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# Sensing based on assessment of non-monotonous effect determined by target analyte: Case study on pore-forming compounds

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ABSTRACT

A new and exciting biosensing avenue based on assessment of the non-monotonous, concentration dependent effect of pore formation is discussed. A novel kinetic model is advanced to relate surface plasmon resonance (SPR) data with actual concentrations of interacting partners. Lipid modified L1 sensor chip provide the accessible platform for SPR exploration of peptide–membrane interaction, with POPC and melittin as model systems. We show that quantitative assessment of the interaction between an antimicrobial peptide and lipid modified sensors is capable to provide both sensing avenues and detailed mechanistic insights into effects of pore-forming compounds. The proposed model combined with appropriate design of the experimental protocol adds a new depth to the classic SPR investigation of peptide–lipid interaction offering a quantitative platform for detection, improved understanding of the manifold facets of the interaction and for supporting the controlled design of novel antimicrobial compounds. This biosensing approach can be applied to an entire set of pore-forming compounds including antimicrobial peptides and exo-toxins.



## 1. Introduction

Assessment of the complete action mechanism of pore-forming compounds on their interaction with (artificial) membranes is fundamental in understanding membrane processes with possible applications in disease diagnosis, toxicology and pharmaceutical research (Kass, 2005; Panchal et al., 2002) and represents an important issue, yet less investigated, in biosensing.

Pore formation and subsequent membrane destabilization is a common feature for the interaction process between a large number of compounds such as peptides, toxins and viruses with lipid membranes (Anderluh et al., 2003; Chah and Zare, 2008; Shai, 1999).

Nevertheless, the process has not been largely exploited for sensing purposes; in a recent report (Wilkop et al., 2008) the development of an electrochemical sensing platform for detecting trace amounts of bacterial toxins via the perforation process has been proposed.

Antimicrobial peptides belong to an important class of compounds with pore-forming capabilities. They offer an attractive solution to the problem of increasing resistance of bacteria to conventional antibiotics, based on direct interaction with membranes and subsequent lysis of the pathogen cell membrane. Despite the already documented efficient antimicrobial activity against a wide range of pathogens and viruses (Asthana et al., 2004; Dempsey, 1990), the potential cytotoxic activity against mammalian cells (Wessman et al., 2008), limits the direct use of these peptides as therapeutics.

Concerted efforts to modify the native antimicrobial peptides or design new peptides to achieve better specificity against microbial infections while limiting host organism cytotoxicity, add everyday new peptides to the ~800 natural antimicrobial peptides identified in eukaryotes (http://www.bbcm.units.it, Antimicrobial Sequences Database).

Elucidation of the complete interaction mechanism represents a key step in peptide design and in detection of new pore-forming compounds, requiring effective appraisal of these compounds and access to lipid platforms for quantitative assessment of the interaction kinetics.

Melittin, the main component of bee venom (Habermann and Jentsch, 1967), is often employed as model (pore-forming) compound in interaction studies with natural and artificial (biomimetic) membranes (Lundquist et al., 2008). It induces membrane disruption and lysis upon spontaneous binding to biological and model membranes (Frey and Tamm, 1991). The mode of action is dependent on the net charge of substrate: it forms transmembrane pores in zwitterionic lipid bilayer via barrel-stave mechanism (Vogel and Jahnig, 1986) and acts as a detergent in negatively charged membranes (Ladokhin and White, 2001). Similar with all pore-forming compounds, its effect occurs via complex, multiphase process

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and exhibits concentration dependency with distinct thresholds (Mozsolits et al., 2003, 2001).

Upon binding on the lipid membrane and reaching a threshold concentration, the insertion phase occurs leading to pore formation and subsequent phospholipid matrix destabilization and disintegration (Shai, 1999).

Each step of this model of interaction has been experimentally validated using different lipid membrane matrices: liposomes, supported lipid bilayers, micelles, phospholipid multilayers (Lundquist et al., 2008; Popplewell et al., 2005) and various methods of investigation: infrared spectroscopy (Frey and Tamm, 1991), fluorescence (Matsuzaki et al., 1997), transmission electron microscopy (Wessman et al., 2008), X-ray diffraction (Lee et al., 2008), circular dichroism (Zhu et al., 2007) and SPR (Mozsolits et al., 2001; Papo and Shai, 2003). As a corollary, this interaction depends on the lipid composition and charge, on the hydration level and on the peptide concentration, orientation on membrane surface and protonation state (Berneche et al., 1998; Lin and Baumgaertner, 2000). Yet, the dynamic assessment of the whole process has never been reported.

Using the advantages offered by surface plasmon resonance (SPR) technique, i.e. label free, real time monitoring of analyte–ligand interaction, we aim to reveal, for the first time, the entire process of interaction between melittin, as a model of a pore-forming compound, and a model (POPC) membrane as well as to highlight related biosensing capability.

Dynamic, quantitative assessment of the concentration dependent of non-monotonous processes associated with the target analyte (i.e. pore-forming compound) interaction with a lipid modified SPR platform is proposed as a novel biosensing approach. To this end, comprehensive SPR measurements on melittin binding to an artificial lipid membrane (POPC) using lipid modified L1 sensor chip (Biacore) are quantitatively analyzed. A numerical routine linking the SPR data with the actual concentrations of interacting partners via a novel kinetic model is developed, substantiating the quantitative parameters governing the interaction. We envisage that this approach is able to support accurate detection and related analysis platform, which could be further extended to membranes with different lipid compositions and other pore-forming compounds, as well.

## 2. Materials and methods

### 2.1. Materials

1-Palmitoil-2-oleyl-sn-glycero-3-phosphocholine (POPC) was purchased from Avanti Lipids (Alabaster, USA) while melittin from honey bee venom (89.4% purity), (3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), bovine serum albumin (BSA), 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), sodium chloride, sodium hydroxide, were purchased from Sigma–Aldrich (München, Germany). All chemical reagents were of analytical grade and were used without further purification. Ultrapure water (MilliQ) and chloroform were used as solvents. HEPES (10 mM Hepes, 150 mM NaCl, pH 7.4) was used as running buffer, 0.22 μm filtered and degassed prior to use.

### 2.2. Methods

Commercial L1 sensor chips and Biacore 3000 analytical system (Biacore AB, Uppsala, Sweden), at 25 ± 0.1 °C operating temperature, were used in all biosensor experiments. The L1 surface, displaying lipophilic anchors in a carboxymethyl dextrane matrix, is optimal for lipid vesicles capture and formation of stable lipid matrices. The sensor surface was prepared for lipid immobilization with two injections of CHAPS.

#### 2.2.1. Lipid vesicles preparation

Lipid vesicles were prepared by dissolving POPC (1.5 mM) in chloroform followed by drying under vacuum using a rotary-evaporator for 3 h. After hydration with running buffer and sonication (5 cycles, 30 min/cycle), the lipid suspension was extruded using the Mini-extruder (Avanti Lipids, Alabaster, USA) by 22–25 passages through a 1 μm-polycarbonate membranes. The extruded stock solution was stored at +4 °C prior to use; before each experiment the samples of different POPC vesicles concentrations were freshly prepared.

#### 2.2.2. SPR measurements

The experimental conditions for each step in the complete SPR experiment: sensor preparation, lipid immobilization, removal of loosely bound structures, melittin injection and sensor regeneration are as follows.

*2.2.2.1. Sensor chip preparation.* The normalized sensor chip L1 was treated with two short (1 min) injections of CHAPS at a flow rate of 20 μL/min (Biacore Handbook, 2003a,b).

*2.2.2.2. Formation of lipid matrix.* Samples of POPC vesicles (80 μL) with different lipid concentrations (0.01 mM, 0.05 mM, 0.15 mM and 0.5 mM) were applied to the sensor surface at a low flow rate of 2 μL/min. Three injections of NaOH (50 mM), each of 5 min, were applied at flow rate of 20 μL/min to remove loosely bound vesicles structures and to stabilize the baseline. The quality of the lipid matrix is indicated by baseline stability for more than two hours. To check the lipid coverage of the L1 chip, a pulse injection of BSA (0.5 mg/mL in HEPES, 10 μL/min) was performed (Papo and Shai, 2003).

The lipid matrix immobilized on the L1 chip was used as a model cell membrane for further investigation of the pore-forming peptide binding.

*2.2.2.3. Peptide binding.* Peptide stock solution (1 mM) was prepared by dissolving melittin in MilliQ water and stored in light protected vials at +4 °C. Melittin spiked samples, with concentrations between 0.35 and 3.62 μM, were freshly prepared before experiments by dilution in running buffer. Since preliminary tests with 7–30 min injection times showed incomplete interaction steps, a long injection, 65 min, was used to reveal the complete mechanism of peptide–lipid interaction. 5 μL/min flux was used to avoid mass transport limitations.

*2.2.2.4. Sensor regeneration.* Injections of CHAPS, 20 mM, were used for sensor regeneration, preparing the surface for another analysis cycle.

#### 2.2.3. Quantitative analysis of the SPR data

Classical SPR analysis relates the SPR sensorgram (i.e. time variation of the reflectance dip position, or SPR angle) to the quantity of interest, assuming one effective layer (characterized by an effective thickness $d_{eff,}$ and dielectric constant $\varepsilon_{eff}$) on top of the SPR chip.

In contrast, our approach relates the evolution of interacting compounds (based on a kinetic model) to the "evolving" layers on the chip. Via repeated application of the Fresnel equation (Reitz et al., 1993), i.e. the construction of a Transfer Matrix (Born and Wolf, 1980) we relate the SPR angle shift to the surface concentration of compounds in the multilayer system associated with the experimental platform. The effective thickness $d_i$ and dielectric constant $\varepsilon_i$ of each layer in the system shape the reflectivity spectrum and influence the SPR angle and are important parameters in the construction of the transfer matrix.

We have designed a home made fitting routine with the following land marks: