



**HAL**  
open science

## **Native lysozyme and dry-heated lysozyme interactions with membrane lipid monolayers: lateral reorganization of LPS monolayer, model of the E. coli outer membrane**

Melanie Derde, Françoise Nau, Valérie Lechevalier-Datin, Catherine Guérin-Dubiard, Gilles Paboeuf, Sophie Jan, Florence Baron, Michel Gautier, Véronique Vie

### ► To cite this version:

Melanie Derde, Françoise Nau, Valérie Lechevalier-Datin, Catherine Guérin-Dubiard, Gilles Paboeuf, et al.. Native lysozyme and dry-heated lysozyme interactions with membrane lipid monolayers: lateral reorganization of LPS monolayer, model of the E. coli outer membrane. *Biochimica et Biophysica Acta: Biomembranes*, 2015, 1848 (4), pp.1065-1073. 10.1016/j.bbamem.2014.10.026 . hal-01090040

**HAL Id: hal-01090040**

**<https://univ-rennes.hal.science/hal-01090040v1>**

Submitted on 4 Dec 2014

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

# **Native lysozyme and dry-heated lysozyme interactions with membrane lipid monolayers: lateral reorganization of LPS monolayer, model of the *E. coli* outer membrane**

*Melanie Derde*<sup>1,2</sup>(\*), *Françoise Nau*<sup>1,2</sup>, *Valérie Lechevalier*<sup>1,2</sup>, *Catherine Guérin-Dubiard*<sup>1,2</sup>,  
*Gilles Paboeuf*<sup>3</sup>, *Sophie Jan*<sup>1,2</sup>, *Florence Baron*<sup>1,2</sup>, *Michel Gautier*<sup>1,2</sup>, *Véronique Vié*<sup>3</sup> (\*)

<sup>1</sup>Agrocampus Ouest, UMR1253 Science et Technologie du Lait et de l'Oeuf, F-35042 Rennes, France

<sup>2</sup>INRA, UMR1253 Science et Technologie du Lait et de l'Oeuf, F-35042 Rennes, France

<sup>3</sup>Université de Rennes 1, Institut de Physique de Rennes, UMR6251, CNRS, F-35042 Rennes, France

Corresponding authors :

## **Melanie Derde**

Agrocampus Ouest

INRA, UMR 1253 STLO

65, rue de St-brieuc

35042 Rennes, France

✉: [melanie.derde@agrocampus-ouest.fr](mailto:melanie.derde@agrocampus-ouest.fr)

Phone : +33/2.23.48.55.74

## **Véronique Vié**

Université de Rennes 1

Institut de Physique de Rennes (IPR)

263, Av Général Leclerc

F-35042 Rennes Cedex

✉: [veronique.vie@univ-rennes1.fr](mailto:veronique.vie@univ-rennes1.fr)

Phone : +33/2.23.23.56.45

**ABSTRACT:** Lysozyme is mainly described active against Gram-positive bacteria, but is also efficient against some Gram-negative species. Especially, it was recently demonstrated that lysozyme disrupts *E. coli* membranes. Moreover, dry-heating changes the physicochemical properties of the protein and increases the membrane activity of lysozyme. In order to elucidate the mode of insertion of lysozyme into the bacterial membrane, the interaction between lysozyme and a LPS monolayer mimicking the *E. coli* outer membrane has been investigated by tensiometry, ellipsometry, Brewster Angle Microscopy and Atomic Force Microscopy. It was thus established that lysozyme has a high affinity for the LPS monolayer, and is able to insert into the latter as long as polysaccharide moieties are present, causing reorganization of the LPS monolayer. Dry-heating increases the lysozyme affinity for the LPS monolayer and its insertion capacity; the resulting reorganization of the LPS monolayer is different and more drastic than with the native protein.

**KEYWORDS:** BAM, AFM, Langmuir film, Dry-heated lysozyme, LPS monolayer

## **ABBREVIATIONS**

AFM, atomic force microscopy; AMP, antimicrobial peptide or protein; BAM, Brewster angle microscopy; DH-L, dry-heated lysozyme; HEPES, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; KLA, Lipid A-(KdO)<sub>2</sub>; LPS, lipopolysaccharides; MIP, maximum insertion pressure; N-L, native lysozyme

## 1 1. INTRODUCTION

2 Antibiotic resistance due to decades of misuse in human and veterinary medicine, is causing an  
3 enormous public health problem. Several pathogens, such as *Staphylococcus aureus* and  
4 *Klebsiella pneumonia*, have developed multiple antibiotic resistance mechanisms. The  
5 consequence is difficult and expensive treatments of several diseases.[1] The number of these  
6 multi-resistant strains is increasing, but only three new antibiotic molecules against Gram-  
7 positive multiresistant strains were registered since 1970, and none for Gram-negative  
8 multiresistant strains.[2] Research for novel antimicrobial compounds is thus needed, besides the  
9 measures of the European Union to limit the spread of antibiotic resistances. Preferably, novel  
10 compounds should decrease the development rate and spread of antibiotic resistance.

11 Several natural proteins and peptides can be considered as potential candidates, especially the  
12 antimicrobial proteins or peptides (AMP) which act on the bacterial membranes. Their target, i.e.  
13 the bacterial cell membrane, is a generalized and vital target, and thus antimicrobial resistance  
14 development remains limited.[3,4] AMP are mostly positively charged molecules, amphiphilic,  
15 flexible, and contain several hydrophobic residues, suggesting electrostatic and hydrophobic  
16 interactions with the bacterial cell membrane.[3] These interactions can then lead to the  
17 membrane disruption, causing bacterial cell death or translocation of the AMP into the cytoplasm  
18 where these can interact with several intracellular targets.[3,5]

19 One of the natural antimicrobial proteins, widely studied, is hen egg white lysozyme. This  
20 small enzyme (129 amino acid residues) was for a long time only known for its antimicrobial  
21 activity against Gram-positive bacteria, due to its muramidase activity.[6,7] However, several  
22 studies suggest other mechanisms of action against both Gram-positive and Gram-negative  
23 bacteria, such as perturbation of DNA or RNA synthesis, activation of autolysin production, and

24 membrane permeabilisation.[7–10] The disruption of both the outer and cytoplasmic membranes  
25 of *E. coli* by native lysozyme has been recently demonstrated in our laboratory.[9] Especially,  
26 pore formation in the outer membrane of *E. coli* has been described.[11] Moreover, pore size  
27 and/or quantity were higher with dry-heated lysozyme as compared to the native protein.[11] The  
28 physicochemical modifications resulting from dry-heating are an increased positive charge at  
29 physiological pH as well as increased flexibility and hydrophobicity while preserving the  
30 secondary and tertiary structure; these modifications could be responsible for the enhanced  
31 antibacterial activity of dry-heated lysozyme, similarly to what has been described for lysozyme  
32 modification by enzyme hydrolysis, heat denaturation, or fusion with several chemical  
33 moieties.[7,12–17]

34 However, the interactions between the outer membrane lipids of Gram-negative bacteria and  
35 both types of lysozyme (native and dry-heated) remain to be investigated. In the presently  
36 reported study, lipopolysaccharide (LPS) monolayers of *E. coli* K12 have been used as a model  
37 for the bacterial outer membrane.[18,19] These LPS monolayers were formed in a Langmuir  
38 trough at a controlled initial surface pressure ( $\pi_{\text{initial}}$ ). Multiscale analysis of the interfacial film  
39 using tensiometry, ellipsometry, Brewster angle microscopy (BAM) and atomic force  
40 microscopy (AFM), enabled to investigate the LPS-lysozyme interactions, to study the  
41 consequences of lysozyme interaction on the LPS monolayer

## 42 2. MATERIALS AND METHODS

### 43 2.1 Proteins and lipids.

44 Native lysozyme (N-L) powder (pH 3.2) was obtained from Liot (Annezin, 62-France). It was  
45 heated for 7 days at 80°C in hermetically closed glass tubes to obtain dry-heated lysozyme (DH-  
46 L). Lysozyme (N-L or DH-L) was solubilized (around 0.5 g/L) in 5 mM 4-(2-  
47 hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) buffer (Sigma Aldrich, Saint-Quentin,  
48 France), pH 7.0, 150 mM NaCl (Fluka, Saint-Quentin, France). The concentration of the  
49 lysozyme stock solution was precisely determined by absorbance at 280 nm (extinction  
50 coefficient = 2.6 L/g)[20]. The protein solution was then diluted in the HEPES buffer to obtain  
51 the desired concentration for used lysozyme solutions.

52 The lipopolysaccharides (LPS) of *E. coli* K12 were obtained from Invivogen (Toulouse,  
53 France). The LPS were solubilized in 2:1 chloroform/methanol mixture at 0.5 g/L. Lipid A-  
54 (KdO)<sub>2</sub> (KLA) were purchased by Avanti Polar Lipids (Alabaster, Alabama, USA) and were  
55 solubilized in a 2:1 chloroform/methanol mixture at 0.67 g/L.

56

### 57 2.2 Lipid/protein monolayers.

58 The experiments were performed in a homemade TEFLON<sup>®</sup> trough of 8 mL at 20°C. Before  
59 each use, the trough was thoroughly cleaned with successively warm tap water, ethanol,  
60 demineralized water, and then boiled for 15 minutes in demineralized water. After cooling the  
61 TEFLON<sup>®</sup> trough was then filled with 8 mL HEPES buffer. The LPS or KLA were spread with a  
62 high precision Hamilton microsyringe at the clean air / liquid interface to obtain an initial surface  
63 pressure between 18 and 30 mN/m. After 1h to allow the solvent to evaporate and the lipids to

64 organize, 50  $\mu\text{L}$  N-L or DH-L solution were injected in the subphase with a Hamilton syringe to  
65 obtain a final protein concentration of 0.02, 0.03, 0.05, 0.1, 0.2, 0.3 or 1  $\mu\text{M}$ .

66

### 67 **2.3 Surface pressure measurements.**

68 The surface pressure was measured following a Wilhelmy method using a 10 mm x 22 mm  
69 filter paper as plate (Whatman, Velizy-Villacoublay, France) connected to a microelectronic  
70 feedback system (Nima PS4, Manchester, England). The surface pressure ( $\pi$ ) was recorded every  
71 4 s with a precision of  $\pm 0.2$  mN/m. The measured surface pressure is the result of the surface  
72 tension of water minus the surface tension of the lipid film.

73

### 74 **2.4 Ellipsometry.**

75 Measurements of the ellipsometric angle value were carried out with an in-house automated  
76 ellipsometer in a “null ellipsometer” configuration.[21,22] A polarised He-Ne laser beam  
77 ( $\lambda=632.8$  nm, Melles Griot, Glan-Thompson polarizer) was reflected on the surface of the  
78 trough. The incidence angle was  $52.12^\circ$ , i.e. Brewster angle for the air/water interface minus  $1^\circ$ .  
79 After reflection on the liquid surface, the laser light passed through a  $\lambda/4$  retardation plate, a  
80 Glan-Thompson analyser, and a photomultiplier. The analyser angle, multiplied by two, yielded  
81 the value of the ellipsometric angle ( $\Delta$ ), i.e. the phase difference between parallel and  
82 perpendicular polarisation of the reflected light. The laser beam probed the  $1\text{ mm}^2$  surface with a  
83 depth in the order of  $1\text{ }\mu\text{m}$ . Initial values of the ellipsometric angle ( $\Delta_0$ ) and surface pressure of  
84 buffer solutions were recorded for at least half an hour to assure that the interface is clean. Only  
85 in the case of a stable, minimal signal, experiments were performed. Values of  $\Delta$  were recorded  
86 every 4 s with a precision of  $\pm 0.5^\circ$ . When measuring the pressure increase induced by lysozyme



87 at the air/liquid interface a lysozyme solution at  $0.1 \mu\text{M}$  is deposited in the trough. When the  
88 pressure increase induced by lysozyme is measured at the LPS/liquid interface a LPS monolayer  
89 is first created as formerly described.

90 For the detection of local ellipsometric angle values, an imaging ellipsometer EP3 (Nanofilm,  
91 Göttingen, Germany) in “null ellipsometer” configuration was used with a 10X objective. A  
92 solid-state laser ( $\lambda=532 \text{ nm}$ ) was used as a light source. Delta/Psi maps were recorded with the  
93 EP3 software for a  $450 \mu\text{m} \times 390 \mu\text{m}$  surface. For delta and psi maps, a polarizer and analyzer  
94 range of  $20^\circ$  was used. Delta/psi maps were based on 20 images taken at different polarizer and  
95 analyzer angles.

96

## 97 **2.5 Brewster angle microscopy.**

98 An ellipsometer EP3 (Nanofilm, Berlin, Germany) with a polarized incident laser ( $\lambda=532.0$   
99 nm) was used with a 10X objective in a Brewster angle configuration (angle of incidence was  
100  $53.1^\circ$ ). The images represented a  $450 \mu\text{m} \times 390 \mu\text{m}$  surface. Different zones of each sample were  
101 evaluated; the images here shown are representative of the whole samples.

102

## 103 **2.6 AFM sample preparation and AFM imaging.**

104 Experiments were performed with a computer-controlled and user-programmable Langmuir  
105 TEFLON<sup>®</sup>-coated trough (type 601BAM) equipped with two movable barriers and of total  
106 surface  $90 \text{ cm}^2$  (Nima Technology Ltd., England). Before starting the experiments, the trough  
107 was cleaned successively with ultrapure water (Nanopure-UV), ethanol, and finally ultrapure  
108 water. The trough was filled with 5 mM HEPES buffer pH 7 150 mM NaCl. LPS were spread  
109 over the clean air/liquid interface at a surface pressure of 25 mN/m or 30 mN/m. The solvent was

110 then left to evaporate for 1 h. Then, a Langmuir-Blodgett transfer was performed onto freshly  
111 cleaved mica plates at constant surface pressure by vertically raising (1 mm/min) the mica  
112 through the air/liquid interface to obtain a sample of the initial LPS monolayer. The LPS  
113 monolayer stability was assured during the Langmuir-Blodgett transfer allowing the injection of  
114 lysozyme in the subphase.

115 Then, 0.1  $\mu\text{M}$  lysozyme was injected in the subphase of the previously sampled LPS  
116 monolayer with a Hamilton syringe. Surface pressure variations were recorded until a stable  
117 surface pressure was reached (after  $\sim 1$  h). Then, a second Langmuir-Blodgett transfer was  
118 performed onto freshly cleaved mica as described above to obtain the sample of the LPS  
119 monolayer after lysozyme interaction.

120 AFM imaging of Langmuir Blodgett films was performed in contact mode using a Pico-plus  
121 atomic force microscope (Agilent Technologies, Phoenix, AZ) under ambient conditions with a  
122 scanning area of  $20 \times 20 \mu\text{m}^2$  and  $5 \times 5 \mu\text{m}^2$ . Topographic images were acquired using silicon  
123 nitride tips on integral cantilevers. The forces were controlled along the imaging process.  
124 Different zones of each sample were scanned; the images here shown are representative of the  
125 whole samples.

## 126 3. RESULTS

### 127 3.1 Insertion capacity of lysozyme into a LPS monolayer.

128 The insertion capacity of N-L and DH-L into a LPS monolayer was determined by  
129 independent tensiometry experiments at different protein concentrations. Insertion can be  
130 detected by a surface pressure increase ( $\Delta\pi = \pi_{\text{final}} - \pi_{\text{initial}}$ ). Here, lysozyme was injected under a  
131 LPS monolayer with an initial surface pressure ( $\pi_{\text{initial}}$ ) of 18 mN/m.

132 In both cases (N-L and DH-L), a surface pressure increase is demonstrated indicating lysozyme  
133 insertion into the LPS monolayer for protein concentrations above 0.02  $\mu\text{M}$  (figure 1A). Below  
134 0.05  $\mu\text{M}$ , no difference can be observed between both lysozymes, while above this protein  
135 concentration, DH-L induces a higher surface pressure increase than N-L (figure 1A). When  
136 increasing the lysozyme subphase concentration, a  $\Delta\pi$ -plateau is obtained at a lysozyme  
137 concentration of 0.2  $\mu\text{M}$  for both N-L and DH-L, indicating saturation of the interface in these  
138 conditions. However, the maximum  $\Delta\pi$  value is higher for DH-L than for N-L (12 mN/m and 8  
139 mN/m, respectively; figure 1A). For further investigation of the insertion capacity of lysozyme,  
140 0.1  $\mu\text{M}$  N-L or DH-L has been used. At this concentration, differences exist between both  
141 proteins, and lipid protein interactions can be observed, while minimizing protein-protein  
142 interactions in the bulk solution (aggregation) or at the lipid interface.

143

### 144 3.2 Affinity of lysozyme for LPS monolayers.

145 To evaluate the affinity of both N-L and DH-L for the LPS monolayer,  $\Delta\pi$  was determined  
146 after lysozyme injection (0.1  $\mu\text{M}$ ) under LPS monolayers previously formed at different initial  
147 surface pressures ( $\pi_{\text{initial}}$ ). Supplementary experiments demonstrated that no phase transition  
148 occurs in the  $\pi$ -range here used (supplementary data S3) comparisons are then valuable. Linear

149 regression analysis of the  $\Delta\pi$  values *versus*  $\pi_{\text{initial}}$  allows calculation of three binding parameters  
150 of lysozyme: maximal insertion pressure (MIP), synergy factor, and  $\Delta\pi_0$  (figure 1B).[23–25]  
151 MIP is the intercept of the straight line with x-axis after extrapolation; it is thus the initial surface  
152 pressure for which no surface pressure increase occurs when lysozyme is injected in the  
153 subphase. The synergy factor is determined as the slope of the linear regression +1. The synergy  
154 factor provides information on the affinity of the protein for the lipid monolayer. High positive  
155 synergy values indicate the existence of strong protein/lipid interactions, since it means that the  
156 protein is able to insert into the lipid film even when initial surface pressure is high.  $\Delta\pi_0$  is the  
157 intercept of the straight line with y-axis after extrapolation; it is thus the theoretical pressure  
158 increase in the absence of lipids ( $\pi_{\text{initial}} = 0$  mN/m).

159 Linear regression for N-L and DH-L resulted in equations 1 and 2, respectively, with  
160 respective determination coefficients ( $R^2$ ) of 0.96 and 0.91.

161 
$$y = -0.21 x + 8.75 \quad (\text{eq. 1})$$

162 
$$y = -0.15 x + 9.10 \quad (\text{eq. 2})$$

163 The MIP is higher with DH-L than with N-L (59.6 and 41.5 mN/m, respectively; table 1). The  
164 synergy factor as introduced by Boisselier et al. (2012) and Calvez et al. (2011) is also higher  
165 with DH-L than with N-L, and is positive for both proteins (0.89 and 0.79, respectively; table  
166 1).[24] Oppositely, the  $\Delta\pi_0$  are similar for N-L and DH-L (8.75 and 9.10 mN/m, respectively;  
167 table 1). It is noticeable that these latter values are smaller than the experimental surface pressure  
168 increase observed for N-L and DH-L lysozymes at the air/liquid interface at the same subphase  
169 concentration (10 and 11 mN/m, respectively).

170 The rate constant of adsorption  $k_{\text{ads}}$  ( $\text{M}^{-1}\cdot\text{s}^{-1}$ ) of a lysozyme solution with a concentration (c) of  
171  $0.1 \mu\text{M}$  at the air/liquid interface and the LPS/liquid interface can be evaluated by fitting the

172 Langmuir equation (eq. 3) for adsorption to the surface pressure measurements. The rate constant  
173 of desorption  $k_{\text{des}}$  ( $\text{M}^{-1}\cdot\text{s}^{-1}$ ) can here be considered negligible.

174 
$$\pi(t) = \pi_{\text{final}} (1 - \exp(-\sigma t)) \quad (\text{eq. 3})$$

175 
$$\sigma = k_{\text{ads}} c + k_{\text{des}} \quad (\text{eq. 4})$$

176 At the air/liquid interface, N-L and DH-L have a  $k_{\text{ads}}$  value of  $6.6 \cdot 10^2 \text{ M}^{-1}\cdot\text{s}^{-1}$  and  $6.4 \cdot 10^2$

177

### 178 **3.3 Changes of surface pressure ( $\pi$ ) and ellipsometric angle ( $\Delta$ ) of LPS monolayer in the** 179 **presence of lysozyme**

180 Kinetics of the  $\pi$  and  $\Delta$  changes after injection of N-L and DH-L in the subphase were  
181 recorded using a LPS monolayer with an initial surface pressure of 25 mN/m and 30 mN/m,  
182 respectively. Different initial surface pressures of the LPS monolayers were chosen because of  
183 the different insertion capacities of N-L and DH-L for this experiment. The aim of this study is to  
184 evaluate the effects of N-L or DH-L on the LPS monolayer after a similar insertion of proteins,  
185 i.e. a similar  $\Delta\pi$ . The initial surface pressures which correspond to this prerequisite is 25 mN/m  
186 and 30 mN/m for a concentration of  $0.1 \mu\text{M}$  N-L and DH-L, respectively (figure 1B); more so,  
187 LPS monolayers with an initial surface pressure of 25 mN/m and 30 mN/m have similar  $\Delta$  values  
188 (supplementary data S1). The injection of N-L and DH-L under the LPS monolayer in these  
189 conditions results in a surface pressure increase of 2.9 mN/m and 3.5 mN/m, respectively (figure  
190 2A), and induces an increase of ellipsometric angle of 8 and  $12^\circ$ , respectively (figure 2B).

191

### 192 **3.4 Changes of surface pressure ( $\pi$ ) and ellipsometric angle ( $\Delta$ ) of KLA monolayer in** 193 **the presence of lysozyme.**

194 To estimate the influence of the polysaccharide moieties on lysozyme interactions with LPS  
195 monolayer, KLA lipids were used. KLA lipids are derivative forms of LPS from which the

196 polysaccharide moiety besides two 3-deoxy-D-manno-octulosonic acid (KdO) groups are  
197 missing (figure 3). The use of KLA was also relevant to test the role of electrostatic interactions  
198 between lysozyme and the negative charge at the interface by making the access to the charge  
199 easier. KLA monolayers are homogeneous lipid films on the contrary to LPS monolayers. This  
200 was confirmed by AFM imaging (supplementary data S2).

201 Kinetics of the  $\pi$  and  $\Delta$  changes after injection of N-L and DH-L in the subphase were  
202 recorded for a KLA monolayer with an initial surface pressure of 25 mN/m and 30 mN/m,  
203 respectively. For N-L, the surface pressure of the KLA monolayer is stable for the first half hour  
204 and then decreases after 3 h (-2.1 mN/m ) (figure 4A). Oppositely, DH-L injection induces an  
205 immediate and more intense decrease (-5 mN/m after 3 h) (figure 4A). Both N-L and DH-L  
206 interact with the KLA monolayer in such a way that the ellipsometric angle increases slightly  
207 after injection of both proteins:  $+0.65^\circ$  and  $+1.5^\circ$  after 3 h, respectively (figure 4B).

208

### 209 **3.5 Microscopic observations of LPS monolayer in the presence of lysozyme.**

210 Brewster angle microscopy (BAM) and ellipsometry were performed to visualize the LPS  
211 monolayer organization on a  $\mu\text{m}$ -scale before and after lysozyme injection in the subphase.  
212 BAM-images give information on the thickness and refraction index of the LPS monolayer.  
213 Thick and/or high refraction index zones will appear lighter (white) than thin and low index  
214 zones (black). Delta maps show the same information as the BAM images, but the differences in  
215 height and/or refraction index are more precisely measured. Blue is the baseline color of the delta  
216 maps and correspond to a small delta value. High delta zones will be represented from green till  
217 red.

218 Before lysozyme injection, the LPS monolayer is heterogeneous, with black and white zones,  
219 at both initial surface pressures (25 mN/m and 30 mN/m), as evidenced by BAM-imaging

220 (figures 5A and 5E). In the absence of literature references, the black colored zones are assumed  
221 to correspond to LPS with short polysaccharide chains (low refractive index and low thickness),  
222 while the white regions are assumed to correspond to LPS with long polysaccharide chains (high  
223 refractive index and low thickness). Such domain-organization is likely considering the optimal  
224 thermodynamic configuration that suggests segregation of LPS with similar polysaccharide chain  
225 lengths. The same information is provided by the delta-maps (figures 5C and 5G).

226 One hour after injection of 0.1  $\mu$ M N-L, the BAM-images and delta-maps do not show any  
227 significant change of the heterogeneity as compared to the initial LPS monolayer (figures 5B and  
228 5D), despite a slight increase of the background  $\Delta$ -value in the delta-map (figure 5D). On the  
229 contrary, after injection of DH-L, an unequivocal change of the LPS monolayer organization is  
230 observed in both BAM-images and delta-maps (figures 5F and 5H). Especially, the small high  $\Delta$ -  
231 domains make place for bigger ones, and the background  $\Delta$ -value increases (figure 5H).

232 Atomic force microscopy (AFM) enables to investigate the LPS monolayer at a nanoscale with  
233 a high resolution. Thus, this technique was used to study more precisely the organization of the  
234 lipid monolayer observed in the background of BAM-images (black zones).  
235

236 The resulting AFM-images show the heterogeneity of the initial LPS monolayer at a nanoscale  
237 at both initial surface pressures (25 mN/m and 30 mN/m; figures 6A, 6C, 6E and 6G). The height  
238 difference between the lower (zone 1) and higher (zone 2) lipid zones is  $1.2$  to  $2.0 \pm 0.2$  nm. By  
239 grating the LPS (data not shown), the monolayer thickness could be measured and corresponds  
240 to 5 nm. The monolayer thickness is in coherence with the one found by Le Brun et al.  
241 (2013).[26]

242 The impact of N-L and DH-L on the lipid monolayer can also be studied by AFM, enabling to  
243 study more carefully the reorganization of the low  $\Delta$ -domains present in the BAM-images.

244 AFM shows that the injection of  $0.1 \mu\text{M}$  N-L or DH-L does not significantly modify the  
245 heterogeneous appearance of the LPS monolayer (figures 6B, 6D, 6F and 6H). However, the  
246 insertion and adsorption of  $0.1 \mu\text{M}$  N-L gives rise to the formation of small domains (object 1)  
247 with a height of  $1.4 \pm 0.4$  nm (figures 6B and 6D). The height of these domains is equivalent to  
248 the height of the dense domains observed in absence of lysozyme (figures 6A and 6C). The  
249 adsorption and insertion of  $0.1 \mu\text{M}$  DH-L induces the formation of two types of clusters (object 2  
250 and 3 with a height of  $25 \pm 5$  and  $57 \pm 12$  nm, respectively) and small domains (object 4) ( $1.4 \pm$   
251  $0.3$  nm height) (figures 6F and 6H).

252 Topographical information shown in the AFM images is representative for the whole sample.  
253 However, the size and shape of the different domains is irregular and heterogeneously distributed  
254 over the sample, making it impossible to quantify the effect of lysozyme on the domain size and  
255 shape.



256 **4. DISCUSSION**

257 Native lysozyme (N-L) has been shown active against Gram-negative bacteria such as *E.*  
258 *coli*. [11,27] Membrane permeabilization has been suggested as one of the mechanisms  
259 responsible for this activity. [8,28] This assumption was recently confirmed by our group who  
260 demonstrated that N-L causes the formation of pores and ion channels in the outer and  
261 cytoplasmic membranes, respectively. [9,11] Pore formation due to N-L implies that interactions  
262 occur between the protein and the *E. coli* outer membrane. Nevertheless, the mode of insertion of  
263 lysozyme into the outer membrane remains unknown.

264 Moreover, dry-heated lysozyme (DH-L) has a higher antimicrobial activity and higher  
265 membrane disruption potential than N-L. [11] This improved activity is supposed to be related to  
266 the modified physico-chemical properties of DH-L. DH-L is more hydrophobic, flexible and  
267 surface active than N-L, but its secondary and tertiary structures remain intact. [29,30] It is thus  
268 relevant to compare the interaction of native and dry-heated lysozymes with LPS, the lipid  
269 components of the outer leaflet of the outer membrane of Gram negative bacteria.

270 Interfacial monolayers are considered as good models to study interactions between  
271 antimicrobial peptides and bacterial membranes. [18,19] In the presently reported study, a LPS  
272 monolayer was used to mimic the outer leaflet of the *E. coli* K12 outer membrane, in order to  
273 explore the first step of lysozyme interaction with bacterial membrane. It is noticeable that wild-  
274 type LPS was here used for the first time to investigate protein-LPS interactions at a macroscopic  
275 and mesoscopic level, using biophysical tools such as tensiometry, ellipsometry, AFM and  
276 BAM.

277

278 **4.1 The affinity of N-L for LPS is very high and makes possible the insertion of the**  
279 **protein into a LPS monolayer.**

280 For the first time, protein insertion into a wild type LPS monolayer is here demonstrated. Until  
281 now, protein insertion was only recorded for LPS-derivative monolayers and lung surfactant  
282 protein D. [31] . The surface pressure increase measured when N-L is injected into the subphase  
283 demonstrates that N-L is able to insert into a LPS monolayer (figure 1A). The ability of  
284 lysozyme to interact with LPS is consistent with the surface activity of the protein at the  
285 air/liquid interface.[30] However, insertion of N-L into the LPS monolayer remains lower than  
286 for antimicrobial peptides such as temporin L, as suggested by the lower surface pressure  
287 increase (table 2).[32,33] The larger molecular size and higher rigidity of lysozyme,[34] as  
288 compared to peptides, could be responsible for the lower efficiency of the protein.

289 The maximal insertion pressure (MIP), determined from measurements of N-L insertion at  
290 different initial pressures, is high (41.5 mN/m, table 1) and similar to MIP recorded for  
291 antimicrobial peptides and phospholipid monolayers (25-45 mN/m).[23,33] Especially, it is  
292 remarkable that the MIP value is higher than the lateral pressure which is supposed to exist in  
293 natural membrane systems in eukaryotic cells (~ 30 mN/m).[35] Unfortunately, no measurements  
294 or theoretical deductions of the lateral pressure in the outer and cytoplasmic membranes of  
295 prokaryotes are available in literature then no comparison is possible with the here observed MIP  
296 value. Moreover, the N-L synergy factor (0.79, table 1) is extremely high as compared to  
297 reported values for protein insertion into phospholipid monolayers (from 0.3 to 0.5).[24] It can  
298 thus be concluded that the protein has a high affinity for the LPS interface between 18 and 30  
299 mN/m and strikingly lysozyme insertion is almost not impacted by the lateral cohesion of the  
300 LPS molecules. These observations suggest a mode of action that is unusual compared to the

301 interaction between protein and phospholipids. This could result from the LPS inherent molecular  
302 structure and from the specificities of a LPS monolayer compared to a phospholipid monolayer;  
303 the LPS molecules have heterogeneous polysaccharide chains in length, thus the monolayer has a  
304 variable thickness induced by the auto-assemblage of similar LPS molecules observed by BAM  
305 and AFM microscopy (figures 5 and 6). Indeed, a LPS monolayer can be divided into two  
306 distinct zones, i.e. a polysaccharide zone and a phospholipid-like zone, on the contrary to a  
307 phospholipid monolayer which is composed of a unique zone.

308

309 **4.2 The polysaccharide moieties of LPS are needed for N-L insertion.**

310 When LPS molecules are depleted from their polysaccharide moieties (KLA), lysozyme is no  
311 longer able to insert into the lipid monolayer, since no increase of the surface pressure occurs  
312 (figure 4A). However, lysozyme adsorption is evidenced by the increase of the ellipsometric  
313 angle ( $\Delta$ ) (figure 4B). Lysozyme adsorption could involve hydrogen bonds between the protein  
314 and the remaining two sugar moieties, or electrostatic interactions between the positive lysozyme  
315 and the negative KLA. The latter assumption is reinforced by the immediate and higher  
316 adsorption of DH-L which is more positively charged than N-L (figure 2B). It is also in  
317 accordance with Brandenburg et al (1998) who reported electrostatic interactions between *S.*  
318 *minnesota* Re LPS and lysozyme in solution.[36]

319 Actually, while N-L adsorption is proceeding for 3 h (figure 4B), the surface pressure of the  
320 lipid monolayer is decreasing (figure 4A). This could be due to a destabilization and partial  
321 solubilization of the lipid monolayer as has been previously described for with the antimicrobial  
322 peptide protegrin-1 at a lipid A monolayer [37]; another hypothesis is the reorganization or  
323 reorientation of the lipid headgroups induced by lysozyme presence beneath the monolayer,  
324 similarly to what has been previously reported for a dystrophin subdomain R20-24 at a  
325 DOPC/DOPS monolayer.[38] If a partial solubilization of the KLA occurs, this should be  
326 reflected in a decrease of the ellipsometric angle ( $\Delta$ ), due to the loss of matter at the interface.  
327 Here, the ellipsometric angle increases (figure 4), meaning that rather than a solubilization of the  
328 KLA monolayer, a reorganization of the KLA head groups takes place leading to a relaxation of  
329 the lipid film. Lysozyme molecules are trapped beneath the KLA monolayer caused by strong  
330 electrostatic attractive forces between lysozyme and the KLA lipids.

331 On the contrary, when N-L interacts with a LPS monolayer, i.e. including polysaccharide  
332 moieties, surface pressure and ellipsometric angle simultaneously increase (figures 2A and 2B).  
333 Undoubtedly, N-L is thus able to insert deeply in the interface, up to the hydrophobic zone of the  
334 LPS monolayer. A hypothesis can be the effect of steric hindrance of the polysaccharides which  
335 prevents total coverage of the interface by the lipid headgroups, thus leaving free space for  
336 lysozyme insertion. Moreover, the polysaccharide chains can also cause simultaneously partial  
337 shielding of the negative charges on the headgroups and therefore prevent the entrapment of the  
338 positive lysozyme molecules at the level of these negative charges as is the case for KLA lipids.  
339 The decreased interaction of the negative charges with positive lysozyme could enable insertion  
340 of the protein between the LPS headgroups. At last, lysozyme and the polysaccharides moieties  
341 could interact and create compact zones as LPS/lysozyme domains and complexes (figure 6)  
342 resulting in lesser density in other areas enabling the remaining free lysozyme to attain the  
343 interface. Such strong hydrophobic interactions have already been reported between LPS and  
344 lysozyme in solution,[39] and LPS/lysozyme complexes have been observed. [36,40]

345

### 346 **4.3 N-L interaction with LPS causes a slight reorganization of the LPS monolayer.**

347 At the same time as the surface pressure increases when N-L is injected under a LPS  
348 monolayer, a strong increase of the ellipsometric angle  $\Delta$  (+7°, figure 2B) is observed, which is  
349 higher than the ellipsometric angle increase for protein/phospholipid monolayers.[41] This  
350 unusually high  $\Delta$  increase can be explained by the LPS/lysozyme complex formation,  
351 polysaccharide reorganization, and/or the presence of N-L at the interface, since the ellipsometric  
352 angle depends on the refraction index and the film thickness.

353 BAM and AFM imaging were performed to evaluate the different hypotheses explaining the  $\Delta$   
354 increase. BAM and AFM imaging show the heterogeneity of the initial LPS monolayer at  
355 micrometer and nanometer scales, respectively (figures 4A, 4C, 5A and 5C), as a result of the  
356 variable lengths of the polysaccharides chains. After N-L injection and interaction with LPS, this  
357 heterogeneity is maintained as can be observed in the BAM (figures 5B and 5D) and AFM  
358 images (figures 6B and 6D). But N-L injection also results in a slight increase of the background  
359  $\Delta$ -value in the delta-map (figure 5D), and in the formation of small domains on the background  
360 zones in AFM-imaging (figures 6B and 6D). It can thus be concluded that N-L reorganizes the  
361 LPS monolayer, even if this reorganization remains limited. The reorganization of the LPS  
362 monolayer and the LPS/lysozyme complex formation could possibly be the preliminary steps for  
363 pore formation by N-L as observed *in vivo* by Derde et al (2013). [9]

364

365 **4.4 DH-L has a stronger affinity for LPS than N-L, and causes more radical**  
366 **reorganization of the LPS monolayer.**

367 Similarly to N-L, DH-L insertion into the LPS monolayer is enabled by the polysaccharides  
368 moieties, and DH-L reorganizes the LPS monolayer. However, differences in the behavior of  
369 DH-L *versus* N-L with the LPS monolayer can be noticed. This modified behavior could be  
370 related to its different physico-chemical properties such as increased hydrophobicity, surface-  
371 activity, positive charge and flexibility.[29,30]

372 DH-L insertion into the LPS monolayer is more efficient than N-L at concentrations higher  
373 than  $0.05 \mu\text{M}$  (figure 1A). This could be due to the higher flexibility of DH-L as compared to N-  
374 L,[29] which could allow more DH-L molecules to insert into the LPS monolayer, and/or to  
375 restructure more efficiently the interface. The increased insertion capacity of DH-L is consistent

376 with its slight increased interfacial behavior ( $\pi_{\text{final}}$ , table 1). Especially, it is noticeable that the  
377 surface pressure increase induced by DH-L insertion into the LPS-monolayer is similar to that  
378 measured with an antimicrobial peptide, i.e. temporin L, in equivalent conditions (table 2). DH-L  
379 has also more affinity for the LPS monolayer than N-L, demonstrated by its higher MIP and  
380 synergy factor (59.6 mN/m and 0.89, respectively; table 1).[23,33] The drastically different  
381 reorganization of the LPS monolayer by DH-L is highlighted by BAM and AFM imaging (figure  
382 5 and 6). The BAM-images show that the many small domains with a high  $\Delta$ -value visible in the  
383 presence of N-L (figures 5B and 5D) are replaced by larger and fewer high  $\Delta$ -value domains in  
384 the presence of DH-L (figures 5F and 5H). Concurrently, more or less thick, and more or less  
385 large clusters appear in the presence of DH-L, as evidenced by AFM images (figures 6F and 6H).  
386 These clusters could be protein aggregates caused by high local concentration of DH-L at the  
387 LPS-monolayer, consistently with the higher sensitivity to aggregation of DH-L as compared to  
388 N-L, previously established by Desfougères et al (2011). [30]

389 **5. CONCLUSIONS**

390 The presently reported study demonstrates the strong interaction between N-L and a LPS  
391 monolayer, usually considered as a relevant model of the outer membrane of Gram-negative  
392 bacteria. Even more, N-L is able to insert leading to a lateral reorganization of the LPS  
393 monolayer, which can explain pore formation into the *E. coli* outer membrane, previously  
394 observed *in vivo*. [11] An original and unexpected result is that lysozyme insertion between the  
395 lipid A of LPS monolayers requires the presence of the polysaccharide moieties. This reveals  
396 specific interactions between lysozyme and the polysaccharide moieties leading to better  
397 insertion and decreased electrostatic attraction. Further experiments are needed in order to settle  
398 between the different hypotheses that could explain this finding.

399 Moreover, dry-heating modifies lysozyme properties in such a way that its affinity for LPS, its  
400 insertion capacity, and its ability for LPS monolayer reorganization are emphasized. These  
401 results are thus consistent with *in vivo* experiments that demonstrated larger and/or more  
402 numerous pores induced by DH-L into the *E. coli* outer membrane, as compared to N-L. [11]

403 The interaction of N-L and DH-L with the outer membrane lipids is now well established and  
404 consistent with the pore formation previously demonstrated *in vivo*. Self-uptake mechanism is  
405 then imaginable meaning that lysozyme molecules involved in pore formation and stabilization  
406 could enable the entrance of free lysozyme in the bacterial cell. Then, it is relevant to further  
407 study the interaction of lysozyme with the cytoplasmic membrane, the final hurdle before access  
408 to the cytoplasm. The findings resulting from this study are currently analyzed and will soon be  
409 published.



## **ASSOCIATED CONTENT**

**Supporting information.** Additional experimental data on the ellipsometric angle of a LPS monolayer (S1), Atomic Force images of LPS or KLA monolayers (S2) and isothermal compression of LPS and KLA monolayers (S3). This material is available free of charge via the Internet at <http://pubs.acs.org>.

## **ACKNOWLEDGMENT**

The authors thank “Conseil Regional de Bretagne” for the funding of this work.

## REFERENCES

- [1] World Health Organization, *Overcoming Antimicrobial Resistance*, Geneva, Switzerland, 2000.
- [2] A. Rosbach, *Report on the Microbial Challenge – Rising threats from Antimicrobial Resistance (2012/2041(INI))*, European parliament, committee on the environment, public health and food safety, 2012.
- [3] L.T. Nguyen, E.F. Haney, H.J. Vogel, The expanding scope of antimicrobial peptide structures and their modes of action, *Trends Biotechnol.* 29 (2011) 464–472.
- [4] A. Peschel, H.G. Sahl, The co-evolution of host cationic antimicrobial peptides and microbial resistance, *Nat.Rev.Micro.* 4 (2006) 529–536.
- [5] H. Jenssen, P. Hamill, R.E. Hancock, Peptide antimicrobial agents, *Clin.Microbiol.Rev.* 19 (2006) 491–511.
- [6] P. Jolles, J. Jolles, *Whats New in Lysozyme Research - Always A Model System, Today As Yesterday*, *Mol. Cell. Biochem.* 63 (1984) 165–189.
- [7] B. Masschalck, C.W. Michiels, Antimicrobial properties of lysozyme in relation to foodborne vegetative bacteria, *Crit.Rev.Microbiol.* 29 (2003) 191–214.
- [8] A. Pellegrini, U. Thomas, P. Wild, E. Schraner, R. von Fellenberg, Effect of lysozyme or modified lysozyme fragments on DNA and RNA synthesis and membrane permeability of *Escherichia coli*, *Microbiol.Res.* 155 (2000) 69–77.
- [9] M. Derde, V. Lechevalier, C. Guérin-Dubiard, M.F. Cochet, S. Jan, F. Baron, et al., Hen egg white lysozyme permeabilizes the *E. coli* outer and inner membranes, *J. Agric. Food Chem.* 61 (2013) 9922-9929.

- [10] K. During, P. Porsch, A. Mahn, O. Brinkmann, W. Gieffers, The non-enzymatic microbicidal activity of lysozymes, *FEBS Lett.* 449 (1999) 93–100.
- [11] M. Derde, C. Guérin-Dubiard, V. Lechevalier, M.-F. Cochet, S. Jan, F. Baron, et al., Dry-Heating of Lysozyme Increases Its Activity against *Escherichia coli* Membranes, *J. Agric. Food Chem.* 62 (2014) 1692–1700.
- [12] Y. Mine, F.P. Ma, S. Lauriau, Antimicrobial peptides released by enzymatic hydrolysis of hen egg white lysozyme, *J. Agric. Food Chem.* 52 (2004) 1088–1094.
- [13] A.M. Abdou, S. Higashiguchi, A. Aboueleinin, M. Kim, H.R. Ibrahim, Antimicrobial peptides derived from hen egg lysozyme with inhibitory effect against *Bacillus* species, *Food Control.* 18 (2007) 173–178.
- [14] H.R. Ibrahim, S. Higashiguchi, L.R. Juneja, M. Kim, T. Yamamoto, A structural phase of heat-denatured lysozyme with novel antimicrobial action, *J. Agric. Food Chem.* 44 (1996) 1416–1423.
- [15] M. Hoq I., K. Mitsuno, Y. Tsujino, T. Aoki, H.R. Ibrahim, Triclosan-lysozyme complex as novel antimicrobial macromolecule: A new potential of lysozyme as phenolic drug-targeting molecule, *Int. J. Biol. Macomol.* 42 (2008) 468–477.
- [16] H.R. Ibrahim, A. Kato, K. Kobayashi, Antimicrobial effects of lysozyme against Gram-negative bacteria due to covalent binding of palmitic acid, *J. Agric. Food Chem.* 39 (1991) 2077–2082.
- [17] H.R. Ibrahim, M. Yamada, K. Kobayashi, A. Kato, Bactericidal action of lysozyme against Gram-negative bacteria due to insertion of a hydrophobic pentapeptide into its C-terminus, *Biosci. Biotechnol. Biochem.* 56 (1992) 1361–1363.

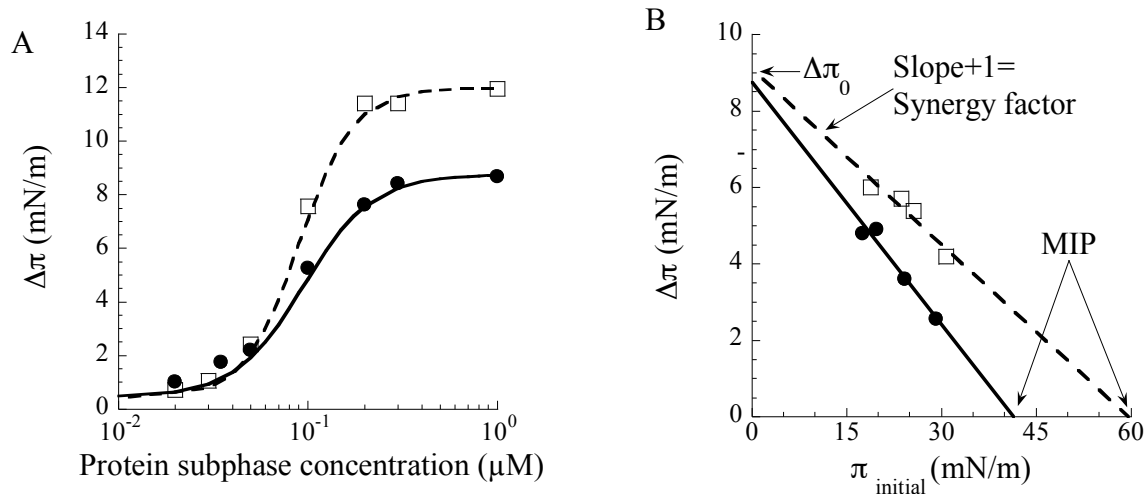
- [18] H. Brockman, Lipid monolayers: why use half a membrane to characterize protein-membrane interactions?, *Curr. Opin. Struct. Biol.* 9 (1999) 438–443.
- [19] S. Roes, U. Seydel, T. Gutschmann, Probing the Properties of Lipopolysaccharide Monolayers and Their Interaction with the Antimicrobial Peptide Polymyxin B by Atomic Force Microscopy, *Langmuir*. 21 (2005) 6970–6978.
- [20] ExPASy, P00698 (Chicken lysozyme), (n.d.). <http://web.expasy.org/cgi-bin/protparam/protparam1?P00698@19-147@> (accessed April 2, 2014).
- [21] B. Berge and A. Renault, Ellipsometry Study of 2D Crystallization of 1-Alcohol Monolayers at the Water Surface, *EPL Europhys. Lett.* 21 (1993) 773.
- [22] R.M.A. Azzam, N.M. Bashara, *Ellipsometry and polarized light*, North-Holland Pub. Co., 1977.
- [23] P. Calvez, S. Bussi eres,  Eric Demers, C. Salesse, Parameters modulating the maximum insertion pressure of proteins and peptides in lipid monolayers, *Biochimie*. 91 (2009) 718–733.
- [24]  . Boisselier, P. Calvez,  . Demers, L. Cantin, C. Salesse, Influence of the Physical State of Phospholipid Monolayers on Protein Binding, *Langmuir*. 28 (2012) 9680–9688.
- [25] P. Calvez, E. Demers, E. Boisselier, C. Salesse, Analysis of the Contribution of Saturated and Polyunsaturated Phospholipid Monolayers to the Binding of Proteins, *Langmuir*. 27 (2011) 1373–1379.
- [26] A.P. Le Brun, L.A. Clifton, C.E. Halbert, B. Lin, M. Meron, P.J. Holden, et al., Structural Characterization of a Model Gram-Negative Bacterial Surface Using Lipopolysaccharides from Rough Strains of *Escherichia coli*, *Biomacromol.* 14 (2013) 2014–2022.

- [27] A. Pellegrini, U. Thomas, R. Vonfellenberg, P. Wild, Bactericidal Activities of Lysozyme and Aprotinin Against Gram-Negative and Gram-Positive Bacteria Related to Their Basic Character, *J. Appl. Bacteriol.* 72 (1992) 180–187.
- [28] P. Wild, A. Gabrieli, E.M. Schraner, A. Pellegrini, U. Thomas, P.M. Frederik, et al., Reevaluation of the effect of lysozyme on *Escherichia coli* employing ultrarapid freezing followed by cryoelectronmicroscopy or freeze substitution, *Microsc.Res.Tech.* 39 (1997) 297–304.
- [29] Y. Desfougères, J. Jardin, V. Lechevalier, S. Pézenec, F. Nau, Succinimidyl Residue Formation in Hen Egg-White Lysozyme Favors the Formation of Intermolecular Covalent Bonds without Affecting Its Tertiary Structure, *Biomacromol.* 12 (2011) 156–166.
- [30] Y. Desfougères, A. Saint-Jalmes, A. Salonen, V. Vié, S. Beaufils, S. Pézenec, et al., Strong Improvement of Interfacial Properties Can Result from Slight Structural Modifications of Proteins: The Case of Native and Dry-Heated Lysozyme, *Langmuir.* 27 (2011) 14947–14957.
- [31] L. Wang, J.W. Brauner, G. Mao, E. Crouch, B. Seaton, J. Head, et al., Interaction of Recombinant Surfactant Protein D with Lipopolysaccharide: Conformation and Orientation of Bound Protein by IRRAS and Simulations, *Biochem.*, 47 (2008) 8103-8113.
- [32] L. Zhang, P. Dhillon, H. Yan, S. Farmer, R.E.W. Hancock, Interactions of Bacterial Cationic Peptide Antibiotics with Outer and Cytoplasmic Membranes of *Pseudomonas aeruginosa*, *Antimicrob. Agents Chemother.* 44 (2000) 3317–3321.
- [33] A. Giacometti, O. Cirioni, R. Ghiselli, F. Mocchegiani, F. Orlando, C. Silvestri, et al., Interaction of Antimicrobial Peptide Temporin L with Lipopolysaccharide In Vitro and in

- Experimental Rat Models of Septic Shock Caused by Gram-Negative Bacteria, *Antimicrob. Agents Chemother.* 50 (2006) 2478–2486.
- [34] R.E. Canfield, A.K. Liu, The disulfide bonds of egg white lysozyme (muramidase), *J.Biol.Chem.* 240 (1965) 1997–2002.
- [35] D. Marsh, Lateral pressure in membranes., *Biochim. Biophys. Acta.* 1286 (1996).
- [36] K. Brandenburg, M.H. Koch, U. Seydel, Biophysical characterisation of lysozyme binding to LPS Re and lipid A, *Eur. J. Biochem.* 258 (1998) 686–695.
- [37] Y. Ishitsuka, D. Pham, A. Waring, R. Lehrer, K. Lee, Insertion selectivity of antimicrobial peptide protegrin-1 into lipid monolayers: Effect of head group electrostatics and tail group packing, *Biochim. Biophys. Acta BBA - Biomembr.* 1758 (2006) 1450–1460.
- [38] V. Vié, S. Legardinier, L. Chieze, O. Le Bihan, Y. Qin, J. Sarkis, et al., Specific anchoring modes of two distinct dystrophin rod sub-domains interacting in phospholipid Langmuir films studied by atomic force microscopy and PM-IRRAS, *Biochim. Biophys. Acta BBA - Biomembr.* 1798 (2010) 1503–1511.
- [39] N. Ohno, D.C. Morrison, Lipopolysaccharide interaction with lysozyme. Binding of lipopolysaccharide to lysozyme and inhibition of lysozyme enzymatic activity., *J. Biol. Chem.* 264 (1989) 4434–4441.
- [40] N. Ohno, N. Tanida, T. Yadomae, Characterization of Complex-Formation Between Lipopolysaccharide and Lysozyme, *Carbohydr. Res.* 214 (1991) 115–130.
- [41] J. Sarkis, J.-F. Hubert, B. Legrand, E. Robert, A. Chéron, J. Jardin, et al., Spectrin-like Repeats 11–15 of Human Dystrophin Show Adaptations to a Lipidic Environment, *J. Biol. Chem.* 286 (2011) 30481–30491.

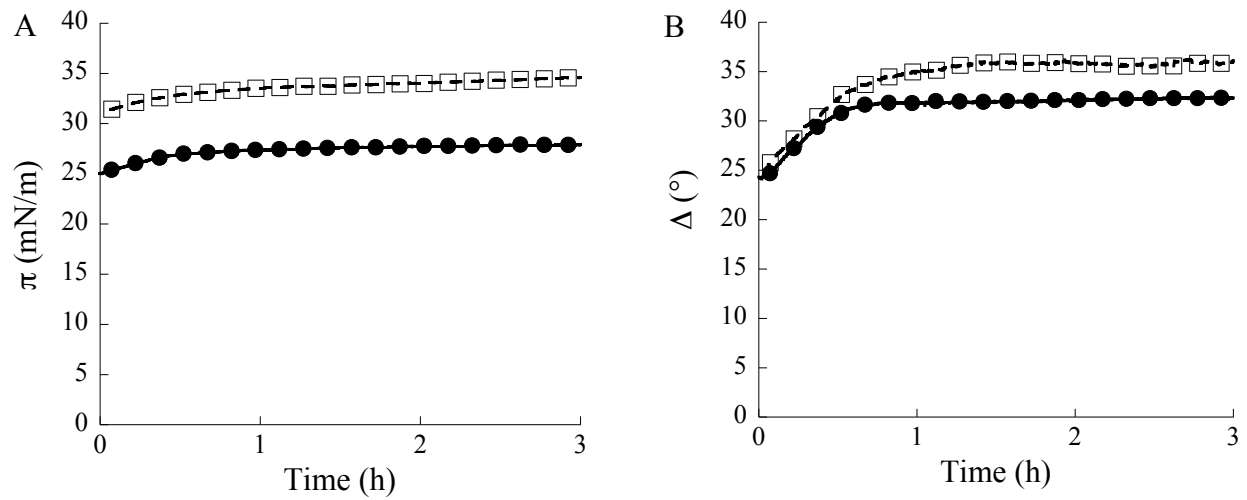
## FIGURES

**Figure 1:**



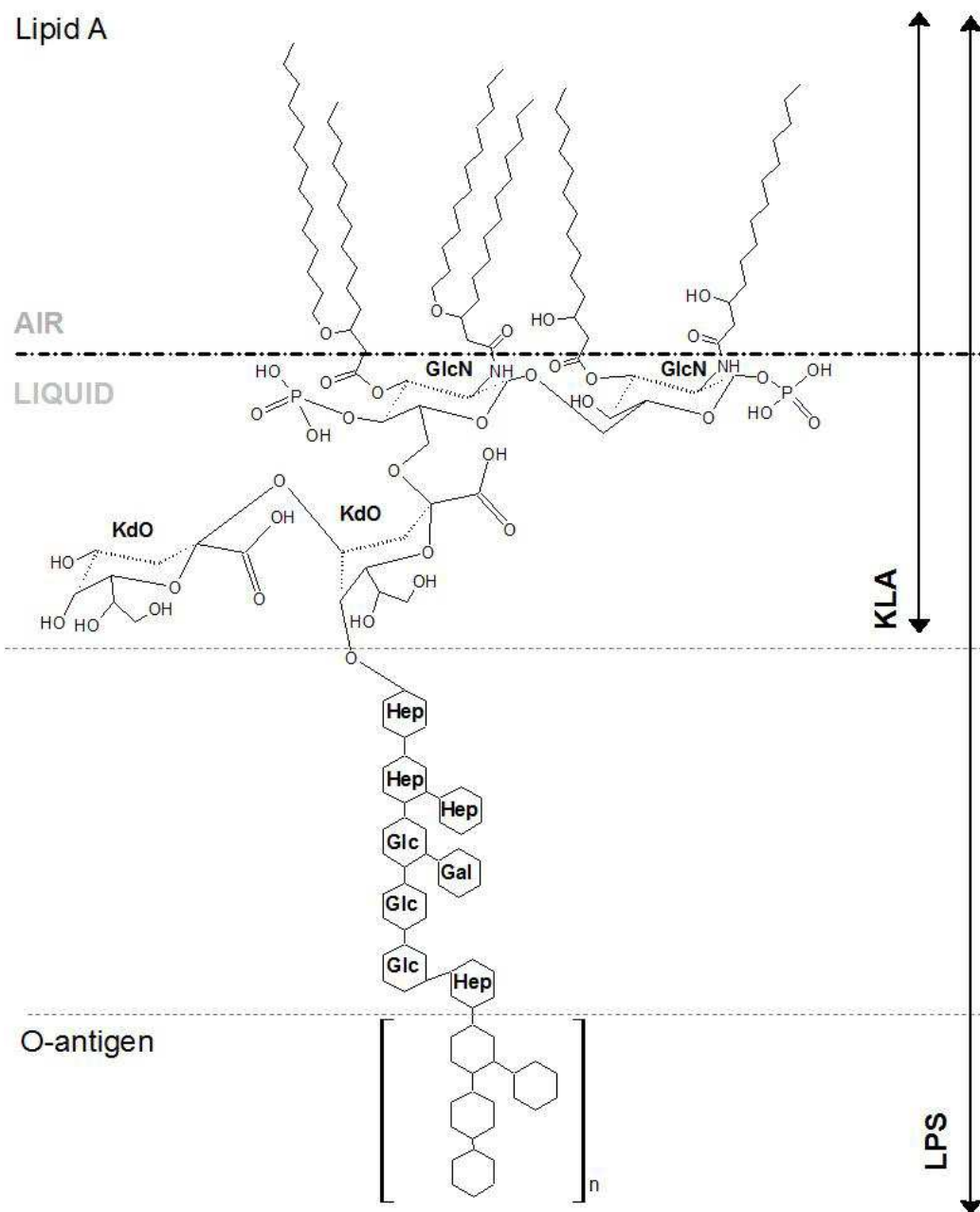
**Figure 1.** A) Surface pressure increase ( $\Delta\pi$ ) of a LPS monolayer ( $\pi_{\text{initial}}=18$  mN/m) induced by different subphase concentrations of native lysozyme (N-L) (□) and dry-heated lysozyme (DH-L) (●); B) Surface pressure increase of a LPS monolayer induced by  $0.1 \mu\text{M}$  N-L (□) and DH-L (●), depending on the initial surface pressure ( $\pi_{\text{initial}}$ ); the maximal insertion pressure (MIP) and the theoretical pressure increase in the absence of lipids ( $\Delta\pi_0$ ) are indicated by arrows.

**Figure 2:**



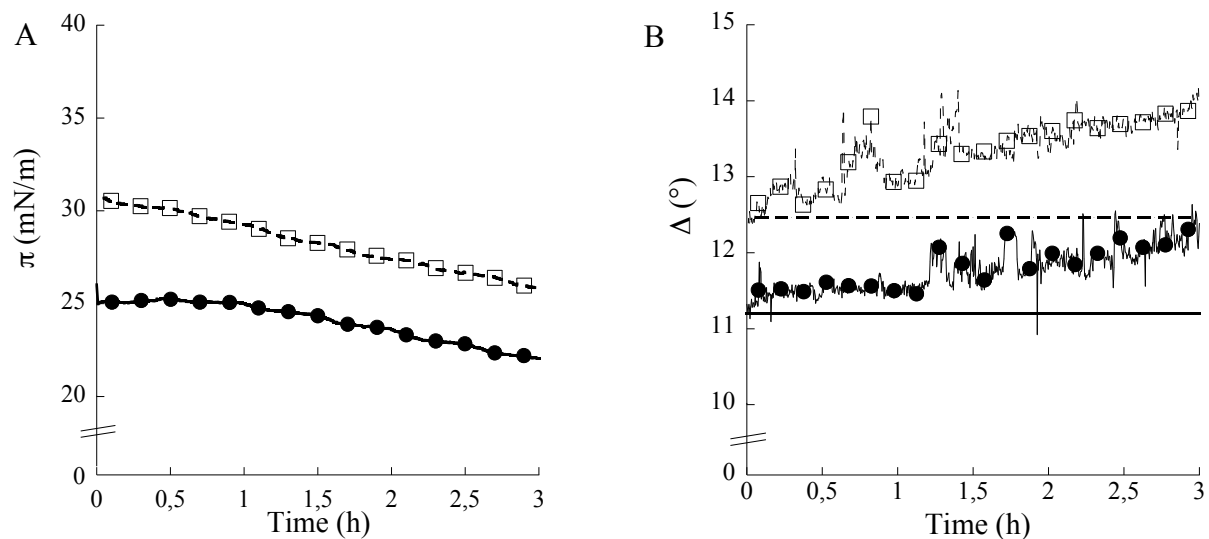
**Figure 2.** Surface pressure  $\pi$  (A) and ellipsometric angle  $\Delta$  (B) changes during N-L (□) and DH-L (●) adsorption at a LPS monolayer having an initial surface pressure of 25 mN/m and 30 mN/m, respectively.





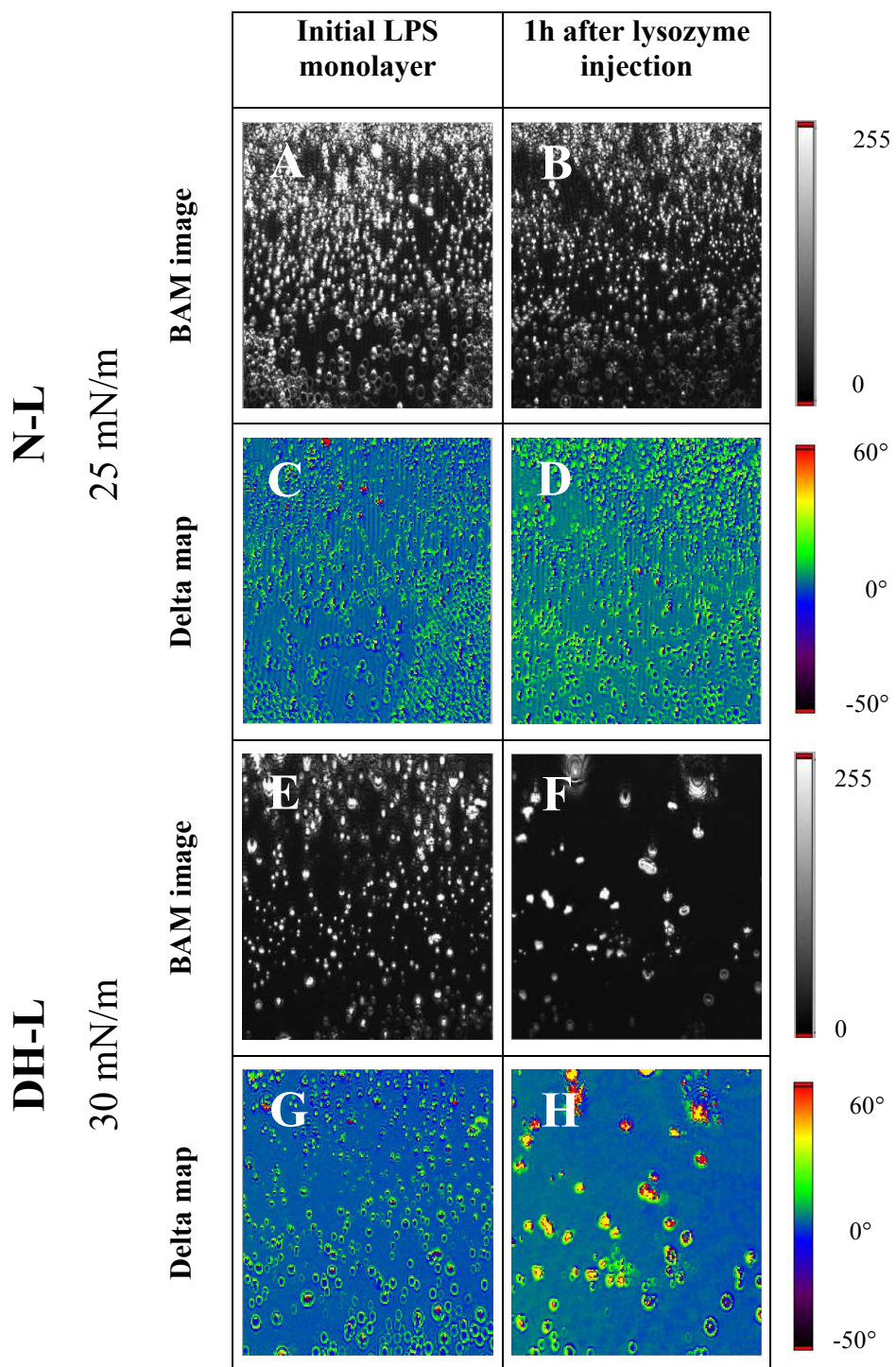
**Figure 3:**  
**Figure 3.** Schematic representation of *E. coli* K12 LPS and KLA lipids. GlcN (N-acetylglucosamine); KdO (3-deoxy-D-manno-octulosonic acid); Hep (L-gycero-D-manno heptose); Gal (galactose); Glc (glucose).

**Figure 4:**



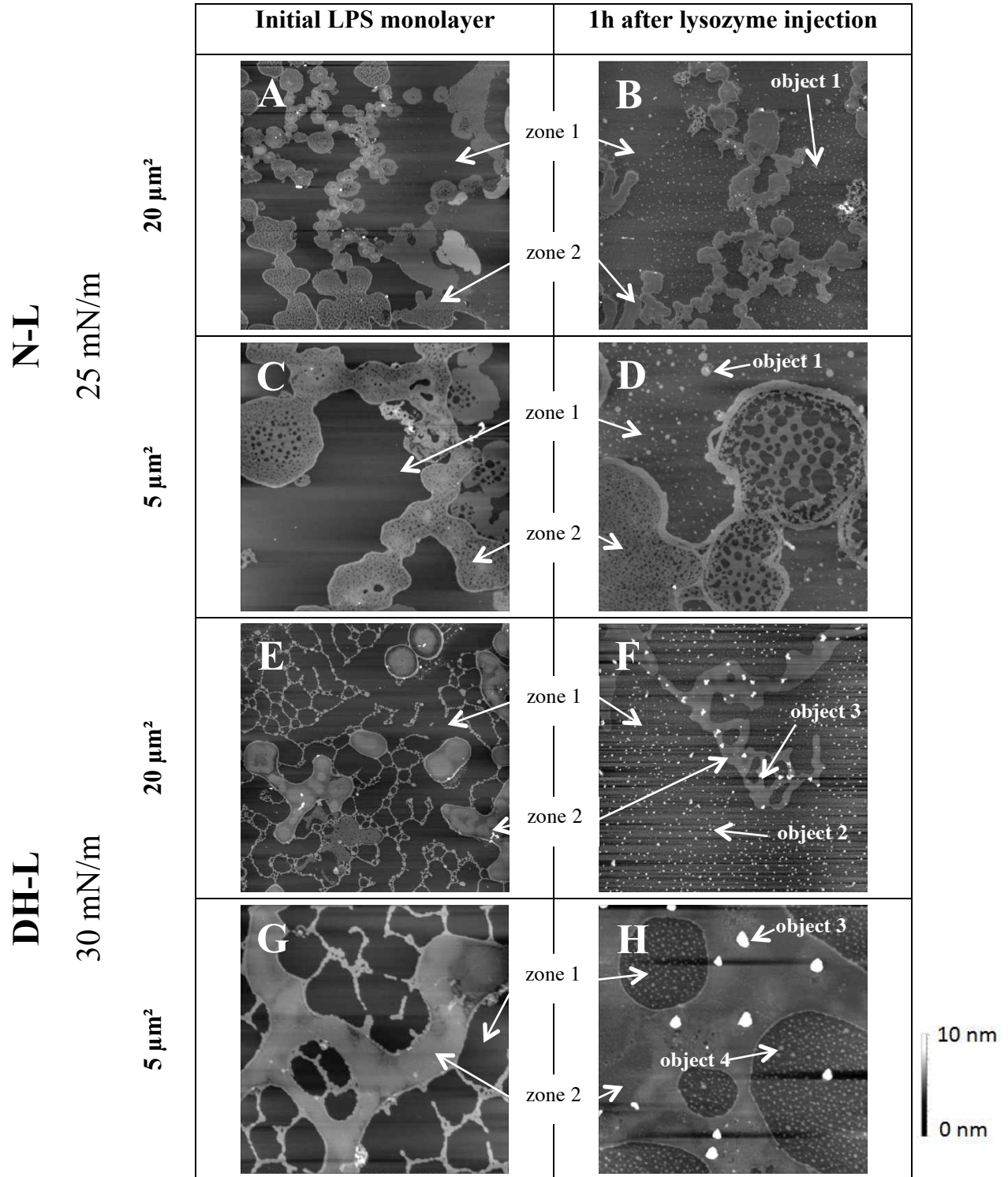
**Figure 4.** Surface pressure  $\pi$  (A) and ellipsometric angle  $\Delta$  (B) changes during N-L ( $\square$ ) and DH-L ( $\bullet$ ) adsorption at a KLA monolayer having an initial surface pressure of 25 mN/m and 30 mN/m, respectively. The initial  $\Delta$  of the KLA lipids at 25 mN/m and 30 mN/m are shown as a full and dashed line, respectively.

**Figure 5:**



**Figure 5.** BAM-images and delta-maps ( $450 \mu\text{m} \times 390 \mu\text{m}$ ) before (A, C, E, G) and after N-L (B, D) or DH-L (F, H) injection under the LPS monolayer. The initial surface pressure was 25 mN/m or 30 mN/m, for N-L or DH-L, respectively.

**Figure 6:**



**Figure 6.** Topographic AFM-images before (A, C, E, G) and after N-L (B, D) or DH-L (F, H) injection under the LPS-monolayer. The initial surface pressure was 25 mN/m or 30 mN/m, for N-L or DH-L, respectively. The Z-range is 10 nm.

## TABLES

**Table 1.** Binding parameters calculated for N-L and DH-L adsorption at a LPS monolayer: maximal insertion pressure (MIP), synergy factor, and theoretical pressure increase in the absence of lipids ( $\Delta\pi_0$ ); these parameters were extrapolated from the  $\Delta\pi$  vs  $\pi_{\text{initial}}$  plots for  $0.1\mu\text{M}$  lysozyme. For comparison, the surface pressure increase resulting from  $0.1\mu\text{M}$  lysozyme adsorption at the air/liquid interface is indicated ( $\Delta\pi_{\text{final}}$ ).

		<b>N-L</b>	<b>DH-L</b>
<b>LPS/liquid interface</b>	<b>MIP (mN/m)</b>	41.5	59.6
	<b>Synergy factor</b>	0.79	0.89
	<b>Theoretical <math>\Delta\pi_0</math> (mN/m)</b>	8.75	9.10
<b>air/liquid interface</b>	<b><math>\Delta\pi_{\text{final}}</math> (mN/m)</b>	10	11

**Table 2.** Surface pressure increase ( $\Delta\pi$ ) of LPS or LPS-derivative monolayers measured after adsorption of antimicrobial peptides and N-L or DH-L. The initial surface pressure was 18 mN/m for both peptides and protein.

	<b>Peptide or protein</b>	<b>Concentration (<math>\mu\text{M}</math>)</b>	<b><math>\Delta\pi</math> (mN/m)</b>	<b>Bacterial species</b>	<b>LPS type</b>	<b>Reference</b>
<b>Peptides</b>	Polymyxin B (1.4 kDa)	0.5	17.5	<i>S. enterica</i>	Re-LPS	[32]
	Polymyxin E1 (1.2 kDa)	0.5	21	<i>S. enterica</i>	Re-LPS	[32]
	Colymycin (1.8 kDa)	0.5	0.5	<i>S. enterica</i>	Re-LPS	[32]
	Gramicidin S (1.1 kDa)	0.15	17	<i>S. enterica</i>	Re-LPS	[32]
	Temporin L (1.6 kDa)	0.1	7.5	<i>E. coli</i>	Wild-type LPS	[33]
<b>Lysozyme</b>	N-L (14.4 kDa)	0.1	5.2	<i>E. coli</i>	Wild-type LPS	This study
	N-L (14.4 kDa)	0.1	-2.1	<i>E. coli</i>	KLA ~ Re-LPS	This study
	DH-L (14.4 kDa)	0.1	7.8	<i>E. coli</i>	Wilt-type LPS	This study
	DH-L (14.4 kDa)	0.1	-5	<i>E. coli</i>	KLA ~ Re-LPS	This study