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1 **B2LiVe, a label-free 1D-NMR method to quantify the binding of amphitropic peptides or**
2 **proteins to membrane vesicles**

3

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14

15 **Summary**

16 Amphitropic proteins and peptides reversibly partition from solution to membrane, a key
17 process that regulates their functions. Experimental approaches, such as fluorescence and
18 circular dichroism, are classically used to measure the partitioning of amphitropic peptides and
19 proteins into lipid bilayers, yet hardly usable when the peptides or proteins do not exhibit
20 significant polarity and/or conformational changes upon membrane binding. Here, we describe
21 B2LiVe (*i.e.*, Binding to Lipid Vesicles), a simple, robust, and widely applicable NMR method
22 to determine the solution-to-membrane partitioning of unlabeled proteins or peptides. The
23 experimental strategy proposed here relies on previously described proton 1D NMR fast pulsing
24 techniques with selective adiabatic pulses. Membrane partitioning induces a large line
25 broadening leading to a progressive loss of protein signals, and therefore, the decrease of the
26 NMR signal directly measures the fraction of membrane-bound protein. The B2LiVe method
27 uses low polypeptide concentrations and has been validated on several membrane-interacting
28 peptides and proteins, ranging from 3 to 54 kDa, with membrane vesicles of different sizes and
29 various lipid compositions.

30

31 **Keywords**

32 Amphitropic proteins, peptides, solution to membrane partitioning, protein membrane binding,
33 affinity, partition coefficient, nuclear magnetic resonance, proton 1D NMR fast pulsing
34 techniques, selective adiabatic pulses

35

36 **Motivation**

37 Characterization of the interaction of peptides and proteins with lipid membranes is involved
38 in various biological processes and is often challenging for polypeptides which do not possess
39 intrinsic fluorophores, do not exhibit significant structural content changes, as well as for those
40 characterized by low affinities for membranes. To meet these challenges, we have developed a
41 simple and robust label-free NMR-based experimental approach, named B2LiVe, to measure
42 the binding of polypeptides to lipid vesicles. The experimental strategy relies on previously
43 described proton 1D NMR fast pulsing techniques with selective adiabatic pulses to excite the
44 amide resonances. B2LiVe is a label-free method based on the observation of amide hydrogen
45 nuclei which are naturally present in all protein and peptide backbones. Our results validate the
46 B2LiVe method and indicate that it compares well with established technics to quantify
47 polypeptide-membrane interactions. Overall, B2LiVe should efficiently complement the

48 arsenal of label-free biophysical assays available to characterize protein-membrane
49 interactions.

50

51 **In brief**

52 We describe a robust label-free NMR-based experimental approach (B2LiVe) to measure
53 interactions between peptides or proteins with membranes. The validity of this approach has
54 been established on several polypeptides and on various membrane vesicles. The B2LiVe
55 method efficiently complements the arsenal of label-free biophysical techniques to characterize
56 protein-membrane interactions.

57

58 **Highlights**

59 B2LiVe is a simple and robust NMR-based method to quantify affinity of proteins and peptides
60 for membranes

61 B2LiVe is a label-free approach that relies on proton 1D NMR fast pulsing techniques with
62 selective excitation of amide resonances

63 B2LiVe has been validated on several membrane-interacting peptides and proteins

64 B2LiVe coupled to DOSY can pinpoint the presence within a membrane-bound protein of
65 polypeptide segments remaining in solution

66

67 **Introduction**

68 Characterization of the interaction of peptides and proteins with lipid membranes often begins
69 with the determination of the affinity, or more appropriately, the partition coefficient K_x . A
70 common preliminary study consists in the identification of the lipid species as well as the lipid
71 properties, such as lipid polymorphism, charge, and acyl chain fluidity, favoring the partitioning
72 of proteins and peptides from solution to membrane. Moreover, knowledge of membrane
73 affinity can be important to decipher molecular mechanisms or mutational analyses to identify
74 regions or amino acid residues critical for membrane binding.

75 Several experimental approaches, such as SPR (Surface Plasmon Resonance), fluorescence,
76 centrifugation and circular dichroism are commonly used to measure the partitioning of soluble
77 peptides and proteins into lipid bilayers. However, some peptides and proteins do not possess
78 intrinsic fluorophores, or their secondary or tertiary structural contents do not change
79 significantly enough to be used as a probe of their partitioning into membranes. Alternatively,
80 they may exhibit a propensity to aggregate, complicating, if not precluding, the use of SPR or
81 centrifugation-based approaches. Besides, techniques based on phase separation (e.g.,
82 centrifugation or membrane flotation assays) are hardly applicable for peptides/proteins
83 showing low affinity for membranes.

84 In this article, we describe B2LiVe (Binding to Lipid Vesicles), a simple and robust NMR-
85 based experimental approach to determine the solution-to-membrane partition coefficient, K_x ,
86 of unlabeled peptides and proteins. The experimental strategy relies on proton 1D NMR fast
87 pulsing techniques with selective adiabatic pulses to excite the amide resonances (HET-
88 SOFAST) developed by Schanda and co-workers¹. Membrane partitioning induces a large line
89 broadening leading to a loss of peptide/protein NMR signals. The decrease of the NMR signal
90 thus directly reports the fraction of membrane-bound peptide/protein. The validity of this
91 approach has been established on several membrane-interacting peptides and proteins, ranging
92 from 3 to 54 kDa, and on membrane vesicles of various sizes and different lipidic compositions.
93 The method does not require any labeling and requires only low amounts of peptide/protein
94 when using high-field spectrometers and sensitive probes. Our results show that the B2LiVe
95 NMR-based method compares well with traditional fluorescence and CD-based techniques.
96 Overall, B2LiVe should efficiently complement the arsenal of label-free biophysical techniques
97 to characterize protein-membrane interactions.

98

99 **Material and Methods**

100 **Reagents**

101 The lipids 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC, reference 850457C), 1-
102 palmitoyl- 2-oleoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (POPG, reference 840457C) and
103 cholesterol (Chol, reference 700000P) were purchased from Avanti Polar Lipids (Alabaster,
104 AL, USA). HEPES-Na (reference H9897), NaCl (reference S5150) and CaCl₂ (reference
105 21115) were purchased from Sigma Aldrich, USA. The D₂O (reference D214) was purchased
106 from Eurisotop, England.

107

108 **Peptides**

109 The synthetic peptides were purchased as powder from Genosphere Biotech (France) and their
110 purity (95 %) and composition were controlled by reverse-phase HPLC and MALDI-mass
111 spectrometry, respectively. The peptides are capped on the N terminus with an acetyl group and
112 on the C terminus with an amide group. The P233 and P414 peptides used in this study were
113 derived from the CyaA protein²⁻⁴. The P233 peptide^{5,6}, which corresponds to the H-helix of
114 ACD (adenyl-cyclase domain), residues 233-254 of the CyaA toxin^{5,7} has the following
115 sequence: LDRERIDLLWKIARAGARSAVG. The P414 peptide is derived from the segment
116 414-440 of CyaA⁶ and contains a F415W mutation
117 (SWSLGEVSDMAAVEAAELEMTRQVLHA). Peptide powders were resuspended in 20 mM
118 HEPES-Na, 150 mM NaCl, pH 7.4. Peptide concentrations were calculated from the
119 absorbance at 280 nm subtracted by the absorbance at 320 nm of absorbance spectra recorded
120 with a Jasco V630 spectrophotometer using Hellma cuvettes. Aliquots of the dissolved peptides
121 were stored at -20°C.

122

123 **Proteins**

124 Bovine serum albumin (BSA, reference A0281), bovine α -lactalbumin (BLA, reference L-
125 6010) and apo-myoglobin (apo-Mb, reference A8673-5X1VL) were purchased as powders
126 from Sigma-Aldrich, USA. BSA was resuspended in H₂O, BLA was resuspended in 20 mM
127 HEPES-Na, 20 mM NaCl, 2 mM EDTA, pH 7.5 and apo-Mb was resuspended in 20 mM
128 HEPES-Na, 20 mM NaCl, pH 7.5. After resuspension, BSA, BLA and apo-Mb were filtered
129 through 0.2 μ m syringe filters (reference F2504-8, Thermofisher Scientific). Anthrolysin O
130 (ALO) production and purification were performed as described in⁸. The Diphtheria toxin
131 translocation domain (T)⁹ was produced and purified as described in¹⁰¹¹. The calcium-free apo-

132 state of calmodulin, apo-CaM, was produced and purified as described elsewhere¹²⁷. The
133 physical-chemical parameters of the proteins and peptides used in this study are reported in
134 Tables S1 and S2, respectively.

135

136 **Lipid vesicle preparation**

137 Multilamellar vesicles (MLVs), large unilamellar vesicles (LUVs) and small unilamellar
138 vesicles (SUVs) were used in this study. Lipid vesicles were prepared by reverse phase
139 evaporation as described elsewhere¹³⁻¹⁶ at a lipid concentration of 40 mM. MLVs produced by
140 reverse phase evaporation were submitted to extrusion through 1.2 μm polycarbonate filters.
141 LUVs were prepared by further extrusion through 0.4 and 0.2 μm polycarbonate filters. The
142 SUVs were obtained by sonication of filtered MLVs. The hydrodynamic diameters and
143 dispersity were checked by dynamic light scattering (DLS) using a NanoZS instrument
144 (Malvern Instruments, Orsay, France). Lipid vesicles were aliquoted and stored under argon at
145 4°C. Various lipid compositions were used: POPC:POPG at a 8:2 molar ratio; POPC:POPG at
146 a 9:1 molar ratio, both in 20 mM HEPES-Na, 20 mM NaCl, pH 4; POPC:cholesterol at a 6:4
147 molar ratio in 20 mM HEPES-Na, 20 mM NaCl, pH 7; POPC:POPG:cholesterol at a 7:2:1 ratio
148 in 20 mM HEPES-Na, 150 mM NaCl, pH 7.

149

150 **Sample preparation**

151 The samples used for NMR, tryptophan fluorescence and far-UV CD were derived from a
152 common stock solution to ensure the comparability of the three methods, when applicable. A
153 typical titration series comprises 19 samples. Protein (3 μM) and peptide (15 μM)
154 concentrations are kept constant for all samples, while the lipid concentration increases from 0
155 up to 10 mM. Apo-Mb and T titrations were performed at 25°C in the presence of POPC/POPG
156 8:2 (apo-Mb) or 9:1 LUV (T) in 20 mM HEPES-Na, 20 mM NaCl, 5% D₂O, pH 4. The titrations
157 of ALO were performed in the presence of POPC/cholesterol 6:4 LUVs in 20 mM HEPES-Na,
158 20 mM NaCl, 2 mM CaCl₂, 5% D₂O, pH 7.5 at 37°C. The titrations of the P233 and P414
159 peptides were performed at 25°C in the presence of POPC/POPG/cholesterol 7:2:1 SUVs in 20
160 mM HEPES-Na, 150 mM NaCl, 2 mM CaCl₂, 5% D₂O, pH 7.5.

161

162 **Nuclear Magnetic Resonance**

163 NMR experiments were performed on an Avance Neo 800 MHz spectrometer (Bruker,
164 Billerica, MA, USA) equipped with a triple resonance cryogenically cooled probe. Mono-

165 dimensional (1D) proton experiments with selective excitation of the amide region ¹⁷ of peptide
166 (5-15 μ M) and protein (1-3 μ M) samples in the presence of varying lipid concentrations were
167 recorded at 25°C or 37°C as indicated. Experiments were based on the proton HET-SOFAST
168 pulse sequence ¹ implemented in the NMRLIB 2.0 package ¹⁸. Selective adiabatic
169 polychromatic PC9 pulses with a 120° pulse centered at 9.5 ppm with a 3-4 ppm bandwidth
170 were used to excite amide resonances. The recycling time between scans was 0.15 ms. Spectra
171 were processed and analyzed with Topspin 4.0.7 (Bruker). The relative amount of
172 peptide/protein remaining in solution at each lipid concentration was evaluated from the
173 integral of the amide proton resonances, after subtraction of the spectrum of lipids at the highest
174 concentration used, considered as the baseline. Integral errors were estimated from the spectral
175 noise standard deviation summed over an equivalent region.

176 Self-diffusion experiments of the T domain (15 μ M) at pH 4.5 were performed in the presence
177 or absence of 15 mM LUV (POPC:POPG 9:1) at 25°C by means of the amide selective self-
178 diffusion experiment 1D_DOSY (Diffusion Ordered Spectroscopy) implemented in the
179 NMRLIB 2.0 package ¹⁸. We used a diffusion delay of 140 ms, bipolar shaped gradients applied
180 during 3.7 ms and 5 gradient strengths that varied from 2 to 98% of the probe maximum gradient
181 (53.5 G/cm). The selective pulses were centered at 9.5 and had a 3.1-ppm width. Water was
182 suppressed with an excitation sculpting scheme incorporated in the 1D_DOSY scheme. To
183 evaluate the effect of the different viscosity of T samples \pm LUV on diffusion, we followed the
184 signal of Hepes at 3.89 ppm contained in the buffer in non-selective experiments. We recorded
185 16 stimulated-echo experiments with a diffusion delay of 60 ms, bipolar gradients applied
186 during 1.6 ms at different gradient strength and excitation sculpting water suppression. Integral
187 errors for each data point were estimated from the noise standard deviation.

188

189 **Intrinsic tryptophan fluorescence**

190 Measurements were performed at 25 or 37 °C with a FP750 spectrofluorimeter (Jasco, Japan)
191 equipped with a Peltier-thermostated cell holder, using a quartz cell (reference 105.251-QS,
192 3x3 mm pathlength) from Hellma (France). The peptide tryptophan emission spectra were
193 recorded from 300 to 400 nm (excitation at 280 nm at a scan rate of 100 nm/min). A bandwidth
194 of 5 nm was used for both, excitation and emission beams. The ratio of fluorescence intensities
195 at 320 and 370 nm extracted from the fluorescence emission spectra was used to monitor
196 solution to membrane partitioning.

197

198

199 **Determination of the partition coefficient K_x and of the free energy of partitioning ΔG_{Kx}**

200 The solution to membrane partitioning, K_x , was determined by analysis of the fluorescence
201 intensity ratios at 320/370 nm, of 1D NMR data and when applicable of CD data. The value of
202 K_x is defined as the ratio of peptide/protein concentrations in membrane and solution phases
203 ^{19,20,14,6}, given by equation 1:

204

$$K_x = \frac{P_L/(P_L + L)}{P_w/(P_w + W)} \quad (Eq.1)$$

205

206 P_w (protein in aqueous phase) and P_L (protein in lipid phase) denote the concentration of soluble
207 and membrane-bound protein, respectively; P_T stands for total protein ($P_T = P_w + P_L$); W is the
208 concentration of water (55.55 M); L is the concentration of lipid. Since $L \gg P_L$ and $W \gg P_w$,
209 equation 1 can be written as follows:

210

$$K_x = \frac{P_L/L}{P_w/W} \quad (Eq.2)$$

211

212 Equation 2 can, in turn, be expressed as follows:

213

$$\frac{P_w}{P_L} = \frac{W}{LK_x} \quad (Eq.3)$$

214

215 The peptide fraction $f(P_L)$ partitioned into membrane is described by the following equation:

216

$$f(P_L) = \frac{P_L}{P_T} = \frac{P_L}{P_L + P_w} = \frac{P_L}{P_L} * \frac{1}{\left(1 + \frac{P_w}{P_L}\right)} \quad (Eq.4)$$

217

218 Based on equations 3 and 4, equation 5 can be expressed as follows:

219

$$f(P_L) = \frac{1}{1 + \frac{P_w}{P_L}} = \frac{1}{1 + \frac{W}{LK_x}} \quad (Eq.5)$$

220

221

222 Adding the experimental data offset (A), the amplitude of the experimental signal (B) and the
223 Hill coefficient (n) for the interaction to Equation 5, leads to Equation 6, which is used to fit all
224 parameters to the experimental data:

225

$$f(P_L) = A + B \left(\frac{1}{1 + \left(\frac{W}{LK_x}\right)^n} \right) \quad (Eq.6)$$

226

227 Equation 6 was fitted to the experimental data with Kaleidagraph (Synergy Software, Reading,
228 USA).

229 The K_x constant can be expressed in terms of the dissociation constant K_D of the peptide-
230 membrane interaction and the water concentration:

231

$$K_D = \frac{W}{K_x} \quad (Eq.7)$$

232

233 From Equation 7, Equation 6 rewrites:

234

$$f(P_L) = A + B \left(\frac{1}{1 + \left(\frac{L}{K_D}\right)^n} \right) \quad (Eq.8)$$

235

236 Finally, the free energy of solution to membrane partitioning ΔG_{Kx} was determined according
237 to Equation 9:

238

$$\Delta G_{Kx} = -RT \ln(Kx) = -RT \ln(W) + RT \ln(K_D) \quad (Eq.9)$$

239

$$\Delta G_{Kx} = -2.4 + RT \ln(K_D)$$

240

241

242 **Results**

243 Protein binding to unilamellar vesicles of lipid bilayers induces a large line broadening in NMR
244 signals, due to the slow tumbling rate of the particles, leading to a loss of protein signals. This
245 observation has been exploited for characterization of the association of some ^{15}N -labeled
246 proteins with membranes ²¹⁻²³. Here, we explored the possibility of quantifying membrane
247 partitioning of unlabeled proteins by using proton 1D NMR fast pulsing techniques with
248 selective excitation of amide protons using adiabatic pulses: the relative amount of protein or
249 peptide remaining in solution at each lipid concentration should be proportional to the integral
250 of the amide proton resonances. Hence, the decrease of the NMR signal should directly report
251 on the fraction of protein bound to membranes.

252
253 To prove the general applicability of this approach, we investigated membrane partitioning of
254 several proteins and peptides. Titrations were performed at constant protein and peptide
255 concentrations and the lipid concentration was increased up to 10 mM lipids. Large and small
256 unilamellar vesicles (LUV and SUV, respectively) of lipid bilayers were used. Protein
257 partitioning into membranes was monitored by selective amide band 1D NMR (proton HET-
258 SOFAST, see Methods), by recording the loss of intensity of the integral of the amide proton
259 resonances. In parallel, protein binding to membranes was measured by intrinsic tryptophan
260 fluorescence changes (following the ratio of fluorescence intensities emitted at 320 and 370 nm
261 as a proxy for polarity change upon membrane binding), and far-UV circular dichroism (for
262 peptides). The partition coefficient, K_x , which is directly related to the affinity constant
263 K_D (Equation 7), is determined by fitting Equation 8 to the experimental data (see Methods).

264
265 We first analyzed the partitioning of three amphitropic proteins into large unilamellar vesicles
266 (LUV): apo-myoglobin (apo-Mb, 16.9 kDa) ^{24,25}, the diphtheria toxin translocation domain (T,
267 22 kDa) ^{26,27}, and anthrolysin O (ALO, 54 kDa) ⁸. Figure 1 shows the 1D NMR spectra of apo-
268 Mb (Figure 1A), T (Figure 1B) and ALO (Figure 1C and 1D) with increasing concentrations of
269 lipids.

270
271 The intensity of the 1D NMR spectra of apo-Mb and T (Figure 1A-B) decreases with the
272 addition of LUVs. ALO is a cholesterol-dependent cytolysin (CDC) that requires the presence
273 of cholesterol in lipid bilayers to partition from solution to membranes. Whereas the 1D NMR
274 spectra of ALO titrated by membranes composed of POPC and cholesterol show a loss of

275 intensity (Figure 1C), the intensities of the 1D NMR spectra do not significantly change for
276 ALO titrated by LUV composed of POPC only (Figure 1D). This clearly establishes that the
277 loss of 1D NMR signal intensity is a straightforward indicator of ALO binding to membranes.

278

279 To estimate the partition coefficients of the different proteins, the fractions of lost intensity of
280 the integral of the amide proton envelope of apo-Mb, T and ALO were plotted as a function of
281 lipid concentrations as reported in Figure 2A. In parallel, membrane partitioning was monitored
282 by recording the ratio of tryptophan fluorescence intensity for the same proteins as a function
283 of lipid concentration (Figure 2B). The fluorescence data indicate that the three amphitropic
284 proteins interact with membranes, as expected from the literature ^{8,10,25}. Furthermore, fitting
285 Equation 8 to 1D NMR and fluorescence experimental data provided similar partition
286 coefficients for each protein (see Table S3). Hence, as NMR and fluorescence provide similar
287 quantitative results, we conclude that 1D ¹H NMR can reliably be used to report protein
288 partitioning into membranes.

289

290 As additional controls, we similarly characterized proteins that are not expected to interact with
291 membranes of various lipid compositions: calmodulin (apo-CaM, 16.7 kDa), bovine holo-
292 alpha-lactalbumin (hBLA, 14.2 kDa) and bovine serum albumin (BSA, 66.5 kDa) (Figure 3).
293 These proteins were chosen to cover a similar range of molecular masses than apo-Mb, T and
294 ALO. The addition of LUV does not affect the NMR signal nor the tryptophan fluorescence
295 (the fluorescence experiment could not be done for CaM as it does not contain tryptophan),
296 indicating that, as expected, these proteins do not interact with membranes under the
297 experimental conditions used ²⁸.

298

299 Taken together, these results indicate that proton 1D NMR fast pulsing techniques with
300 selective adiabatic pulses is a sensitive approach to quantitatively monitor protein partitioning
301 into membranes. The decrease of the NMR signal directly reports on the fraction of membrane-
302 bound proteins and can be easily recorded for unlabeled samples.

303

304 We then extended the study to peptide:membrane interactions. For this, we selected two
305 peptides from the *Bordetella pertussis* adenylate cyclase toxin (CyaA)^{3,4}, which were previously
306 characterized in our lab: P233 that was shown to interact with membranes and P414 that has no
307 membrane binding activity ⁶.

308

309 The NMR data shown in Figure 4 clearly indicate that P233 strongly interacts with
310 POPC/POPG/Cholesterol membranes with a complete loss of NMR signal at high lipid/peptide
311 ratios, while in the same conditions the P414 peptide does not show any signal decrease and
312 therefore does not bind to membranes. Membrane partitioning of P233 was monitored in
313 parallel by tryptophan fluorescence and far-UV circular dichroism (Figure 5). Overall, the
314 experimental data revealed an excellent correspondence between the different techniques that
315 provide similar quantitative parameters for the peptide membrane partitioning process (see
316 Table S4). Hence, our data indicate that 1D ^1H NMR is also readily applicable to quantify
317 membrane partitioning of standard, unlabeled peptides.

318
319 Interestingly, we noticed that, while in many cases (*e.g.*, apo-Mb, ALO or peptide P233) no
320 residual NMR signal is observed at saturating lipid concentrations (*i.e.*, after reaching a
321 plateau), a significant NMR signal remains for the T domain at the highest lipid concentrations
322 tested. This signal might be due to either a fraction of protein unable to bind to membranes and
323 remaining in solution or, alternatively, it might arise from fully membrane-attached
324 polypeptides containing flexible regions not directly bound to the lipids and floating above the
325 membrane (disordered regions or ordered regions linked to the membrane-bound region(s) by
326 a flexible linker, see figure 6). To discriminate between these two possibilities, we performed
327 NMR self-diffusion experiments as diffusion is expected to be different for proteins in solution
328 or bound to the lipid vesicles.

329
330 Diffusion experiments were performed with the T domain ($15\ \mu\text{M}$) alone or in the presence of
331 a 1000-fold excess of lipids in LUV at pH 4.5. Under these conditions, in amide-selective 1D
332 spectra, we observed that in the presence of LUV, the signal was $\sim 40\%$ ($39 \pm 3\%$) that of the
333 protein without lipid vesicles (Figure 7A). We then submitted the samples to amide-selective
334 diffusion experiments. In diffusion experiments in which all delays (diffusion delay, gradient
335 pulses) are kept constant and only the gradient strength is varied, the signals show a gaussian
336 decay that depends on the diffusion coefficient and the applied gradient. As can be observed in
337 Figures 7B and 7C, the relative decay on intensity of the spectra at high gradient strength is
338 much higher for the T domain alone than in the presence of LUV. This can also be visualized
339 on the diffusion curves (Figure 7D), which show the signal intensity as a function of the gradient
340 strength. These data indicate that the diffusion coefficient of the species giving rise to the
341 residual signal at high lipid concentration are bound to lipid vesicles. To rule out the possibility
342 that the slower diffusion of the T domain at high lipid concentration could be due to a higher

343 viscosity resulting from the presence of LUV rather than a consequence of membrane binding,
344 we used a buffer signal (Hepes resonance) as a control and showed that Hepes displays very
345 similar diffusion decays with or without lipid vesicles in samples containing the T domain
346 (Figure 7E). Hence, the residual signal pertains to membrane-bound protein, in agreement with
347 previous results ¹⁰. In summary, in addition to quantifying membrane partitioning, the B2LiVe
348 method coupled to diffusion-ordered spectroscopy (DOSY) can pinpoint toward the presence,
349 within a membrane-bound protein, of polypeptide segments that are not imbedded into
350 membrane, but remain in the aqueous phase.

351

352 **Discussion**

353 We have shown that 1D ¹H NMR with selective excitation of the amide region is a simple and
354 robust approach to quantify membrane partitioning of unlabeled peptides or proteins. Selective
355 excitation of the amide region allows one to efficiently filter-out the proton signals of water
356 (111 M), buffers (tenths of millimolar range), lipid vesicles (from 0 to 10 mM in this study) as
357 well as most of the impurities that are usually observed at lower frequencies, without the need
358 of ¹⁵N-labeling the proteins. In addition, the fast relaxation of the amide protons in the HET-
359 SOFAST experiment ¹ warrants the use of short inter-scan repetition delays leading to high
360 sensitivity. With high-field spectrometers and high-sensitivity, cryogenically cooled probes,
361 data acquisition on low protein-concentration samples can indeed be very short. In the
362 experiments presented here, run on an 800 MHz spectrometer equipped with a cold probe, one
363 data point was recorded with 2048 scans in ~12 minutes for 3 μ M protein in 180 μ L samples.

364

365 The B2LiVe method requires low concentrations and low amounts of unlabeled proteins, is
366 independent of the protein/peptide composition and can be applied to a large range of protein
367 sizes, which can go well-above the range of the protein sizes shown in this study (> 54 kDa,
368 unpublished data). Importantly, the capacity to perform the experiments at low protein
369 concentrations and high lipid/protein ratios, ensures the conditions of protein ‘infinite’ dilution
370 required to determine the thermodynamic partitioning constants (see Equation 2). On the other
371 hand, given that the determination of the membrane-bound fraction is based on the
372 disappearance of the solution protein signals, which is due to the large size of the lipid vesicles,
373 the method is independent of the lipid bilayer particle size (SUV or LUV) and is not negatively
374 affected by high lipid concentration, which can cause light scattering problems in optical
375 techniques like fluorescence and CD. This tolerance to particle size and high concentration

376 allows the technique to quantify the solution-to-membrane partitioning for systems with low
377 affinity.

378

379 Furthermore, as we showed here for the T domain, and in contrast to other techniques, NMR
380 can directly indicate if certain regions from a membrane-bound protein remain in the aqueous
381 phase. In its molten globule state at acidic pH, the T domain is able to penetrate membranes on
382 a pH dependent manner²⁶. Here, we showed that at pH 4.5, *ca.* 40% of the membrane-bound
383 protein residues (39 ± 3 % residual amide signal) are not directly attached to the membranes
384 and remain flexible enough in solution so that their NMR signals can be observed. Previous
385 works on the T domain, as well as with peptides encompassing its four N-terminal helices (TH1
386 to TH4), indicate that under the conditions used in this study (pH 4.5, POPG:POPC 9:1 LUV),
387 the N-terminal region does not interact with membranes^{10,11}; membrane insertion of the N-
388 terminal helices of T is only observed for more acidic pH (pH ≤ 4)^{26,11}. Remarkably, this N-
389 terminal amphiphilic region corresponds to ~ 40 % of the residues of the T domain, in close
390 agreement with our NMR results (39 ± 3 % residual amide signal).

391

392 In conclusion, the proposed NMR-based B2LiVe method should be valuable to quantify the
393 affinity of unlabeled peptides and proteins for lipid bilayers in the context of structural biology
394 and biophysical studies.

395

396 **Limitations of Study**

397 The B2LiVe method may be difficult to apply for proteins or peptides that have a strong
398 propensity to aggregate. However, for such peptides/proteins, other classical approaches such
399 as SPR, reflectometry-based or centrifugation-based partitioning assays will also be severely
400 impeded and only membrane flotation assays might potentially be applicable to demonstrate
401 their membrane binding capacity. However, it should be stressed that because B2LiVe can be
402 carried out at high lipid:polypeptide molar ratio (*i.e.*, in the low micromolar range for proteins
403 and in the tens of millimolar range for lipids), the aggregation-propensity of most proteins
404 should be rather limited in such conditions. Also, if there is intermediate exchange, affinity
405 could be overestimated but this effect should be easily detectable by visual inspection of the
406 NMR spectra. In this latter case, the B2LiVe method will still provide a good estimation of the
407 affinity range (*i.e.* order of magnitude of K_D) that should be useful for performing most
408 comparative studies (e.g. effects of mutations, different lipid compositions, ...).

409

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421

422 **Author contributions**

423 Conceptualization, J.I.G. and A.C., Methodology, J.I.G. and A.C., Software, B.V. and J.I.G.,
424 Validation, B.V., D.L., J.I.G. and A.C., Formal Analysis, M.S., N.C., B.V., J.I.G. and A.C.,
425 Investigation, M.S., N.C., B.V. and J.I.G., Resources, D.L., J.I.G. and A.C., Writing – Original
426 Draft, M.S., D.L., J.I.G. and A.C., Visualization, M.S., N.C., J.I.G. and A.C., Supervision, J.I.G.
427 and A.C., Project Administration, J.I.G. and A.C., Funding Acquisition, J.I.G. and A.C.

428

429 **Declaration of interests**

430 The authors declare not competing interests.

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434

435 **Figure legends**

436

437 Figure 1. Membrane partitioning of amphitropic proteins monitored by 1D ¹H amide-selective
438 NMR spectroscopy. The concentration of lipids ranges from 0 mM (black) to 2 mM (dark
439 yellow). The spectrum of lipid vesicles in the absence of protein at the highest lipid
440 concentration used is indicated as a dashed line (yellow). Each protein is present at a
441 concentration of 3 μM. Membrane partitioning and corresponding NMR spectra upon titration
442 of apo-Mb with POPC:POPG 9:1 LUV (A), T with POPC:POPG 9:1 LUV (B), ALO with
443 POPC:cholesterol 6:4 LUV (C) and ALO with POPC LUV (D). Membrane partitioning is
444 followed by the disappearance of the amide envelope signal (see main text for details). The
445 sharp signal at 8.43 ppm arises from a trace impurity.

446

447 Figure 2. Membrane partitioning of amphitropic proteins. Solution-to-membrane partitioning
448 of apo-Mb (cyan), T (violet) and ALO (green) at a concentration of 3 μM, each in the presence
449 of increasing lipid concentrations monitored by (A) 1D ¹H NMR (signal loss of the amide
450 envelope) and (B) fluorescence (ratio of tryptophan fluorescence intensities at 320 nm and 370
451 nm). POPC:POPG 9:1 LUV were used for apo-Mb and T, POPC:Chol 6:4 LUV for ALO
452 (green, closed circles) and 100% POPC LUV for ALO (green, open circles). The error bars in
453 (A) are calculated from the integral of the amide envelope (8.4-7.7 ppm) and from the spectral
454 noise standard deviation, respectively.

455

456 Figure 3. Selective amide spectra of soluble proteins that do not partition into membranes
457 monitored by 1D ¹H NMR spectroscopy. The concentration of lipids ranges from 0 mM (black)
458 to 10 mM (light blue), the spectrum of lipid vesicles in the absence of protein at the highest
459 lipid concentration used is indicated as a dashed line (yellow). Each protein is present at a
460 concentration of 3 μM. 1D ¹H NMR spectra of proteins at increasing lipid concentrations are
461 shown for apo-CaM in the presence of POPC:POPG 9:1 LUV (A), holo-BLA in the presence
462 of POPC:POPG 9:1 LUV (B) and BSA in the presence of POPC:cholesterol 6:4 LUV (C). The
463 sharp signals at 8.43 ppm in (A), (B) and (C) arise from an impurity. The plot in (D) represents
464 the fraction of membrane-bound apo-CaM (brown), holo-BLA (grey) and BSA (black) in the
465 presence of increasing lipid concentration. The fraction of membrane-bound proteins and the
466 errors in (D) are calculated from the integral of the amide envelope (8.4-7.7 ppm) and from the
467 spectral noise standard deviation, respectively.

468

469 Figure 4. Membrane interaction of peptides followed by 1D ^1H NMR spectroscopy. NMR
470 amide spectra upon titration of the P233 (A) and P414 (B) peptides with
471 POPC:POPG:cholesterol 7:2:1 SUV. The lipid concentrations range from 0 (black) to 10 mM
472 (dark yellow). The concentrations of the P233 and P414 peptides are 15 μM and 5 μM ,
473 respectively. The spectrum of lipid vesicles in the absence of peptides at the highest lipid
474 concentration used is represented as a dashed line (yellow).

475
476 Figure 5. Membrane partitioning of the P233 and P414 peptides followed by 1D ^1H NMR,
477 tryptophan fluorescence and far-UV CD. The experiments were performed in the presence of
478 POPC:POPG:chol 7:2:1 SUV. Data for P233 and P414 as a function of lipid concentration are
479 displayed in magenta and blue, respectively. Membrane partitioning followed by 1D ^1H NMR
480 is represented on a linear scale (A) and on a logarithmic scale (B). The ratio of fluorescence
481 intensity (320 nm/370 nm) followed by tryptophan fluorescence (C) and circular dichroism in
482 the far-UV region monitored by far-UV CD (D) are shown. The signal loss in (A) and (B) of
483 the tryptophan indol proton (open circles) followed by NMR is similar to that of the amide
484 envelope (closed circles).

485
486 Figure 6. Schematic model of three potential conformational states of an amphitropic protein
487 exposed to membranes. The experimental results of the B2LiVe method report on the fraction
488 of amide signal (red traces) that is lost upon titration of the protein (black line) titration by
489 membranes. The total loss of the NMR signal upon protein titration by lipid membranes
490 indicates that all proteins populate the B state at the expense of the A state. If the NMR signal
491 does not fully disappear and reaches an intermediate plateau, this indicates that a fraction of the
492 proteins remains in solution. The remaining amide signal may arise either from a fraction of the
493 population of proteins remaining in solution (A state) or from fully membrane-bound proteins
494 that contain specific regions remaining in solution (C state). The DOSY experiment allows to
495 discriminate between these possibilities.

496
497 Figure 7. Self-diffusion of the T domain in the absence and presence of LUV monitored by 1D
498 ^1H amide-selective diffusion NMR experiments. A. 1D ^1H amide-selective spectra of domain T
499 (15 μM) at pH 4.5 and 25°C in the absence (blue) or presence (violet) of LUV (15 mM
500 concentration), and of LUV (POPC:POPG 9:1) without protein (orange). The envelope integral
501 decays to $39 \pm 3\%$ in the presence of LUV. (B to D) diffusion experiments recorded with a 140
502 ms diffusion delay. 1D Spectra at 2% and 98% of the maximum gradient available (53.5 G/cm)

503 of T alone (B) or in the presence of LUV (C) displayed on the same scale. (D) Diffusion rate-
504 dependent decay of the intensity (integral) of the signal at 8.55 ppm of T with (violet) or without
505 (blue) LUV. The much lower decrease in relative intensity of the signal in the presence of LUV
506 indicates a slower diffusion rate. (E) diffusion rate-dependent decays of the Hepes signal (3.86
507 ppm) in samples (used in A to D) with (violet) or without (blue) lipid vesicles. Experiments in
508 (E) were recorded with a 60 ms diffusion delay; data in (E) were fitted to a gaussian decay of
509 the intensity (I) from an I_0 value (without gradients) as a function of the gradient strength G
510 and an apparent diffusion coefficient d ($I = I_0 e^{-dG^2}$). The similarity of the diffusion decays in
511 (E) indicates that the viscosity of the sample is not significantly increased by the LUV and is
512 not at the origin of the slower diffusion of T in the presence of lipid vesicles shown in B, C and
513 D. The sharp signal at 8.43 ppm arises from a low molecular weight trace impurity.

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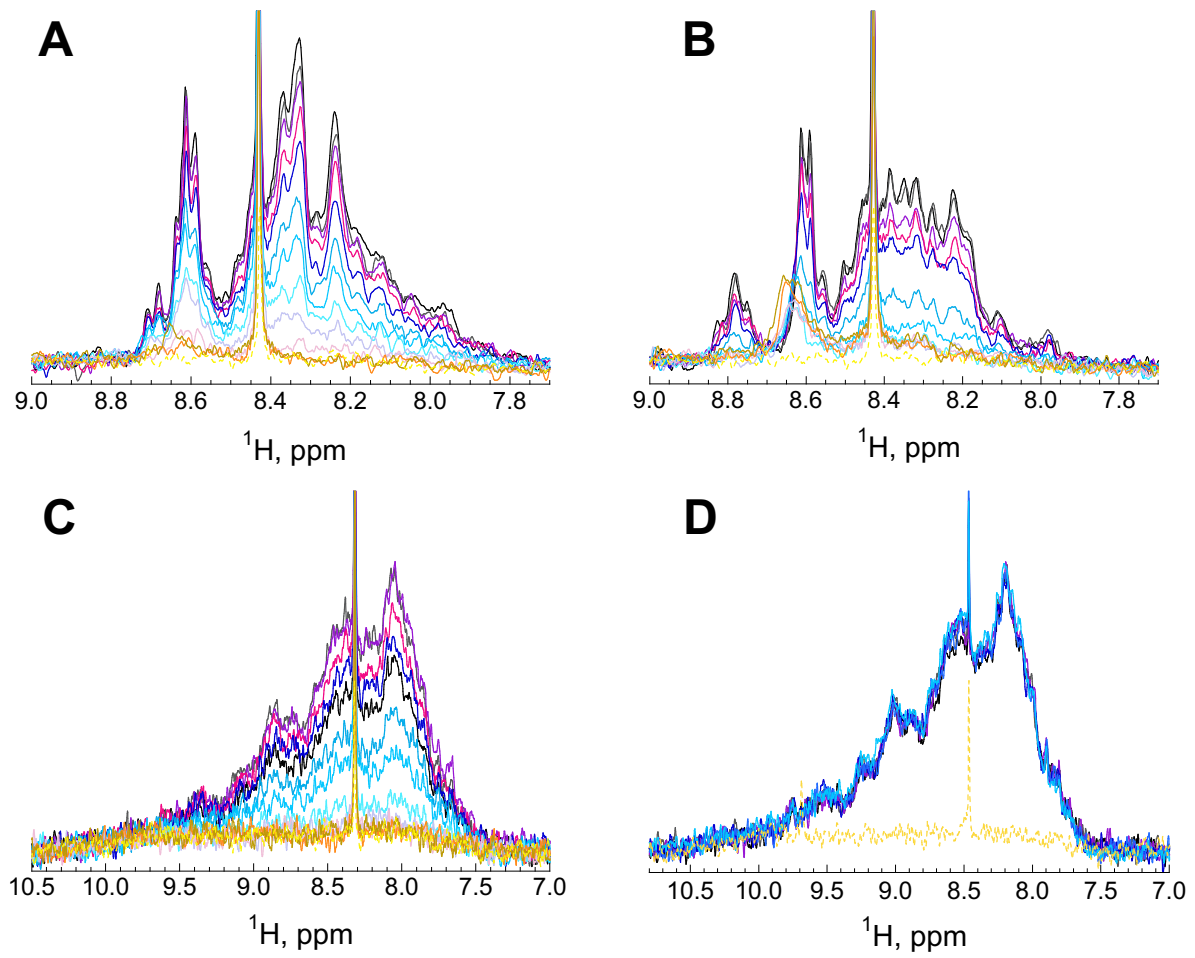


Figure 1

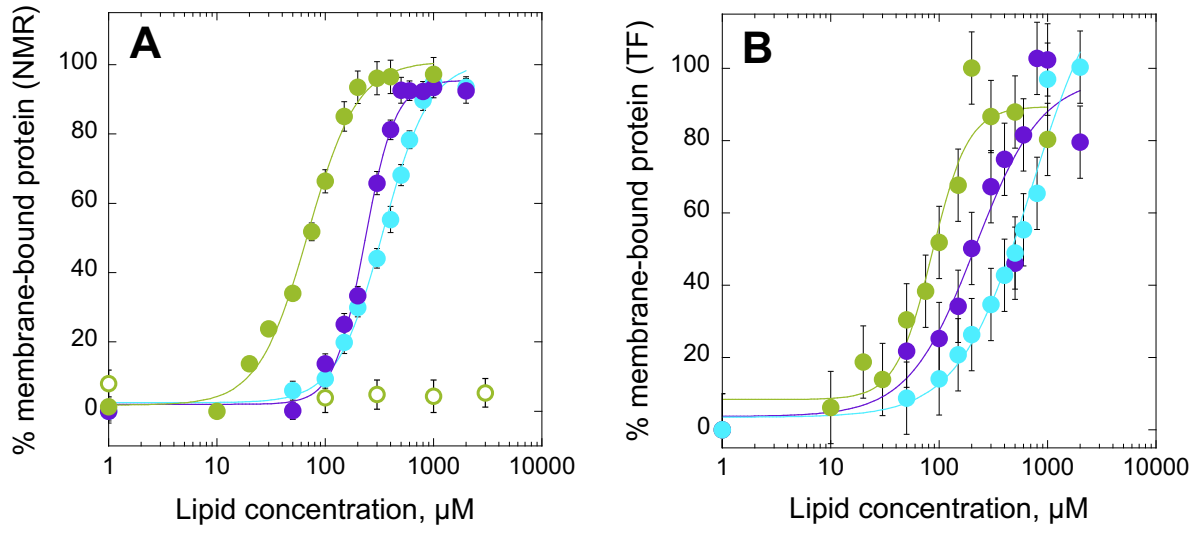


Figure 2

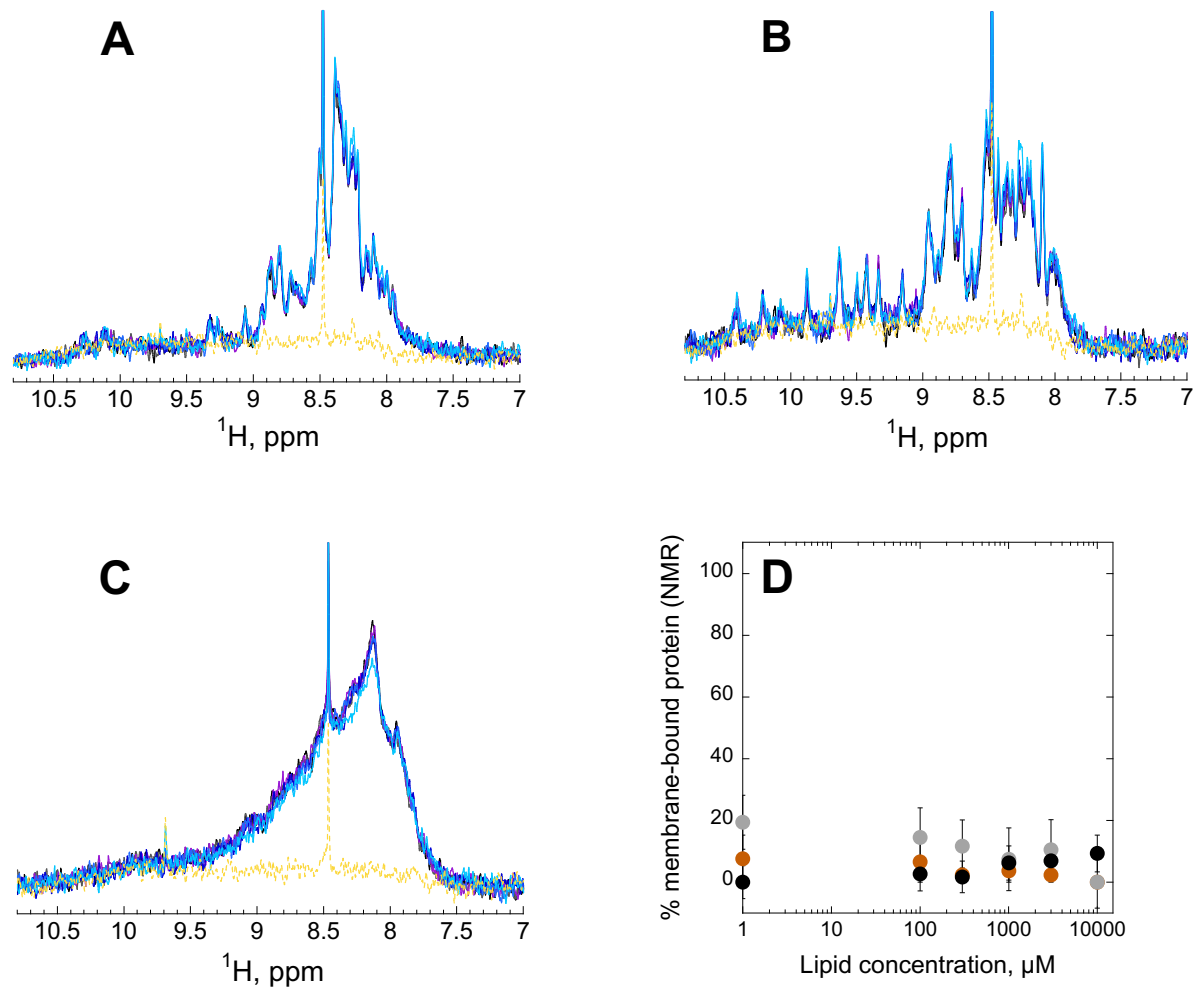


Figure 3

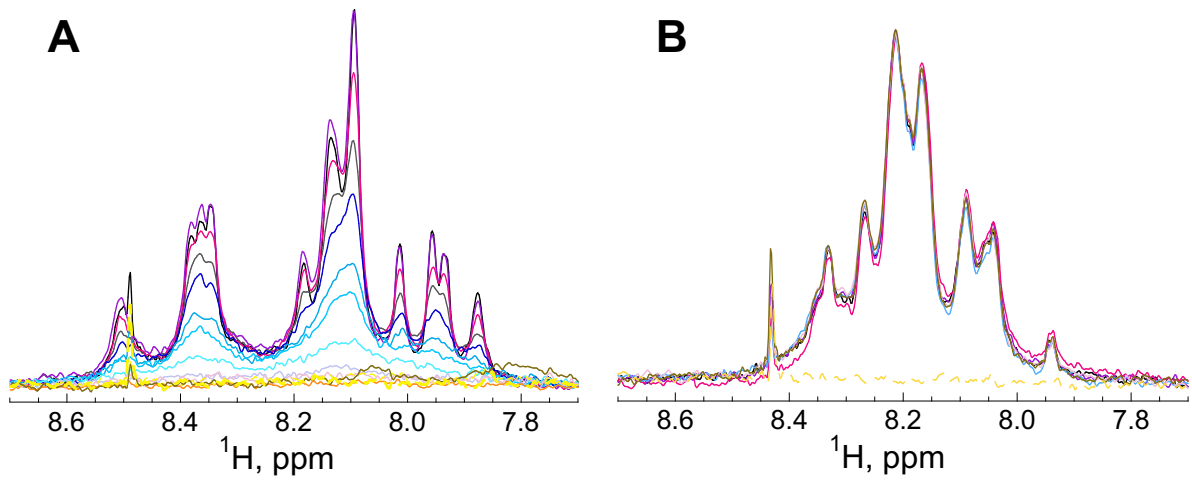


Figure 4

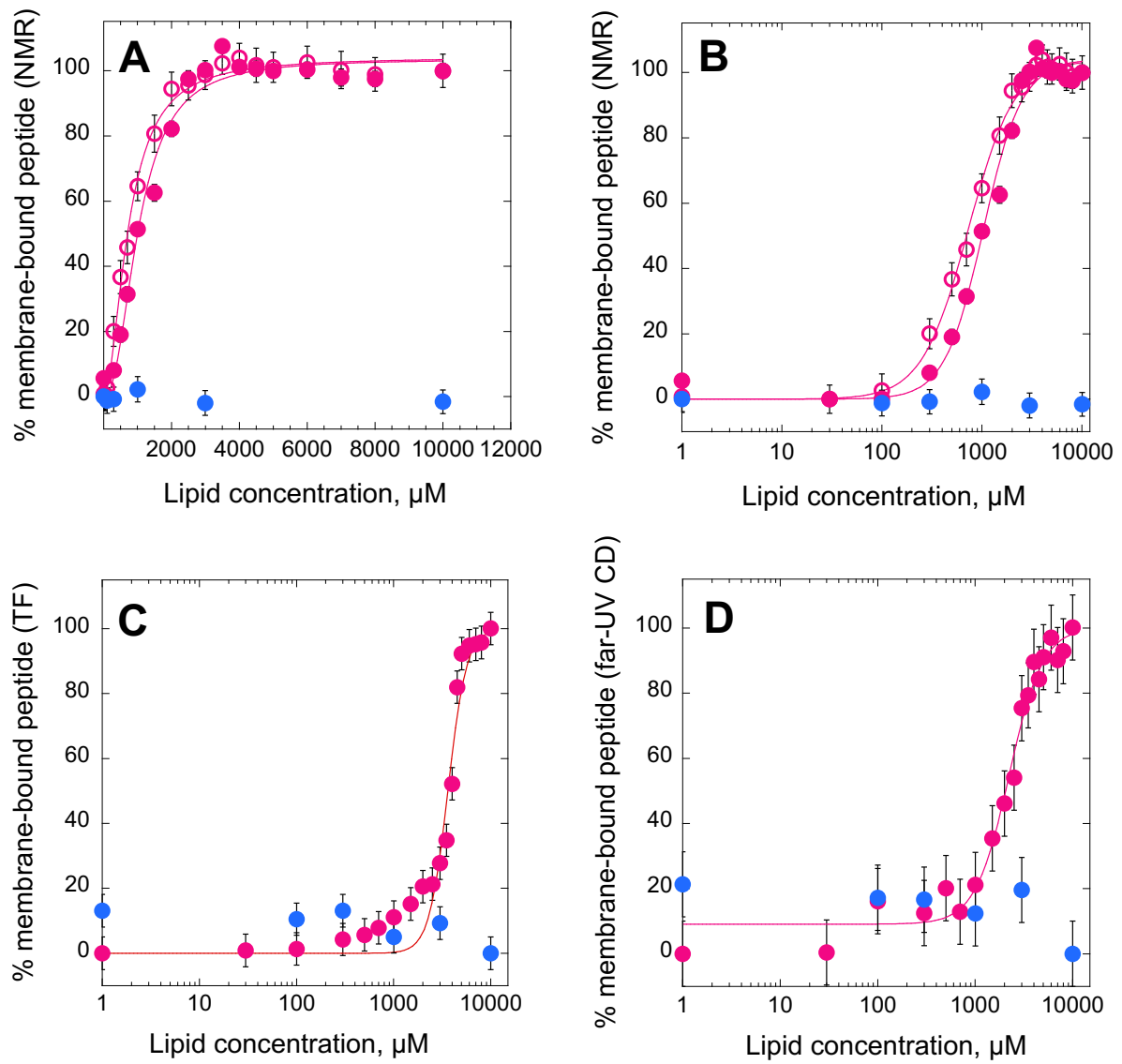


Figure 5

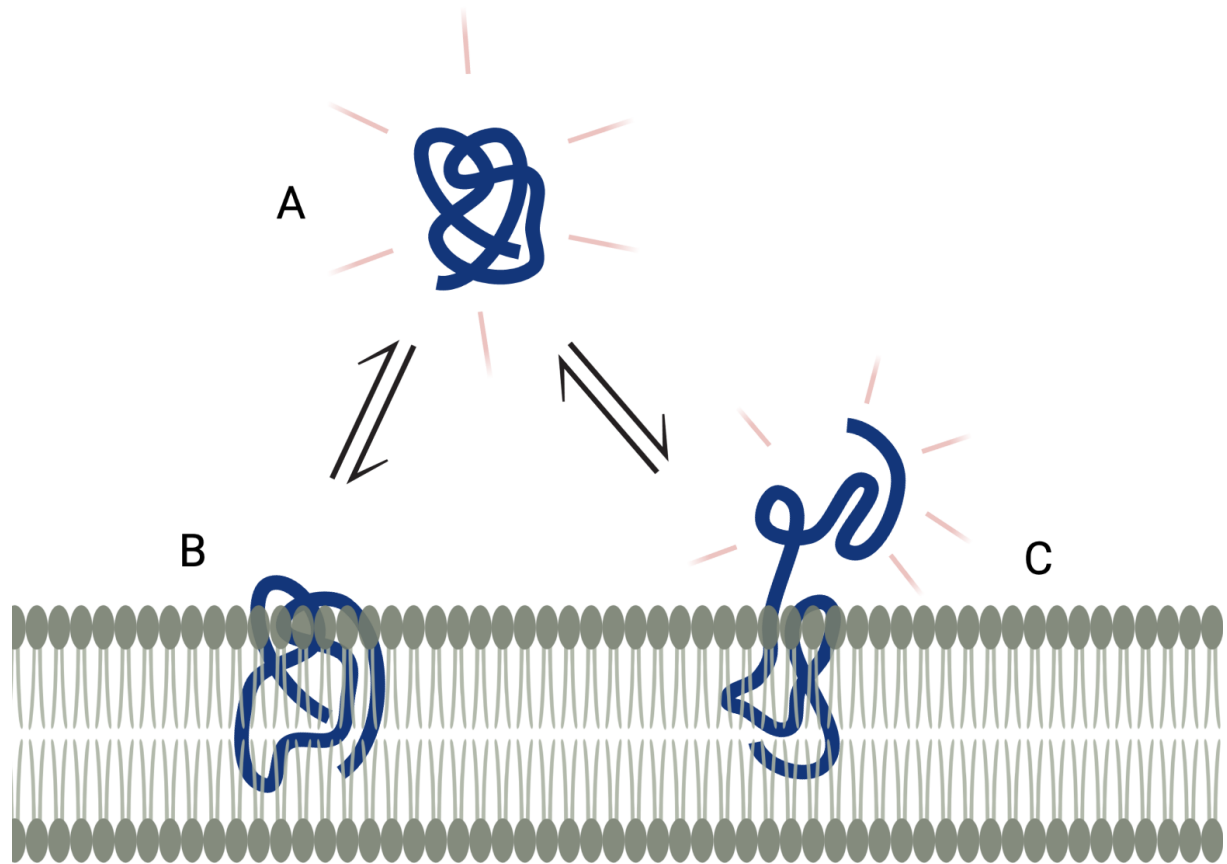


Figure 6

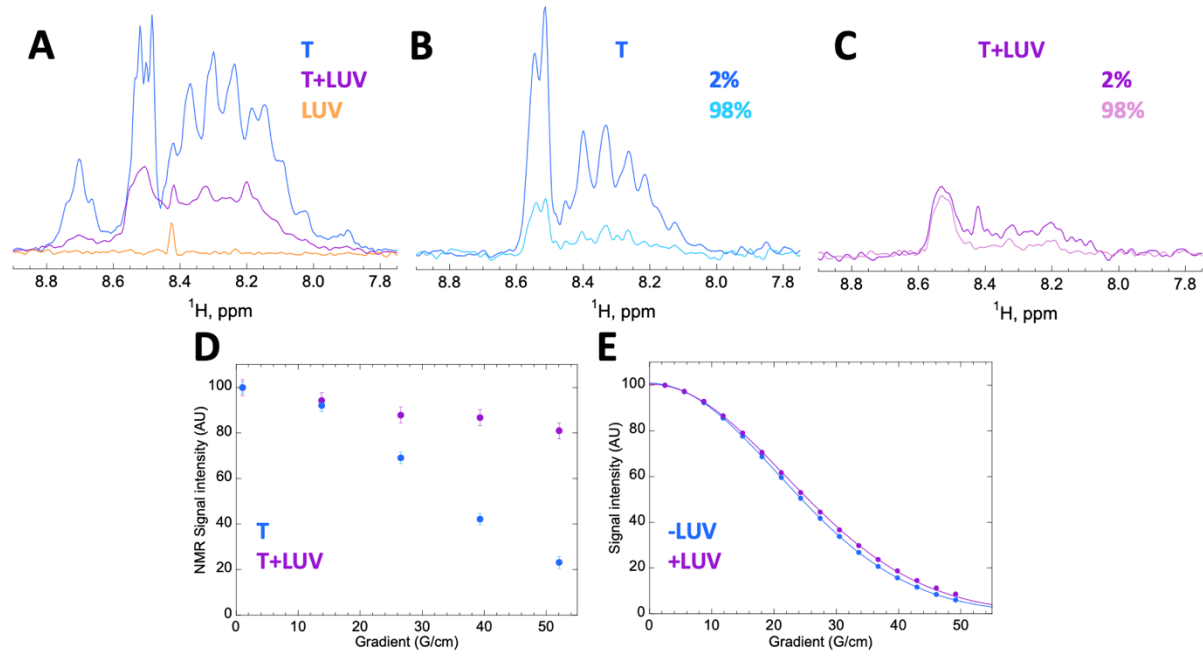


Figure 7