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The interaction of antimicrobial peptide LL-37 with artificial biomembranes: epifluorescence and impedance spectroscopy approach

Frances Neville[1], **Marjolaine Cahuzac**[1], **Andrew Nelson**[2] **and David Gidalevitz**[1,3,4]

[1] Institute for Materials Research and Department of Chemical Engineering, University of Leeds, Leeds, LS2 9JT, UK
[2] Centre for Self Organising Molecular Systems and School of Chemistry, University of Leeds, Leeds, LS2 9JT, UK
[3] Department of Chemical and Environmental Engineering, Illinois Institute of Technology, Chicago, IL 60616, USA

E-mail: gidalevitz@iit.edu

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Abstract

Membrane interactions of the human antimicrobial peptide LL-37 have been studied by a variety of techniques including insertion assay, epifluorescence microscopy and impedance spectroscopy. This study makes use of lipid monolayers at the air–aqueous interface to mimic bacterial or eukaryotic membranes. It was found that LL-37 readily inserts into phosphatidylglycerol (PG) and lipid A monolayers, significantly disrupting their structure. In contrast, the structure of phosphatidylcholine (PC) monolayers remains virtually unaffected by LL-37, which is evident both from epifluorescence and electrochemical measurements. Impedance spectroscopy showed that the LL-37 rich PC monolayer remains an ideal capacitor while LL-37 enriched lipid A capacitance decreases significantly, suggesting an increase in layer thickness from peptide–lipid binding.

1. Introduction

Antimicrobial peptides are part of the innate immune system and have the ability to fight infection [1, 2]. They can be found naturally in a range of living organisms from bacteria to mammals. Antimicrobial peptides have recently become a matter of increasing interest because of their excellent potential for treating very common, as well as sometimes fatal, illnesses which may not be cured by conventional antibiotics. Antibiotic resistance is a

[4] Author to whom any correspondence should be addressed.

common problem with the number of bacterial infections caused by multi-drug-resistant bacteria increasing steadily [3–5]. Commonly used antibiotics target bacterial protein receptors in cell membranes. However, resistance occurs when these receptors are changed after DNA mutations or alterations occur and this therefore means the antibiotic cannot bind at all or binds but cannot function. Antimicrobial peptides have enormous potential with regard to bacterial resistance because they interact not only with specific membrane protein receptors, but also with the lipid matrix of cell membranes, whose lipid composition is highly unlikely to change as a result of bacterial mutation.

Several antimicrobial peptides are already in clinical trials and the pharmaceutical industry has a great interest in them for human therapeutics [1]. For example, MBI-594AN [6] is in phase IIb trials and iseganan hydrochloride [7, 8] is in phase II/III clinical trials. One of the major drawbacks for any serious pharmaceutical application of antimicrobial peptides is a continuing controversy with regard to their mechanism of action.

Recently solid-state nuclear magnetic resonance and differential scanning calorimetry have been used to investigate lipid bilayer disruption by LL-37 [9]. Vacuum-dried samples were used to decrease interference and facilitate the acquisition of spectra. However, this eliminates any motion of the peptide that occurs in fluid bilayer systems. Thus the model is less able to mimic the natural *in vivo* situation in which molecules may freely move around in membranes. Their results ruled out the barrel stave mechanism [10] of LL-37 action in favour of the carpet/toroidal approach [11, 12].

One of the most interesting properties of antimicrobial peptides is their ability to differentiate between foreign and native cells. Eukaryotic and bacterial membranes have very different lipid compositions, with the outer leaflet of the former comprising mainly of phosphatidylcholine (PC), sphingomyelin and cholesterol, and the latter including substantial amounts of negatively charged phospholipids, such as phosphatidylglycerol (PG), cardiolipin or lipopolysaccharides (LPS).

In this work we model the outer leaflet of the red blood cell (RBC) membrane and an external layer of the outer cell wall of Gram-negative bacteria with a Langmuir monolayer composed of phospholipid molecules at the air–aqueous interface—zwitterionic dipalmitoyl-phosphatidylcholine (DPPC) or dioleoyl-phophatidylcholine (DOPC) is used to simulate the RBC, and negatively charged dipalmitoyl-phosphatidylglycerol (DPPG) or lipid A to imitate the external layer of the bacterial membrane. Lipid A is a major component of lipopolysaccharides isolated from the *E. coli* outer cell wall. For insertion assay and epifluorescence measurements these monolayers are compressed to a surface pressure [13] where the area per lipid molecule value roughly corresponds to values found in actual cell membranes [14]. This pressure is kept constant throughout the experiments. Therefore any changes in the recorded lipid area per molecule values result in corresponding changes of the overall monolayer area. Molecular areas are monitored throughout the experiments and, when coupled with *in situ* epifluorescence measurements, give a quantitative description of peptide–lipid interactions, leading to a better understanding of the mechanism of action of antimicrobial peptides. Previously, this approach has been successfully applied to study membrane interactions of protegrin-1, a β-sheet antimicrobial peptide isolated from pig leukocytes [15].

In this paper we further extend this approach looking at membrane interactions of LL-37, an α-helical antimicrobial peptide of human origin. LL-37 (LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES(–NH_2) [12]) is a 37 amino acid peptide from the cathelicidin family, originally isolated from neutrophil-specific granules but also found at the mucosal linings in the body and skin [16, 17]. It is actively secreted at these locations during inflammation, which suggests an active role in the immune system [18].

Impedance spectroscopy has been applied to study lipid–peptide interactions only relatively recently [19–21]. Research has mainly been carried out on the phospholipid dioleyl-phosphatidylcholine (DOPC) and the pore-forming peptide gramicidin A [19, 22, 23]. In this research the impedance data were used to show the effect of the peptide on the lipid capacitance, which is related to the thickness of the lipid monolayer.

The aim of this study is to further investigate the interaction of LL-37 with model membranes using a unique combination of insertion assay, epifluorescence and electrochemical techniques in a complementary manner in order to probe the question of LL-37 selectivity between prokaryotic and eukaryotic cells and its possible mechanism of action.

2. Experimental details

All surface pressure–area isotherms were collected by using a custom-built two-barrier Teflon Langmuir trough equipped with a Wilhelmy plate as described previously [24]. Dulbecco's phosphate buffered saline (Invitrogen Life Technologies) without calcium and magnesium ions was used as the subphase both for insertion and impedance spectroscopy measurements. The temperature of the subphase was maintained at $30 \pm 0.5\,^{\circ}\mathrm{C}$ for insertion experiments and a resistively heated indium tin oxide-coated glass plate was placed over the trough to minimize interference by contamination, air currents and evaporative losses and also to prevent condensation of water on the microscope objective. Excitation between 530 and 590 nm and emission between 610 and 690 nm was gathered through the use of a HYQ Texas red filter cube; 0.5 mol% of lipid-linked Texas-red (TR-DHPE) dye from Molecular Probes was incorporated into the spreading phospholipid solutions. Due to steric hindrance, the dye partitions into the disordered phase, rendering it bright and the ordered phase dark. Images from the fluorescence microscope were collected at a video rate of 30 frames s^{-1} using a silicon intensified target (SIT) camera and recorded on Super-VHS formatted videotape with a recorder. This assembly permits the monolayer morphology to be observed over a large lateral area while isotherm data is obtained concurrently. The entire apparatus is set on a vibration isolation table.

DPPC (dipalmitoyl-phosphatidylcholine), DOPC (dioleyl-phosphatidylcholine), DPPG (dipalmitoyl-phosphatidylglycerol) (Avanti Polar Lipids Inc.) and lipid A ((diphosphoryl, from *E. coli* F583), Sigma-Aldrich) were used without further purification. For insertion assay and epifluorescence microscopy measurements monolayers were deposited quantitatively from chloroform (Fisher Spectranalysed) solution while a pentane (HPLC grade) solution was used for impedance spectroscopy. Measurements were first obtained with the lipids only and then the desired amount of peptide was subsequently injected into the subphase. The final concentration of LL-37 in the subphase used was 0.4×10^{-7} g ml^{-1}.

For the impedance spectroscopy experiments, a three-electrode system was used, consisting of a platinum counterelectrode, an Ag/AgCl (versus 3.5 mol dm^{-3} KCl) reference electrode and a hanging mercury drop electrode (HMDE) as the working electrode as described previously [20, 25]. The subphase was deaerated before each experiment with special grade argon and a blanket of the gas was maintained above the liquid phase throughout the experiments. Measurements of the impedance (Z) of the electrode systems using frequencies (f) logarithmically distributed from 65 000 to 0.1 Hz, 0.005 V_{rms} at potentials from -0.4 V were carried out on the coated electrode systems.

The monolayer was deposited onto the mercury drop (area 0.0088 cm^2) by exuding a drop of mercury in the gas phase and slowly lowering it through the spread layer until the coated mercury was completely immersed in the electrolyte. After the chosen monolayer was established and sufficient coverage of the electrode had been confirmed by cyclic voltammetry, LL-37 was injected into the subphase. The subphase was stirred very slowly to facilitate the

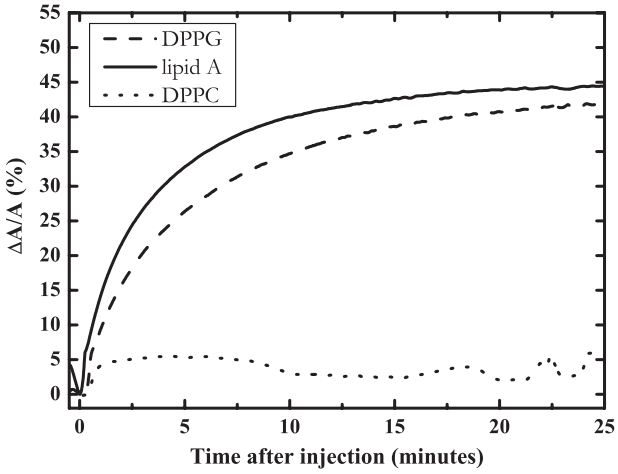

Figure 1. Percentage change of area during a 25 min period after injection of 0.4×10^{-7} g ml^{-1} LL-37 into the aqueous buffer subphase at 30 mN m^{-1}. Dotted curve: very small insertion was observed in the DPPC monolayer (<5%). Full curve: much more pronounced insertion into lipid A (45%). Broken curve: substantial insertion into DPPG (43%).

equilibrium of the peptide so as not to disturb the lipid at the interface. Stirring was terminated before a new mercury drop was used with the LL-37 modified monolayer. The amount injected gave a final concentration of LL-37 in the subphase of 0.4×10^{-7} g ml^{-1}. An Autolab general purpose electrochemical system (Echo Chemie) equipped with a frequency response analyser module was utilized for voltammetry and impedance measurements.

3. Results and discussion

In order to examine the selectivity of LL-37 towards eukaryotic and bacterial cells, constant pressure peptide insertion experiments were conducted with different lipids. Zwitterionic DPPC or DOPC was used to examine the influence of LL-37 on the membrane integrity of red blood cells, while negatively charged DPPG and lipid A were used to test for the mechanism of action of LL-37 towards bacteria.

In order to examine the nature of the interaction between the peptide and the lipid monolayer, a series of constant pressure insertion experiments were performed. Generally during such experiments a phospholipid monolayer spread over a buffer subphase is compressed to a certain pressure, which is maintained constant via a built-in feedback system of the apparatus. An aliquot of the peptide solution is injected by a syringe into the buffer subphase. With the surface pressure held constant, any increase in the percentage change in area per lipid molecule ($\Delta A/A$) after injection indicates that insertion of the peptide into the phospholipid monolayer occurs and that the peptide is taking up space at the interface. The level of peptide insertion therefore correlates with the level of increase in $\Delta A/A$. The surface morphology of these insertion events was monitored using epifluorescence microscopy (EFM). Image contrast arises due to different phase densities, partitioning characteristics of the dye molecules in coexisting phases. Thus, one can obtain information about the structure of the monolayer by imaging the lateral fluorescence distribution.

Constant pressure insertion isotherms ($\Pi = 30$ mN m^{-1}) indicate little or no LL-37 insertion into the DPPC monolayer (figure 1, dots), showing a less than 5% area increase

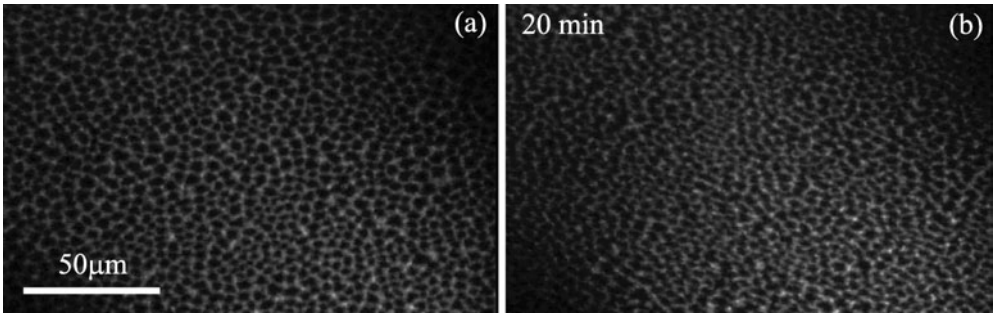

Figure 2. Epifluorescence micrographs of DPPC at 30 mN m^{-1}. (a) Before injection and (b) 20 min after injection with 0.4×10^{-7} g ml^{-1} LL-37. Both images were taken at the same magnification.

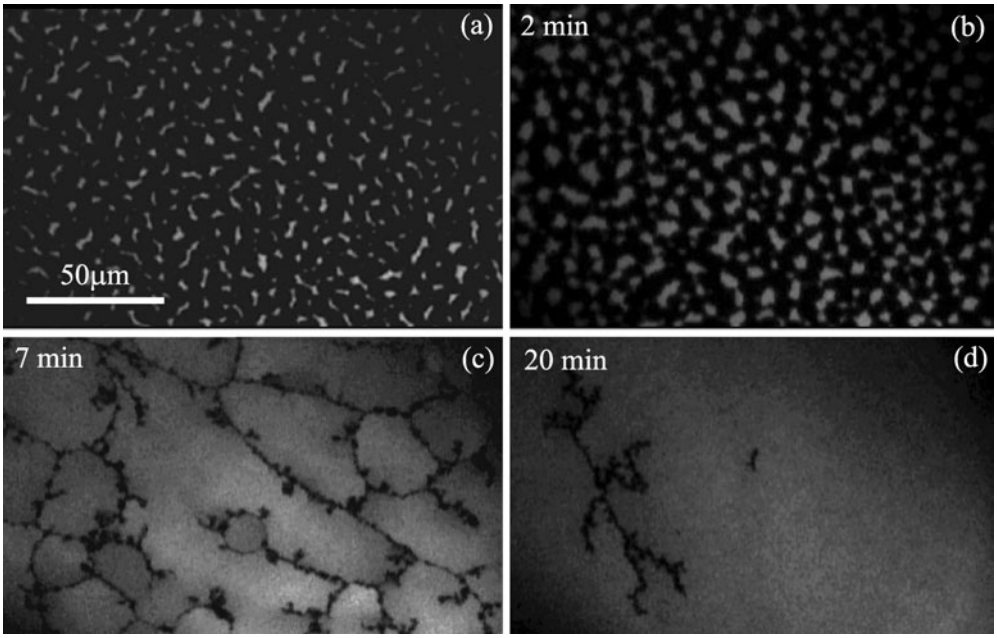

Figure 3. Epifluorescence micrographs of DPPG at 30 mN m^{-1}: (a) before injection, (b) 2 min after injection, (c) 7 min after injection and (d) 20 min after injection. All images were taken at the same magnification.

following peptide injection underneath the monolayer. In contrast, LL-37 causes a substantial increase in DPPG (43%, figure 1, broken curve) and lipid A (46%, figure 1, full curve) area per molecule, indicating incorporation of peptide molecules into the monolayer structure. Increasing the peptide concentration from 0.4×10^{-7} to 1.0×10^{-7} g ml^{-1} (not shown) does not affect DPPC but leads to an almost four times insertion increase for DPPG and lipid A.

Epifluorescence microscopy measurements were performed simultaneously with the insertion isotherms to monitor the effect of peptide binding on the morphology of the monolayer. No change in DPPC morphology following LL-37 injection was observed (figure 2). In contrast, DPPG displays profound changes with a significant increase in the bright disordered phase domain area, and a decrease in the dark condensed phase area (figure 3)

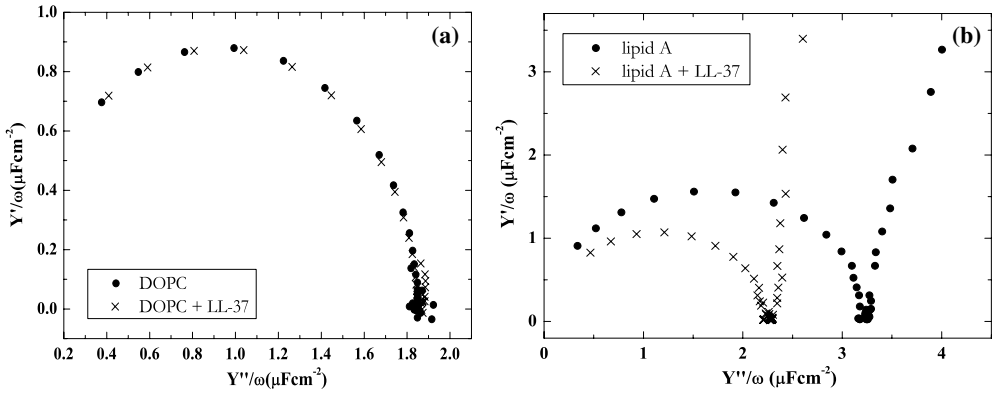

Figure 4. Cole–Cole plots of (a) DOPC with (crosses) and without (dots) LL-37 and (b) lipid A with (crosses) and without (dots) LL-37.

indicating a substantial insertion of the peptide into the fluid domains of the monolayer. These results agree well with insertion assay data, showing peptide insertion into DPPG and lipid A, but not into DPPC (figure 1).

Impedance spectroscopy measurements used DOPC and lipid A to investigate the ability of LL-37 to differentiate between eukaryotic and prokaryotic cells, respectively. Measurements were carried out to examine the interaction of LL-37 with the monolayer after injection of the peptide into the subphase. The data were transformed to normalized admittance values and plotted as Cole–Cole plots. In this case a Cole–Cole plot showing a perfect semicircle represents an ideal capacitor in a linear resistor–capacitor circuit. Figure 4(a) shows that such Cole–Cole plots, derived from impedance data of DOPC before and after LL-37 adsorption, conform to near-'perfect' semicircles [20] with zero-frequency capacitances (C_0) of approximately 1.85 μF cm^{-2}, which agree well with previously published data for pure DOPC [25]. The point of zero-frequency capacitance (C_0) is the point where the data cross the Y''/ω axis. This indicates that the layer acts as a simple capacitor (C) in series with the resistance of the electrolyte (R), whether or not peptide is present in the subphase. Very different results were obtained with the lipid A system (figure 4(b)). The Cole–Cole plot for pure lipid A gives a zero-frequency value for C_0 of approximately 3.2 μF cm^{-2} and a separate low frequency capacitative element or 'tail'. This shows that lipid A does not act as a simple capacitor. This was expected as lipid A is a complex lipid which would not pack as closely as DOPC and the separate tail corresponds to the presence of inhomogeneities or defects. When LL-37 is added C_0 decreases to approximately 2.3 μF cm^{-2}, suggesting an increase in layer thickness from possible peptide–lipid binding. This result is in agreement with lipid A constant pressure insertion assay measurements which indicate a substantial insertion of LL-37 into the lipid A monolayer (figure 4).

Although previous work has used a variety of techniques, such as attenuated total reflectance Fourier-transform infrared spectroscopy [12], solid state NMR and circular dichroism [9], to investigate the mechanism of action of the antimicrobial peptide LL-37, the exact mechanism of action of LL-37 is still debatable. This is due to the fact that the results of different research groups suggest varying mechanisms of action depending on the method used. We must stress that in this study we investigate interactions of the antimicrobial peptide with models of outer leaflets of red blood cells and bacteria. It has been shown [26] that the inner leaflet of the membrane sometimes has a significant contribution in peptide–membrane

interactions. Therefore, it is important to consider the methodology presented as a powerful, but complementary, approach to studies involving live cells, vesicles and bilayers.

Overall our data suggest that LL-37 preferentially disrupts the lipid components of bacterial membranes (DPPG, lipid A) rather than the eukaryotic lipid membrane components (DPPC, DOPC) at the concentrations of LL-37 used here. This is concurrent with data from bacterial cytotoxicity tests and haemolysis assays carried out previously [27]. The degree of insertion into anionic DPPG or lipid A monolayers is significantly larger than that of zwitterionic DPPC or DOPC. The degree of disorder caused by the peptide insertion corresponds to the extent of peptide incorporation and constant pressure insertion experiments and epifluorescence have been used to observe this effect. This is the first study where electrochemistry techniques have been used to corroborate the effects of the interaction of membrane components with the antimicrobial peptide LL-37. The impedance spectroscopy results imply that the phosphatidylcholine headgroup of the lipid does not interact with LL-37 whereas there is a clear interaction between LL-37 and lipid A. This approach shows that a lipid monolayer at the gas–aqueous interface can serve as a tool for the specific detection of ions and peptides in solution and is an alternative to the supported lipid bilayer which is also frequently used.

In addition to studying peptide–membrane interactions and demonstrating that the LL-37 can differentiate between eukaryotic and bacterial cell membrane types, this work uses techniques that exploit an interface on a liquid substrate, enabling the system to be fluid. The discrimination of the LL-37 peptide for different cell types may have possible future pharmaceutical applications, helping to combat the problem of resistance with conventional antibiotic drugs.

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