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# Atomic Force Microscopy and Electrochemical Studies of Melittin Action on Lipid Bilayers Supported on Gold Electrodes

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ABSTRACT

Melittin is an amphipathic helical peptide which shows strong membranolytic activity against bacterial cells as well as human erythrocytes. It is often considered as a good model for general studies on interactions of antimicrobial peptides with biological membranes since the detailed mechanisms of their action on biological membranes are still subject of intense debate. In this paper we have used electrochemical methods combined with in situ AFM imaging in order to evaluate the mechanisms involved in melittin membranolytic activity. We have observed that high concentration of the peptide causes rapid degradation of a single component DMPC bilayer supported on gold electrode. The lipid membrane undergoes micellization and subsequent dissolution. This indicates that under such conditions melittin acts according to detergent-like mechanism. The mode of melittin action differs substantially when the peptide concentration is lowered. In this case, the changes in DMPC bilayer structure are less rapid and at the initial stages the peptide adsorbs on top of the membrane. This process is followed by fluidization of the DMPC film, which facilitates further reorientation and insertion of melittin into the bilayer. As a result, the permeability of the membrane is increased. AFM data shows that sharp differentiation between carpet and toroidal pore mechanism is difficult and it is very likely that melittin acts according to mixed mechanism. Yet different behavior of melittin was observed for the mixed DMPC/Cholesterol bilayer. The susceptibility of the membrane to melittin action was reduced probably due to the increased packing within the hydrocarbon chain region of the bilayer. We have also noticed that the fluidization of the membrane is a common feature for all systems studied here. Thus, it seems to be a crucial step for melittin action which enables the peptide molecules to adopt proper orientation either for pore formation or disruption of the membrane.



## 1. INTRODUCTION

Rapidly increasing number of multi-drug resistance pathogens resulted in a continuous need for new active compounds with strong activity. One of the most promising groups of compounds includes antimicrobial peptides [1]. These are small naturally occurring peptides with molecular mass less than 10 kDa and usually positively charged. Antimicrobial peptides are functional substances in the innate immune system of virtually all forms of life and they constitute a first line of defense against pathogens [2]. They display a broad range of activity on bacteria, fungi and parasites. Therefore, they have enormous potential as novel therapeutic agents. Based on the targets of AMPs action, they can be divided in two major groups: membrane disruptive AMPs which cause the increase in membrane permeability and non-membrane disruptive AMPs which can pass through the membrane to reach the intracellular targets and inhibit the synthesis of cell wall, proteins, RNA or DNA [3]. Irrespective of the targets of AMPs action, the key step is always their interactions with cell membrane driven by electrostatic or hydrophobic forces. Until now, there are four main models describing the possible way of interactions of AMPs with membranes [4–6]. In the barrel-stave model peptides bind to the membrane surface as monomer, next reorient perpendicularly to the surface during the insertion process and, after reaching the threshold peptide–lipid (P/L) ratio, form barrel-stave-like aggregates with the hydrophobic regions interacting with hydrophobic tails of membrane and hydrophilic regions facing each other and forming the transmembrane pore or channel. In this case, lipids are not involved in a pore structure and the membrane does not bend during formation of the pore. The latter is the biggest difference between barrel-stave and toroidal model, in which hydrophilic regions of peptide remain in contact with polar lipid head groups during the pore formation. As a

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consequence, membrane bends sharply inward to form a pore lined by both lipids head groups and peptides. The third, micellar-aggregate model assumes that after insertion into lipid membrane, peptides associate into micelle-like complexes without any fixed stoichiometry. Such aggregates are formed in a concentration- and voltage-dependent manner and contribute to the disruption in permeability. This way transmembrane transport of the molecules including lipids and peptides is enabled [7,8]. Different model, that is carpet model, involves adsorption of the peptides parallel to the membrane surface and covering the membrane in a carpet-like manner. When the threshold peptide concentration on the surface is reached, the membrane breaks down and lipid aggregates surrounded by peptide molecules are formed. The membrane disruption, therefore, is not associated with peptide insertion and pore formation; it is rather dissolved by peptide in a dispersion-like fashion.

Melittin is an example of membrane active peptide. It is water-soluble amphipathic helical peptide consisting of 26 amino acids, which is isolated from the honeybee *Apis mellifera*. The overall net charge of melittin molecule is +6 and most of the charge is localized at the C-terminus of the peptide [9]. Melittin interacts with biological as well as artificial membranes. For example, it causes hemolysis of cells and leakage of entrapped dyes in lipid vesicles. Melittin is a non-selective peptide. In other words, it is a toxin that displays strong lytic activity against bacterial cells as well as human erythrocytes [10]. This feature precludes melittin for a direct therapeutic use but it offers a great model for general studies on interactions of antimicrobial peptides with biological membranes. The mode of action of melittin depends on the properties of lipid bilayer, therefore numerous aspects have to be considered, including structural requirements of peptide, the length and unsaturation of lipid acyl chains, charge of polar headgroup, role of cholesterol, melittin concentration and its spatial orientation in lipid membranes [11,12].

Supported lipid membranes constitute an attractive model of biological membrane. Therefore, such systems are often used as biomimetic architectures, which enable investigation of various membrane-related biological processes [13–15]. Properties of solid supported lipid membranes are determined by number of factors including the lipid composition, size, shape and presence of unsaturated bonds. Equally important is the nature of the substrate. Most widely used are glass, quartz, silicon and mica [16–18]. However, metal surfaces were demonstrated to be useful as well [19]. Importantly, they show certain advantage, since the structure of the supported film as well as lipid-lipid and lipid-protein interactions can be probed in full electrochemical conditions. This enables investigation of voltage-dependent membrane processes including structural changes as well as lipid-lipid and lipid-protein interactions.

In this work, we demonstrate an electrochemical approach combined with in situ AFM imaging, which enabled the evaluation of the mode of melittin action in the presence of artificial lipid membranes supported on gold electrodes. Electrochemical methods were demonstrated to be useful for monitoring the structural changes in supported lipid bilayers occurring upon action of membrane disrupting peptides [20–23]. We use cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) in order to verify the permeability of intact lipid films and then after their exposure to melittin. AFM imaging allowed in situ observation of structural changes within the bilayers. It is well known that the modification of the electrode with blocking dielectric layer leads to significant decrease of the capacitance accompanied by increase of charge transfer resistance [24]. The latter results from the fact, that the electrode surface cannot be directly accessed by redox probe. Consequently, electron transfer between electroactive species and the electrode surface is inhibited and it occurs from certain distance defined by the thickness of the immobilized film. As it was already mentioned, action of melittin affects the structure of lipid membranes. This should result in significant changes in a permeability of the membrane to ions and water molecules. Since capacitance as well as charge transfer resistance are sensitive to the presence of the defects in a bilayer, both could be used for evaluation of the mechanism of melittin action. Using such approach, we have investigated how the mode of melittin action is determined by the nature of the lipid membrane. For this purpose, single-component membrane composed of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) was compared with two-component system containing DMPC and cholesterol. DMPC is broadly used for preparation of biomimetic membranes. It was chosen as a model system since the structure of gold supported bilayers of this particular lipid are very well characterized using photon polarization modulation infrared reflection absorption spectroscopy (PMIRRAS) [25,26], atomic force microscopy (AFM) [27], and neutron reflectivity (NR) [28]. Cholesterol is a common component of the membranes of eukaryotic cells [29]. It affects the properties of the lipid membranes, providing enhanced stiffness and rigidity due to the increased packing density within the hydrophobic region [30]. Since the natural target for melittin is the membrane of erythrocyte that contains up to 30 mol% of cholesterol [10], the understanding of interactions of melittin with membrane cholesterol becomes an important issue. Several studies have revealed that the role of cholesterol in melittin activity depends on the lipid composition: in case of homogeneous lipid bilayers (i.e. composed of a single lipid component), cholesterol inhibits the action of melittin [31,32], whereas in heterogeneous systems like lipid rafts or a mixture of zwitterionic lipids cholesterol does not provide any additional protection against melittin [33,34].

## 2. EXPERIMENTAL

All chemicals were purchased from Sigma-Aldrich with exception of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), which was purchased from Avanti Polar Lipids Inc. The distilled water used in all experiments was passed through a Milli-Q water purification system and its final resistivity was 18.2 MΩ/cm. Monolayers were prepared at the air-water interface using a KSV LB trough 5000 (KSV Ltd., Finland). Before each experiment, trough and barriers were washed using the mixture of chloroform and methanol and finally rinsed with Milli-Q water. The compression of the monolayers was performed at the barriers speed of 10 mm/min at a constant temperature of $22 \pm 1^{\circ}$C. The spreading solutions of lipids were prepared by dissolving DMPC and cholesterol samples in chloroform. Lipid bilayers were transferred on gold substrates ($11 \times 11$ mm slides, Arrandee), which were 200-300 nm thick gold films evaporated onto borosilicate glass precoated with a 4 nm thick adhesive layer of chromium. Prior to the deposition of the lipid bilayer, gold substrates were cleaned in the mixture of $H_2O_2/NH_3/H_2O$ (1:1:5 v/v/v) at 70°C, rinsed with water, dried and then flame annealed. The latter involved heating of the gold electrode in a propane/butane flame until the dark red glowing was observed. Then the substrate was quenched with water and the procedure was repeated for 6-7 times. As a result, large Au(111) terraces were obtained with the length of the edge in the order of few hundred nanometers. For atomic force microscopy experiments gold beads prepared according to Clavilier procedure were used [35]. The quality of both types of gold substrates was evaluated using AFM and the representative images are shown in Figure 3S in supporting information. Lipid bilayers were transferred from the air-water interface (with pure water as a subphase) onto the solid supports at the pressure of 35 mN/m. This particular pressure was chosen