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# Assemblies of lauryl maltose neopentyl glycol (LMNG) and LMNG-solubilized membrane proteins

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## **Keywords**

Membrane proteins – Detergent – LMNG – homogeneity – crystallization – rods

## **Footnotes**

AUC: Analytical Ultracentrifugation ; DSF: Differential Scanning Fluorimetry; SEC-LS: Size Exclusion Chromatography coupled to Light Scattering; SAXS: Small Angle X-Ray Scattering; SANS: Small Angle Neutron Scattering; TLC: Thin Layer Chromatography; CMC: critical micelle concentration ; DDM: dodecyl- $\beta$ -D-maltopyranoside; DDAO: decyldimethylamine-N-oxide; LDAO: lauryldimethylamine-N-oxide; LMNG: lauryl maltose neopentyl glycol; OTG: octyl- $\beta$ -D-thioglucopyranoside; bR: bacteriorhodopsin from *Halobacterium*; BmrA: Bacillus multidrug resistance ATP; SpNOX: *S. pneumoniae* NOX protein; FhuA: *E. coli* outer membrane ferrichrome-iron transporter. Vi: orthovanadate;

## 1 Abstract

2  
3 Laurylmaltose neopentylglycol (LMNG) bears two linked hydrophobic chains of equal length  
4 and two hydrophilic maltoside groups. It arouses a strong interest in the field of membrane  
5 protein biochemistry, since it was shown to efficiently solubilize and stabilize membrane  
6 proteins often better than the commonly used dodecylmaltopyranoside (DDM), and to allow  
7 structure determination of some challenging membrane proteins. However, LMNG was  
8 described to form large micelles, which could be unfavorable for structural purposes. We thus  
9 investigated its auto-assemblies and the association state of different membrane proteins  
10 solubilized in LMNG by analytical ultracentrifugation, size exclusion chromatography  
11 coupled to light scattering, centrifugation on sucrose gradient and/or small angle scattering.  
12 At high concentrations (in the mM range) LMNG forms long rods, and it stabilized the  
13 membrane proteins investigated herein, *i.e.* a bacterial multidrug transporter, BmrA; a  
14 prokaryotic analogous of the eukaryotic NADPH oxidases, SpNOX; an *E. coli* outer  
15 membrane transporter, FhuA; and the halobacterial bacteriorhodopsin, bR. BmrA, in the Apo  
16 and the vanadate-inhibited forms showed reduced kinetics of limited proteolysis in LMNG  
17 compared to DDM. Both SpNOX and BmrA display an increased specific activity in LMNG  
18 compared to DDM. The four proteins form LMNG complexes with their usual quaternary  
19 structure and with usual amount of bound detergent. No heterogeneous complexes related to  
20 the large micelle size of LMNG alone were observed. In conditions where LMNG form  
21 assemblies of large size, FhuA crystals diffracting to 4.0 Å were obtained by vapor diffusion.  
22 LMNG large micelle size thus does not preclude membrane protein homogeneity and  
23 crystallization.

## 24 Introduction

25  
26  
27 Detergents are amphipatic molecules that auto-assemble into micelles above the critical  
28 micelle concentration (CMC). Above the CMC, they are able to solubilize lipids and  
29 membrane proteins, forming protein-detergent complexes of small and well-defined size [1].  
30 Detergents are essential for membrane protein solubilization and purification steps, as well as  
31 functional and structural studies. However, they often lead to membrane protein inactivation.  
32 One of the mechanisms leading to inactivation (for a recent review, see [2]) is the dissociation  
33 of subunits, loss of essential lipids or other hydrophobic co-factors. As an example,  
34 cytochrome *b<sub>6</sub>f* inactivation and monomerization was related to lipid loss [3]. Detergent  
35 dynamics, *i.e.* detergent exchange between the free monomers, or between micelles, and  
36 bound detergent at the surface of the transmembrane protein surface, could trigger transient  
37 exposure of this hydrophobic surface leading to irreversible protein aggregation. Detergent  
38 binding can also alter protein structure: molecular dynamics simulation indeed showed, for a  
39 thermostable mutant of the adenosine receptor, that the harsh octylglucoside detergent  
40 molecules intercalate between trans-membrane helices, moving them apart in the 200 ns  
41 simulation [4]. The structure and dynamics of different  $\alpha$ -helical membrane proteins  
42 solubilized in alkyl phosphocholines appear deeply altered [5]. For example, NMR, molecular  
43 dynamics simulations, and functional studies, amongst other techniques, show that  
44 mitochondrial carriers are incorrectly folded in these detergents. Some parts of the  
45 transmembrane segments are disordered, with molecules of detergent penetrating between the  
46 helices; the proteins are in a highly dynamic state with unstable tertiary contacts; and weak  
47 ligand binding results from non-specific interactions [5]. In the challenging aim to obtain high  
48 resolution structure of membrane proteins by crystallography, a usual strategy is to select

49 detergents that not only preserve membrane protein stability, function and solubility, but also  
50 form small micelles (*e.g.* by adding compounds decreasing the micelle size), in order to favor  
51 protein-protein contacts [6,7].

52  
53 The propensity of detergents to inactivate membrane proteins drove the search for new  
54 amphiphilic environments (for a review, see [6,8,9]). Peptides that form an  $\alpha$ -helix or a  $\beta$ -  
55 sheet [10–13], amphipatic polymers with multiple hydrophobic tails [14,15], fluorinated  
56 surfactants (reviewed in [8,16,17]), Calixarene based detergents (*e.g.* [18]) or detergents with  
57 varying architectures: tripod amphiphiles (*e.g.* [19,20]), facial amphiphiles [21–23],  
58 polycyclic based [24], bi-tails [25–29], tri-tails [30], and other compounds [31–33] have been  
59 synthesized to decrease their exchange kinetics, and/or reinforce interactions with membrane  
60 protein surfaces. This is done noticeably by increasing the rigidity as well as the surface of  
61 their hydrophobic moieties. Among these compounds, neopentyl glycols (MNG) constitute a  
62 class of detergents with notable interest [25,26]. Members with different chain lengths or with  
63 different sugar head groups have been described and are commercialized (Anatrace). We will  
64 focus here on the most commonly used one, lauryl maltose neopentyl glycol (LMNG), also  
65 named MNG3 (MNG3, however, designates the related family of compounds in [26]). Its  
66 structure (Scheme 1) is formed by two molecules of n-dodecyl- $\beta$ -D-maltopyranoside (DDM)  
67 linked at the junction between the hydrophilic and hydrophobic moieties of the molecule. The  
68 presence of the two hydrophobic chains of equal length mimics better the structure of lipids  
69 than classical detergents having a single hydrophobic tail. According to the authors who  
70 designed this detergent in 2010, the central quaternary carbon, was “intended to place subtle  
71 restraints on conformational flexibility” [26]. In this work, LMNG was shown to display a set  
72 of very interesting features: to extract integral membrane proteins from membranes; to  
73 improve substantially the stability of various membrane proteins, including G protein-coupled  
74 receptors and respiratory complexes. It also allowed diffraction up to 3.4 Å resolution of  
75 LMNG-solubilized cytochrome *b<sub>6</sub>f* crystals obtained by vapor diffusion [26]. Shortly after this  
76 first publication, high resolution structures of the challenging  $\beta$ 2 adrenergic receptor, in  
77 complex with G-proteins [34] or with covalently bound agonist [35], were obtained. The  
78 proteins were solubilized and purified in LMNG followed by crystallization in mesophase.  
79 LMNG was then described as successfully used for various applications, including membrane  
80 solubilization and membrane protein stabilization (*e.g.* [26,36–40] ), excited state  
81 intermediates [37,41–44], NMR studies [44], H/D exchange coupled to mass spectroscopy  
82 (*e.g.* [45,46]), negative stain electron microscopy (*e.g.* [43,46,47]), or reconstitution in lipid  
83 vesicles [48]. Impressively, LMNG has now been used to solve the structure of more than ten  
84 G-protein coupled receptors (*e.g.* refs in [49]; in all cases, LMNG was used for protein  
85 purification and crystallization was performed in mesophase). Other proteins, *e.g.* ABC  
86 transporters [41,50,51] or channels [52], were purified and crystallized in LMNG by vapor  
87 diffusion. Furthermore, LMNG was also successfully used to solve the high resolution  
88 structures of membrane proteins by single particle cryo-electron microscopy (*e.g.* [47,53–  
89 55]).

90  
91



131 SANS), Analytical Ultracentrifugation Sedimentation Velocity (AUC-SV) and Size exclusion  
132 chromatography coupled to light scattering (SEC-LS) and dynamic light scattering (DLS). We  
133 showed that, although LMNG alone forms increasingly large assemblies with increasing  
134 concentration, the protein-LMNG complexes are of comparable size to those formed with  
135 other detergents, with overall the same weight amount of bound detergent, and the oligomeric  
136 state of each protein remains unchanged. Furthermore, FhuA solubilized in LMNG showed an  
137 overall increased number of hits when screened for crystallization, suggesting that the large  
138 size of LMNG micelles does not prevent protein crystallization.

139

## 140 **Materials and method**

141

### 142 **Chemicals and buffers**

143 Detergents are from Anatrace, except octyl- $\beta$ -D-thioglucopyranoside (OTG) from Acros,  
144 other chemicals are typically from Sigma-Aldrich.

145 Buffer A : 50 mM Tris-HCl pH 7.0, 300 mM NaCl

146

### 147 **FhuA purification**

148 FhuA was purified from the *E. coli* strain AW740 transformed with a plasmid encoding the  
149 *fhuA* gene in which a 6xHis.tag has been inserted in the extracellular loop L5 [56]. Cells were  
150 grown in LB medium at 37 °C in the presence of 100 mM of the iron-chelating agent  
151 dipyrindine, and broken with a microfluidizer (10 passages at 15 kpsi) in a 20 mM Tris-HCl  
152 pH 8, 150 mM NaCl buffer with a pinch of DNase I. Unbroken cells were removed by a first  
153 centrifugation (15 min, 6000 rpm, rotor SX4250), and the membrane fraction was recovered  
154 by ultracentrifugation (20 min, 35 000 rpm, 45Ti). A first solubilization with 2 % OPOE  
155 (octylpolyoxyethylene), 20 mM Tris-HCl pH 8.0, 30 min at 37 °C under gentle shaking  
156 solubilized the inner membranes, and the outer membrane pellet (20 min, 35 000 rpm, 45Ti)  
157 was solubilized in 1 % lauryldimethylamine-N-oxide (LDAO), 20 mM Tris-HCl pH 8, 1 mM  
158 EDTA for 1 h at 37 °C. Insoluble material was removed (20 min, 35 000 rpm, 45Ti). The  
159 protein was purified by Nickel affinity chromatography (HiTrap Chelating, 5 mL, GE  
160 Healthcare). The LDAO supernatant was supplemented with 5 mM MgCl<sub>2</sub> and 5 mM  
161 Imidazole, loaded onto the column and thoroughly washed (20 mM Tris-HCl pH 8, 150 mM  
162 NaCl, 0.1 % LDAO). A delipidation step was achieved by washing with 10 mL of the same  
163 buffer containing 1% LDAO. The protein was eluted with 20 mM Tris-HCl pH 8.0, 200 mM  
164 Imidazole, 0.1 % LDAO. Fractions containing the protein were pooled and loaded onto an  
165 anion-exchange chromatography (HiTrap Q, 1 mL, GE Healthcare) equilibrated with 20 mM  
166 Tris-HCl pH 8.0, 0.05 % LDAO and the protein was eluted by a linear gradient of NaCl in 20  
167 mM Tris-HCl pH 8, 0.05 % (2.2 mM) LDAO (final NaCl concentration ~150 mM). The  
168 concentration of FhuA was determined using  $\epsilon_{280\text{ nm}} = 103,690\text{ M}^{-1}\text{ cm}^{-1}$ .

169

### 170 **FhuA detergent exchange**

171 For LDAO - LMNG exchange, the protein sample was diluted 50 times with water, to reach a  
172 detergent concentrations far below the LDAO CMC. Protein aggregation was checked by  
173 spectrometry (scattering), and the protein was recovered as a pellet by ultracentrifugation (20  
174 min, 35 000 rpm, 45 Ti or 20 min, 80 000 rpm TLA 100.3 depending on the volume). The  
175 protein pellet was rinsed with water, and resuspended in 11.9 mM LMNG, 20 mM Tris-HCl  
176 pH 8.0 to reach a final protein concentration of 7-10 mg mL<sup>-1</sup>. A different protocol is  
177 described below for the thermal denaturation assays.

178

### 179 **FhuA crystallization**

180 Crystallization screening was carried out using commercial screens (Qiagen and Hampton  
181 Research). Sitting drops, consisting of 100 nL protein and 100 nL crystallization buffer, were

182 dispensed in 96-well plates (Greiner Crystal Quick plates) using a Cartesian PIXSYS 4200  
183 robot (Genomic Solutions) and equilibrated at 20 °C against 100 mL of crystallization buffer.  
184 Hits were then manually reproduced and improved using the vapour diffusion hanging drop  
185 technique. The drops, consisting of 0.8 mL protein and 0.8 mL crystallization buffer, were  
186 equilibrated against 250 mL of crystallization buffer at 20 °C in 48-well plates (Hampton  
187 Research). Crystals were transferred to the crystallization buffer supplemented with 20% (v/v)  
188 glycerol for 30 s, flash-cooled and stored in liquid nitrogen.

189

### 190 **FhuA LMNG data processing**

191 The final data set was collected at 4 Å on beamline FIP-BM30A at the European Synchrotron  
192 Radiation Facility, Grenoble, France [57]. Data reduction was performed using the XDS  
193 program [58]. The space group was C222<sub>1</sub> (a = 149.53 Å, b = 210.96 Å, c = 188.70 Å) with  
194 two molecules per asymmetric unit (solvent content of 73 %). The structure was solved by  
195 molecular replacement performed with the Phaser program [59] using the model of the *E. coli*  
196 protein available in the Protein Data Bank (code 2FCP). A refinement was carried out with  
197 the *phenix.refine* program [60,61] in the 48-4 Å resolution range.

198

### 199 **SpNOX production**

200 SpNOX was overexpressed and purified in typically 0.025 mM LMNG, as previously  
201 described [62]. When using DDM as the detergent, the same purification protocol was used  
202 but with adjustments for DDM concentrations in the purification buffers corresponding to  
203 each step. Indeed, the solubilization step was performed using 5.1 mM DDM, the loaded Ni-  
204 HisTrap column was washed in the corresponding buffer containing 2 mM DDM, and eluted  
205 with 0.3 mM DDM in the elution buffer. Final size exclusion was performed in 50 mM Tris-  
206 HCl pH 7.0, 300 mM NaCl, 0.3 mM DDM.

207

### 208 **BmrA production**

209 Typical bacterial culture protocol: pET23b-*bmrA* [63] was used to transform C41(DE3) strain  
210 of *E. coli*. For preculture, 200 mL LB medium containing 100 µg mL<sup>-1</sup> ampicillin was  
211 inoculated with a freshly transformed colony and growth allowed overnight at 37 °C and 180  
212 rpm. The next morning, enough preculture was added into 1 L 2X YT medium, containing 100  
213 µg mL<sup>-1</sup> ampicillin, to get an optical density of 0.1, and growth then allowed at 37 °C 180  
214 rpm. When the optical density reached 0.6, the culture was induced for the overexpression of  
215 BmrA for 4 h at 25 °C and 180 rpm by the addition of 0.7 mM IPTG (final concentration).  
216 Thereafter, the cells were harvested by centrifugation at 4,000 g at 4 °C for 20 min and the  
217 bacterial pellet was frozen at -80 °C until further use. Inverted Membrane Vesicles  
218 preparation : The bacterial pellet was resuspended in 50 mM Tris-HCl pH 8, 5 mM MgCl<sub>2</sub>, 1  
219 mM dithiothreitol, with an anti-protease cocktail from Roche. The bacterial cells were lysed by  
220 passing them thrice through Microfluidizer<sup>TM</sup> at 18,000 psi. Next, the cellular debris was  
221 removed by centrifugation at 15,000 g for 30 min at 4 °C. The membrane fraction was  
222 subsequently obtained by centrifugation at 150,000 g for 1 h at 4 °C. The membrane fraction  
223 was resuspended and centrifuged again, with the same conditions as before, after resuspension  
224 in 50 mM Tris-HCl pH 8, 1.5 mM EDTA, 1 mM dithiothreitol. Finally, the obtained membrane  
225 vesicles were resuspended in 20 mM Tris-HCl pH 8, 300 mM sucrose, 1 mM EDTA and flash  
226 frozen in liquid nitrogen before storage at -80 °C. The total protein concentration in  
227 membrane was analysed by BCA assay. BmrA purification: BmrA enriched Inverted  
228 Membrane Vesicles were solubilized for 1 h at 4 °C in 50 mM Tris-HCl pH 8, 10% glycerol,  
229 100 mM NaCl, with 1% detergent (LMNG or DDM). The soluble fraction obtained after  
230 ultra-centrifugation, at 150,000 g for 1 h at 4 °C, was injected into a 1 mL HisTrap HP  
231 column (GE Healthcare) which was pre-equilibrated with the same buffer with 20 mM  
232 imidazole, and 0.1% (2 mM) DDM or 0.01% (0.1 mM) LMNG. The column was washed with

233 20 column volumes of the same buffer. Gradient elution was eventually performed with the  
234 same buffer with imidazole gradient from 20 mM to 500 mM. The protein fractions were  
235 dialyzed overnight at 4 °C against 50 mM Hepes pH 8, 10% glycerol, 50 mM NaCl, 2 mM  
236 DDM or 0.1 mM LMNG. The protein concentration, 2.2 mg mL<sup>-1</sup> for both BmrA in 2 mM  
237 DDM and BmrA in 0.1 mM LMNG, was determined from UV absorbance at 280 nm by using  
238 Nanodrop spectrophotometer, and a  $\epsilon_{280\text{ nm}} = 38850\text{ M}^{-1}\text{ cm}^{-1}$ . Reconstitution of BmrA in  
239 proteoliposomes was performed as previously described [64].

240

#### 241 **Limited Proteolysis of BmrA**

242 Apo form: Purified BmrA in detergent was added to buffer 50 mM Hepes-KOH pH 8, 50 mM  
243 NaCl, 10% glycerol and the specified detergent, in case of trypsin, or 100 mM ammonium  
244 bicarbonate pH 7.8 with the specified detergent, in case of protease V8. After 15 min of  
245 incubation, either trypsin (1 µg/250 µg of protein) or protease V8 (1 µg/20 µg of protein) was  
246 added. Samples of 10 µL (5 µg BmrA) were withdrawn at 0, 2, 5, 15, 30, 60, 120, 180 and  
247 300 minutes. 2.5 µL of TFA 5% was added immediately to each sample to stop the reaction. 3  
248 µL of Laemmli 5x was then added and the samples were placed in ice before resolving them  
249 on SDS-PAGE. Vanadate-inhibited form: For the Vi-inhibited form, before the addition of  
250 purified BmrA, the buffer was supplemented with 3 mM MgCl<sub>2</sub>, 2 mM ATP and 1 mM Vi.  
251 The samples were then incubated for 15 min before the addition of protease. Samples of 10  
252 µL were withdrawn and treated thereafter in the same way as the apo form.

253

#### 254 **bR solubilization and detergent exchange by sucrose gradient**

255 Purified purple membrane was solubilized for 40 h at 4 °C with 89 mM OTG (CMC = 9 mM)  
256 at a membrane concentration of 1.5 g L<sup>-1</sup> in 20 mM sodium phosphate buffer, pH 6.8.  
257 Samples were diluted to reach a final OTG concentration of 15 mM, supplemented with  
258 2 mM of the surfactant to be tested, and incubated 15 min prior to being loaded onto a 10–30  
259 % (w/w) sucrose gradient containing 20 mM sodium phosphate buffer pH 6.8 and 0.2, 2 or  
260 20 mM LMNG. A control gradient contained 1 mM DDM. Gradients were centrifuged for  
261 15 h at 55,000 rpm (200,000 g) in the TLS55 rotor of a TL100 ultracentrifuge (Beckman).  
262 Bands containing the colored protein were collected with a syringe, and protein samples were  
263 kept at 4 °C in the dark for UV-visible spectrophotometry. To check for detergent exchange,  
264 10 µl of each recovered bR band was deposited onto a Thin Layer Chromatography (TLC)  
265 silica plate (Macherey-Nagel, ref. 818423), and migration performed in a  
266 Chloroform/Methanol/Water solvent (65/35/5). After drying, the plate was first stained with  
267 iodine vapour and then with orcinol (0.1 % orcinol in 3 % H<sub>2</sub>SO<sub>4</sub> and 72.5% ethanol) and  
268 charring at 120 °C.

269

#### 270 **Thermal denaturation and activity assays**

271 Thermal unfolding experiments and analysis were performed by differential scanning  
272 fluorimetry coupled to back scattering using a Prometheus NT.48 instrument (Nanotemper  
273 Technologies, Munich, DE), and the provided software PR.thermocontrol v2.0.4. Up to 48  
274 capillary containing 10 µL of sample are sequentially illuminated at 280 nm, and fluorescence  
275 intensity at 350 (F350) and 330 (F330) nm, and back scattering measured as a function of  
276 temperature. The derivatives of F350/F330 and of the back scattering were used to estimate  
277 the melting temperature,  $T_m$ , and the onset of aggregation,  $T_{agg}$ , respectively. For FhuA :  
278 samples were prepared by diluting 48 times a stock sample of 1.92 mg mL<sup>-1</sup> FhuA, in 2.2 mM  
279 LDAO, 20 mM Tris pH 8.0, ~150 mM NaCl, into 20 mM Tris pH 8.0 with the appropriate  
280 detergent, leading to a final residual LDAO concentration of  $\approx 0.05$  mM (0.05 CMC), with  
281 FhuA at 0.04 mg mL<sup>-1</sup>. For SpNOX : samples were prepared after purification in LMNG, and  
282 eventual detergent exchange in DDM, and concentration. Buffer is 50mM Tris HCl pH 7.0,  
283 300 mM NaCl, 10 µM FAD, with 0.025 mM LMNG or 0.2 mM DDM initial concentrations,



284 0.21 LMNG and 1.27 mM DDM final estimated concentrations, considering co-concentration  
285 of protein and detergent, and SpNOX at 8.3 mg mL<sup>-1</sup>, in the  $T_m$  assays. For activity assays,  
286 SpNOX was diluted to 1 µg mL<sup>-1</sup> in 50 mM Tris HCl pH 7.0, 300 mM NaCl, 10 µM FAD ,  
287 with 0.025 mM LMNG or 0.2 mM DDM. Activity assays were performed in the presence of  
288 100 µM Cytochrome C, 10 µM FAD, 200 µM NADPH, following Cytochrome C absorbance  
289 at 550 nm. For BmrA : for  $T_m$  measurements, samples were prepared at a final protein  
290 concentration of 1 mg mL<sup>-1</sup>, from dilution of the purified protein in 50 mM Hepes pH 8, 10%  
291 glycerol, 50 mM NaCl, with 2 mM DDM or 0.1 mM LMNG. For Vi-inhibited form of BmrA,  
292 the dilution buffer also contained 10 mM ATP, 10 mM MgCl<sub>2</sub> and 1 mM Vi (all final  
293 concentrations). BmrA samples were incubated for 15 min at room temperature before  
294 allowing them to be filled in the capillaries for Nano DSF analysis. The activity assays of  
295 BmrA were done in a quartz cuvette with a final volume of 700 µL. The buffer, containing 50  
296 mM Hepes/KOH pH 8, 10 mM MgCl<sub>2</sub>, 4 mM phosphoenolpyruvate, 0.3 mM NADH, 32 µg  
297 mL<sup>-1</sup> lactate dehydrogenase, 60 µg mL<sup>-1</sup> pyruvate kinase, 10 mM ATP and either 2 mM DDM  
298 or 0.1 mM LMNG, was added into the cuvette and was allowed to attain the desired  
299 temperature i.e. 37 °C for 5 min, before adding 3 µg protein, and measuring the absorbance at  
300 340 nm for 20 min at 37 °C. For bR : to perform  $T_m$  measurements, after the solubilization  
301 step (see above), solubilized bR was incubated 40 mins at 4 °C in the presence of biobeads  
302 (10 g/g OTG), that reduced the concentration of OTG to 70 mM estimated from TLC, from  
303 the comparison with OTG aqueous solution at 10, 25, 50, 75 and 100 mM. TLC was  
304 performed as described above in bR preparation, but the mobile phase was  
305 Chloroform/Methanol 2/1, and the volume deposited was 1 µL. Imaging and quantification  
306 were done with the Gel Doc XR system and Image lab software (Biorad). The bR was then  
307 diluted 18 times in 0.1 M NaCl, 0.02 M Na phosphate buffer supplemented with the  
308 appropriate detergent, residual OTG concentration being estimated as 0.4 its CMC, and final  
309 bR concentration to 0.14 mg mL<sup>-1</sup>. In the thermal denaturation assays, the temperature was  
310 increased by 1 (FhuA, bR, SpNOX) or 0.5 (BmrA) °C/min from 15 °C (FhuA, bR, SpNOX)  
311 or 20 °C (BmrA) up to 95 °C.

312

### 313 **Density measurements**

314 31.8 mg of dried LMNG was dissolved in 1.99948 g of water. From the density of water  
315 (0.998205 +/- 0.000001 g mL<sup>-1</sup>) and sample (1.001416 +/- 0.000002 g mL<sup>-1</sup>) determined from  
316 triplicate measurements at 20 °C using a DMA 58 density meter (Anton Paar, Graz, Austria),  
317 we derived [65] a LMNG concentration of 15.68 mg mL<sup>-1</sup> and a partial specific volume  $\bar{v} =$   
318 0.797 mL g<sup>-1</sup>.

319

### 320 **AUC-SV**

321 AUC-SV experiments were conducted in an XLI analytical ultracentrifuge (Beckman, Palo  
322 Alto, CA) using an ANTi-50 rotor, using double channel Ti centre pieces (Nanolytics,  
323 Germany) of 12, 3, or 1.5 mm optical path length equipped with sapphire windows, with the  
324 reference channel being typically filled with solvent without detergent. For BmrA, LMNG  
325 was in the buffer in the reference channel, and we used double sector capillary type cells  
326 (Beckman, Palo Alto, CA) allowing perfect matching of the channel heights upon  
327 centrifugation 90 min at 7,000 rpm (3,600 g). Acquisition was done at 42,000 rpm (130,000  
328 g), overnight, using absorbance (280 nm, and additionally for SpNOX, 412nm) and  
329 interference detection. Data processing and analysis was done using the program SEDFIT  
330 [66] from P. Schuck (NIH, USA), REDATE [67] and GUSSE [68] from C. Brautigam (USA),  
331 and using standard equations and protocols described in [65,69–71]. LMNG samples in water  
332 were prepared from precise weight dilution from a stock solution at 9.88 mM. The  
333 sedimentation velocity profiles for LMNG at 23.2 and 35 mM in Buffer FhuA showed an

334 optical artefact, and  $s$ -values were determined from  $(r_b/r_m)=s\omega^2t$ , with  $r_b$  the radial position at  
335 half the plateau signal,  $r_m$  the meniscus position,  $\omega$  the angular velocity, and  $t$  the time.  
336 Corrected  $s$ -values for solvent density and viscosity,  $s_{20w}$ , are calculated, for membrane  
337 proteins, considering for their partial specific volume, the mean value between protein and  
338 detergent.

339

### 340 **Dynamic light scattering (DLS) of LMNG**

341 LMNG at 20 mM in water was measured using the DynaPro NANOSTAR (Wyatt, Santa-  
342 Barbara, USA) instrument, at room temperature, and analyzed with the associated software  
343 DYNAMICS.

344

### 345 **SEC-LS**

346 SEC-LS experiments were conducted at 4 or 6 °C on a HPLC consisting of a degasser DGU-  
347 20AD, a LC-20AD pump, an autosampler SIL20-AC<sub>HT</sub>, a communication interface CBM-  
348 20A and a UV-Vis detector SPD-M20A (Schimadzu, Kyoto, Japan), a column oven XL-  
349 Therm (WynSep, Sainte Foy d'Aigrefeuille, France), a static light scattering miniDawn Treos,  
350 a dynamic light scattering DynaPro NANOSTAR, and a refractive index Optilab rEX  
351 detectors. The analysis was made with the software ASTRA, v5.4.3.20 (Wyatt, Santa-  
352 Barbara, USA). Samples of 10 to 60  $\mu\text{L}$  were injected at  $0.5 \text{ mL min}^{-1}$  on a Superdex 200  
353 10/300 GL (GE Healthcare), equilibrated with the elution buffer. Bovine serum albumin at  
354  $2 \text{ mg mL}^{-1}$  in PBS buffer was injected as a control in each experiment. The extinction  
355 coefficient and refractive index increments for the proteins were calculated from the amino-  
356 acid sequences using the program SEDFIT described above. For LMNG, we used  
357  $\partial n/\partial c=0.146 \text{ mL g}^{-1}$  determined by AUC.

358

### 359 **SAXS and SANS samples**

360 A first series of SAXS measurements was carried out with 14 samples of LMNG in  $\text{H}_2\text{O}$ , at  
361 concentrations between 0.31 and 20 mM, and a second series, measured by SAXS and SANS,  
362 with four samples of LMNG in  $\text{H}_2\text{O}$  and in  $\text{D}_2\text{O}$ , at 20 and 10 mM. SANS contrast variation  
363 determination was achieved for LMNG at 20 mM in 25 mM Tris pH 8, 150 mM NaCl, 10%  
364 glycerol, with different  $\text{D}_2\text{O}$  %s. LMNG concentrations were precisely determined using  
365 weighing-controlled solubilization and dilutions.

366

### 367 **SAXS experiment**

368 SAXS experiments were conducted on the BM29 beamline at the European Synchrotron  
369 Radiation Facility (Grenoble, France). The data were recorded for  $0.004 < Q < 0.5 \text{ \AA}^{-1}$   
370 ( $Q=(4\pi/\lambda)\sin\Theta$  is the modulus of the scattering vector, with  $2\Theta$  being the scattering angle,  
371 and  $\lambda$  the wavelength), using a two-dimensional 1M Pilatus detector, at 20 °C, with a  
372 monochromatic X-ray beam with  $\lambda = 0.9919 \text{ \AA}$  and a sample to detector distance of 2.864 m.  
373 Measurements were performed with 50  $\mu\text{L}$  loaded sample, in a quartz capillary, with a  
374 continuous flow. In the first series, 10 acquisitions with 1 s irradiation per acquisition (flow of  
375  $2.7 \mu\text{L/s}$ ), in the second series, 10 and 30 acquisitions with 0.5 s irradiation (flows of 5 and 1  
376  $\mu\text{L/s}$ ), were recorded for the samples and the solvents. The scattering curves were  
377 indistinguishable with the two flows. Data reduction was performed using the automated  
378 standard beamline software (BSxCuBE) [72], and data processing, including the elimination  
379 of data suffering from radiation damage, averaging, buffer subtraction, Guinier plots, using  
380 PRIMUS of the software suite ATSAS [73]. Absolute scales were obtained using the  
381 scattering of water.

382

### 383 **SANS experiment**

384 SANS experiments were carried out on the small-angle diffractometer D22 at the Institut  
 385 Laue Langevin (Grenoble, France), at 20 °C, using neutron wavelengths  $\lambda$  of 6 and 12 Å, with  
 386 samples measured in Hellma quartz cells 100QS with 1-mm optical path length. Scattering  
 387 data for LMNG at 10 and 20 mM in H<sub>2</sub>O and D<sub>2</sub>O were recorded at 20 °C for  $0.003 < Q <$   
 388  $0.45 \text{ \AA}^{-1}$ , using a neutron wavelength  $\lambda$  of 6 Å, and sample-detector/collimation distances (SD  
 389 / coll) of 17.6 m / 17.6 m, 5.6 m / 5.6 m, and 1.4 m / 2.8 m for 15, 5 and 5 mins, respectively.  
 390 For LMNG at 20 mM in D<sub>2</sub>O, additional data were recorded at (SD/coll) of 17.6 m / 17.6 m at  
 391 12 Å, leading to a total  $Q$ -range of 0.0012-0.45 Å<sup>-1</sup>. Transmissions were measured at 17 m for  
 392 1 min. For the determination of the contrast match point, samples were measured at 10 °C,  
 393 with SD / coll of 5.6 m / 5.6 m, 1.4 m / 2.8 m, for 5 and 1 min. respectively, and transmissions  
 394 were measured at 5.6 m for 1 min. LMNG at its match point, in 21.4 % D<sub>2</sub>O, was measured at  
 395 15 °C, with SD / coll of 8 m / 8 m and 1.4 m / 2 m for 60 and 15 min, respectively. Data  
 396 reduction to obtain scattering curves in absolute scale, and manipulation -merging, buffer  
 397 subtraction- were done in the usual way (see *e.g.*[74]) using Grasp (a Matlab<sup>TM</sup> script  
 398 application produced by ILL) and SANS reduction macros provided by NIST Center running  
 399 on IGOR (Wavemetrics)[75]. Absolute intensities are obtained using the direct beam  
 400 measurement.

401

#### 402 **SAXS and SANS Guinier analysis**

403 The radii of gyration ( $R_g$ ) and the intensities scattered in the forward direction ( $I(0)$ ) were  
 404 extracted by the Guinier approximation, with  $R_g Q \leq 1.3$ . Aggregation number were derived  
 405 from the molar mass of LMNG micelle,  $M$  (g mol<sup>-1</sup>) obtained from the absolute forward  
 406 intensity,  $I(0)$  (cm<sup>-1</sup>), using  $M = (I(0)/c)N_A/(\partial\rho_{el}/\partial c)^2$ , in SAXS, and  $M = (I(0)/c)N_A/(\partial\rho_N/\partial c)^2$ ,  
 407 in SANS.  $N_A$  is Avogadro's number,  $c$  the concentration (g mL<sup>-1</sup>), and  $\partial\rho_{el}/\partial c$  and  $\partial\rho_N/\partial c$  (cm  
 408 g<sup>-1</sup>) the increment of electron and neutron, respectively, scattering length density per g of  
 409 LMNG.  $\partial\rho_{el}/\partial c = (\rho_{el} - \rho_{el}^o) r_{el} \bar{v}$ , with  $\rho_{el} = 4.105 \cdot 10^{23}$  (from composition and  $\bar{v}$ ) and  $\rho_{el}^o =$   
 410  $3.297 \cdot 10^{23}$  the numbers of electron per mL of LMNG and solvent, respectively, and  $\rho_{el} =$   
 411  $2.818 \cdot 10^{-13}$  cm the scattering length of an electron.  $\partial\rho_N/\partial c$  was calculated to be  $1.108 \cdot 10^{10}$  cm  
 412 g<sup>-1</sup> in H<sub>2</sub>O, and  $-3.509 \cdot 10^{10}$  cm g<sup>-1</sup> in D<sub>2</sub>O.

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#### 416 **SAXS and SANS combined analysis for LMNG shape**

417 SAXS and SANS data were analyzed using shape-dependent models in SASview [76]. The  
 418 scattered intensity from an homogeneous dispersion of particles is written as:

$$419 \quad I(Q) = N V^2 (\Delta\rho)^2 P(Q) S(Q)$$

420 Where  $N$  is the number density of particles,  $V$  their volume,  $\Delta\rho$  (cm<sup>-2</sup>) is the scattering  
 421 contrast,  $P(Q)$  is the inter particle form factor and  $S(Q)$  is the interparticle structure factor.

422 In the present case, SAXS and SANS data have been fitted simultaneously with a core shell  
 423 cylinder form factor and hard sphere potential with beta approximation using SASview  
 424 software. The cylinder form factor included size polydispersity on radius (fixed at 5 %) using  
 425 a gaussian distribution. The scale factor was fixed by the surfactant concentration. The head  
 426 groups SLD or ED were fitted using the same hydration level of the anhydrous polar head.  
 427 Radius, length and shell thickness of the cylinder were constrained and kept equal for X-ray  
 428 and neutrons. Fitting was performed using the optimizer DREAM which allows to set  
 429 simultaneous fits as well as proper error determination.

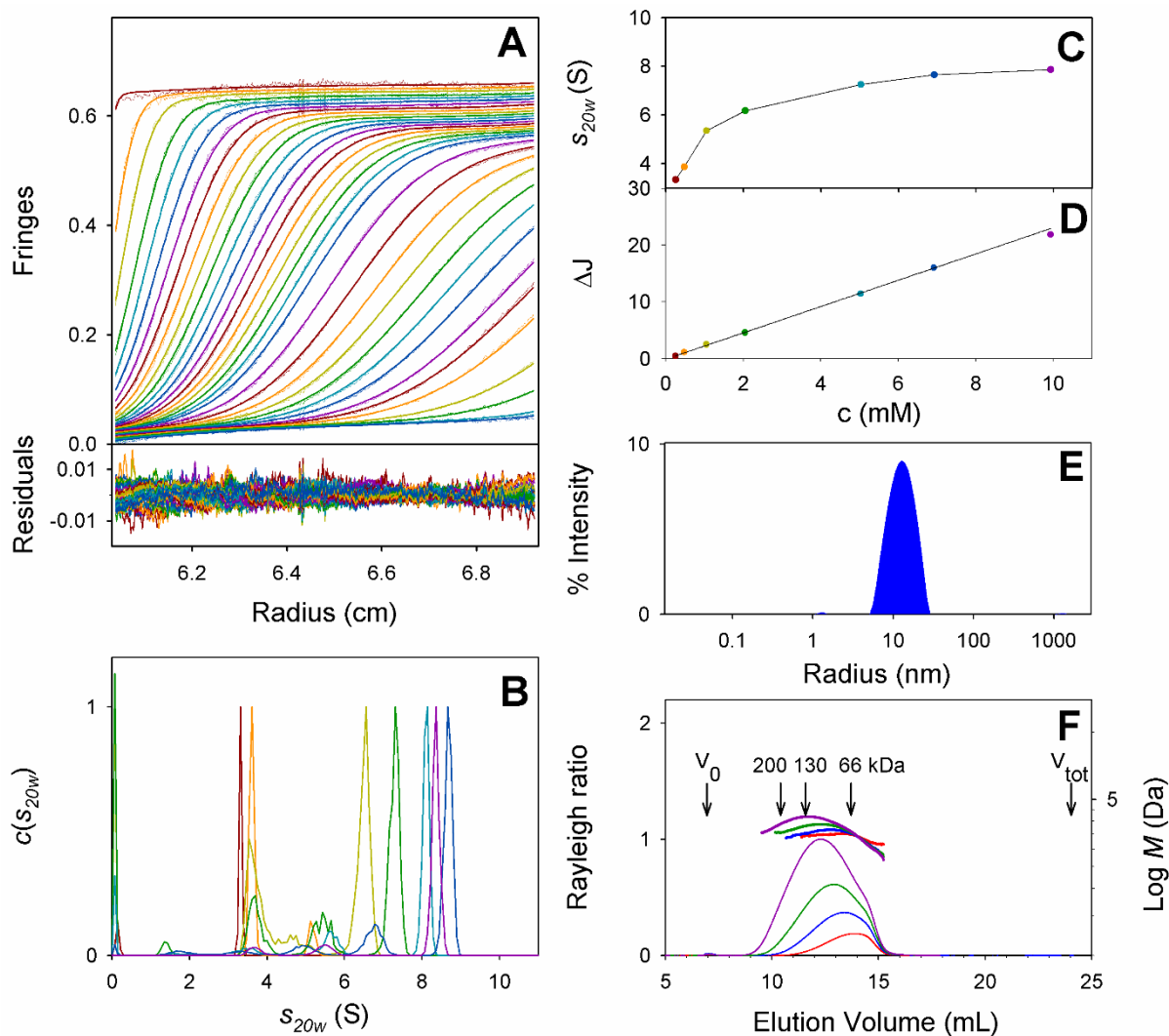
430

## 431 **Results**

432

### 433 ***Hydrodynamic characterization of LMNG***

434  
435 Our first aim was to characterize the assembly properties of LMNG molecules by itself.  
436 AUC-SV experiments were done for LMNG solubilized in water at different concentrations.  
437 The SV profiles and analysis in terms of continuous distributions  $c(s_{20w})$  of sedimentation  
438 coefficients,  $s_{20w}$ , showed a unique or main boundary. The mean  $s_{20w}$ - value increased with  
439 concentration, from 3.3 S at 0.25 mM to 7.9 S at 9.8 mM (Fig 1A-C). This indicates that  
440 LMNG micelle aggregation number increases with concentration, an atypical behaviour  
441 compared to usual detergents. Complementary AUC-SV in a dilute buffer (20 mM Tris-HCl  
442 pH 8.0, 150 mM NaCl), at larger LMNG concentrations of 23 and 35 mM, gave estimates for  
443  $s_{20w}$  of 7.7 and 8.7S, respectively. By integrating the signal for the micelles at the different  
444 LMNG concentrations (Fig 1D), we determine, in water, a refractive index increment for  
445 LMNG  $\partial n/\partial c=0.146 \text{ mL g}^{-1}$  (close to that of DDM,  $0.143 \text{ mL g}^{-1}$  [65]), and a CMC of  $40 \mu\text{M}$ .  
446 The knowledge of the partial specific volume,  $\bar{v}$ , of LMNG is required to correlate  $s_{20w}$  and  
447 molar mass, or aggregation numbers  $N_{agg}$ . We measured, with density measurement,  $\bar{v} = 0.797$   
448  $\text{mL g}^{-1}$ , a value close to that of DDM:  $0.82 \text{ mL g}^{-1}$  from [65] and  $0.81\text{-}0.837 \text{ mL g}^{-1}$  from [1].  
449  
450 Minimum aggregation numbers  $N_{agg}$  of 63 and 230 are calculated at 0.25 and 10 mM  
451 considering globular compact micelles (frictional ratio of 1.25), but  $N_{agg}$  are larger, at least for  
452 the larger micelles, since their shape is anisotropic (see below). Dynamic light scattering  
453 (DLS) of 20 mM LMNG in water showed essentially one population with a polydispersity  
454 index of 35% and a hydrodynamic radius,  $R_H$ , of 14 nm (Fig 1E), in line with the reported  
455 value of 7.2 nm at 5 mM [49]. Combining  $s_{20w}$  and  $R_H$  values at 5 and 20 mM provides  $N_{agg}$   
456 estimates of 300 and 620, corresponding to frictional ratios of 1.65 and 2.4, indicating  
457 moderately and strongly elongated shapes, respectively.  
458  
459 In SEC-LS experiments with 0.05 mM LMNG in the equilibration and elution buffer  
460 (bufferA), LMNG injected at 2, 5, 10 and 20 mM eluted as a very large peak covering the  
461 elution volumes of BSA monomer (65 kDa) to BSA trimer (Fig 1F). When LMNG  
462 concentration increased, the elution volume decreased. However, the molar masses  
463 determined from coupled refractive index and light scattering detections did not change  
464 significantly, neither along the elution, nor with the injected LMNG concentration: from 67  
465 kDa ( $N_{agg} = 67$ ) at 2 mM to 76 kDa ( $N_{agg} = 76$ ) at 20 mM (Fig 1F). This suggests the  
466 dissociation of the largest LMNG complexes upon sample dilution on the column.  
467



468  
 469 **Fig 1. LMNG in AUC-SV, DLS, and SEC-LS**  
 470 AUC-SV: **A:** Superposition of experimental and fitted sedimentation velocity profiles (top) and residuals  
 471 (bottom), obtained at 130,000 g and 20 °C during  $\approx$  5 hours using interference optics and 12 mm optical path  
 472 centerpiece, for LMNG at 0.25 mM in water. **B:** Superposition of the normalized  $c(s)$  distributions for LMNG at  
 473 0.25 (brown), 0.5 (orange), 1 (yellow-green), 2 (green), 5 (cyan), 7 (blue), and 10 (purple) mM. **C:** plot of the  
 474 mean sedimentation coefficient  $s_{20w}$  versus LMNG concentration. Note that the mean  $s_{20w}$  is obtained from the  
 475 integration of the  $c(s_{20w})$  over the whole  $s_{20w}$ -range; At 10 mM compared to 7 mM, as seen in Panel B,  $s_{20w}$   
 476 for the main species is decreased, while, as seen in Panel C, the mean  $s_{20w}$  is slightly increased. **D:** Fringe  
 477 number  $\Delta J$ , normalized to 1 cm optical path-length, for the micelle contributions vs total LMNG concentration,  
 478 and linear fit. DLS: **E:** Hydrodynamic radius distribution of LMNG at 20 mM in water. SEC-LS: **F:** Rayleigh  
 479 ratio measured along the elution profiles, for 30  $\mu$ L LMNG injected at 2, 5, 10, and 20 mM (red, blue, green, and  
 480 purple thin lines, respectively) on a Superdex 200 10/300 GL (GE Healthcare) column eluted with 50 mM Tris-  
 481 HCl pH 7.0, 300 mM NaCl, 0.05 mM LMNG, and molar masses (thick lines, right axis) derived from the light  
 482 scattering and refractive index detections.  $V_0$  is the dead volume,  $V_{tot}$  the total volume of the column, and the  
 483 arrows indicate the elution volumes of BSA as a monomer, dimer, and trimer (66, 130, and 200 kDa) from a  
 484 different injection.

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### 487 **Structural characterization of LMNG by small angle scattering**

488 SAXS and SANS experiments were performed to get additional information on the size and  
 489 shape of LMNG micelles. A first set of SAXS experiments were done with LMNG between  
 490 0.3 and 20 mM (0.03 and 2%) (Fig 2A). From the Guinier analysis, the size of the micelle  
 491 increases with  $R_g$  and aggregation numbers increasing from 3.4 nm to 10 nm, and from 55 to  
 492 240, respectively, when LMNG concentration is raised from 0.3 to 10 mM. (Fig 2B and 2C).

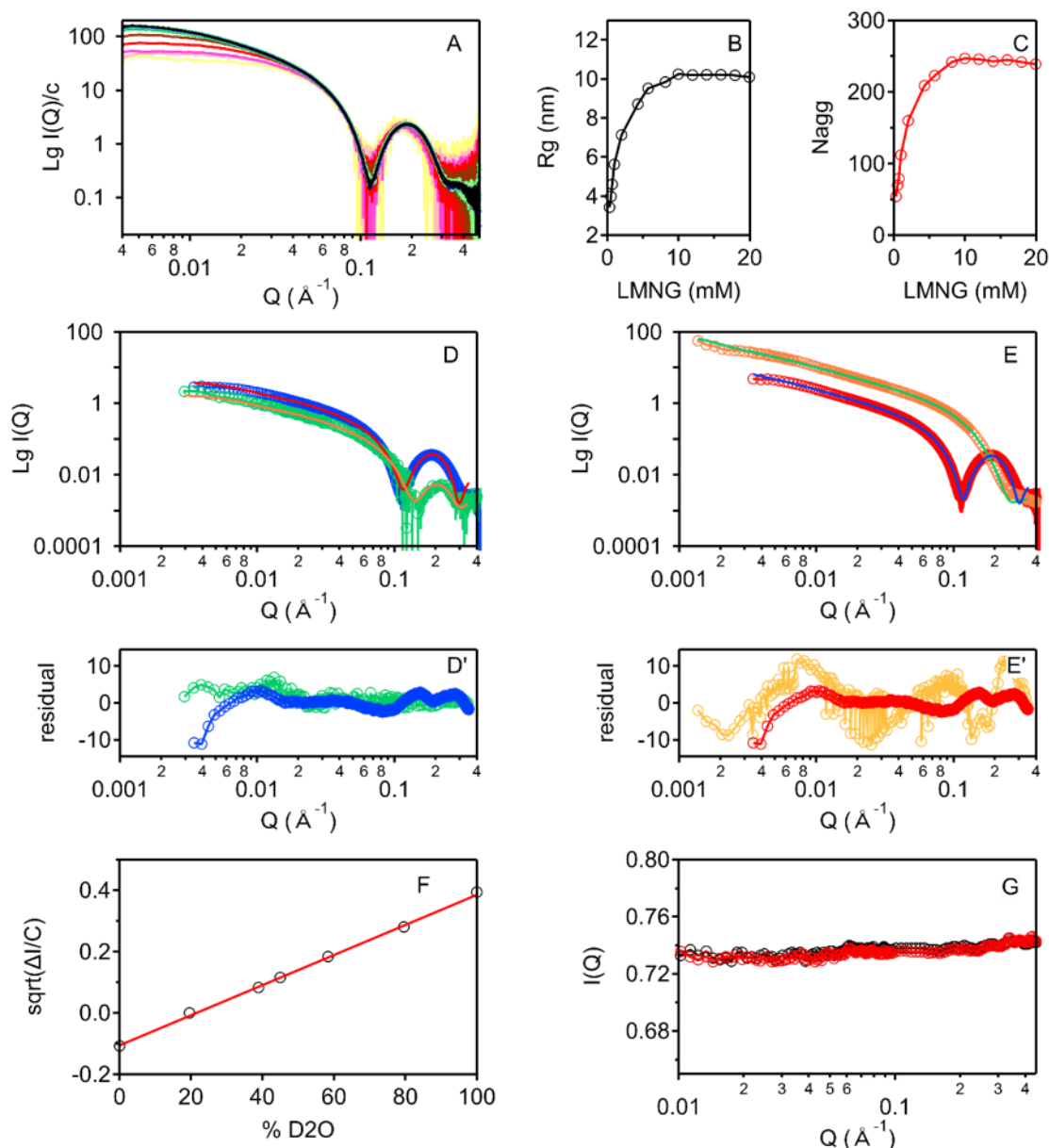
493 These values remain constant for LMNG concentrations increasing from 10 to 20 mM, and  
494 indeed the scattering curves are superimposed (Fig 2A). All curves are superimposed at the  
495 largest angles, representing the smallest distances in the real space, compatible with a circular  
496 cross section. To ascertain the shape of the LMNG micelles, 10 and 20 mM LMNG samples  
497 were measured by SAXS and SANS, in H<sub>2</sub>O and D<sub>2</sub>O. In the same buffer, the scattering  
498 curves normalized by concentration were superimposable for the 10 and 20 mM LMNG  
499 samples. From Guinier plots, the radii of gyration from SAXS were 11.5 and 11.6 nm at 10  
500 and 20 mM in H<sub>2</sub>O, and 17.7 and 17.2 nm at 10 and 20 mM in D<sub>2</sub>O. The aggregation numbers  
501 were 285 in H<sub>2</sub>O, and 528 in D<sub>2</sub>O. The  $R_g$  and aggregation numbers from SAXS, in H<sub>2</sub>O were  
502 slightly larger but similar to those of the first set of experiments. The size of the micelles  
503 appears significantly larger here for LMNG in D<sub>2</sub>O than in H<sub>2</sub>O, but, as will be discussed  
504 below, the significance of this difference is not ascertained.

505  
506 The SAXS and SANS scattering curves of 20 mM LMNG in H<sub>2</sub>O and D<sub>2</sub>O were analyzed  
507 globally. We used a model of a core shell cylinder with hard sphere interaction, defined by a  
508 core representing the hydrophobic tails (*i.e.* the hydrocarbon chains) covered by a shell  
509 representing the hydrated polar heads. The scattering length densities (SLD) of the core and of  
510 the solvent were fixed to the theoretical values. The SLD of the polar head (different in SAXS  
511 and SANS), and the dimensions of the micelles (radius of the cylinder core, thickness of the  
512 shell, and length, common in SAXS and SANS) were globally fitted keeping the same  
513 hydration level in X-ray and neutron. The results are presented in Table 1. The fitted values  
514 for the SLD of the anhydrous shell values are in the same order of magnitude as those  
515 calculated for the anhydrous polar head. We extracted reasonable hydration of 30 % in H<sub>2</sub>O  
516 and 44 % in D<sub>2</sub>O. The radius of the hydrophobic core, 14 Å, and that of the shell thickness, 7-  
517 9 Å, are in line with those expected from the LMNG size.

518  
519 Finally, we investigated whether LMNG could be totally matched out in SANS. This is  
520 required to be able to focus on protein contribution. The differential distribution of detergent  
521 hydrophobic tails and hydrophilic heads in the detergent micelles and bound detergent indeed  
522 corresponds to ordered scattering density fluctuations, which makes the scattering curve of  
523 the micelles and bound detergent possibly not flat even at the match point [77]. We thus  
524 measured for LMNG a set of contrast variation curves, using a solvent with different %s of  
525 D<sub>2</sub>O. We determined a contrast match point of 21.4 % D<sub>2</sub>O (Fig 2F). The scattering curve of  
526 the 2 mM LMNG sample in 21.4 % D<sub>2</sub>O perfectly superimposes that of the solvent (Fig 2G).  
527 Thus LMNG can be homogeneously masked. The analysis of the neutron scattering curves of  
528 membrane proteins solubilized in LMNG, in 21.4 % D<sub>2</sub>O will not require the fastidious step  
529 of detergent modelling to access membrane protein envelope structure. Because the contrast  
530 match point of hydrogenated proteins is  $\approx$  44 % D<sub>2</sub>O, membrane proteins should be deuterated  
531 to improve their scattering signal, but this is not problematic, since deuterating proteins, in  
532 general, is not an issue.

533  
534 To summarise, SAXS and SANS showed that LMNG forms large elongated rod-like micelles  
535 above the mM concentration. Given this observation, the question of the homogeneity of  
536 different membrane proteins at various LMNG concentrations was addressed below.

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**Fig 2. SAXS and SANS analysis of LMNG**

A: Superposition of 13 SAXS profiles obtained at 0.3 (yellow), 0.5 (pale pink), 0.7 (pink), 1 (red), 2 (brown), 6 (green), 8 (light green), 10 (light blue), 12 (blue), 14 (navy), 16 (purple), 18 (grey), and 20 (black) mM LMNG, in H<sub>2</sub>O, at 10 °C. B: corresponding  $R_g$ , and, C: aggregation numbers, versus LMNG concentrations. D,E: Superposition, for LMNG at 20 mM in H<sub>2</sub>O (D), and D<sub>2</sub>O (E) of the experimental scattering curves obtained by SAXS (blue and red dots, respectively) and SANS (green and orange dots, respectively), and of the fitted curves, in the model of a core-shell cylinder, in SAXS (red and blue lines in H<sub>2</sub>O and D<sub>2</sub>O, respectively), and in SANS (orange and green lines lines in H<sub>2</sub>O and D<sub>2</sub>O, respectively); D',E': respective normalized by data error residuals. SAXS and SANS data in each solvent were globally fitted (Table 1). F: Match point determination from SANS scattering curves obtained for 20 mM LMNG in 0, 20, 40, 45, 60, 80, and 100 % D<sub>2</sub>O. G: Superposition of raw scattering curves obtained in a solvent with 21.4 % D<sub>2</sub>O for LMNG at 2 mM (black) and of the solvent alone (red).

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**Table 1. Rod analysis of SAXS and SANS data for 20 mM LMNG**

	LMNG 20 mM in H <sub>2</sub> O		LMNG 20 mM in D <sub>2</sub> O	
	SAXS	SANS	SAXS	SANS
$Q$ range ( $\text{\AA}^{-1}$ )	0.0035-0.35	0.0030-0.35	0.0035-0.35	0.0015-0.35
$\chi^2_{\text{R}}$	3.2	3.2	2.3	26.7
Background, fixed ( $\text{cm}^{-1}$ )	0.0008	0.001	0.0012	0.0015
SLD solvent, fixed ( $10^{-6} \text{\AA}^{-2}$ )	9.4	-0.56	9.4	6.4
SLD core, fixed ( $10^{-6} \text{\AA}^{-2}$ )	8.3	-0.3	8.3	-0.3
SLD shell, fixed ( $10^{-6} \text{\AA}^{-2}$ )	14.75	1.88	14.75	3.99
SLD shell, fitted ( $10^{-6} \text{\AA}^{-2}$ )	13.12 $\pm$ 0.02	1.134*	12.42 $\pm$ 0.02	5.048*
Fitted shell hydration (%)	30		44	
Radius ( $\text{\AA}$ )**	14.51 $\pm$ 0.02		13.29 $\pm$ 0.01	
Thickness ( $\text{\AA}$ )	7.33 $\pm$ 0.04		9.25 $\pm$ 0.03	
Length ( $\text{\AA}$ )	603 $\pm$ 7		5376 $\pm$ 16	
Vol fraction	0.02 $\pm$ 0.00001		0.01951 $\pm$ 0.00006	

$\chi^2_{\text{R}}$ : reduced  $\chi^2$ . \*: linked parameter. \*\*: a polydispersity on radius width was fixed to 5%.

### ***FhuA-LMNG assemblies***

FhuA is an *E. coli* outer membrane ferrichrome-iron transporter involved in bacteriophage infection [78,79]. The structure of this robust  $\beta$ -barrel protein was solved in 1998 [80]. We investigated FhuA stability, homogeneity and association state at different LMNG concentrations, and whether the protein can crystallize in LMNG.

We first compared the thermal stability of FhuA in LDAO and in LMNG by DSF, allowing to measure the melting temperature ( $T_m$ ) of the protein by probing the fluorescence emission ( $F_{350\text{nm}}/F_{330\text{nm}}$  ratio) of the aromatic residues upon increasing temperature. Simultaneous light back-reflection measurements permit to detect qualitatively aggregation events. Detergent was exchanged by a 48 times dilution, leading to a final residual concentration of LDAO that was much below its CMC (20 times). The melting curves are similar whatever the final detergent concentration, corresponding to CMC + 0.2, 0.5 mM and 1-2 mM, *i.e.* LDAO at 1.2, 1.5, and 2.2 mM and LMNG at 0.21, 0.51 and 2.01 mM. They show (Fig 3A) two unfolding events, attributed to, first, the unfolding of the cork, and then of the barrel [81] (both domains contain tryptophans). From light back-reflection, the onset of protein aggregation coincides with the first  $T_m$ , and significant aggregation to the second  $T_m$ . In LDAO, the  $T_m$  at 63 and 69 °C are in line with the published values of 60-65 and 74-75 °C, from scanning calorimetry [81], or synchrotron radiation circular dichroism [78] in slightly different buffers with 1.3 or 4.4 mM LDAO. In LMNG, unfolding is shifted to higher temperatures. The first  $T_m$  is at 67 °C, showing a moderate stabilization (+ 4 °C) of the cork, the second one at 80 °C indicating a significant stabilization (+ 11°C) of the barrel.  $T_m$  values are reported in Table 2. For comparison, binding of ferrichrome, the natural ligand, was described to have a strong stabilizing effect on FhuA cork, shifting the first transition by 6 °C [81]; binding of phage T5 Receptor Binding Protein pb5 leads to one unique unfolding event at 89 °C (+ 15 °C vs  $T_m$  of barrel for FhuA alone) [78]. Thus, LMNG significantly stabilizes both the flexible parts and the rigid domain of this  $\beta$ -barrel protein.



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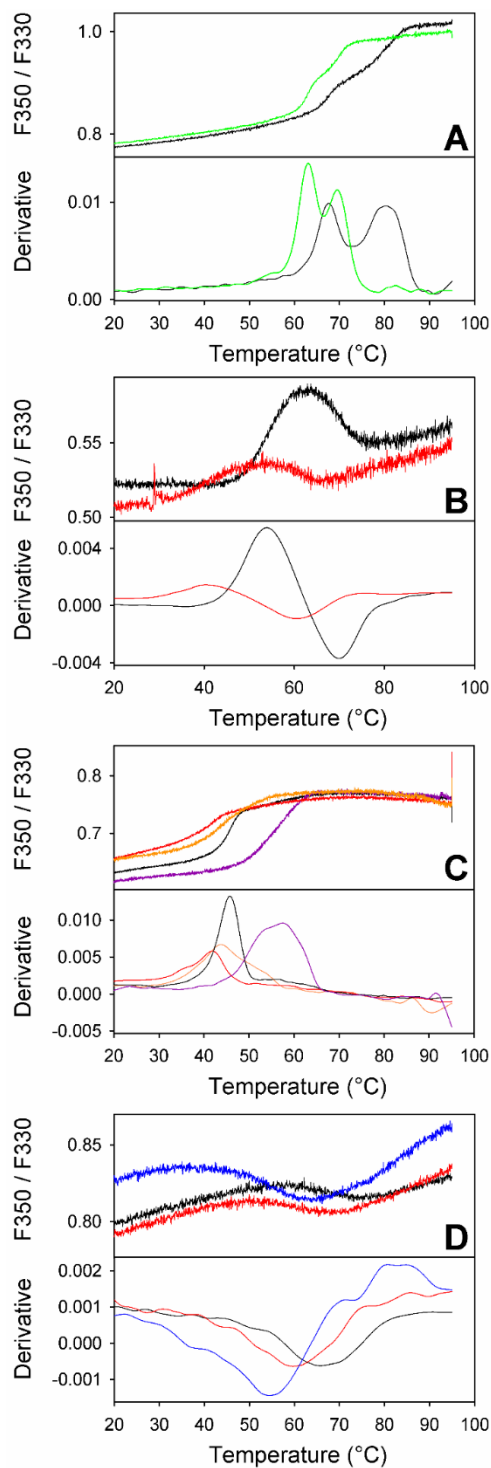
**Table 2. Melting temperatures of FhuA, SpNOX, BmrA, and bR**

Protein	Detergent type	detergent conc. (mM)	Micelle conc. (mM)	$T_m$ (°C)	$T_{agg}$ (°C)
FhuA	LDAO	1.2, 1.5, 2.2	0.2, 0.5, 1.2	63.0 + 69.0	65
FhuA	LMNG	0.21, 0.51, 2.0	0.2, 0.5, 1.0	67.0 + 80.0	74
SpNOX	DDM	1,27	1.1	40.5	56
SpNOX	LMNG	0.21	0.2	53.9	60
BmrA Apo	DDM	2	1.8	41.2	54
BmrA Apo	LMNG	0.1	0.1	45.7	70
BmrA Vi-inh	DDM	2	1.8	46.4	45
BmrA Vi-inh	LMNG	0.1	0.1	59.3	50
bR	OTG	11	2.0	53.6	43
bR	DDM	2.01	1.8	59.9	52
bR	LMNG	2.17	2.2	65.9	63

632  $T_m$ : melting temperature measured by differential scanning fluorimetry;  $T_{agg}$ : onset temperature for aggregation from light  
633 back reflexion. BmrA Vi-inh, Vi-inhibited forms were incubated with 10 mM ATP, 10 mM MgCl<sub>2</sub>, and 1 mM vanadate.  
634 Standard deviations of typically 0.2 were determined from triplicates experiments in BmrA, otherwise the precision on  $T_m$  is  
635 estimated at 1°C. Micelle concentrations (*i.e.* above the CMC concentrations) were calculated considering CMC-values of 1,  
636 0.01, 0.17 and 9 mM for LDAO, LMNG, DDM, and OTG, respectively.

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For FhuA, detergent exchange can easily be done, by dilution of the purified protein solution until FhuA precipitates and re-solubilization of the pellet in the desired detergent. The resulting sample in LMNG, also used for crystallization assays, contained  $\approx 7.5 \text{ mg mL}^{-1}$  FhuA and 12 mM (total concentration) LMNG. SV-AUC analysis shows a main contribution ( $\approx 75\%$  of the absorbance) at  $s_{20w} = 7.8 \text{ S}$ , *i.e.* the same value as that published for FhuA in DDM [82]. Additional contributions are detected at  $s_{20w} = 10.7 \text{ S}$  ( $\approx 5\%$ ) and  $6.2 \text{ S}$  (15-20%), which correspond reasonably to FhuA dimer and LMNG free micelles, respectively (Fig 4AB). Analysis of the absorbance of interference fringe signals provides an estimate of  $1 \text{ g g}^{-1}$  ( $80 \text{ mol mol}^{-1}$ ) of bound LMNG, and a free micelle concentration of 4.5 mM. In order to investigate the effect of LMNG concentration on FhuA homogeneity, complementary SV-AUC experiments were done on a similarly prepared sample with nominal concentrations of  $5.4 \text{ mg mL}^{-1}$  FhuA and 13 mM LMNG, and two derived samples, with FhuA diluted twice, and increased LMNG nominal concentrations to 18 and 24 mM. For these three samples too, only one main boundary is observed ( $> 80\%$ ) at about 7 S (Table 3). Probably, due to the large total concentrations of LMNG and FhuA, the fit is poor and the  $s_{20w}$  values imprecise (data not shown). The data can be interpreted by the non-ideal co-sedimentation of FhuA complexes and of LMNG free micelles, sedimenting at nearly the same  $s_{20w}$  value (Table 3). Nevertheless the membrane protein appears to remain monomeric in the presence of even very large concentrations of LMNG, where the latter forms large micelles. SEC-LS performed on a similar sample injected after 10 times dilution, showed traces of aggregates, FhuA dimer as a shoulder, FhuA monomer as the main contribution at 12.3 mL, and LMNG micelles at 14.5 mL (Fig 4C). The molar mass analysis gives for the main peak 79.3 kDa for FhuA molar mass contribution, in agreement with the protein sequence (79.9 kDa), and 98.5 kDa for LMNG contribution, corresponding to  $1.2 \text{ g g}^{-1}$  ( $95 \text{ mol mol}^{-1}$ ) bound detergent.



**Fig 3. Thermal denaturation of FhuA, SpNOX, BmrA, and bR, by differential scanning fluorimetry**

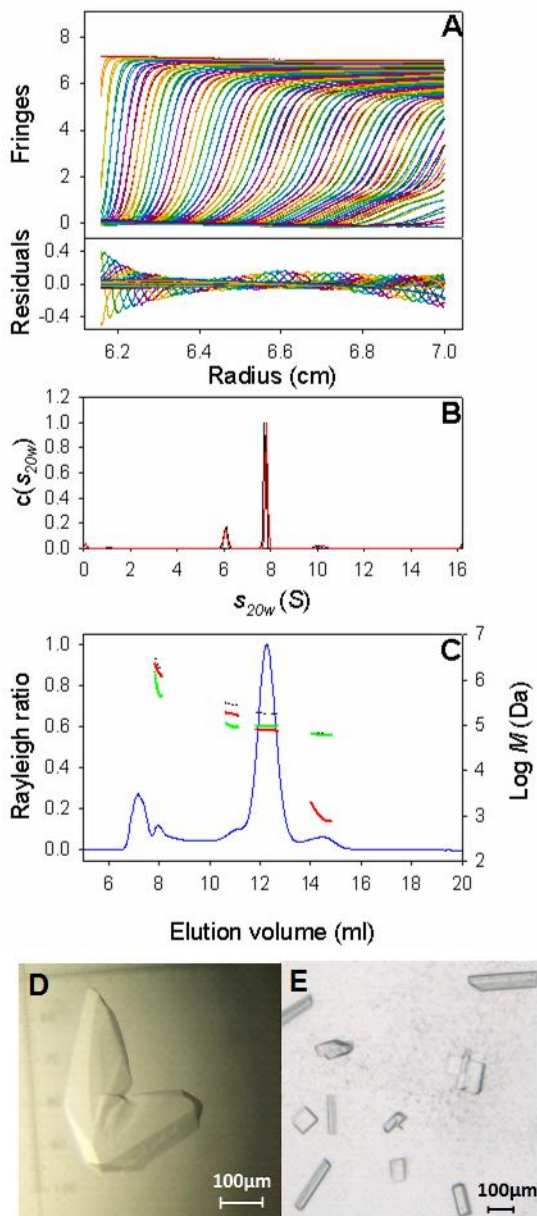
Ratio of the fluorescence emitted at 350 and 330 nm (top panels), and derivative (bottom panels) for **A:** FhuA at  $0.04 \text{ mg mL}^{-1}$  in the presence of LDAO  $1.2 \text{ mM}$  (green) or LMNG  $0.21 \text{ mM}$  (black); **B:** SpNOX at  $8.3 \text{ mg mL}^{-1}$  in the presence of DDM  $1.27 \text{ mM}$  (red) or LMNG  $0.21 \text{ mM}$  (black); **C:** BmrA at  $1 \text{ mg mL}^{-1}$ , in DDM  $2 \text{ mM}$ , in the Apo (red), or Vi-inhibited (orange) forms, and in LMNG  $0.1 \text{ mM}$ , in the Apo (black), or Vi-inhibited (purple) forms. The Vi-inhibited forms were incubated with  $1 \text{ mM}$  Vi,  $10 \text{ mM}$  ATP and  $10 \text{ mM}$   $\text{MgCl}_2$  for 15 minutes. **D:** bR at  $0.14 \text{ mg mL}^{-1}$  in OTG  $11 \text{ mM}$  (blue), DDM  $2.17 \text{ mM}$  (red), or LMNG  $0.21 \text{ mM}$  (black).

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**Table 3. LMNG solubilized FhuA in AUC-SV**

exp	FhuA ( $\text{mg mL}^{-1}$ )	total LMNG (mM)	free LMNG <sup>a</sup> (mM)	Main $s_{20w}$ (S) from $A_{280}$ <sup>b</sup>	Main $s_{20w}$ (S) from $\Delta J$ <sup>b</sup>	$s_{20w}$ (S) for LMNG <sup>c</sup>
1	7.5	12	4.5	7.8	7.8	6.8
2	5.4	13	7.6	6.8	6.8	7.6
3	2.7	18	15.3	6.4	7.1	7.6
4	2.7	24	21.3	7.0	7.5	7.7

LMNG free micelles and FhuA complexes sediment separately (Fig 4) in exp 1, but are not resolved in exp 2-4. <sup>a</sup>calculated with the hypothesis of  $1 \text{ g g}^{-1}$  LMNG bound to FhuA, except for exp. 1, where free LMNG micelle concentration was



measured in AUC-SV. <sup>b</sup>for FhuA complex in exp. 1, and unresolved LMNG free micelles and FhuA complex in exp. 2-4. <sup>c</sup>from AUC-SV experiments of pure LMNG (Fig 1) for the LMNG concentrations reported on the fourth column.

**Fig 4. Characterisation and crystallization of LMNG solubilized FhuA**

AUC-SV: **A:** Superposition of experimental and fitted sedimentation velocity profiles (top) and residuals (bottom), obtained at 130 000 g and 4 °C during  $\approx$  4.5 hours using interference optics and 1.5 mm optical path centerpiece, for 7.5 mg mL<sup>-1</sup> FhuA solubilized with 12 mM LMNG in 20 mM Tris-HCl pH 8.0, 150 mM NaCl. Reference channel was filled with solvent without LMNG. **B:** Superposition of the normalized  $c(s_{20w})$  distributions obtained at 280 nm (red line) and with interference optics (black line). SEC-LS: **C:** Rayleigh ratio measured along the elution profiles, for 35  $\mu$ L of FhuA at 1 mg mL<sup>-1</sup> and LMNG at 1.2 mM injected on a Superdex 200 10/300 GL (GE Healthcare) column equilibrated at 6 °C with 100 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.5 mM LMNG, and molar masses (thick lines, right axis) for the whole complex (black), and LMNG (green) and FhuA (red) contributions, derived from combining light scattering, refractive index, and absorbance at 280 nm detections. **D.** Typical DDAO-solubilized FhuA crystals grown in 0.1 M MES pH 6.0, 10% PEG 6000, 2% DDAO. **E.** Typical LMNG-solubilized FhuA crystals grown in 0.1 M ADA pH 6.5, 12% PEG 6000, 1.2% LMNG.

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#### 755 **FhuA crystallization**

756 480 conditions were screened for the crystallization of FhuA solubilized in 1.2% (12 mM)  
 757 LMNG or, for comparison, 1.6% (79.4 mM) decyldimethylamide-N-oxide (DDAO). DDAO  
 758 is the detergent in which the protein was historically crystallized [56], and in our hands,  
 759 FhuA-DDAO crystals diffracting to 2.2 Å could be obtained (Arnaud and Breyton,  
 760 unpublished data).

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762 The FhuA-LMNG complex is clearly more susceptible to precipitating conditions: only  
 763 53.7% of the drops remained clear (*vs.* 86% for FhuA-DDAO). In addition, 13.3% of the  
 764 conditions with LMNG yielded unambiguous crystal formation *vs.* only 3% in DDAO, and 10  
 765 *vs.* 8 conditions, respectively showed single crystals. Whereas conditions allowing single  
 766 crystal growth in DDAO were closely related (high molar mass PEG, pH 6-7 and MgCl<sub>2</sub>),  
 767 those in LMNG displayed more differences: different precipitants, including wide ranges of  
 768 PEG molar mass (400-8000) and pH (4-9) and a variety of co-crystallization salts. The shapes  
 769 of the crystals were also very different, with feather-like shapes for FhuA-DDAO and more

770 rod for FhuA-LMNG crystals (Fig 4D). X-ray diffraction data were collected on FhuA-  
 771 LMNG crystals (Table 4). Crystals grown in the screening assay, with little optimization,  
 772 diffracted up to 4 Å, which allowed to get a model at low resolution and to determine the  
 773 crystal packing. Interestingly, these crystals have a different space groups (C222<sub>1</sub>) than  
 774 crystals obtained with DDAO (P6<sub>1</sub>).

775  
 776 Thus, LMNG largely expanded the number of potential conditions in which FhuA crystals are  
 777 grown, possibly by allowing different crystal packing. These data seemingly support the idea  
 778 that LMNG enhances successful crystallization of membrane proteins [26].

779

**Table 4. Characteristics of LMNG solubilized FhuA crystals**

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**Crystal data collection statistics for FhuA – LMNG**

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782	<b>Crystal data collection statistics for FhuA – LMNG</b>	
783	Space group	C 2 2 2 <sub>1</sub>
784	Unit cell dimension (Å)	149.53 210.96 188.7 90 90 90
785	Number of reflections	371461 (37282)
786	Number of unique reflections	25522 (2481)
787	Resolution limits (Å)	40.61 - 4.0
788	Higher resolution shell (Å)	4.143 - 4.0
789	Completeness	98.13 % (98.06 %)
790	Redundancy	14.6 (14.8)
791	Rmerged	0.1771 (7.384)
792	Rmeasured	0.1838 (7.648)
793	I/(I)	9.51 (0.50)

794

**Molecular replacement with Phaser**

795 LLG = 3698.6

796 TFZ = 16.4

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**Crystallographic refinement statistics**

799		
800	Resolution range (Å)	40.61 - 4.0
801	Number of reflections	25098
802	R / FreeR	0.3141 / 0.3546
803	Number of atoms (total)	11024
804	Mean B value (Å <sup>2</sup> )	209.52
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806	rms deviation from ideal values:	
807	bond length (Å)	0.007
808	bond angle (degree)	1.52

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***SpNOX-LMNG assemblies***

811 We then investigated the prokaryotic enzyme SpNOX [62]. This protein is a recently  
 812 identified analog to the eukaryotic NOX membrane proteins. They are the central catalytic  
 813 component, involved in transmembrane electrons transfer, within NADPH oxidase  
 814 complexes, which play essential roles in *e.g.* immunity, cardiovascular physiology, etc.  
 815 Despite the progress done in the characterization of the soluble subunits of the NADPH  
 816 oxidase complexes [83,84], there were very few structural information on the NOX α-helical  
 817 membranous component up to the recent composite structure of a NOX5 isoform from a  
 818 cyanobacteria [85]. Apart from its fundamental biological interest, this protein displays  
 819 several features that make it a convenient model to test and develop new methods or  
 820 processes in membrane protein biochemistry. Indeed, the red color brought by the presence of  
 821 two hemes *b* within the transmembrane protein constitutes a good reporter in all steps of  
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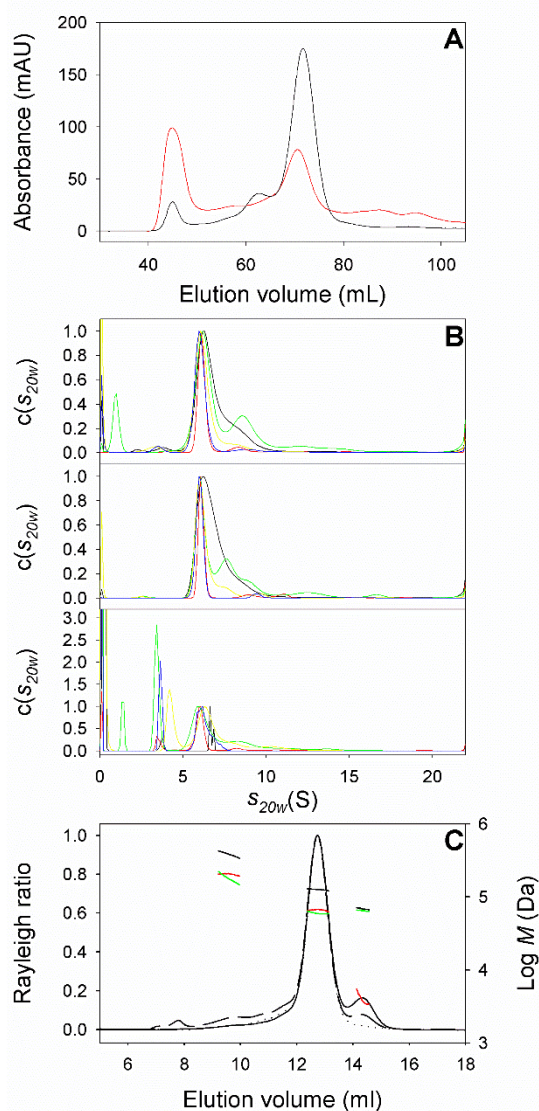
823 protein manipulation as well as a precious indicator of the correct folding of the protein.  
 824 Moreover, SpNOX has an enzymatic activity that can be measured spectrophotometrically in  
 825 a fast and convenient way.

826  
 827 For SpNOX solubilization and purification, maltoside detergents were identified as essential  
 828 in order to preserve protein activity [62]. However, several lines of improvement have been  
 829 observed by using LMNG instead of DDM. First, from the elution profile from size exclusion  
 830 chromatography of the second purification step (Fig 5A), the peak of SpNOX aggregates, at  
 831 45 mL, decreases drastically from DDM to LMNG while, in addition to minor amounts of  
 832 dimers detected in the two conditions, that of monomeric SpNOX, at 70 mL, increases.  
 833 SpNOX is obtained with an improved homogeneity in LMNG compared to DDM. Moreover,  
 834 SpNOX shows a significantly increased specific activity in LMNG compared to DDM (Table  
 835 5). We evaluate the relative thermal stability of SpNOX in LMNG compared to DDM.  $T_m$  in  
 836 LMNG is drastically increased (+13°C) (Table 2).

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839 **Table 5. Compared activities, in LMNG and DDM, of SpNOX and BmrA**

Protein	Environment	Activity
841 SpNOX	DDM 1.27 mM	5.9 +/- 0.5 mol Cyt c reduced s <sup>-1</sup> mol <sup>-1</sup> SpNOX
	LMNG 0.21 mM	9.3 +/- 0.2 mol Cyt c reduced s <sup>-1</sup> mol <sup>-1</sup> SpNOX
BmrA	DDM 2 mM	0.8 +/- 0.1 μmol ATP hydrolyzed min <sup>-1</sup> mg <sup>-1</sup> BmrA
BmrA	LMNG 0.1 mM	1.4 +/- 0.2 μmol ATP hydrolyzed min <sup>-1</sup> mg <sup>-1</sup> BmrA
BmrA	liposome	5.0 +/- 0.6 μmol ATP hydrolyzed min <sup>-1</sup> mg <sup>-1</sup> BmrA



**Fig 5. LMNG solubilized SpNOX: preparative SEC, AUC-SV and SEC-LS**

**A:** Superdex 200 16/60 elution profiles, in the second step of SpNOX purification, performed in 50 mM Tris-HCl pH 7, 300 mM NaCl, with either 0.3 mM DDM (red line) or 0.03 mM LMNG (black line). Aggregates elute at 45 mL, while monomeric SpNOX elutes at 70 mL. **B:** Superposition of normalized  $c(s_{20w})$  distributions obtained from AUC-SV at 280 nm (top), 412 nm (middle) and with interference optics (bottom), for SpNOX samples *c0.1*, *c3*, *c0.25d*, *c0.1d*, *c0.25dLMNG*, described in the text (black, red, green, yellow, and blue lines, respectively). The vertical dashed line at 6 S indicates the main SpNOX complex. **C:** SEC-LS normalized elution profiles obtained at 280 nm (dotted line), by refractive index (continuous line), and light scattering (dashed line), for 30 μL of SpNOX at 2.9 mg mL<sup>-1</sup> injected on a Superdex 200 10/300 GL (GE Healthcare) column equilibrated at 4 °C with 50 mM Tris-HCl pH 7.0, 300 mM NaCl, 0.05 mM LMNG (buffer A) and molar masses (thick lines, right axis) for the whole complex (black), and LMNG (green) and SpNOX (red) contributions, derived from combining static light scattering, refractive index, and absorbance

877 at 280 nm detections. A hydrodynamic radius of 4.0 nm was measured by DLS at the maximum of the main  
878 peak.

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886 We then investigated, using SV-AUC, homogeneity and association state of SpNOX  
887 solubilized in LMNG, at different concentrations of SpNOX and LMNG. After a final SEC  
888 purification step with 0.03 mM LMNG in the elution buffer (buffer A, see legend to Fig 5),  
889 SpNOX was obtained at 0.1 mg mL<sup>-1</sup> (sample *c0.1*). Then the protein was concentrated 30  
890 times by ultrafiltration with a 30-kDa cut off membrane (sample *c3*). To further investigate  
891 the effects of protein and LMNG concentrations on the size and composition of the  
892 LMNG/SpNOX complexes, part of *c3* was rediluted 12 or 30 times in buffer A (containing  
893 0.03 mM LMNG) and 12 times with the same buffer but containing LMNG at 1.5 mM  
894 (samples *c0.25d*, *c0.1d*, *c0.25dLMNG*, respectively). AUC-SV of these samples was followed  
895 at 412 nm, in addition to the usual 280 nm and interference detection, which allows following  
896 the heme b cofactors. The normalized  $c(s)$  curves (Fig 5B) at 280 and 412 nm superimpose  
897 showing that the different SpNOX complexes in all samples have the same heme content. The  
898 measured ratio  $A_{412}/A_{280}=1.75 \pm 0.05$  is that expected for pure SpNOX [62]. The main  
899 SpNOX species (typically 85%) sediments at  $s_{20w} = 6.0 \pm 0.1$  S. A minor contribution  
900 (typically 8%) is detected at  $\approx 8.2$  S, in addition to poorly-defined aggregates (typically 7 %).  
901 *c0.25dLMNG* appears to be more homogeneous, and *c0.25d* more heterogeneous, but we  
902 cannot ascertain the relevance of these subtle variations. We conclude that SpNOX main  
903 assemblies have clearly the same composition in all samples, and increasing LMNG or  
904 protein concentration does not lead to larger complexes. Interference optics allows the  
905 additional detection of LMNG micelles below 4 S, in the expected  $s$ -range, since we  
906 measured  $s$  at 2.5 and 5 S for pure LMNG at 0.05 and 1.5 mM in buffer A. From the  
907 integration of the signal, we derived a concentration of 1.2 mM free micelles in *c3*, which  
908 indicated an almost stoichiometrical co-concentration of LMNG and SpNOX. For the main  
909 SpNOX complex, the ratio of the interference fringes and  $A_{280}$  signals is the same for *c0.1*, *c3*,  
910 *c0.25d*, suggesting that no or few LMNG micelles co-sediment (the ratio is however larger for  
911 *c0.25dLMNG* and *c0.1d*, but it may be related to the uncertainty of the analysis), and provides  
912 a rough estimate of  $1 \pm 0.1$  g g<sup>-1</sup> LMNG bound to SpNOX (this value is imprecise, as  
913 discussed below).

914

915 Complementary SEC-LS-DLS in buffer A with 0.05 mM LMNG was performed on four  
916 samples of SpNOX from a different purification. One at 0.16 mg mL<sup>-1</sup> is from SEC, the three  
917 others are from subsequent concentration to 1.0, 2.9 and 8.7 mg mL<sup>-1</sup> (concentration by a  
918 factor of 8, 18 and 54, respectively) by ultrafiltration. The elution profiles of the four SpNOX  
919 samples are very similar, and one example is shown on Fig 5C. The main SpNOX complex  
920 elutes at 12.8 mL with a  $R_H = 4.0$  nm measured from DLS. The analysis provides molar  
921 masses for the main complex of  $68 \pm 3$  kDa and  $59 \pm 3$  kDa for SpNOX and LMNG-  
922 components, remarkably constant for the four samples. These values correspond to a  
923 detergent/protein ratio similar to that estimated from AUC. The protein molar mass, however  
924 is intermediate between the theoretical ones for a monomer (48 kDa) and a dimer. This  
925 suggests a systematical error in the input value, used in AUC and SEC-LS, of the SpNOX  
926 extinction coefficient. We thus rather consider the total molar mass of the complex of 126  
927 kDa, derived from LS and RI detection to derive, in the hypothesis of a monomeric protein,

928 an amount of bound detergent of 1.6 g/g. Combining  $s$  from AUC and  $R_H$  from DLS, a  
929 buoyant molar mass of 26.2 kDa is derived, which corresponds to a globular compact  
930 monomer of SpNOX binding 1.6 g.g<sup>-1</sup> of LMNG, with a frictional ratio of 1.2, corresponding  
931 to a globular compact shape.

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### 933 *BmrA-LMNG assemblies*

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935 BmrA (for ‘Bacillus multidrug resistance ATP’) is a member of the ABC (“ATP-Binding  
936 Cassette”) transporters family. It is capable to export multiple drugs with no chemical  
937 relationship [86]. In human, its closest homologue MDR1 confers resistance to  
938 chemotherapeutic treatments [63]. Crystal structures of homologs stabilized in different states  
939 [87,88] suggest that, to permit drug translocation, large conformational changes must occur in  
940 an alternating access model. BmrA could be purified in a functional homodimeric form in  
941 0.05% DDM [89,90], and, indeed it was shown to be flexible and adopt different  
942 conformations [91–94].

943

944 Since then, the protein was purified in LMNG [95]. It was noticed that LMNG, in contrast to  
945 DDM, significantly reduced BmrA aggregation with time. Moreover, the ATPase activity of  
946 BmrA was significantly increased, by a factor of about 1.5 to 2, in LMNG compared to DDM  
947 (Table 5). The activity in LMNG is thus getting closer to that of the protein reconstituted into  
948 liposomes: 1.4 vs. 5, respectively,  $\mu\text{mol ATP hydrolyzed /min/mg protein}$  (Table 5).

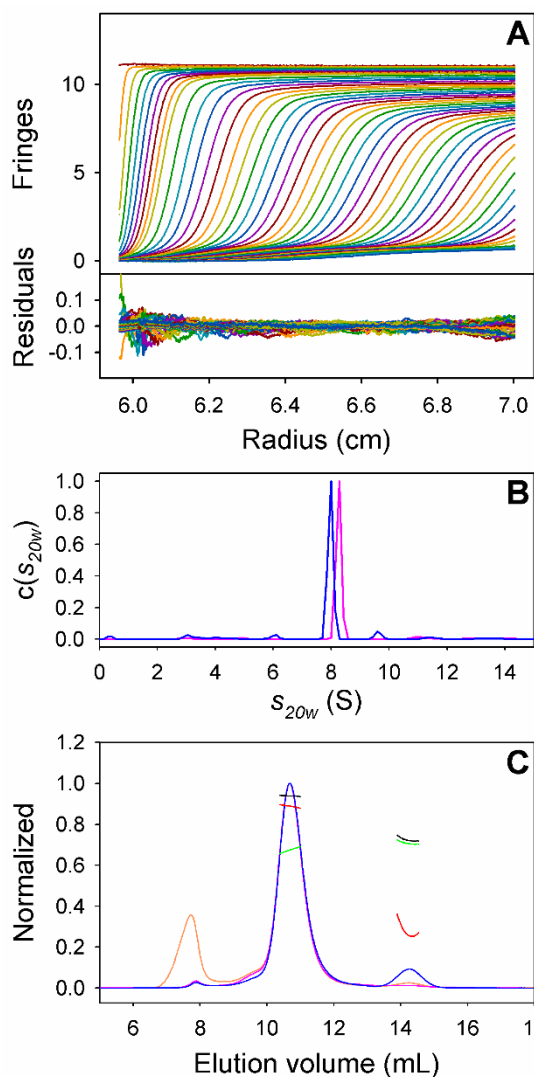
949

950 We evaluated by DSF the protein’s thermal stability for two freshly purified -in either DDM  
951 or LMNG- batches of BmrA. Measurements were done on the Apo and the vanadate (Vi)  
952 inhibited forms. The Apo-form is expected to be in an open conformation, the Vi-inhibited  
953 form would exist in a closed conformation ([64,93] and unpublished results). Fig 3 shows the  
954 measurements for BmrA Apo and Vi-inhibited forms, and Table 2 reports the melting  
955 temperatures  $T_m$ . In DDM and in LMNG, the  $T_m$  increases steadily from the less stable Apo to  
956 the more stable Vi-inhibited form. For the two forms, BmrA unfolds at higher temperature  
957 when purified in LMNG compared to DDM: by 4.5 °C for BmrA Apo, and  $\approx 13$  °C for the  
958 Vi-inhibited BmrA (Table 2).

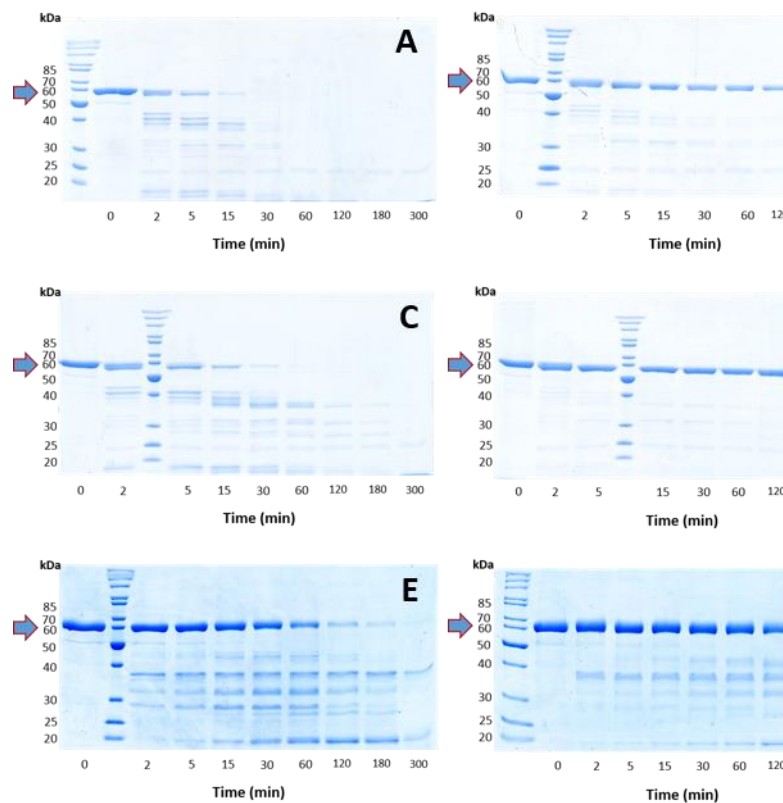
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960 Limited proteolysis experiments were performed on the Apo- and Vi-inhibited BmrA forms,  
961 to evaluate differences in protein flexibility between the two forms, in DDM and in LMNG.  
962 After incubation for different times with a protease, BmrA samples were run on SDS-PAGE.  
963 Fig 6A-D show clearly, for BmrA purified in DDM and LMNG, respectively, and incubated  
964 with trypsin, the increased robustness of Vi-inhibited BmrA compared to the Apo form. In  
965 DDM, the 55 kDa band representing the intact protein essentially disappeared after 30 min of  
966 incubation for the Apo form, and more than 300 min for the Vi-inhibited form, in agreement  
967 with previously published results [93]. In LMNG, 60 min are required for an almost full  
968 disappearance of the intact Apo BmrA, and the Vi-inhibited form is essentially preserved for  
969 the entire time period tested. The reduced kinetics of proteolysis in LMNG compared to DDM  
970 is also observed with protease V8 (Fig 6E-F). We checked that the detergent type had no  
971 effect on protease activity by itself.

972



983 adding the proteases. The arrows indicate intact BmrA.



**Fig 6. BmrA limited proteolysis experiments**

A-D: incubation with trypsin at  $1 \mu\text{g}/250 \mu\text{g}$  of protein, for A: Apo BmrA in DDM, B: Vi-inhibited BmrA in DDM, C: Apo BmrA in LMNG, D: Vi-inhibited BmrA in LMNG. E-F: incubation with protease V8 at  $1 \mu\text{g}/20 \mu\text{g}$  of protein, for Apo BmrA, in E: DDM, and F: LMNG. DDM and LMNG concentrations are 2 and 0.1 mM, respectively. Time zero is taken after 15 min of pre-incubation before

983  
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 985  
 986 In order to evaluate protein homogeneity in LMNG, samples of BmrA were purified with a  
 987 last step of SEC in a buffer containing LMNG at 0.1 mM, concentrated using a centrifugal  
 988 filter device by a factor of 4.4 for a final BmrA concentration of  $1.3 \text{ mg mL}^{-1}$ . SV-AUC  
 989 revealed a very homogeneous preparation, with one protein complex at  $s_{20w} = 8.2 \text{ S}$   
 990 (experimental value of 4.2S, at  $10^\circ \text{C}$ , in the buffer containing glycerol) (Fig 7A). SEC-LS  
 991 experiments, done with a BmrA sample at  $2.7 \text{ mg mL}^{-1}$ , prepared in a similar way, stored  
 992 frozen at  $-80^\circ \text{C}$  and injected on a column equilibrated with buffer A, showed minor (total  $\approx$   
 993 5%) contributions of large and small aggregates eluting at 7.8 mL and as a shoulder at 9.7  
 994 mL, in addition to the main complex, eluting at 10.7 mL, with a  $R_H = 5.5 \text{ nm}$  measured from  
 995 DLS, and a total molar mass of 240 kDa, corresponding to a dimer with 0.8 g/g bound  
 996 detergent (Fig 7B). Combining  $R_H$  and the  $s$ -values, we derived a buoyant molar mass of 45.9  
 997 kDa, corresponding to a dimer with 0.6 g/g bound LMNG, and a frictional ratio of 1.35.  
 998 Excess LMNG micelles were undetected in the AUC experiments; in SEC-LS their amount  
 999 corresponds to an injected LMNG concentration of 0.2 mM. In conclusion, very  
 1000 homogeneous BmrA samples can be prepared in LMNG with protein concentration in the mg  
 1001  $\text{mL}^{-1}$  range, which remains essentially homogeneous after storage at  $-80^\circ \text{C}$ .

**Fig 7. LMNG solubilized BmrA in AUC-SV and SEC-MALS**



1005 AUC-SV: **A:** Superposition of experimental and fitted sedimentation velocity profiles (top) and residuals  
1006 (bottom), obtained at 130,000 g and 10 °C during  $\approx$  8 hours using interference optics and 12 mm optical path  
1007 centerpiece, for BmrA at 2.3 mg mL<sup>-1</sup> and free LMNG at 0.44 mM (putative concentration) in 25 mM Tris-HCl  
1008 pH 8.0, 150 mM NaCl, 10% glycerol. Reference channel was filled with solvent without LMNG. **B:**  
1009 Superposition of the normalized  $c(s_{20w})$  distributions obtained at 280 nm (pink line) and with interference optics  
1010 (blue line). SEC-LS: **C:** Rayleigh ratio, refractive index, and absorbance at 280 nm (orange, blue, and pink lines,  
1011 respectively) measured along the elution profiles, for 50  $\mu$ L of BmrA at 2.7 mg mL<sup>-1</sup> and free LMNG at 0.06  
1012 mM, this concentration being experimentally determined in SEC-LS, injected on a Superdex 200 10/300 GL (GE  
1013 Healthcare) column equilibrated at 4 °C with 50 mM Tris-HCl pH 7.0, 300 mM NaCl, 0.05 mM LMNG (buffer  
1014 A), and molar masses (thick lines, right axis) for the whole complex (black), and LMNG (green) and BmrA (red)  
1015 contributions, derived from combining the three detections. The protein elutes as a main species at 10.7 mL,  
1016 together with large and small aggregates, in minor amounts, in the void volume (7.8 mL) and as a shoulder at 9.7  
1017 mL, respectively; free LMNG elutes at 14.2 mL.

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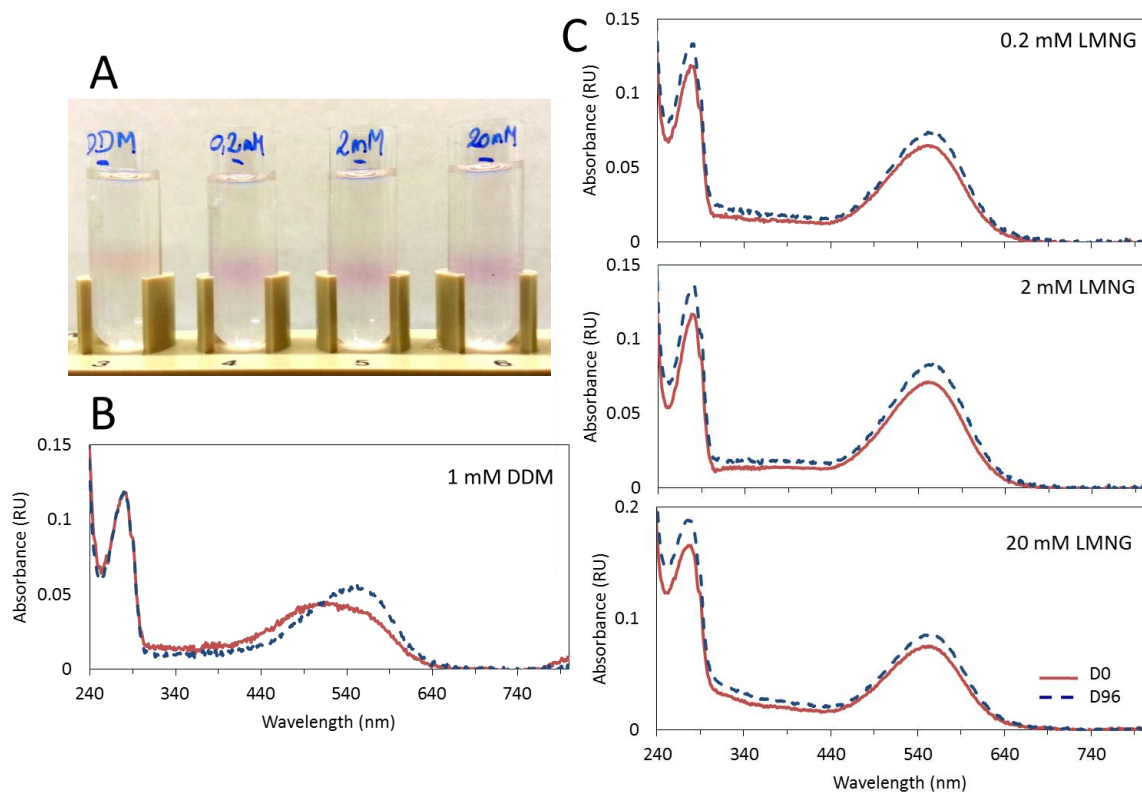
#### 1024 ***bR homogeneity, and time and thermal stability***

1025 Lastly, we investigated the homogeneity and time stability of the bR as a model protein. bR is  
1026 a light-driven proton pump purified from the archaea *Halobacterium*. It is composed of seven  
1027 transmembrane  $\alpha$ -helices and binds a covalent cofactor, a retinal molecule that confers purple  
1028 color to the protein.

1029

1030 Sucrose gradients are a convenient means to perform both detergent exchange and evaluate  
1031 the colloidal homogeneity of the protein-detergent complex. In the case of colored proteins,  
1032 this latter information is directly visible after centrifugation, by the broadness of the protein-  
1033 detergent band. We routinely use this method to evaluate the potentialities of fluorinated  
1034 surfactants in the biochemistry of membrane proteins, using the bR. Fig 8A shows the results  
1035 of sucrose density gradient experiments in the presence of 1 mM DDM, or 0.2, 2 and 20 mM  
1036 LMNG. After 15 h centrifugation, the protein migrated past the middle of the gradient,  
1037 insuring total detergent exchange, as checked by TLC. In all LMNG conditions, the protein  
1038 band migrates at the same depth and appears to have the same width/broadness as that in  
1039 DDM, suggesting that the bR-LMNG complexes are homogeneous monomers as the bR-  
1040 DDM complexes. The retinal molecule, whose visible absorption spectrum is very sensitive to  
1041 its local environment, is a convenient reporter of the state of the protein: the trimeric protein  
1042 in its native membrane reveals a visible absorption spectrum with a maximum at  $\lambda_{\max} =$   
1043 570 nm; when solubilized in detergent, the protein monomerises and displays  $\lambda_{\max} = 554$  nm;  
1044 in both cases, the protein appears purple. When the protein denatures, the retinal is released,  
1045 and  $\lambda_{\max}$  shifts to 400–380 nm: the protein solution turns yellow. Fig 8B and 8C report the  
1046 absorption spectra of the protein recovered just after centrifugation and three months after,  
1047 stored on ice in the dark. The DDM sample, which appears pinkish in the gradient, displays a  
1048 broad absorption peak, with a  $\lambda_{\max} \sim 530$  nm (Fig 8B), suggesting a poorly folded protein.  
1049 After three months however, the spectrum shows a usual peak, with  $\lambda_{\max} = 555$  nm. The  
1050 protein probably started to denature in OTG, the detergent used for its solubilization, but  
1051 returned to its native fold when transferred to DDM. In all LMNG samples, the absorption  
1052 peak displays a  $\lambda_{\max} = 554$  nm (Fig 8C), suggesting that the dynamics of the bR monomer in  
1053 LMNG is different than in classical detergents. After three months incubation, the absorption  
1054 spectra are unchanged, indicating that the protein is very stable.

1055



1056  
1057 **Fig 8. bR sucrose gradient**

1058 **A:** Migration of bR in 10-30% sucrose gradients in the presence of either 1 mM DDM, 0.2, 2 or 20 mM LMNG.  
1059 Gradients were centrifuged 15 h at 200,000 x g. bR was recovered from the gradients and its UV-visible  
1060 spectrum measured immediately (D0, solid line) or three months after incubation at 4°C in the dark (D96, dashed  
1061 line). **B:** bR in 1 mM DDM, **C:** bR in 0.2, 2 and 20 mM LMNG.

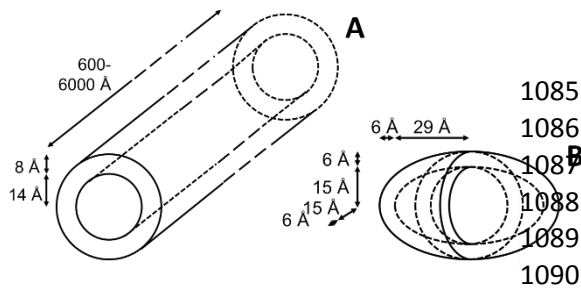
1062  
1063 At last, bR thermal denaturation assays were performed. Solubilized bR samples were  
1064 incubated with biobeads to decrease the OTG concentration, and diluted 18 times (leading to  
1065 a residual OTG concentration of 0.4 times the CMC) in OTG, DDM, or LMNG, at a  
1066 concentration of 2 mM above the CMC of each detergent, for thermal denaturation assays.  
1067 Result are presented on Fig 3 and Table 2. bR has an increased thermostability in DDM  
1068 compared to OTG, and LMNG is even more stabilizing, with  $T_m$  increased by 6 and 12 °C,  
1069 respectively.

1070  
1071  
1072 **Discussion and conclusion**

### 1073 **On the self-assembly of LMNG**

1074 From AUC experiments, we determined the CMC of LMNG to be 40  $\mu$ M. This is coherent  
1075 with previously published value from hydrophobic dye solubilization measurements (10  
1076  $\mu$ M) [26], while isothermal titration calorimetry measurements yielded a significantly lower  
1077 concentration (11.3 nM) [44]. The CMC is not an absolute value, rather a range of  
1078 concentrations, and different techniques used to determine the CMC probe different properties  
1079 of micelle formation. Thus, it is not unusual to find different values depending on the  
1080 technique used, and discrepancies become exacerbated when the CMC is very low.

1081  
1082  
1083 Regarding the size of the micelles, the  $s_{20w}$ -value of LMNG at 0.25 mM (0.025 %) is 3.3 S.  
1084 This value is in the same range than that of DDM (3.12 S) that forms globular compact



micelles of about 60 kDa in a large range of detergent concentration [65]. If LMNG forms quite small micelles at 0.25 mM, AUC, DLS, SAXS, and SANS, clearly indicate it forms larger micelles, rods of increasing length, when increasing the

1091 concentration above 0.25 mM. The lateral dimension of the hydrophobic core radius is 14 Å,  
1092 and that of the shell thickness is 7 Å in H<sub>2</sub>O (9 Å in D<sub>2</sub>O). These values correspond to that of  
1093 DDM micelles, described from SAXS to form oblate ellipsoids with, in the minor axis, a  
1094 hydrophobic core of ≈ 15 Å surrounded by a hydrophilic layer of ≈ 6 Å thickness [96,97]  
1095 (Scheme 2). The differences of 1 Å between our LMNG and the published DDM dimensions  
1096 are not significant since different techniques and buffers, thus contrasts (in terms of electron  
1097 density or neutron scattering length densities), were used. To this date, this is only one  
1098 membrane protein pdb file (4b4a) containing LMNG as a ligand (pdf identifier “LMN”). The  
1099 maximum C-O, C-C and O-O distances in the whole molecule, in the two tails, and in the two  
1100 heads, respectively, are 21.9, 13 and 11, 9.8 and 9.7 Å, respectively (we note that in this  
1101 unique structure, the hydrophilic head is quite open: we measure a maximal distance between  
1102 the O of the two sugars of LMNG of 19 Å). For DDM as a ligand (pdf identifier “LMT”), in  
1103 an arbitrary selection of membrane protein pdb files (6haw, 6hqb, 4kfm, 6cnn), we measure  
1104 maximal distances for the whole molecule, the tail, and the head, of 20.5 +/- 1, 12 +/- 1, and  
1105 9.2 +/- 0.8 Å, respectively. These values are logically similar to that of LMNG. The  
1106 maximum length of the tail is logically lower than 16.7 Å corresponding to the dimension of  
1107 an extended alkyl chain with 12 carbons [97].  
1108

1109 If DDM is described to form slightly elongated micelles, for LMNG at 20 mM however, we  
1110 measured lengths of 600 Å in H<sub>2</sub>O and more than 5000 Å in D<sub>2</sub>O. It corresponds to LMNG  
1111 rods with ratios of the total length over the total diameter, of 14 and 120. For LMNG in H<sub>2</sub>O,  
1112 the rod length value coincides with the maximum distances estimated between 10 and 20 mM  
1113 LMNG, from the pair distribution analysis of the – measured independently – SAXS data of  
1114 Fig 2A. In a preliminary independent SANS experiment, however, rods of only 1200 Å long  
1115 were modelled for LMNG micelles at 20 mM in D<sub>2</sub>O. It corresponds to a length over diameter  
1116 ratio of 28, 4-5 times smaller than that presented above. It is likely that the precise length of  
1117 LMNG rods depends on uncontrolled parameters during sample preparation. It is possible that  
1118 LMNG forms longer rods in D<sub>2</sub>O, compared to H<sub>2</sub>O, but we cannot ascertain it. For both  
1119 SAXS and SANS, the shape and size of the micelles does not change between 10 and 20 mM.  
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## 1127 **Scheme 2. Schematic representation of LMNG and DDM micelles**

1128 **A:** LMNG micelles at 20 mM in H<sub>2</sub>O or D<sub>2</sub>O, from modelling of combined SANS and SAXS data (this work) **B:**  
1129 DDM micelles from modelling of SAXS data [96,97].  
1130  
1131

1132 Israelachvili et al. [98,99] have provided a rationale to explain how the molecular geometry of  
1133 individual surfactant molecules affects and controls the shape and size of the assemblies they  
1134 form. The geometry of the molecule can be described by three parameters: the area of the  
1135 hydrated polar head,  $a_0$ , the volume,  $v$ , and the extension,  $l_c$ , of the hydrophobic tail. The

1136 value of the related critical packing parameter,  $v/(a_0 l_c)$ , representing the ratio of the mean area  
1137 of the hydrophobic tail on that of the hydrophilic head, determines the geometry of the  
1138 assemblies: for  $v/(a_0 l_c) < 1/3$  (large hydrophilic surface), spherical micelles are formed; for  
1139  $1/3 < v/(a_0 l_c) < 1/2$ , rods are favored; then, for  $v/(a_0 l_c) < 1$ ,  $\sim 1$ , and  $> 1$  (with increasingly  
1140 large hydrophobic lateral extensions), flexible or planar bilayers, and inverted micelles,  
1141 respectively, are stabilized. The LMNG molecule corresponds to two linked DDM molecules.  
1142 The linkage is done through a quaternary carbon, localized on the lauryl chain, at the second  
1143 carbon following the ether group belonging to the maltose moiety. It is thus at the junction  
1144 between the hydrophilic head and the hydrophobic tail of the detergent. The constraints of this  
1145 link on each moiety dimensions are hardly intuitively predictable. The fact that DDM forms  
1146 globular micelle while LMNG forms rod means that the packing parameter  $v/(a_0 l_c)$  increases  
1147 from DDM to LMNG, which can be related to an increase, in LMNG compared to DDM of  
1148 the hydrophilic surface per maltose group, and/or of the mean length of the hydrophobic  
1149 chain, corresponding to changes in their landscape of the molecular conformational stability  
1150 and/or dynamics.

1151  
1152 Rod formation has noticeable consequences for biochemists. When using ultrafiltration  
1153 centrifugal devices to concentrate proteins, LMNG co-concentrates with the membrane  
1154 protein by the same factor. Depending on the initial detergent concentration, typically from  
1155 0.02 to 0.1 mM, LMNG in the concentrated sample will form small to very large assemblies.  
1156 Concentrated LMNG dissociates upon SEC, but elutes as a very broad peak, preventing  
1157 membrane protein in concentrated LMNG to be recovered with a well-defined LMNG  
1158 concentration.

1159  
1160 **On the relative stabilization of proteins in LMNG compared to DDM**  
1161 Our work corroborates the stabilizing propensity of LMNG towards membrane proteins,  
1162 already described in the literature (see the introduction). All four proteins investigated here  
1163 are thermally stabilized in LMNG compared to DDM. The temperature shift is very large,  
1164 both for FhuA (+11 °C), a very stable  $\beta$ -barrel protein, and for SpNOX (+13 °C), a fragile  $\alpha$ -  
1165 helical protein. For the flexible BmrA, temperature shifts between 4 and 13 °C are observed  
1166 depending on the presence or absence of ligands. For bR, the gain is 6 °C. SpNOX solubilized  
1167 and partially purified in LMNG shows a significantly decreased content of aggregates,  
1168 corresponding to an increased stability. The reduced kinetics of limited proteolysis in LMNG  
1169 compared to DDM for the Apo and the Vi-inhibited forms of BmrA, expected to represent the  
1170 open and closed conformations, could have suggested some restricted flexibility of the  
1171 protein. However, the protein is more active in LMNG as compared to DDM thereby  
1172 indicating that the flexibility required for ATPase activity is not impaired in LMNG. Indeed,  
1173 in LMNG, BmrA seems to be more prone to reach or stabilize the closed conformation which  
1174 is required to get a high ATPase activity. Hence, BmrA is in a more stable and perhaps, more  
1175 physiologically relevant conformations in LMNG as compared to DDM. Restricted protein  
1176 flexibility is also suggested for bR, which UV-visible spectrum displays a maximum  
1177 absorption peak close to that of the constrained trimer in the purple membrane, far from that  
1178 of the more flexible monomer in DDM. These results are in the line with the restraint of  
1179 protein inter-conversion dynamics measured for GPCRs. The reduced dynamics of proteins  
1180 may be related to the strong binding of the LMNG correlated to its low CMC, deriving from  
1181 the connection of the two hydrophobic tails in its structure. As for BmrA, SpNOX displays an  
1182 increased specific activity in LMNG compared to DDM, which may reflect a significantly  
1183 higher proportion of inactive protein in DDM compared to LMNG, or be related to a  
1184 difference in SpNOX flexibility, or to the stabilization of some conformations.

1185

1186 **On the assembly of LMNG-membrane proteins**

1187 The solubilized membrane proteins investigated in this work have the same quaternary  
1188 structure in LMNG as in their usual detergent: FhuA, SpNOX, and bR, are monomeric and  
1189 BmrA is dimeric. In the case of SpNOX, dimers of the main species –probably inactivated  
1190 forms– are detected in LMNG, for typically 10%, the proportion varying slightly depending  
1191 on the purification. These species are also detected in DDM. In the AUC experiments where  
1192 LMNG concentration was voluntarily raised to 24 mM, FhuA and SpNOX association states  
1193 were not affected. The amount of bound detergent did not vary too, despite rod micelles of  
1194 LMNG are expected at these large concentrations. This behavior was not anticipated. We  
1195 previously investigated fluorinated surfactants (for reviews, see [8,16,17]), and, in a series of  
1196 surfactants whose polar head size was modulated by the presence of one, two, or three  
1197 glucose moieties, homogeneous complexes with bR and cytochrome *b<sub>6</sub>f* were obtained only  
1198 with the compounds that form homogeneous micelles, *i.e.* the surfactants with the more  
1199 voluminous head. In the surfactant forming long rods, with only one glucose, the membrane  
1200 proteins were soluble and active, but formed heterogeneous detergent protein complexes. We  
1201 anticipated it could be related to the heterogeneity in the amount of bound detergent [100].  
1202 Other fluorinated surfactants with relatively small head groups (lactoside, maltoside,  
1203 octaethylene glycol ether) formed large and polydisperse, probably rod-like assemblies, and  
1204 indeed formed also large and heterogeneous complexes with membrane proteins (refs in  
1205 [100]). By contrast, the fluorinated surfactant F-TAC (now commercialized by Calixar),  
1206 which has a voluminous polymeric hydrophilic head, was shown to assemble into small  
1207 micelles and form well defined complexes with membrane proteins. The present work on  
1208 LMNG shows that large size of surfactant micelle does not necessarily translate into protein  
1209 detergent complex heterogeneity.

1210

1211 **On the assembly of LMNG-membrane proteins: bound detergent**

1212 In the case of FhuA, previous measurements determined 1.2 g of bound DDM per g of  
1213 protein, using SEC in the presence of radiolabeled DDM [81]. This value is close to 1 and 1.2  
1214 g g<sup>-1</sup> determined in the present work for the protein solubilized in LMNG by AUC and SEC-  
1215 LS. For BmrA, the amount of bound LMNG (0.6 g g<sup>-1</sup>) we determined by combining *s*- and  
1216 *R<sub>H</sub>*-values, is lower but in the same order of magnitude than that determined for bound DDM  
1217 by the same technique (0.9 g g<sup>-1</sup>)[101]. A similar trend, in the comparison of DDM and  
1218 LMNG bound to BmrA, was reported using MALDI/TOF for the evaluation of bound  
1219 detergent [7]. These estimates of bound DDM to BmrA are much lower than that determined  
1220 by SEC in the presence of radiolabeled DDM (1.5 g g<sup>-1</sup>)[89], which emphasizes the difficulty  
1221 to determine precisely bound detergent. Nevertheless, these comparisons presented in Table 6,  
1222 show that the amounts of bound detergent are similar whether the protein is solubilized in  
1223 DDM or LMNG. It means that the amount in mol/mol is nearly twice for DDM than for  
1224 LMNG, and it is possible that the amount (in g g<sup>-1</sup>) of bound LMNG is slightly less compared  
1225 to that of DDM.

1226

1227

1228 **Table 6. Bound LMNG and DDM to FhuA, BmrA, and SpNOX**

1229

Protein	detergent	Bound detergent g/g	mol/mol*	technique	reference
FhuA	DDM	1.2	188	SEC/[ <sup>14</sup> C]DDM	[81]
FhuA	LMNG	1.0	80	AUC	present work
FhuA	LMNG	1.2	95	SEC-LS	[81]

1237

1238	BmrA	DDM	0.9	234	AUC+ SEC-LS	[101]
1239	BmrA	DDM	1.3-1.6	388-416	MALDI/TOF	[7]
1240	BmrA	DDM	1.5	390	SEC/[ <sup>14</sup> C]DDM	[89]
1241						
1242	BmrA	LMNG	0.6	79	AUC+ SEC-LS	present work
1243	BmrA	LMNG	1.1-1.3	145-171	MALDI/TOF	[7]
1244						
1245	SpNOX	LMNG	1.6	77	AUC+ SEC-LS	present work
1246	* considering FhuA and SpNOX as monomers, and BmrA as a dimer.					

1247  
1248

### On the crystallization of LMNG-membrane protein

1250 The high resolution structures of several GPCRs were obtained thanks to purification in  
1251 LMNG (cf Introduction) followed by crystallization in mesophase, a process in which the  
1252 membrane protein in the crystal is stripped off from its bound detergent [102]. Crystallization  
1253 in LMNG using vapor diffusion was described for *e.g.* the cytochrome *b<sub>6</sub>f* complex [26], the  
1254 TatC core of the twin-arginine protein transporter [50], an ABC transporter lipid-linked  
1255 oligosaccharide flippase [41], a borate efflux transporter [51], and a plant voltage-gated two-  
1256 pore channel [52]. FhuA is yet another one. One of our motivations, in crystallizing this well  
1257 described protein, was to check whether the ability to obtain crystals was prevented by the  
1258 presence of large LMNG assemblies in the membrane protein sample. It is clearly not the  
1259 case, and on the contrary, comparing the behavior of the protein in crystallization screens, we  
1260 observed that the number of initial hits was larger when the protein was solubilized in LMNG  
1261 when compared to the classically used DDAO. The space group of crystals of FhuA grown in  
1262 LMNG is not the same as that of the protein grown in DDAO. However, this is not  
1263 remarkable, as the same space group is found when the protein is crystallized in LDAO.

1264

1265 To summarize, the interest of structural biologists for LMNG is very strong, since it stabilizes  
1266 noticeably most membrane proteins compared to other detergents. Due to its low desorption  
1267 kinetics, it would stabilize membrane proteins even in sub-CMC concentrations. It forms  
1268 large rod assemblies at concentration above the sub mM range, but, for the examples we  
1269 investigated, the solubilized proteins remain as homogeneous as in usual detergents, with  
1270 similar amounts of bound detergent, and the presence of the large LMNG micelles does not  
1271 prohibit membrane protein crystallization using vapor diffusion or mesophase.

1272

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1288

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