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1 **Topological connection between vesicles and nanotubes in single-**
2 **component lipid membranes driven by head-tail interactions**

3

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18 **Abstract**

19 Lipid nanotube-vesicle networks are important channels for intercellular communication and
20 transport of matter. Experimentally observed in neighboring mammalian cells, but also
21 reproduced in model membrane systems, a broad consensus exists on their formation and
22 stability. Lipid membranes must be composed of at least two components, each stabilizing low
23 (generally a phospholipid) and high curvatures. Strong anisotropy or enhanced conical shape
24 of the second amphiphilic component is crucial for the formation of nanotunnels. Anisotropic
25 driving forces generally favor nanotube protrusions from vesicles. In the present work, we
26 report the unique case of topologically-connected nanotubes-vesicles obtained in the absence
27 of directional forces, in single-component membranes, composed of an anisotropic bolaform
28 glucolipid, above its melting temperature, T_m . Cryo-TEM and fluorescence confocal
29 microscopy show the interconnection between vesicles and nanotubes in a single-phase region,
30 between 60° and 90°C under diluted conditions. Solid-state NMR, necessarily recorded on the
31 dry powder, yet demonstrates that the glucolipid can simultaneously assume two distinct
32 configurations, head-head and head-tail. These arrangements, seemingly of comparable energy
33 above the T_m , could explain the existence and stability of the topologically-connected vesicles
34 and nanotubes, which are generally not observed for classical single-component phospholipid-
35 based membranes above their T_m .

36

37 **Keywords:** *Nanotube vesicle networks; Tunnelling nanotubes; Block liposomes; Liposomes;*
38 *Lipid nanotubes; Biosurfactants; Microbial glycolipids;*

39

40 **Introduction**

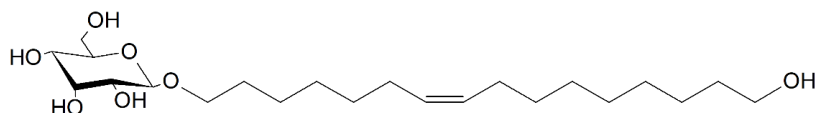
41 Topological connections between closed lipidic compartments through nanotubes¹⁻³
42 have been shown to play a crucial role in the transfer of matter and communication in
43 neighboring mammalian cells.⁴ These singular nanosystems, observed since the '90s as
44 spontaneous non-equilibrium structures in electroformed model liposome membranes,^{5,6} have
45 since been largely studied, both experimentally and theoretically.⁷⁻⁹ Addressed in the literature
46 by different terms, tunnelling nanotubes (TNT),^{1,4,9} block liposomes¹⁰⁻¹² or nanotube-vesicle
47 networks,¹³⁻¹⁶ (instead of tubes, some work speak of tethers^{6,17}) all refer to a similar
48 phenomenon, driven by various internal or external forces. The latter must overcome the energy
49 barrier needed to bend a phospholipid bilayer from low positive mean and gaussian curvatures
50 (vesicle) to a high mean and zero Gaussian (tube) curvatures.

51 A large body of both experimental and theoretical work has shown that budding and
52 eventual nanotube formation from an existing membrane can only occur spontaneously for
53 membranes of at least two-components^{9,10,24,25,11,12,18-23} and below a vesicle-nanotube phase
54 transition event.^{26,27} Otherwise, external anisotropic driving forces like electroformation,<sup>5-
55 8,28,29</sup> osmotic pressure,⁶ laser “tweezers”³⁰ or electrodynamics¹³⁻¹⁶ must be employed to obtain
56 similar results with, in some cases, an impressive degree of 2D and 3D organization.¹³⁻¹⁶ The
57 origin of spontaneous nanotube formation has been shown to be related to a nanoscale phase
58 separation between two membrane components, stabilizing low and high curvatures
59 respectively,^{10-12,31} with at least one component being highly anisotropic.^{20,32,33} Theoretically,
60 this behavior has been explained by deviations in the elastic properties of membranes due to in-
61 plane orientational ordering of membrane inclusions composed of anisotropic amphiphiles,
62 these referring to a non-symmetrical shape upon a 90° tilt along the amphiphile axis.^{8,9,19,24,25,33}

63 In this work, we show unexpected nanotubing of membranes prepared from a single-
64 component lipid, in the absence of external directional forces and above the lipid’s T_m . This
65 phenomenon is observed for a novel anisotropic double amphiphile (bolaform amphiphile, or
66 bolaamphiphile), a glucolipid composed of β -D-glucose and a C18:1-*cis* fatty alcohol (G-
67 C18:1-OH, Figure 1). This compound is obtained by microbial fermentation of a genetically-
68 modified *S. bombicola* yeast in the presence of oleyl alcohol³⁴ and is developed in the broader
69 context of extending the library of new biobased surfactants and lipids, in view of replacing
70 petrochemical low molecular weight amphiphiles.³⁵⁻⁴¹ The structure of G-C18:1-OH is
71 analogous to that of other microbial glycolipids developed through genetic engineering.^{42,43}

72 Topological connections between nanotubes and vesicles are observed by means of
73 cryogenic transmission electron microscopy (cryo-TEM), fluorescence microscopy and wide
74 angle X-ray scattering (WAXS) above the melting temperatures, $T_m = 48.3^\circ\text{C}$ under strong
75 dilution (0.5 wt%). Spin diffusion and ^1H double-quantum single-quantum (DQ-SQ) 2D solid-
76 state nuclear magnetic resonance (ssNMR) spectroscopy under magic angle spinning (MAS),
77 necessarily performed on the dry glucolipid powder, provides a spectroscopic ground to
78 understand the vesicle-nanotube coexistence. The bolaform glucolipids could be in a head-
79 head/tail-tail configuration in the vesicles, but rather in a head-tail configuration in the
80 nanotubes. In the dry glucolipid, both configurations are energetically comparable in the single-
81 phase region above T_m and their coexistence is evidenced by the splitting of some ^{13}C NMR
82 signals. These facts could explain the stability of nanotubes, while the following hypotheses are
83 formulated for their formation: membrane inclusions with different orientational ordering¹³⁻¹⁶
84 possibly driven by inter-vesicle collisions.

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Figure 1 – Non acetylated C18:1 alcohol glucoside, G-C18:1-OH, is obtained by a bioprocess performed with modified *S. bombicola* yeast.

93 **Experimental Section**

94 *Synthesis of non acetylated C18:1 Alcohol Glucosides (G-C18:1-OH).* G-C18:1-OH (Mw=
95 418.56 g.mol⁻¹) was produced by aerobic whole cell bioprocess with a modified *S. bombicola*
96 strain as described by Van Renterghem et al. (Fig. S4 in Ref. ³⁴). The molecule was purchased
97 from the Bio Base Europe Pilot Plant (Gent, Belgium) and has the generalized chemical
98 structure given in figure Figure 1. The HPLC and ¹H NMR spectrum (MeOD-d4) with peak
99 assignment are shown in Figure S 1. High purity levels (99%) and high degree of uniformity
100 were obtained, as can be derived from HPLC-ELSD chromatogram, ¹H NMR and table of
101 contaminant given in Figure S 1.

102

103 *¹H solution Nuclear Magnetic Resonance (NMR).* ¹H solution NMR experiments were
104 performed on a Bruker Avance III 300 spectrometer using a 5 mm ¹H-X BBFO probe using
105 methanol-d4 as solvent. The number of transients is 8 with 3 s recycling delay, an acquisition
106 time of 5.46 s and a receiver gain of 362. The ¹H NMR spectrum and relative assignment are
107 shown in Figure S 1 and Table S 1. ¹³C solution NMR were performed on the same probe using
108 DMSO-d6. Corresponding chemical shifts are given in Table 1. Referencing is done with
109 respect to TMS. $\delta_{1H} = 0$ ppm, $\delta_{13C} = 0$ ppm.

110

111 *Sample preparation.* The sample was dissolved in milliQ-grade water at the concentration of 5
112 mg/mL (0.5 wt%). Lack of pH-sensitive probes (e.g., COOH groups) in G-C18:1-OH, as
113 otherwise found in other microbial amphiphiles,⁴⁴ but also the will to avoid ion-specific
114 effects,⁴⁵ exclude the use of buffer. The solution was heat at the given temperature using a CH3-
115 150 Combitherm-2 dry block heating device. For the cryo-TEM experiments, the vials were
116 heat at the given temperature next to the cryofixation device and plunged into liquid ethane as
117 fast as possible. For temperatures of 25°C and 70°C, the sample was heat at 90°C and then

118 cooled at to the desired temperature. For the SAXS experiment, the sample was heat at 90°C in
119 the block heating device and cooled at to 25°C before analysis. For the SANS experiment, the
120 sample was heat at 90°C directly in front of the beam using the thermalized sample holder
121 available.

122

123 *Differential Scanning Calorimetry (DSC).* DSC was performed using a DSC Q20 apparatus
124 from TA Instruments equipped with the Advantage for Q Series Version acquisition software
125 (v5.4.0). Acquisition was performed on both hydrated and dry powder sample (~ 3-5 mg) sealed
126 in a classical aluminium cup and using an immediate sequence of heating and cooling ramps at
127 a rate of 10°C.min⁻¹. Melting temperatures, T_m , 1 and 2, T_{m1} and T_{m2} , were taken at the minimum
128 of the endothermic peak.

129

130 *Small and Wide Angle Scattering experiments.* Small angle neutron scattering (SANS)
131 experiments were performed at the D11 beamline of Institut Laue Langevin (Grenoble, France)
132 during the run No. 9-13-778. Four q-ranges have been explored and merged using the following
133 wavelengths, λ , and sample-to-detector (StD) distances. 1) ultra-low q: $\lambda= 13.5\text{\AA}$, StD= 39 m;
134 2) low-q: $\lambda= 5.3\text{\AA}$, StD= 39 m; 3) mid-q: $\lambda= 5.3\text{\AA}$, StD= 8 m; 4) high-q: $\lambda= 5.3\text{\AA}$, StD= 1.4 m.
135 The sample ($C= 5 \text{ mg.mL}^{-1}$) was prepared in 99.9% D₂O to limit the incoherent background
136 scattering. The sample solution was analyzed in standard 1 mm quartz cells. Direct beam, empty
137 quartz cell, H₂O (incoherent scatterer) within the quartz cell were recorded and boron carbide
138 (B4C) was used as neutron absorber. The sample acquisition was measured at 90°C, where
139 temperature was controlled through the controller thermalized sample holder available at the
140 beamline. The background sample (D₂O) signal was subtracted from the experimental data.
141 Absolute values of the scattering intensity were obtained from the direct determination of the
142 number of neutrons in the incident beam and the detector cell solid angle. The 2D raw data were
143 corrected for the ambient background and empty cell scattering and normalized to yield an
144 absolute scale (cross section per unit volume) by the neutron flux on the samples. The data were
145 then circularly averaged to yield the 1D intensity distribution, $I(q)$. The software package Grasp
146 (developed at ILL and available free of charge) was used to integrate the data, while the
147 software package SAXSUtilities (developed at ESRF and available free of charge) was used to
148 merge the data acquired at all configurations and subtract the background.

149 Wide-angle X-ray scattering (WAXS) was performed under temperature control at the
150 SWING beamline of SOLEIL synchrotron facility (Saint-Aubin, France) during the run
151 20201747 (energy: 14 keV, sample-to-detector distance: 0.5 m). Two sample concentrations in

152 H₂O were probed, C= 5 mg.mL⁻¹ and C= 25 mg.mL⁻¹. The 2D data were integrated azimuthally
153 at the beamline using the software Foxtrot and in order to obtain the $I(q)$ vs. q spectrum after
154 masking the beam stop shadow. Silver behenate ($d_{(100)} = 58.38 \text{ \AA}$) was used as a standard to
155 calibrate the q -scale. Sample solutions were inserted in borosilicate capillaries of 1.5 mm in
156 diameter. Capillaries were flame-sealed. A capillary oven with controlled temperature ($\pm 0.5^\circ\text{C}$),
157 provided at the beamline, was used to control the sample temperature between 25°C and 111°C .
158 Data were normalized by the transmission and calibrated to the SAXS signal of H₂O at large q -
159 values ($I = 0.0163 \text{ cm}^{-1}$) in order to obtain an absolute intensity scale. The water signal was
160 measured by subtracting the signal of the empty capillary from the signal of a water-filled
161 capillary. The signal of (water + capillary) was used as background for the samples and it was
162 subtracted after integration of the 2D data.

163

164 *Cryogenic transmission electron microscopy (cryo-TEM)*. Cryo-TEM experiments were carried
165 out on an FEI Tecnai 120 twin microscope operating at 120 kV and equipped with a Gatan
166 Orius CCD numeric camera. The sample holder was a Gatan Cryoholder (Gatan 626DH,
167 Gatan). Digital Micrograph software was used for image acquisition. Cryofixation was
168 performed using a homemade cryofixation device. The solutions were deposited on a glow-
169 discharged holey carbon coated TEM copper grid (Quantifoil R2/2, Germany). Excess solution
170 was removed and the grid was immediately plunged into liquid ethane at -180°C before
171 transferring them into liquid nitrogen. All grids were kept at liquid nitrogen temperature
172 throughout all experimentation. Cryo-TEM images have been treated and analyzed using Fiji
173 (is just ImageJ) software, available free of charge at the developer's website.⁴⁶

174 The sample's concentration was C= 5 mg.mL⁻¹. For technical reasons, the temperature
175 during cryofixation could not be controlled. For this reason, the temperature associated with
176 each cryo-TEM image in the text was related to the equilibration temperature just before
177 cryofixation, the device being as close as possible to the dry bath used for temperature control.
178 Since temperature control during cryofixation was not possible in our device, and practically
179 impossible at high temperatures, one should take the T^* values as indicative of the given value.
180 Although the sample transfer between the dry bath and the cryofixation device was performed
181 as fast as possible, it is more than likely that the actual temperature of the sample at the moment
182 of cryofixation was lower than the one indicated here, although impossible to know precisely.

183

184 *Fluorescence microscopy*. Images were recorded using a 40x objective on a Leica SP5 upright
185 confocal microscope with 405 nm laser excitation. The sample was marked with 6-dodecanoyl-
186 N,N-dimethyl-2-naphthylamine (Laurdan, Mw= 353.54 g.mol⁻¹) fluorophore as follows. A
187 stock solution of 3 mg of Laurdan in 100 μL of acetone was initially prepared (C= 30 mg.mL⁻¹
188 ¹). A volume of 0.28 μL of the stock Laurdan solution was diluted in 1 mL of a 5 mg.mL⁻¹ G-
189 C18:1-OH water solution temporary set at 110°C in a sealed Eppendorf. The latter was
190 important so to enhance inclusion of Laurdan in the membrane constituted by the glucolipid,
191 while in its vesicle phase. The final Laurdan concentration was approximately 24 μmol, with a
192 molar Laurdan-to-G-C18:1-OH molar ratio of approximately 1-to-500. Fluorophore-to-lipid
193 ratios above 1:200 were generally considered as non-interfering with the lipid phase behavior,
194 as also verified in this work.

195 Laurdan can be excited between 365 nm and 410 nm and its emission wavelength can
196 vary according to the rigidity of the membrane: intercalation within a rigid or flexible
197 membrane generates emissions at 440 nm or 490 nm, respectively.⁴⁷ In the present experiment,
198 we have flame-sealed a solution of G-C18:1-OH at 5 mg.mL⁻¹ containing Laurdan (1-to-500 in
199 lipid-to-Laurdan ratio) in flat optical capillaries of 0.1 mm thickness. Capillaries were set on a
200 Linkam support for temperature control, eventually placed under the microscope. The sample
201 was excited at 405 nm and detection was made in spectral mode with a 5 nm window sliding
202 every 5 nm from 412 nm to 602 nm in 16 bits, which allows quantify the signal and compare
203 the different conditions. Quantitative analysis was performed according to the literature:^{48,49}
204 several ROI (range of interest) have been drawn on several images using the ROI manager of
205 Fiji (is just ImageJ) software. The integrated intensity of each ROI at 440 nm and 490 nm was
206 eventually recorded and used to calculate the value of the generalized polarization, GP, as
207 follows of

$$208 \quad GP = \frac{(\lambda_{440} - \lambda_{490})}{\lambda_{440} + \lambda_{490}}$$

209 A negative GP indicates a flexible membrane while a positive GP indicates a rigid membrane.

210
211 *Solid-state Nuclear Magnetic Resonance (ssNMR)*: ¹H and ¹³C ssNMR experiments have been
212 performed using an Avance III HD Bruker 7.05 T (ν_{1H}= 300 MHz) spectrometer and a 4 mm
213 magic angle spinning (MAS) probe, except for the BACK-to-BAck (BABA) experiment, which
214 was performed on an Avance III Bruker 16.44 T (ν_{1H}= 700 MHz) spectrometer equipped with
215 2.5 mm MAS probe. Temperature was controlled through a Smart cooler BCUII/a BCU-xtreme
216 unit, using 10 min of equilibration time after each temperature variation. Temperature was

217 always varied from high (110°C) to low (70°C) before analysis. Due to combined use of fast
218 MAS rates, necessary to achieve high resolution spectra, and heating close to the boiling point
219 of water, neither diluted nor concentrated samples could be used for ssNMR experiments. All
220 tests employing up to 50 wt% G-C18:1-OH in water resulted in sample centrifugation, drying
221 but also expulsion from the rotor, causing sudden stop of rotor spinning. For these reasons, all
222 attempts to study hydrated G-C18:1-OH failed and only the dry powder could be analyzed. The
223 dry sample powder was analyzed as such in zirconia rotors (4 mm or 2.5 mm) spinning at MAS=
224 10 kHz (4 mm) or 22 kHz (2.5 mm). and Chemical shifts were calibrated with respect to
225 adamantane (^{13}C : $\delta_{13\text{C}}(\text{CH}_2)= 38.48$ ppm; $\delta_{1\text{H}}= 1.91$ ppm) and glycine alpha ($\delta_{13\text{C}}(\text{CO})= 176.03$
226 ppm). Details on the acquisition parameters are given in the Supporting Information.

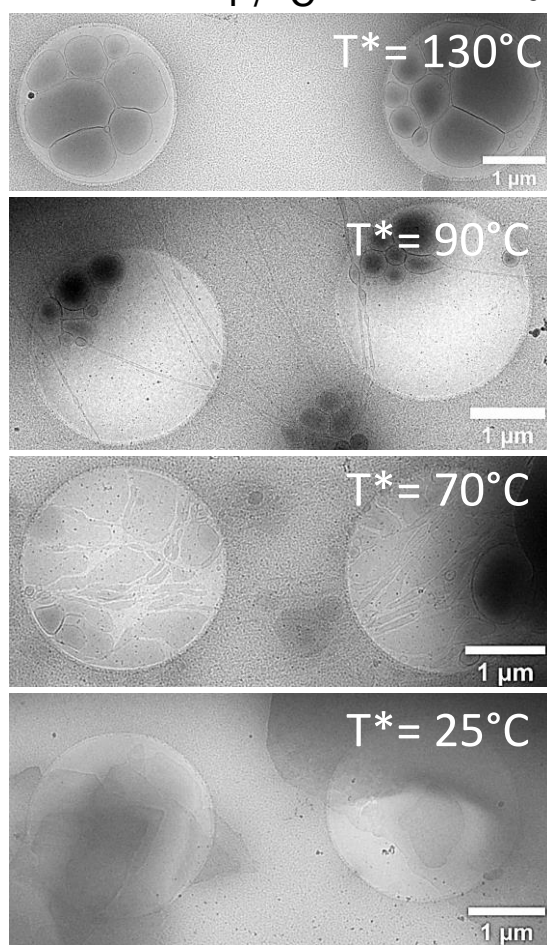
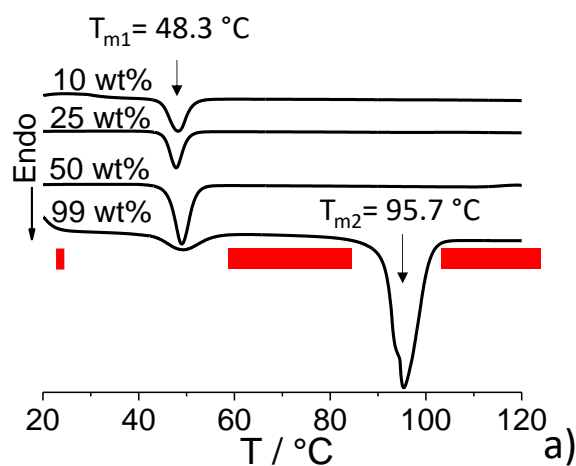
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228 **Results and discussion**

229 **Topologically-connected nanotubes and vesicles**

230 G-C18:1-OH is a biobased glucolipid, water-insoluble at room temperature, and of
231 which the physicochemical properties are poorly known. The DSC thermogram performed at
232 10°C/min of G-C18:1-OH as a function of the water content (Figure 2a) shows two endothermic
233 peaks on the dry powder (99.6 wt% glucolipid). They are attributed to two melting phenomena
234 occurring at the following temperatures, T_m : 48.3°C (T_{m1}) and 95.7°C (T_{m2}), the enthalpy of the
235 former being lower than that of the latter. Interestingly, DSC also shows that T_{m1} is hydration
236 independent and reversible (the associated exothermic peak is systematically observed), while
237 the transition at T_{m2} is simply suppressed (here tested up to 130°C) on hydrated powders and
238 never observed on the cooling profile, even at slow cooling rates (1°C/min). The thermogram
239 recorded on the dry powder is typical of single-component lipids having an intermediate,⁵⁰
240 metastable, phase (often referred to the ripple phase, $P_{\beta'}$, in reference to the periodic undulation
241 of the bilayer),⁵⁰⁻⁵⁴ between the classical lamellar gel (L_{β}) and liquid crystalline (L_{α}) phases⁵⁰⁻
242 ⁵⁴ and characterized by regions of both liquid order and disorder.^{54,55} The effect of water does
243 not influence the low-temperature crystallization at T_{m1} , but it contributes to disrupt the ordered
244 array of hydrocarbon chains in the metastable phase.

245 On the basis of the DSC thermogram, the aqueous phase behavior of G-C18:1-OH under
246 diluted conditions (5 mg.mL⁻¹) was studied at four different temperatures (red marks on Figure
247 2a): below and above T_{m1} , but also between T_{m1} and T_{m2} , corresponding to the thermogram of
248 the dry powder.

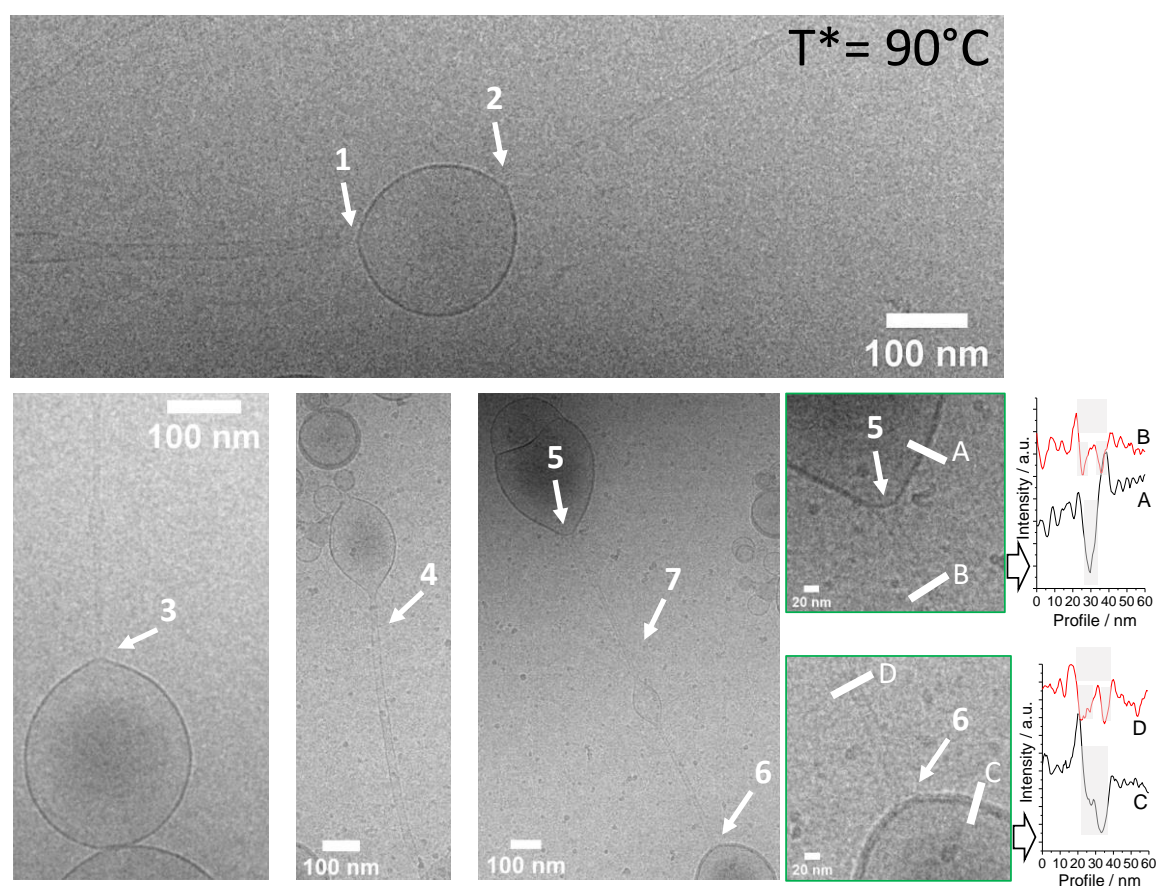


b)

249
 250 **Figure 2 – a) DSC thermogram of G-C18:1-OH under dry (0.4 wt% water, estimated by thermogravimetric**
 251 **analysis) and hydrated conditions recorded at a heating rate of 10°C/min. Red marks indicate the**
 252 **temperatures at which cryo-TEM experiments are performed on the corresponding 5 mg/mL aqueous**
 253 **solutions. b) Cryo-TEM images recorded for a 5 mg/mL G-C18:1-OH aqueous solution heat at $T^*= 130^\circ\text{C}$,**
 254 **90°C , 70°C and 25°C . T^* indicates the temperature of the sample solution before cryofixation. $T^* \neq T$, T**
 255 **being the temperature of the sample upon plunging in liquid ethane. Please refer to the materials and**
 256 **methods section for more information.**

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The series of cryo-TEM images in Figure 2b show an overview of the most relevant structures observed on G-C18:1-OH solution from $T^*=130^\circ\text{C}$ to $T^*=25^\circ\text{C}$, whereas T^* is indicative of the equilibrium temperature before cryofixation (refer to materials and methods section for more information on the sense and interpretation of T^*). At $T^*>100^\circ\text{C}$, the sample is essentially composed of polydisperse single wall vesicles, massively shown by the complementary pictures presented in Figure S 2. In between T_{m1} and T_{m2} , at $T^*=90^\circ\text{C}$ and $T^*=70^\circ\text{C}$ (Figure 2b, Figure 3, Figure 4, complemented by Figure S 3 and Figure S 4), vesicles are connected by nanotubes. At $T^*<T_{m1}$, flat crystals are otherwise observed (Figure 2b and Figure S 5).



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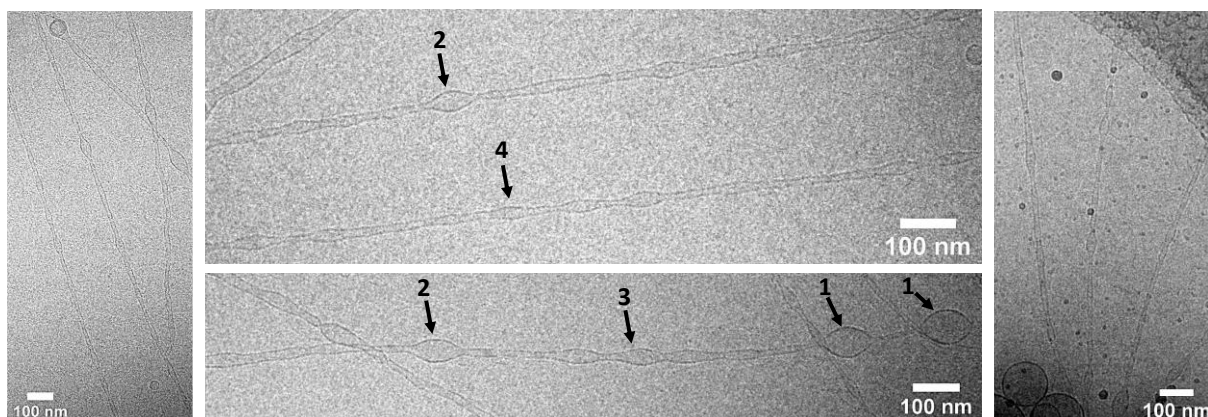
Figure 3 – Cryo-TEM images recorded for a 5 mg/mL G-C18:1-OH aqueous solution heat at $T^*=90^\circ\text{C}$.

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A deeper insight at $T^*=90^\circ\text{C}$ is given in Figure 3 (more images are provided in Figure S 3). Arrows 1 through 6 mark very clearly the typical continuity in the glucolipid membrane between the vesicle and nanotubes. Arrow N° 7 shows that a single nanotube connects the outer membrane of two vesicles several hundred nanometers apart. The connection is shown by

275 arrows N°5 and N°6. The corresponding profiles, A through D, confirm the tubular nature of
276 the elongated objects. Profiles B and D are both characterized by typical symmetrical thick
277 walls (low intensity depths highlighted by shaded rectangles) surrounding a hollow region (high
278 intensity peak, between the shaded rectangles). The wall thickness varies between 3.5 and 4.0
279 nm for profile B and between 4.1 and 6.1 nm for profile D, while the total nanotube diameter
280 varies between 14 nm at B and 20 nm at D. At the same time, the thickness of the vesicle wall
281 is about 6 nm at A and 13.5 nm at C. Considerations about the bilayer or interdigitated structure
282 of the membrane will be presented below.

283 The connection between nanotubes and vesicles seems to be multiple. For instance,
284 arrow N°1 (Figure S 3) and N°4 (Figure 3) show a single-wall vesicle, of which the membrane
285 is extruded into a nanotube. Similarly, a closer look at arrows N°1, 2 and 3 in Figure 3 show
286 that the vesicle is single-wall and fully closed, while the nanotube nucleates at the vesicle outer
287 surface, with no apparent topological continuity. In many regions of the sample at $T^*=90^\circ\text{C}$,
288 one can find exvaginations or buds^{23,25,31} in the nanotube wall. This is shown, for instance, by
289 arrows N°7 and particularly put in evidence in Figure 4 by arrows N°1 through N°4, where the
290 amplitude of the oscillation varies between 20-25 nm (arrow N°1) and 6-8 nm (arrows N°3-4).



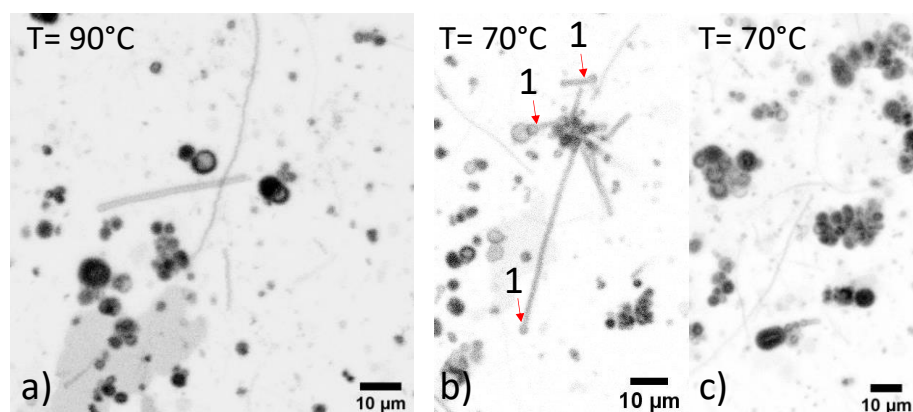
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292 **Figure 4 - Cryo-TEM images recorded for a 5 mg/mL G-C18:1-OH aqueous solution heated at $T^*=90^\circ\text{C}$.**
293

294 Still between T_{m2} and T_{m1} , but at lower temperature ($T^*=70^\circ\text{C}$), the nanotubes seem to
295 vary from single threads (arrow N°1, Figure S 4) to nanotubes of diameter below 15 nm, both
296 connecting nanotubes of diameter ranging between 50 nm and 100 nm (arrows N°2, Figure S
297 4). The larger tubes are most likely formed by the inflation and flattening of the nanotubes,
298 probably after assimilation of surrounding vesicles and fusion of the exvagination discussed
299 above. The transition between vesicles, tubes and flat membranes is certainly more accentuated.
300 Arrow N°3 in Figure S 4 shows distinct vesicles while arrow N°4 identifies a flat structure
301 evidently formed from fused vesicles. In particular, arrow N°5 points at the fusion of two

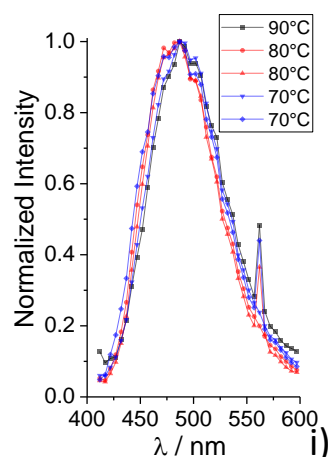
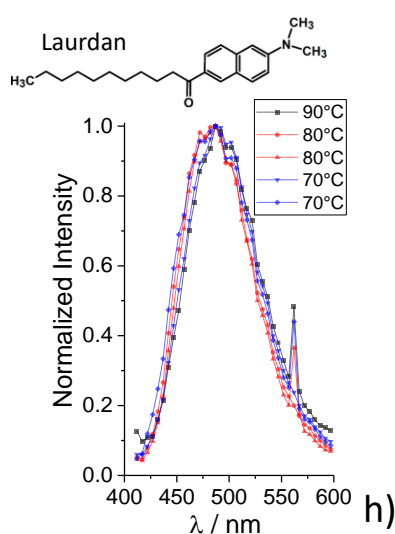
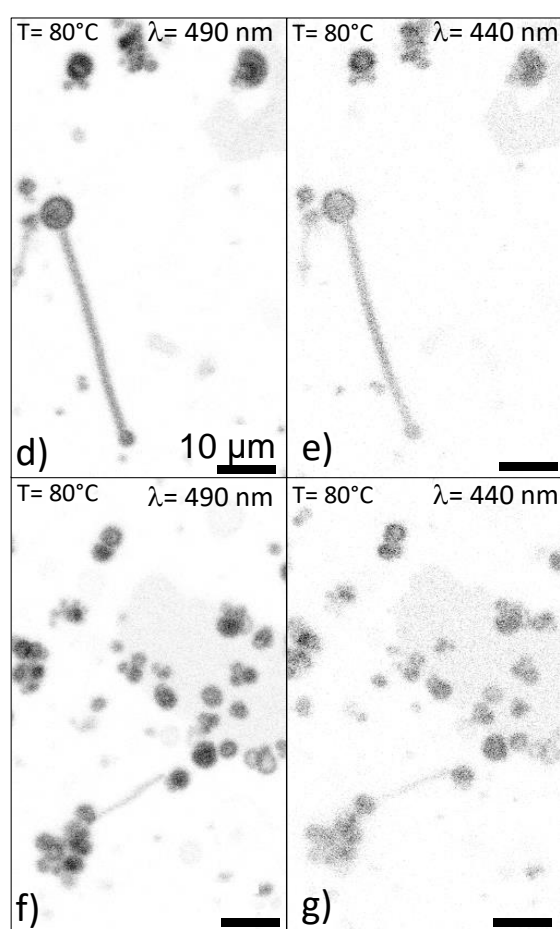
302 vesicles while arrow N°6 points at two vesicles fusing into the flat membrane. Additional areas
303 where vesicle fusion is prominent are indicated by the oscillations at the outer skirt of broad
304 lamellae and pointed by arrow N°7.

305 Finally, below T_{m1} , at $T^*= 25^\circ\text{C}$ for instance, mainly flat crystals, with no visible
306 exvaginations or tubes, are visible (Figure S 5) by cryo-TEM and the material turns into an
307 insoluble precipitate. Massive presence of flat crystalline structures is also clear from the bulk
308 iridescent behavior of a G-C18:1-OH solution under manual shear.

309 In summary, despite the uncertainty related to the actual state of equilibrium of the
310 glucolipids at the moment of cryofixation, cryo-TEM depicts a vesicle phase at $T > 100^\circ\text{C}$, a
311 flat lamellar phase below T_{m1} and an interconnected vesicle-nanotube network between T_{m1} and
312 about 100°C . The combination of statistically-relevant fluorescence microscopy and
313 scattering/diffraction experiments provides an additional proof of the coexistence of
314 vesicle/nanotube systems between the two transition temperatures. Please note that the
315 experiments reported below have been performed under controlled conditions of temperature,
316 which is then indicated by the use of T , instead of T^* .



$$GP = \frac{(\lambda_{440} - \lambda_{490})}{\lambda_{440} + \lambda_{490}}$$



317
 318 **Figure 5 – a-c) Confocal fluorescence microscopy images recorded at 90°C and 70°C for a 5 mg/mL G-**
 319 **C18:1-OH aqueous solution. d-g) Typical fluorescence microscopy images recorded at T= 80°C using**
 320 **Laurdan (refer to materials and methods section for more details on this fluorophore). Excitation**
 321 **wavelength is 405 nm for all the images, while emission wavelength is 490 nm in (d,f) and 440 nm in (e,g).**
 322 **h) Full emission spectra obtained from the integration of various fluorescence images recorded from 412**
 323 **nm to 600 nm (step: 5 nm) at 90°C, 80°C and 70°C. i) Box plot of GP, defined as $GP = \frac{(\lambda_{440} - \lambda_{490})}{\lambda_{440} + \lambda_{490}}$ with λ_{440}**
 324 **and λ_{490} being the emission wavelengths at 440 nm and 490 nm, at 90°C, 80°C and 70°C. Each data point**

325 corresponds to a set of regions of interest (ROIs) (54 at 90°C, 65 at 80°C and 75 at 70°C) selected in the
326 corresponding fluorescence images. ROIs were representatively selected so to include the entire image but
327 also vesicles alone, tubes alone and mixtures of vesicles and tubes.

328

329 Figure 5a,b shows two typical confocal fluorescence microscopy images recorded on a
330 G-C18:1-OH solution (5 mg/mL) in a flame-sealed capillary heat at $T= 90^{\circ}\text{C}$ and $T= 70^{\circ}\text{C}$.
331 Although the resolution of optical microscopy is not comparable with cryo-TEM, the sample is
332 broadly composed of vesicles coexisting with nanotubes, whereas in some cases the nanotube
333 starts from the vesicle itself (arrow N°1), in good agreement with cryo-TEM data. Furthermore,
334 SANS recorded at $T= 90^{\circ}\text{C}$ (Figure S 6a) shows a scattering profile with a pronounced -2
335 dependence of the scattering intensity against the wavevector q in the log-log scale. This
336 indicates the massive presence of a flat interface and it is expected typical of bilayers in
337 vesicular morphologies.⁵⁶ However, the typical signature of nanotubes cannot be observed in
338 the SANS profile, and this can be explained by both an argument related to the relative lower
339 amount of nanotubes with respect to vesicles at 90°C but also to the steeper slope of flat
340 morphologies (-2) compared to tubes (-1), the signal of the former masking the tube signal at
341 low wavevector values. The SANS profile is characterized by a diffraction peak at $q= 0.167 \text{ \AA}^{-1}$
342 ¹, of which the assignment is refined by WAXS data.

343 Temperature-driven WAXS experiments in Figure S 6b,c (5 mg.mL⁻¹ and 25 mg.mL⁻¹,
344 respectively) show a lamellar order, possibly attributed to a multilamellar structure of the
345 vesicles (d_v , Figure S 6d) at high temperature and to the interplanar arrangement of the
346 crystalline flat structures (d_L , Figure S 6d) at $T= 25^{\circ}\text{C}$. The sharp (001) reflection indicates
347 typical interlamellar distance of 35.3 Å at 25°C, although this value varies with temperature
348 (40.0 Å at 111°C, 37.2 Å at 88°C), thus confirming its assignment to a hydration-dependent
349 interlamellar order. More intriguing is the second, broader, peak at $q= 0.403 \text{ \AA}^{-1}$ ($d= 15.6 \text{ \AA}$)
350 and its reflection, both clearly visible at 88°C, below T_{m2} . We attribute it to the inter-glucolipid
351 arrangement inside the tubular phase (d_t , Figure S 6d), coexisting with the vesicle phase,
352 between T_{m1} and T_{m2} and to the planar lipid arrangement (d_i , Figure S 6d) in the rigid flat
353 lamellar structure, below T_{m2} . Its temperature-, hence hydration-, independent position also
354 confirms its structural origin. Finally, besides the water-structure peak at about 2 \AA^{-1} , we also
355 observe a broad peak at 1.44 \AA^{-1} , characteristics of the lateral packing (d^* , Figure S 6d) of lipids
356 in bilayers. While its position could be consistent with an L_{α} phase^{57,58} above T_{m2} , its broadness
357 and positional invariance as well as the coexistence between vesicles and tubes do not allow a
358 precise assignment.

359 Does G-C18:1-OH assume a bilayer or interdigitated structure, the latter being the one
360 found for other similar bolaform glucolipids?^{42,43} The size of G-C18:1-OH can be estimated to
361 be at the most 32 Å, given by the length, L, of the C16 tail ($L = 22 \text{ Å}$, from the Tanford formula,
362 $L = 1.54 + 1.265 \times n$, ($n = 16$)⁵⁹) and the sugar (about 10 Å).^{42,43} However, the *cis* conformation
363 of the C=C bond imposes a “kink” of 60° in the aliphatic chain, which reduces the effective
364 length of the oleic backbone to about 17 Å, for an effective total G-C18:1-OH length of about
365 27 Å. Cryo-TEM provides a tube thickness varying from 35 Å to 60 Å and a vesicle membrane
366 thickness of at least 60 Å. WAXS suggests an interlamellar thickness (membrane and water)
367 between 40 Å and 37 Å and an inter-lipid distance in the tube of about 16 Å. In the absence of
368 a clear form factor oscillation profile, as found for similar glucolipids,^{42,43} one must
369 acknowledge that these data pledge neither in favor of fully interdigitated nor a double layer
370 structure and might suggest a tilted lipid arrangement with partial interdigitation.⁶⁰

371

372 **¹³C ssNMR demonstrates that G-C18:1-OH can assume two coexisting, head-head and**
373 **head-tail, configurations**

374 Vesicles are characterized by low positive mean and gaussian curvatures, while
375 nanotubes are described by a combination of a high positive mean and zero gaussian curvatures.
376 In this regard, the coexistence of vesicles and nanotubes requires glucolipids to adapt
377 themselves to both environments at the same time. For this reason, the formation of nanotube-
378 vesicle networks generally requires a specific driving force, able to impose a high mean and
379 zero gaussian curvature to the membrane. In many systems, insertion of at least a second
380 constituent (lipid, surfactant, protein) in the membrane becomes a necessary internal driving
381 force for the nucleation and growth of so-called membrane inclusions, from which
382 topologically-connected nanotubes can form,^{10–12,32,61} as explained by orientational ordering
383 arguments.⁶² It has also been shown that external driving forces, such as use of laser
384 “tweezers”,³⁰ osmotic pressure,⁶ electroformation^{5–8,28,29} or controlled suction,^{13–16} are
385 otherwise necessary, as also supported by theoretical studies.¹⁹

386 The bending energy of tubes is higher than that of a vesicle membrane,⁸ due to the
387 dependence of the membrane free energy from the principal curvatures. For this reason,
388 isotropic (rotational symmetry along the main axis), but also anisotropic,³³ lipids generally
389 stabilize low-curvature shapes, like flat membranes or vesicles. Lipid nanotubes are also a
390 common phase formed by amphiphiles, but generally for lipids with a certain complexity in
391 their molecular structures, as exemplified by diacetylenic derivatives of phospholipids, among
392 others.^{26,27,63–65} Negative contributions to the bending energy favoring the simultaneous

393 presence of interconnected vesicles and tubes was shown to be driven by the presence of
394 anisotropic lipids in a membrane continuum. Spontaneous deviations to the elastic theory in the
395 presence of surfactants, lipids and proteins with anisotropic structures favor the formation of
396 protrusions with radius of the order of the fraction of a micron.^{9-12,19-23,62}

397 In the absence of both internal and external driving forces mentioned above, G-C18:1-
398 OH should behave as a standard lipid. Its DSC profile (Figure 2)^{53,66,67} under both hydrated and
399 dry conditions identifies a hydration-independent transition (T_{m1}) slightly above room
400 temperature; this is followed by one main transition at higher temperature (T_{m2}) in the dry
401 sample.⁵⁰ For such a profile, one expects a gel phase (L_{β}) below T_{m1} and a liquid crystalline
402 structure, possibly in the morphology of vesicles (L_4 phase), possibly above T_{m1} , and most likely
403 above T_{m2} ,³¹ as experimentally found. On the basis of the thermogram recorded on the dry
404 powder, one may also expect a “ripple” (P_{β}) phase between T_{m1} and T_{m2} characterized by
405 periodic undulations of the bilayer (generally addressed to as regions with both liquid order and
406 disorder).^{54,55}

407 In the present work, instead of a L_{β} phase, we observe flat crystals below T_{m1} , and a
408 vesicle-nanotube network instead of the “ripple” phase. The latter is particularly unexpected
409 because the membrane is composed of a single-component (G-C18:1-OH) and no anisotropic
410 external force (osmotic, hydrodynamic, electric) is used to pull and stabilize the tubes. In
411 addition, the nanotubes are observed at fairly high temperatures, above T_{m1} (between about
412 60°C and 90°C), while this morphology is generally favored at low temperature (proportionality
413 with $1/kT$, figure 5 in Ref. ⁶²), commonly below a given transition temperature, characteristics
414 for each specific amphiphile.²⁷ Furthermore, nanotubes of G-C18:1-OH seem to be stable in
415 time, thus differing from what is found in electroformed vesicles.^{7,8} They are also observed
416 away from phase transition events (may them be at T_{m1} or T_{m2}), as otherwise reported before
417 for a diacetylenic derivative of phosphatidylcholine²⁶ and many other systems.²⁷

418 A stable nanotube-vesicle network for a single-component phospholipid in the absence
419 of external driving forces is not expected and has never been reported before. It could be
420 explained by the coexistence of two micro-separated phases of different rigidity. This
421 hypothesis is excluded by confocal microscopy on Laurdan-labelled membranes and magic
422 angle spinning (MAS) solid-state NMR (ssNMR) experiments.

423 Laurdan is a well-known fluorophore used to put in evidence the coexistence of rigid
424 and fluid domains in both model and biological membranes.⁴⁷⁻⁴⁹ Laurdan was used here to label
425 the G-C18:1-OH membranes between T_{m1} and T_{m2} and to verify the possible micro-segregation
426 and coexistence of rigid (e.g., L_{β} , λ_{em} = 440 nm) and fluid (e.g., L_{α} , λ_{em} = 490 nm) domains, in

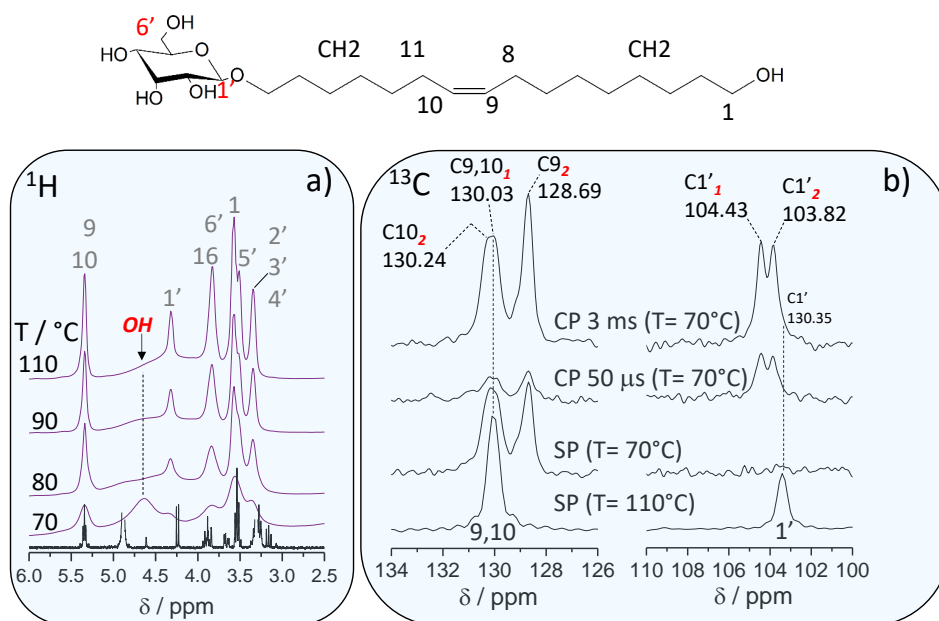
427 tubes and vesicles, respectively. A set of quantitative experiments performed on a 5 mg.mL⁻¹
428 solution, summarized in Figure 5, shows an excess of fluorescence at only $\lambda_{em}= 490$ nm for
429 temperature between 90°C and 70°C (Figure 5h). The GP value, which quantifies the rigid/fluid
430 character of the membrane, is systematically negative (Figure 5i), indicating an overall fluid
431 membrane at all temperatures. GP is quantified on more than 50 regions of interest (ROIs) at
432 each temperature, including vesicles and tubes alone as well as connected nanotube-vesicles
433 (Figure 5d-g). The dispersion of GP at 80°C and 70°C between -0.8 and -0.2 could suggest the
434 spurious presence of membranes with various degrees of flexibility, although fluidity is always
435 predominant. The combination of WAXS (broad peak, $d^*= 1.44 \text{ \AA}^{-1}$, invariant with
436 temperature, Figure S 6b,c) and confocal fluorescence microscopy using Laurdan confirms the
437 single-phase of the membrane composed of G-C18:1-OH, both in vesicles and nanotubes. This
438 is also consistent with the lack of phase transitions in DSC experiments between ~85°C and
439 ~60°C, especially in hydrated samples. We conclude that the vesicle-nanotube transition is not
440 driven by the local segregation of gel phase (L_{β}) microdomains, nor that the nanotubes are
441 composed of such phase.

442 ssNMR is the best-suited approach to investigate the origin of the local driving force for
443 the nucleation of anisotropic inclusions, of which the average orientation depends on the local
444 membrane and inclusion curvatures and was shown to promote tubing.⁶² ssNMR can provide
445 information on the local molecular mobility, e.g. through the nuclear spin relaxation behavior,
446 but also on intermolecular interactions and stereochemistry, e.g. through variations in the
447 chemical shift.⁶⁸⁻⁷⁰

448 Ideally, ssNMR should be performed on diluted G-C18:1-OH samples above T_{m1} .
449 However, fast MAS, necessary for high resolution, centrifuges the sample. As a first
450 approximation, hydrated G-C18:1-OH samples, for instance at 50 wt% as classically done for
451 phospholipids,⁷¹ could be studied. Unfortunately, permeability of the sample holder combined
452 to heating (close to the boiling point of water) and MAS provoke dehydration and even sample
453 ejection. After experiencing both of the above, this approach was abandoned. As a second
454 approximation, the study of the dehydrated G-C18:1-OH powder avoids centrifugation and
455 evaporation. If studying dried lipids may not reflect their exact behaviour under diluted
456 conditions, valuable structural information can still be obtained in dehydrated molecular
457 systems, like proteins, thus illustrating that this approach is not unreasonable. Furthermore, if
458 DSC (Figure 2a) shows that hydration suppresses the T_{m2} , it also shows that the value of T_{m1} is
459 not affected by water at concentrations as low as 10 wt%. For this reason, performing a
460 structural analysis of dehydrated G-C18:1-OH by ssNMR above T_{m1} may help understanding

461 the local arrangement of G-C18:1-OH in two morphologies, vesicles and nanotubes, having
462 sensibly different curvatures.

463 To explore the local mobility of the most relevant chemical groups of G-C18:1-OH
464 above T_{m2} ($T = 110^\circ\text{C}$) and between T_{m2} and T_{m1} ($70 < T / ^\circ\text{C} < 90$), we combine a series of ^1H
465 and ^{13}C experiments. Single-pulse (SP) MAS experiments (^1H and ^{13}C), when recorded under
466 quantitative conditions, allow the detection of all chemical species, while cross-polarization
467 (CP) MAS (^{13}C only) experiments are sensitive only to constrained groups, for which the ^1H -
468 ^{13}C dipolar coupling is not averaged to zero by the molecular motions.



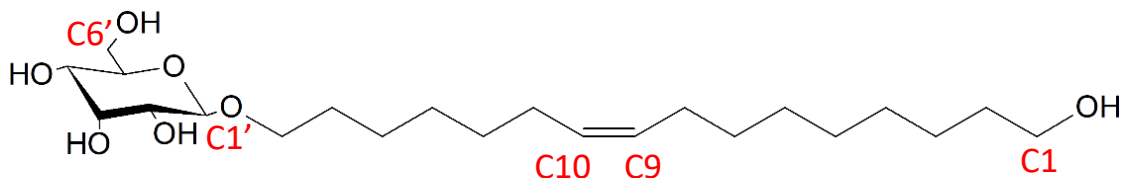
469
470 **Figure 6 – a) ^1H MAS (10 kHz) ssNMR spectra recorded from 110°C to 70°C . b) ^{13}C MAS (10 kHz) ssNMR**
471 **spectra recorded using high power decoupling single pulse (SP) and cross-polarization (CP) pulse sequences.**
472 **Contact time for CP experiments and corresponding temperatures are given on each spectrum. Full spectra**
473 **are given in Figure S 7.**

474 The most relevant ^1H and ^{13}C spectra are given in Figure 6 (full range in Figure 7 and
475 Figure S 7). As expected, the ^1H solid-state SP/MAS spectra show broader peaks compared to
476 the solution-state NMR spectrum (Figure 6a), reflecting the obvious solid-state character of the
477 sample at any temperature. The sugar group and the C=C bond resonate in the 2.5-6 ppm range
478 and 5.35 ppm, respectively, while the OH groups of the sugar moiety are found below 4.5 ppm
479 (Figure 6a, full assignment in Table S 1). Very broad (~ 1 ppm) at 100°C , the position of the
480 OH peak varies with the temperature value and becomes narrower ($< \sim 0.5$ ppm) and visible at
481 $\delta_{\text{H}} = 4.64$ ppm at $T = 70^\circ\text{C}$. Line narrowing and chemical shift variations of the OH resonance
482 in ssNMR is always a direct sign of H-bonding interactions, whereas the strength of the H-
483 bonding, hence its $\text{OH}\cdots\text{H}$ distance, is correlated to a dispersion in chemical shifts.⁷²⁻⁷⁵ All other

484 resonances broaden from 110°C to 70°C, thus indicating slower and/or more anisotropic
 485 reorientational motions of all chemical groups. The specific dynamic behaviour of the sugar
 486 headgroup is also reflected by its ¹H transverse relaxation time, *T*₂, whereas the shorter the *T*₂,
 487 the higher the rigidity. Experiments performed between 110°C and 70°C, shown and
 488 commented in Figure S 8 in the Supporting Information, globally show a single, short (< 1 ms
 489 below 100°C), *T*₂ relaxation component for H1', H5' and H6' and two, both short and long
 490 (between 4 ms and 12 ms) *T*₂ for the acyl chain (CH₂, H9,10, H8,11), even below 100°C.

491 At 110°C, ¹³C SP/MAS experiments (Figure 6b, full range in Figure 7 and Figure S 7,
 492 assignments in Table 1) show a single peak at δ_C= 103.35 ppm for C1' and at δ_C= 130.03 ppm
 493 for C9,10, as expected for a symmetrical conformation with two chemically-equivalent
 494 carbons.⁷⁶ At 70°C, the C1' peak disappears, while C9,10 now shows two peaks around 128
 495 ppm and 130 ppm (Table 1; full temperature series in Figure S 7b), attributed to C9₂ (δ_C= 128.69
 496 ppm) and C10 in a non-symmetric configuration respectively, for which these two carbons are
 497 chemically inequivalent.⁷⁶ More precisely, the resonance at 130 ppm is actually the
 498 superposition of two close resonances, C9,10₁ (δ_C= 130.03 ppm) and C10₂ (δ_C= 130.24 ppm),
 499 where subscripts 1 and 2 stand for two different conformations of the *cis*-olefin group, as
 500 reported for various phases of oleic acid in the vicinity of 0°C.⁷⁶

501
 502 **Table 1 – ¹³C Chemical shifts of G-C18:1-OH in solution and solid-state. Solid-state experiments are**
 503 **recorded at MAS= 10 kHz. All spectra are reported in Figure 7 and Figure S 7. The contact time for cross**
 504 **polarization is 3 ms. In all ssNMR experiments, temperature is first raised at 110°C and then lowered to**
 505 **either 90°C or 70°C. Attribution of the ¹³C resonances in Table 1 is performed on the basis of the solution**
 506 **spectrum (Figure S 1b). Additional peaks observed in both single pulse and cross polarization ssNMR**
 507 **experiments are attributed, when possible, to a configuration effect of the given atom, e.g., C9,10₁ and C9₂**
 508 **indicate two configurations of C9. All new peaks observed in solid-state and not detected in solution are**
 509 **highlighted in red for convenience.**



510
 511

Peak	Liquid (DMSO-d6)	δ / ppm			
		Solid Single Pulse T= 110°C	Solid Single Pulse T= 90°C	Solid Single Pulse T= 70°C	Solid Cross Polarization T= 70°C
C10₂		-	-	130.24	130.24
C9,10/C9,10₁	130.09	130.03	130.03	130.03	130.03

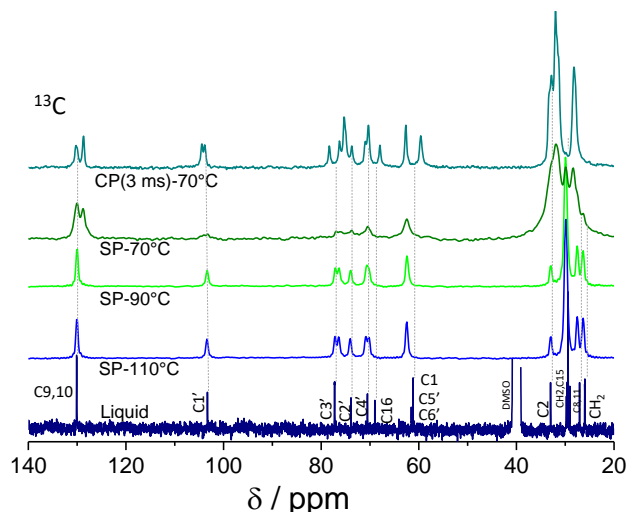
C9₂	-	-	-	128.69	128.69
C1'/C1'₁	103.29	103.35	103.35	-	104.43
C1'₂	-	-	-	-	103.82
C3'	77.24	77.09	77.02	-	78.29
?	-	76.34	76.29	-	76.21
?					75.25
C2'	73.88	73.98	73.90	73.70	73.70
C4'	70.52	70.77	70.54	70.41	70.98
C16	69.00	70.16	-	-	70.29
?	-	-	-	-	67.93
C1,C5',C6'	61.53	62.41	62.36	62.39	62.62
C1,C5',C6'	61.17	-	-	-	59.56
C2'	33.00	32.90	32.90	32.83	32.79
CH2(trans)	-	-	-	31.98	31.96
CH2(gauche),C15	29.41	29.81	29.87	30.04	-
C8,11	27.06	27.47	27.47	28.32	28.17
CH2	25.97	26.25	26.28	26.30	28.17

512

513 ¹³C CP/MAS spectra at 70°C (Figure 6b, full range in Figure 7 and Figure S 7d) at
514 increasing contact times confirm the presence of two *cis*-olefin environments: one for which
515 C9,10 are equivalent, labelled here C9,10_I, and one for which they are inequivalent, labelled
516 C9₂, C10₂. Both environments are rigid enough to be observed at short contact time (t_c= 50 μs).
517 A similar double environment is also observed for the sugar C1': CP/MAS at 70°C shows two
518 peaks (C1'₁ at δ_C= 104.43 ppm, C1'₂ at δ_C= 103.82 ppm, Figure 6b, Figure S 7d). These are
519 significantly shifted downfield from the C1' peak observed at 110°C and are detected at contact
520 times as short as 50 μs, thus indicating their rigid character. One also finds that CP/MAS is
521 totally inefficient at 100°C for short contact times (50 μs) and poorly efficient even at 3 ms,
522 typical of fast-tumbling molecules (Figure S 7c); also, both *gauche* (δ_C= 30.04 ppm) and all-
523 *trans* (δ_C= 31.98 ppm) conformations of the CH₂ carbons coexist at 70°C (Figure S 7b).⁷⁷

524 According to ssNMR, one main, mobile, molecular environment exists at 110°C, as
525 shown by the well-resolved ¹H spectrum, single C=C (C9,10_I) and anomeric (C1') resonances,
526 *gauche* conformation of the aliphatic CH₂ and single T₂ of the aliphatic CH₂ chain and sugar
527 protons. The sugar group is certainly mobile (inefficient ¹H→¹³C CP/MAS) but still more
528 constrained than the acyl chain (shorter T₂ (¹H)). As the temperature is lowered, the overall
529 rigidity increases (broadening of ¹H NMR peaks, faster ¹H transverse relaxation and efficient
530 ¹H→¹³C magnetization transfer during CP/MAS), presumably driven by H-bonding
531 interactions (downfield shift and line narrowing of the OH ¹H signal, very short OH T₂, loss of
532 the C1' signal in SP/MAS). At the same time, a number of spectroscopic data (two concomitant

533 resonances for: anomeric C1'₁ and C1'₂, *cis*-olefin C9,10_I and C9₂, C10₂, and CH₂, *gauche* and
 534 *all-trans*, environments) and relaxation behaviour (fast and slow *T*₂ relaxation components)
 535 strongly suggests the coexistence of two molecular environments. Further proofs of the
 536 coexistence of two distinct molecular domains are given by a number of chemical shift
 537 variations in the ¹³C CP/MAS spectrum at 70°C compared to the one at 110°C, 90°C and
 538 solution. This is shown in Figure 7 and in the list of ¹³C chemical shifts in Table 1.
 539



540
 541 **Figure 7 - Comparison between solution- and solid-state ¹³C NMR spectra of G-C18:1-OH. Solid-state**
 542 **experiments are recorded directly on the sample powder at a MAS of 10 kHz (4 mm zirconia rotor). SP:**
 543 **Single Pulse; CP: Cross Polarization. In all the ssNMR experiments, temperature is first raised at 110°C**
 544 **and then lowered to either 90°C or 70°C. Segmented lines are meant to highlight the known peak position**
 545 **in solution, thus stressing the strong chemical shift variations in the solid-state.**
 546

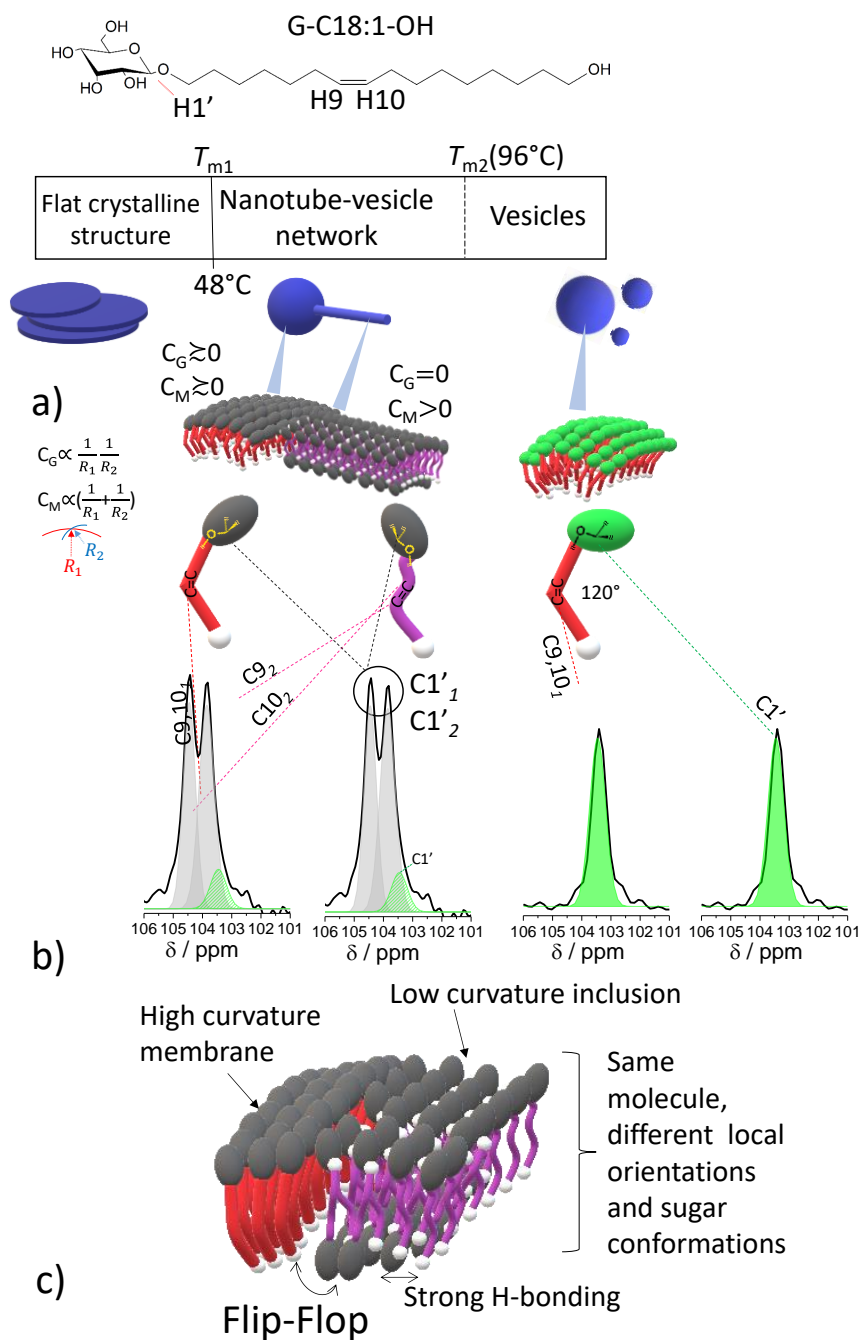
547 NMR experiments above *T*_{m2} suggest that vesicles could contain G-C18:1-OH with a
 548 single family of mobile conformations, characterized by chemically-equivalent C=C (red,
 549 Figure 8b) and a single C1' conformation (green, Figure 8b). Experiments between *T*_{m1} and *T*_{m2}
 550 otherwise suggest that two coexisting conformations, characterized by chemically-equivalent
 551 (red, Figure 8b) and inequivalent (magenta, Figure 8b) C=C, could be associated to nanotubes.
 552 Two distinct C1' sites (grey, Figure 8b) also indicate two configurations of the sugar headgroup.
 553 A comment on this point will be given in the following paragraphs.

554 The local (Å-scale) variation of molecular mobility below *T*_{m2} drives the coexistence of
 555 two distinct molecular environments of the same G-C18:1-OH molecule. This occurs within a
 556 single-phase domain (as deduced by WAXS) of equivalent membrane rigidity (as derived from
 557 Laurdan probe in confocal microscopy). Although never reported before, the difference in the
 558 local mobility of the same amphiphile (here, G-C18:1-OH) could satisfy the theoretically-

559 required conditions, that induce local lipid segregation and formation of membrane inclusions
560 (Figure 8c). In case of their non-zero average orientation, the local membrane curvature
561 increases,⁶² and nanotubes can spontaneously form (Figure 8b,c). More intriguing, this
562 mechanism occurs at least 10°C above T_{m1} , although it should not be favored above the T_m .²⁷

563 From both experimental and theoretical backgrounds, the conditions of topologically-
564 connected vesicles-nanotubes systems are met only when either internal (two components), or
565 external, driving forces are applied.¹⁹ In the present single-lipid system, of which the phase
566 behavior is summarized in Figure 8a, the most plausible driving force could be explained by
567 the NMR results collected on the dry sample. G-C18:1-OH concomitantly occupies two
568 molecular environments, characterized by: 1) two distinct conformations of the sugar
569 displaying equivalent mobility and 2) two conformations of the acyl chain unsaturation.

570



571
 572 **Figure 8 – a) Thermotropic phase behavior of G-C18:1-OH at 5 mg.mL⁻¹. Note that T_{m2} is observed on the**
 573 **dry powder, only. b) Possible relationship between the molecular environment, as derived from NMR, and**
 574 **membrane curvatures. All the molecular drawings refer to the same G-C18:1-OH molecule (bulky ellipsoid**
 575 **≡ sugar headgroup), while each color corresponds to a given configuration. Grey of $C_1'_{1,2}$ indicates**
 576 **the uncertainty about the assignment of the specific NMR peak. The curvature, C , is defined as $1/R$, R being**
 577 **the curvature radius. R_1, R_2 : radius of curvature; C_G : Gauss curvature; C_M : Mean curvature. c) Free**
 578 **interpretation of the anisotropic, high-curvature ($C_M > 0$; $C_G = 0$), inclusions in the membrane (low C_M and**
 579 **C_G) continuum driven by inter-sugar H-bonding and reduced intramembrane diffusion for a G-C18:1-OH**
 580 **glucolipid.**

581

582 Two related questions are unanswered at the moment. What actually drives nanotube
583 formation and is the organization of G-C18:1-OH within the vesicles and tubes the same? Lipid
584 nanotubes have been studied for at least three decades, over which broad experimental and
585 theoretical insight has been generated. Despite this fact, the actual understanding is still matter
586 of debate.²⁷ Micelle-to-nanotube and vesicle-to-nanotube phase transitions are generally
587 reported for single amphiphiles below a given T_m and intermediate phase composed of twisted
588 and flat fibers is concomitantly reported. Initially thought to be a chirality-driven phenomenon,
589 recent studies propose a more general mechanism involving crystallization and symmetry
590 breaking in the lipid packing driven by optimal packing.²⁷ If twisted ribbons were observed
591 before for a number of microbial glycolipid bolaamphiphiles,⁴⁴ they are not found for G-C18:1-
592 OH. This fact, as well as the large body of cryo-TEM images and the observation of nanotubes
593 above T_{m1} , rather pledge in favour of a direct topological change from positive (vesicles) to
594 zero (nanotubes) Gaussian curvatures. In this case, vesicle-to-nanotube transitions could be
595 driven by external forces.^{5,6,30,7,8,13-16,28,29}

596 Considering the absence of external forces in the present system, there are only two
597 possibilities left, spontaneous budding of the membrane or nanotube pulling after inter-vesicle
598 collisions due to Brownian motion. Both are possible, as inclusions composed of a different
599 arrangement of the same molecule in the membrane continuum can theoretically explain
600 spontaneous nanotube formation in an isotropic medium.^{6,7,22,23,62,9-12,18-21} However, the second
601 one is probably more likely to prevail, because it could explain the large number of nanotubes
602 connecting two distant vesicles. Interestingly, similar events occur for any phospholipid-based
603 vesicular solution, but nanotubes are extremely rare unless at least two lipids of different
604 molecular structure are mixed together. The spectroscopic, ^1H and ^{13}C , and relaxation (^1H)
605 ssNMR data, even if collected on a dry powder, demonstrate that the coexistence of two G-
606 C18:1-OH configurations, involving both the sugar headgroup and fatty alcohol chain, are
607 possible for this molecule. As a matter of fact, this is not so surprising, as more than one energy
608 minima, each corresponding to a given conformation, are known for glucose, its non-reducing
609 derivative, methylglucose, and carbohydrates in general.⁷⁸⁻⁸⁰

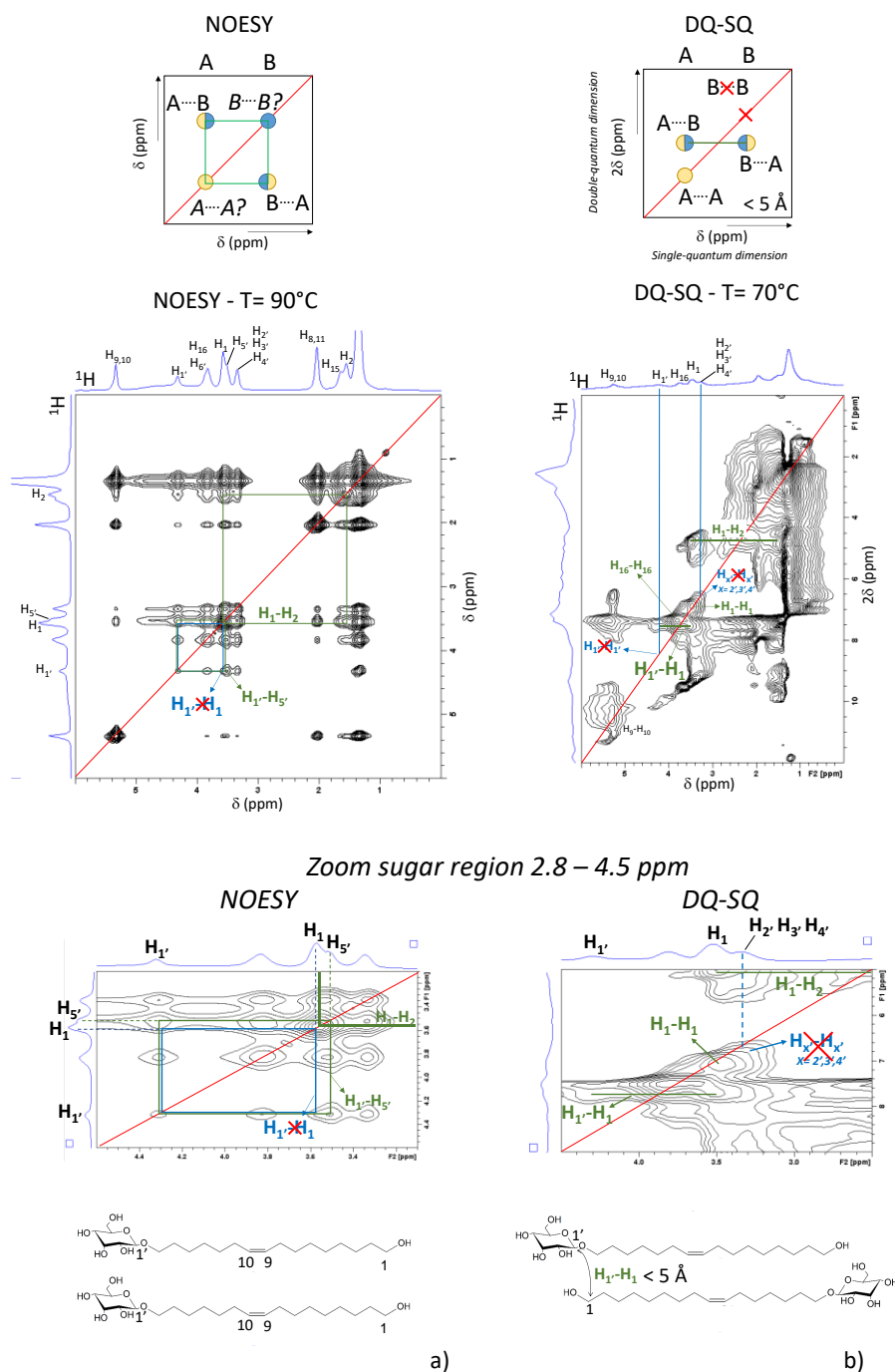
610 The presence of two configurations could explain the coexistence between two
611 structures of different curvature, but the data presented so far do not explain the origin of these
612 two configurations. In other words, what drives the change in the local structure of the sugar
613 headgroup and fatty alcohol within the membrane?

614 The association of known self-assembled structures to spectroscopic ^{13}C ssNMR data is
615 known for years, in particular for gluconamides, lipids having a linear sugar headgroup.⁸¹⁻⁸⁴ It

616 was found that *gauche* effects, in some cases up to the γ position, in the sugar chain induce
617 upfield chemical shifts, while *anti* and *trans* conformations result in downfield shifts. Each
618 structure (monolayer crystal, bilayer crystal or micellar fiber) was then characterized by a given
619 organization of the gluconamide and a characteristic ^{13}C ssNMR fingerprint, which could be
620 explained by *gauche*, γ -*gauche*, *anti* and *trans* conformations of the headgroup. Similar
621 structural-spectroscopic correlations have been collected over the years for much simpler cyclic
622 sugars,⁸⁵ whereas pyranoses can undergo important conformational changes (chair, half-chair,
623 boat, skew), when the appropriate energy barriers are crossed.^{78-80,86} A quantitative
624 conformational study based on the ^{13}C chemical shift variations shown in Table 1 would be
625 certainly helpful, but very delicate in the context of the present state of the art, because a
626 rigorous approach requires at least one known crystal structure,^{81,82} which is currently missing
627 for this compound. A qualitative approach will then be adopted.

628 G-C18:1-OH is a bolaform amphiphile and flip-flop could be a possible mechanism
629 involved in the nanotube stabilization, whereas head-tail interactions have long been considered
630 as important in nanotube-forming bolaform amphiphiles.^{60,63} From mere considerations based
631 on T_2 (^1H) relaxation arguments, one could exclude head-tail configurations: if the R- CH_2OH
632 endgroup (H1) was involved in a direct interaction with the sugar group, its corresponding T_2
633 would probably be comparable to the sugar's one (OH and H1'), but this does not seem to be
634 the case. However, this hypothesis would be based on the comparison of the relaxation from
635 CH and CH_2 protons, additionally involved in different kind of motions. Besides, the relaxation
636 of H1 is influenced by the strong overlap between H1 and H5'. For these reasons, relaxation
637 arguments are not solid enough and will not be used.

638 We then address this question by exploring intermolecular interactions in a direct
639 manner, using 2D ^1H - ^1H homonuclear ssNMR correlation spectroscopy. At 90°C, in the fast-
640 tumbling domain of molecular motion (narrow ^1H NMR peaks, Figure 6a) and corresponding
641 to the vesicle-rich region, spin diffusion (NOESY) experiments are preferred over dipolar
642 coupling based experiments, because such interaction is averaged out by molecular motion.
643 However, when the molecular motion is more constrained, in the domain of nanotube stability
644 at 70°C (broad ^1H spectra, Figure 6a), Double-Quantum Single-Quantum (DQ-SQ) correlation
645 experiments are best suited to probe interaction between close molecular neighbors. These
646 experiments are reported in Figure 9 and the related interpretation is only briefly reported
647 below, while a more detailed discussion can be found on Page 12 in the Supporting Information.



648

649 **Figure 9 – a) 2D ^1H - ^1H NOESY NMR experiment performed at MAS= 10 kHz (4 mm probe) on a Bruker**
 650 **300 MHz Avance III HD spectrometer using 300 ms of mixing time. Sample temperature is regulated to**
 651 **90°C , reached by cooling from 110°C . b) 2D ^1H - ^1H DQ-SQ (Back-to-Back, BABA, pulse sequence used for**
 652 **the DQ excitation scheme) NMR experiment performed at MAS= 22 kHz (2.5 mm probe) on a Bruker 700**
 653 **MHz Avance III spectrometer using a DQ excitation time equal to 2 rotor periods. Sample temperature is**
 654 **regulated to 70°C , reached by cooling from 110°C . The typical schemes illustrating how to interpret these**
 655 **2D experiments are reported above each experimental map. Zooms of the sugar region and configurational**
 656 **interpretation are given below each 2D map. More information are given in the text below.**

657 Head-tail or head-head configurations could be probed by the existence of H1'-H1
658 interactions, whereas H1 ($\delta_{\text{H}}= 3.56$ ppm) is associated to the glucolipid tail and H1' ($\delta_{\text{H}}= 4.32$
659 ppm) to the sugar headgroup. These atoms are more than 15 Å apart, and a direct interaction
660 could not be observed in both NOESY and DQ-SQ experiments. NOESY experiments (Figure
661 9a) show that H1'-H1 correlations are not observed. Meanwhile, clear-cut, direct, H1'-H1
662 interactions are indeed observed are detected at $T= 70^{\circ}\text{C}$ (DQ cross-peaks related to H1'-H1
663 correlation in DQ-SQ experiment shown in Figure 9b). Interestingly, the absence of auto peaks
664 at $\delta_{\text{H}}= 4.32$ ppm and $\delta_{\text{H}}= 3.35$ ppm in the SQ dimension excludes H1'-H1' and Hx'-Hx' ($x= 2,$
665 3, 4) interactions respectively, strengthening the idea that sugar headgroups from two nearest-
666 neighbor G-C18:1-OH molecules are not adjacent in the nanotube-vesicle region.

667 Homonuclear 2D ssNMR supports the idea, according to which G-C18:1-OH can
668 undergo molecular flip-flops and a head-tail arrangement in the dry product. Extrapolating these
669 conclusions in the lower concentration range, lack of H1'-H1 proximity at $T= 90^{\circ}\text{C}$ suggests a
670 head-head configuration in the vesicle-rich region and head-tail configuration within the
671 nanotubes. It is however still unclear whether flip-flops are driven by spontaneous, temperature-
672 induced, conformational changes in the glucolipid packing, stimulating inclusions within the
673 membrane itself,^{6,7,22,23,62,9-12,18-21} or by defects generated by inter-vesicle collisions. In fact, a
674 combination of both is not unlikely. Nanotubes are not stable when temperature is 20 to 30
675 degrees above T_{m1} , indicating that inter-vesicle collisions alone cannot explain the unusual
676 phenomenon. In an energy landscape approach,⁸⁷ the head-head configuration is certainly
677 favored at high temperature, while both head-head and head-tail configurations could be
678 favored in the mid-temperature region, corresponding to the range between T_{m1} and T_{m2} in the
679 dry powder. Both statistical and defect-induced flip-flop events contribute to form high mean,
680 zero Gaussian, curvature morphologies (nanotubes), for which the head-tail conformation,
681 limiting the proximity between bulky sugar headgroups, is probably more adapted (Figure 8c).
682 The flip-flop interpretation now reasonably explains the strong differences in the sugar and fatty
683 alcohol conformations, deduced by the multiple NMR signals attributed to chemically-
684 equivalent groups. The existence of various low-energy conformations, each having specific
685 NMR signature, for sugars in solution^{78-80,86} and solid-state⁸¹⁻⁸⁴ further supports our
686 interpretation.

687 One last point should be clarified. Why, between T_{m1} and T_{m2} , are two molecular
688 environments able to coexist, instead of a single one of average rigidity, as found in the case of
689 “ripple” phases?⁵⁰⁻⁵⁴ We believe that this question can be answered based on the NMR results:
690 the relatively short T_2 values of the H1' and OH protons shows the constrained mobility of the

691 sugar headgroup, despite a certain flexibility of the chain (long T_2 component). This trend may
692 suggest restrictions in translational mobility, probably due to the sugar-OH hydrogen bonding
693 (Figure 8c), meaning that lipids are not able to diffuse within the membrane, thus keeping two
694 different configurations stable.

695

696 **Conclusions**

697 Topologically-connected nanotube-vesicle lipidic systems are commonly observed for
698 self-assembled membranes composed of at least two amphiphiles, and spontaneously driven by
699 anisotropic inclusions and/or by external directional forces. This work shows that strong
700 differences in membrane curvature can spontaneously occur in single-lipid systems without
701 external anisotropic forces. Coexistence and interconnection between nanotubes and vesicles
702 are evidenced via cryo-TEM and fluorescence confocal microscopy in a single-phase region at
703 temperature above a transition temperature, $T_{m1} = 48.3^\circ\text{C}$, determined by DSC on the hydrated
704 sample, but below the second transition temperature, T_{m2} (95.7°C) determined by DSC on the
705 dry powder. X-ray diffraction (WAXS) also shows the coexistence of two lattice periods, one
706 attributed to an inter-membrane repeating distance and the other possibly related to the intra-
707 nanotube glucolipid arrangement. Labelling the systems with Laurdan, a fluorophore of which
708 the emission strongly varies with the local membrane rigidity, excludes the presence of rigid
709 micro-separated phases (e.g., L_β).

710 ^1H and ^{13}C ssNMR, despite the fact that it can only be performed on the dry powder,
711 confirm the presence of one main fluid G-C18:1-OH environment above T_{m2} . Below T_{m2} , the
712 splitting of some ^{13}C peaks and the coexistence of short and long ^1H relaxation components
713 demonstrate that G-C18:1-OH can simultaneously pack into structurally and dynamically
714 inequivalent environments. Combining advanced 2D ^1H - ^1H correlation experiments based on
715 spin diffusion or double-quanta excitation suggests that vesicles could contain a preferred head-
716 head configuration of the bolaform lipid, while nanotubes a preferred head-tail assembly.

717 The presence of two environments composed of the same molecule characterized by
718 different molecular configurations and rigidity fulfills the theoretical conditions necessary to
719 spontaneously nucleate a membrane inclusion of non-zero average orientation, described as
720 necessary to observe topologically-connected nanotube-vesicle structures in the absence of
721 external forces. This hypothesis could also be enriched by the possibility of nanotubes to be
722 pulled away between two vesicles after a collision event.

723 This work expands the actual theoretical and experimental framework on lipid networks
724 stabilized by a different radius of curvature. It could also motivate the synthesis, or isolation,

725 of new lipids providing a higher level of 3D architectural control in soft networks, possibly in
726 the range of room temperature.

727

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743

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- 903

904 **Supporting Information**

905

906 **Topological connection between vesicles and nanotubes in single-**
907 **component lipid membranes driven by head-tail interactions**

908

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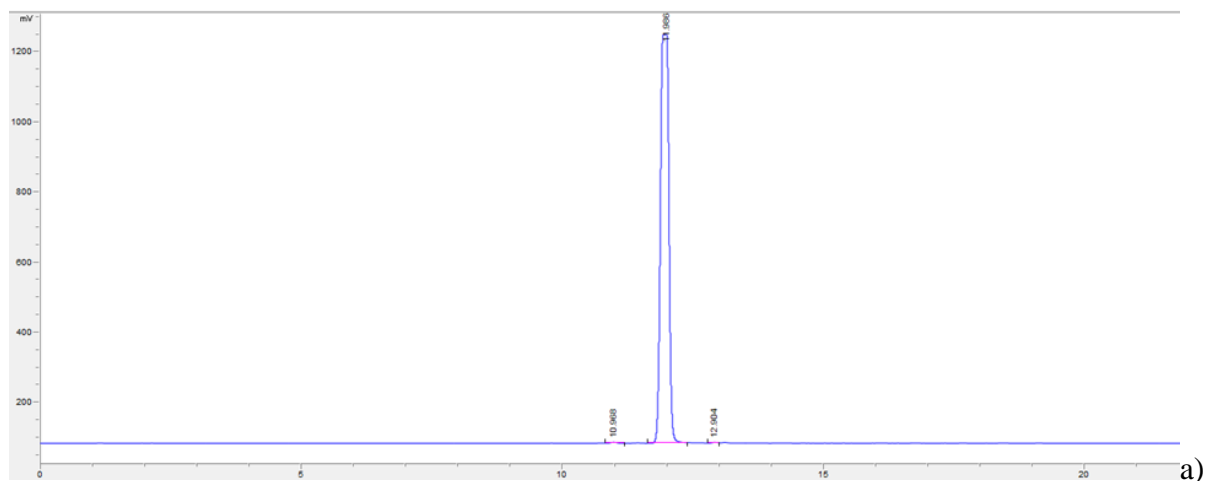
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Parameter		Method
Dry Matter (DM %)	99.6	Infrared balance 105°C
Glucose (%)	0.12	HPLC-Metacarb
Glycerol (%)	n.d.*	HPLC-Metacarb
Free Fatty Acid content (%)	<0.01	Internal method BBEPP: GC with FID Detector
Oil (%)	<0.01	Internal method BBEPP: GC with FID Detector
Moulds (CFU/g)	<10	3M-Nordval n°16 B
Yeasts (CFU/g)	<10	3M-Nordval n°16 B
Anaerobic count (CFU/g)	<10	SP-VG M005 B
Protein (%)	t.b.d.*	BCA protein assay

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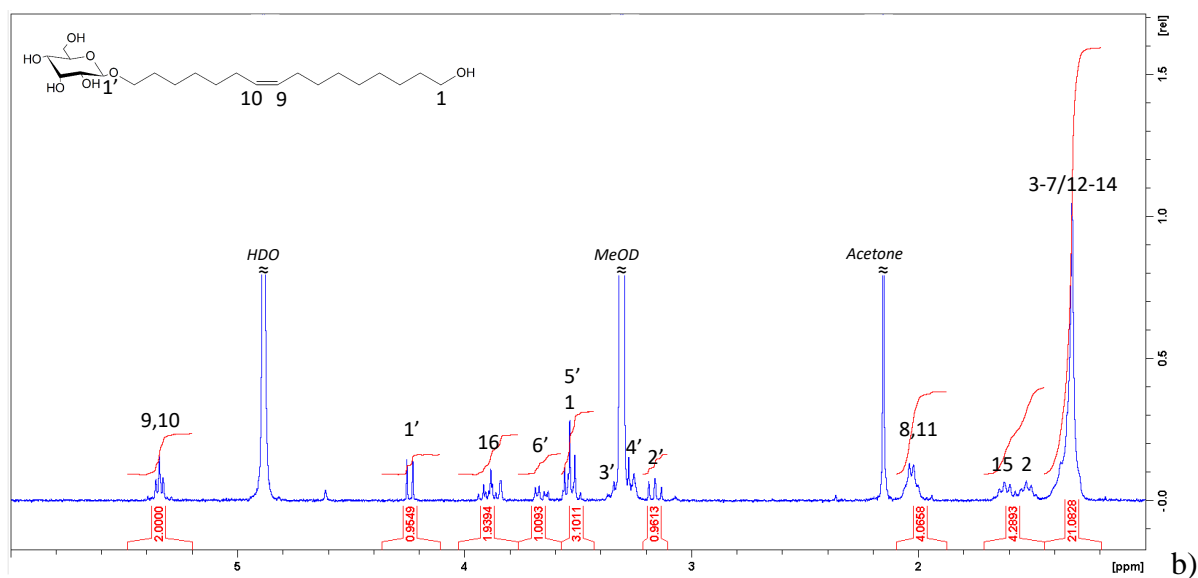
929 **Figure S 1 - a) HPLC-ELSD chromatogram and composition table.**

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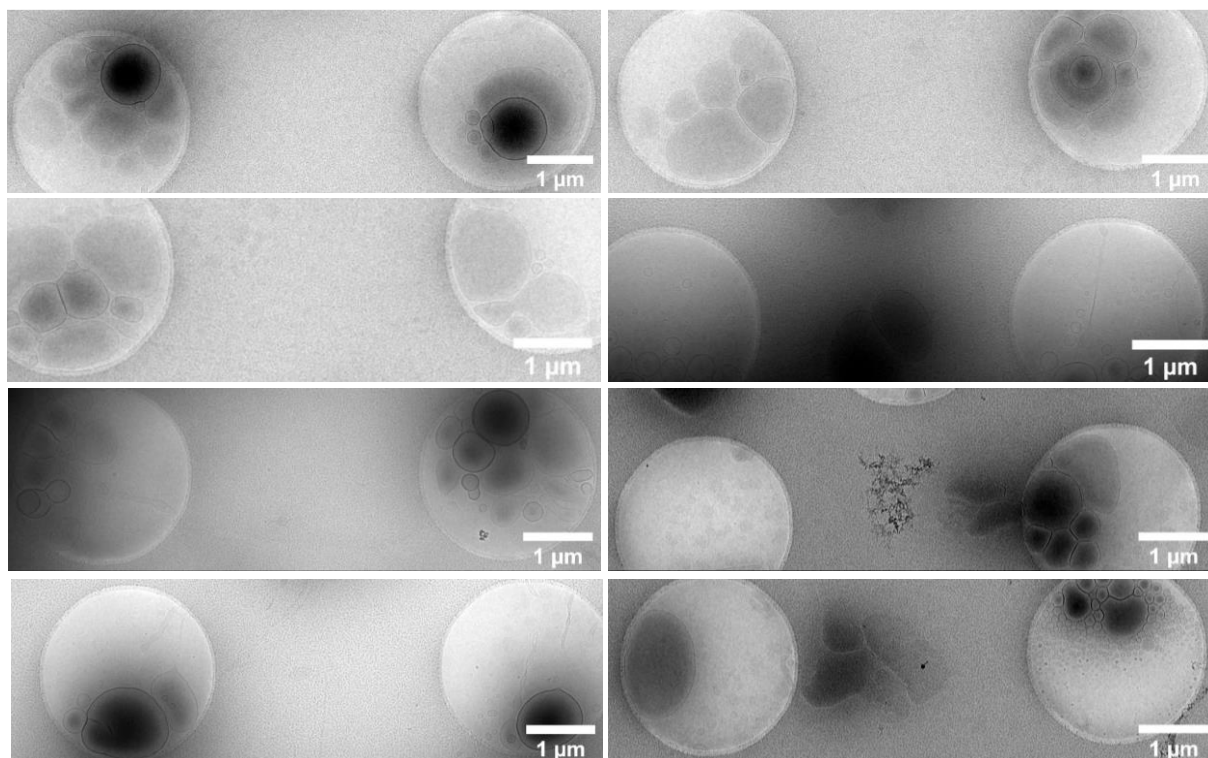
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Group	N°	δ / ppm
CH ₂	1	3.54
CH ₂	2	1.53
CH ₂	3-7/12-14	1.32
CH ₂	8,11	2.03
CH	9,10	5.34
CH ₂	15	1.62
CH ₂	16	3.88
CH	1'	4.24
CH	2'	3.16
CH	3'	3.34
CH	4'	3.29
CH	5'	3.54
CH ₂	6'	3.66

936 Figure S 1 (next) – b) ¹H NMR spectrum (MeOD-d₄) recorded on the purified G-C18:1-OH compounds
 937 and related assignment

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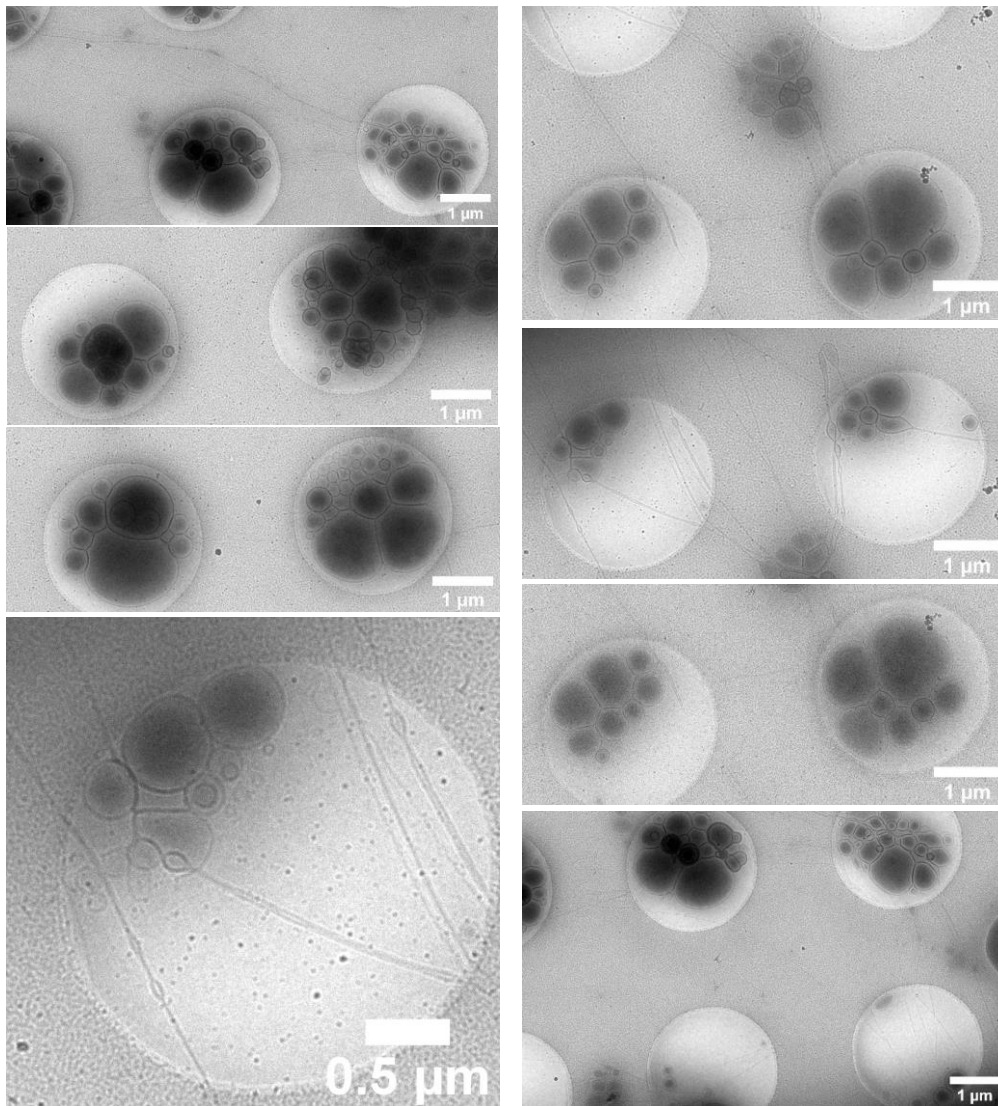
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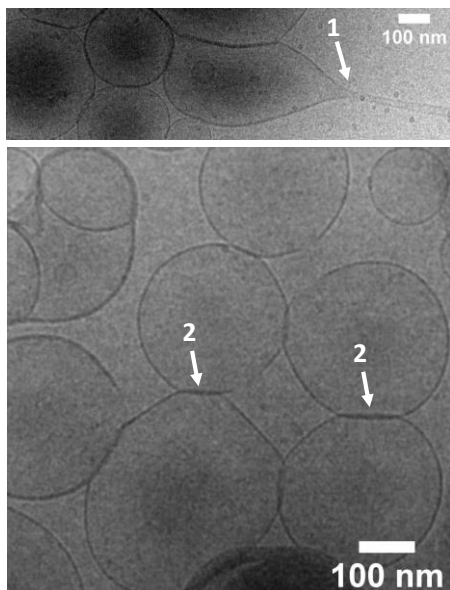
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Figure S 2 – Cryo-TEM images recorded for a 5 mg/mL G-C18:1-OH aqueous solution heat at $T^*= 130^\circ\text{C}$.



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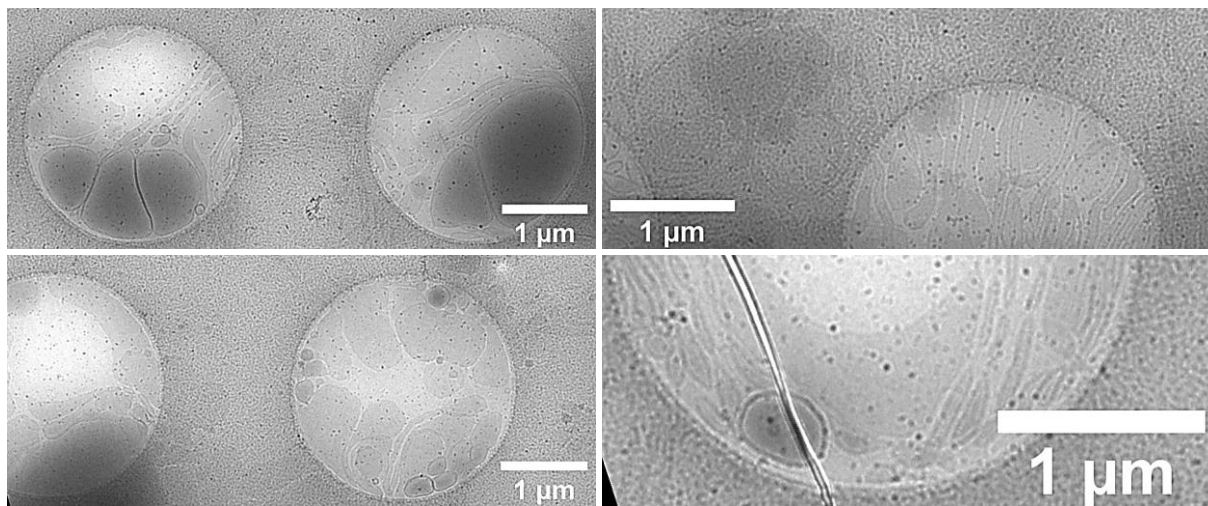


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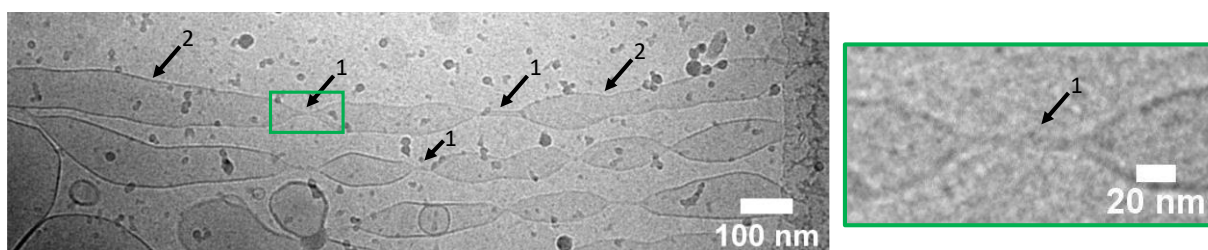
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Figure S 3 - Cryo-TEM images recorded for a 5 mg/mL G-C18:1-OH aqueous solution heat at $T^* = 90^\circ\text{C}$.

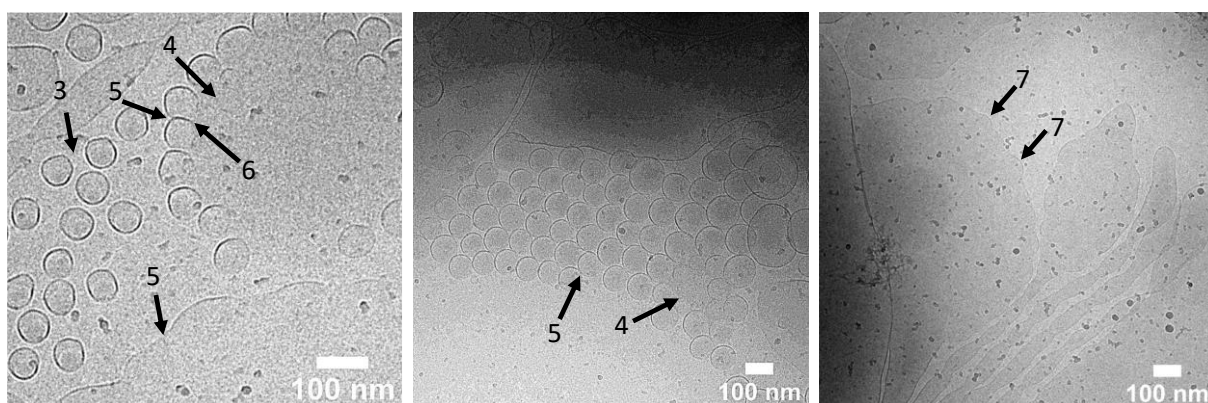


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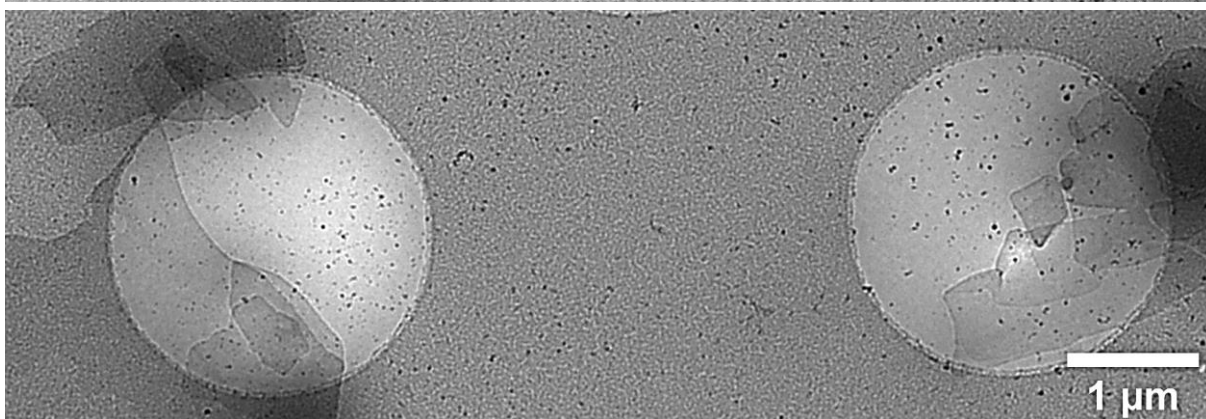
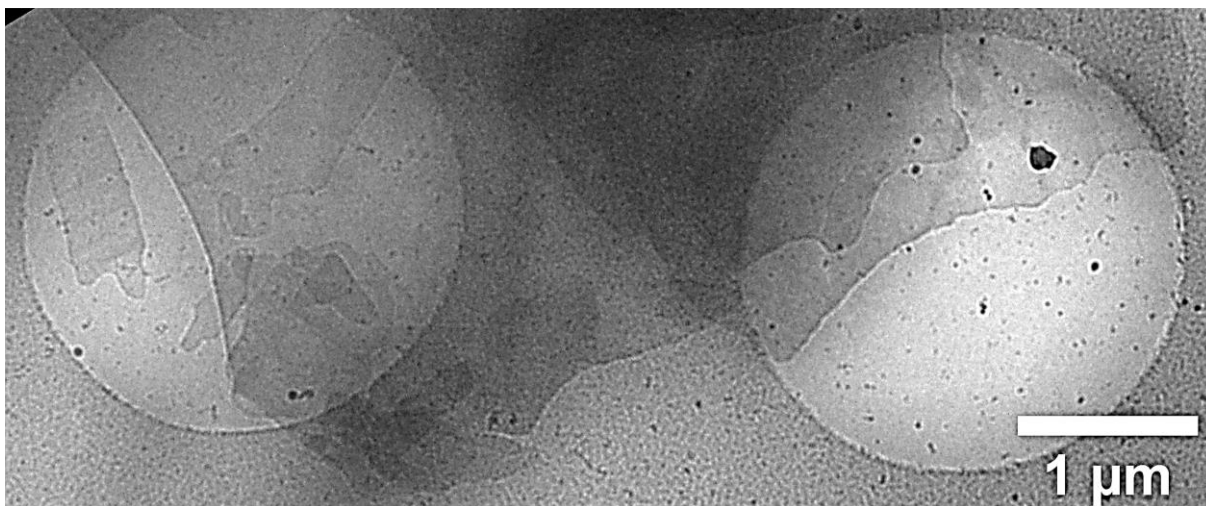


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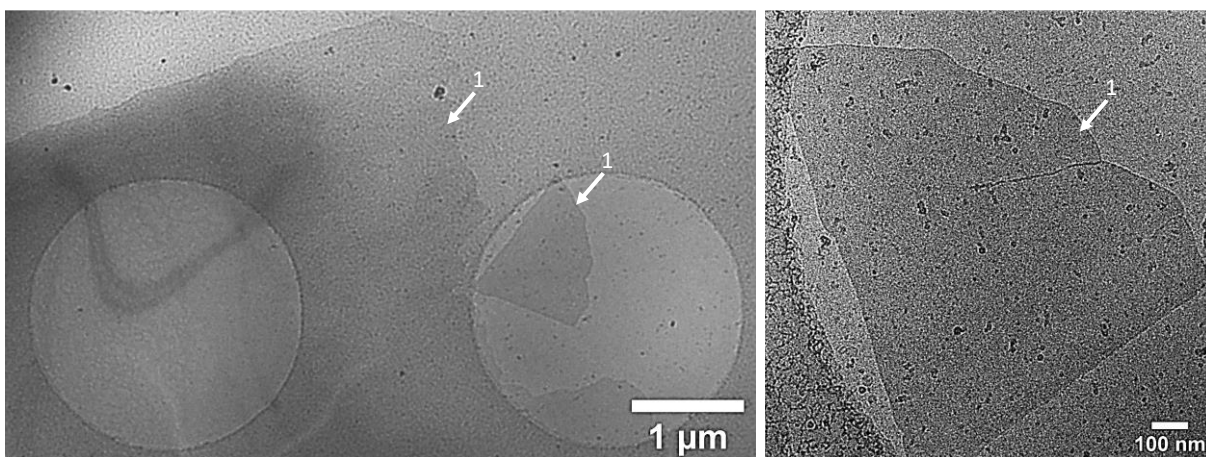
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Figure S 4 – Cryo-TEM images recorded for a 5 mg/mL G-C18:1-OH aqueous solution heat at $T^*= 70^\circ\text{C}$.

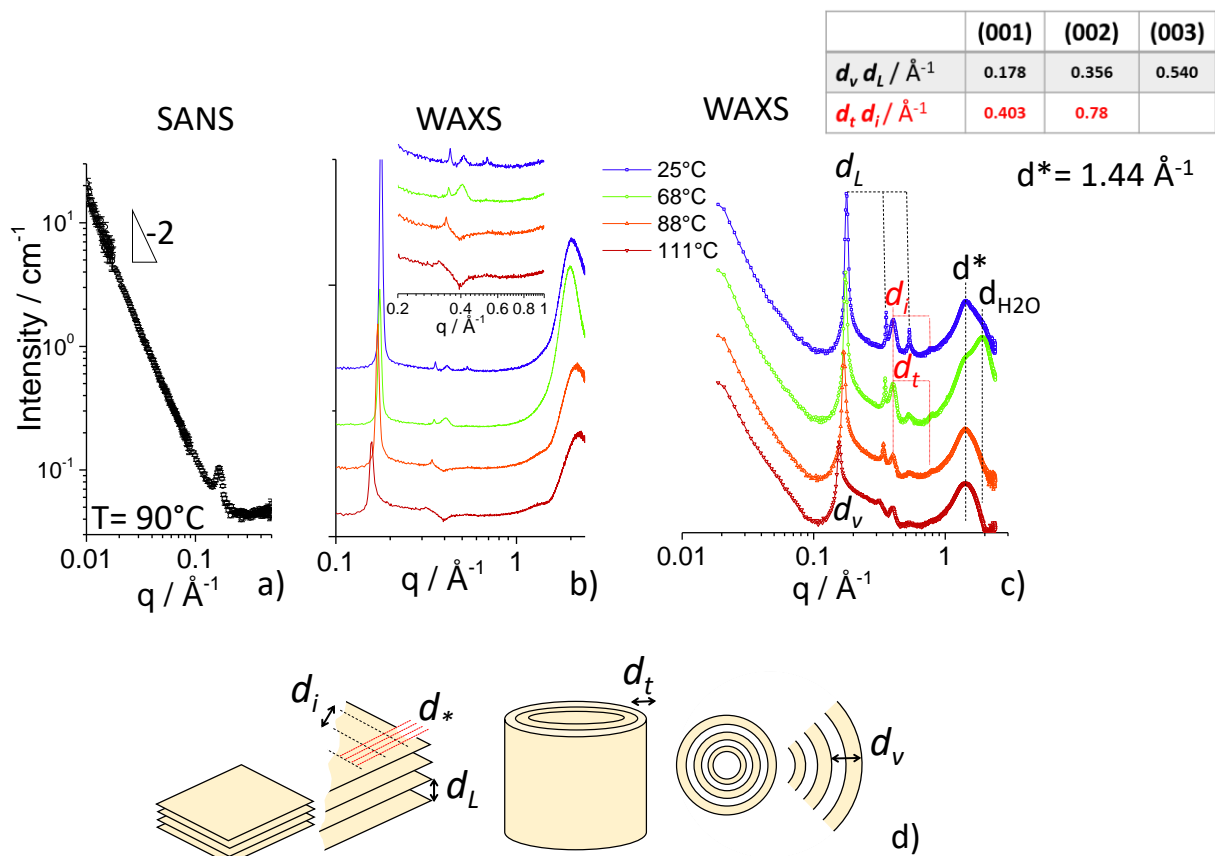


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Figure S 5 – Cryo-TEM images recorded for a 5 mg/mL G-C18:1-OH aqueous solution heat at $T^*= 25^\circ\text{C}$.



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959 Figure S 6 – a) SANS experiment recorded at 90°C for a 5 mg/mL G-C18:1-OH aqueous solution. b-c)

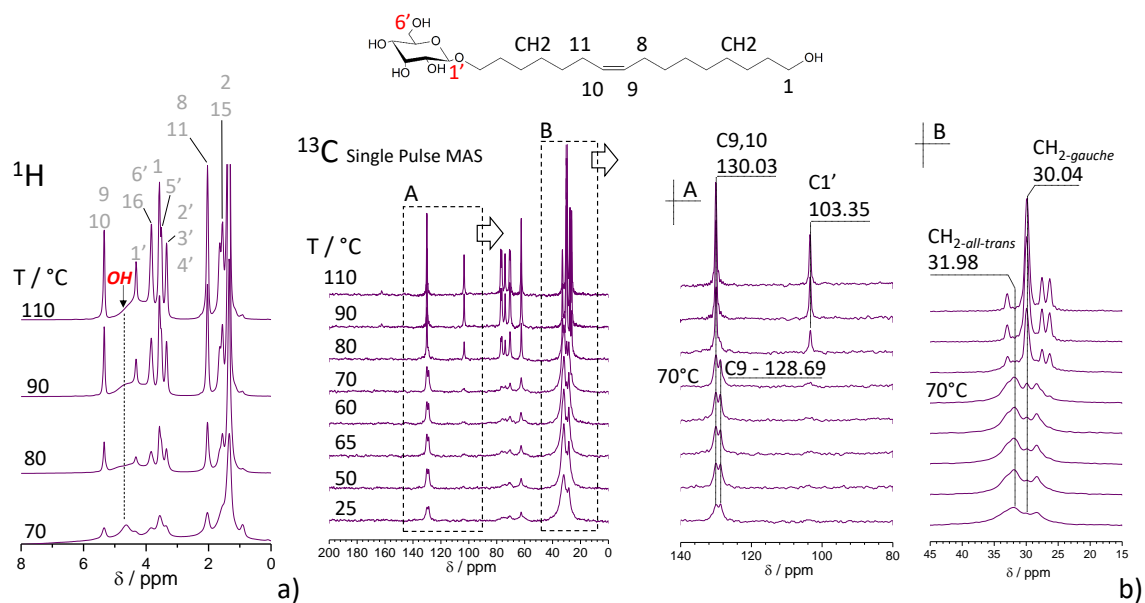
960 WAXS experiments recorded on aqueous solutions of G-C18:1-OH at b) 5 mg/mL and c) 25 mg/mL. d)

961 Structural interpretation of the repeating distances pointed out in c).

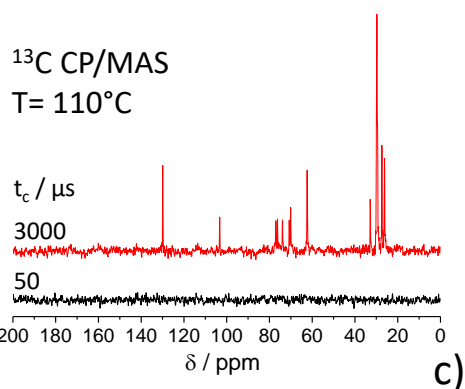
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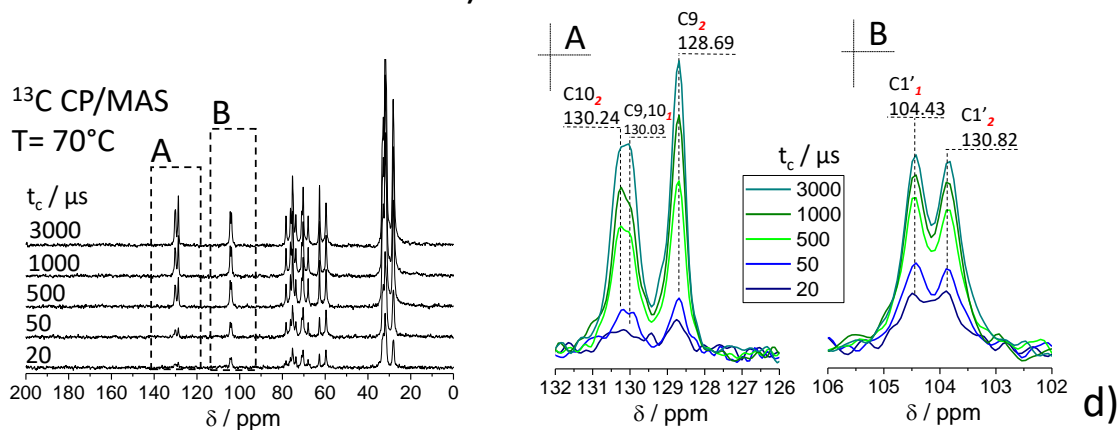
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c)

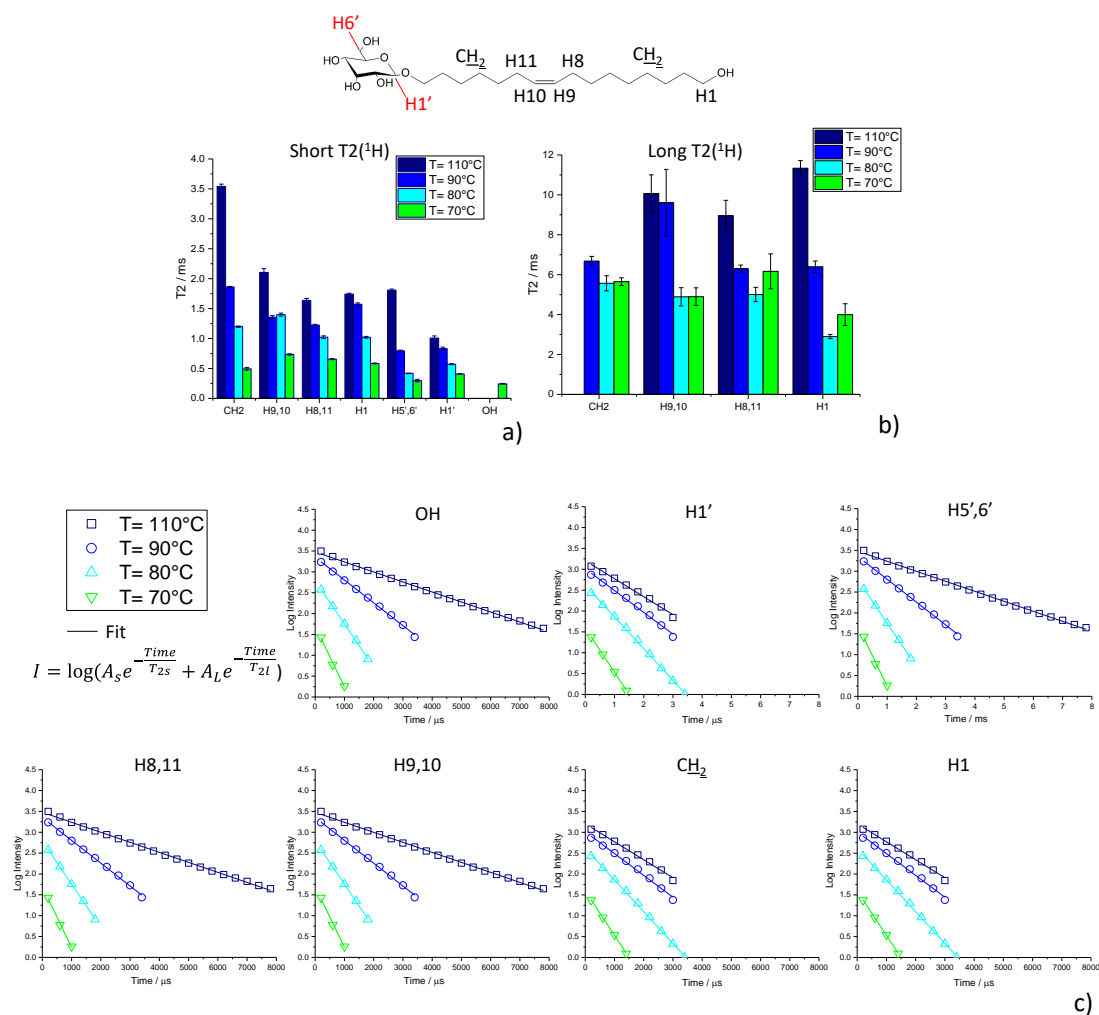


d)

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967 **Figure S 7 – ssNMR MAS (10 kHz) experiments recorded on a G-C18:1-OH dry powder. a) ^1H spectra**
 968 **recorded from 110°C to 70°C. b) ^{13}C single pulse (SP) spectra recorded with high power ^1H decoupling from**
 969 **110°C to 70°C. c) ^{13}C cross polarization (CP) spectra recorded at 110°C and contact time, t_c , of 50 μs and**
 970 **3000 μs . d) ^{13}C CP spectra recorded at 70°C and for 20 < t_c / μs < 3000**

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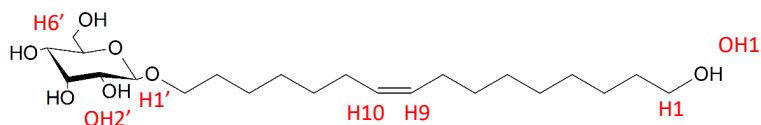
973 **Figure S 8 – a) Short and d) long T_2 components displayed by the ^1H transverse relaxation signal. c) ^1H**
 974 **transverse relaxation signals recorded on a G-C18:1-OH dry powder from 110°C to 70°C. The area of each**
 975 **given resonance is plotted (log-lin) as a function of the echo time (CPMG pulse sequence) for each**
 976 **temperature and fitted using the equation given above. T_{2s} and T_{2l} indicate the short and long T_2 relaxation**
 977 **times respectively obtained by describing the ^1H transverse relaxation signal using a biexponential decay.**
 978 **For H1', H5' and H6', the ^1H relaxation function could be described by a monoexponential decay.**

979 - Protons from the sugar group (H1', H5', H6') show a single T_2 relaxation component (Figure
 980 6c and Figure S 8), while protons of the acyl chain (CH₂, H9,10, H8,11) show both short (Figure
 981 6c) and long (Figure 6d) T_2 relaxation component.

982 - The short T_2 relaxation time systematically decreases from 110°C to 70°C for all species by a
 983 factor ranging from two to seven, depending on the specific chemical group. Very long T_2 are
 984 observed at 110°C for all acyl species, while nearly unchanged values are measured below
 985 90°C, without any clear trend.

986 - The sugar protons (H1', H5', H6') have the shortest set of T_2 values (< 1 ms) below 100°C.
 987 The long component of T_2 generally ranges between 4 ms and 12 ms.

988 Table S 1 – ¹H chemical shifts of G-C18:1-OH in solution and in the solid-state. Solid-state spectra are
 989 recorded at a MAS frequency of 10 kHz. *: in DMSO-d₆ ; #: broad peak.



990

Group	N°	δ_H / ppm		
		Liquid (MeOD-d ₄)	Solid (T= 110°C)	Solid (T= 70°C)
CH ₂	H1	3.54	3.57	
CH ₂	H2	1.53	1.56	
CH ₂	H3-7/12-14	1.32	1.35	
CH ₂	H8,11	2.03	2.03	
CH	H9,10	5.34	5.34	
CH ₂	H15	1.62	1.64	
CH ₂	H16	3.88	3.83	
CH	H1'	4.24	4.32	
CH	H2'	3.16	3.35	
CH	H3'	3.34	3.35	
CH	H4'	3.29	3.35	
CH	H5'	3.54	3.51	3.57
CH ₂	H6'	3.66	3.83	
OH*	OH1	4.32	4.5 [#]	4.63
OH*	OH2'	4.92		
OH*	OH3'	4.90		
OH*	OH4'	4.87		
OH*	OH6'	4.45		

991

992

993 *Detailed discussion about the NOESY and DQ-SQ 2D ¹H-¹H ssNMR experiments presented in*
994 *Figure 9*

995

996 2D ¹H-¹H ssNMR experiments are a unique tool to explore through-space proximities
997 between ¹H nuclei. ssNMR offers a large number of possibilities, each adapted to a given
998 system. The advantage of 2D maps with respect to a dynamics approach is their direct
999 readability; in clear, two nuclei which exchange their magnetization through-space (whichever
1000 mechanism may be responsible for this exchange) will display a specific cross-peak. However,
1001 the way cross-peaks are presented and interpreted strictly depends on the nature of the pulse
1002 program, which in turn is chosen according to the physical nature of the sample.

1003 1D, temperature-dependent, ¹H NMR experiments performed on G-C18:1-OH (Figure
1004 4a in the main text) indicate sharp peaks at 90°C and broad peaks at 70°C. Narrow peaks
1005 indicate fast and/or large-amplitude molecular tumbling, averaging the strong ¹H-¹H dipolar
1006 interactions. This excludes the use of pulse sequences, like DQ-SQ, which is not efficient under
1007 these conditions. For this reason, we employ spin-diffusion-based pulse sequences, like
1008 NOESY, which is more adapted for samples with high molecular mobility. When mixing times
1009 are chosen to be short (< 1 s), one generally explores through-space proximities within about 5
1010 10 Å. On the contrary, when molecular motions are restricted, dipolar coupling becomes
1011 significant, ¹H NMR peaks are broader and pulse sequences, like DQ-SQ, can now be used,
1012 with a spatial resolution of less than 5 Å.

1013 Whenever possible, DQ-SQ experiments are preferred over spin diffusion-based pulse
1014 sequences, because they are more selective, in particular for likewise interactions, which are
1015 not discriminated in NOESY-type experiments. This is illustrated by the typical interaction
1016 schemes for NOESY (top Figure 9a) and DQ-SQ (top Figure 9b). For two given A and B
1017 protons, 2D NOESY maps will display two on-diagonal and two square-shaped symmetrical
1018 off-diagonal cross-peaks. Off-diagonal cross-peaks illustrate the mutual A-B and B-A
1019 interaction, but the on-diagonal peaks are systematic and do not prove any A-A or B-B
1020 interaction. Similarly to NOESY, 2D DQ-SQ maps display off-diagonal peaks (symmetric
1021 across the diagonal, top Figure 9b) upon A-B and B-A interaction, but in contrast they will
1022 display on-diagonal peaks only in the presence of self-interaction. In the tutorial example of
1023 Figure 9b top, the on-diagonal A-A peak indicates mutual A-A interaction while lack of on-
1024 diagonal B-B peak evidences the absence of B-B proximities. Combining NOESY and DQ-SQ
1025 experiments is capital to study the local arrangement of G-C18:1-OH and its evolution with
1026 temperature.

1027 At $T=90^{\circ}\text{C}$, the H1 and H5' contributions are very close but possible to discriminate
1028 them ($\delta(\text{H1})=3.54\text{ ppm}$, $\delta(\text{H5}')=3.51\text{ ppm}$) according to ^1H NMR chemical shifts in Table S
1029 1. 2D ^1H - ^1H NOESY in Figure 9a also discriminates between the two components, attributed
1030 on the basis of the interactions with their mutual neighbors (green H1-H2 and H5'-H1' squares
1031 in Figure 9a). On this basis, 2D NOESY does not show a direct H1'-H1 correlation (blue, red-
1032 crossed), but rather an extensive network of intra-sugar interactions. These features exclude
1033 suggest intra- and inter-sugar interactions and they exclude head-tail arrangements as a major
1034 molecular configuration at 90°C .

1035 At 70°C , the ^1H NMR peaks are broader, indicating a reduction of the T_2 (^1H) relaxation
1036 times and/or residual homonuclear dipolar interactions, excluding the use of NOESY. In this
1037 case, ^1H - ^1H DQ-SQ correlation experiments are best suited. Figure 9b presents the ^1H - ^1H DQ-
1038 SQ experiment on G-C18:1-OH at 70°C . Peaks are broader than in NOESY and attribution may
1039 be cumbersome. However, the experiment is performed at 700 MHz and MAS= 22 kHz, which
1040 improves spectral resolution with respect to the same experiment performed at 300 MHz and
1041 MAS= 10 kHz. The auto-peak at $\delta_{\text{H}}=3.56\text{ ppm}$ in the SQ dimension is attributed to H1-H1 spin
1042 pairs, mainly resulting from intra CH_2 interaction, while the DQ cross-peaks at $\delta_{\text{H}}=1.56\text{-}3.56$
1043 ppm and $\delta_{\text{H}}=4.32\text{-}3.56\text{ ppm}$ respectively origin from H2-H1 and H1'-H1 interactions (Figure
1044 9b). DQ-SQ map does not show any auto-peak corresponding to H1'-H1' or Hx'-Hx' ($x=2',$
1045 $3', 4'$) interactions (red-crossed, blue, Figure 9b), thus excluding any proximity ($<5\text{ \AA}$) between
1046 close glucose neighbors. On the contrary, DQ-SQ maps (recorded both at 700 MHz, Figure 9b,
1047 or at 300 MHz, not shown) systematically show DQ cross-peaks, which can only be attributed
1048 to H1'-H1 interactions ($\delta_{\text{H}}=4.32\text{-}3.56$). The presence of these H1'-H1 interactions can only be
1049 explained by a head-tail arrangement of the G-C18:1-OH molecules, as illustrated below the
1050 DQ-SQ map in Figure 9b.