Colloids and Surfaces B: Biointerfaces 167 (2018) 229–238

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

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

A QCM-D study of the concentration- and time-dependent interactions of human LL37 with model mammalian lipid bilayers

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ARTICLE INFO

Article history:
Received 17 October 2017
Received in revised form 5 March 2018
Accepted 4 April 2018
Available online 5 April 2018

Keywords:
Antimicrobial peptide
Cathelicidin LL37
Peptide-bilayer interactions
Quartz crystal microbalance with dissipation
Voigt-Kelvin Model

ABSTRACT

The human antimicrobial peptide LL37 is promising as an alternative to antibiotics due to its biophysical interactions with charged bacterial lipids. However, its clinical potential is limited due to its interactions with zwitterionic mammalian lipids leading to cytotoxicity. Mechanistic insight into the LL37 interactions with mammalian lipids may enable rational design of less toxic LL37-based therapeutics. To this end, we studied concentration- and time-dependent interactions of LL37 with zwitterionic model phosphatidylcholine (PC) bilayers with quartz crystal microbalance with dissipation (QCM-D). LL37 mass adsorption and PC bilayer viscoelasticity changes were monitored by measuring changes in frequency (Δf) and dissipation (ΔD), respectively. The Voigt-Kelvin viscoelastic model was applied to Δf and ΔD to study changes in bilayer thickness and density with LL37 concentration. At low concentrations (0.10–1.00 μM), LL37 adsorbed onto bilayers in a concentration-dependent manner. Further analyses of Δf, ΔD and thickness revealed that peptide saturation on the bilayers was a threshold for interactions observed above 2.00 μM, interactions that were rapid, multi-step, and reached equilibrium in a concentration- and time-dependent manner. Based on these data, we proposed a model of stable transmembrane pore formation at 2.00–10.0 μM, or transition from a primarily lipid to a primarily protein film with a transmembrane pore formation intermediate state at concentrations of LL37 > 10 μM. The concentration-dependent interactions between LL37 and PC bilayers correlated with the observed concentration-dependent biological activities of LL37 (antimicrobial, immunomodulatory and non-cytotoxic at 0.1–1.0 μM, hemolytic and some cytotoxicity at 2.0–13 μM and cytotoxic at >13 μM).

1. Introduction

Overcoming antimicrobial resistance presents a grand challenge in the healthcare industry, and requires the development of antibiotic alternatives [1]. Among current alternatives, antimicrobial peptides (AMPs) show significant therapeutic promise [2]. AMPs are short (10–50), cationic (+2 to +9), amphiphilic, and broad-spectrum antimicrobial proteins that are a part of the innate immune systems of many species [3], including humans [4]. Their diverse structures promote biophysical membrane interactions that are difficult for bacteria to develop resistance against [5].

LL37 (LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES) is the only human-derived cathelicidin (a class of mammalian AMPs [6]), and is of clinical interest because of its broad antimicrobial activity [7,8] and immunomodulatory properties [9–11]. These functions have been observed at different concentration ranges (Fig. S1):

- Immunomodulatory, 0.05–1.0 μM [9],
- Antimicrobial, 0.50–10 μM [7],
- Hemolytic and cytotoxic, 2.50–13 μM [12], and
- Cytotoxic threshold, >13 μM [13].

Unfortunately, LL37 is not as selective as other cathelicidins toward charged bacterial membranes over neutral (or zwitterionic) mammalian membranes [14,15]. Thus, improvement of its therapeutic ratio, defined as the ratio of antimicrobial concentration to cytotoxic concentration, is required for LL37 to achieve clinical utility. This will be enabled by an improved understanding of LL37 interaction mechanisms with zwitterionic lipids at different concentrations within its bioactive concentration range.

At low concentrations, LL37 interacts with zwitterionic membranes via electrostatic interactions facilitated by its charge (+6 at neutral pH [15]) and amphiphilicity (demonstrated by the separa-

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https://doi.org/10.1016/j.colsurfb.2018.04.016
0927-7765/

tion of its polar and non-polar residues in the peptide helix, Fig. S1). However, the exact mechanism of LL37-lipid interactions at hemolytic and cytotoxic concentrations are still debated. Proposed mechanisms include the carpet (detergent-like [14] and non-pore forming [16]) and toroidal pore [17,18] models. In the detergent-like carpet model, LL37 adsorbs to the bilayer surface, followed by insertion at a critical concentration [14]. At high concentrations, massive bilayer disruption occurs by the formation of peptide-lipid micellular aggregates that lift off the surface [19]. Sevcsik et al. suggested that disk-like micelles, 270 Å in diameter, formed after exposure to 4 mol% LL37 under certain experimental conditions, such as bilayers composed of lipids with shorter acyl chain lengths [20,21]. Porcelli et al. later suggested an alternative model due to the lack of evidence of peptide-lipid aggregates [16]. They proposed a non-pore carpet mechanism using nuclear magnetic resonance (NMR), in which concentration-dependent adsorption of LL37 in a parallel orientation occurred accumulatively, eventually causing a rigid transition of the bilayer into cubic phase prior to its full disruption [16,22,23]. Later studies using higher resolution NMR observed bilayer leakage, increased disorder, and induced positive curvature strain, and thus suggested that LL37 forms transmembrane pores [24,25]. The toroidal pore model has been proposed for LL37 [26,27], a specific model of transmembrane pore formation defined by increased bilayer disorder, leakage, and importantly, membrane thinning, leading to a critical concentration where toroid-shaped pores are formed [28].

Both the carpet and toroidal pore models are concentration-dependent, and include aspects that are time-dependent, such as removal of disk-like aggregates or formation of unstable transient pores over time; however, it is difficult to measure these properties in a cohesive, non-destructive way using a single technique. Recently, Shahmiri et al. demonstrated the usefulness of quartz crystal microbalance with dissipation (QCM-D) in studying the real-time interactions of LL37 (at concentrations of 1.00–20.0 μM) with different lipid compositions [29]. In the current study, we further elucidated the real-time interactions of LL37 with zwitterionic phosphatidylcholine (PC) lipid bilayers by exploring a wider range of LL37 concentrations representative of its different functions, and implemented viscoelastic modeling of system properties with the overall goal of understanding the mechanisms directing the various functions of LL37. This study contributes to a better understanding of the concentration-dependent interaction of LL37 with zwitterionic lipids as models for mammalian membranes. Results from this study will allow a clearer understanding of LL37 cytotoxicity and new insight into creating clinically relevant LL37 peptides as antimicrobial alternatives that will aid in solving the grand challenge of antibiotic resistance.

2. Materials and methods

2.1. Materials

Cathelicidin LL37 (LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES) (Anaspec, Fremont, CA) was synthesized at >95% purity, as confirmed by high-performance liquid chromatography, and stored according to manufacturer recommendations at −20 °C. Egg L-α-phosphatidylcholine (PC) lipids were purchased from Avanti Polar Lipids (Alabaster, AL) and were stored at 100 mg/mL in ethanol at −20 °C. Experiments were performed in filter-sterilized Tris buffer (100 mM sodium chloride and 10 mM tris(hydroxymethyl)aminomethane), corrected to pH 7.8, to allow for comparison to our previous work [30–32]. All other chemicals were purchased from Sigma Aldrich (St. Louis, MO).

2.2. Methods

2.2.1. PC vesicle preparation

Stock solutions (2.5 mg/mL) of small unilamellar vesicles of PC were prepared in batches as described elsewhere [30], and stored under N_2 at 4 °C. PC vesicles were stable for up to 1 month.

2.2.2. LL37-PC bilayer interactions monitored using QCM-D

Supported lipid bilayers (SLBs) were formed over piezoelectric SiO_2-coated quartz crystal sensors in QCM-D (Q-Sense E4, Biolin Scientific, Stockholm, Sweden), as described previously [30–32]. QCM-D is a real-time, acoustic method that measures changes in frequency (Δf) and energy dissipation (ΔD) of viscoelastic films deposited on the crystal sensors, along different overtones (multiples of the fundamental frequency, f_0 = 5 MHz). The Δf represents changes in mass of all deposited material and associated water molecules on the sensor surface; mass adsorption and mass loss are reflected by negative Δf and positive Δf, respectively. The ΔD is a measure of film viscoelasticity; higher ΔD represents more flexible and viscoelastic films while lower ΔD represents more rigid films. The overtones (1st, 3rd, 5th, 7th, 9th and 11th) indicate phenomena occurring at different penetration depths into the viscoelastic film [30,33]; high overtones suggest phenomena occurring closer to the sensor surface while lower overtones indicate phenomena occurring closer to the bulk solution.

All QCM-D experiments were performed as described previously [30–32], with a flow rate of 0.15 mL/min. Vesicles were diluted to 0.1 mg/mL in Tris and flown through QCM-D. SLBs spontaneously formed within minutes due to the bursting of the vesicles after reaching a critical coverage over the sensor surface, achieving a consistent equilibrium $\Delta f = -25$ Hz and $\Delta D \approx 0$. This process has been well-characterized [34–36]. The SLBs were rinsed with Tris for 10 min, exposed to 1.5 mL LL37 (concentrations from 0.01–15.0 μM in Tris), incubated for 1 h in a static condition to allow LL37-bilayer interactions, and rinsed with Tris again. A total of $n > 4$ replicates were collected for all experimental conditions.

Two variations in this experiment were studied. First, in order to differentiate Δf and ΔD responses specifically due to peptide-bilayer interactions, we compared the Δf and ΔD profiles of LL37 peptides physically adsorbed directly onto SiO_2 crystal sensors. In these experiments, 1.5 mL of LL37 (concentrations from 0.10–10.0 μM in Tris) were directly adsorbed onto SiO_2, followed by the 1-h static incubation and final Tris rinse as described above.

In the second type of experiment, we were interested in determining the composition of the peptide-bilayer film after finding unique LL37-bilayer interactions at high LL37 concentrations (e.g. whether it was removed or still remained on the crystal sensor). To do this, we utilized 0.1% (w/v) sodium dodecyl sulfate (SDS), which completely removes lipid bilayers from QCM-D crystal sensors [37]. In these experiments, SLBs were formed, exposed to 10.0 μM LL37 and incubated for 1 h. Then, the film was exposed to 1.5 mL SDS, and the resulting Δf and ΔD responses to SDS were compared with those of pure PC bilayers, pure films of physically adsorbed LL37 (10.0 μM), and bare SiO_2 crystal sensors.

2.2.3. Modeling viscoelastic film properties in QCM-D

2.2.3.1. QCM-D theory. The viscoelastic properties of the deposited film and associated water molecules (reflected by ΔD) influence the type of analyses that can be performed on QCM-D data. In the case of rigid systems, where $\Delta D \approx 0$ and the different overtones nearly overlap, then Δf is inversely proportional to mass adsorption and the Sauerbrey constant (C), according to the Sauerbrey equation [38] (Eq. (1)). The formation of PC bilayers falls within this applicable range [30,35]; thus, the equilibrium Δf for a bilayer, −25 Hz, corresponds to approximately 445 ng/cm^2 [32].