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Apolar Polyisoprenoids Located in the Midplane of the Bilayer Regulate the Response of an Archaeal-like Membrane to High Temperature and Pressure

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Author contribution statement

PO, MS, and JP conceived the project. MS, BD, JP, and PO carried out the experiments. JL performed the data analysis and wrote the initial draft. All authors contributed to the final manuscript.

Keywords

Archaea, Archaeal lipids, high pressure, Phase coexistence, membrane architecture, adaptation, membrane

Abstract

Word count: 189

Archaea are known to inhabit some of the most extreme environments on Earth. The ability of archaea possessing membrane bilayers to adapt to high temperature ($>85^{\circ}\text{C}$) and high pressure (>1000 bar) environments is proposed to be due to the presence of apolar polyisoprenoids at the midplane of the bilayer. In this work, we study the response of this novel membrane architecture to both high temperature and high hydrostatic pressure using neutron diffraction. A mixture of two diether, phytanyl chain lipids (DoPhPC and DoPhPE) and squalane was used to model this novel architecture. Diffraction data indicate that at high temperatures a stable coexistence of fluid lamellar phases exists within the membrane and that stable coexistence of these phases is also possible at high pressure. Increasing the amount of squalane in the membrane regulates the phase separation with respect to both temperature and pressure, and also leads to an increase in the lamellar repeat spacing of both phases. The ability of squalane to regulate the ultrastructure of an archaea-like membrane at high pressure and temperature supports the hypothesis that archaea can use apolar lipids as an adaptive mechanism to extreme conditions.

Contribution to the field

We provide an exhaustive characterization of a model membrane with a novel membrane bilayer ultrastructure which midplane is populated by apolar polyisoprenoids. Physical parameters were determined from neutron diffraction on multilayered ordered stacks of reconstructed membranes, in presence or absence of the apolar lipids. We characterized the localization of the apolar molecule, the order parameters, size, shape and domain formation of the lipid membranes. We confirm the predicted presence of the apolar polyisoprenoid molecules within the bilayer midplane, and validate the shift of the stability domain of the bilayer to higher temperatures and pressures. The lipid system is present as a coexistence of two lamellar phases. The apolar intercalant allows the switch between the two phases as a function of P and T. We demonstrate that the presence of the apolar molecules increase membrane properties and stability shifting the biologically relevant, functional, domain significantly to consider the quantity of apolar intercalant inside the midplane of the bilayer as an adaptive route of extremophiles to thrive under extreme P and T conditions. These results are directly transposable to artificial membrane systems to increase membrane/liposomes stability without increase costs since it would not require the synthesis of ipolar, membrane spanning lipids.

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In review

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In review

Apolar Polyisoprenoids Located in the Midplane of the Bilayer Regulate the Response of an Archaeal-like Membrane to High Temperature and Pressure

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14 **Keywords:** Archaea, archaeal lipids, high pressure, phase coexistence, membrane architecture,
15 **adaptation**

16

17

18 **Abstract**

19 Archaea are known to inhabit some of the most extreme environments on Earth. The ability of archaea
20 possessing membrane bilayers to adapt to high temperature (>85°C) and high pressure (>1000 bar)
21 environments is proposed to be due to the presence of apolar polyisoprenoids at the midplane of the
22 bilayer. In this work, we study the response of this novel membrane architecture to both high
23 temperature and high hydrostatic pressure using neutron diffraction. A mixture of two diether, phytanyl
24 chain lipids (DoPhPC and DoPhPE) and squalane was used to model this novel architecture.
25 Diffraction data indicate that at high temperatures a stable coexistence of fluid lamellar phases exists
26 within the membrane and that stable coexistence of these phases is also possible at high pressure.
27 Increasing the amount of squalane in the membrane regulates the phase separation with respect to both
28 temperature and pressure, and also leads to an increase in the lamellar repeat spacing of both phases.
29 The ability of squalane to regulate the ultrastructure of an archaea-like membrane at high pressure and
30 temperature supports the hypothesis that archaea can use apolar lipids as an adaptive mechanism to
31 extreme conditions.

32

33

34 **1 Introduction**

35 According to the Singer-Nicolson model, cell membranes are composed of a “mosaic” of
36 proteins embedded in a fluid, lipid bilayer (Singer and Nicolson, 1972). Our understanding of cell
37 membranes has developed further since then, for example we now recognize the importance of other
38 lipid phases in addition to the fluid lamellar phase (bilayer phase), and that there can be substantial
39 lateral heterogeneity within the membrane (Goñi, 2014). Membrane structural lipids, typically
40 phospholipids, are diverse and display varied properties owing to the differences in both the lipid polar
41 head groups and the hydrophobic tails. In aqueous solution, these lipids, driven by the hydrophobic
42 effect, self-assemble into structures in which the hydrophilic heads can interact with the solution and
43 the hydrophobic tails are excluded. The most biologically common phases, seen in familiar lipid bilayer
44 structures, are lamellar phases in which the lipids assemble into flat sheets (zero membrane curvature)
45 (Cullis et al., 1991; Perutková et al., 2009; Frolov et al., 2011; Goñi, 2014). However, the ability of
46 membrane to form phases with non-zero curvature, such as cubic or inverted hexagonal phases, is also
47 important and plays a role in many cellular processes such as membrane fission and fusion (Jouhet,
48 2013; McMahan and Boucrot, 2015; Jarsch et al., 2016). Membrane lipids can prefer different
49 membrane curvatures based on their shape, and this preferred curvature can vary with environmental
50 conditions such as pressure and temperature. The presence of diverse lipids within a membrane can
51 even promote phase separation and domain formation within the membrane (Jouhet, 2013). Domains
52 are laterally organized membrane regions with distinct lipid compositions and specialized functions
53 such as interacting with specific proteins, or adopting a specific curvature (Tayebi et al., 2012;
54 Arumugam and Bassereau, 2015; Marquardt et al., 2015). Such lateral membrane domains have been
55 well-characterized in eukaryotic and bacterial cells (Baumgart et al., 2003; Heberle and Feigenson,
56 2011; Heberle et al., 2013; McCarthy et al., 2015; Schmid, 2017).

57 Life has been found at some of the most extreme conditions on Earth such as temperatures
58 above 100°C and pressures up to 120 MPa (Yayanos et al., 1981; Takai et al., 2008; Zeng et al., 2009;
59 Dalmasso et al., 2016; Siliakus et al., 2017). All aspects of these organisms must be specially adapted
60 to tolerate such conditions. Cell membranes, in particular, are highly sensitive to pressure and
61 temperature (Winter and Jeworrek, 2009; Oger and Jebbar, 2010; Brooks and Seddon, 2014). In order
62 to maintain functionality of the membrane under extreme conditions cells adjust the composition of
63 their membranes to cope with environmental changes, in a process known as homeoviscous adaptation
64 (Sinensky, 1974). High temperature tends to increase membrane fluidity, permeability, and promote
65 more negative membrane curvature whereas increasing pressure tends to have the opposite effects
66 (Brooks, 2014). To compensate, bacteria and eukaryotes are known to regulate the length, saturation,
67 and branching of the hydrophobic chains as well as the proportion of different polar headgroups (Jebbar
68 et al., 2015; Siliakus et al., 2017). In archaea the mechanisms of adaptation are less well understood,
69 in part due to their unique membrane lipids. Archaeal lipids have methyl-branched phytanyl chains
70 rather than straight chain fatty acids which are linked via ether rather than ester bonds to the glycerol
71 backbone (De Rosa et al., 1986; Gambacorta et al., 1995). These lipids have higher temperature
72 stability, and lowered proton permeability (Gliozzi et al., 1983; Yamauchi et al., 1993) compared with
73 typical bacterial/eukaryotic lipids.

74 In addition to bilayer forming lipids, archaea are known to produce bipolar, tetra-ether lipids
75 capable of forming lipid monolayers with high stability and low permeability (De Rosa et al., 1983;
76 Elferink et al., 1994). Archaea have been shown to increase the quantity of tetra-ether lipids in the
77 membrane in response to temperature (Matsuno et al., 2009; Cario et al., 2015). Even some bacteria
78 have shown to produce membrane spanning tetraether or tetraester lipids in response to high-
79 temperature conditions (Damsté et al., 2007; Schouten et al., 2007; Siliakus et al., 2017). Despite the
80 link to high temperature adaption, tetra-ether lipids are not found solely in thermophiles but have also

81 been observed in mesophilic archaea. In addition, not all organisms living at high temperatures produce
 82 large quantities, if any tetra-ether lipids (Tornabene and Langworthy, 1979; Hafenbradl et al., 1996;
 83 Sako et al., 1996; Sprott et al., 1997; Siliakus et al., 2017). Some insight into the ability of archaea with
 84 bilayer forming lipids to live at temperatures up to 100°C can be found in the study by Cario et al.
 85 (2015) on the piezo-hyperthermophilic archaeon, *Thermococcus barophilus*. The quantity and
 86 saturation of isoprenoid derivatives (such as lycopane, squalane) were shown to vary in response to
 87 temperature and pressure. In order to explain the ability of *T. barophilus* to live at high temperature in
 88 presence of bilayer forming lipids, it was hypothesized that apolar lipids sit at the midplane of the
 89 bilayer and provide enhanced stability (Cario et al., 2015). The ability of apolar lipids such as squalane
 90 to localize to the midplane of the lipid bilayer has been confirmed in model membranes of both
 91 bacterial-like and archaea-like lipids (Hauß et al., 2002; Salvador-Castell et al., 2020b). The presence
 92 of apolar molecules is capable of modulating membrane physicochemical properties, for example, the
 93 presence of squalane at the bilayer midplane has been shown to increase permeability to water and
 94 decrease proton permeability (Haines, 2001). Apolar molecules at the midplane have been shown to
 95 increase the tendency towards negative membrane curvature by reducing packing frustration
 96 (Salvador-Castell et al., 2020b, 2020a) and to play a role in phase separation and domain formation
 97 (Gilmore et al., 2013; Salvador-Castell et al., 2020b). Such isoprenoid hydrocarbons are found in all
 98 archaea (Langworthy et al., 1982) suggesting this hypothesis could be extended to explain adaptation
 99 to high temperature and pressure in other archaea processing lipid bilayers.

100 In this work we studied the behavior of an archaeal-like bilayer with the proposed novel
 101 architecture composed of 1,2-di-O-phytanyl-*sn*-glycero-3-phosphocholine (DoPhPC) and 1,2-di-O-
 102 phytanyl-*sn*-glycero-3-phosphoethanolamine (DoPhPE) and 2,6,10,15,19,23-hexamethyltetracosane
 103 (squalane) under high temperature and high pressure conditions, mimicking the extreme conditions in
 104 which some archaea live. Using neutron diffraction, we are able to see the localization of squalane in
 105 the midplane bilayer, and to detect the presence of coexisting lamellar phases within the membrane.
 106 The structure of each phase as well as the phase coexistence were shown to vary in response to
 107 temperature and pressure. The response of the membrane ultrastructure to temperature and pressure
 108 could be modulated by varying the quantity of the apolar lipid squalane present in the membrane.

109 **2 Material and Methods**

110 **2.1 Chemicals**

111 1,2-di-O-phytanyl-*sn*-glycero-3-phosphocholine (DoPhPC) and 1,2-di-O-phytanyl-*sn*-glycero-3-
 112 phosphoethanolamine (DoPhPE) were both purchased from Avanti Polar Lipids (Alabaster, USA) in
 113 the lyophilized form and utilized without further purification. The isoprenoid used, 2,6,10,15,19,23-
 114 hexamethyltetracosane (squalane) was bought from Sigma – Aldrich Co (Montana, USA) in its
 115 hydrogenated form and from CDN Isotopes (Pointe-Claire, Canada), in its deuterated form.

116 **2.2 Sample Preparation**

117 3 mg of DoPhPC:DoPhPE (9:1 molar) and the corresponding amount of either hydrogenated squalane
 118 (h-squalane) or deuterated squalane (d-squalane) were dissolved in chloroform:methanol (2:1) and
 119 were spread on a silicon wafer using the “rock and roll” method (Tristram-Nagle, 2007) and dried
 120 overnight under high vacuum. Next, the sample was hermetically sealed inside an aluminum sample
 121 holder containing a 1:1 ratio of H₂O:D₂O (50% D₂O). The sample was left at 50 °C for 48h to allow
 122 for complete hydration.

123 **2.3 Neutron Diffraction**

124 Neutron diffraction experiments were performed on D16 (Cristiglio et al., 2015) at the Institut Laue-
 125 Langevin (Grenoble, France). The incident wavelength was 4.52 Å. The accessible q-range was from
 126 0.06 Å⁻¹ to 0.51 Å⁻¹. The H₂O/D₂O contrast was 50% D₂O. 50% D₂O was previously found to give
 127 both strong diffraction signal and good resolution between the first order peaks of the two lamellar
 128 phases. The temperature was carefully controlled by placing the sample holder in a cryostat. Samples
 129 were either measured in a high temperature (HT) sample holder or in a HT-high hydrostatic pressure
 130 (HHP) cell (Peters et al., 2018). Data obtained at ILL are identified by doi: 10.5291/ILL-DATA.8-02-
 131 852 (Salvador-Castell et al., 2018).

132 Data treatment was performed by LAMP (Richard et al., 1996) and Origin Pro (Version 2019,
 133 OriginLab Corporation, Northampton, MA, USA). The integrated intensities of the Bragg peaks were
 134 corrected according to the absorption and analyzed by a Gaussian function, as done previously
 135 (Salvador-Castell et al., 2020c). The angle (θ) of a Bragg peak is related to the scattering vector (q)
 136 by:

$$137 \quad q = \frac{4\pi \sin(\theta)}{\lambda} \quad (1)$$

138 where λ is the wavelength. In cases where many orders of diffraction were visible, a linear fit of the
 139 form $y = a + bx$ was performed on a plot of peak location (q) vs. diffraction order (h). The slope of the
 140 line (Δq) was used to determine the d-spacing using the following equation:

$$141 \quad d = \frac{2\pi}{\Delta q} \quad (2)$$

142 In cases where only a single Bragg order was visible, the d-spacing was calculated using the first order
 143 peak and corrected based on the y-intercept determined for each sample under conditions where
 144 multiple peaks were present.

145 To locate squalane in the membrane we used the method described in (Hauß et al., 2002, 2005)
 146 taking advantage of difference scattering density between hydrogen and deuterium. Neutron scattering
 147 length density (NSLD) profiles are constructed from the sum of the neutron scattering lengths per unit
 148 volume (Marquardt et al., 2015). The NSLD profiles were calculated from a discrete set of Fourier
 149 coefficients (F_h) using the following equation (Katsaras, 1995):

$$150 \quad \rho(z) = \frac{2}{d} \sum_{h=1}^{h_{max}} |F_h| v_n \cos\left(\frac{2h\pi}{d} z\right), \quad (3)$$

151 where d is the lamellar spacing of the bilayer in the z direction, $z \in \left[-\frac{d}{2}, \frac{d}{2}\right]$, $|F_h| = \pm \sqrt{I_h Q_z}$, Q_z is the
 152 Lorentz correction factor equal to q for oriented bilayers, I_h is the integrated intensity of the h^{th} Bragg
 153 peak and v_n is the phase of the structure factor. The assigned phases of structure factors 1 to 4 (-, +, +,
 154 -) were based on those previously determined for this (DoPhPC:DoPhPE(9:1) + squalane) (Salvador-
 155 Castell et al., 2020a).

156 3 Results and Discussion

157 3.1 Stable phases coexist within archaeal-like membrane at high temperature and pressure

158 Cario et al. (2015) proposed a novel membrane architecture to explain the stability of archaea lipid
 159 bilayers under high pressure and temperatures. In this novel membrane architecture, apolar lipids act
 160 as structural lipids, sitting at the midplane of the lipid bilayer and leading to enhanced membrane
 161 stability under extreme conditions (Oger and Cario, 2013; Cario et al., 2015; Salvador-Castell et al.,
 162 2020a). A synthetic archaeal-like membrane composed of a mixture of diphytanyl lipids (DoPhPC and

163 DoPhPE in a 9 to 1 molar ratio) and the apolar lipid squalane was used to model this novel architecture.
 164 The chemical structures of these lipids are shown in Figure S1. In order to probe how this membrane
 165 behaves under the extreme conditions of temperature and pressure faced by archaea, neutron diffraction
 166 was performed on oriented stacked bilayers at temperatures up to 85°C and pressures up to 1000 bar.
 167 Experiments were performed with both hydrogenated squalane (h-squalane) and deuterated squalane
 168 (d-squalane) in order to take advantage of the differential neutron scattering between hydrogen and
 169 deuterium (Hauß et al., 2002, 2005). The neutron scattering length density (NSLD) for membranes
 170 containing h-squalane and d-squalane was plotted as a function of distance (Figure S2). For
 171 convenience, 0 Å represents the midplane of the bilayer. These plots exhibit two characteristic maxima
 172 corresponding to the glycerol backbone and a minimal intensity near the terminal methyl groups. The
 173 spectra for the membrane containing h-squalane and d-squalane overlap fairly well except within the
 174 region corresponding to the midplane of the bilayer (-10 to 10 Å) where the sample containing d-
 175 squalane shows an excess of scattering density. This indicates that squalane is located in the midplane
 176 of the bilayer and is in agreement with the findings of Salvador-Castell et al. (2020b). In this work we
 177 see that this localization of squalane is seen in both Phase_{LT} and Phase_{HT} at low (20 bar) and high (1000
 178 bar) pressure.

179 The stacked multilayers were sufficiently ordered in the membrane to give rise to Bragg peaks
 180 in the diffraction data. Figure 1A shows the 1D neutron diffraction profiles for a membrane composed
 181 of DoPhPC:DoPhPE (9:1) + 5 mol% h-squalane at 20 bar (black spectra). The temperature increased
 182 from 25°C (bottom) to 85°C (top). At 25°C, there were three orders of diffraction for the sample at low
 183 pressure (20 bar). The first order diffraction peak was located at $q = \sim 0.12 \text{ \AA}^{-1}$, the second order peak
 184 at $q = \sim 0.23 \text{ \AA}^{-1}$, and the third order peak at $q = \sim 0.34 \text{ \AA}^{-1}$. The location of peaks in the ratio of (1, 2,
 185 3...) indicates a lamellar phase (Tyler et al., 2015). As the temperature increased, the peaks
 186 corresponding to the original phase were still present at similar values of q (marked with an 'x'),
 187 although with diminished intensities. At higher temperatures (above 40 °C) a second set of peaks
 188 appeared at lower values of q corresponding to a new phase (marked with an 'o'). The first order
 189 diffraction peak of this new phase is located at $q = \sim 0.09 \text{ \AA}^{-1}$ and the second order diffraction peak at
 190 $q = \sim 0.18 \text{ \AA}^{-1}$. The spacing of the peaks indicates that this new phase is also lamellar. We will refer to
 191 the new phase as Phase_{HT} because it appeared at high temperatures (HT) compared to the original
 192 phase, which is also present at low temperatures (LT) and will therefore be referred to as Phase_{LT}.
 193 Increasing temperature leads to an increase in the intensity of the Phase_{HT} peaks indicating that
 194 temperature stabilizes Phase_{HT}, and leads to a decrease in the intensity of Phase_{LT} indicating that
 195 temperature destabilizes Phase_{LT}. Upon returning the membrane to 25 °C and 20 bar, the initial state,
 196 is restored indicating that this change in phase is fully reversible (Figure 1A).

197 At each temperature, the diffraction of the DoPhPC:DoPhPE (9:1) + 5 mol% h-squalane
 198 membrane was measured at 20, 250, 500, and 1000 bar. The Figure 1 insets show the first order
 199 diffraction peaks, indicated by the red boxes, seen at pressures of 20 bar (black), 250 bar (red), 500 bar
 200 (blue) and 1000 bar (green). At 85 °C and 55 °C, two first order diffraction peaks are seen, representing
 201 the two phases. The area of the Phase_{HT} peak (found at lower q) decreases and the area of the Phase_{LT}
 202 peak (found at higher q) increases as a function of pressure (Figure 1B, Figure 1C). This indicates that
 203 high pressure has a stabilizing effect on Phase_{LT} and a destabilizing effect on Phase_{HT}. Although it is
 204 well-established that increasing pressure causes an effect similar to decreasing the temperature, our
 205 results show that even 1000 bar is not enough to completely replace Phase_{HT} and return to the lipid
 206 organization present at lower temperatures. At 25 °C, the membrane is exclusively in Phase_{LT}. Again,
 207 there is a slight increase in the intensity of the Phase_{LT} peak with pressure indicating that pressure
 208 stabilizes Phase_{LT} (Figure 1D).

209 Both pressure and temperature play an important role in the stability of each phase and their
 210 ability to coexist. In order to better quantify the temperature and pressure range at which these phases
 211 coexist, the change in the integrated peak intensities was monitored as a function of temperature at
 212 each pressure (20, 250, 500 and 1000 bar). The changes in the peak intensities of the first order peaks
 213 of both Phase_{LT} and Phase_{HT} have a linear dependence on temperature (Figure 2 A-D, Figure S3). A
 214 linear fit could be used to extrapolate/interpolate when the peak intensity would be zero and thus to
 215 determine the temperature at which the corresponding phase was no longer present in the membrane
 216 for each pressure, as shown in Figure 2.

217 The integrated peak intensities of Phase_{LT} (blue) and Phase_{HT} (green) at 20 bar were analyzed
 218 as a function of temperature (Figure 2A). A linear fit of the integrated Phase_{HT} peak intensities
 219 determined that the peak intensity would equal zero at 34.3 ± 3.9 °C, signifying that Phase_{HT} appears
 220 above this temperature. Below this temperature, the membrane is exclusively in Phase_{LT}. A linear fit
 221 of the Phase_{LT} peak intensities determined that the peak intensity would reach zero at 85.3 ± 1.2 °C.
 222 Above this temperature, Phase_{LT} would disappear and the membrane would be entirely in Phase_{HT}.
 223 Between these two temperatures, both phases coexist. This analysis was repeated with the data taken
 224 at 250, 500, and 1000 bar (Figure 2 B-D), allowing the determination of a pressure-temperature
 225 diagram of the phase coexistence of Phase_{LT} and Phase_{HT} (Figure 2E). The temperature at which
 226 Phase_{HT} appears (T_{HT}) increased with increasing pressure from 34.3 ± 3.9 °C at 20 bar to 37.4 ± 3.2 °C
 227 at 250 bar, 37.7 ± 3.0 °C at 500 bar and to 38.9 ± 2.6 °C at 1000 bar. The increase in T_{HT} with pressure
 228 was small (~ 5 °C/kbar) and was not statistically significant. The temperature at which Phase_{LT}
 229 disappeared (T_{LT}) also increased with increasing pressure from 85.3 ± 1.2 °C at 20 bar, to 90.2 ± 2.3
 230 °C at 250 bar, 99.7 ± 5.7 °C at 500 bar and finally to 108.0 ± 6.9 °C at 1000 bar. The increase in T_{LT}
 231 as a function of pressure was much larger (~ 24 °C/kbar) (Figure 2E). Here we demonstrate that
 232 although the phases are pressure sensitive, phase coexistence is possible at pressures up to, and
 233 presumably above, 1000 bar. Based on the phase diagram, at high temperatures (> 85 °C), the
 234 application of pressure is predicted to induce phase separation from solely Phase_{HT} at low pressure to
 235 a coexistence of Phase_{HT} and Phase_{LT} at high pressure and high temperature. Pressure induced phase
 236 separation is not a novel concept, as high pressure has been previously shown to be capable of inducing
 237 phase separation and domain formation (McCarthy et al., 2015). Our results suggest that this is also
 238 possible in membranes composed of archaeal lipids. It should be noted that the T_{HT} and T_{LT} reported
 239 here were calculated only as the sample being heated, and that the transition temperature upon cooling
 240 was not explicitly tracked. Membranes are known to exhibit hysteresis near phase transitions meaning
 241 that the transition temperatures upon heating and cooling are different. A similar effect has also been
 242 seen in membranes upon pressurization/depressurization (Trovaslet-Leroy et al. 2016).

243 From the changes in peak intensity, it could be determined that pressure destabilizes Phase_{HT}
 244 and stabilizes Phase_{LT}. In addition to changes in the integrated intensities of the peaks, there were also
 245 changes in the location of the peaks with pressure that reflect changes in the lamellar repeat structure.
 246 The multilayer organization of the membranes makes it simple to determine the repeat spacing (d),
 247 which includes the thickness of the bilayer and its associated water layer (Nagle and Tristram-Nagle,
 248 2000), from the location of the Bragg peaks of each phase. For a lamellar phase, the repeat spacing (d)
 249 can be calculated simply by equation (3), where q is the location of the first order lamellar peak. The
 250 d-spacing of both Phase_{LT} (solid symbols) and Phase_{HT} (open symbols) increase as a function of
 251 pressure (Figure 3A). The d-spacing of Phase_{LT} with increasing pressure is shown again in Figure 3B
 252 using a different scale to better visualize the changes. Phase_{LT} exhibits either a small increase or
 253 negligible change in d-spacing with increasing pressure. Linear fitting of the data determined that the
 254 d-spacing of Phase_{LT} increases at a rate of 0.23 ± 0.32 Å/kbar at 25 °C, 0.61 ± 0.13 Å/kbar at 40 °C,
 255 0.53 ± 0.11 at 55 °C, 0.09 ± 0.30 at 70 °C and 0.40 ± 0.16 at 85 °C. An increase in membrane d-spacing
 256 with pressure has been observed previously (Winter and Jeworrek, 2009; Trapp et al., 2013; Brooks

257 and Seddon, 2014). Increasing pressure provokes a lateral compression of membrane lipids and an
 258 increase in membrane thickness due to extension of the hydrocarbon chains. The d-spacing of Phase_{HT}
 259 also increases with pressure (Figure 3A). The d-spacing increased at a rate of 5.84 ± 0.81 Å/kbar at 40
 260 °C, 5.78 ± 0.19 Å/kbar at 55 °C, 3.91 ± 0.36 Å/kbar at 70 °C, and 2.62 ± 0.22 Å/kbar at 85 °C. The
 261 changes in d-spacing for Phase_{HT} were almost an order of magnitude greater than that seen in Phase_{LT}
 262 showing that Phase_{HT} is much more sensitive to changes in pressure. It is interesting to note that the
 263 swelling of Phase_{HT} decreases with temperature.

264 The formation of a new phase, Phase_{HT}, is most likely due to the presence of lipids with different
 265 preferred curvatures within the membrane as seen by Salvador-Castell et al., (2020b). The bilayer is
 266 made up of two phytanyl lipids with different polar head groups (DoPhPC and DoPhPE) and the apolar
 267 isoprenoid squalane. The phosphoethanolamine (PE) headgroup is small, so the lipid has a conical
 268 geometry and favors a negative curvature (favoring the formation of non-lamellar structure such as
 269 inverted hexagonal phases/cubic phases/inverted micelles). The phosphocholine (PC) headgroup is
 270 larger and the lipid has a cylindrical geometry which favors zero-curvature (favoring the formation of
 271 lamellar structures such as bilayers). The PC headgroup can stabilize the lamellar phase of PE lipids
 272 (Kates and Manson, 1984). The phase separation can be explained by partitioning of DoPhPC and
 273 DoPhPE into different phases based on preferred curvature which can be triggered, for example, by
 274 temperature which favors more negative membrane curvatures. The presence of squalane also
 275 promotes negative lipid curvature and was found to forward the formation of non-lamellar phases in a
 276 DoPhPC:DoPhPE (9:1) membrane (Salvador-Castell et al., 2020b, 2020a). Increasing pressure, on the
 277 other hand, tends to increase the preferred membrane curvature (Shearman et al., 2006; Brooks, 2014).
 278 In our model membrane, phase separation was triggered by increasing temperature, which could be
 279 due to the differences in curvature between the lipids becoming too great to remain within the same
 280 phase. Increasing pressure hinders the phase separation and favors Phase_{LT}, lending further support to
 281 the idea that the phase separation is driven by components of different curvature.

282 Increasing negative curvature favors a transition from a lamellar phase to a non-lamellar phase.
 283 Although no such phases were seen by neutron diffraction, non-lamellar phases in this membrane
 284 system have been detected by SAXS (Salvador-Castell et al., 2020a). Neutron scattering was performed
 285 on multi-stacks of bilayers on a silicon wafer, whereas for the SAXS the sample was not ordered on a
 286 substrate but rather was self-assembled in bulk. These differences in lipid preparation may explain why
 287 in one case the DoPhPC:DoPhPE (9:1) + 5 mol% squalane membrane separates into two lamellar
 288 phases and in the other case the membrane separates into a lamellar phase and an inverted hexagonal
 289 phase. The ordering of the lipids on a solid support for neutron diffraction may impede the formation
 290 of non-lamellar phases. For both phases seen by neutron diffraction, increasing pressure leads to an
 291 increase in the membrane d-spacing, however, the magnitude of the pressure-induced swelling was
 292 significantly different between the phases. The changes in Phase_{LT} are small, generally within the error
 293 of the measurements for the d-spacing. The changes in Phase_{HT} are quite large (2-6 Å/kbar). The
 294 pressure-induced swelling of the Phase_{HT} seen by neutron diffraction is unusually large for a lamellar
 295 phase which is typically < 2 Å/kbar (Brooks, 2014). Interestingly, the magnitude of the pressure-
 296 induced swelling seen in Phase_{HT} is similar to that seen in the inverted hexagonal phase seen by SAXS
 297 (Salvador-Castell et al., 2020a). Figure S4 illustrates how the membrane lattice parameter (equal to d
 298 for lamellar phase and $2/3$ d for inverted hexagonal phase) increases as a function of pressure. The
 299 similarities in the response to pressure of Phase_{HT} and the inverted hexagonal phase seen in SAXS
 300 could be due to the partitioning of lipid headgroups and squalane in a similar manner.

301 3.2 Squalane regulates membrane ultrastructure under high temperature and pressure

302 Archaea are thought to regulate their membranes response to extreme conditions with apolar lipids.
 303 For this reason, the neutron diffraction experiments were repeated with two additional concentrations
 304 of hydrogenated squalane, 2.5 mol% and 10 mol% in order to determine what effect the amount of
 305 squalane would have on the membrane structure at high temperatures and pressures. Two coexisting
 306 phases are also present in membranes containing 2.5 mol% or 10 mol% squalane at elevated
 307 temperatures (Figure S5, Figure S6).

308 The repeat spacing of Phase_{LT} and Phase_{HT} was calculated for the membranes containing 2.5
 309 mol%, 5 mol% and 10 mol% squalane and the values are displayed in Table 1. In all membranes, there
 310 is an increase in the membrane d-spacing with increasing pressure, and Phase_{HT} is again found to be
 311 the more pressure-sensitive phase (Figure 4). The pressure induced swelling of Phase_{LT} remains small
 312 ($\leq 1 \text{ \AA/kbar}$) for all squalane concentrations and is not significantly different with changes in
 313 temperature. The pressure induced swelling of Phase_{HT} is much larger (2-8 \AA/kbar) and the swelling
 314 of Phase_{HT} is temperature dependent. The swelling is significantly larger at low temperatures and
 315 smaller at high temperatures. Although the percentage of squalane does not change the swelling of
 316 Phase_{HT}, the amount of squalane does play an important role in affecting the temperature range at
 317 which Phase_{HT} is present. At $\geq 55 \text{ }^\circ\text{C}$, Phase_{HT} is seen in membranes containing 2.5 mol%, 5 mol%
 318 and 10 mol% squalane. At 40°C , Phase_{HT} is no longer capable of forming the membrane containing
 319 2.5 mol% squalane and at 25°C , Phase_{HT} is only seen in the membrane containing 10 mol% squalane.

320 The temperatures at which Phase_{HT} appears for a membrane containing either 2.5 mol% or 10
 321 mol% squalane are determined from a linear fit of integrated peak intensity vs temperature (Figure S3).
 322 As seen previously with the membrane containing 5 mol% squalane, pressure leads to an increase in
 323 T_{HT} and T_{LT} (Figure S7). Increasing the quantity of squalane promotes the formation of Phase_{HT} at
 324 lower temperatures. For example, at 20 bar Phase_{HT} appears at $44.7 \pm 4.0 \text{ }^\circ\text{C}$ for the membrane
 325 containing 2.5 mol% squalane, $34.3 \pm 3.9 \text{ }^\circ\text{C}$ for the membrane containing 5 mol% squalane and 23.9
 326 $\pm 3.4 \text{ }^\circ\text{C}$ for the membrane containing 10 mol% squalane. The concentration of squalane has a similar
 327 effect on T_{HT} at elevated pressures (Table 2).

328 This agrees with previous findings that squalane promotes negative membrane curvature,
 329 favoring the formation of Phase_{HT} and phase separation at lower temperatures (Salvador-Castell et al.,
 330 2020a, 2020b). Here we show that squalane is also capable of promoting the formation of Phase_{HT} at
 331 high pressures. Previously at ambient pressure, it was shown that the phase coexistence and lateral
 332 organization within a DoPhPC:DoPhPE membrane was squalane dependent leading to the hypothesis
 333 that squalane may promote the formation of membrane domains (Salvador-Castell et al., 2020b). That
 334 would mean that the ability of squalane to favor phase separation at high pressures, may indicate that
 335 squalane could also promote domain formation in archaea that live at such high-pressure conditions.

336 In addition to regulating the phase coexistence within the membrane, increasing the amount of
 337 squalane in the membrane also affects the d-spacing. The d-spacing of the membrane is typically higher
 338 when the membrane contains a higher quantity of squalane for Phase_{HT} (Table 1, Figure S8). For
 339 example, in Phase_{HT} at 20bar/ 55°C the d-spacing is 67.1 \AA when the membrane contains 2.5 mol%
 340 squalane compared with 68.1 \AA when the membrane contains 5 mol% squalane and 74.7 \AA when the
 341 membrane contains 10 mol% squalane. In this experiment, we did not see a significant change in the
 342 d-spacing of Phase_{LT} with increasing squalane concentration which was seen previously, at ambient
 343 pressure and temperature (Salvador-Castell et al., 2020b). The increase in d-spacing with increasing
 344 squalane for Phase_{LT} was only seen up to 5 mol% squalane at which point the phase is thought to reach
 345 saturation. It is conceivable that the amount of squalane required to reach saturation within membrane
 346 could change with pressure and temperature and this may be one of the reason we did not see any
 347 change in the d-spacing of Phase_{LT} with increasing squalane in this experiment.

348 At temperatures at which Phase_{HT} was present, Phase_{HT} was always found to increase in d-
349 spacing with increasing squalane. Previously it was shown that increasing the percentage of squalane
350 leads to an increase in the d-spacing in Phase_{LT} due to an increase in the hydrophobic core thickness
351 (Salvador-Castell et al., 2020b). The localization of squalane to the midplane of the bilayer is confirmed
352 for Phase_{HT} as well as Phase_{LT} (Figure S2). This could indicate that the increase in the d-spacing for
353 Phase_{HT} is also due to an increase in hydrophobic core thickness although this cannot be directly
354 confirmed from this data. Increasing squalane promotes changes in membrane structure of both phases,
355 even at high pressures and temperatures, indicating that squalane could act as a membrane regulator
356 under the extreme conditions at which many archaea live.

357 **4 Conclusions**

358 It was proposed by Cario et al., (2015) that apolar lipids, such as squalane, sit at the midplane of the
359 lipid bilayer, and provide a means of enhancing the stability of archaeal membrane bilayers under
360 extreme conditions. This localization of apolar lipids to the midplane of an archaeal-like bilayer was
361 then confirmed by the work of Salvador-Castell et al., (2020b). The aims of this study were to determine
362 how an archaeal-like membrane with this novel membrane architecture behaves in response to the high
363 temperatures and high hydrostatic pressures and to determine how the quantity of apolar lipid present
364 in the membrane modulates this behavior. To model the proposed membrane architecture in which
365 apolar molecules sit in the midplane of the bilayer, we used an artificial archaea-like membrane
366 composed of DoPhPC, DoPhPE and squalane. The use of neutron diffraction allowed us to confirm the
367 localization of the apolar lipid squalane and to further study the coexistence of two distinct lamellar
368 phases previously reported by Salvador-Castell et al., (2020b). The phase separation within the
369 membrane is most likely due to the partitioning of lipids (DoPhPC/DoPhPE) with different preferred
370 curvatures and the presence of the apolar molecule (squalane). The two phases are capable of coexisting
371 at a wide range of temperatures and pressure. High temperature favors the formation of a swollen
372 lamellar phase (Phase_{HT}) and high pressure favors a thinner lamellar phase (Phase_{LT}). The temperature
373 and pressure range of the phase coexistence is regulated by the percentage of squalane present in the
374 membrane. Increasing the percentage of squalane favors the formation of Phase_{HT} at lower
375 temperatures, for pressures up to 1000 bar. Coexistence and lateral organization of these phases was
376 seen previously at ambient pressure (Salvador-Castell et al., 2020b). Here we have shown that phase
377 coexistence is also seen at high pressure which could indicate the possibility of domain formation in
378 archaea living at high pressure in addition to high temperature. Increasing the amount of squalane also
379 leads to an increase in the membrane lamellar repeat spacing for Phase_{HT}. The ability of squalane to
380 modify the membrane ultrastructure at both high pressure and temperature supports the hypothesis that
381 apolar lipids play a role in adaptation of archaea to extreme conditions.

382 **5 Conflict of Interest**

383 The authors declare that the research was conducted in the absence of any commercial or financial
384 relationships that could be construed as a potential conflict of interest.

385 **6 Author Contributions**

386 PO, MS, and JP conceived the project. MS, BD, JP, and PO carried out the experiments. JL performed
387 the data analysis and wrote the initial draft. All authors contributed to the final manuscript.

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394 **1 Data Availability Statement**

395 The raw data supporting the conclusions of this article will be made available by the authors, without
396 undue reservation.

397 **9 References**

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565

566 **Figure 1.** 1D Neutron diffractograms of DoPhPC:DoPhPE (9:1) + 5 mol% h-squalane membrane. (A)
 567 Diffractograms at 20 bar and temperatures increasing from 25°C to 85°C and after returning to 25°C.
 568 Diffraction peaks corresponding to Phase_{LT} are denoted with an ‘x’ and peaks corresponding to Phase_{HT}
 569 are denoted with an ‘o’. First order diffraction peaks at 85°C (B), 55°C (C) and 25°C (D) measured at
 570 20 bar (black), 250 bar (red), 500 bar (blue) and 1000 bar (green).

571 **Figure 2.** Coexistence of phases in DoPhPC:DoPhPE (9:1) membrane containing 5 mol% h-squalane.
 572 (A-D) Plots of peak intensities of Phase_{LT} (blue circles) and Phase_{HT} (green squares) as a function of
 573 temperature. Pressures were 20 bar (A), 250 bar (B), 500 bar (C) and 1000 bar (D). Error bars are
 574 within symbols. (E) Pressure-Temperature diagram of the coexistence of Phase_{LT} and Phase_{HT}. Error
 575 bars indicate standard errors.

576 **Figure 3.** (A) + (B) Membrane d-spacing as a function of pressure for Phase_{LT} (solid symbols) and
 577 Phase_{HT} (open symbols) in DoPhPC:DoPhPE (9:1) membrane containing 5 mol% h-squalane.
 578 Temperature was 25 °C (black squares), 40 °C (red circles), 55 °C (blue triangles), 70 °C (green
 579 inverted triangles), or 85 °C (purple diamonds). Error for d-spacing measurements is $\pm 0.5 \text{ \AA}$.

580 **Figure 4.** Pressure induced swelling of DoPhPC:DoPhPE (9:1) membrane containing different
 581 quantities of h-squalane. Swelling of Phase_{LT} containing 2.5 mol% (dark gray), 5 mol% (gray), or 10
 582 mol% (light gray) h-squalane. Swelling of Phase_{HT} containing 2.5 mol% (dark blue), 5 mol% (blue),
 583 or 10 mol% (light blue) h-squalane.

584 Table 1: Membrane d-spacing for Phase_{LT} (left table) and Phase_{HT} (right table) for DoPhPC:DoPhPE
 585 (9:1) membrane containing h-squalane. N.P.: not present. Error is ± 0.5 Å.

Phase _{LT}	+2.5% squalane	+5% squalane	+10% squalane	Phase _{HT}	+2.5% squalane	+5% squalane	+10% squalane
25°C 20bar	55.6 Å	55.2 Å	56.0 Å	25°C 20bar	N.P.	N.P.	82.3 Å
25°C 250 bar	56.2 Å	55.5 Å	57.0 Å	25°C 250 bar	N.P.	N.P.	86.7 Å
25°C 500 bar	56.1 Å	55.8 Å	57.1 Å	25°C 500 bar	N.P.	N.P.	88.9 Å
25°C 1000bar	56.2 Å	55.5 Å	57.2 Å	25°C 1000bar	N.P.	N.P.	90.6 Å
40°C 20bar	55.2 Å	54.6 Å	55.7 Å	40°C 20bar	N.P.	70.8 Å	78.6 Å
40°C 250 bar	55.2 Å	54.7 Å	55.8 Å	40°C 250 bar	N.P.	72.2 Å	79.8 Å
40°C 500 bar	55.0 Å	54.7 Å	56.0 Å	40°C 500 bar	N.P.	74.6 Å	82.6 Å
40°C 1000bar	55.6 Å	55.2 Å	56.4 Å	40°C 1000bar	N.P.	76.5 Å	83.6 Å
55°C 20bar	54.1 Å	54.0 Å	55.3 Å	55°C 20bar	67.1 Å	68.1 Å	74.7 Å
55°C 250 bar	54.8 Å	54.0 Å	55.7 Å	55°C 250 bar	68.5 Å	69.3 Å	75.9 Å
55°C 500 bar	55.2 Å	54.3 Å	55.3 Å	55°C 500 bar	69.4 Å	70.6 Å	77.3 Å
55°C 1000bar	55.2 Å	54.4 Å	56.1 Å	55°C 1000bar	72.2 Å	73.7 Å	80.0 Å
70°C 20bar	53.8 Å	53.5 Å	54.4 Å	70°C 20bar	65.7 Å	66.8 Å	72.4 Å
70°C 250 bar	54.0 Å	53.9 Å	55.3 Å	70°C 250 bar	65.7 Å	67.5 Å	74.3 Å
70°C 500 bar	54.2 Å	53.9 Å	55.2 Å	70°C 500 bar	67.7 Å	68.2 Å	74.1 Å
70°C 1000bar	54.8 Å	53.7 Å	55.2 Å	70°C 1000bar	70.1 Å	70.6 Å	75.3 Å
85°C 20bar	53.5 Å	53.6 Å	N.P.	85°C 20bar	64.9 Å	65.0 Å	74.1 Å
85°C 250 bar	53.5 Å	53.6 Å	54.3 Å	85°C 250 bar	65.4 Å	65.9 Å	75.4 Å
85°C 500 bar	53.6 Å	53.9 Å	54.5 Å	85°C 500 bar	65.9 Å	66.4 Å	74.8 Å
85°C 1000bar	53.7 Å	54.0 Å	54.8 Å	85°C 1000bar	67.3 Å	67.6 Å	76.3 Å

586

587 Table 2: Temperature at which Phase_{HT} appears in DoPhPC:DoPhPE (9:1) membrane containing h-
 588 squalane.

Pressure	2.5% squalane	5% squalane	10% squalane
20 bar	44.7 \pm 4.0 °C	34.3 \pm 3.9 °C	23.9 \pm 3.4 °C
250 bar	49.3 \pm 1.5 °C	37.4 \pm 3.2 °C	28.8 \pm 3.3 °C
500 bar	49.2 \pm 0.1 °C	37.7 \pm 3.0 °C	31.8 \pm 1.6 °C
1000 bar	50.8 \pm 1.2 °C	38.9 \pm 2.6 °C	32.2 \pm 2.6 °C

589

Figure 1.TIF

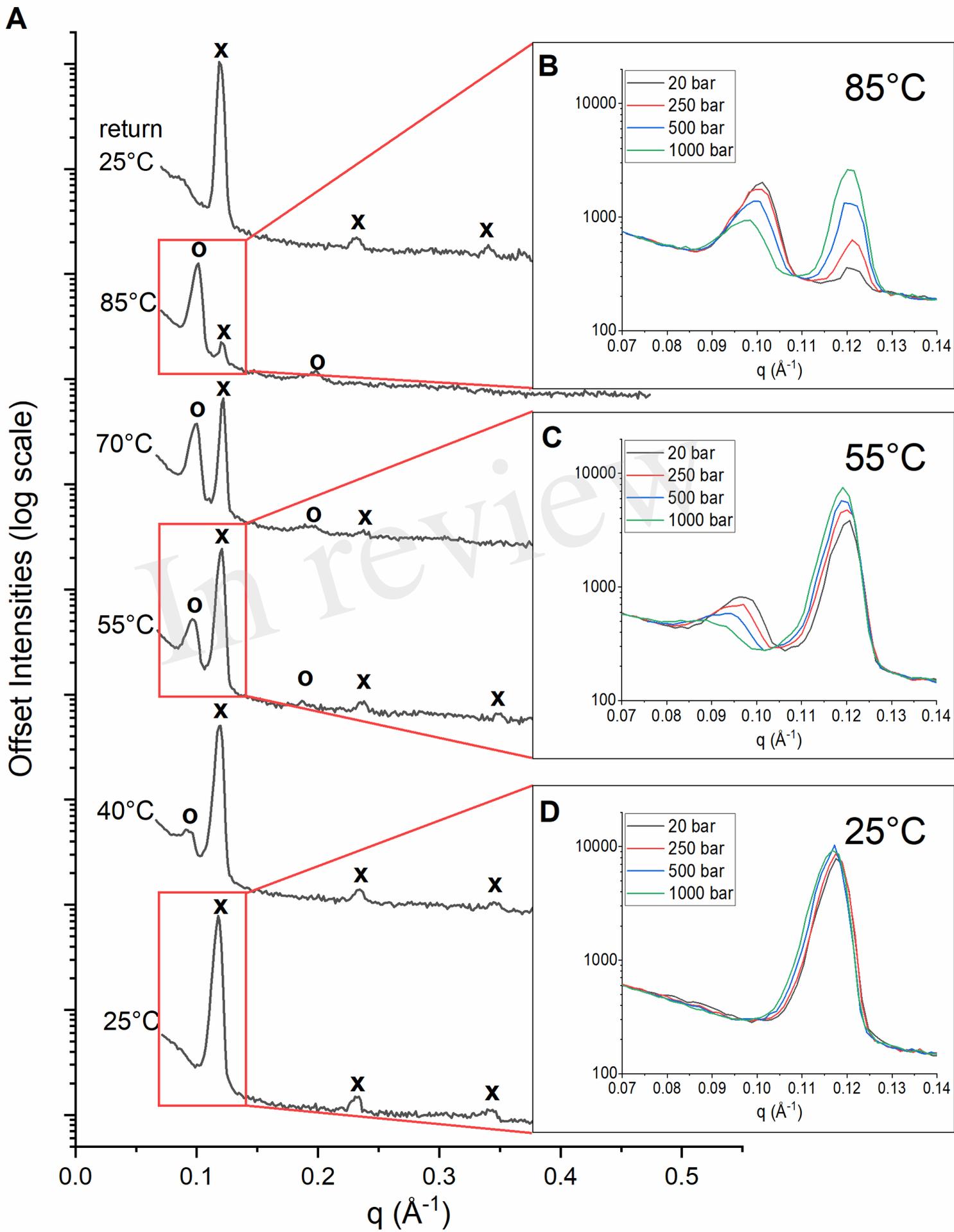


Figure 2.TIF

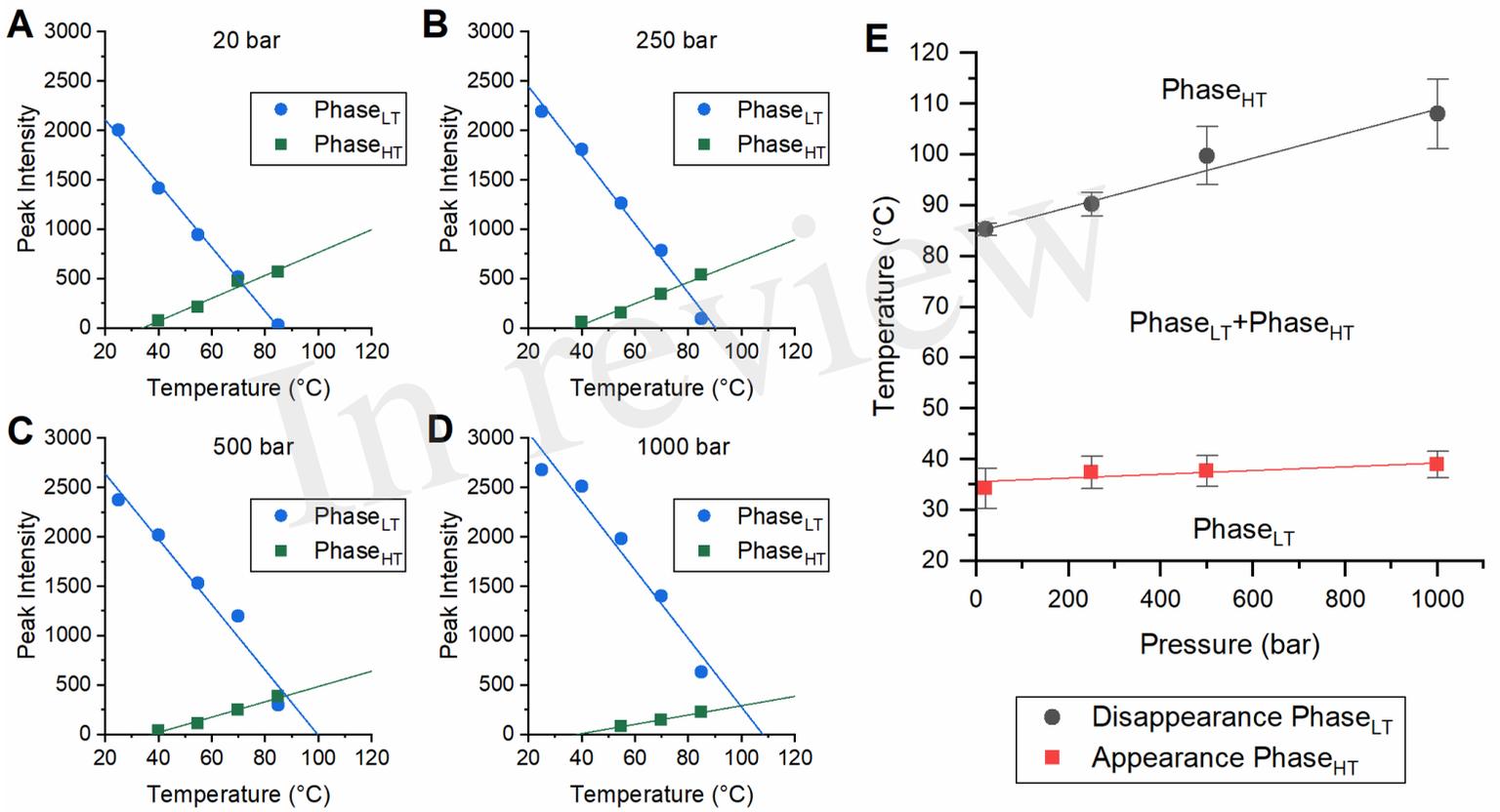


Figure 3.TIF

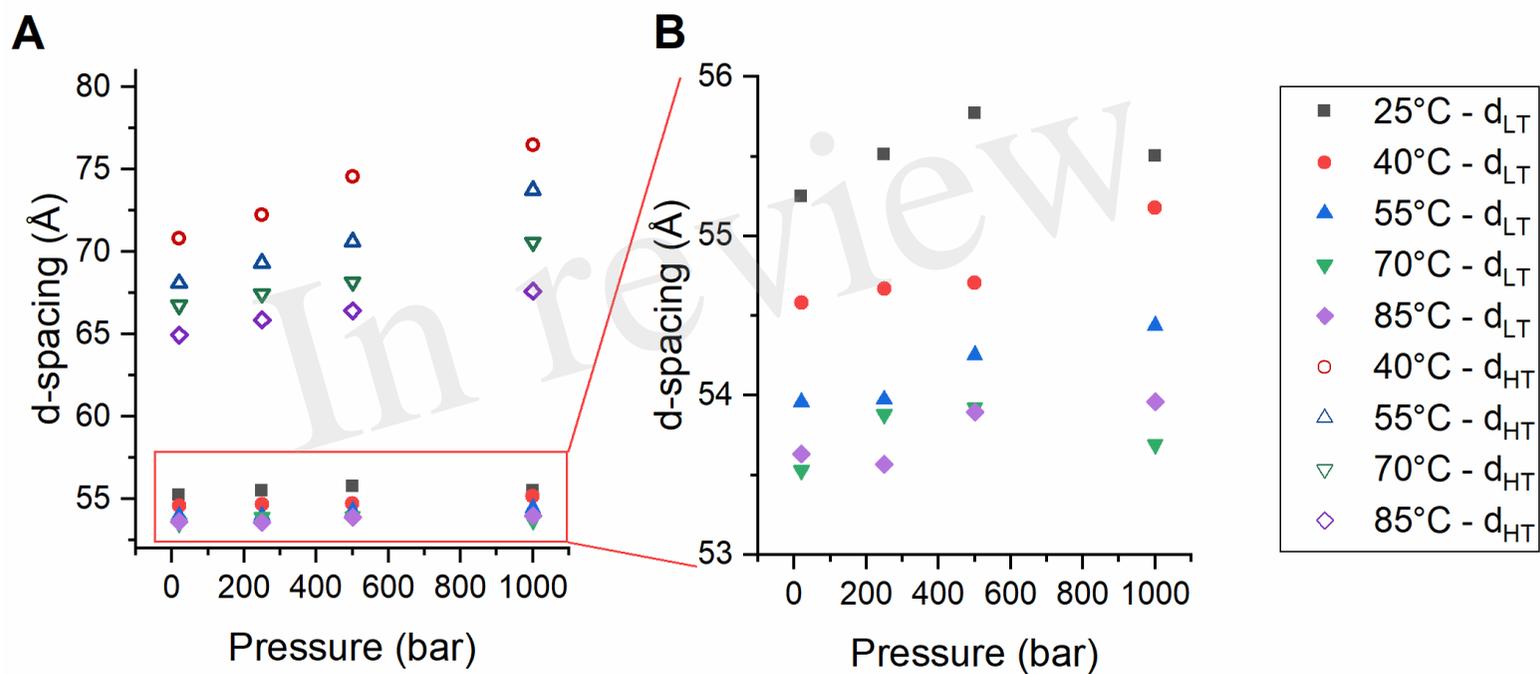


Figure 4.TIF

