Electrochimica Acta 267 (2018) 195–205

Contents lists available at ScienceDirect

Electrochimica Acta

journal homepage: www.elsevier.com/locate/electacta

Direct visualization of alamethicin ion pores formed in a floating phospholipid membrane supported on a gold electrode surface

Fatemeh Abbasi, J. Jay Leitch, ZhangFei Su, Grzegorz Szymanski, Jacek Lipkowski*

Department of Chemistry, University of Guelph, Guelph, Ontario, N1G 2W1, Canada

ARTICLE INFO

Article history:
Received 3 January 2018
Received in revised form
8 February 2018
Accepted 10 February 2018
Available online 13 February 2018

ABSTRACT

Unilamellar DMPC/DMPG vesicles in the absence and presence of alamethicin were fused onto the surface of a gold electrode modified with a 1-thio-β-D-glucose self-assembled monolayer. The resulting floating bilayer lipid membranes (fBLMs) were investigated using atomic force microscopy (AFM) and electrochemical impedance spectroscopy (EIS). A corrugated film structure was observed for the pure DMPC/DMPG fBLMs due to surface stress between the tightly packed lipids. These corrugations are removed by the addition of alamethicin suggesting the lipid-peptide interactions alleviate the overall surface stress creating a more uniform bilayer. Both DMPC/DMPG films in the absence and presence of alamethicin had thickness of 5.5 ± 0.9 nm demonstrating that alamethicin has a minimal effect on the overall bilayer thickness. However, a significant decrease in membrane resistivity was observed when alamethicin was inserted into the fBLM indicating that the peptides are forming ion conducting pores. A direct visualization of the alamethicin pores was obtained by molecular resolution AFM images revealing that the pores are not randomly dispersed throughout the bilayer, but instead form hexagonal aggregates. The diameter of an individual pore within the aggregates is equal to 2.3 ± 0.3 nm, which is consistent with the size of a hexameric pore predicted by molecular dynamics simulations. Additionally, the image revealed a broad size distribution of alamethicin aggregates, which explains the origin of multiple conductivity states observed for the incorporation of alamethicin into free standing bilayer lipid membranes.

1. Introduction

Alamethicin, a 20 amino acid residue antimicrobial peptide, was first discovered by Meyer and Reusser in the culture broth media of the fungus Trichoderma viride in 1976 [1]. Alamethicin belongs to the peptaibol family with an acetylated N-terminus and a phenylalaninol (Pheol) residue at the C-terminal end [2]. Peptaibol peptides are characterized by the presence of 2-aminoisobutyric acid (Aib), which induces a helical secondary structure. A proline residue at position 14 divides the peptide into a 13-residue N-terminal α-helix and a 7-residue 3_{10}-helix at C-terminus. The total length of the alamethicin peptide is 3.2 nm, which is comparable to the thickness of the lipid of a biological cell membrane. The positions of the Aib and Gln residues on the alamethicin helices make the peptide amphipathic where one side is hydrophobic and the other side is more hydrophilic [3]. The difference in hydrophobicity provides a driving force for peptide adsorption at the biological membrane surface and insertion into the membrane core upon further aggregation with other alamethicin peptides [3].

The adsorption and insertion of the alamethicin depend on its peptide-to-lipid ratio. At low ratios, alamethicin adsorbs to the membrane surface with its helical axis oriented in the direction parallel to the surface (S state). At sufficiently high peptide-to-lipid ratios ($P/L \geq 1/15$ for most lipids), alamethicin inserts into bilayer (I state) [4,5]. In I state, the alamethicin molecules span the hydrophobic region of the bilayer and aggregate around a central pore with their hydrophobic domains in contact with the phospholipid tails. The polar regions of the peptides are oriented towards the center of the pore creating an aqueous channel that permits the transport of small ions across the bilayer [6]. This pore structure is referred to as the barrel-stave model and is responsible for the antimicrobial activity of alamethicin by allowing for the indiscriminate transport of ions across the bacterial cell membrane leading to cell lysis [6,7].

* Corresponding author.
E-mail address: jlipkows@uoguelph.ca (J. Lipkowski).

https://doi.org/10.1016/j.electacta.2018.02.057
0013-4686/

Electrochemical measurements (I–V curves) of the alamethicin-incorporated free standing bilayers (black lipid membranes) show multiple conductance steps providing evidence that the alamethicin pore consists of a varying number of peptide monomers [6]. A voltage-dependent conductance is observed in the alamethicin inserted black lipid membrane where the conductance is proportional to the ninth power of the bulk alamethicin concentration [8,9]. This voltage-dependent conductance has been recently explained by Guidelli and Becucci [10] using model that assumes peptide insertion driven by the electric field-dipole interactions, followed by nucleation of peptide molecules into clusters forming transmembrane ion pores.

The structure of the alamethicin aggregates in model membranes has been extensively investigated by X-ray crystallography. Pan et al. [11] showed that the composition of phospholipid bilayers has an overall effect on the size of the aggregated alamethicin pore. In bilayers composed of 1,2-dioleoyl-*sn*-glycero-phosphatidylcholine (DOPC) and 10% alamethicin, the pore consists of five alamethicin monomers with an outer radius of 1.37 nm, while in membranes of 1,2-dierucoyl-*sn*-glycero-phosphatidylcholine (DEPC) with the same peptide concentration, the alamethicin pore is composed of 8–9 monomers with an outer radius of 1.9 nm. The length of the DEPC lipid chain is four carbons longer than the DOPC lipid implying that the aggregation is driven by the length of the lipid tails. These two lipids are unsaturated meaning that their corresponding bilayers are in the liquid crystalline state. He et al. [12] tested the effects of peptide aggregation in saturated phospholipid bilayers, specifically 1,2-dilauroyl-*sn*-glycero-3-phosphatidylcholine (DLPC) and 1,2-diphytanoyl phosphatidylcholine (DPhPC). The alamethicin pore consisted of 8–9 monomers with a radius of 2.0 nm in the DLPC gel state bilayer and 11 monomers with an outer pore radius of 2.5 nm in the DPhPC liquid crystalline bilayer. Salditt et al. [13] used X-ray scattering to measure the aggregation of alamethicin peptides in bilayers composed of both saturated and unsaturated phospholipid bilayers with variable acyl chain lengths. These studies show that alamethicin aggregation is largely dependent on the phase state and thickness of the phospholipid bilayer [13]. The Chen research group applied sum frequency generation (SFG) and the attenuated total reflection (ATR) spectroscopy to study alamethicin in POPC bilayers supported on a CaF_2 prism [14,15]. They found that alamethicin was inserted into the lipid bilayer in the fluid phase, but remained on the external surface when the bilayer was in a gel state. Quartz crystal microbalance with dissipation monitoring (QCM-D) was used to study the interactions of alamethicin with the egg phosphatidylcholine (egg PC) supported lipid bilayer. QCM-D results support the model that the inserted peptides form a cylindrical pore [16].

The electrochemical impedance spectroscopy (EIS) was used to study the voltage-gated channel formation by alamethicin incorporated in the mercury-supported tethered lipid bilayer [17]. Most recently, Su et al. [18] performed impedance spectroscopy studies on alamethicin incorporated into DPhPC bilayers supported at gold electrode surfaces and in tethered bilayer lipid membranes [19]. They supplemented the EIS result with parallel photon polarization modulation infrared reflection absorption spectroscopy (PM-IRRAS) measurements and were able to provide molecular level interpretation of the conductivity through pores formed by alamethicin molecules.

The direct visualization of alamethicin aggregation into channels was achieved by Pieta et al. [20] using the electrochemical scanning tunneling microscopy EC-STM. The EC-STM images showed that alamethicin molecules form porous nanoclusters where an individual peptide participates in the pore formation of two adjacent channels. Unfortunately, these images were only acquired for monolayer films because the tunneling barrier is too large to allow for STM imaging of a bilayer. Therefore, the structure of the peptide aggregates may be influenced by direct contact with the solid gold support.

The objective of this work is to provide direct visualization of alamethicin aggregation in a floating bilayer (fBLM) consisting of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine/1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol (DMPC/DMPG) (1:1) bilayer supported on a 1-thiol-β-D-glucose modified gold (111) electrode by atomic force microscopy (AFM). The combination of the fBLM architecture with the mixed phospholipid matrix (neutral DMPC and negatively charged DMPG) are used to better represent the membrane environment of a Gram-positive bacteria for alamethicin incorporation and channel formation. The modification of the gold surface with a monolayer of 1- thiol- β-D-glucose separates the DMPC/DMPG membrane from the gold surface by creating water-rich spacer region [21]. The AFM images of alamethicin aggregates are complemented by EIS measurements of conductivity through alamethicin transmembrane pore. This approach provides unique understanding of the nature of alamethicin antimicrobial activity and insight into the design of novel antimicrobial peptides to combat the growing number of antibiotic resistant bacteria.

2. Experimental section

2.1. Chemicals and solutions

1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) and 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol (DMPG) were purchased from Avanti Polar Lipids (99% pure; Alabaster, AL, US). Alamethicin (Alm) and 1-thio-β-D-glucose (Tg) were purchased from Sigma-Aldrich (St. Louis, MO, US). These molecules were used as purchased without further purification. HPLC-grade chloroform was purchased from Sigma-Aldrich (St. Louis, MO, US) and used to prepare all vesicle solutions. Sodium fluoride powder, 99% purity (Sigma-Aldrich, St. Louis, MO, US), was cleaned in a UV-ozone chamber (Jelight, Irive, CA, US) for 20 min to oxidize and remove any organic impurities prior to use. The sodium fluoride electrolyte solutions were prepared by dissolving the pre-cleaned powder in Milli-Q UV plus ultra-pure water, resistivity 18.2 MΩ cm, (EMD Millipore, Billerica, MA, US) to give a final concentration of 100 mM and 1 mM for the EIS and AFM experiments, respectively.

2.2. Sample preparation and bilayer formation

All glassware was cleaned in a hot mixed acid bath (1-part HNO_3: 3-parts H_2SO_4) for 60 min and then thoroughly rinsed and soaked in Milli-Q water for 3–4 h. The Teflon pieces were soaked in a Piranha solution (1-part H_2O_2: 3-parts H_2SO_4) and then rinsed with Milli-Q ultra-pure water.

The gold substrates for the AFM studies were produced in the AXXIS vacuum system (Kurt J. Lesker, PA, US) by magnetron sputtering technique (Torus, 3" dia., DC). A 3–5 nm titanium adhesion layers were first deposited onto a clean, standard glass microscope slides. Next ~200-nm of gold was deposited onto the titanium adhesion layer to create gold substrates that are highly uniform and stable. The gold-coated glass slides were then annealed in a muffle furnace at 675 °C for 70 s to produce large and well-ordered gold crystallites with (111) facets preferentially oriented in parallel to the electrode surface [21,22]. The gold slides were then immersed into a 2 mM aqueous solution of Tg for 20 h to ensure that a uniform self-assembled monolayer (SAM) is deposited on the gold surface. The Tg SAM is highly hydrophilic and acts as a conditioning layer to assist with vesicle fusion to form model bilayers that better mimics biological conditions [21]. Afterwards, the Tg-modified gold (111)