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Native and dry-heated lysozyme interactions with membrane lipid monolayers: Lipid packing modifications of a phospholipid mixture, model of the *Escherichia coli* cytoplasmic membrane

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ABSTRACT

Antimicrobial resistance is currently an important public health issue. The need for innovative antimicrobials is therefore growing. The ideal antimicrobial compound should limit antimicrobial resistance. Antimicrobial peptides or proteins such as hen egg white lysozyme are promising molecules that act on bacterial membranes. Hen egg white lysozyme has recently been identified as active on Gram-negative bacteria due to disruption of the outer and cytoplasmic membrane integrity. Furthermore, dry-heating (7 days and 80 °C) improves the membrane activity of lysozyme, resulting in higher antimicrobial activity. These *in vivo* findings suggest interactions between lysozyme and membrane lipids. This is consistent with the findings of several other authors who have shown lysozyme interaction with bacterial phospholipids such as phosphatidylglycerol and cardiolipin. However, until now, the interaction between lysozyme and bacterial cytoplasmic phospholipids has been in need of clarification. This study proposes the use of monolayer models with a realistic bacterial phospholipid composition in physiological conditions. The lysozyme/phospholipid interactions have been studied by surface pressure measurements, ellipsometry and atomic force microscopy. Native lysozyme has proved able to absorb and insert into a bacterial phospholipid monolayer, resulting in lipid packing reorganization, which in turn has lead to lateral cohesion modifications between phospholipids. Dry-heating of lysozyme has increased insertion capacity and ability to induce lipid packing modifications. These *in vitro* findings are then consistent with the increased membrane disruption potential of dry heated lysozyme *in vivo* compared to native lysozyme. Moreover, an eggPC monolayer study suggested that lysozyme/phospholipid interactions are specific to bacterial cytoplasmic membranes.

1. Introduction

The discovery of new antimicrobial molecules is greatly necessary as a means to counterbalance the prominent public health problem of antimicrobial resistance [1]. In order to limit the development of bacterial resistance, peptides or proteins which target the bacterial cell membranes should be considered as relevant antimicrobial molecules. These antimicrobial peptides or proteins generally permeate the bacterial outer and/or cytoplasmic membranes leading to bacterial cell death [2].

The hen egg white lysozyme is one of the antimicrobial proteins that has been widely used in pharmaceutical applications. This protein is historically known for its enzymatic hydrolysis of peptidoglycan, causing its antimicrobial activity on Gram-positive bacteria [3]. However, this protein is more than just an enzyme; it is also able to disrupt the bacterial membranes, to inhibit the synthesis of DNA or RNA and to induce autolysin production [4–7]. Hence, lysozyme is active against both Gram-negative and Gram-positive bacteria [4,7]. In particular, it has been recently established that lysozyme permeates both the outer and inner membranes of *Escherichia coli*, respectively with and without perforations [6,7]. Moreover, lysozyme depolarizes the cytoplasmic membrane and causes cytosol leakage [7]. However, the antimicrobial effect of lysozyme on Gram-negative species remains limited [4,7].

Abbreviations: AMP, antimicrobial peptide or protein; CA, cardiolipin; CMEC, *Escherichia coli* cytoplasmic phospholipid mixture; DH-L, dry-heated lysozyme; DOPE, 1,2-di-(9Z-octadecenoyl)-*sn*-glycero-3-phosphoethanolamine; DOPG, 1,2-di-(9Z-octadecenoyl)-*sn*-glycero-3-phospho-(1′-*rac*-glycerol); DPPE, 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine; DPPG, 1,2-dihexadecanoyl-*sn*-glycero-3-phospho-(1′-*rac*-glycerol); EggPC, hen egg L-α-phosphatidylcholine; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; LC, liquid condensed; LE, liquid expanded; N-L, native lysozyme

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Dry-heating, a common practice to decontaminate pharmaceutical products, has been shown to improve lysozyme antimicrobial activity on *E. coli* [7–9]. A higher membrane disruption potential is at the basis of this improvement. Increased protein/membrane interactions are due to the more favorable physicochemical characteristics of dry-heated lysozymes [7]. Dry-heating lysozymes result in succinimide derivatives, making dry-heated lysozymes more basic and more flexible; these chemical modifications also induce higher surface hydrophobicity [10–13].

These *in vivo* findings thus indicate that lysozyme interacts with bacterial membrane lipids. To investigate lysozyme/lipid interactions and the impact thereof on the bacterial membrane, *in vitro* models such as lipid monolayers can be used. Interfacial monolayers are considered as good models to evaluate protein/lipid interactions, since the initial lateral lipid pressure can be controlled and multiple lipid compositions can be used [14,15]. In this context, our group had previously investigated the lysozyme/lipopolysaccharide interactions in order to have a better understanding of the lysozyme outer membrane disruption. We established that it was possible to insert lysozyme into LPS monolayers, a model for the *E. coli* outer membrane, and reorganize this lipid film laterally and vertically [16]. In this study, the aim is to investigate lysozyme interactions with bacterial cytoplasmic membrane phospholipids. Previous studies established that the lysozyme/phospholipid interactions are highly dependent on the pH, ionic strength and lipid nature [17–20]. To obtain a maximal significance from the results, environmental conditions and phospholipid composition should be as close as possible to the natural situation. In particular, the use of a complex lipid mixture is key, due to the complexity of electrostatic and hydrophobic interactions between the different phospholipids, and due to the impact of the lipid geometry and structure on the later lipid packing [21]. Thus, lysozyme interactions with bacterial cytoplasmic lipids were studied here for the first time in physiological conditions (pH 7 and ionic strength of 155 mM) using a phospholipid monolayer constituted of a lipid mixture close to the natural *E. coli* K12 composition as described by Lugtenberg et al. (1976) [22].

Since electrostatic and hydrophobic interactions are considered as the major interactive forces between lysozyme and the phospholipids [18,19], the comparison between native and dry-heated lysozymes could possibly reveal interesting differences. Indeed, dry-heating of lysozyme induces physicochemical modifications as mentioned above. These modifications should enhance lysozyme/phospholipid interactions such as protein adsorption and insertion, explaining the increased membrane depolarization and ion channel formation observed *in vivo* after dry-heating [7].

In this study, interactions between lysozyme and the phospholipid monolayer membrane model were investigated using biophysical tools such as ellipsometry, surface pressure measurements and atomic force microscopy (AFM).

2. Material and methods

2.1. Proteins and lipids

Native lysozyme (N-L) powder (pH 3.2) was obtained from Liot (Annezin, 62-France). It was heated for 7 days at 80 °C in hermetically closed glass tubes to obtain dry-heated lysozyme (DH-L). Lysozyme (N-L or DH-L) was solubilized (around 0.5 g/L) in 5 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) buffer (Sigma Aldrich, Saint-Quentin, France) pH 7.0 with 150 mM NaCl (Fluka, Saint-Quentin, France). The concentration of the lysozyme stock solution was precisely determined by absorbance at 280 nm (extinction coefficient = 2.6 $g^{-1} \cdot L$) [23]. The protein solution was then diluted in the HEPES buffer to obtain the desired lysozyme concentration.

A mixture of different lipids (Avanti Polar Lipids, Alabaster, USA) was prepared in order to obtain a composition close to the natural one present in the cytoplasmic membrane of *E. coli* as detected by Lugtenberg et al. [22]; it contained 2.6% 1,2-di-(9Z-octadecenoyl)-sn-glycero-3-phospho-(1′-rac-glycerol) (DOPG), 3.9% 1,2-dihexadecanoyl-sn-glycero-3-phospho-(1′-rac-glycerol) (DPPG), 11.8% cardiolipin (CA), 32.3% 1,2-di-(9Z-octadecenoyl)-sn-glycero-3-phosphoethanolamine (DOPE) and 49.4% 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (DPPE). This lipid mixture (CMEC) was prepared in 2:1 chloroform/methanol mixture at 0.25 mM. Hen egg L-α-phosphatidylcholine (eggPC) (Avanti Polar Lipids, Alabaster, USA) was also prepared in 2:1 chloroform/methanol mixture at 1 mM.

2.2. Lipid/protein monolayers

The experiments were performed in a homemade TEFLON® trough of 8 mL at 21 °C. Before each use, the trough was thoroughly and successively cleaned with warm tap water, ethanol and ultra-pure water, then boiled for 15 min in ultra-pure water. After cooling, the TEFLON® trough was filled with an 8 mL HEPES buffer. The CMEC was spread with a high precision Hamilton microsyringe at the clean air/liquid interface to obtain an initial surface pressure of 20 ± 1 or 30 ± 1 mN/m. The eggPC was spread as described for the CMEC to obtain an initial surface pressure of 30 mN/m. After 15 min, *i.e.* a duration necessary to allow solvent evaporation and lipid organization, 50 μL N-L or DH-L solution was injected into the subphase with a Hamilton syringe in order to obtain a final protein subphase concentration between 0.02 and 3 μM.

2.3. Surface pressure measurements

The surface pressure was measured following a Wilhelmy method using a 10 mm × 22 mm filter paper as plate (Whatman, Velizy-Villacoublay, France) connected to a microelectronic feedback system (Nima PS4, Manchester, England). The surface pressure (π) was recorded every 4 s with a precision of ±0.2 mN/m. The measured surface pressure was the result of the surface tension of water minus the surface tension of the lipid film.

2.4. Ellipsometry

Measurements of the ellipsometric angle value were carried out with an in-house automated ellipsometer in a "null ellipsometer" configuration [24,25]. A polarized He–Ne laser beam (λ = 632.8 nm, Melles Griot, Glan-Thompson polarizer) was reflected on the liquid surface. The incidence angle was 52.12°, *i.e.* Brewster angle for the air/water interface minus 1°. After reflection on the liquid surface, the laser light passed through a λ/4 retardation plate, a Glan-Thompson analyzer, and a photomultiplier. The analyzer angle, multiplied by two, yielded the value of the ellipsometric angle (Δ), *i.e.* the phase difference between parallel and perpendicular polarization of the reflected light. The laser beam probed the 1 mm^2 surface with a depth in the order of 1 μm. Initial values of the ellipsometric angle (Δ_0) and surface pressure (π_0) of buffer solutions were recorded for at least half an hour to assure that the interface was clean. Only in the case of a stable and minimal signal experiments were performed. Values of Δ were recorded every 4 s with a precision of ±0.5°.

2.5. AFM sample preparation and AFM imaging

Experiments were performed with a computer-controlled and user-programmable Langmuir TEFLON®-coated trough (type 601BAM) equipped with two movable barriers and of total surface 90 cm^2 (Nima Technology Ltd., England). Before starting the experiments, the trough was cleaned successively with ultrapure water (Nanopure-UV), ethanol, and finally ultrapure water. The trough was filled with a 5 mM HEPES buffer pH 7 with 150 mM NaCl. CMEC was spread over the clean air/liquid interface at a surface pressure of 20 ± 1 mN/m. The solvent was then left to evaporate for 15 min. Then, a Langmuir–

Blodgett (L–B) transfer was performed onto freshly cleaved mica plates at constant surface pressure by vertically raising (1 mm/min) the mica through the air/liquid interface to obtain a sample of the initial CMEC monolayer.

To investigate the influence of lysozyme on the CMEC monolayer, two lysozyme subphase concentrations (0.1 and 0.3 μM) were used. After lysozyme injection with a Hamilton syringe, surface pressure variations were recorded until a stable surface pressure was reached in order to obtain the lysozyme adsorption kinetics on the CMEC monolayer. Then, a L–B transfer of the lysozyme/lipid film was performed onto freshly cleaved mica as described above.

AFM imaging of LB films was performed in contact mode using a Pico-plus atomic force microscope (Agilent Technologies, Phoenix, AZ) under ambient conditions with scanning areas of 25 μm^2. Topographic images were acquired using silicon nitride tips on integral cantilevers with a nominal spring constant of 60 mN/m (Bruker, CA, USA). The forces were controlled and minimized along the imaging process. Three different areas were imaged for each sample to assure that the images here shown are representative of the whole sample.

3. Results

3.1. Insertion capacity of lysozyme into CMEC monolayers

The insertion capacity of lysozyme was evaluated by injecting different concentrations of native lysozyme (N-L) or dry-heated lysozyme (DH-L) into the subphase of CMEC monolayers with an initial surface pressure ($\pi_{initial}$) of 20 mN/m. The surface pressure was continuously measured until a stable surface pressure was reached. A surface pressure increase $\Delta\pi$ ($\Delta\pi = \pi_{max} - \pi_{initial}$) indicates lysozyme insertion into the CMEC monolayer.

N-L inserts into a CMEC monolayer when the lysozyme subphase concentration is higher than 0.2 μM (Fig. 1). Above this concentration, the $\Delta\pi$ value increases when the N-L concentration increases in the subphase, up to a $\Delta\pi$-plateau value of 6 mN/m. This plateau is reached at 1 μM, indicating that no more N-L insertion is possible from this subphase concentration onwards.

DH-L inserts into the CMEC monolayer at lysozyme subphase concentrations higher than 0.03 μM, and a $\Delta\pi$-plateau value of 8 mN/m is attained at 0.3 μM DH-L in the subphase. It is thus noticeable that DH-L inserts into the CMEC monolayer and reaches a $\Delta\pi$-plateau at lower concentration than N-L, and DH-L leads to a $\Delta\pi$-plateau value that is 2 mN/m higher than that of N-L.

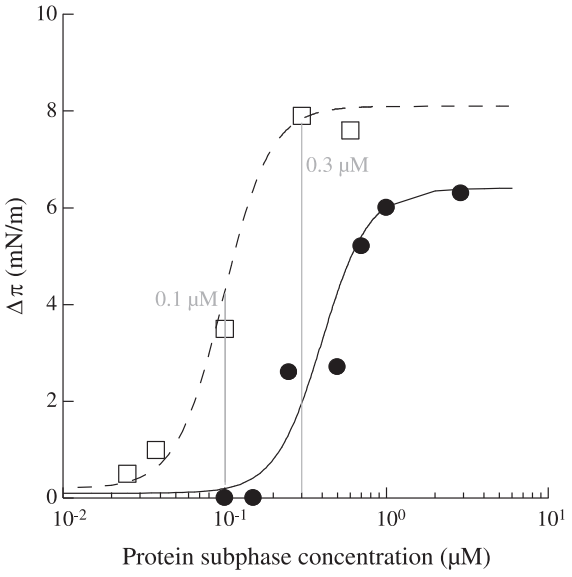

Fig. 1. Surface pressure increase ($\Delta\pi$) of a CMEC monolayer ($\pi_{initial} = 20$ mN/m) induced by different subphase concentrations of native (N-L) (●) or dry-heated lysozyme (DH-L)(□).

Further investigation of lysozyme insertion and adsorption kinetics, and of the impact of the lipid organization was performed at 0.1 μM and 0.3 μM lysozyme in the subphase. These subphase concentrations were chosen so that differences existed between both lysozymes, while minimizing protein/protein interactions in the bulk solution (aggregation) or at the lipid interface, thus allowing lipid protein interactions to be observed.

3.2. Changes of ellipsometric angle (Δ) and surface pressure (π) of CMEC monolayer in the presence of lysozyme

The ellipsometric angle and surface pressure were monitored during 3 h after 0.1 μM lysozyme subphase injection in order to evaluate the adsorption and insertion of N-L and DH-L onto and into a 20 mN/m CMEC monolayer, respectively (Fig. 2). Indeed, the ellipsometric angle reflects changes of two film parameters, the reflective index and the film thickness, while the surface pressure is related to the lateral molecular cohesion in the interfacial film.

After a 0.1 μM N-L subphase injection, the surface pressure remains stable for the first hour. It is thus obvious that N-L does not insert into a 20 mN/m CMEC monolayer, when injected in the subphase at 0.1 μM (Fig. 2A). However, a slight ellipsometric angle increase (+0.3°) is observed after 0.5 h indicating moderate lysozyme adsorption (Fig. 2A). After 1 h, the surface pressure decreases severely until 16.7 mN/m is reached, and remains stable afterwards (Fig. 2A). Meanwhile, the ellipsometric angle decreases slightly, but remains stable at 9.7° from 1 h to 3 h (Fig. 2A). It should be noticed that the ellipsometric angle is never lower than the ellipsometric angle of the CMEC monolayer before lysozyme injection.

On the contrary, a surface pressure increase (+4.3 mN/m) is observed 1 h after injection of 0.1 μM DH-L, demonstrating DH-L insertion into a 20 mN/m CMEC monolayer (Fig. 2B). Simultaneously, the ellipsometric angle increases with +1.5°. Beyond 1 h after the DH-L subphase injection, the surface pressure decreases, while the ellipsometric angle remains constant (Fig. 2B).

When increasing the concentration of N-L or DH-L in the subphase (0.3 μM *vs* 0.1 μM), the ellipsometric angle increases (+0.8° and +2.8°, respectively), indicating lysozyme adsorption onto the CMEC monolayer. Simultaneously, surface pressure increases (+1.5 and +9.0 mN/m, respectively), indicating insertion into the CMEC monolayer with initial surface pressure of 20 mN/m (Fig. 1). Contrary to the data shown in Fig. 2, no surface pressure decrease beyond 1 h of lysozyme injection is detected for 0.3 μM lysozyme (data not shown).

To evaluate the influence of the initial surface pressure of the CMEC monolayer, a 30 mN/m CMEC monolayer was used. In these conditions, no insertion is detected for 0.1 μM subphase concentration of N-L. Even more, surface pressure decreases immediately after N-L subphase injection (Fig. 3). However, the ellipsometric angle measurements increase slightly (+0.4°) at 0.5 h after the 0.1 μM N-L subphase injection, indicating that N-L adsorbs onto the CMEC monolayer. Afterwards the ellipsometric angle decreases slightly, but the Δ-value is never lower than the initial value. On the contrary, DH-L induces a surface pressure increase (+1.1 mN/m), meaning that DH-L insertion still occurs in these conditions. Simultaneously, DH-L also induces an ellipsometric angle increase (+0.7°), indicating DH-L adsorption occurs (Fig. 3). After 0.5 h the surface pressure increase induced by DH-L is maximal and decreases afterwards; the ellipsometric angle remains constant after 0.5 h (Fig. 3).

3.3. Atomic force microscopy observations of CMEC monolayers in the presence of lysozyme

AFM imaging is a highly sensitive technique, allowing the visualization of topographic surfaces of transferred interfacial films. Thus, in a

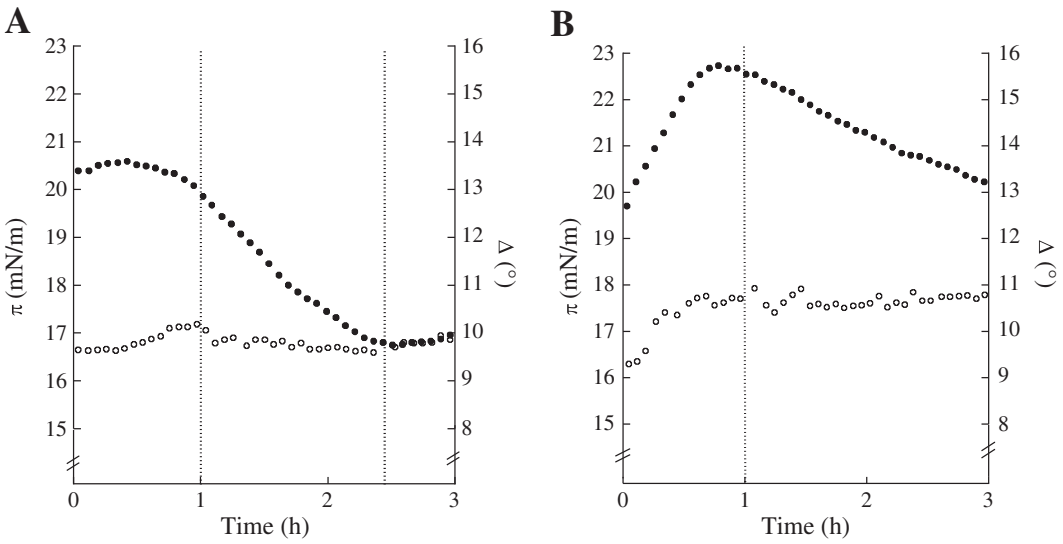

Fig. 2. Surface pressure (π) and ellipsometric angle (Δ) measurements for a 20 mN/m CMEC monolayer after 0.1 μM N-L (A) or DH-L (B) subphase injection. The full circles (●) and open circles (○) represent the surface pressure and ellipsometric angle measurements, respectively.

20 mN/m CMEC monolayer, lighter domains are visible on a dark background, indicating that two lipid phases coexist: liquid condensed (LC) and liquid expanded phases (Fig. 4A) [26]. Then, the lighter domains, whose diameters range from 0.9 to 1.7 μm, are attributed to the liquid condensed (LC) phase, while the dark background corresponds to a liquid expanded (LE) phase as described by Vié et al. for DPPC/DOPC monolayer [26,27]. The height difference between these two phases is 1.1 ± 0.1 nm (Fig. 4A and A′). This height difference is similar to the one measured between LE and LC phase domains in DPPC/DOPC films as described by Vié et al. [27].

Two lysozyme subphase concentrations were used to investigate the effect of N-L and DH-L on the CMEC monolayer. A general view of the topographic images of each protein subphase concentration shows that both phases still co-exist. The height difference between the two phases is similar to the one observed in the initial CMEC monolayer (Fig. 4A′–E′). Nevertheless, changes in the size and shape of the LC domains can be noticed after N-L and DH-L interaction.

After injection of 0.1 μM N-L in the subphase (Fig. 4B), diameter changes of the LC domains are clearly observed through a division into two groups. When considering the whole set of images taken for 0.1 μM N-L, the largest domains have diameters ranging from 0.6 to 4.0 μm, and the smallest ones have diameters ranging from 0.1 to 0.3 μm. It is also noticeable that the domains are more circular than in the initial situation. A 0.3 μM (Fig. 4D and D′) N-L injection induces no change in the LC domains size compared to the initial situation (diameter ranging from 0.7 to 2.1 μm). But a high number of small objects with a height of 1.2 ± 0.1 nm and diameter of 31 ± 13 nm appear in the LE phase. It should be noticed that these small objects only appear at the higher N-L subphase concentration (0.3 μM) and that at this subphase concentration lysozyme insertion occurs (Fig. 1). The small objects could thus be lysozyme/phospholipid clusters.

When the subphase concentration of DH-L is 0.1 μM, the LC domain size increases up to 7.3 μm, and many small objects (1.4 ± 0.8 nm height; 66 ± 20 nm diameter) appear in the LE phase (Fig. 4C and C′).

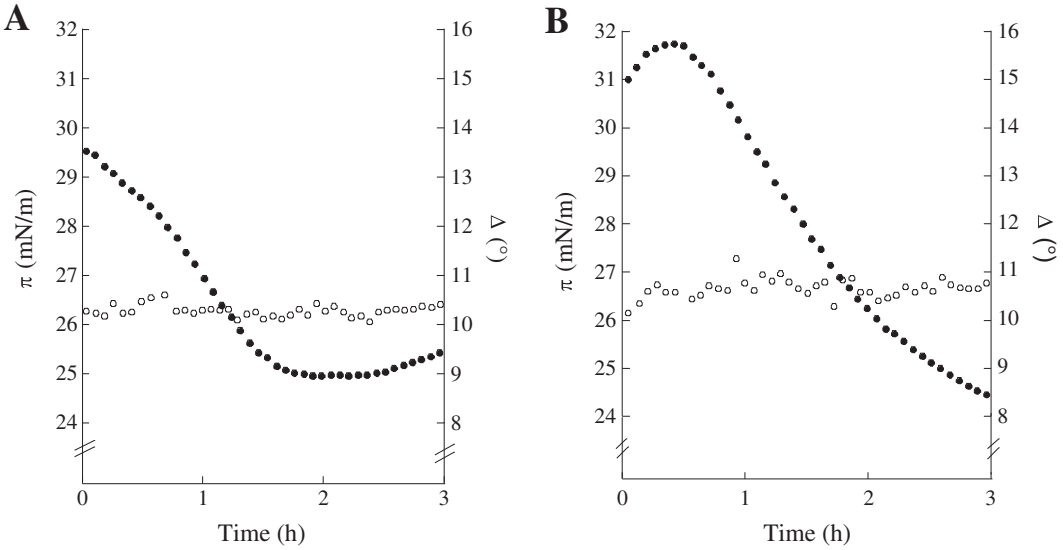

Fig. 3. Surface pressure (π) and ellipsometric angle (Δ) measurements for a 30 mN/m CMEC monolayer after 0.1 μM N-L (A) or DH-L (B) subphase injection. The full circles (●) and open circles (○) represent surface pressure and ellipsometric angle measurements, respectively.

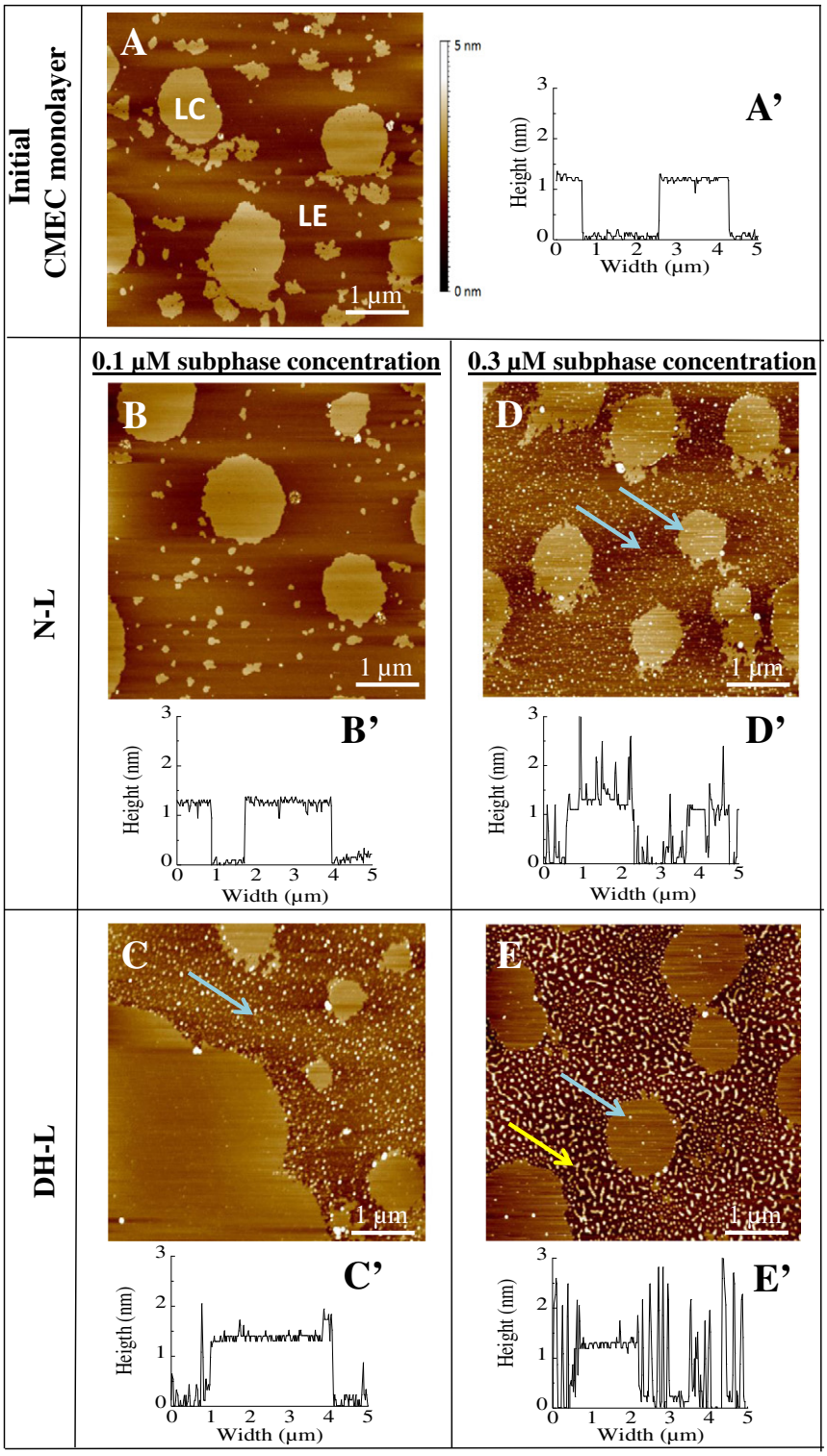

Fig. 4. AFM topographical images (25 µm^2) of CMEC monolayers without lysozyme (A) and with lysozyme after 0.1 µM N-L (B) or DH-L (C) subphase injection and after 0.3 µM N-L (D) or DH-L (E) subphase injection. Transversal cross-sections of the CMEC monolayers without (A′) and with lysozyme after 0.1 µM N-L (B′) or (C′) DH-L subphase injections and after 0.3 µM N-L (D′) or DH-L (E′) subphase injection. Small and large objects are respectively indicated with blue and yellow arrows.

The most spectacular modifications of the CMEC monolayer organization are observed after injection of 0.3 µM DH-L. Here, the edges of the domains are smooth, and long objects with a height of 2.5 ± 0.5 nm are observed in the LE phase; small objects (2.6 ± 0.6 nm height; 45 ± 10 nm diameter) are also visible in the LC phase domains (Fig. 4E and E′). Small and large objects are observed at both DH-L subphase concentrations and DH-L insertion occurs in both cases. The observed objects could be lysozyme or lysozyme/phospholipid clusters. Finally, it should be noticed that the division of the small objects between the LE and LC phases is different at 0.1 and 0.3 µM DH-L in the subphase:

at the lower concentration, the small objects are only observed in the LE phase, contrary to the higher concentration where in both LE and LC phases objects are observed (Fig. 4C and E).

3.4. Changes of the surface pressure (π) and ellipsometric angle (Δ) in the presence of lysozyme for an eggPC monolayer: a simplified model for the eukaryotic plasma membrane

Phosphatidylcholine (PC) is the most abundant phospholipid in eukaryotic cells and is absent in the model bacteria *E. coli* [22,28]. EggPC monolayer was used in this study as a simplified model for the eukaryotic plasma membrane. The initial surface pressure of the eggPC monolayer was 30 mN/m, which is the theoretical initial surface pressure of the eukaryotic plasma membrane according to Marsh et al. (1996) [29]. It is important to test the insertion and adsorption capacities of N-L and DH-L into and onto an eggPC monolayer model in order to evaluate the selectivity of the antimicrobial proteins against bacterial cell membranes. The highest concentration used in this study (0.3 μM) was tested, because a higher concentration is more prone to induce interactions with the phospholipid monolayer. The surface pressure and ellipsometric angle changes were thus recorded after injection of 0.3 μM of lysozyme (N-L and DH-L) in the eggPC monolayer subphase.

Surface pressure (π) remains stable after lysozyme injection for both N-L and DH-L, meaning both lysozymes are unable to insert into eggPC monolayer (Fig. 5). Similarly, the ellipsometric angle Δ stays constant, equal to the initial value (7.8°) measured for the eggPC monolayer, meaning that both N-L and DH-L do not adsorb onto the eggPC monolayer (Fig. 5).

4. Discussion

Native lysozyme (N-L) has been shown active against Gram-negative bacteria such as *E. coli* [4,6,7]. The mechanism of this interaction has been confirmed to be membrane permeabilization [6]. This suggests that lysozyme interacts with the bacterial membrane lipids. Consistently, N-L has been proven able to insert into LPS monolayers, *i.e.* a model of the *E. coli* outer membrane, and then to reorganize latterly and vertically this lipid film [16]. Thus, in this study we aimed to clarify the interactions between lysozyme and the *E. coli* cytoplasmic membrane phospholipids using a CMEC monolayer as a model system.

Furthermore, the limited antimicrobial activity of N-L was shown to be increased by dry-heating (7 days, 80 °C). This increased activity is the result of an increased membrane disruption potential due to the modified physicochemical properties of DH-L [7]. It was then interesting to compare the interactions of N-L and DH-L on the bacterial cytoplasmic phospholipids to get a better understanding of the interaction mechanism on the one hand, and to identify major lipid interaction factors inducing antimicrobial activity on the other hand.

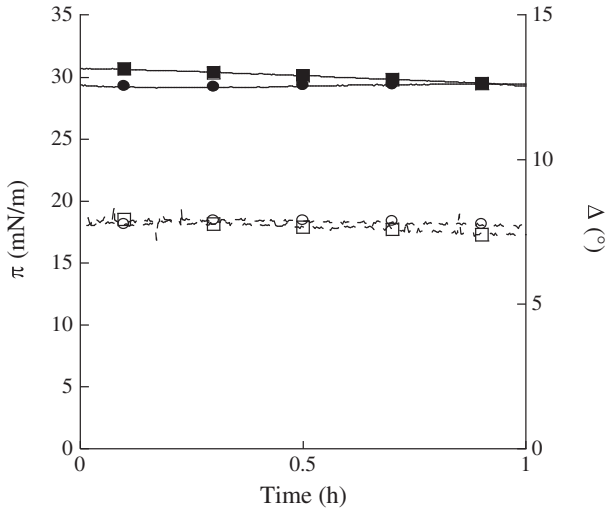

Fig. 5. Surface pressure (π), represented as full symbols and full lines, and ellipsometric angle (Δ), represented as open symbols and dashed lines, after injection of 0.3 μM N-L (circles) or DH-L (squares) in the subphase of an eggPC monolayer with an initial surface pressure of 30 mN/m.

4.1. Hen egg white lysozyme is able to insert into the CMEC monolayer

Native lysozyme insertion into the complex bacterial phospholipid mixture mimicking the *E. coli* cytoplasmic membrane is demonstrated in this study by means of surface pressure measurements; it is noticeable that lysozyme insertion is concentration-dependent (Fig. 1). This result completes earlier work on the interactions between antimicrobial peptides and monolayer of pure (DPPG or DPPC), simpler bacterial phospholipid mixtures (POPE:POPG, PG/CA, PE/PG) and complex bacterial phospholipid mixtures (PE/PG/CA) [30–34].

The surface pressure increase due to lysozyme insertion into a CMEC monolayer remains moderate compared to similar experiments with antimicrobial peptides such as bombinins, protegrin 1, Phd 1 or 2, or Gramicidin S (Table 1) [30–34]. However, it should be noticed that among the literature data, the highest surface pressure increases are obtained with highly simplified monolayer models in which only negatively charged phospholipids (PG and CA) or high proportions of such phospholipids are used. Thus, most of the results obtained for these antimicrobial peptides could potentially be biased. This emphasizes the importance of phospholipid mixtures that mimic the composition of bacterial cytoplasmic membranes to be as true-to-nature as possible.

Similarly, Mudgil et al. observed native lysozyme insertion at a subphase concentration of 0.1 μM into PE or PG monolayers ($\pi_{initial}$ = 20 mN/m) inducing a surface pressure increase of 2 and 3 mN/m, respectively (Table 1) [35], whereas at the same subphase concentration, N-L does not insert into a CMEC monolayer (PE/PG/CA). The lower negative charge density of CMEC compared to the pure PG monolayer could be responsible for these different behaviors.

4.2. Native lysozyme adsorption or insertion changes lipid packing of the CMEC monolayer

At low N-L concentration (0.1 μM), only adsorption (Δ increase) and no insertion (π constant) occurs at the CMEC/liquid interface; after the adsorption step, a severe decrease of surface pressure is observed independently of the initial surface pressure of the CMEC monolayer (Figs. 2A and 3A). The surface pressure decrease does not result from the lipid monolayer solubilization by lysozyme since the ellipsometric angle (Δ) never drops below its initial value (Figs. 2A and 3A), excluding any matter desorption from the CMEC/liquid interface. Moreover, in the absence of lysozyme, the CMEC monolayer is a stable lipid film. The surface pressure decrease observed in this study then proves that lysozyme adsorption affects the lateral cohesion of the CMEC lipids and causes lipid headgroup reorganization as described by Vié et al. for the dystrophin subdomain R20-24 at a DOPC/DOPS monolayer [36]. These findings are consistent with the changes induced by lysozyme on the lipid packing density of cardiolipin/phosphatidylglycerol bilayers [37]. The lipid packing changes could also be confirmed by AFM topographical images. When N-L only adsorbs onto the CMEC monolayer (0.1 μM), larger LC phase domains are visible (Fig. 4B) compared to the initial CMEC monolayer (Fig. 4A). This means that lipid film relaxation takes place even at low lysozyme concentration. Moreover, the reorganization and lateral cohesion decrease of CMEC lipids induced by N-L adsorption onto the lipid film is independent of the initial surface pressure (20 or 30 mN/m) (Fig. 3A).

When increasing the N-L concentration (0.3 μM), adsorption and insertion occur in the first hour after lysozyme injection under CMEC monolayer, without a subsequent surface pressure decrease and thus without loss of the later cohesion between lipids. In these conditions

Table 1
Maximal surface pressure increase (Δπ) of bacterial cytoplasmic phospholipid monolayers ($\pi_{initial}$ = 20 mN/m) measured after insertion of antimicrobial peptides.

	Peptide or protein	Concentration (μM)	Δπ (mN/m)	Phospholipid composition	Reference
Peptides	Protegrin 1	0.05	24	DPPG	[31]
	(2.2 kDa)	0.05	2	DPPE	
	Phd1	0.6	10	POPE:POPG (7:3)	[34]
	(2.1 kDa)	0.15	8	POPE:POPG (7:3)	
	Phd 2	0.6	6	POPE:POPG (7:3)	[34]
	(2.2 kDa)	0.15	2	POPE:POPG (7:3)	
	Bombinin H2	1	18	PG/CA (6:4)	[30]
	(1.9 kDa)	1	15	PE/PG (7:3)	
	Gramicidin S	0.1	17	PE/PG/CA (8:0.5:1.5)	[32]
	(1.2 kDa)	0.8	24	PG	
		0.8	6	POPE	
		0.8	18	CA	
	Polymyxin B	0.7	8	PG	[32]
	(1.4 kDa)	0.7	7	CA	
	Polymyxin E1	0.8	8	PG	[32]
	(1.2 kDa)	0.8	7	CA	
	Polyphemusin 1	0.1	2	POPC/PG/CA (8:0.5:1.5)	[33]
	(2.4 kDa)	1	7	POPC/PG/CA (8:0.5:1.5)	
		0.4	13	PG	
		0.4	5	CA	
Lysozyme (14.3 kDa)	N-L	0.1	2	PG	[35]
		0.1	3	PE	[35]
		0.1	–	PE/PG/CA (8.2:0.6:1.2) PE/PG/CA (8.2:0.6:1.2)	This study
		0.3	2		This study
	DH-L	0.1	4	PE/PG/CA (8.2:0.6:1.2)	This study
		0.3	8	PE/PG/CA (8.2:0.6:1.2)	

(0.3 μM), making insertion into CMEC monolayer possible, small objects are visible on the AFM images (Fig. 4D). These objects could be lysozyme aggregates, consistent with lysozyme aggregation already described on liposomes [18,38]. These objects could also be phospholipid/lysozyme clusters as lysozyme is positively charged at physiological pH and anionic phospholipids are present in the monolayer. Thus, lipid packing changes also take place at higher lysozyme concentration.

Finally, the present study establishes that N-L adsorbs onto a CMEC monolayer, thus changing lipid packing of the monolayer in different ways, depending if lysozyme insertion occurs or not. These findings are consistent with ion channel formation observed *in vivo* [7], since more permeable zones could be created when N-L adsorbs onto the cytoplasmic membrane and forms lipid/protein clusters. Moreover, as N-L is a positively charged molecule, its adsorption onto the cytoplasmic membrane could disturb the transversal charge equilibrium of the membrane, resulting in the membrane potential dissipation observed *in vivo* [7].

4.3. Dry-heated lysozyme highly adsorbs and inserts into CMEC monolayer, resulting in severe lipid packing modifications

DH-L adsorbs more efficiently than N-L onto CMEC monolayer, as evidenced by ellipsometric angle increase (Fig. 2), independently of initial surface pressure and even at low lysozyme concentration. The higher positive charge of DH-L at physiological pH [13] could increase electrostatic interactions with the negatively charged CMEC monolayer, as compared to N-L.

DH-L insertion into a 20 mN/m CMEC monolayer is detectable at a sixfold lower subphase concentration compared to N-L insertion; moreover, DH-L generates a higher Δπ-plateau value than N-L (Fig. 1). Also, dry-heating increases the lysozyme insertion capacity: either more DH-L molecules than N-L molecules insert into the CMEC monolayer, or DH-L spreads out more extensively than N-L at the CMEC/liquid interface, creating higher lateral pressure. This is consistent with the higher amphiphilic character and higher flexibility of DH-L compared to N-L [10,13]. Furthermore, the higher surface hydrophobicity combined with the higher flexibility of DH-L compared to N-L [13] may favor the insertion of the hydrophobic parts of the protein between the lipid chains of the CMEC monolayer.

Finally, and similarly to N-L, a surface pressure decrease is observed while DH-L adsorbs onto the CMEC monolayer, indicating that DH-L interaction with CMEC lipids decreases the lateral cohesion of the CMEC monolayer (Fig. 2B and 3B). At low concentration, DH-L induces fusion of LC domains (Fig. 4C), suggesting preferential insertion of lysozyme into LE phase of the CMEC monolayer (Fig. 4C). This is consistent with the higher fluidity of the LE phase. But considering the equal repartition of negative charges into both phases, this finding highlights that DH-L insertion into a CMEC monolayer is not only guided by electrostatic attractions, but also by the local phospholipid packing. At high concentration, DH-L induces the formation of high and long objects (Fig. 4E), which could be protein clusters or lipid/protein complexes in the LE phase. In particular, the positively charged DH-L could recruit the anionic lipids (PG or CA) for subsequent lipid/protein cluster formation. Since these objects are visible in both phospholipid phases, it can be assumed that DH-L insertion at high concentration is no longer guided by lipid packing.

Overall, DH-L disrupts more severely the organization of CMEC monolayer, probably due to its modified physicochemical properties [10,13]. This is consistent with its higher efficiency compared to N-L for *in vivo* disruption of the cytoplasmic membrane of *E. coli*.

4.4. Native and dry-heated lysozymes specifically adsorb onto and insert into bacterial monolayer models

An eggPC monolayer, model of the eukaryotic plasma membrane, was used in this study to verify the specificity of the N-L and DH-L interactions with bacterial membrane models. A subphase injection of 0.3 μM N-L or DH-L beneath an eggPC monolayer did not modify the surface pressure or ellipsometric angle values (Fig. 5). Thus, N-L and DH-L do neither insert into nor adsorb onto the eggPC monolayer. Similarly Mudgil et al. [35] and Matsumura and Dimitrova [39] did not observe any insertion of lysozyme into DPPC monolayers (20 mN/m) and eggPC monolayers (30 mN/m), respectively. Because the net charge of eggPC is null at physiological pH, the present study thus suggests that electrostatic attractions are essential for first interaction between

lysozyme and a CMEC monolayer model, as previously established for interactions between lysozyme and anionic phospholipid vesicles [19].

The lack of interaction between N-L and DH-L on the one hand, and eggPC on the other hand is a promising property for the development of innovative antimicrobial drugs. Indeed, the lack of interactions between antimicrobial molecules and eukaryotic cell walls are necessary to limit cell toxicity.

4.5. Conclusions

N-L insertion into CMEC monolayers is a concentration-dependent phenomenon as usually described for protein/lipid interactions. Remarkably, N-L causes a lateral cohesion decrease of the CMEC lipids even in the absence of insertion, as observed at low lysozyme concentration. This finding demonstrates that a protein can modify the lipid packing simply by adsorbing onto the lipid headgroups. Thus, protein insertion is not a prerequisite for monolayer packing modifications. At high lysozyme concentration, this phenomenon is accentuated and completed by insertion. Moreover, the phospholipid/lysozyme interactions observed in the present study were specific for the bacterial cytoplasmic membranes compared to the eukaryotic plasma membranes, highlighting the importance of electrostatic attraction forces.

Dry-heating, a common decontamination practice for pharmaceutical products, increases the adsorption and insertion capacity of lysozyme towards CMEC monolayers, as well as the extent of lipid packing modifications of CMEC monolayers. The increased effect of DH-L compared to N-L is due to its modified physicochemical characteristics such as increased positive charge and flexibility.

Finally, the present results are consistent with the *in vivo* disruption of the *E. coli* cytoplasmic membrane previously reported [7]. N-L and DH-L adsorption should induce the dissipation of the cytoplasmic membrane potential, caused by its disorganizing effect as here evidenced by AFM. Furthermore, the creation of ion channels in the cytoplasmic membrane could be due to lipid packing defects similar to those here highlighted. Thus, such modifications in membrane permeability have an impact on bacterial cell viability and growth.

Another striking result of the present study is that the processing of natural pharmaceutical molecules, such as dry-heating, could have a significant impact on their antimicrobial activity. The impact of processing methods on the biological activity of molecules should thus be at least considered, at most exploited.

Conflict of interest

I state that none of the authors has a conflict of interest.

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