

# *The effects of native and modified clupeine on the structure of gram-negative model membranes*

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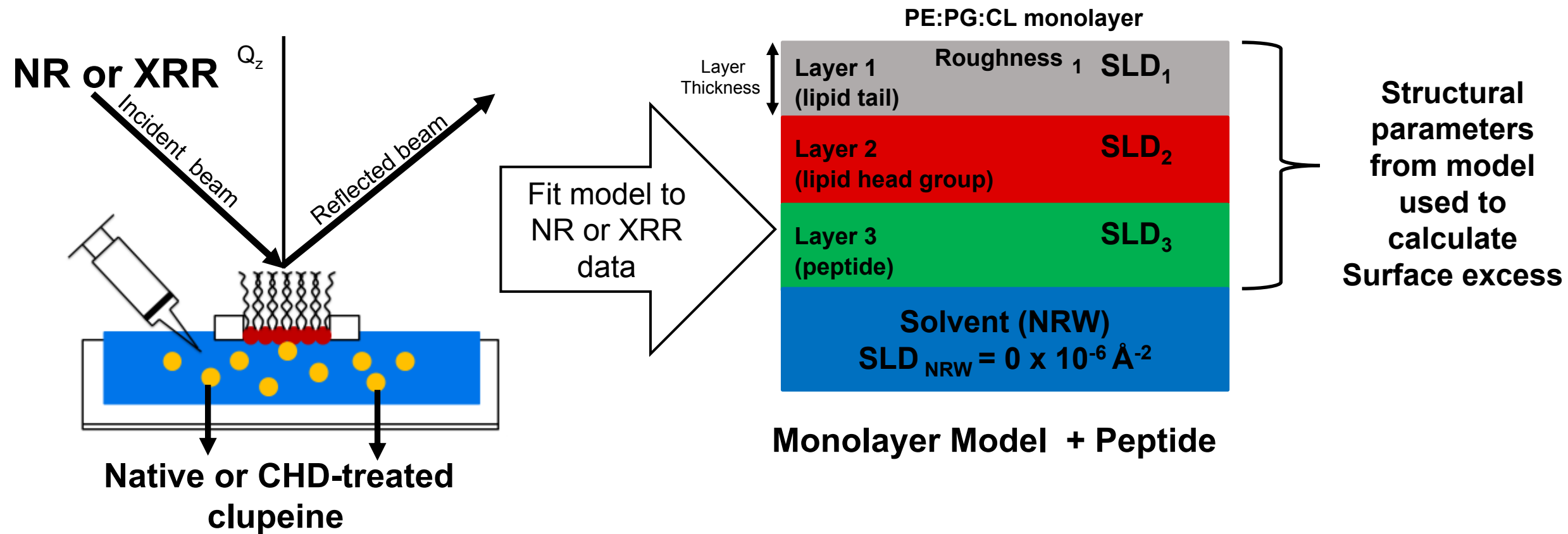
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## Highlights

- Similar structural effects were observed for both peptides in the monolayer and bilayer models, however, the magnitude of the effect was greater in the presence of the chemically modified peptide.
- Improved hydrophobicity and electrostatic interactions with lipid head groups resulting in thickening of the peptide layer, along with lipid translocation in the inner tail region of the bilayer, strongly suggests that the modified clupeine may use the carpet mechanisms to exert its effect on model membranes.
- Simultaneous fitting of neutron reflectometry and x-ray reflectometry data from PE:PG:CL monolayer model systems, resulted in quantitative determination of surface excess values for both native and modified clupeine.

# Graphical Abstract

March 3, 2019



1 **Title:** The **effects** of native and modified clupeine on the **structure** of Gram-negative model  
2 membranes.

3  
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21  
22 **Word count of text: “7,254 words”**

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24 **Short version of title:** Structural effects of clupeine in model membranes.  
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31 **ABSTRACT:** Clupeine, a cationic antimicrobial peptide found in fish, is of interest as a food  
32 additive but non-specific binding of the peptide to anionic molecules reduces its antimicrobial  
33 activity. The overall positive charge of clupeine can be reduced by blocking 10% of its arginine  
34 residues with 1,2-cyclohexanedione (CHD). The modified peptide retains antimicrobial activity  
35 but it is not known if its effect on the structure of Gram-negative model membranes is the same as  
36 the native peptide. In the presented paper, neutron reflectometry (NR) and X-ray reflectometry  
37 were used to investigate the effect of native and modified clupeine on the structure of model  
38 monolayer membranes composed of Phosphatidylethanolamine (PE), Phosphatidylglycerol (PG),  
39 and Cardiolipin (CL). The effect of the peptides on the structure of 1,2-dipalmitoyl (d62)-sn-  
40 glycerol-3-phosphocholine (DPPC)/PE:PG:CL bilayers were also examined by NR. In both model  
41 systems, modified clupeine demonstrated a greater effect on the lipid structure. **Charge reduction**  
42 **in the modified sample also resulted in improved hydrophobicity, and the formation of thicker**  
43 **peptide layers in the membrane models. Some lipid translocation** was observed in the inner tail  
44 region ( $\sim 69 \pm 0.24\%$  DPPC and  $\sim 24 \pm 0.02\%$  PE:PG:CL); and in the outer tail region ( $\sim 24 \pm 0.02\%$   
45 DPPC and  $\sim 56 \pm 0.01\%$  PE:PG:CL). **Improved hydrophobicity and electrostatic interactions with**  
46 **lipid head groups, strongly suggests that the modified clupeine may use the carpet mechanisms to**  
47 **exert its effect on model membranes. These findings suggest that changing the charge on the native**  
48 **peptide changes the way in which the modified peptide disrupts Gram-negative model membranes.**

49

50 **Keywords:** Clupeine, cationic antimicrobial peptide, Gram-negative bacteria, neutron  
51 reflectometry, and X-ray reflectometry, and protamine.

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## 55 **1 Introduction**

56

57 Bacteria can have both beneficial and harmful effects in food systems. For example, their  
58 use as probiotics (lactic acid bacteria) in fermented foods provide beneficial effects on human  
59 health (Ohashi & Ushida, 2009; Doyle, Steenson, & Meng, 2013). On the other hand, approaches  
60 to ensure the safety of food components and to combat illnesses caused by food-borne pathogens  
61 must confront the global problem of bacterial resistance (Manyi-Loh, Mamphweli, Meyer, &  
62 Okoh, 2018). Molecular studies have emphasized that the remarkable ability of bacteria to  
63 undermine the efficacy of antimicrobial agents is due in part to their ability to adapt under selective  
64 pressure and develop resistance through mutations or by acquiring genes from other bacteria  
65 (Canu, A., Malbruny, B., Coquemont, M., Davies, T., Appelbaum, P., & Leclercq, 2002; Spratt,  
66 Bowler, Zhang, Zhou, & Smith, 1994). Thus, for the past three decades, a major scientific priority  
67 has been the pursuit of new sources of antimicrobial agents with alternate mechanisms of action,  
68 which can limit the development of bacterial resistance (Munita & Arias, 2016).

69 In this context, cationic antimicrobial peptides (CAPs) have attracted interest as potential  
70 alternatives to conventional antimicrobial agents because they have exhibited broad spectrum  
71 inhibitory activity against several foodborne pathogens, and there have only been a few reports of  
72 developed resistance (Anaya-López, López-Meza, J., & Ochoa- Zarzosa, 2013). CAPs are found  
73 in many organisms including plants and fish (Omardien, Brul, & Zaat, 2016), and some can be  
74 cheaply extracted from waste streams (Gill, Singer, & Thompson, 2006). **In spite** of differences in  
75 their overall structure and sequence, many CAPs are characterized by their amphipathic domains,  
76 and their polycationic nature due to the presence of lysine, arginine or histidine residues (Wu,  
77 Maier, Benz, & Hancock, 1999).

78 **Several membrane disruption models including the barrel-stave, carpet, and the toroidal**  
79 **pore models have been proposed for CAPs. The validity of these models, and therefore**



80 antimicrobial activity largely depend on the cationic charge and amphipathic nature of CAPs  
81 (Straus & Hancock, 2006). In the barrel-stave model (BSM), the amphipathic nature of CAPs is  
82 utilized, here their hydrophobic peptide regions align into the lipid environment, whereas the  
83 hydrophilic side chains are aligned inward to form trans-membrane pores (Brogden, 2005). It is  
84 through these pores that cytoplasmic contents can leak from the cell, and result in cell death.  
85 Similarly, in the toroidal pore model, CAPs are inserted into the bilayer and cause the latter to  
86 bend and form a pore. As a result, phospholipid head groups and polar peptide surfaces line the  
87 pore lumen and local aggregations of varied numbers of peptide molecules within the membrane  
88 provide a route of passage of ions (Brogden, 2005). On the other hand, in the carpet model, the  
89 peptides bind to the cell surface in an electrostatic manner, and form a layer that alters membrane  
90 fluidity and or reduces the barrier properties of the membrane (Pelegriani, del Sarto, Silva, Franco,  
91 & Grossi-de-Sa, 2011).

92         Among CAPs, protamine, is a small peptide (4112 Da) which may be extracted from the  
93 sperm cells of fish such as herring (clupeine) and salmon (salmine). Similar to most CAPs  
94 protamine is very cationic and consists of 31 amino acids, with 20 of those residues being arginine  
95 (Suzuki & Ando, 1972). However, unlike most CAPs, protamine is not amphipathic, and lacks  
96 secondary structure due to the even distribution of positive charges along the peptide backbone  
97 (Bonora, Ferrara, Paolillo, Toniolo, & Trivellone, 1979). Protamine has also exhibited  
98 antimicrobial activity toward food-borne pathogenic bacteria but widespread applications in foods  
99 are made difficult due to non-specific interactions with food components (Truelstrup Hansen &  
100 Gill, 2000; Ueno, Fujita, Yamamoto, & Kozakai, 1988). These non-specific interactions can be  
101 overcome by chemically blocking arginine residues with 1,2-cyclohexanedione (CHD), which also  
102 reduces the surface charge of the peptide (Potter et al., 2005). The CHD-treated peptide also has

103 improved **antimicrobial activity** as demonstrated by reduced growth of *Listeria monocytogenes* in  
104 milk as well as in ground beef (Potter, Truelstrup Hansen, & Gill, 2005) but the effects of the  
105 peptides on bacterial membrane structure is not fully known. Accordingly, our objective was to  
106 use two complementary biophysical techniques, neutron reflectometry (NR) and X-ray  
107 reflectometry (XRR), to investigate the effect of native and modified clupeine on the structure of  
108 model monolayer membranes composed of zwitterionic (Phosphatidylethanolamine, PE), and  
109 anionic phospholipids (Phosphatidylglycerol, PG and Cardiolipin, CL). These phospholipids are  
110 present in the natural, cytoplasmic membrane of Gram-negative bacteria in an approximate 79:17:4  
111 mole % ratio (Sohlenkamp & Otto, 2016). The effect of the peptides on the structure of 1,2-  
112 dipalmitoyl (d62)-sn-glycero-3-phosphocholine (DPPC)/PE:PG:CL bilayers was also investigated  
113 by NR. Understanding the initial steps involved in native and modified clupeine membrane  
114 interactions will begin to define characteristics of the peptides and the target bacteria that will be  
115 useful in understanding the peptides' mode of action.

## 116 **2 Materials and Methods**

### 118 **2.1 Materials**

119  
120 DPPE, 1,2-dipalmitoyl-glycero-3-phosphoethanolamine, MW 691.97 (zwitterionic and  
121 synthetic purity > 99%); DPPG, 1,2-dipalmitoyl-sn-glycero-3-[phosphor-rac-1-glycerol] (anionic  
122 sodium salt), MW 744.96; and 1,1',2,2'-tetramyristoyl cardiolipin (anionic sodium salt), MW  
123 1,285.62 were all purchased from Avanti Polar Lipids (Alabaster, AL, USA). Stock solutions of  
124 all lipids were prepared using a 3:1 mixture of HPLC grade chloroform to methanol (Sigma-  
125 Aldrich, Oakville, ON, Canada) in a ratio (PE:PG:CL; 79:17:4 mole %), **hereafter referred to as**  
126 **PPC** and stored at -20°C.. Native clupeine (clupeine sulfate (MW 4112 Da, P4505)), L-arginine,  
127 0.1 M HCL solution, CHD (MW 112.13 g/mol), 8-hydroxyquinoline, sodium hydroxide, liquid

128 bromine, and HPLC grade chloroform were obtained from Sigma-Aldrich (Oakville, Oakville,  
129 ON, Canada):

## 130 **2.2 Clupeine Modification**

131  
132 To reduce the surface charge of arginine CHD (2.8 g) was dissolved in 500 mL of 0.2 M  
133 boric acid buffer (pH 8.5) then 2.5 g of native clupeine was added and the contents of each flask  
134 stirred for 20 s (Potter et al., 2005). The samples were incubated at 37°C for 2.5 min and then 500  
135 mL of cold 5 % (v/v) acetic acid was added. Control samples were prepared in a similar manner  
136 except that no CHD was added to the reaction flasks. The modified samples were concentrated to  
137 200 mL, and then exhaustively dialyzed in a Prep/Scale Millipore Model P34404 ultrafiltration  
138 apparatus (Millipore, Toronto, ON, Canada) equipped with 900 cm<sup>2</sup>, 1000 Da tetrafluoroethylene  
139 (TFE) filters and flushed with five volumes of 1% (v/v) acetic acid and ten volumes of distilled,  
140 deionized water (DDW) and concentrated once again to 200 mL as described by Potter et al.  
141 (2005). Finally, the purified samples were frozen at -30°C and then freeze dried (Labconco,  
142 Missouri, USA). Stock solutions were prepared by dissolving 0.1 g of the powder in 40 mL of 1%  
143 (v/v) acetic acid. Working solutions were prepared by diluting the stock solutions 1:50 with DDW.  
144 The Sakaguchi reaction (Sakaguchi, 1950; Potter et al., 2005), which is specific for arginine, was  
145 used to determine the unmodified arginine residues in the CHD-treated clupeine. The percent  
146 modification of arginine residues was determined using an arginine-HCl standard curve and taking  
147 into account that ~20 of the 30 amino acid residues in clupeine are arginine (Ando et al., 1973).  
148 Only CHD-treated clupeine with 10% of the arginine residues modified was chosen for further  
149 testing because it has been reported that moderate reductions in charge led to improved  
150 antimicrobial efficacy (Potter et al., 2005).

## 151 **2.3 Peptide Surface Hydrophobicity**

152

153 The surface hydrophobicity ( $S_o$ ) of the native and modified samples was measured using  
154 a fluorescent probe, 6-propionyl-2-(N,N-dimethylamino) naphthalene (PRODAN) as outlined by  
155 Alizadeh-Pasdar and Li-Chan (2000) with modifications. A PRODAN standard curve was  
156 developed using concentrations ranging from 0 to 0.95  $\mu\text{M}$ . Using this PRODAN binding curve,  
157 it was possible to measure the amount of PRODAN bound to the peptide samples. PRODAN (20  
158  $\mu\text{L}$ , 7.6 mM) was added to 4 mL of peptide in a 0.01 M phosphate buffer (pH 7). After 15 min  
159 incubation in the dark, the relative fluorescent intensity (RFI) was measured using a Photo  
160 Technology International (PTI) fluorescence spectrophotometer, with excitation and emission  
161 wavelengths set at 390 and 470 nm, respectively.

## 162 **2.4 Zeta Potential**

163 The net charge density of the peptides was measured as zeta potential (mV) using a Zetasizer  
164 Nano Model ZS (Malvern Instruments, Derbyshire, UK) as outline by Paulson and Tung (1987)  
165 with modifications. Measurements were made at 20°C in triplicate. The zeta potential was  
166 calculated from the electrophoretic mobility of individual particles, measured using laser Doppler  
167 velocimetry (Malvern Instruments Ltd, 2004).

## 168 **2.5 Surface Pressure Measurements**

169  
170 Surface pressure measurements on a Langmuir trough (model 611 Nima Technology,  
171 Coventry, England) interfaced with a computer data acquisition system were carried out by the  
172 Wilhelmy plate method as described by Lad, Birembaut, Clifton, Frazier, Webster, & Green,  
173 (2007). Clean troughs were filled with 80 mL of 0.02 M phosphate buffer (pH 7), and 20  $\mu\text{L}$  of  
174 the lipid solution in chloroform was spread dropwise using a Hamilton syringe (Hamilton  
175 Company, Reno, NV) on the surface of the buffer to form a monolayer. The lipid monolayer was  
176 compressed to a target surface pressure of  $\sim 25 \text{ mN m}^{-1}$ . Control checks were carried out for  $\sim 4.2$

177 h on the bare **PPC** monolayers to determine their stability. For each experiment, the compressed  
178 film was relaxed for 20 min at  $\sim 25 \text{ mN m}^{-1}$  prior to the addition of 1 mL of native or modified  
179 clupeine solution to the subphase (final peptide concentration of  $0.48 \mu\text{M}$ ). Compression isotherms  
180 were recorded as surface pressure ( $\pi$ ) vs. area ( $A$ ) curves prior to the addition of the peptides and  
181 on addition of the peptide to the subphase, and plots of surface pressure vs. time were recorded to  
182 follow adsorption of the peptides to the lipid layer. All compressions were repeated until a  
183 reproducible trace was obtained and the final surface pressure values had a standard deviation of  
184  $\pm 1 \text{ mN m}^{-1}$ . Similar experiments were carried out using the negatively charged phospholipid,  
185 DPPG, as a control.

## 186 **2.6 Neutron Reflectometry Measurements on **PPC****

187  
188 NR measurements were carried out using the white beam SURF reflectometer at the  
189 Rutherford Appleton Laboratory (Didcot, Oxfordshire, UK), using neutron wavelengths from 0.5  
190 to  $6.5 \text{ \AA}$ . The beam intensity was calibrated with respect to a clean  $\text{D}_2\text{O}$  surface. The sample  
191 preparation and NR method were carried out as described by Clifton, Sanders, Hughes, Neylon,  
192 Frazier, & Green (2011) with some modifications. Briefly, all the NR experiments were performed  
193 at room temperature and the lipid films were prepared by spreading the **PPC** lipid mix (from the  
194 stock solution) in a  $200 \times 400 \text{ mm}$  Langmuir trough (Nima Technology, Coventry, UK) containing  
195 a  $20 \text{ mM}$  phosphate buffer (pH 7.0). Films were compressed to a surface pressure of  $23 \text{ mN m}^{-1}$   
196 and the films were relaxed for 20 min at  $23 \text{ mN m}^{-1}$  prior to the addition of native or CHD-treated  
197 clupeine solutions ( $0.48 \mu\text{M}$ ) to the lipid monolayer. NR curves were recorded at two angles of  
198 incidence ( $\theta = 1.5$  and  $0.8^\circ$ ) to yield a momentum transfer range of  $\sim 0.01 - 0.6 \text{ \AA}^{-1}$  both before and  
199 after the addition of native or CHD-treated clupeine. NR was measured under multiple isotopic  
200 contrasts and this was achieved by using hydrogenated and deuterated lipids in a **non-reflecting**

201 water subphase compared to air, NRW (8% D<sub>2</sub>O, 92% H<sub>2</sub>O), and D<sub>2</sub>O. Measurements using  
202 hydrogenated lipids (h-lipids) on NRW were done to observe protein binding since the h-lipid will  
203 be largely non-reflecting ( $\rho(h - lipid) = -0.39 \times 10^{-6} \text{ \AA}^{-2}$ ), where  $\rho$  represents the scattering  
204 length density (SLD). Repeat experiments using isotopic contrasts with d-lipid ( $\rho(d - lipid) =$   
205  $7.5 \times 10^{-6} \text{ \AA}^{-2}$ ) on NRW were also done to reveal any changes in lipid layer structure caused by  
206 the interaction. Contrasts of h-lipid on D<sub>2</sub>O were also done to enable differentiation between  
207 peptide adsorbed beneath the interface and the lipid head group (Clifton, Neylon, & Lakey 2013a).

## 208 **2.7 X-Ray Reflectometry Measurements on PPC**

209 X-ray reflectometry experiments were performed at the I07 beamline at the Diamond Light  
210 Source (Harwell Science and Innovation Campus, Didcot, Oxfordshire, UK). The sample  
211 preparation and method described by Clifton et al. (2012) was carried out. Experiments were  
212 performed at room temperature and the lipid films were prepared by spreading the PPC lipid mix  
213 (from the stock solution) in a 200 x 400 mm Langmuir trough containing a 20 mM phosphate  
214 buffer (pH 7.0). The films were compressed to a surface pressure of 23 mN m<sup>-1</sup> and then relaxed  
215 for 20 min at 23 mN m<sup>-1</sup> prior to the addition of native or CHD-treated clupeine solutions (0.48  
216  $\mu\text{M}$ ). A monochromatic X-ray wavelength of 0.992  $\text{\AA}$  (corresponding to a photon energy, E of  
217 12.5keV) was used and a fast shutter was applied to avoid over-exposure to the X-ray beam.

219 The experiments were also performed in a helium atmosphere, the reflectivity profiles were  
220 measured in a  $Q_z$  range of 0.01 to 0.8  $\text{\AA}^{-1}$  and data were collected on a Dectris Pilatus 100 k  
221 detector. XRR data were reduced by performing a normalisation and a “footprint correction” step.  
222 There were three parts to the normalisation, the first part involved dividing by the incident flux  
223 since this varies with the incident angle. The second part involved stitching the three regions  
224 together; by overlapping points at the extremes of each region. The third part involved scaling the

225 data so that reflectivity at the critical edge was equal to one. The detector also used two ‘regions  
226 of interest’ (ROI) to simultaneously measure the signal, and this background was subtracted from  
227 all the data sets (Clifton et al., 2012).

## 228 **2.8 Bilayer Deposition and Neutron Reflectometry Measurements**

229  
230 Gram-negative model, single bilayer membranes were prepared at the ISIS Biological  
231 Sample Laboratory (Rutherford, England) as outlined by Clifton et al. (2013b). NR measurements  
232 were carried out using the white beam SURF reflectometer, using neutron wavelengths from 0.5  
233 to 6.5 Å. The collimated neutron beam was reflected from the silicon-liquid interface at three  
234 different glancing angles of incidence, 0.35°, 0.65° and 1.5°.

235 A neutron flow-cell was placed at the bottom of a clean Langmuir-Blodgett (LB) trough  
236 (KSV-Nima, Biolin Scientific, Finland) and the cell was flushed with ultrapure water (Millipore,  
237 18.2 MΩ cm<sup>-1</sup>) to remove air bubbles and was then filled with 20 mM phosphate buffer (pH 7.0)  
238 A Piranha-cleaned (H<sub>2</sub>O<sub>2</sub>/H<sub>2</sub>SO<sub>4</sub>/H<sub>2</sub>O 1:4:1) silicon (SiO<sub>2</sub>) crystal was then mounted onto the  
239 dipping mechanism of the trough in a vertical position and with the active face away from the  
240 center, then the block was submerged under the buffer. Two bilayers were prepared and 150 μL  
241 of tail-hydrogenated or deuterated 1,2-dipalmitoylphosphatidylcholine (h-DPPC and d-DPPC) in  
242 1 mg/mL in chloroform, was spread onto the clean water surface. The lipid was compressed to an  
243 initial pressure of 10 mN m<sup>-1</sup> and then equilibrated for 15 min. The lipid layer was then compressed  
244 to 35 mN m<sup>-1</sup> at a rate of 3 mm min<sup>-1</sup>. Pressure-area isotherms were recorded to confirm the  
245 homogeneity of the film.

246 For LB deposition of the inner bilayer leaflet, the submerged silicon crystal was lifted  
247 through the air-water interface at a rate of 3 mm/min and at a constant pressure of 35 mN m<sup>-1</sup>. The  
248 entire LB deposition procedure took 45 min. For Langmuir Schaefer (LS) transfer, a clean neutron

249 flow-cell was placed in the bottom of the trough before it was filled with cold 20 mM Hepes buffer  
250 (pH 7.2). A monolayer was formed on the surface by adding 150  $\mu\text{L}$  of the PE:PG:CL (79:17:4  
251 mole %) lipid mix, and the latter was compressed to 35  $\text{mN m}^{-1}$ . The silicon crystal containing the  
252 LB-deposited DPPC monolayer was placed on the dipping mechanism of the trough, with the  
253 crystal face parallel to the water surface. The silicon crystal with the deposited LB film was then  
254 dipped through the interface at a constant rate of 3  $\text{mm min}^{-1}$  and lowered into the neutron flow-  
255 cell at the bottom of the trough. Native or CHD-treated clupeine (0.48  $\mu\text{M}$ ) were added to the cell  
256 in a 20 mM Hepes buffer (pH 7).

## 257 **2.9 Reflectivity Data Analysis for Monolayers**

258 NR and XRR data were analyzed using a Matlab version of RasCal (version 1.1.2, Hughes,  
259 A., ISIS Spallation Neutron Source, Rutherford, Appleton Laboratory). In RasCal, structures  
260 across the interface were modeled as a series of layers and each layer was described by three main  
261 parameters: thickness ( $\tau$ ), SLD ( $\rho$ ), and roughness (Clifton et al., 2013a). The SLD of the lipids  
262 (head groups and tails), solvents and peptides were calculated using equation 1:  
263

264 Eq. 1

$$265 \quad \rho = \frac{\sum b}{V}$$

266 Where  $b$  represented the SL for each element and  $V$  represented the molecular volume (Lad, 2006).  
267 The XRR and NR data were first fitted individually then fitted simultaneously as described by  
268 Nelson (2006) and Clifton et al. (2012) to place restrictions on the NR fit. The thickness and  
269 roughness parameters were linked in a single model and the SLD and background values were  
270 allowed to vary (Nelson, 2006).

271 Bare lipid monolayers with no peptides were divided into two layers, the first, a lipid chain  
272 layer containing  $\text{CH}_3$  and  $\text{CH}_2$  groups and the second, a head group layer containing the lipid head



273 groups (Dabkowska, Fragneto, Hughes, Quinn, & Lawrence, 2009). This classification was based  
274 on two assumptions: (1) the first layer contained only lipid component and the second layer  
275 contained only the head group; (2) the second assumption was related to the area per molecule and  
276 assumed that this value was the same for both the lipid head group and the tail region (Clifton et  
277 al., 2011). However, in order to measure peptide binding to the monolayer, a third layer was  
278 included in the model to represent the presence of the peptides below the lipid monolayer  
279 (Saunders, Clifton, Frazier, & Green, 2016). A set of reflectivity profiles measured under the three  
280 isotopic contrasts hydrogenated (h)-lipid in NRW; h-lipid in D<sub>2</sub>O and deuterated (d)-lipid in NRW  
281 were fitted together and the large difference between the scattering lengths of hydrogen (-0.56 x  
282 10<sup>-6</sup> Å<sup>-2</sup>) and deuterium (6.35 x 10<sup>-6</sup> Å<sup>-2</sup>) was used to detect the location of different components  
283 in the monolayer. The parameters of the measured data were then fitted to the theoretical model  
284 until the best fit was achieved. The quality of the fit was also assessed visually. The fitted SLD for  
285 each isotopic contrast was related to the volume fraction of each component using equation 2,  
286 where  $\Phi$  represented the volume fraction,  $\rho$  represented the SLD,  $\rho_{(D)}$  and  $\rho_{(H)}$  represented the  
287 fitted SLD and  $\rho_{(D-L)} - \rho_{(H-L)}$  represented the calculated SLD.

288 Eq. 2

$$289 \quad \Phi (lipid) = \frac{\rho(D) - \rho(H)}{\rho(D - L) - \rho(H - L)}$$

290 The SLDs and the molecular volume for the native and CHD-treated peptides were calculated as  
291 outlined in the ISIS Biomolecular SLD Calculator (<http://psldc.isis.rl.ac.uk/Psldc/>). To calculate  
292 the SLD for the lipid mixture of PPC, the SLD of each individual lipid head and tail was calculated  
293 and then multiplied by its fraction in the mixture. The molecular volumes of the lipid components  
294 were calculated using the Molinspiration Property Calculator  
295 (<http://www.molinspiration.com/cgi-bin/properties>). The area per molecule ( $A$ ) occupied by the

296 peptide and the lipid in each layer and the surface excess ( $\Gamma$ ) for each component in the system  
297 were calculated using equations 3 and 4, where  $b$  represented the scattering length,  $\rho$  represents  
298 the SLD, and  $\tau$  represented the layer thickness obtained from the model fit (Clifton et al., 2011).

299

$$300 \quad A = \frac{\sum b}{\tau \phi \rho} \quad \text{Eq. 3}$$

301

$$302 \quad \Gamma = \frac{MW}{A * 6.02 \text{ g/mol}} \quad \text{Eq. 4}$$

303

## 304 **2.10 Reflectivity Data Analysis for Bilayers**

305

306 Model biomembranes systems composed of either tail deuterated or tail hydrogenated  
307 DPPC as the inner leaflet and hydrogenated-PPC as the outer leaflet were prepared, then NR  
308 experiments were carried out using three different solution subphases; (1) D<sub>2</sub>O (100%,  $\rho=6.35 \times 10^{-6} \text{ \AA}^{-2}$ );  
309 (2) silicon matched water (SMW, 38% D<sub>2</sub>O and 62% H<sub>2</sub>O,  $\rho=2.07 \times 10^{-6} \text{ \AA}^{-2}$ ); and (3)  
310 100% water ( $\rho=-0.56 \times 10^{-6} \text{ \AA}^{-2}$ ). Each deuterated and hydrogenated lipid bilayer was measured  
311 under all three isotopic contrasts (D<sub>2</sub>O; SMW and H<sub>2</sub>O) thus resulting in a total of six different  
312 reflectivity profiles. The large difference between the SLD for deuterated-DPPC ( $7.45 \times 10^{-6} \text{ \AA}^{-2}$ )  
313 and hydrogenated-DPPC ( $-0.39 \times 10^{-6} \text{ \AA}^{-2}$ ) tail regions made it possible to determine structural  
314 parameters from the tail region within each individual bilayer. Reflectivity data were obtained for  
315 the six contrasts before and after the addition of native and CHD-treated clupeine and the data  
316 were analyzed as described in Clifton et al. (2013) using a Matlab version of RasCal. The three  
317 membrane components in the bilayer were DPPC, PPC and water and their individual  
318 contributions to the bilayer were determined from the fitted values obtained for the tail deuterated-

319 DPPC SLDs in the three subphase mixtures (100% D<sub>2</sub>O, SMW (30% D<sub>2</sub>O and 100% water). The  
 320 SLD ( $\rho$ ) of a given layer was related to the three membrane components by the following equation:

$$321 \quad \rho = (\rho_{DPPC})(\phi_{DPPC}) + (\rho_{PPC})(\phi_{PE:PG:CL}) + (\rho_{Water})(\phi_{Water}) \quad \text{Eq. 2}$$

322  
 323  
 324 Where  $\rho$  represented the SLD of a given layer and  $\rho_{DPPC}$ ,  $\rho_{PPC}$  and  $\rho_{Water}$  represented the SLD  
 325 of DPPC, PPC and water respectively, while  $\phi_{DPPC}$ ,  $\phi_{PPC}$  and  $\phi_{Water}$  represented the volume  
 326 fractions of the same components. Because the DPPC and PPC lipid tail regions do not contain  
 327 labile hydrogens and would not undergo solvent-contrast-related changes in SLD (Clifton et al.,  
 328 2013b), the volume fraction of water was determined from the following equation:

$$329 \quad \phi_{Water} = \frac{\rho_{water\ contrast1} - \rho_{water\ contrast2}}{\rho_{water1} - \rho_{water2}} \quad \text{Eq. 6}$$

330  
 331 Where  $\rho_{water\ contrast1}$  and  $\rho_{water\ contrast2}$  represented the SLDs of the same layer in any two of  
 332 the three contrasts (H<sub>2</sub>O, SMW or D<sub>2</sub>O) used, while  $\rho_{water1} - \rho_{water2}$  represented the SLDs of  
 333 each solvent mixture. Once the volume fraction of water ( $\phi_{Water}$ ) was determined, then the DPPC  
 334 fraction in the d-DPPC/h-PPC bilayer system was determined using equation 6.  
 335

$$336 \quad \rho - (\rho_{water}\phi_{water}) = (\rho_{DPPC\ tails})(\phi_{DPPC\ tails}) + (\rho_{PPC\ tails})(\phi_{PPC\ tails}) \quad \text{Eq. 7}$$

337  
 338 Equation 7 was used to find the value of  $\rho - (\rho_{water}\phi_{water})$ , which was needed in order to fully  
 339 complete equation 8:  
 340

$$341 \quad \phi_{DPPC\ tails} = \left( \frac{(\rho - (\rho_{(water)}\phi_{water}) - (\rho_{PPC\ tails}(1 - \phi_{water})))}{(\rho_{d-DPPC\ tails} - \rho_{PPC\ tails})} \right) \quad \text{Eq. 8}$$

343

344 Once the relative contribution of the  $\phi_{DPPC\ tails}$  were determined, then the relative contributions  
345 of the PPC tails to the bilayer were determined by using equation 9:

346  
347 Eq. 9  
348 
$$\phi_{PPC} = 1 - (\phi_{DPPC\ tails} + \phi_{water})$$

### 349 **2.11 Model to Experimental Data Fitting Analyses**

350  
351 The ‘bootstrap’ error analysis function in RasCal was used to obtain model to experimental data  
352 fitting errors as previously described by (Clifton et al., 2012; Clifton et al., 2013b). The original  
353 data set was resampled, then new data sets were fitted using the methods described earlier. “The  
354 parameter value distributions obtained across these fits were used to estimate errors, and these  
355 values were then propagated through the calculations of the derived parameters according to  
356 error treatment methods” (Clifton et al., 2013b).

## 357 **3 Results and Discussion**

### 358 **3.1 Net charge density and surface hydrophobicity**

359  
360  
361 The native peptide was far less hydrophobic than the modified sample (P=0.02, n=3) at the  
362 pH level tested (Figure 1A ). Since the native clupeine is highly cationic in nature, the use of an  
363 anionic probe such as 1-anilinonaphthalene-8-sulfonic acid (ANS) would have resulted in greater  
364 interaction with the positively charged sites on the peptide, thus overestimating the  
365 hydrophobicity. This supports the use of the uncharged probe PRODAN which eliminated the  
366 possible electrostatic contributions in the hydrophobicity measurements (Alizadeh-Pasdar & Li-  
367 Chan, 2000). The measured zeta potential of the native clupeine was  $7.2 \pm 0.2$  mV, which was  
368 similar to the value reported by Arbab et al. (2004). Conversely, the modified sample registered a  
369 zeta potential of  $5.3 \pm 0.1$  mV.

### 370 **3.2 Peptide binding to lipid monolayer using surface pressure measurements**

371

372 The surface pressure change on addition of clupeine to a compressed **PPC** monolayer at  
373 the air/water interface was investigated as a function of time (Figure 1). The data showed an  
374 increase in surface pressure for CHD-treated clupeine that was not seen for the native peptide. The  
375 maximum increase seen after 300 min from addition of the treated clupeine to the subphase was  
376 approximately 11 mN/m. This suggests that the CHD-treated clupeine had penetrated into the lipid  
377 layer leading to increase compression of the layer, an effect previously reported by (Abuillan et  
378 al., 2013; Oliveira et al., 2009). For the native clupeine no increase in surface pressure was  
379 observed, although a gradual decrease was seen that could be due to lipid removal at the surface,  
380 but was more likely a consequence of the stability of the lipid layer and not an indication of any  
381 clupeine interaction. Importantly, this could have been resolved if an equivalent volume of peptide-  
382 free buffer was added to the subphase and the same decrease in surface pressure was observed.  
383 Conversely, if no effect on surface pressure was observed over the same time period, this would  
384 suggest that the peptide did not sit at the air-water surface (Dabkowska et al., 2009).

### 385 **3.3 Impact of peptide on lipid monolayer structure**

386 NR and XRR reflectivity data were fitted simultaneously to provide characterisation of the  
387 interfacial layer structure before and after peptide addition. The **PPC** monolayer characterization  
388 prior to peptide addition was determined using two reflectivity profiles, the d-**PPC** on an NRW  
389 buffer subphase (NR) and the h-**PPC** on a H<sub>2</sub>O phosphate buffer subphase (XRR). **Models of the**  
390 **XRR fits are not shown.** A two layer model was used to fit the data, where layer 1 was the acyl  
391 chain region with a thickness ( $\tau$ ) of  $15 \pm 0.64 \text{ \AA}$  and a volume fraction ( $\Phi_L$ ) of  $0.97 \pm 0.02$ , whereas  
392 layer 2 was the lipid head group of the condensed PE:PG:CL monolayer, with a  $\tau$  of  $12.9 \pm 1.2 \text{ \AA}$   
393 (Table A2). Ciumatic et al. (2017) and Dabkowska et al. (2009) have also reported similarly thin  
394

395 hydrophobic chain regions for DPPC or DPPG and for 1,2-dioleoyl-sn-glycero-3-phosphocholine  
396 or palmitoyl-oleoyl-glycero-3-phosphoserine (DOPC/ POPS) monolayers.

397 A third layer was included into the model to allow for fitting of clupeine adsorbed below the  
398 lipid layer (Figure 2). In addition, the hydrogenated contrasts in NRW proved to be informative in  
399 identifying the contribution of the peptide to the monolayer. As shown in Figure 2 A, the three  
400 layer model proposed for native clupeine adsorbed to the condensed phase PPC monolayer, fitted  
401 the data well. Peptide binding in the presence of native clupeine showed minimal adsorption in the  
402 lipid layer (surface excess ( $\Gamma$ ) =  $0.005 \pm 0.02$ ), but a greater effect was observed in the lipid head  
403 group region ( $\Gamma$  =  $0.297 \pm 0.02$ ) and a thickening of the peptide layer ( $\tau$ , increased from  $15.0 \pm$   
404  $0.01 \text{ \AA}$  to  $15.3 \pm 0.07 \text{ \AA}$ ). Conversely, in the presence of the modified peptide a greater effect on  
405 the structure of the monolayer was observed (Figure 3). For example, there was a 25% and 15%  
406 increase in surface excess and a peptide layer thickness, respectively, compared to the measured  
407 values in the presence of the native peptide (Table 1). Slight increases in SLD,  $1.07 \pm 0.06 \times 10^{-6}$   
408  $\text{\AA}^{-2}$  or  $1.69 \pm 0.05 \times 10^{-6} \text{\AA}^{-2}$  in the presence of native or CHD-treated clupeine, respectively, were  
409 also observed (Table 1). Notable, the difference in the fitted SLDs and the total adsorbed peptide  
410 was almost two-fold in the presence of the modified peptide compared to the native peptide.

411 The requirement of a third layer below the monolayer supports the observation from the  
412 surface pressure studies for the modified clupeine, and confirms that the peptide interacted with  
413 the PE:PG:CL monolayer (Figure 1). More importantly, the advantage of using two different  
414 techniques to characterize peptide interaction with the PPC monolayer is emphasized since, NR is  
415 sensitive to the total amount of material at the interface. Thus although the presence of native  
416 clupeine on the PPC monolayer led to a decrease in surface pressure change, NR measurements  
417 clearly revealed a thickening of the layer (Table1). Work with Puroindoline-b (pin-b) protein

418 mutants has also shown little change in surface pressure when the proteins were inserted onto  
419 DPPC or DPPG monolayers, however, similar to native and CHD-treated clupeine, NR revealed  
420 most of the peptide situated below the lipid region (Clifton, Lad, Green, & Frazier, 2007; Clifton,  
421 Green, Hughes, & Frazier, 2008). Moreover, NR and XRR methods were advantageous since  
422 differences in the radiation source (XRR versus NR) result in different scattering length densities  
423 (SLD), and selective SLD modification with deuterium (D<sub>2</sub>O) labeling made it possible to reveal  
424 subtle changes in membrane structure in the presence of the peptides (Lopez-Rubioa, & Gilbert,  
425 2009).

### 426 **3.4 Impact of Peptides on Bilayer Structure**

427  
428 To validate that the trends observed with the monolayer work were not dependent on the  
429 lipid layer model used, bilayer studies were performed. Figure 4 shows the NR profiles and data  
430 fits of bilayers in the presence of native (4A), and modified clupeine (4B) examined under three-  
431 solution contrasts (D<sub>2</sub>O, SMW and H<sub>2</sub>O). In the outer lipid head group region there was a change  
432 in SLD from 2.5 to 2.2 or 2.3 x 10<sup>-6</sup>Å<sup>-2</sup> in the presence of native or modified clupeine, respectively  
433 (Table 2). The decrease in SLD may be explained by lipid removal from the bilayer in the presence  
434 of the peptides. Lipid loss was also accompanied by an increase in hydration of the lipid head  
435 group, from 17.9 ± 12.7% on the bare bilayer compared to 26.9 ± 5.5% in the presence of native  
436 clupeine and 48.2 ± 11.5% in the presence of the modified clupeine. The greater degree of  
437 hydration in the lipid head group region in the presence of modified peptide compared to the native  
438 peptide is observed as a broader peak in Figure 4 D compared to Figure 4 F and may also indicate  
439 greater solvent penetration.

440 The model used to fit the reflectivity data from the deuterated lipids (Figure 4B) showed  
441 that it was possible to form asymmetric bilayers with ~90% DPPC inner leaflet composition and

442 an outer layer of ~80% PPC. Although it is now known how closely the model membrane fits the  
443 real membrane, similar percent coverages have been reported by Fernandez et al. (2013). Lipid  
444 translocation was also observed in the inner tail region ( $\sim 69 \pm 0.24\%$  DPPC and  $\sim 24 \pm 0.02\%$  PPC)  
445 and in the outer tail region ( $\sim 24 \pm 0.02\%$  DPPC and  $\sim 56 \pm 0.01\%$  PPC) (Table 2). Lipid  
446 translocation may have resulted due to lateral heterogeneity in the bilayer which leads to the  
447 formation of domains (Epanand, 2013). Vorobyov and Allen (2011) discussed the importance of  
448 bilayer charge in mediating peptide interaction and showed that adsorption of cationic peptides to  
449 anionic bilayers is significantly higher than in zwitterionic membranes. Importantly, electrostatic  
450 interactions between peptides and anionic lipids has also been postulated as another factor that  
451 supports the formation of domains (Epanand, 2013). In the present study it is possible that the  
452 peptides could exert part of their effect by changing lateral organization in the membrane.  
453 Increased hydrophobicity of the modified clupeine may also explain the increased magnitude of  
454 the effect on the lipid structure. Furthermore, thicker peptide layers in the presence of the modified  
455 peptide ( $11.04 \pm 6.0 \text{ \AA}$  versus  $4.15 \pm 2.9 \text{ \AA}$  in the presence of the native peptide) (Table 2), implies  
456 the accumulation of peptides to form a layer that can interact with negatively charged components  
457 in the membrane. Thus, it appears that both hydrophobic and electrostatic interactions may govern  
458 the mode of action of the modified clupeine, and strongly suggests that the modified clupeine may  
459 use the carpet mechanisms to exert its effect on model membranes. These observations support the  
460 findings of Pink, Hasan, Quinn, Winterhalter, Mohan, and Gill (2014) who reported that native  
461 clupeine can internalize and kill some Gram-negative bacteria without lysis or pore formation.

## 462 **Conclusion**

463 The initial interactions of native and CHD-treated clupeine in model membranes has been  
464 investigated by combining NR and XRR techniques. In the less complex monolayer system,



465 quantitative amounts of peptides could be determined as surface excess values in the presence of  
466 both peptides. Lipid translocation was observed in the inner acyl chains of the bilayer membrane  
467 however, but the peptides were not able to penetrate the bilayer membrane. Similar effects on the  
468 model membrane structure were observed, although peptide perturbation of the membranes  
469 appeared different. Increased hydrophobicity along with electrostatic interactions of the modified  
470 peptide were attributed to the improved peptide-lipid interactions. A more comprehensive  
471 understanding of the safety and toxicology of these peptide is required before they can be  
472 considered for food applications in Canada.

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### 478 **Author Contributions**

479 M. English analysed the data and drafted the manuscript. L. Clifton and O. Florek contributed to  
480 the acquisition of NR data and the interpretation of the results. T. Arnold assisted with the  
481 collection of the X-ray data. A. Paulson, R. Green and R. Frazier contributed to critically  
482 revising the manuscript and giving final approval of the version to be submitted.

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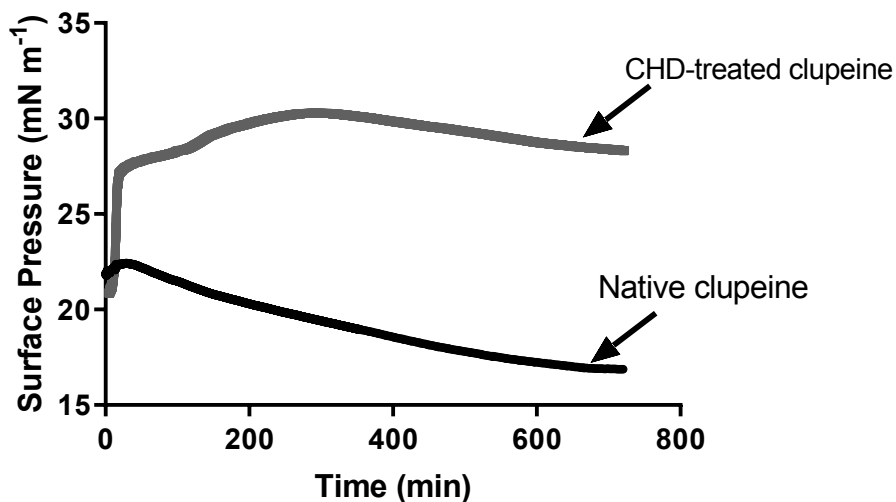
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718 **Figure 1.** Surface pressure versus time plot for CHD-treated clupeine and native clupeine adsorbed  
719 on a **PPC** monolayer. There was a general increase (4.6%) in surface pressure after adding the  
720 CHD-treated peptide. On the other hand, the addition of the native peptide resulted in a decrease  
721 (2.3%) in surface pressure. These experiments were repeated twice.

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723 \*Note that **PPC** is the abbreviation of PE:PG:CL.

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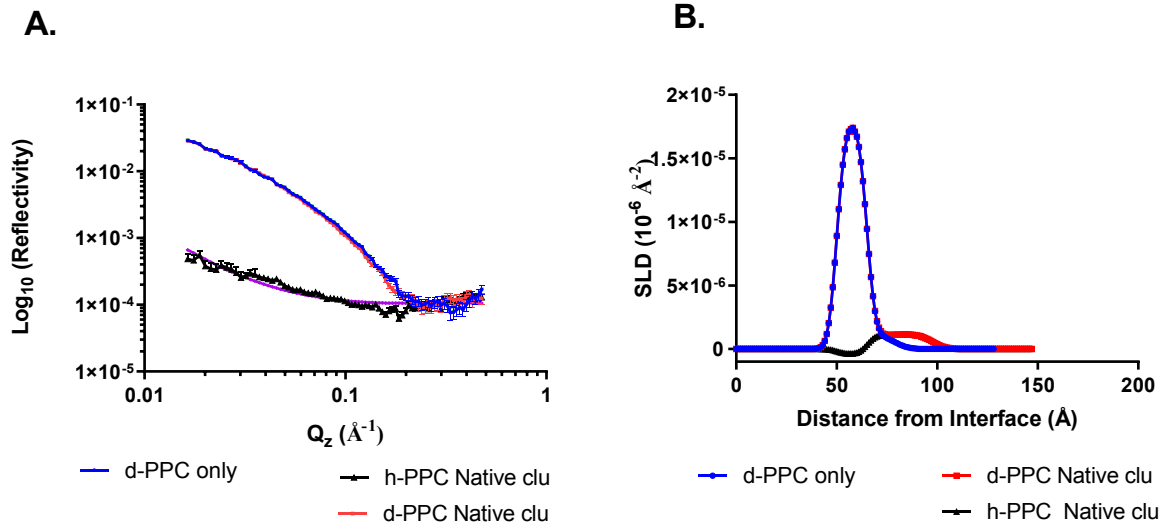
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735 **Figure 2.** Neutron and X-ray reflectometry profiles and model data fits, and corresponding SLD  
 736 profiles after equilibrium adsorption of native clupeine. (A) Reflectivity of **PPC** lipid monolayer  
 737 in NRW with adsorbed native clupeine on the deuterated lipid in (red) and the hydrogenated lipid  
 738 in (black) is plotted against  $Q_z (\text{\AA}^{-1})$ , the momentum transfer. The bare lipid with no peptide is  
 739 shown in blue and the experimental data are represented with error bars whereas the best fit  
 740 simulated data are represented as continuous lines. The SLD profile as a function of distance from  
 741 the interface as determined from the fit is shown in (B).

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\*Note that **PPC** is the abbreviation of PE:PG:CL.

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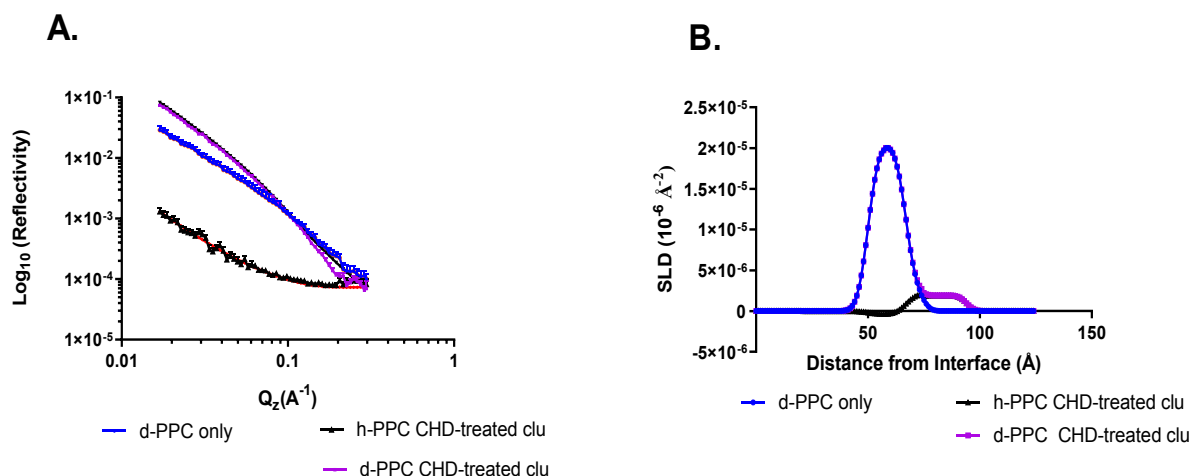
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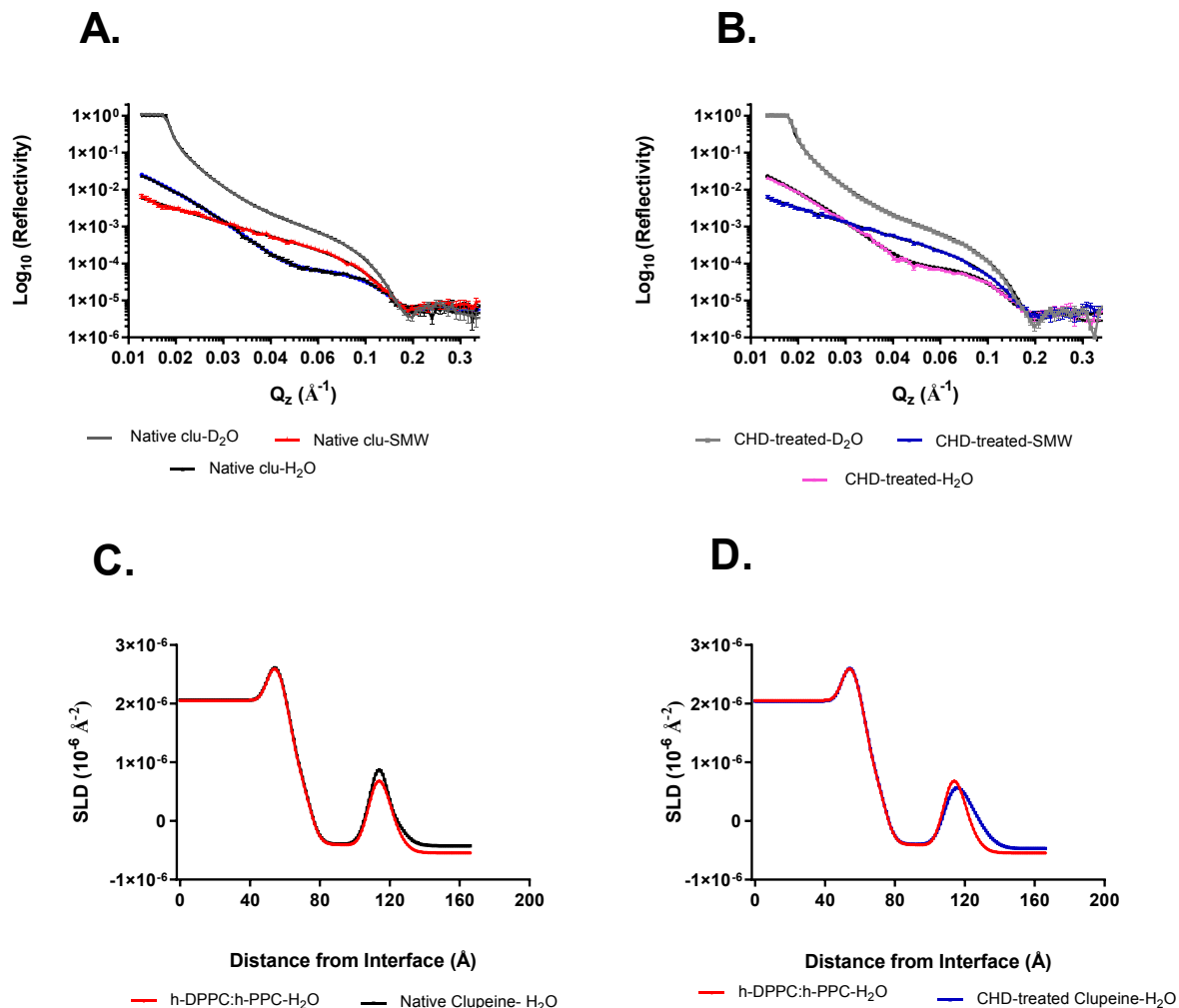




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 760 **Figure 3.** Neutron and X-ray reflectometry profiles and model data fits, and corresponding SLD  
 761 profiles after equilibrium adsorption of CHD-treated clupeine. (A) Reflectivity of PE:PG:CL  
 762 monolayer in NRW with adsorbed CHD-treated clupeine on the deuterated lipid in (purple) and  
 763 the hydrogenated lipid in (black). The bare lipid with no peptide is shown in blue and the  
 764 experimental data are represented with error bars whereas the best fit simulated data are  
 765 represented as lines. The SLD profile as a function of distance from the interface as determined  
 766 from the fit is shown in (B).

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 768 \*Note that PPC is the abbreviation of PE:PG:CL.  
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 786 **Figure 4.** Reflectivity curves and SLD profiles from d/h-DPPC:h-PPC lipid bilayer. **A.**  
 787 Reflectivity data for the h-DPPC:h-PPC bilayer lipids in D<sub>2</sub>O (gray), SMW (red), and H<sub>2</sub>O (black)  
 788 containing native clupeine. The corresponding fits are shown as lines, D<sub>2</sub>O (black), SMW (black),  
 789 and H<sub>2</sub>O (blue). **B.** Reflectivity data for the h-DPPC:h-PPC bilayer lipids in D<sub>2</sub>O (grey), SMW  
 790 (blue), and H<sub>2</sub>O (pink) containing CHD-treated clupeine. The fits are shown as black lines for all  
 791 contrasts. **C.** SLD profiles for the bilayer in water contrast in the presence of native clupeine. The  
 792 data are plotted as points with error bars and the fits are represented as a black line. SLD profile  
 793 for bilayer in water contrast in the presence of CHD-treated clupeine. The data are plotted as points  
 794 with error bars and the fits are represented as a blue line. The greater degree of hydration in the  
 795 lipid head group region in the presence of CHD-treated clupeine compared to the native peptide is  
 796 observed as a broader peak in Figure 4 D compared to Figure 4 C.

797 \*Note that PPC is the abbreviation of PE:PG:CL.

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800 **Table 1** Structural parameters obtained from the three layer model fits of native and CHD-  
 801 treated clupeine (0.48  $\mu\text{M}$ ) adsorbed to **PPC** monolayers. The fits were repeated three times.

Parameters	Thickness $\tau$ ( $\text{\AA}$ )	SLD ( $10^{-6}\text{\AA}^{-2}$ )	Layer roughness ( $\text{\AA}$ )	$\Gamma$ Surface excess ( $\text{mg}/\text{m}^2$ )	( $\Phi_L$ ) Lipid volume fraction
<b>Layer 1, acyl chain</b>					
d- <b>PPC</b> , NRW	15.0 $\pm$ 0.01	1.60 $\pm$ 0.01	3.51 $\pm$ 0.15	0.005 $\pm$ 0.02	0.59 $\pm$ 0.02
h- <b>PPC</b> , NRW	15.0 $\pm$ 0.01	-0.37 $\pm$ 0.01			
h- <b>PPC</b> , XRR	15.0 $\pm$ 0.01	9.69 $\pm$ 0.03			
<b>Layer 2, head group</b>					
d- <b>PPC</b> , NRW	12.7 $\pm$ 0.01	1.07 $\pm$ 0.06		0.297 $\pm$ 0.02	
h- <b>PPC</b> , NRW	12.7 $\pm$ 0.01	1.07 $\pm$ 0.06			
h- <b>PPC</b> , XRR	12.7 $\pm$ 0.01	12.9 $\pm$ 0.40			
<b>Layer 3, peptide layer (native)</b>					
d- <b>PPC</b> , NRW	15.3 $\pm$ 0.07	1.00 $\pm$ 0.09	3.88 $\pm$ 0.32	0.364 $\pm$ 0.02	
h- <b>PPC</b> , NRW	15.3 $\pm$ 0.07	1.00 $\pm$ 0.01			
h- <b>PPC</b> , XRR	15.3 $\pm$ 0.07	10.9 $\pm$ 0.01			
<b>Layer 1, acyl chain</b>					
d- <b>PPC</b> , NRW	16.5 $\pm$ 0.14	2.08 $\pm$ 0.05	3.83 $\pm$ 0.06	0.007 $\pm$ 0.03	0.69 $\pm$ 0.03
h- <b>PPC</b> , NRW	16.5 $\pm$ 0.14	-0.37 $\pm$ 0.01			
h- <b>PPC</b> , XRR	16.5 $\pm$ 0.14	8.64 $\pm$ 0.01			
<b>Layer 2, head group</b>					
d- <b>PPC</b> , NRW	8.27 $\pm$ 0.06	1.69 $\pm$ 0.05		0.372 $\pm$ 0.03	
h- <b>PPC</b> , NRW	8.27 $\pm$ 0.06	1.69 $\pm$ 0.05			
h- <b>PPC</b> , XRR	8.27 $\pm$ 0.06	12.5 $\pm$ 0.06			
<b>Layer 3, peptide layer (CHD)</b>					
d- <b>PPC</b> , NRW	17.6 $\pm$ 0.05	1.42 $\pm$ 0.44	3.50 $\pm$ 0.44	0.59 $\pm$ 0.14	
h- <b>PPC</b> , NRW	17.6 $\pm$ 0.05	1.22 $\pm$ 0.25			
h- <b>PPC</b> , XRR	17.6 $\pm$ 0.05	9.25 $\pm$ 0.05			

802  $\tau$ , represents layer thickness;  $\Gamma$ , represents, clupeine surface excess; and  $\Phi_L$  represents lipid  
 803 volume fraction

804 \*Note that **PPC** is the abbreviation of PE:PG:CL.

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810 **Table 2.** Best fit values and error estimates of asymmetrically deposited bare h-DPPC (inner  
811 leaflet) *E. coli* PPC (outer leaflet) bilayer deposited on a silicon surface and the bilayer in the  
812 presence of native and CHD-treated clupeine.  
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Parameters of the Bilayer	Bare h-bilayer	h-bilayer + native clupeine	h-bilayer + CHD- treated clupeine
Oxide layer thickness (Å)	11.9 ± 2.6	nf	nf
Oxide layer hydration (%)	15.6 ± 2.4		
Oxide layer roughness (Å)	3.58 ± 0.95		
Inner head gp SLD (10 <sup>-6</sup> Å <sup>-2</sup> )	1.53 ± 0.01	nf	nf
Inner head group hydration (%)	31.3 ± 5.5		
Inner head group thickness (Å)	11.9 ± 3.3		
Inner tail SLD (10 <sup>-6</sup> Å <sup>-2</sup> )	-0.39	nf	nf
Inner tail hydration (%)	8.18 ± 1.5		
Inner tail thickness (Å)	15.7 ± 2.2		
Outer tail SLD (10 <sup>-6</sup> Å <sup>-2</sup> )	-0.39	nf	nf
Outer tail hydration (%)	4.45 ± 0.93		
Outer tail thickness (Å)	19.2 ± 0.89		
Outer head gp SLD (10 <sup>-6</sup> Å <sup>-2</sup> )	2.51 ± 0.30	2.17 ± 0.50	2.27 ± 0.48
Outer head group hydration (%)	17.9 ± 12.7	26.9 ± 5.5	48.2 ± 12
Outer head group thickness (Å)	7.94 ± 0.54	8.52 ± 0.04	8.13 ± 0.66
Bilayer roughness (Å)	4.99 ± 0.01	nf	nf
Clupeine hydration (%)	n.a.	48.8 ± 3.1	58.9 ± 15
Clupeine thickness (Å)	n.a.	4.15 ± 2.9	11.0 ± 6.0
Clupeine roughness (Å)	n.a.	3.15 ± 2.7	6.91 ± 1.6

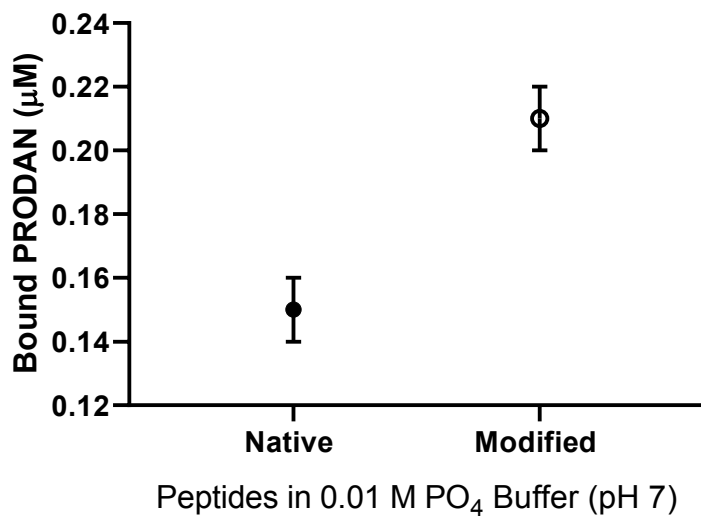
814 nf = not fitted and n.a. = not applicable

815 \*Note that PPC is the abbreviation of PE:PG:CL.

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## Appendix A



**Figure 1A.** The binding of PRODAN to native and modified clupeine. The surface hydrophobicity of the native and modified clupeine was measured using an uncharged probe, PRODAN. A PRODAN standard curve was developed which was used to measure the amount of probe bound to the clupeine samples.

**Table A1.** Summary of Scattering length scattering length densities, molecular weights, and molecular volumes of the lipids (PPC and DPPC) and peptides used in the present study.

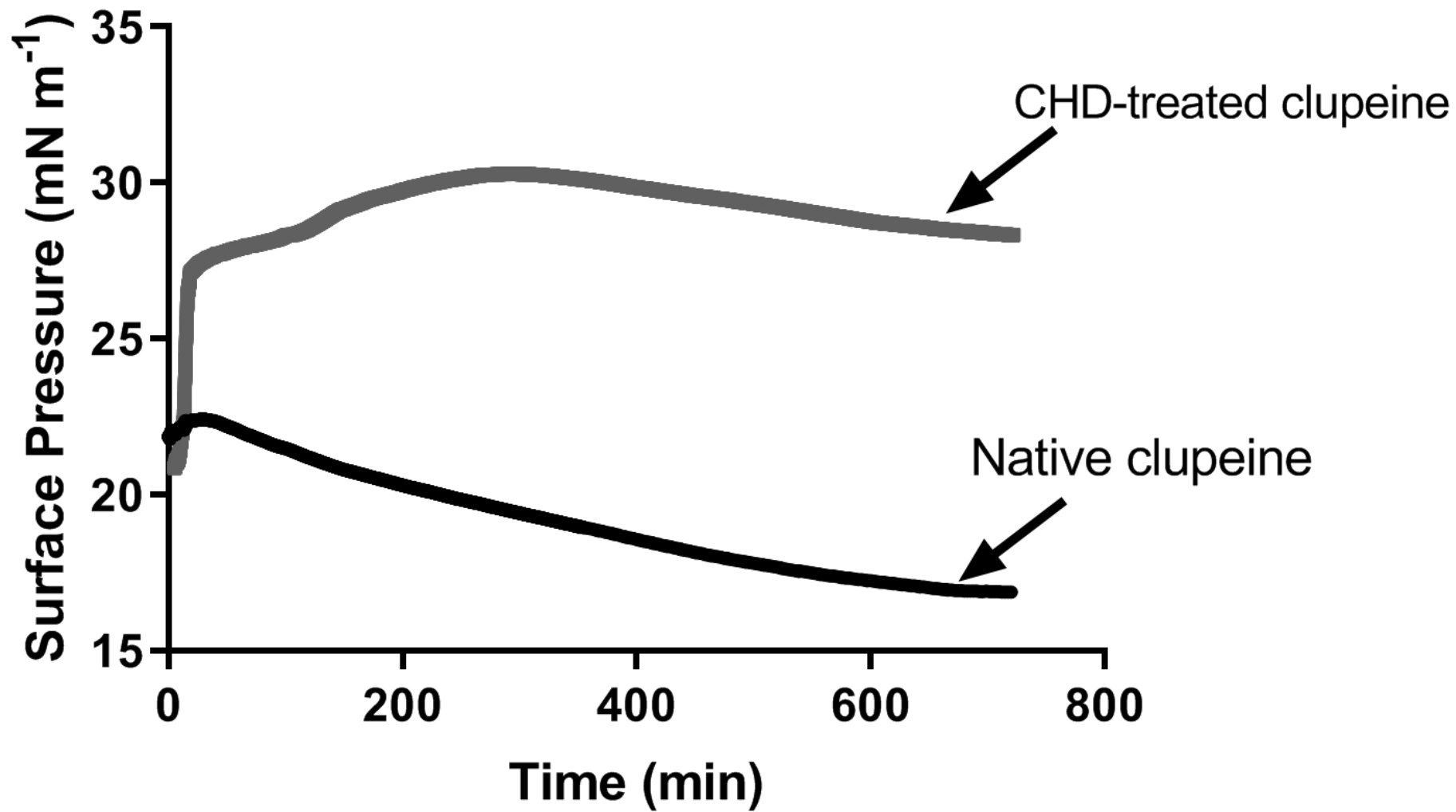
Parameters	Scattering length $\Sigma b$ ( $10^{-3}\text{\AA}$ )	SLD ( $10^{-6}\text{\AA}^{-2}$ )	Molecular Weight (g/mol)	Molecular Volume ( $\text{\AA}^3$ )
h-PPC (head + tail)	0.339	0.300	720	1128
h-PPC (hd. group)	0.598	2.06	273	288
d-PPC tail	6.24	7.49	496	838
h-PPC tail	-0.326	-0.394	434	838
Native clupeine in NRW	29.0	2.02	4200	
CHD-treated clupeine in NRW	29.0	2.02	4200	
h-DPPC (head + tail)	0.277	0.241	734	1152
h-DPPC (hd. group)	0.597	1.74	311	342
h-DPPC tail		-0.39 <sup>a</sup>		
d-DPPC tail		7.45 <sup>a</sup>		

<sup>a</sup> These values were obtained from Clifton et al. (2013 b).

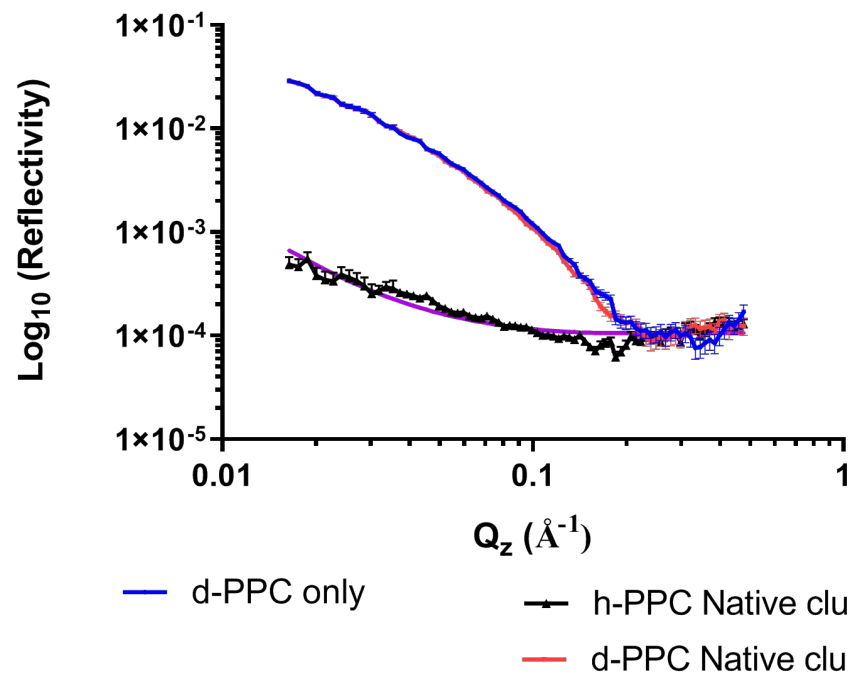
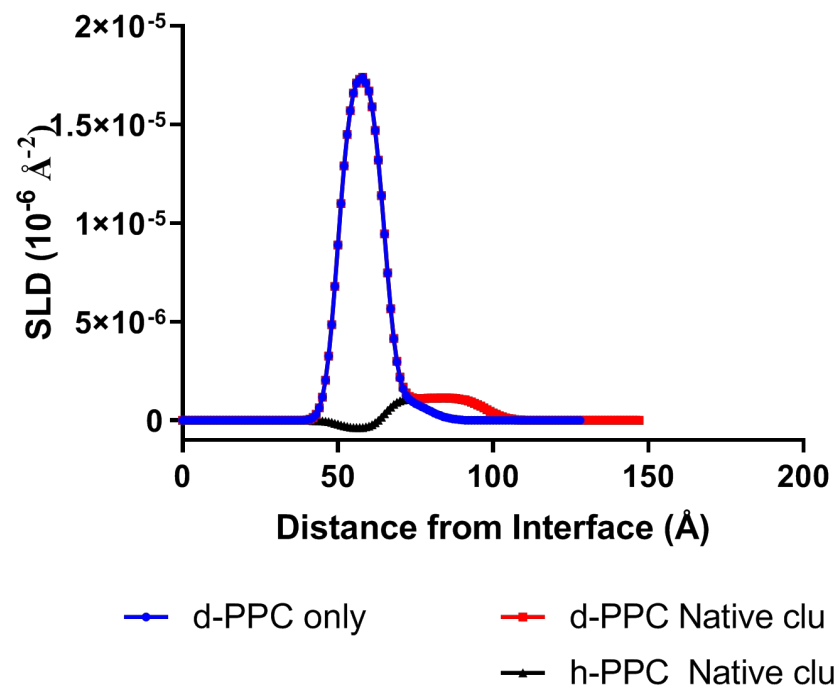
**Table A2** Structural parameters obtained from a two-layer model fit of a condensed phase d-PE:PG:CL monolayer obtained from simultaneously fitting NR and XRR profiles. The structural parameters described for each layer are the layer thickness ( $\tau$ ), the SLD ( $\rho$ ) and the corresponding layer roughness. The fits were repeated three times.

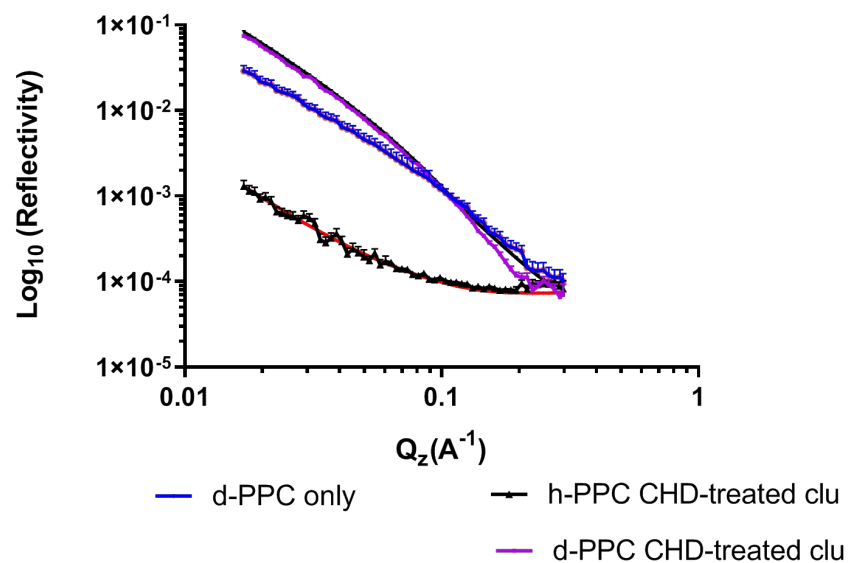
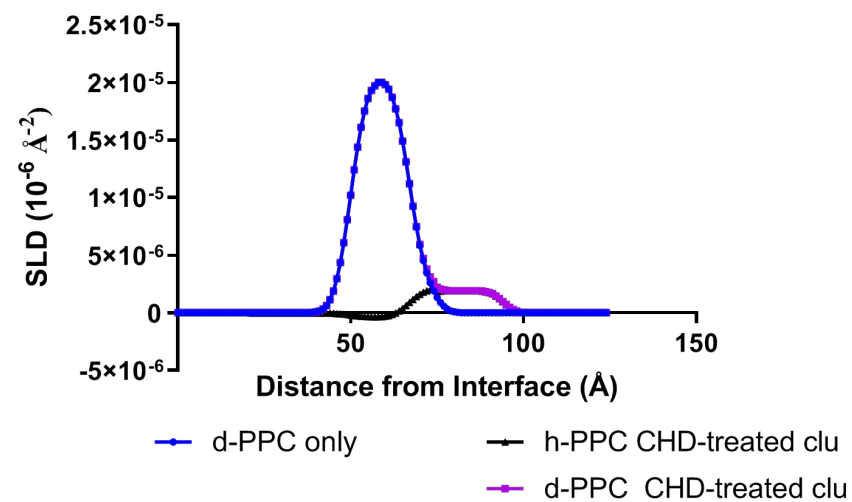
<b>Parameters</b>	<b>Thickness <math>\tau</math> (Å)</b>	<b>SLD (<math>10^{-6}\text{Å}^{-2}</math>)</b>	<b>Layer roughness (Å)</b>	<b>Lipid volume fraction (<math>\Phi_L</math>)</b>
<b>Layer 1, acyl chain</b>				
d-PE:PG:CL, NR	$15.0 \pm 0.64$	$7.28 \pm 0.76$	$3.93 \pm 1.1$	$0.97 \pm 0.02$
h-PE:PG:CL, XRR	$15.0 \pm 0.64$	$9.55 \pm 0.49$		
<b>Layer 2, head group</b>				
d-PE:PG:CL, NR	$12.9 \pm 1.2$	$0.46 \pm 0.25$		
h-PE:PG:CL, XRR	$12.9 \pm 1.2$	$13.2 \pm 0.07$		

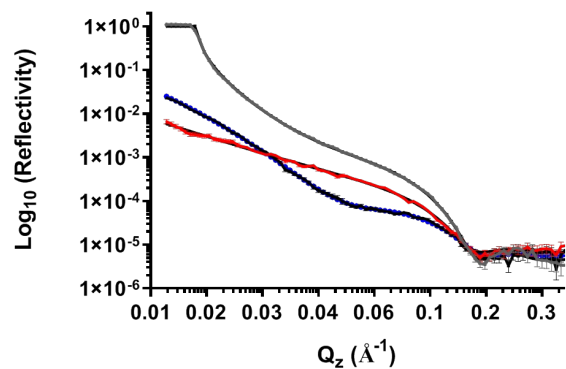
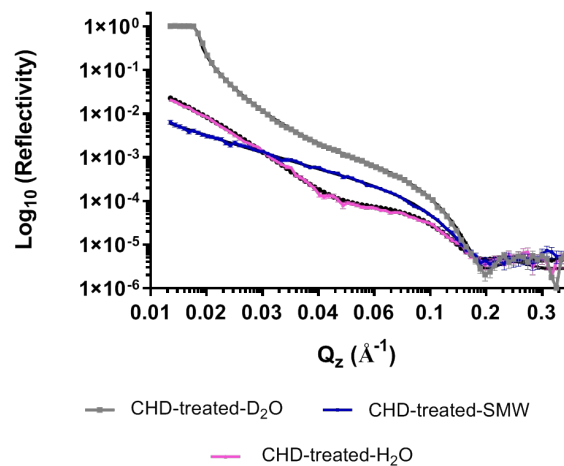
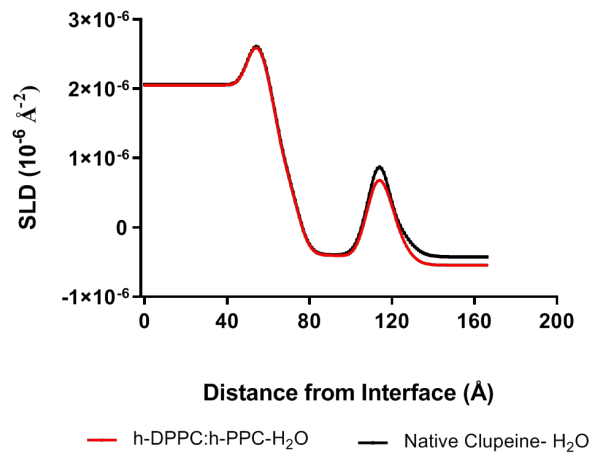
$\tau$ , represents layer thickness and  $\Phi_L$ , represents lipid volume.





**A.****B.**

**A.****B.**

**A.****B.****C.****D.**