form of a cosine function, with an example depicted in Figure 4 and as detailed in the “Materials and Method” section) with the proper amplitude and phase. Figure 5 shows the NSLD profiles of the samples measured during the contrast variation experiments at 8% D<sub>2</sub>O/H<sub>2</sub>O contrast. It allows easy definition of the position of the headgroup centres, at the local maxima located at  $z \approx \pm 11.5$  Å from the bilayer center.

By superposing the NSLD profiles in the water regions for the samples containing the hydrogenated and deuterated form of the same alkane (h- and d-eicosane; h- and d-squalane), one can calculate the difference profiles and get information about the average alkane location inside the membrane. As shown in Figure 5, both alkanes are found to be incorporated and sit inside the bilayer in the hydrocarbon region, preferentially in the midplane in between the monolayers.



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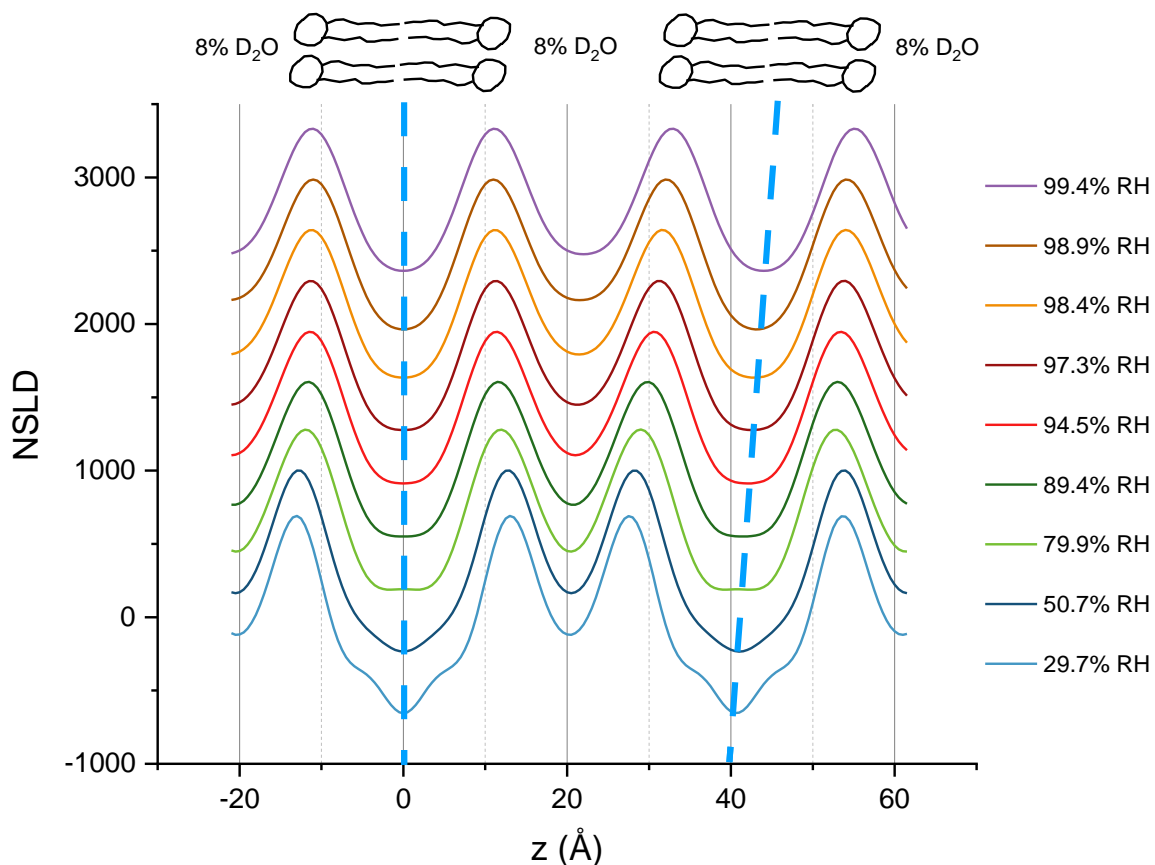
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**Figure 5.** NSLD profiles at 8% D<sub>2</sub>O contrast of samples measured at 97% RH. The y-axis scale refers to the alkane containing samples, while the other NSLD profiles were shifted for clarity. The top image shows a sketch of the bilayer with a molecule of squalane in the mid-plane, as mapped by the NSLD profiles within the dotted lines defined by  $d_{HH}$  the position of the maxima.

324 Membrane swelling

325 In addition to screening the phase of the membrane structure factors as a function of  
326 humidity, swelling experiments (at 8% D<sub>2</sub>O contrast, to easily locate the lipid headgroup  
327 position) provided further information on the membrane characteristics and properties. The  
328 samples measured here were a) DCPC; b) DCPC + 4% h-eicosane; c) DCPC + 4% h-  
329 squalane; d) DCPC + 4% h-triacontane. All numerical values of the structure factor terms  
330 can be found in Table 2 in the ESI. Figure 6 shows an example of the NSLD profiles  
331 obtained at each measured RH for the DCPC sample. Some qualitative features are visible  
332 from the plot:

- 333 - The lowest humidity values of 30% and 50% show a more pronounced minimum in  
334 the centre of the bilayer. This can be attributed to the higher density of hydrogen  
335 atoms at the terminal methyl group position, a feature that disappears at higher  
336 humidity values presumably because of an increase in membrane static and/or  
337 dynamic disorder.
- 338 - The repeat distance (d-spacing) increases with increasing RH, as expected because  
339 of the water layer thickening between bilayers.
- 340 - The bilayer thickness decreases with increasing RH, shown in the plot as a shift of  
341 the headgroup maxima position.



342  
343 **Figure 6.** NSLD profiles of a DCPC sample during swelling experiments showing 2 unit cells. The  
344 blue dotted lines show a perfect vertical line as reference on the left side and the effect of swelling  
345 with a minimum shifted more and more to the right side. Top image represents the location of the two  
346 bilayers mapped by the NSLD profiles. Water contrast was 8% D<sub>2</sub>O.

347 The last two features can be used for quantitative comparison between the different samples  
348 investigated. Figure S1 in the ESI shows the bilayer thickness changes as a function of  
349 humidity for all samples. We use here the same nomenclature applied in former diffraction  
350 studies carried out on DLPC and DMPC samples [12]. As a first definition of the bilayer  
351 thickness, we define  $d_{HH}$  as the distance between the two maxima in the NSLD profiles at  
352 8%  $D_2O$  contrast, that corresponds to the distance between the phosphate groups of the PC.

353 As shown in figure S1,  $d_{HH}$  decreases for all samples when humidity increases. Interestingly,  
354 the membrane thickness of the samples containing eicosane and squalane appears to be  
355 offset by a value of approximately  $[0.5 - 1] \text{ \AA}$ , while this is not observed for the sample  
356 containing triacontane. The lipid molecular volume ( $V$ ) being constant, the decrease in  
357 bilayer thickness ( $d_{HH} = 2l$  where  $l$  is the lipid length), is consistent with an increase of the  
358 area per headgroup ( $A$ ) upon increasing hydration, according to:

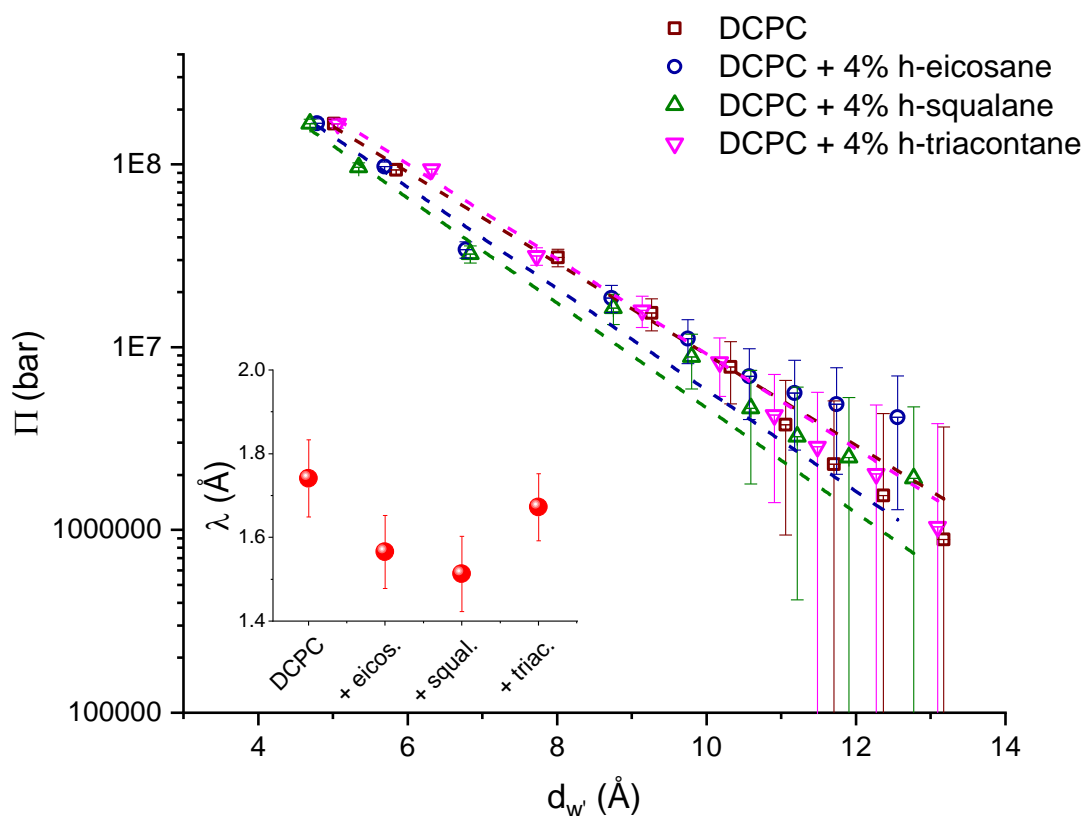
$$359 \quad A = \frac{V}{l}. \quad (6)$$

360 From previous data on DMPC and DLPC [12], since the headgroup area should not change  
361 significantly between DMPC, DLPC and DCPC, we assume an average area  $A \approx 62 \text{ \AA}^2$   
362 between the reported values [12]  $A = 60.6 \text{ \AA}^2$  (DMPC) and  $63.2 \text{ \AA}^2$  (DLPC). Also,  
363 considering the lipid volume  $V_L$  scaling linearly with the chain length ( $V_L = 1101$  and  $991 \text{ \AA}^3$   
364 for DMPC and DLPC, respectively), we obtain  $V_L \approx 881 \text{ \AA}^3$  and a calculated  $d_{HH} \approx 20.3 \text{ \AA}$  for  
365 DCPC at full hydration. As our reported value at maximum hydration is  $d_{HH} \approx 22.5 \pm 0.5 \text{ \AA}$ ,  
366 this result suggests that the DCPC membranes at the highest measured RH were close to  
367 full hydration, but not fully hydrated, (Figure S1).

368 The feature of interest, namely the water layer thickness as a function of humidity, is shown  
369 in the more common pressure – distance plot in Figure 7. In order to estimate the water layer  
370 thickness  $d_w$ , we used existing data from the literature on PC lipids to estimate the thickness  
371 of the headgroups [12]. This led to the following formula for our calculation of  $d_w$ :

$$372 \quad d_w = d - d_{b'} = d - d_{HH} - 8.1 \text{ \AA}. \quad (6)$$

373 where  $d_{b'}$  is the bilayer thickness calculated from the interfaces water-headgroup.



374

375 **Figure 7.** Pressure-distance curves: osmotic pressure  $\Pi$  as a function of the water layer thickness  $d_w$   
 376 calculated for all samples. Inset: decay lengths found from exponential fits for the four samples.

377 The data show very small qualitative differences probably due to the humidity range  
 378 explored, still far away from the expected values for maximum swelling in bulk with excess  
 379 water (the water layer thickness at full swelling for PC lipids is known to be at  $d_w \approx 20\text{-}22 \text{ \AA}$   
 380 [12] and the bilayer thickness did not shrink to the expected minimum value, as previously  
 381 reported). Exponential fits of  $\Pi$  as a function of  $d_w$  yield an estimation of the decay length  $\lambda$   
 382 (inset of Figure 7). Although the differences are small, the samples containing eicosane and  
 383 squalane show slightly lower decay lengths which could imply a lower swelling limit for these  
 384 two samples. The obtained  $\lambda$  values are slightly lower than the value reported in the  
 385 literature for DLPC membranes (around  $\lambda = 2.1 \text{ \AA}$ ) [37].

## 386 Discussion

387 Our results allow us to gain numerous insights regarding the structure of a short chain  
 388 membrane model, with and without insertion of additional apolar molecules (linear and  
 389 branched alkanes).

390 First, contrast variation measurements clearly show that both the alkanes, eicosane and  
 391 squalane, are included in the hydrocarbon region of the DCPC bilayer (Figure 5). Compared  
 392 to earlier studies by Hauss et al. [18] and Salvador-Castell et al. [38], we find an alkane layer  
 393 of almost identical size, so that we can conclude that the alkanes are likely oriented  
 394 perpendicular to the lipid acyl chains. In addition, the bilayer thickness values calculated  
 395 from fitting the maxima of the NSLD profiles at 8%  $D_2O$  contrast (Figure S1) show an offset  
 396 of  $\approx 0.5\text{-}1 \text{ \AA}$  compared to the alkane-free DCPC membrane, confirming further the  
 397 incorporation of the alkanes in the mid-plane.

398 Such finding is of primary importance for the protomembrane model suggested here. A  
 399 similar strategy was forwarded already for modern membranes in archaeal cells, which

400 withstand the most extreme conditions. We found a similar architecture and investigated in  
401 many details the consequences of the presence of the apolar lipids [19, 38]. In particular,  
402 they are able to extend the stability domain, to reduce the membrane's permeability to  
403 protons but to increase that of water, and to induce a negative curvature in the membrane,  
404 allowing the transition to novel non-lamellar phases. As these characteristics are mandatory  
405 for the good functioning of the membrane in extreme conditions, they can be taken as  
406 indicative for the architecture of protomembranes, and we found already positive impact of  
407 alkanes on the stability of protomembranes under high temperature and pressure conditions  
408 [39, 40] and on their molecular dynamics [41]. In this context the experimental proof of the  
409 presence of the apolar lipids is an important step.

410 Interestingly, a slight increase of the bilayer thickness offset with humidity increase  
411 (differences in Figure S1) might suggest a small effect of the hydration in increasing the  
412 portion of alkane molecules accommodating in the mid-plane normal to the bilayer plane.  
413 The thickness offset is not observed on the sample containing triacontane, which suggests  
414 that it is not included in the bilayer and that size might play a role in the ability of such  
415 molecules to be successfully inserted within the bilayer. The long-axis length of eicosane  
416 (C20) and squalane (C30) being similar (because of the branched structure of the squalane)  
417 and of the same order of twice the lipid chain length of DCPC are capable of inserting within  
418 the membrane, however the triacontane size could not be accommodated in the  
419 hydrocarbon region by intercalating parallel to the lipid chains (see Figure 1). Perhaps due to  
420 the need for the membrane to assume a certain undulated in-plane arrangement (governed  
421 by the lipid intrinsic geometry and the membrane bending/compression moduli), the  
422 arrangement of triacontane perpendicular to the lipid chain is not possible, so that these  
423 molecules are excluded from the membrane and are not observed in the calculated NSLD  
424 profiles. Such a conclusion remains, however, speculative.

425 All membrane stacks investigated show a clear effect of the humidity increase in thinning the  
426 bilayer up to  $\approx 4$  Å in the humidity range  $\approx 30 - 100\%$ , which is consistent with the increase  
427 of the headgroup area observed in PC lipids of longer chain lengths [12]. The extent of such  
428 decrease in bilayer thickness is not affected by the inclusion of alkane molecules. The decay  
429 length of the samples containing eicosane and squalane is slightly lower than that of  
430 pure DCPC, implying a possible lower swelling limit induced by the alkane presence. The  
431 compatible value of  $\lambda$  between DCPC and DCPC + triacontane samples gives an additional  
432 indication that the latter alkane did not incorporate into the membrane.

433 To summarize, we have performed an extensive study of DCPC oriented membrane stacks  
434 as a function of the relative humidity (RH) and at different  $D_2O/H_2O$  contrasts. This allowed  
435 us to determine the bilayer NSLD profile and to define unambiguously the phase of each of  
436 the structure factor terms  $F_h$ .

437 From that, and by using deuterium labelled alkanes, we found that eicosane and squalane  
438 are inserted inside the model membrane in the hydrocarbon region. At least a significant  
439 portion of the alkane molecules inserts perpendicular to the lipid chains, causing a small but  
440 significant increase in the bilayer thickness  $d_b$ , consistent with a confined elongated position  
441 of the compound in the mid-plane. The membrane swelling induced by the increase in  
442 humidity causes, on one hand, an increase of the intermembrane water thickness (as  
443 expected), while on the other hand it leads to a slight decrease in the bilayer thickness,  
444 which can be explained by a concomitant volume increase of the lipid headgroup upon  
445 hydration. The decrease of  $d_{HH}$  is not affected by the eicosane or squalane presence inside  
446 the membrane.



447 The sample containing the longer linear hydrocarbon alkane triacontane does not show any  
448 increase in the bilayer thickness. This, together with physical considerations about the  
449 alkane and lipid molecule sizes, implies that the triacontane molecules do not incorporate  
450 into the membrane, unlike eicosane and squalane. This is consistent with the selective  
451 exclusion of triacontane (and inclusion of squalane) molecules that has been reported in a  
452 former study with different membrane compositions [19]. The slight differences in osmotic  
453 pressure decay with water thickness  $d_w$  indicate that the eicosane and squalane may  
454 decrease the membrane maximum swelling limit of the membrane.

455 Therefore, the incorporation of certain alkanes within short chain lipid membranes could  
456 have also occurred in an origin of life scenario, where both these alkanes and membrane-  
457 forming SCA could have been synthesized abiotically. This membrane enrichment, coupled  
458 with the existence of a preferential structural arrangement of the n-alkanes in the  
459 hydrophobic core, can effectively increase the membrane thickness and possibly lead to a  
460 lower membrane swelling capability. Together with previously observed effects in increasing  
461 the membrane thermal and pressure stability [21], the results suggest a role of the n-alkanes  
462 in modifying significantly the membrane structural and functional properties, constituting one  
463 of the possible survival strategies against the extreme prebiotic environmental conditions for  
464 the first membrane compartments at the origin of life.

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