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## 1 Functional Characterization of Cell-Free Expressed OprF Porin from 2 *Pseudomonas aeruginosa* Stably Incorporated in Tethered Lipid 3 Bilayers

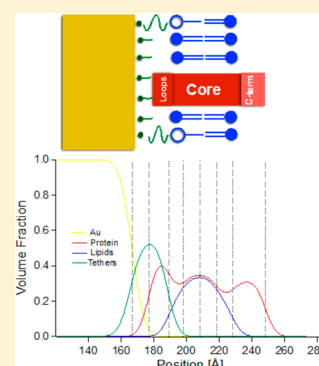
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8 **S** Supporting Information

9 **ABSTRACT:** OprF has a central role in *Pseudomonas aeruginosa* virulence and thus provides a  
10 putative target for either vaccines or antibiotic cofactors that could overcome the bacterium's  
11 natural resistance to antibiotics. Here we describe a procedure to optimize the production of  
12 highly pure and functional OprF porins that are then incorporated into a tethered lipid bilayer.  
13 This is a stable biomimetic system that provides the capability to investigate structural aspects  
14 and function of OprF using and neutron reflectometry and electrical impedance spectroscopy.  
15 The recombinant OprF produced using the optimized cell-free procedure yielded a quantity of  
16 between 0.5 to 1.0 mg/mL with a purity ranging from 85 to 91% in the proteoliposomes. The  
17 recombinant OprF is capable of binding IFN- $\gamma$  and is correctly folded in the proteoliposomes.  
18 Because OprF proteins form pores the biomimetic system allowed the measurement of OprF  
19 conductance using impedance spectroscopy. The neutron reflectometry measurements showed  
20 that the OprF protein is incorporated into the lipid bilayer but with parts of the protein in both  
21 the regions above and below the lipid bilayer. Those structural aspects are coherent with the  
22 current assumed structure of a transmembrane N-terminal domain composed by eight stranded  
23 beta-barrels and a globular C-terminal domain located in the periplasm. Currently there are no crystal structures available for  
24 OprF. The experimental model system that we describe provides a basis for further fundamental studies of OprF and particularly  
25 for the ongoing biotechnological development of OprF as a target for antibacterial drugs.



### 26 ■ INTRODUCTION

27 *Pseudomonas aeruginosa* is a human opportunistic pathogen  
28 responsible for severe nosocomial infections of hospital patients  
29 who are difficult to treat due to the resistance of *P. aeruginosa* to  
30 most of the common antibiotics.<sup>1</sup> It has been hypothesized  
31 since the 1980s that the low permeability of the outer  
32 membrane of *P. aeruginosa* is responsible for such resistance  
33 to antibiotics,<sup>2</sup> which has been confirmed by more recent  
34 studies which showed that *P. aeruginosa* strains are resistant to  
35 most of the common antibiotics used currently in the clinic.<sup>3–6</sup>  
36 The outer membrane of *P. aeruginosa* contains an abundance of  
37 the protein OprF, which is a 38 kDa porin with a large diameter  
38 conducting pore. The permeability of the outer membrane is  
39 thought to be controlled by the OprF, which is the major  
40 nonspecific outer-membrane porin, but with the paradox that  
41 OprF produces a large channel that allows only a slow diffusion  
42 of various solutes.<sup>8</sup> That paradox is explained by the existence  
43 of an open and a closed conformer of OprF in the outer  
44 membrane of wild-type *P. aeruginosa* strains, with a relatively  
45 high proportion of OprF being the nonconducting closed  
46 conformer.<sup>7</sup> A proposed model for the OprF envisages a high  
47 conductance conformer in which the C-terminal of the protein  
48 is contained in the membrane, and a low conductance  
49 conformer in which the C-terminal is located in the periplasmic

space. A functional fractionation technique confirmed that the  
50 open-channel conformer composed only 4.2–5.3% of the OprF  
51 population.<sup>8</sup> Furthermore, it is thought that the closed-channel  
52 conformer has a role in anchoring the peptidoglycan layer to  
53 the outer membrane via its C-terminus, whereas the C-terminus  
54 inserts into the outer membrane to form the open-channel  
55 conformer.<sup>8</sup>

OprF also has a role in the interaction with a variety of  
56 components of the host cells such as peptidoglycans for  
57 adhesion to and invasion of the cell, gamma interferon for  
58 evasion of the host immune system.<sup>9</sup> These interactions result  
59 in the stimulation of the expression of factors involved in the  
60 quorum-sensing dependent virulence. This includes the  
61 secretion of ExoT and ExoS toxins through the type III  
62 secretion system (T3SS) and lectin genes including LecB  
63 complex responsible for the inhibition of ciliary beating in  
64 cystic fibrosis.<sup>10</sup>

Recent studies have demonstrated that in addition to the role  
65 of the closed-channel conformer in the interaction with  
66 peptidoglycans, OprF may impact the biogenesis of outer  
67

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70 membrane vesicles (OMV), which is a process known to  
71 package proteins, small molecules, and nucleic acids for  
72 delivering them to the host cells.<sup>11,12</sup> These vesicles are a  
73 common process used by many bacteria for communicating  
74 from cell-to-cell and for allowing the trafficking of hydrophobic  
75 signals within a bacterial population. Wessel et al.<sup>11</sup> recently  
76 demonstrated that inactivation of OprF results in the increase  
77 of vesicle formation and in the *P. aeruginosa* quinolone signal  
78 (PQS), which is a process known to be required for outer  
79 membrane vesicle (OMV) formation. It was demonstrated that  
80 an OprF mutant strain made by a homologous recombination  
81 resulted in a decrease of virulence of *P. aeruginosa*, in an  
82 absence of adhesion to animal cells and in the production of  
83 some virulence factors.<sup>13</sup> Through these pathways OprF is  
84 directly involved in biofilm formation and plays a role as a  
85 sensor of the host immune system.<sup>14</sup>

86 OprF has a central role in the biology of *P. aeruginosa* and  
87 thus provides a putative target for either vaccines or antibiotic  
88 cofactors that could overcome the bacterium's natural  
89 resistance to antibiotics. However, ongoing research is limited  
90 currently because no crystallographic structure of OprF is  
91 available and no robust OprF/biomimetic membrane system  
92 has been described for stable measurements of OprF function.  
93 Nonetheless, some insights can be gained from the molecular  
94 dynamics simulation of OprF based on the crystal structure of  
95 the homologous protein OmpA of *Escherichia coli*.<sup>15,16</sup>  
96 Moreover, all existing measurements of OprF have utilized a  
97 black lipid membrane formed in conjunction with hexane or  
98 pentane.<sup>7</sup> The lipids used to form such black lipid membranes  
99 include diphytanoylphosphatidylcholine,<sup>17,10</sup> oxidized choles-  
100 terol,<sup>18,4</sup> and egg lecithin.<sup>4</sup> Even though such a "solvent-free"  
101 black lipid membrane has advantages over a "painted" black  
102 lipid membrane, the surface for such membranes is only around  
103 several hundreds of micrometers in diameter and the  
104 membrane is stable for only a few hours.<sup>19</sup>

105 We have recently developed and optimized a cell-free  
106 expression system capable to overcome the problem of classical  
107 overexpression systems that cannot provide sufficient yields for  
108 functional and structural studies of highly complex membrane  
109 proteins.<sup>20,21</sup> This *E. coli* based cell-free expression system is  
110 capable of producing milligram amounts of functional  
111 membrane proteins of different complexities and from diverse  
112 origins. In this paper, we used our optimized cell-free  
113 expression system to obtain, in a one-step reaction,  
114 recombinant proteoliposomes containing the OprF porin in a  
115 native conformation. We then incorporated those functional  
116 OprF porins by fusing those proteoliposomes into preformed  
117 tethered lipid bilayer membranes. Such tethered lipid bilayer  
118 membranes provide a system to incorporate and study  
119 membrane proteins that is more stable than the classical  
120 black lipid bilayer.<sup>33</sup> We used this system to determine the low-  
121 resolution structure of the OprF conformation within the  
122 membrane using neutron reflectometry (NR). The function of  
123 the OprF incorporated into those supported lipid bilayer  
124 membranes was confirmed by measuring the channel  
125 conductance. These results provide the basis for further  
126 fundamental studies of OprF and particularly for the ongoing  
127 biotechnological development of OprF as a target for  
128 antibacterial drugs.

## 129 ■ MATERIALS AND METHODS

130 **Construction of the Prokaryotic Recombinant OprF Ex-**  
131 **pression Vector.** The recombinant vector pIVEX2.4-OprF with an

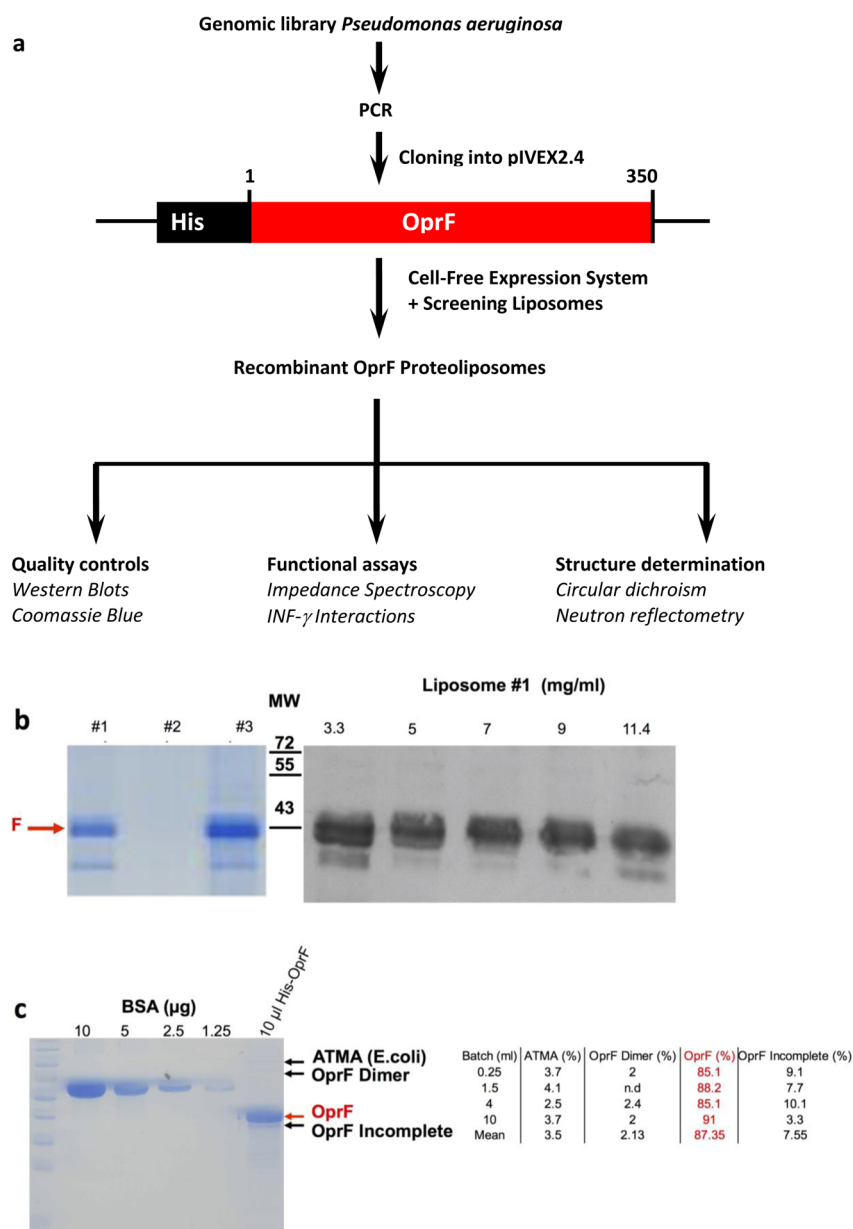
N-terminal His Tag was constructed by cloning the PCR-amplified  
132 OprF gene from genomic DNA of *Pseudomonas aeruginosa* (forward  
133 primer, 5'-GGAATTCCATATGAAACTGAAGAACACCTTAG-3';  
134 reverse primer 5'-GTAGAAGCTGAAGCCAAGTAACTCGAG-  
135 TAACGC-3') into the expression vector pIVEX2.4d (Roche  
136 Diagnostics). The resulting PCR fragment was isolated, purified  
137 (QIAquick gel extraction kit, Qiagen), digested with NdeI and XhoI  
138 (Roche Diagnostics) and ligated (Rapid DNA ligation kit, Roche  
139 Diagnostics) into the digested pIVEX2.4d plasmid to obtain the  
140 pIVEX2.4-OprF recombinant plasmid, which was checked by  
141 sequencing (LGC Genomics).  
142

**Production and Purification of OprF in a Cell-Free**  
**Expression System.** The expression of the full length OprF from  
143 *P. aeruginosa* was performed using a cell-free expression system<sup>20,21</sup>  
144 from a lysate purchased from Syntheliss SAS. Briefly, the recombinant  
145 OprF membrane protein was synthesized in the presence of synthetic  
146 liposomes (liposome #1: cholesterol, 1,2-dioleoyl-*sn*-glycero-3-phos-  
147 phocholine (DOPC), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine  
148 (DOPE), 1,2-dimyristoyl-*sn*-glycero-3-phosphate (sodium salt) molar  
149 ratio [2-4-2-2]); liposome #2: HSPC, cholesterol, DSPE-mPEG2000  
150 molar ratio [56-39-5]; liposome #3: cholesterol, DOPC, DOPE,  
151 DOPE-mPEG2000 molar ratio [2-4-3.8-2]) directly added into the  
152 cell-free reaction mix. The plasmid pIVEX2.4-OprF and the synthetic  
153 liposomes were added at the final concentrations of 24  $\mu\text{g}/\text{mL}$  and 5  
154  $\text{mg}/\text{mL}$ , respectively. Protein was expressed for 16 h at 25 °C and 300  
155 rpm in a 15 mL tube for a 4 mL batch. The solution was then  
156 centrifuged at 30 000g for 20 min at 4 °C to pellet the  
157 proteoliposomes. The pellet was washed twice for 30 min at 4 °C  
158 with NaCl 5 M. The protein concentration during the different steps  
159 of the purification procedure was determined using a Coomassie gel  
160 with comparison to BSA as a standard. Analysis of the purity of OprF  
161 recombinant proteoliposomes was performed by Matrix-assisted laser  
162 desorption/ionization-time-of-flight (MALDI-TOF) mass spectroscopy  
163 (MS) after scale-up of the cell-free expression reactions and  
164 trypsin digestion of the stained bands.  
165

**Interferon- $\gamma$  Binding.** Binding assays of OprF proteoliposomes to  
166 INF- $\gamma$  were performed using 2 protocols. In the first protocol,  
167 MaxiSorp 96-well plates (Nunc MaxiSorp, 442404) were coated for 6  
168 h at 4 °C with INF- $\gamma$  (Abcam: ab9659, 1  $\mu\text{g}$  at 1  $\text{mg}/\text{mL}$ ). The second  
169 protocol was to coat MaxiSorp 96-well plates with OprF  
170 proteoliposomes. The plates coated with OprF proteoliposomes  
171 were then incubated with INF- $\gamma$  (0.01  $\text{mg}/\text{mL}$ ) for 2 h at 37 °C in  
172 PBS. In those plates, the presence of bound INF- $\gamma$  was detected using  
173 a mouse INF- $\gamma$  primary antibody (Abcam: ab9657) and an antimouse  
174 IgG-HRP labeled antibody (GE-Healthcare: NA9310 V). The plates  
175 coated with INF- $\gamma$  were incubated with OprF proteoliposomes (0.15  
176  $\text{mg}/\text{mL}$ ) for 2 h at 37 °C in PBS. The bound OprF was revealed by  
177 using an anti-His-HRP antibody (Sigma, A7058). Protein interactions  
178 were detected after coloration using Tetramethylbenzidine (Sigma,  
179 T0440) for 10 min at room temperature and absorbance was measured  
180 at 450 nm. In separate experiments to test nonspecific binding of the  
181 antibody, BSA was used as a control to replace INF- $\gamma$ .  
182

**Circular Dichroism.** OprF proteoliposomes (0.5  $\text{mg}/\text{mL}$ ) were  
183 suspended in a 10 mM phosphate buffer (pH 7.4) in a Quartz Suprasil  
184 absorption cell (0.1 cm) and the absorbance spectrum was measured  
185 at a bandwidth of 10 nm in the range between 190 and 300 nm with a  
186 scan-rate of 10 nm/min (one measurement each nanometre), using a  
187 commercial Circular Dichroism dispersion meter (Jasco, J-810). The  
188 measurements were performed at 25 °C under a flow of nitrogen (10  
189 l/min, 1.5 bar). The blank negative control was a preparation of  
190 proteoliposomes as for the complete OprF but without DNA during  
191 production. The instrument produced a single spectrum after  
192 deconvolution and subtraction of the OprF proteoliposome and  
193 blank negative control spectra using the K2D2 model.<sup>22</sup>  
194

**Incorporation of recombinant OprF into tethered lipid**  
**bilayer systems.** A tethered lipid bilayer membrane (tLBM) is a  
195 planar membrane composed of mobile lipids and molecules tethered  
196 to a solid planar substrate that provide a realistic model of a  
197 biomembrane. The tLBM is stable due to the chemical attachment to  
198 the substrate.<sup>23</sup> Moreover, the tethering molecules provide a physical  
199



**Figure 1.** (a) Flow-chart of the production and characterization of the recombinant OprF protein. (b) Small-scale optimization for OprF production. The left panel shows Coomassie blue staining of OprF produced in the presence of liposomes with different lipidic composition (#1, #2, and #3). The right panel shows Western blotting (antiHis antibody) of OprF synthesized in liposome #1 at different concentrations. (c) Coomassie blue staining of OprF liposomes after scale-up production and tabulated results of the MALDI-TOF-MD analysis (right panel). ATMA is an abbreviation for  $Mg^{2+}/Ni^{2+}$  transporting P-type ATPase.

202 separation between the bilayer and the planar substrate, which forms a  
203 hydrated reservoir space between the bilayer and the substrate. This  
204 space enables greater mechanical flexibility and fluidity to the  
205 membrane and, analogous to the intracellular space of a cell, provides  
206 a volume that may serve as a reservoir for ions involved in transport  
207 across the membrane or to accommodate transmembrane protein  
208 residues.<sup>24</sup>

209 The tLBM system<sup>33</sup> used was obtained by coadsorption, onto a  
210 gold-coated silicon block, of benzyl disulfide undecaethylene glycol  
211 phytanol (DLP) and benzyl disulfide tetraethylene glycol polar spacer  
212 molecules dissolved in ethanol at a concentration of 400  $\mu$ M and  
213 mixed with a ratio of 10:90.<sup>25</sup> The surface area of the tethered lipid  
214 bilayer membrane was 2.1 mm<sup>2</sup> for the Electrical Impedance  
215 Spectroscopy (EIS) and around 15 cm<sup>2</sup> for the NR experiments. On  
216 the 2.1 mm<sup>2</sup> area of the EIS electrode, the tLBM was completed by

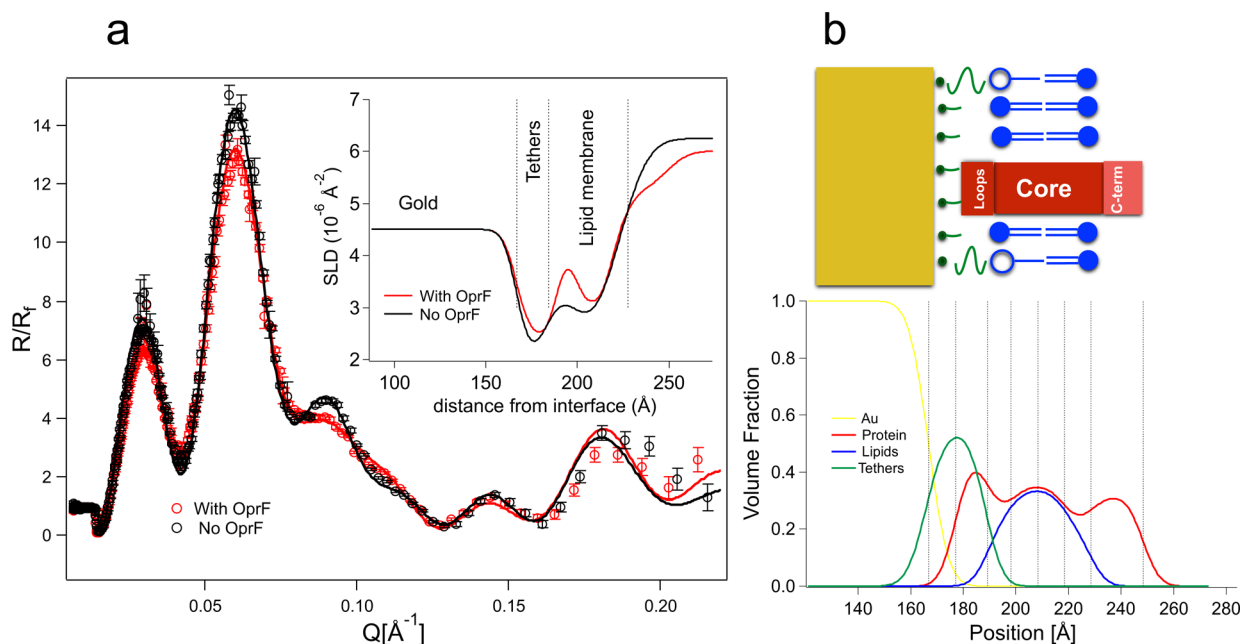
using a 3 mM ethanolic solution of 30:70 (mole ratio) of 217  
glycerodiphtanylether:diphtanyletherphosphatidylcholine (AM199). 218

For the NR experiment, due to the large surface area ( $\sim$ 15 cm<sup>2</sup>) of 219  
the Si substrate (and of the significant volumes involved) a 220  
commercially available DMPC lipid was used to complete the lipid 221  
bilayer with the technique of fast solvent exchange instead of the lipid 222  
AM199 used for the impedance spectroscopy essay. 223

#### Structural Measurements Using Neutron Reflectometry.

224 Reflectivity measurements were performed on the D17 reflectometer 225  
at the Institut Laue Langevin (Grenoble, France) in the time-of-flight 226  
mode, average resolution  $dQ/Q = 7\%$ ,  $\lambda$  range between 2 and 30  $\text{\AA}$ , 227  
with two incoming angles of 0.8 and 3.2°. The solid–liquid interface 228  
cell was oriented vertically and kept in position while changing 229  
solvents. Measurements were performed at the silicon–gold/water 230  
interface with the beam transmitted through the silicon block. The 231  
data taken at two contrasts  $D_2O$  and  $H_2O$  and were analyzed with 232





**Figure 2.** (a) Reflectivity profiles  $R/R_f$  (symbols) obtained for the tethered lipid bilayer system in  $D_2O$  without (black) and with (red) incorporation of the OprF. The lines correspond to the best fit to the model. In the inset, the scattering length density profiles corresponding to the  $D_2O$  contrast are shown. (b) Volume occupancy profile of the different molecular component present in the system. The blue shape with solid head corresponds to the DMPC or the AM199 free lipids. The blue shape with empty head corresponds to the part of the tethering molecule that colocalizes with the lipid bilayer. The green shapes correspond to the part of the tethering molecule that are between the lipid bilayer and the gold substrates. Details are described in ref 25.

233 Motofit<sup>26</sup> allowing simultaneous fitting of data sets from the same  
234 sample under different contrast conditions.

### 235 Conductance of OprF Using Impedance Spectrometry.

236 Electrical Impedance Spectroscopy (EIS) applied to planar lipid  
237 bilayer membrane allows the measurement of the conductance of  
238 incorporated ion channels.<sup>27</sup> We performed EIS measurements on the  
239 tLBM using the TethaPod system (SDX Tethered Membranes,  
240 Australia). The TethaPod is a swept frequency ratiometric impedance  
241 spectrometer that determined the low-voltage (20 mV) AC impedance  
242 spectroscopy measurement of basal membrane conduction of the  
243 tLBM system. A sequential 20 mV excitation was applied over the  
244 frequencies 1000, 500, 200, 100, 50, 20, 10, 5, 2.5, 1.25, 0.5, 0.25, and  
245 0.125 Hz.<sup>28</sup> For those measurements, recombinant OprF was  
246 incorporated into the tLBM system by the fusion of proteoliposomes  
247 (1 mg/mL). The negative control was empty liposomes produced in  
248 the same way as for the OprF proteoliposomes. The fusion of OprF  
249 proteoliposomes or empty liposomes was assisted by Dodecyl- $\beta$ -D-  
250 maltoside (DDM) used at a concentration below one-fifth of its critical  
251 micellar concentration (CMC). At that low concentration, the DDM  
252 did not alter the conductance or capacitance of the tethered lipid  
253 bilayers. The recording solution for the experiments contained NaCl  
254 (140 mM) and HEPES (10 mM) at pH7.

## 255 ■ RESULTS

256 **Expression of Recombinant OprF.** We expressed OprF  
257 using an optimized cell-free expression system in the presence  
258 of synthetic liposomes at different lipid compositions to  
259 enhance the yields and to provide an appropriate lipid  
260 environment for the OprF membrane protein (Figure 1).  
261 Compared to the other liposome preparations, the liposome  
262 composition #1 (DOPC:DOPE:DMPA:cholesterol; 4:2:2:2)  
263 used at 3.3 mg/mL provided the best yield of expressed OprF  
264 protein, with a yield ranging from 0.5 to 1.0 mg/mL (Figure  
265 1b). Variations in liposome concentrations, sucrose, or glycerol  
266 have no influence on protein expression (Figure S1). The  
267 expression efficiencies and purities of recombinant OprF

268 proteoliposomes were analyzed by Coomassie Blue staining (Figure 1c, left panel) and Western blotting. Scale-up  
269 production and purification resulted in highly pure recombinant  
270 OprF proteoliposomes with purity ranging from 85 to 91%.  
271 Analysis by MALDI-TOF-MS after scale-up production and in-  
272 gel trypsin digestion confirmed that the OprF membrane  
273 protein represented the main population either as in monomer  
274 or in dimer conformations (Figure 1c right panel). Circular  
275 dichroism experiments indicated that, after subtraction of the  
276 signal from empty liposomes, OprF embedded into the lipid  
277 bilayer comprised (38 ± 3)% of beta sheets and (15 ± 3)% of  
278 alpha helices (Figure S2) in accordance with the data obtained  
279 by Sugawara (40% beta sheets and 18%  $\alpha$  helix).<sup>8</sup>  
280

### 281 Structural Study of OprF Incorporated in Planar Biomimetic Membranes.

282 We performed NR experiments  
283 in a tLBM environment in order to quantify and characterize  
284 the transfer and incorporation of OprF from the liposomes into  
285 the tLBM. These measurements provide low-resolution  
286 structural information on the lipid/protein system that  
287 confirms the incorporation of OprF into the lipid bilayer.  
288 This is important because no conclusive high-resolution  
289 crystallographic study has yet been done on this protein.

290 The reflectivity profiles measured on the tLBM before and  
291 after the injection of the OprF proteoliposome ( $D_2O$  contrast)  
292 are shown in Figure 2a. We performed the measurements at  
293 two contrasts by replacing the  $D_2O$  present in the measuring  
294 cell with  $H_2O$ . The complete set of measurements at the two  
295 contrasts is shown in the Supporting Information. The data in  
296 Figure 2a are displayed in terms of ratio between the reflectivity  
297 and the Fresnel reflectivity (FR) to emphasize the differences.  
298 The addition of the proteoliposomes causes significant changes  
299 in the  $Q$  range between 0.05 and 0.10  $\text{\AA}^{-1}$ .

300 The analysis of the NR data was obtained by simultaneous  
301 corefinement of the NR curves obtained on the system at the

two different contrasts  $D_2O$  and  $H_2O$ . The model built to fit the reflectometry data is based on the closed conformer of the protein, which is the most probable conformation.<sup>10</sup> The protein spans across the lipid bilayer, which is subdivided into four layers (the head and tail regions of the two leaflets). The intramembranous core of the protein is colocalized with the lipid bilayer<sup>29,30</sup> and it is modeled having a cylindrical shape occupying a volume fraction  $\varphi$  constant across the different slabs belonging to the lipid bilayer. Furthermore, two extra slabs with part of the protein were included in the model (the C-terminus and the extra-membranous loops) at the opposite side of the lipid bilayer (Table S1). The scattering length densities (SLDs) of each term were calculated based on the atomic composition of the protein and of the estimated volume of the amino acid by means of the Biomolecular Scattering Length Density Calculator.<sup>31</sup> The effect of D-H exchange of labile hydrogen atoms was included in the calculations. In the simultaneous corefinement of the NR curves the SLD of the protein was set to that corresponding to the given contrast. The data were analyzed as follows: (i) an hypothesis on the structural properties of the system was made; (ii) the system was divided in slabs each characterized by a thickness ( $T$ ), a roughness ( $\sigma$ ); a SLD ( $\rho$ ), and a volume fraction of solvent in the layer ( $\Psi$ ) (Figure S3); (iii) a SLD profile was reconstructed based on the average in-plane molecular composition and structural property of each slab; (iv) the properties of each slab were optimized and refined by fitting the reflectivity obtained from the reconstructed SLD profile to the measured reflectivity.

The best-fitting model that describes the experimental reflectivity profiles after the injection of proteoliposomes in the solution indicates that the majority of the OprF protein is incorporated into the bulk of the lipid bilayer but with parts of the protein in both the regions above and below the lipid bilayer. That best-fitting model describes the closed OprF channel conformer that contains a transmembrane N-terminal domain composed by eight stranded beta-barrels and a globular C-terminal domain located in the periplasm.

To verify that the best-fitting model uniquely represents the data, we tested different structural hypotheses to build the SLD profile such as (i) no incorporation of protein; (ii) adsorption of protein on top of the bilayer; (iii) incorporation of the protein in the lipid bilayer without the extra-membranous layers. All the models based on these different hypotheses failed to properly fit the reflectivity data and resulted in significantly increased values of  $\chi^2$  (see the Supporting Information). According to our analysis it is therefore necessary to include parts of the protein into the lipid bilayer as well as at the two extra-membranous region adjacent to the bilayer to properly account of the reflectivity data. This configuration is compatible with the natural conformation hypothesized for OprF.

In Figure 2b, the SLD profiles refined from the fitting analysis are represented in terms of volume occupancy of the gold surface, the tethering molecules, the lipids and the proteins. The volume occupancies were obtained thanks to the simultaneous corefinement of the reflectivity curves at different contrasts. As a matter of fact, the change in the isotopic composition of the liquid phase helps to distinguish the scattering contributions due to the lipids and proteins from those due to the hydration molecules present in the bilayer. In Table 1, we list the optimized parameters obtained for the lipid system before and after the OprF injection. This incorporation manifests as an increase of the SLD of the tail region from

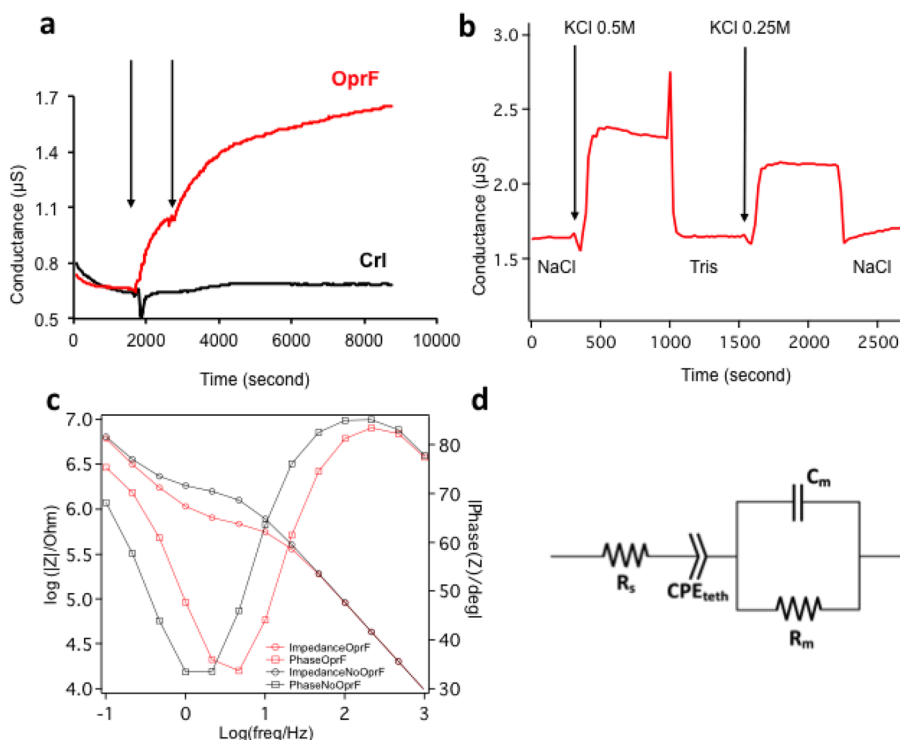
**Table 1. Model Parameters Obtained for the Tethered Lipid Bilayer System in  $D_2O$  before and after the Incorporation of the OprF Protein<sup>a</sup>**

layer	thickness (Å)	SLD ( $\times 10^{-6} \text{Å}^{-2}$ )	solvent (%)	roughness (Å)
before OprF				
interlayer	17 ± 2	0.6 ± 0.1	23 ± 7	8 ± 5
head inner	8 ± 2	1.2	66 ± 20	11 ± 5
tails inner	13 ± 2	-0.35	40 ± 5	11 ± 5
tails outer	13 ± 2	-0.37	40 ± 5	11 ± 5
head outer	8 ± 2	1.8	64 ± 20	11 ± 5
after OprF				
interlayer 1	10 ± 2	0.6	34 ± 10	6 ± 5
interlayer 2	12 ± 2	2.1 (1.4) ± 0.1 <sup>b</sup>	8 ± 10	7 ± 5
head inner	9 ± 2	2.3 (1.4) ± 0.1 <sup>b</sup>	52 ± 20	7 ± 5
tails inner	10 ± 2	1.7 (0.9) ± 0.1 <sup>b</sup>	31 ± 9	7 ± 5
tails outer	10 ± 2	1.7 (0.9) ± 0.1 <sup>b</sup>	31 ± 9	7 ± 5
head outer	10 ± 2	2.7 (1.9) ± 0.1 <sup>b</sup>	58 ± 20	7 ± 5
extra mem. layer	20 ± 5	3.5 (2.0)	77 ± 10	9 ± 5

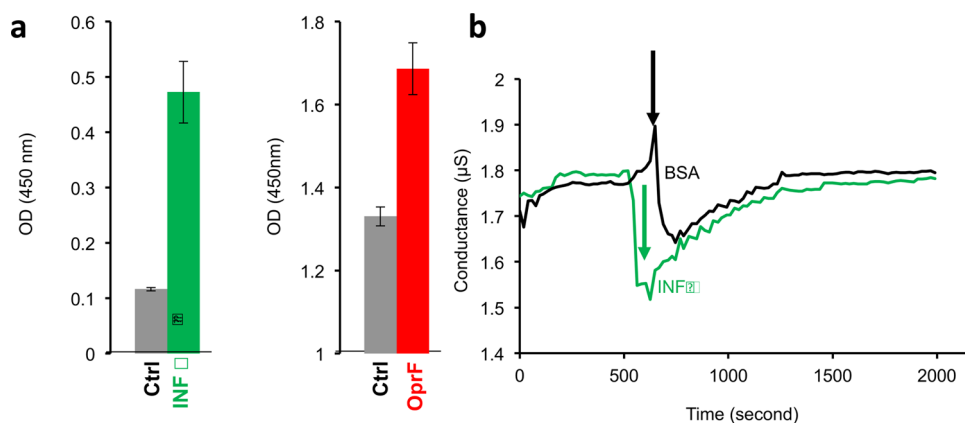
<sup>a</sup>The values without error were fixed or kept to their nominal value. Note that in the layers in which the OprF is present, we list two values of SLD in view of the presence of labile hydrogen atoms that exchange with deuterium. The first value corresponds to the  $D_2O$  contrast, whereas the value in parentheses corresponds to the  $H_2O$  contrast. The extra membranous layer is due to the presence of the C-terminus that protrudes outside the membrane. <sup>b</sup>The error on the SLD in of the bilayer in the presence of OprF is obtained by the fitted value of the volume fraction of the protein ( $\varphi$ ). Refer to Table S1.

approximately  $-0.3 \times 10^{-6} \text{Å}^{-2}$  to a positive value of  $0.9 \times 10^{-6} \text{Å}^{-2}$  ( $H_2O$  contrast) or  $1.7 \times 10^{-6} \text{Å}^{-2}$  ( $D_2O$  contrast). This value is compatible with the incorporation of OprF in the membrane with a volume fraction of  $\varphi = 0.56 \pm 0.03$ , that can be obtained directly by the fitted value of the SLD in this region (see Table S1). Furthermore, the two extra-membranous layers on the two side of the bilayer, extend for around 12 Å in the region of the tethering molecules, and around 20 Å in the solution. The incorporation is associated with a  $\sim 25\%$  reduction of the hydration of the lipid bilayer. In order to test the dependence of the fitting results from selected parameters, we tested how the quality of the fit varied with respect to the change of the volume fraction of the incorporated protein and the thickness of the layer (containing the C-term of OprF). The information can be found in Figures S7 and S8 and Tables S4 and S5.

**Impedance Spectroscopy.** The steady-state conductance of the tethered membrane system was measured using the Tethapod in the presence of recombinant OprF proteoliposomes and of the control (empty liposomes). The Tethapod system used an AC impedance spectroscopy technique that was adapted to the tethered lipid bilayer so as to provide a measurement of the conductance of pore-forming proteins.<sup>33</sup> Such impedance spectroscopy provides a sensitive measurement of the current flows across the membrane that are driven by the ion channels which were incorporated, in this case the OprF.<sup>32</sup> In Figure 3a, we see that the conductivity of the membrane with OprF incorporated ( $\sim 1.6 \mu\text{S}$ ) is greater than the control (tLBM alone). Furthermore, the experiment shown in Figure 3b indicates that the OprF is functioning as an ion channel. The incorporation of OprF in the presence of Na ions resulted in a steady-state conductance value around  $1.6 \mu\text{S}$ . The NaCl (140 mM) was replaced in a sequence of KCl (500 mM) then TrisCl (50 mM) then KCl (250 mM) and then a return to



**Figure 3.** (a) Steady-state conductance of the tethered membrane after the insertion of OprF proteoliposomes (at the times marked with the arrows) compared to the control membrane without OprF (black curve). The arrows correspond to injections of OprF proteoliposomes. (b) Steady-state conductance of the membrane previously modified with the incorporation of OprF, when the NaCl (140 mM) is replaced by KCl (500 mM), TrisCl (50 mM) and KCl (250 mM). (c) Bode plots of the tBLM before and after the incorporation of OprF. (d) Equivalent circuit model used to analyze the EIS measurements of the tBLM, for the resistance of the solutions ( $R_s$ ), the capacitance of the tBLM ( $C_m$ ), the resistance of the membrane to account for the OprF ion channels ( $R_m$ ), and a constant phase element to account for the impedance of the tether region below the tBLM ( $\text{CPE}_{\text{teth}}$ ). The parameters of the fitting are provided in Table S7. The EIS measurements were also fit with an alternative analytical equivalent circuit model, with the parameters of that alternative model provided in Table S6.



**Figure 4.** (a) Demonstration of the binding capacity of the recombinant OprF proteoliposomes to INF- $\gamma$  using ELISA assays where INF- $\gamma$  bound to coated OprF proteoliposomes (green bar) and OprF proteoliposomes bound to coated INF- $\gamma$  (red bar). (b) Binding of INF- $\gamma$  did not have a long-term effect on the conductance of OprF. There was a transient response, which was not different to the effect of BSA. (OD refers to optical density.)

399 NaCl (140 mM). The conductance was greatest with 500 mM  
 400 (2.3  $\mu\text{S}$ ) compared to 250 mM (2.1  $\mu\text{S}$ ) of KCl. The  
 401 conductance in TrisCl ( $\sim 1.6 \mu\text{S}$ ) was similar to that measured  
 402 in NaCl (140 mM). The only concentration-dependent  
 403 increase in the conductance was observed with KCl, whereas  
 404 no change in the conductance was observed with NaCl and  
 405 TrisCl at different concentrations.

406 In Figure 3c, we show the Bode plot of the tBLM before and  
 407 after the incorporation of OprF. The equivalent circuit model  
 408 used to fit the data is displayed in Figure 3d. This equivalent

circuit model was applied previously to this tBLM.<sup>28</sup> The  
 409 parameters of the equivalent circuit model obtained by the  
 410 fitting analysis are listed in Table S7 (Supporting Information).  
 411 By comparing the values obtained before and after the  
 412 incorporation of the OprF we see that only the membrane  
 413 resistance ( $R_m$ ) is affected, with the membrane resistance  
 414 reducing from  $\sim 1.5 \text{ M}\Omega$  to  $\sim 0.6 \text{ M}\Omega$  due to the permeation of  
 415 ions through its pores.  
 416

To confirm the veracity of the analysis, we also used an  
 417 alternative model, commonly used in the literature, based on an  
 418



analytical description of a tethered bilayer that contains natural or artificially induced defects, such as ion channels.<sup>33</sup> In agreement with the previous analysis, the results from fitting the alternative model indicated that the only significant difference between before and after the incorporation of the OprF is that the resistance of the membrane reduces from  $\sim 1.8$  to  $\sim 0.8$  M $\Omega$ . The comparison between the two analyses is reported in the Figures S9 and S10 and Tables S6 and S7.

**Binding Assays with Recombinant OprF Proteoliposomes.** We examined the binding capacity of recombinant OprF proteoliposomes to INF- $\gamma$  by ELISA assays. Those measurements of absorbance at 450 nm indicated that recombinant OprF and INF- $\gamma$  directly interact (Figure 4a). The control experiment with BSA showed that there was no nonspecific binding of the antibody.

We performed impedance spectroscopy (EIS) using the TethaPod in order to measure if the binding of INF- $\gamma$  interfered with the function of the OprF pore. The EIS showed that in the presence of INF- $\gamma$  we observed a slight decrease in the conductance of OprF followed by a rapid return to the original conductance level (Figure 4b). However, this response was not different to the effect of BSA on the conductance of the OprF. These results indicate that the binding of INF- $\gamma$  on the porin did not affect the channel function of OprF.

## DISCUSSION

It has been demonstrated that OprF was capable of binding directly to the cytokine INF- $\gamma$ , which is a probable molecular mechanism used by *Pseudomonas aeruginosa* to thwart the immune system activation of the host.<sup>9</sup> Our results confirm that the recombinant OprF we produced for the current measurements is capable of binding INF- $\gamma$ , which indicates the OprF retained its biological role and was most likely oriented in a natural configuration in the proteoliposomes. Those INF- $\gamma$  binding results were corroborated by circular dichroism measurements that identified that OprF was correctly folded in the proteoliposomes.

Furthermore, native OprF proteins form pores at the cytoplasmic membrane of the bacteria. The impedance spectroscopy experiments described in this article indicate that OprF protein embedded into the lipid bilayer was functional and not just simply associated with the lipid bilayer, as shown by the increase of the conductance of the tethered membrane compared to the control system.

These results are further reinforced by the low-resolution structural information obtained by NR, which revealed that a considerable amount of OprF proteins incorporated in the bilayer ( $\sim 50\%$  of the volume) after incubation with the proteoliposomes. This is a powerful technique for the study of planar model membranes systems with a resolution of a fraction of nanometer along the normal depth direction, and has been used to elucidate nanostructural details of planar lipid membrane/protein systems.<sup>34</sup> By averaging along the in-plane direction, NR provides a one-dimensional structural map in terms of the SLD profiles along the direction orthogonal to the surface. From the SLD profiles the distribution of the molecular components and their volume occupancy at the interface were determined.

Using neutrons as a probe provides the advantage of “gentle” interactions with the nuclei of the atoms, thus minimizing the risk of ionizing radiation damage for delicate biological materials. At the same time, the scattering contrast can be enhanced by the use of isotope substitution. This is particularly

relevant for hydrogenous materials such as lipids and protein in water because replacing hydrogen with deuterium atoms has dramatic effect on the contrast, but minimal effects on the samples. For systems immersed in liquid phases such as the biomimetic membranes studied here, changing the isotopic composition of the liquid allows the scattering contribution from the aqueous components and those coming by other interfacial component to be disentangled.

Neutron reflectometry gave a precise nanostructural characterization of the tethered bilayer system and identified differences occurring before and after the incubation with the OprF proteoliposomes. The precise modeling of the reflectivity data confirms that OprF is incorporated in the lipid bilayer in the predicted configuration. That is, the core of the protein is in the lipid bilayer with extra membranous parts above and below the lipid bilayer. Although the model is based on the most likely conformation of the incorporated OprF (the closed state conformer) with the C-terminus outside the membrane, we cannot exclude that some of the proteins are in the open conformation. In the open conformation, although the C-terminus is thought to become embedded in the intramembranous region,<sup>10</sup> extra-membranous loops are still present on both sides of the membrane. Furthermore, the structural information obtained by NR is averaged over a time of hours, in which the conformation of the porin fluctuates between the open and closed conformers. However, the results show that the protein is more extended on the side of the membrane pointing toward the solvent ( $\sim 20$  Å) compared to the side pointing toward the substrate ( $\sim 12$  Å). This might suggest a preferred orientation of the protein with the voluminous C-terminus preferentially oriented toward the solvent. For example, this orientation might be favored due to the larger space available in the direction of the solvent compared to the side closer to the substrate, where the C-terminus would compete for space with the tethered molecules. The incorporation of the OprF is linked also to a reduced hydration of the lipid bilayer, since the incorporation of the protein is accompanied by the displacement some water molecules from the lipid bilayer. In this regard it is important to note our tethered system formed by the DMPC is characterized by a low hydrated interlayer and a partially hydrated tail region. Further, it is important to note that our work demonstrates that in such conditions where the tethered bilayer presents a significant hydration of the tail region, important nanostructural information on incorporated OprF can be obtained by careful analysis of neutron reflectometry data.

## CONCLUSIONS

In summary, we showed that recombinant OprF, obtained with an optimized cell-free protocol, was incorporated into tethered lipid bilayers to yield a controllable biomimetic system constructed in vitro that was measurable at the nanoscale. The several complementary techniques we utilized together provide a unified set of data for an innovative approach to investigate the function of membrane proteins. This approach was used to study the function of OprF by combining low-resolution structural data from neutron reflectometry with functional measurements of OprF in a tethered lipid membrane environment. The cell-free protocol produces OprF that folds according to the characteristics of the native porins. This experimental approach we describe provides a robust means of incorporating recombinant OprF into tethered lipid bilayer membranes, with this system stable for long periods of time.

543 The system is adaptable for both large-area membranes, such as  
544 needed for structural measurements using neutron reflectom-  
545 etry, and for smaller-area membranes, such as needed for  
546 functional conductance measurements using electrochemical or  
547 electrophysiological measurements. These results demonstrate  
548 that the experimental model system that we describe also  
549 provides the basis for further fundamental studies of OprF and  
550 particularly for the ongoing biotechnological development of  
551 OprF as a target for antibacterial drugs.

## 552 ■ ASSOCIATED CONTENT

### 553 ● Supporting Information

554 The Supporting Information is available free of charge on the  
555 ACS Publications website at DOI: 10.1021/acs.lang-  
556 muir.7b01731.

557 Supporting materials, methods, and results (PDF)

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### 564 Author Contributions

565 <sup>†</sup>M.M. and L.G. contributed equally and are considered co-first  
566 authors. M.M., L.G., J.L.L., and D.K.M. designed the research.  
567 L.G. and L.L. produced recombinant OprF proteoliposomes.  
568 M.M., B.S., J.P.A., E.B.W., and D.K.M. performed neutron  
569 reflectometry experiments. L.G. and J.P.A. performed electrical  
570 impedance spectroscopy experiments. L.G. performed OprF  
571 binding and circular dichroism experiments. M.M. and E.B.W.  
572 analyzed and modeled the neutron reflectometry data. M.M.,  
573 L.G., J.P.A., E.B.W., J.L.L., and D.K.M. wrote the manuscript.

### 574 Notes

575 The authors declare no competing financial interest.

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