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1 **Unveiling the binding and orientation of the antimicrobial peptide Plantaricin 149 in zwitterionic and negatively**
 2 **charged membranes**

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28

29 **Abstract**

30 Antimicrobial peptides are a large group of natural compounds which present promising properties for the
31 pharmaceutical and food industries, such as broad-spectrum activity, potential for use as natural preservatives, and
32 reduced propensity for development of bacterial resistance. Plantaricin 149 (Pln149), isolated from *Lactobacillus*
33 *plantarum* NRIC 149, is a peptide with the ability to inhibit bacteria from the *Listeria* and *Staphylococcus* genera, which
34 is capable of promoting inhibition and disruption of yeast cells. In this study, the interactions of Pln149 with model
35 membranes composed of zwitterionic and/or anionic phospholipids were investigated using a range of biophysical
36 techniques, including isothermal titration calorimetry, surface tension measurements, synchrotron radiation circular
37 dichroism spectroscopy, oriented circular dichroism spectroscopy, and optical microscopy, in order to elucidate their
38 mode of interactions and provide insight into their functional roles. In anionic model membranes, the binding of Pln149
39 to lipid bilayers is an endothermic process and induces a helical secondary structure in the peptide. The helices bind
40 parallel to the surfaces of lipid bilayers and can promote vesicle disruption, depending on peptide concentration.
41 Although Pln149 has relatively low affinity for zwitterionic liposomes, it is able to adsorb at their lipid interfaces,
42 disturbing the lipid packing, assuming a similar parallel helix structure with a surface-bound orientation, and promoting
43 an increase in the membrane surface area. Such findings can explain the intriguing inhibitory action of Pln149 in yeast
44 cells whose cell membranes have a significant zwitterionic lipid composition.

45

46 **Keywords:** antimicrobial peptide; oriented circular dichroism spectroscopy; mechanism of action; peptide-lipid
47 interactions; synchrotron radiation circular dichroism spectroscopy.

48

49 **Introduction**

50 Antimicrobial peptides (AMPs) are natural compounds which have been extensively studied for their potential
51 use in the pharmaceutical industry (Kumar et al. 2018; Ageitos et al. 2017; Marshall and Arenas 2003; Bahar and Ren
52 2013) due to their broad spectrum of targets ranging from viruses to parasites (Gordon et al. 2005), and their reduced
53 propensity for inducing the development of resistance by microorganisms (Bechinger and Gorr 2017). To date, a large
54 number of AMPs produced by lactic acid bacteria have also been investigated for their potential application in the food
55 industry as promising candidates for natural preservatives (Singh 2018; Wang et al. 2016; Rai et al. 2016), capable of
56 inhibiting the growth of other bacteria taxonomically related to the producing strain.

57 The antimicrobial peptide (AMP) Plantaricin 149 (Pln149), with the sequence
58 YSLQMGATAIKQVKKLFKKKGG (Kato et al. 1994), isolated from *Lactobacillus plantarum* NRIC149, has been
59 described as a bacteriocin, capable of inhibiting important pathogenic bacteria such as *Listeria monocytogenes*,
60 *Staphylococcus aureus*, *Enterococcus*, *Salmonella typhimurium*, with no effect on human cells. In addition, it has high
61 sequence homology to the peptide Plantaricin A (Pln A) (Hauge et al. 1998; Sand et al. 2007) which has been described
62 as a pheromone with anti-bacterial properties.

63 Previous studies performed with the synthetic amidated peptide Pln149 (Müller et al. 2007; Lopes et al. 2009),
64 have shown that this peptide is completely unordered/unstructured in aqueous solution. However, as with many AMPs,
65 when exerting its antimicrobial effect, Pln149 appears to adopt a highly structured form (an amphipathic α -helix) upon
66 interaction with negatively charged lipids (Lopes et al. 2009). The electrostatic attraction between the cationic Pln149
67 (net charge +7 at pH 7.0) and the negative net surface charge of target bacteria is important for promoting peptide
68 structuring and permeabilizing action on membranes, possibly through a carpet-like mechanism (Lopes et al. 2009; Sani
69 and Separovic 2016).

70 Pln149 has also been demonstrated to inhibit growth and induce cell disruption in *Saccharomyces cerevisiae*,
71 promoting severe morphological changes in the yeast cells (Lopes et al. 2009). However, yeast cell membranes, unlike
72 the membranes of the target organisms listed above, are not predominantly comprised of negatively-charged lipids. A
73 zwitterionic lipid composition and a complex cellular wall are found instead (Kenneth and Rose 1972), so previously
74 proposed mechanisms/structures based on peptide adsorption and accumulation at the cell membrane guided by
75 electrostatic attraction between the cationic peptide and the negative surface charge density at the microorganism cell
76 membrane may not adequately explain how Pln149 impacts on *Saccharomyces* cells.

77 In this paper, we use a combination of biophysical methods (isothermal titration calorimetry, surface tension
78 measurements, synchrotron radiation circular dichroism and oriented circular dichroism spectroscopies, and optical
79 microscopy) to investigate the binding of Pln149 to different types of model membranes (monolayers, oriented lipid
80 bilayers, and giant unilamellar vesicles). The membrane models were composed either entirely of zwitterionic
81 phospholipids or of phospholipids with a net negative charge density. The prevalent negatively-charged lipid found in
82 bacterial cells, phosphatidylglycerol (PG), and one of the most abundant phospholipids in eukaryotic cells,
83 phosphatidylcholine (PC), were employed to mimic their negatively-charged and zwitterionic membrane surfaces,
84 respectively. In addition, as *S. cerevisiae* cells usually contain low amounts of PG in their plasma membranes, and
85 higher amounts of phosphatidylserine (PS) (Lindberg et al. 2013), the interactions of Pln149 with PS phospholipids were

86 also studied to better understand the potential action of the peptide on yeast cells. The results described advance our
87 understanding of how this AMP is oriented both in negatively-charged membrane such as found in pathogenic bacteria,
88 and in zwitterionic membranes, such as found in yeast cells.

89

90 **Materials and methods**

91 *Materials* – Synthetic Pln149 was produced by solid phase peptide synthesis using Fmoc chemistry, and then purified by
92 reverse phase chromatography, as described in Lopes et al. (2009). The purity (>95%) and molecular weight (2423.7
93 Da) of Pln149 were determined by mass spectrometry (Lopes et al., 2009). Phospholipids 1-palmitoyl-2-oleoyl-sn-
94 glycerol-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphoglycerol (POPG), and 1-palmitoyl-2-
95 oleoyl-sn-glycerol-3-phospho-L-serine (POPS) were purchased from Avanti Polar Lipids and reagents were P.A. grade.

96 *High sensitivity isothermal titration calorimetry (ITC)* - The partitioning of the peptide Pln149 into liposomes was
97 examined using a high-sensitivity MicroCal VP-ITC instrument (Northampton, MA) at 25°C, using an experimental
98 approach based on that of Seelig (2004): an aqueous solution of the peptide Pln149 (28 µM) was loaded into the sample
99 cell (reaction volume of 1.417 mL), and multilamellar vesicles of pure POPC, pure POPG or pure POPS (vesicles at 7
100 mM) were injected via the titration syringe in 20 aliquots of 10 µL, under continuous stirring at 300 rpm. The change in
101 heat rate during the titration steps was registered in real time. All the solutions were degassed under moderate vacuum
102 (ThermoVac, Northampton, MA, USA) prior to use, to eliminate air bubbles. The heats of dilution were determined in
103 control experiments and subtracted from the corresponding peptide-lipid measurements. The results were analyzed using
104 Origin 7.0 software.

105 *Surface tension measurements* – The surface activity of Pln149 was assayed with a Wilhelmy wire attached to a Delta Pi
106 Langmuir-tensiometer (Kibron, Finland) interfaced to a computer. Measurements were made, for 30 min at 25°C over a
107 concentration range from 0.16 to 10 µM in pure MilliQ water. Subsequently, the adsorption kinetics of Pln149 on
108 (separate) Langmuir monolayers of POPG, POPS, and POPC were determined by spreading a small amount of each
109 lipid (pure POPC, POPS or POPG, and a mixture POPC/POPG 1/1 molar ratio) in methanol/hexane (10/1, v/v, at a 1
110 mg/mL) on top (air/water interface) of a microplate well filled with 1.2 mL MilliQ water (or containing 0.25M or 0.5 M
111 NaCl), generating lipid monolayers at the well surface with their lateral packing density controlled from 10 to 45 mN/m.
112 Pln149 (5.0 µM) was injected to the aqueous subphase and subsequent changes in surface pressure due to the peptide
113 adsorption were monitored for 30 min.

114 *Synchrotron radiation circular dichroism (SRCD) spectroscopy* – The SRCD spectra of Pln149 (400 μM) were collected
115 at the UV-CD12 (Bürck et al. 2015) beamline of the KARA synchrotron (formerly known as ANKA), Karlsruhe
116 Institute of Technology (KIT, Germany), and at the AU-CD beamline of the ASTRID2 synchrotron at ISA (Aarhus
117 University, Denmark). Samples were examined in aqueous solution, in the presence of 10 mM sodium dodecyl sulfate
118 (SDS), or liposomes comprised of POPG, POPS and POPC at 1/50 peptide-to-lipid (P/L) molar ratios. The binding of
119 the peptide to POPG liposomes was also investigated in aqueous solutions containing 0.25 M or 0.5 M sodium fluoride.
120 SRCD spectra were measured over the wavelength range from 270 to 170 nm in either 0.5 nm or 1 nm intervals, using
121 quartz glass Suprasil cuvettes (Hellma) with either 97 or 100 μm pathlengths, at 25°C. Baselines for each sample
122 (containing all the components, except the peptide) were collected in the same cell. Data processing was performed
123 using CDTool software (Lee et al. 2004) and consisted of averaging the three successive scans accumulated for each
124 sample, subtraction of the cognate averaged baselines, smoothing with a Savitzky-Golay filter, calibration with
125 camphorsulfonic acid, and conversion to delta epsilon units (using mean residue weights of 115.4).

126 Dehydrated samples were produced as follows: Aliquots (1 nmol) of Pln149 in MilliQ water were deposited on
127 the surface of a quartz glass plate and transferred to a desiccator under moderate vacuum for 2 hours to yield a peptide
128 film on the plate. The SRCD spectra were measured over the wavelength range from 270 to 155 nm in 1 nm intervals, at
129 25°C, collecting and averaging over four different rotation angles (90 degrees each, perpendicular to the direction of the
130 beam) for each scan, in order to examine for possible orientation effects. The same quartz glass plate (without sample)
131 was used for collecting the baseline spectrum.

132 Studies in lipid bilayers were done as follows: large unilamellar vesicles (LUVs) composed of either POPC or
133 POPG, prepared by solubilizing the dried lipids in a 4/1 (v/v) chloroform/methanol mixture, evaporating the solvent
134 under a moderate N_2 stream, followed by 2 hours in a SpeedVac system, and then rehydration by vortexing in 2 mL of
135 aqueous solution. The multilamellar vesicles were extruded through a polycarbonate filter (100 nm pore diameter) for a
136 minimum of 11 times to yield LUVs with an average diameter of 100 nm (the average diameter was confirmed by
137 dynamic light scattering).

138 *Oriented circular dichroism (OCD) spectroscopy* – Oriented lipid bilayers were prepared as follows: aliquots of a stock
139 solution (5 mg/mL) of either POPG, POPS, or POPC solubilized in a mixture of chloroform/methanol (1/1, v/v) were
140 mixed with Pln149 (0.3 mg/mL) solubilized in methanol/TFE (95/5, v/v) to yield peptide-to-lipid molar ratios of 1:25,
141 1:50, 1:100 and 1:200 (mol/mol), and then vortexed to homogeneity. The peptide-lipid mixtures (~30-60 μL) were

142 slowly deposited in a circular spot of 12 mm diameter on the surface of a quartz glass Suprasil plate (Hellma) using a
143 gastight syringe, to promote the formation of homogeneous films on the plate. The plates were then placed in a
144 desiccator chamber kept under moderate vacuum for 2 h to ensure complete removal of the organic solvents. Prior to the
145 measurements, the plates with the deposited samples were maintained for 16 h in a home-built OCD chamber (Bürck et
146 al. 2008) with controlled humidity (97.6% relative humidity, maintained using a saturated solution of K_2SO_4) at 20°C
147 temperature to enable the rehydration of the lipid bilayers on the plate and alignment of the peptides within the oriented
148 lipid bilayers. During the OCD measurements, the humidity and temperature within the sample cell were monitored by a
149 capacitive relative humidity and temperature sensor (SHT 75, Sensirion, Zurich, Switzerland), which was fixed in the
150 immediate vicinity of the sample. The cell design used is as described in Burck et al. (2008). OCD measurements were
151 carried out on a Jasco J-810 spectropolarimeter, over the wavelength range from 260 to 180 nm, at 0.1 nm intervals, at a
152 controlled temperature of 20°C (above the phase transition temperature of the phospholipids), taking 2 successive
153 spectra in 8 different positions of the plate (0, 45, 90, 135, 180, 225, 270 and 315° rotations), which were then averaged.
154 The spectra of lipid bilayers alone were measured under the same conditions, and the averaged lipid reference spectrum
155 was subtracted from the corresponding averaged sample spectrum. The plates were maintained under controlled
156 humidity throughout the measurement to avoid dehydration. For each P/L ratio, at least two measurements were
157 averaged, and finally the data of from a given series were normalized to the same spectral magnitude at the long-
158 wavelength minimum around 220 nm.

159 *Optical microscopy of giant unilamellar vesicles (GUVs)* – GUVs composed of POPC, POPC/POPG (1/1), or
160 POPC:POPS (1/1) containing 0.2% Rhodamine-DOPE, were produced by the electroformation method (Angelova and
161 Dimitrov 1986). Briefly, 20 μ L aliquots of a 1 mg/ml phospholipid solution in chloroform/methanol were spread on the
162 surface of two conductive glasses coated with indium tin oxide (ITO). After solvent evaporation, a Teflon spacer was
163 placed between the two coated glasses to form a closed chamber which was filled with the swelling solution (0.2 M
164 sucrose). The plates were submitted to an alternating electric field of 2 V and 10 Hz frequency for 1 hour. The solution
165 containing the GUVs was diluted 10-fold into a 0.2 M glucose solution (osmolarity measured in an Osmomat Gonotec
166 030 osmometer) containing increasing concentrations of Pln149 (from 16 to 250 nM). Samples were immediately
167 observed by optical microscopy using an inverted microscope Zeiss Axiovert 200 (Jena, Germany) equipped with a
168 Zeiss AxioCam digital camera (Jena, Germany). A Ph2 63x objective was used either in phase contrast or in fluorescent

169 mode (illumination with a mercury lamp HBO 103W with the filter set for excitation at 540-552nm and emission at 575-
170 640 nm).

171

172 **Results and discussion**

173 *Calorimetry studies of Pln149 binding to liposomes* - ITC is a useful technique for studying peptide-lipid interactions,
174 since it detects the heat absorbed or released during molecular interactions. The binding enthalpy reflects the overall
175 process of peptide binding, with contributions from both the peptide adsorption and from the structuring of the lipid
176 environment. In contrast to chemical reactions, peptide-lipid physical interactions do not involve formation/breakage of
177 covalent bonds, and can be divided into at least three different processes: (i) electrostatic attraction of the peptide to the
178 membrane surface; (ii) adsorption/insertion of the peptide into the polar headgroup region or into the hydrophobic core
179 of the membrane; and (iii) conformational change of the peptide, usually associated with the initial binding process
180 (Seelig 1997, 2004; Abraham et al. 2005; Voievoda et al. 2015). Because calorimetric measurements produce results that
181 arise from a range of heat-generating mechanisms in peptide-lipid binding, complementary techniques are required in
182 order to enable their interpretations.

183 Lipid-into-peptide titrations were used in order to evaluate the partitioning of Pln149 into model membranes
184 with different net charges (zwitterionic POPC, and negatively charged POPG and POPS vesicles). The ITC binding
185 isotherms of Pln149 to POPG vesicles (Figure 1a, red) displayed a typical profile of endothermic reactions, where
186 initially each injection promoted the absorption of the heat flow (~1.1 μcal per injection). The integration of these peaks
187 produced a calculated enthalpy of reaction (ΔH) of about 0.5 kcal/mol. The binding was of this magnitude for 7
188 injections, but gradually decreased until the heat of partitioning remained constant, indicating a possible saturation of
189 Pln149-membrane interactions (at a point where all peptides in the sample cell were considered to be bound to the
190 liposomes). Similar behavior of Pln149 was observed in POPS vesicles, but the heat of partition and, consequently, the
191 enthalpy, was twice that obtained for POPG (Figure 1a, green). For both negatively-charged vesicles, the heat of
192 partition was initially increased to a maximum value, and then it gradually decreased to reach the heat of dilution of the
193 system. Such behavior could be observed for both POPG and POPS, but it was more pronounced in POPS.

194 The disorder-to-helix transition due to membrane interaction is a well-understood exothermic reaction
195 (Wieprecht et al. 1999, 2000), and a large exothermic enthalpy is expected to be associated with helix formation.
196 However, only small exothermic or even endothermic binding enthalpies are observed in most cases (Seelig 1997;
197 Abraham et al. 2005; Wieprecht et al. 1996), because the heat for helix formation is usually compensated for other

198 physical processes, such as pore formation, micellization, peptide aggregation, or formation of lipid clusters (Henriksen
199 and Andresen 2011).

200 In contrast, during the titration of POPC vesicles into Pln149 (Figure 1a, in blue), almost no heat change was
201 detectable, being on the order of the dilution heat determined by vesicle titration into aqueous solution and/or aqueous
202 solution into peptide solution. This suggests that no interactions, or only minor interactions (undetectable by the
203 instrument), were occurring between Pln149 and the zwitterionic vesicles.

204 In order to check whether the Pln149 partitioning into the POPG negatively-charged vesicles was driven by
205 electrostatic attraction, the titration of POPG vesicles into the peptide was made in a salt solution (containing 250 mM
206 NaCl). Figure 1b shows the difference between the titration in the aqueous (red) and salt (grey) solutions: The
207 partitioning of the peptide into POPG vesicles in the salt solution was lower than that in the aqueous solution (~0.5
208 $\mu\text{cal}/\text{injection}$). The heat of partition remained constant throughout the experiment (Figure 1b, grey), indicating some
209 “competition” of the charges, i.e., the ions coming from the salt may shield the peptide and/or the membrane surface
210 charge, thus hampering the electrostatic attraction between Pln149 and negatively-charged membranes.

211
212 *Surface tension of Pln149 and its adsorption on Langmuir monolayers* - The antimicrobial peptide Pln149 produced no
213 observable surface activity over the entire concentration range from 0.16-10 μM (Figure 2a), suggesting that no peptide
214 adsorption occurred at the air-water interface; the surface tension value monitored throughout the measured time was
215 ~72.8 mN/m, which corresponds to the expected value for pure water. However, when a lipid monolayer was spread at
216 the air-water interface, the peptide exhibited the ability to migrate from the subphase to the surface, thus adsorbing in the
217 lipid monolayer. The adsorption of Pln149 onto the POPG, POPS and POPC monolayers exhibited significant changes
218 in the lateral pressure due to the induced surface activity of Pln149 (Figure 2a). The adsorption of Pln149 on POPG and
219 POPS monolayers produced higher values of $\Delta\pi$ than those observed for the zwitterionic phospholipid, suggesting
220 stronger peptide-lipid interactions when electrostatic attractions are present. However, such a result on the zwitterionic
221 POPC monolayer is clear evidence that the antimicrobial peptide was also able to migrate from the aqueous subphase to
222 adsorb and, albeit slowly, disturb the lipid packing of an entirely zwitterionic lipid surface formed by the POPC
223 monolayer.

224 Comparing the curves for the binding of Pln149 on lipid monolayers of POPG (or POPS), and POPC (Figure
225 2b) at different initial surface pressures (π_0), the peptide adsorption was clearly enhanced in the presence of an
226 electrostatic attraction between the peptide and negatively charged monolayers. As the maximum insertion pressures

227 (MIP) of Pln149 in both POPG and POPS monolayers were determined to be ~46 mN/m, well above the value estimated
228 for the lateral pressure of a biological membrane (30-35 mN/m) (Marsh 1996), this indicated that Pln149 is likely to be
229 able to insert into biological membranes with a negative surface charge density. In contrast, the MIP on the zwitterionic
230 POPC was ~35 mN/m, suggesting that the peptide Pln149 is less efficient at penetrating into zwitterionic cell
231 membranes.

232 However, it can be seen that Pln149 can also interact with zwitterionic phospholipids and disturb lipid packing
233 effectively, causing more significant changes in $\Delta\pi$ when the lipid packing of the POPC lipid monolayer was in looser
234 molecular packing (10-25 nM/m). This is consistent with observations that AMPs can bind defective membranes or
235 loosely packed lipid layers of a particular lipid composition but not interact with well-formed membranes of the same
236 lipid composition. The antimicrobial peptide TRP3 (Bozelli et al. 2012) has been reported to produce similar preferential
237 binding to LUVs containing negatively-charged lipids, but apparently does not bind to zwitterionic vesicles, whereas it
238 binds to micelles irrespective of their headgroup charge. The different peptide behavior of TRP3 was attributed to the
239 predominance of intermolecular interactions (hydrophobic, hydrogen bonding, van der Waals interactions, hydration
240 forces) other than electrostatic ones in a system where a loose molecular packing of lipids is present. Similarly to the
241 surface behaviour of Pln149, the peptides maculatin and citropin (Ambroggio et al. 2004) interact with both zwitterionic
242 POPC monolayers and anionic POPG bilayers, with a stronger interaction with the anionic lipid.

243 The action of the pheromone Plantaricin A (PlnA) on lipid membranes has also been demonstrated to be highly
244 dependent upon lipid composition (Zhao et al, 2006). A 22-mer version of PlnA
245 (KSSAYSLQMGATAIKQVKKLFKKWGW) (Sand et al., 2007; 2013) is a very close homolog of Pln149 (they differ
246 only in their C-terminal residues, KGG for Pln149 and WGW for PlnA), which also presents the ability to bind to
247 monolayers and liposomes comprised solely of zwitterionic PC lipids. However, there does not appear to be significant
248 penetration of Pln A into these types of membranes under similar conditions. As we found for Pln149, when the
249 negatively-charged POPG was present, the association of PlnA with lipid membranes was significantly enhanced.
250 Interestingly, PlnA is reported to be a peptide pheromone (Kristiansen et al. 2005), which displays antimicrobial activity
251 in addition to its pheromone activity. PlnA was first classified as a bacteriocin (an AMP) but later discovered to
252 pheromone-like activity (Sand et al. 2010; 2013) inducing production of other plantaricin molecules in *L. plantarum* C11
253
254 *SRCD spectroscopy characterizing the structural properties of Pln149 bound to model membranes* – SRCD spectroscopy
255 is a valuable method for determining the spectra in the far ultraviolet and the vacuum ultraviolet wavelength ranges (the

256 latter at wavelengths below 200 nm where the disordered proteins/peptides structures can have a significant contribution
257 to the spectra) (Lopes et al. 2014; Kumagai et al. 2017), was used to provide information on the protein secondary
258 structure. The lower wavelength measurements [relative to those obtainable by conventional CD methods] not only
259 contain additional valuable information on peptides in disordered conformations, but because of the high intensity of the
260 light and the geometry of the instrument, also enable the examination of samples, such as membranes, which scatter
261 light (a feature that can otherwise result in distortion of peptide spectra) (Miles and Wallace, 2016). The SRCD spectrum
262 of Pln149 in aqueous solution (Figure 3, in black) primarily consists of a negative peak centered at 198 nm. This
263 indicates it is predominantly in an unordered conformation.

264 Incubation of Pln149 with zwitterionic (POPC) or negatively-charged (POPG) liposomes produced contrasting
265 results: while no conformational changes were observed in the Pln149 SRCD spectrum in the presence of zwitterionic
266 vesicles (Figure 3a), an increase in the two characteristic negative peaks at 222 and 208 nm, and the appearance of a
267 positive peak around 192 nm suggest that in the presence of POPG liposomes, the peptide includes some helical
268 structure. In the presence of POPS, although their peak magnitudes were much smaller, these bands can also be clearly
269 seen. A similar disorder-to-helix change also appears in the presence of the negatively-charged surfactant SDS,
270 suggesting formation of some helical structure. Such conformational changes are consistent with the electrostatic
271 attraction between the cationic peptide and the negative membrane, which could produce peptide re-structuring and the
272 reported higher activity of the peptide in Gram(+) bacteria (Müller et al. 2007; Lopes et al. 2009). Helical wheel
273 projections (Figure 3c) for residues A7 to K20 of Pln149 indicate the region with highest helical propensity and the
274 predicted location of an amphipathic helix in the peptide.

275 A similar disorder-to-helix transition has previously been observed in PlnA (Hauge et al. 1998) using
276 conventional circular dichroism spectroscopy. Those studies showed that the unstructured peptide in aqueous solution
277 was able to adopt a helical structure in the presence of trifluoroethanol (TFE) or model membranes composed of
278 negatively-charged phospholipids, but not in the presence of zwitterionic liposomes. This led the authors to conclude
279 that the negative charge on the membrane is important for structuring of the similarly positively-charged PlnA peptide as
280 an α -helix.

281 In order to determine whether the binding of Pln149 to the negatively-charged vesicles is driven by electrostatic
282 attraction, the peptide was incubated with POPG vesicles prepared in salt solutions (containing 0.25 or 0.5 M of NaF).
283 Figure 3b shows that as the concentration of NaF in the solution increased, the 222 nm band associated with helical
284 structures was dramatically reduced, and the magnitudes and the position of the 208 nm and 192 nm bands were reduced

285 and shifted to 198 and 184 nm, respectively, producing peaks characteristic of disordered conformations (as seen for the
286 peptide in aqueous solution). Thus, the partition of the peptide into the negatively-charged vesicles appears to be driven
287 by electrostatic interactions.

288 The removal of bulk water from a dehydrated sample containing Pln149 (Figure 3a) also appeared to promote a
289 similar transition from a disordered to a helical state. We propose, as it has been noticed for other disordered proteins,
290 that this can be attributed to the removal of water molecules surrounding the peptide, favoring intramolecular hydrogen
291 bonds rather than peptide-solvent ones (Cammers-Goodwin et al. 1996).

292
293 *OCD spectroscopy to determine peptide orientation in lipid bilayers* - Although the induction of the helical structure in
294 Pln149 in the presence of negatively-charged liposomes has been revealed by its SRCD spectrum, no information about
295 the orientation of the helix relative to the membrane bilayer direction had yet been determined. Therefore, oriented
296 circular dichroism spectroscopy was employed to characterize the conformation of Pln149 in macroscopically-oriented
297 lipid bilayers and to evaluate its alignment as a function of peptide concentration in such systems (using different
298 peptide/lipid molar ratios). The OCD spectrum of Pln149 in oriented POPG bilayers (Figure 4a) at a 1/200 P/L molar
299 ratio exhibits the two characteristic α -helix minima at 208 nm and 220 nm, but with a more pronounced minimum at 208
300 nm. This spectral lineshape corresponds to a surface-bound state, or simply S-state (Bürck et al. 2016), and indicates
301 peptide insertion aligned parallel to the plane of the lipid bilayers, with the amphipathic Pln149 helix possibly
302 intercalated at the interface between lipid head groups and the hydrophobic core of the lipid membrane. It could be
303 speculated that the charged lysine residues point towards the aqueous phase while the leucine, isoleucine and valine
304 residues of the hydrophobic sector of the amphipathic Pln149 helix interact with the hydrocarbon region of the model
305 membrane.

306 The OCD spectra for Pln149 in POPG oriented bilayers exhibited no significant dependence on peptide
307 concentration, i.e., P/L ratio used. When the OCD spectra were normalized to the long-wavelength minimum of this
308 sample at 220 nm for other P/L molar ratios (up to 1/25), they also indicated the peptide orientation was in the S-state,
309 except for the 1/25 P/L molar ratio, where the somewhat decreased intensity of the 208 nm “fingerprint” band indicated
310 a small increase in tilt angle of the α -helix with respect to the membrane normal, suggesting a possible slight insertion of
311 Pln149 into the lipid bilayer, but still mostly in a surface-bound state. Although at P/L molar ratios higher than 1/25 the
312 helix tilt angle of Pln149 appeared to be further increased, assuming a more pronounced obliquely- tilted orientation
313 (Burck et al. 2016), which is described as the T-state (not seen for any of the other molar ratios used here), this AMP

314 needs rather high concentrations to undergo any re-alignment, indicating that the S-state is really the most prevailing
315 orientation of the helix in such systems. It is possible that the membrane tilt angle changing at the P/L ratio of 1/25 may
316 be due to a crowding effect. Assuming all residues of Pln149 interact with the lipid surface (part of the residues in
317 helical conformation and part unstructured), the whole area of the outer lipid surface is estimated to be fully covered by
318 Pln149. Estimation of the area of the lipid surface covered by Pln149 was done from simple geometrical calculations of
319 lipid area and area occupied by peptides. Hence, as opposed to what is seen for other AMPs like PGLa and MAP (Bürck
320 et al. 2008), Pln149 does not have appear to have a high tendency to re-align in POPG oriented lipid bilayers. As a
321 further note, whilst the variation of the spectral magnitude of the positive band around 190 nm can be attributed to
322 absorption flattening effects in some samples (Miles and Wallace 2016), these variations do not affect the orientation
323 determination, because the alignment of the peptide helices was inferred from the unperturbed band around 208 nm,
324 whose spectral magnitude is the “fingerprint” for helix alignment in OCD measurements (Bürck et al. 2008, 2016).

325 A relatively similar spectrum was observed for the binding of Pln149 to POPS oriented bilayers (Figure 4b), i.e.
326 a 208 nm band with significant negative intensity. In the averaged spectra for POPS, the 208 nm band was also slightly
327 more intense when compared to the 222 nm band, suggesting the peptide insertion is more like that in the S-state. At the
328 P/L ratios of 1/100, 1/50 and 1/25, alignment of the peptide is more consistent with a T-state, slightly more tilted
329 compared to that in POPG (even at low concentration). However no pure I-state (a fully inserted transmembrane
330 orientation) could be observed at any of the measured P/L ratios (i.e. the same as in the case for POPG). The OCD
331 spectra of Pln149 in lipid bilayers are in agreement with the model proposed for the insertion and membrane orientation
332 of PlnA in negatively-charged membranes (Kristiansen et al. 2005). Like the PlnA helix, the Pln149 helix is oriented
333 parallel to the membrane with the hydrophobic residues penetrating into the hydrophobic part of the membrane and the
334 hydrophilic residues into and partly through the membrane-water interface.

335 Interestingly, the OCD spectrum of Pln149 in oriented POPC bilayers clearly indicates a helical conformation
336 for the peptide (Figure 4c), suggesting the peptide is in the S-state, and once again, there is a very small re-alignment of
337 peptide orientation at the P/L molar ratio of 1/25, less than what was observed for the negatively-charged POPG and
338 POPS bilayers. Therefore, at the P/L molar ratio of 1/25, the negative charge alone influences the peptide molecules to
339 tilt a little more than in the zwitterionic POPC bilayers. In the OCD assays, the interaction of the peptide Pln149 with the
340 zwitterionic oriented lipid bilayers was probed in a system where hardly any bulk water phase was present. In this case,
341 the peptide bound to the model membrane and the equilibrium was shifted towards the hydrated planar lipid bilayers.
342 That is, once the bulk water was reduced, a significant disorder-to-helix transition was observed in Pln149. Although no

343 evidence of the interaction of Pln149 with the zwitterionic POPC vesicles had been seen by SRCD spectroscopy in
344 aqueous solution, such results are not contradictory, since the partitioning of Pln149 in a zwitterionic liposome
345 environment with a large excess of bulk water (which shifts the equilibrium towards the water phase in the absence of
346 any Coulombic interactions) and in the oriented zwitterionic bilayers are different. Such a finding has already been
347 reported in other peptide-to-lipid systems, and the partitioning of membrane-active peptides into membrane interfaces
348 has been shown to promote the formation of regular secondary structure (Wimley and White 1996).

349
350 *Optical microscopy with giant unilamellar vesicles* – GUVs composed of POPC, POPC/POPG (1:1), and POPC/POPS
351 (1/1) were spherical in shape with an average diameter of ca. ~20-60 μm . All three types of giant vesicles without
352 peptide were very stable, showing no perturbation after 35 min (Figure 5A-C). When a small amount of Pln149
353 (concentration less than 64 nM) was added to the GUV solution, there was an increase in the membrane surface area,
354 evident in the formation of buds from the giant vesicles as the main feature of membrane-peptide interaction (Figure
355 5D). Interestingly, the addition of 64 nM Pln149 to either POPC/POPG (Figure 5E) or POPC/POPS (Figure 5F) GUVs
356 promoted membrane disruption and collapse after circa 8 min. This finding is in agreement with our observation that
357 peptide addition has a larger effect on negatively-charged membranes than on zwitterionic ones. No pore formation was
358 observed on the vesicles prior to their disruption (Figures 5E-F).

359 Of note, in the range of 64 nM to 250 nM Pln149, while the GUVs composed of POPC retained emitting buds
360 (without being disrupted), the disruption of negatively-charged giant liposomes was increased and observed to occur in
361 shorter time periods (data not shown). The disruption of some GUVs composed entirely of POPC could only be
362 observed with 20 μM Pln149 after 9 min of incubation with the giant vesicles (data not shown).

363
364 **Conclusions**

365 The binding of the antimicrobial peptide Pln149 to vesicles is an endothermic process that can be
366 guided/modulated by the presence of the negative surface charge density of model membranes. Moreover, for vesicles
367 composed exclusively of zwitterionic lipids, the partitioning of the peptide is minimal, with little or no binding detected
368 by isothermal titration calorimetry and no conformational changes observed by SRCD spectroscopy.

369 In contrast, Langmuir monolayer interactions and OCD spectroscopy indicated that Pln149 can significantly
370 adsorb onto zwitterionic lipid surfaces with loose molecular packing and with spectra consistent with mostly helical
371 structure. The antimicrobial peptide causes pronounced disruptive effects on GUVs containing negatively-charged

372 phospholipid (POPG or POPS) in response to increasing peptide concentration, as is evident in the increase of surface
373 area and buds formation in zwitterionic GUVs. Taken together, these studies indicate for the first time that the
374 antimicrobial peptide Pln149 can interact with zwitterionic phospholipids, which could explain the previously reported
375 peptide activity on *Saccharomyces cerevisiae* cells (Lopes et al. 2009).

376 The binding of Pln149 to zwitterionic model membranes may have been affected by the different membrane
377 curvatures of the two systems used: the highly curved POPC vesicles in the SRCD and the planar POPC lipid bilayers in
378 the OCD assays. A membrane curvature-dependent binding has previously been demonstrated for other amphipathic α -
379 helical antimicrobial peptides, where the different behaviors on small and giant unilamellar vesicles and the differences
380 in kinetics were attributed to the curvature dependent binding affinities (Tabaei et al. 2012; Iwamoto et al. 2007; Koller
381 and Lohner 2014; Strandberg et al. 2012). Also, the different degrees of hydration of LUVs and oriented bilayers have
382 been already reported to promote differences in the insertion of the AMPs aurein 1.2 and carein 1.1 in model membranes
383 (Fernandez et al. 2013).

384 Membrane curvature, hydration, and lipid organization were shown to play roles in modulating the interactions
385 of Pln149 with lipid membranes, especially in the absence of electrostatic attraction. Therefore, as the model membranes
386 used in the Langmuir monolayers and the surface of GUVs are similar to horizontal planes for the adsorption of Pln149,
387 insertion between the lipid headgroups appear to alter the lipid molecular packing and may induce curvature strain in the
388 planar lipid membranes that can contribute to the formation of the curved morphologies seen by microscopy.
389 Alternatively, bilayers formed from the unsaturated zwitterionic lipid POPC are known to present a slight negative
390 spontaneous curvature (Strandberg et al. 2012), hence the adsorption of Pln149 within this bilayer could initially release
391 such lipid stress, but then, as more peptide accumulates at lipid surface, the membrane lateral pressure is increased .

392 In the “carpet-like” model of interaction (Shai 2002), helical peptides are in contact with lipid headgroups
393 during the whole process of membrane permeation and do not insert into the hydrophobic core of the membrane. Here,
394 Pln149 was shown to produce such types of interactions with both negatively-charged and zwitterionic lipid headgroups.
395 It is possible that Pln149 might use a very similar mechanism of action on Gram(+) bacteria, i.e., with the peptide
396 accumulation at the surface of the membrane, eventually inducing an α -helix in an environment where the amount of
397 water is reduced, and this helix is aligned parallel to the membrane surface and can realign as a function of peptide
398 accumulation at the membrane.

399 The N-terminal residues of Pln149 are not expected to contribute to the electrostatic attraction of the peptide by
400 charged membranes and are not predicted to be part of the amphipathic helix. However, this region affects the peptide

401 hydrophobic moment, which could contribute to the PIn149 adsorption, causing additional strain in the membrane, as the
402 N-terminus region must be located at the lipid-aqueous interface. The participation of this segment in the action of
403 PIn149 has previously been demonstrated (Lopes et al. 2013); it showed the reduced leakage action, an increase of
404 minimum inhibitory concentration (MIC) values, reduced membrane binding, and a change of the peptide action from
405 bactericidal to bacteriostatic. The modulation of PIn149 action due to its N-terminus may occur in either zwitterionic or
406 negatively charged membranes.

407 Therefore, this study has suggested that a carpet-like model may be the mechanism of action for PIn149 in both
408 types of lipid systems: either those with a negative surface charge density (such as the cell membrane of Gram(+)
409 bacteria) or those with zwitterionic features (as in the POPC membrane models used here and in *Saccharomyces*
410 systems).

411

412 **Conflict of Interest:** The authors declare that they have no conflict of interest.

413

414 **References**

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547

548 **Figure Captions**

549 **Figure 1** – Isothermal titration calorimetry: a) Lipid-into-Pln149 titration of POPC (blue), POPG (red) and POPS
550 (green), in aqueous solution and b) POPG with/without (grey/red) 250 mM NaCl. For the assays containing the salt
551 solutions, the concentrations of the ions were the same in both liposome and peptide solutions at each measurement).
552 The heat of injection obtained by integration of the heat flow peaks as a function of P/L molar ratio, at 25°C is shown in
553 the lower diagram. In each measurement, 7 mM of vesicle suspensions were injected into a cell containing 28 μM of
554 Pln149. Each peak refers to the injection of a 10 μl aliquot of the vesicles, which produced an endothermic heat of
555 reaction. The first injection (only 2 μl injected) is prone to artifacts and was discarded from these analysis.

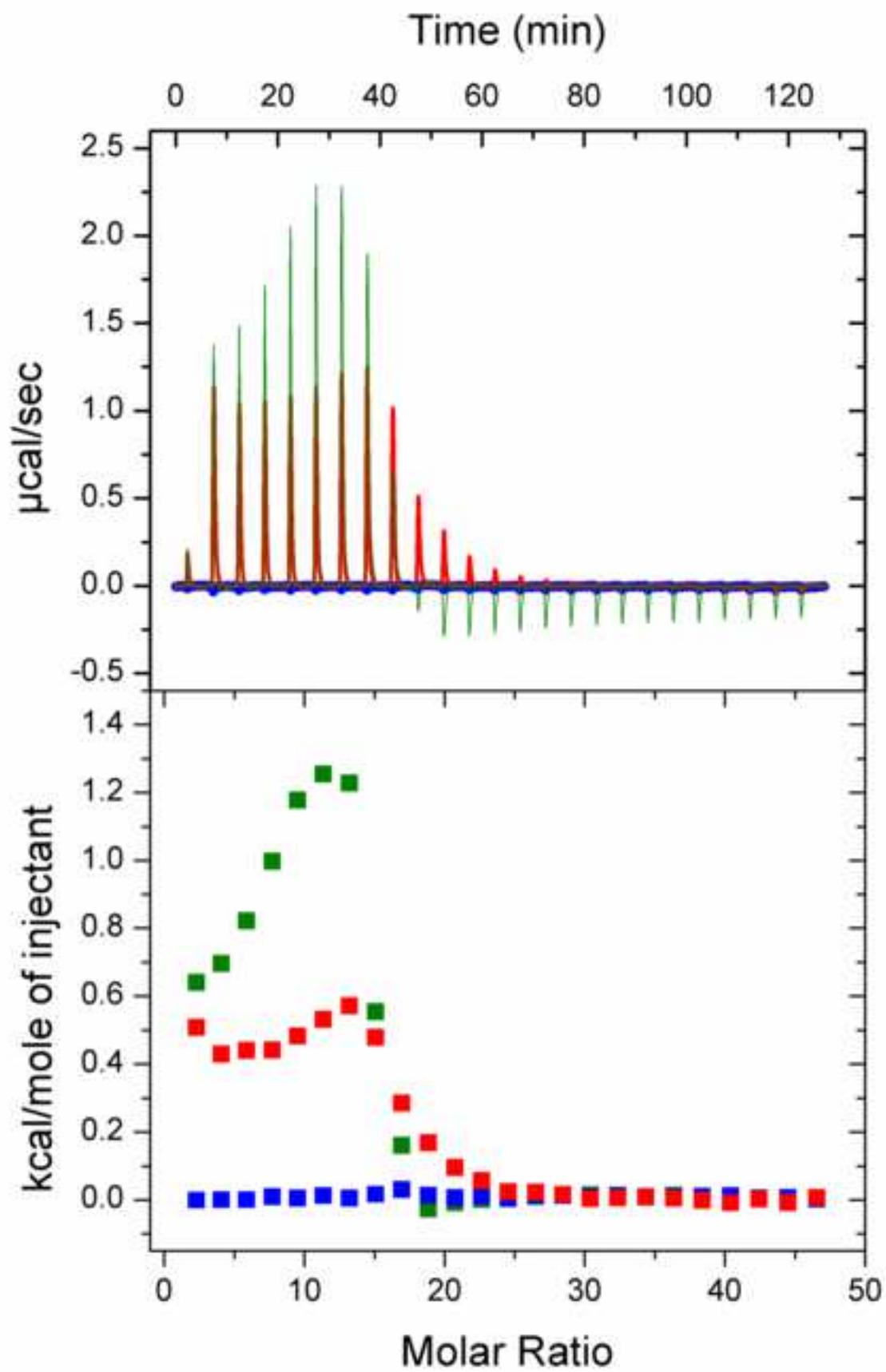
556
557 **Figure 2** – Surface pressure studies: a) Surface activity of Pln149 (10 μM) on an air-water interface (black) and on
558 monolayers of POPC (blue), POPG (red) or POPS (green) with lateral pressure of 25 mN/m, and b) adsorption of Pln149
559 (5 μM) onto POPC (blue), POPG (red), and POPS (green) monolayers. Lipid monolayers were formed at the air-water
560 interface under different initial surface pressures (π_0), then peptide was injected into the water subphase. After
561 equilibrium was reached, surface pressure changes ($\Delta\pi$), induced by Pln149 adsorption into each lipid monolayer were
562 monitored.

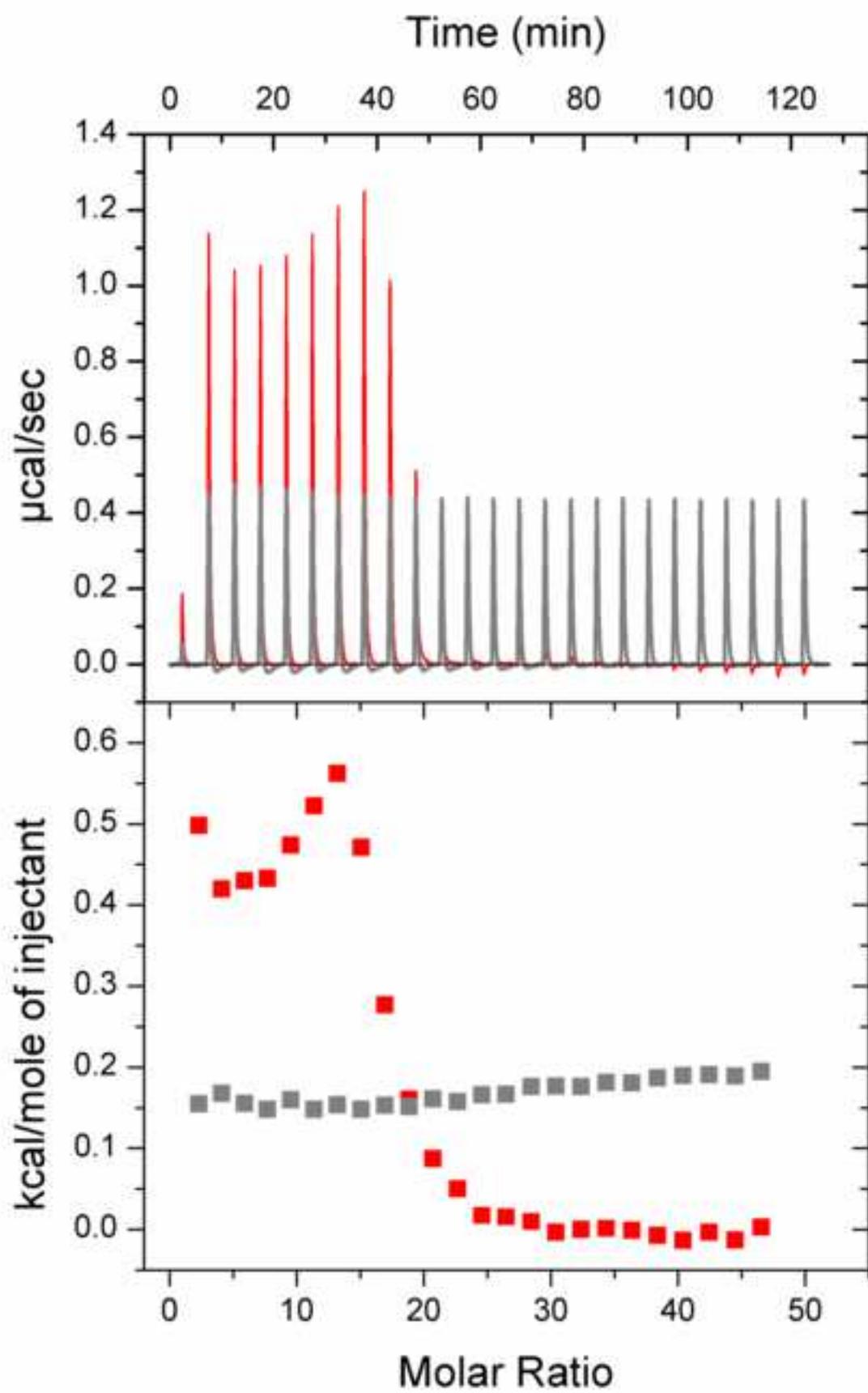
563
564 **Figure 3** – SRCD Spectroscopy: a) spectra of Pln149 in aqueous solution (black), in the presence of 10 mM of SDS
565 (magenta), POPC (blue), POPG (red), and POPS (grey) vesicles at a 1/50 P/L molar ratio, and in a dehydrated film
566 deposited on a quartz plate (green). b) Spectra of Pln149 in water (black) and in the presence of POPG (red) vesicles
567 containing 0.25M (blue), or 0.5M (green) NaF. c) Helical wheel projection for the Pln149 sequence. Projection
568 performed using residues A7 to K20, the region with a calculated helical propensity. Non-polar residues are labeled in
569 yellow, positively-charged residues are in blue, and polar residues are in cyan.

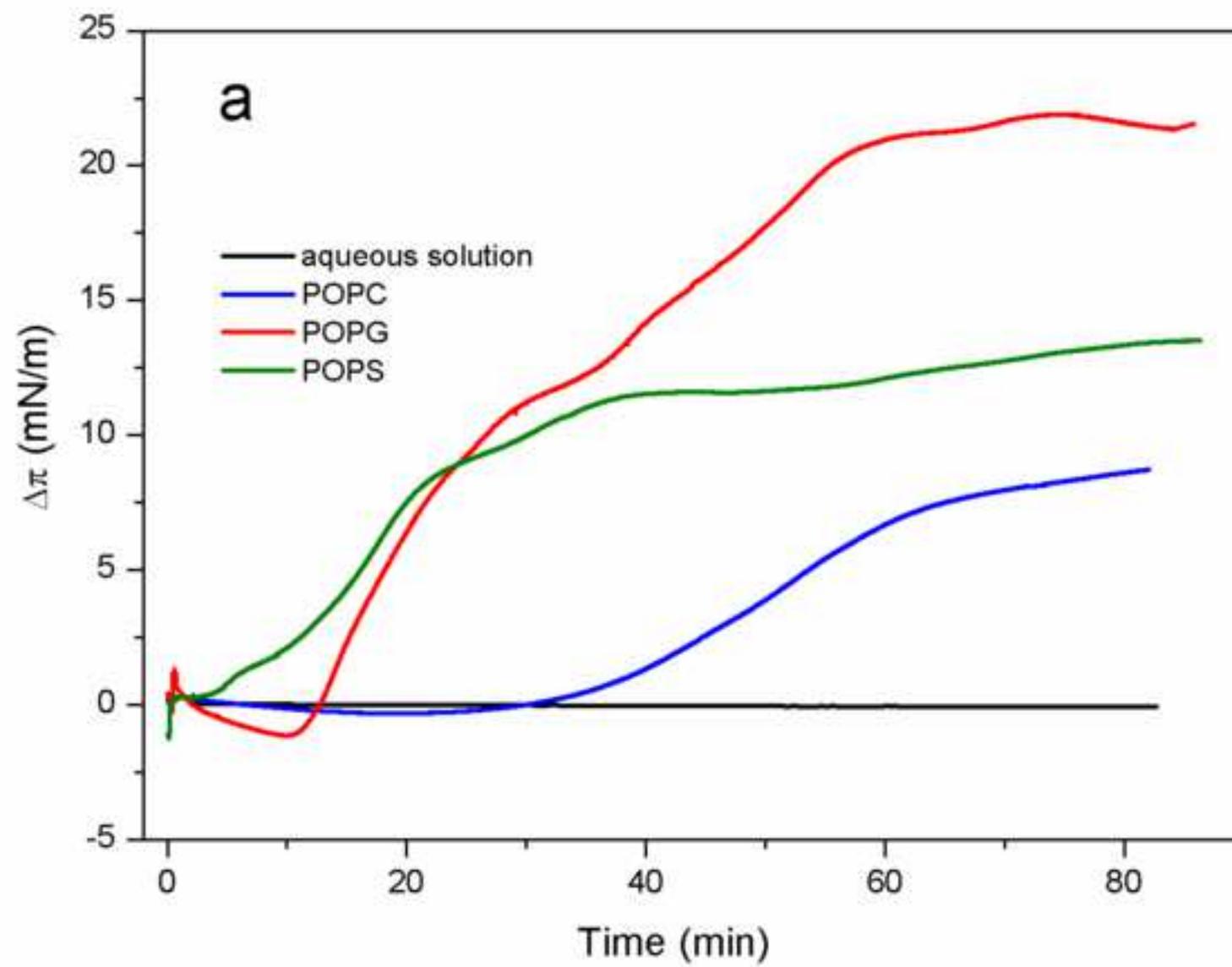
570
571 **Figure 4** – Oriented CD Spectroscopy: Pln149 as a function of P/L molar ratio in a) POPG (spectra were normalized to
572 the sample of P/L 1:200, using the ellipticity value at 220 nm), b) POPS (spectra were normalized to the sample of P/L
573 1:100, using the value at 220 nm), and c) POPC (spectra were normalized to the sample of P/L 1:100, using the value at
574 220 nm) oriented bilayers. The dotted line indicates the wavelength at 208 nm, which is used for the OCD analyses.

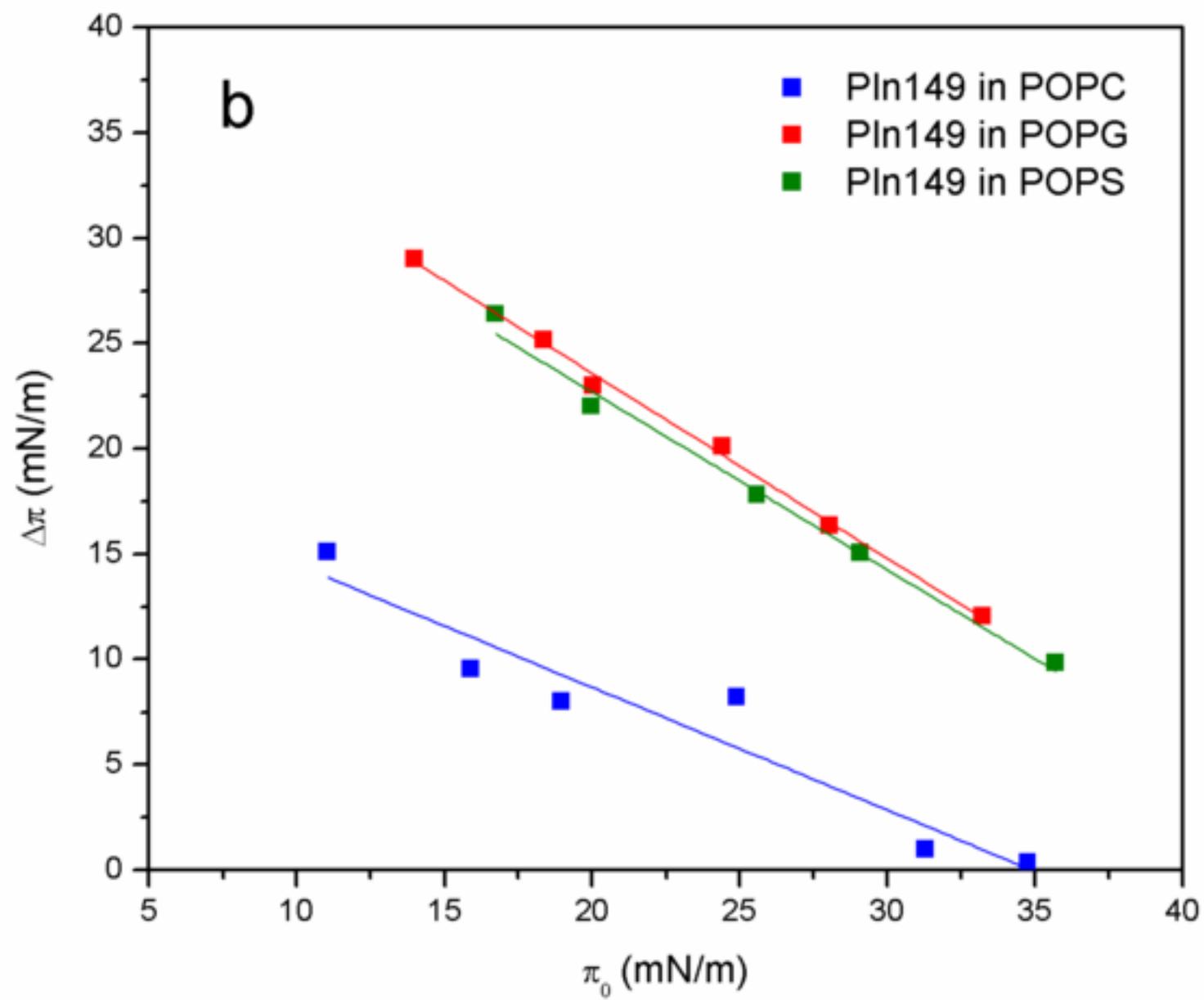
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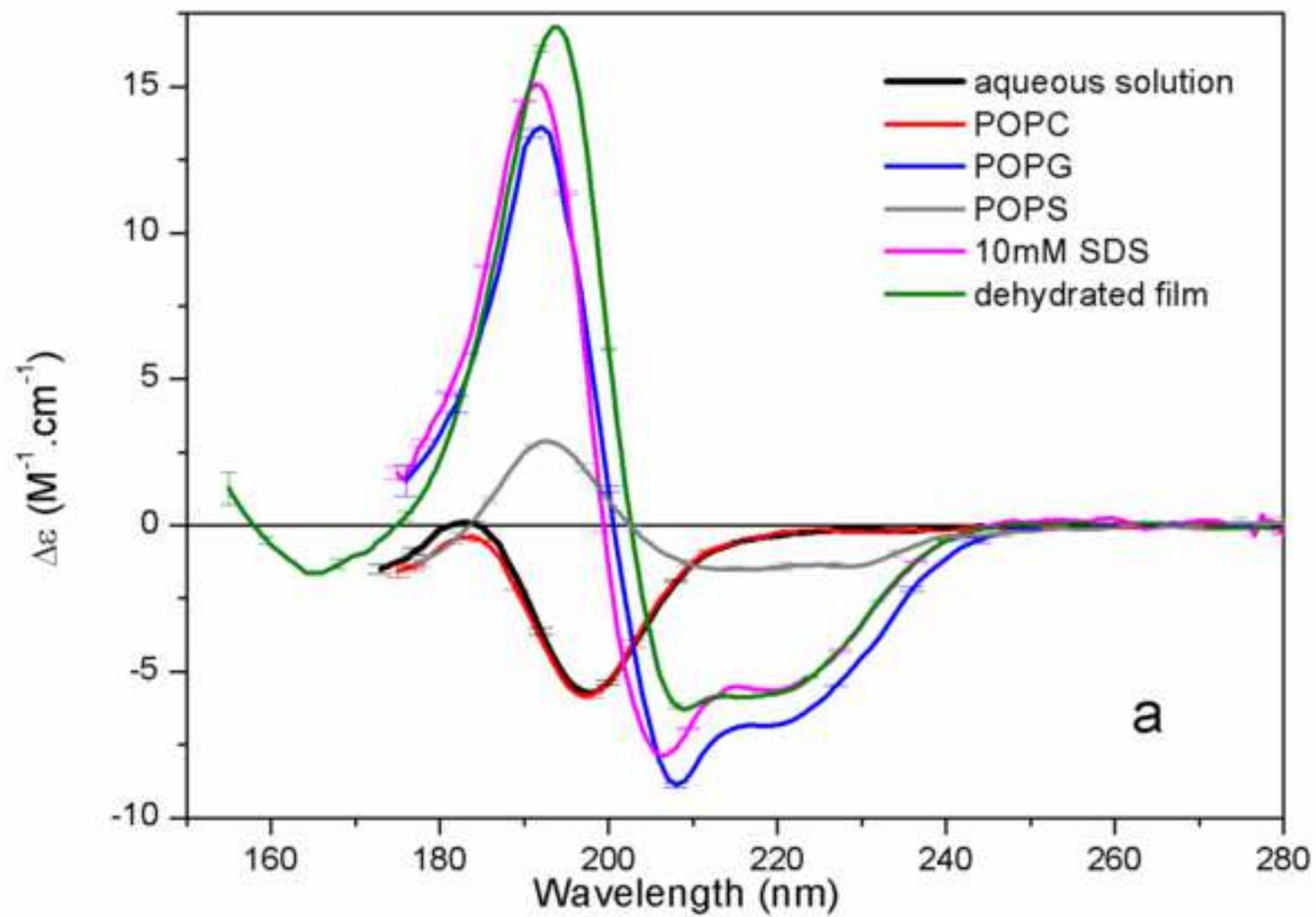
576 **Figure 5** – Optical Microscopy: Images of GUVs in the presence of Pln149 obtained using fluorescence (first and last
577 images of each row) or phase contrast (middle images on each row) modes. Vesicle compositions and peptide
578 concentrations are as follows: A- Giant vesicles composed of POPC (control); B- giant vesicles composed of POPC:
579 POPG, 1:1 (control); C- giant vesicles composed of POPC: POPS, 1:1 (control); D- Giant vesicles composed of POPC
580 with 64nM of Pln149; E- Giant vesicles composed of POPC: POPG (1:1) with 64 nM of Pln149; F- Giant vesicles
581 composed of POPC: POPS (1:1) with 64 nM of Pln149.

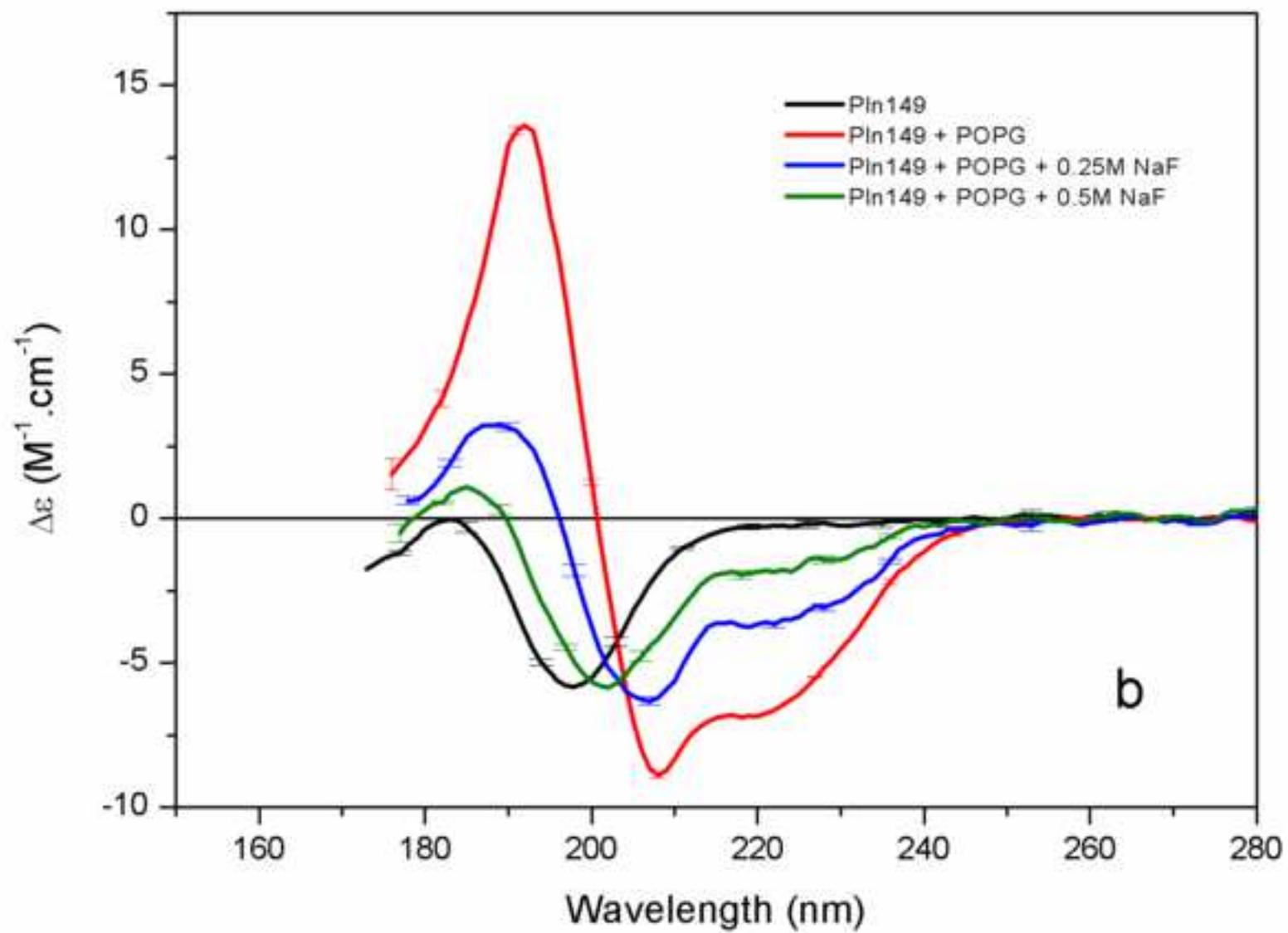


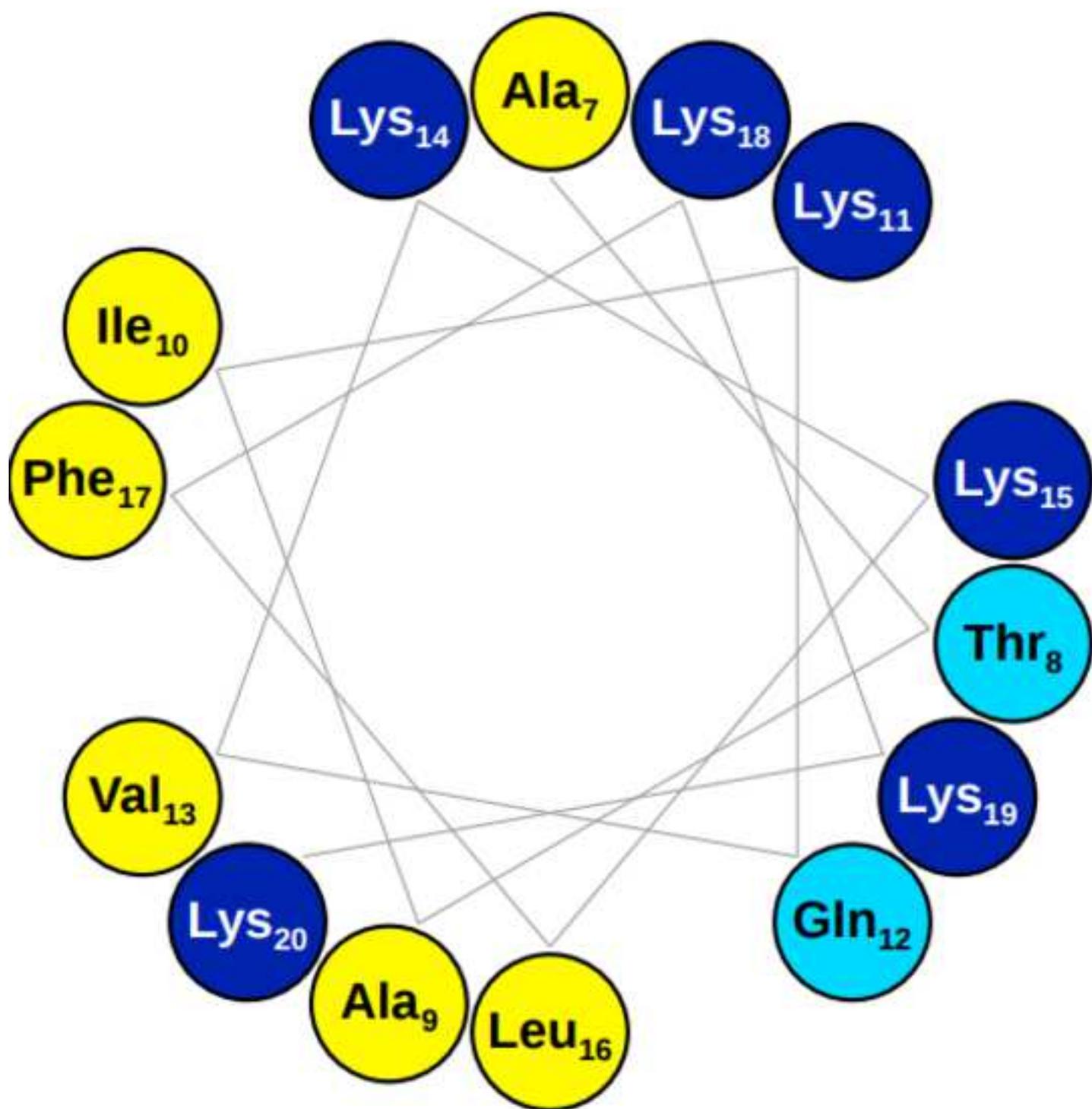


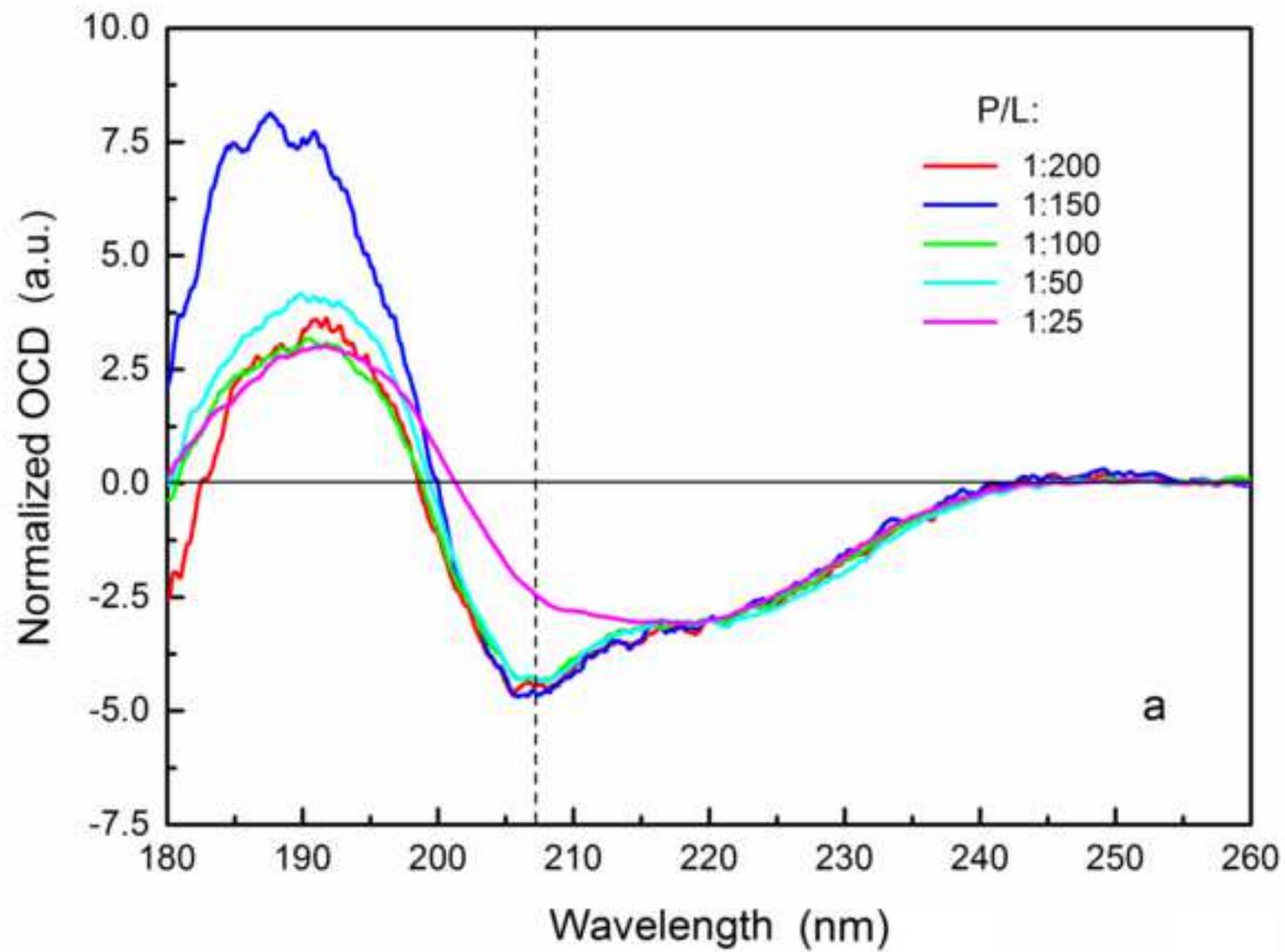












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