Chapter 9

Mass-Sensitive Biosensor Systems to Determine the Membrane Interaction of Analytes

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Abstract

Biosensors are devices that transform a biological interaction into a readout signal, which is evaluable for analytical purposes. The general strength of biosensor approaches is the avoidance of time-consuming and cost-intensive labeling procedures of the analytes. In this chapter, we give insight into a mass-sensitive surface-acoustic wave (SAW) biosensor, which represents an elegant and highly sensitive method to investigate binding events at a molecular level. The principle of SAW technology is based on the piezoelectric properties of the sensors, so as to binding events and their accompanied mass increase at the sensor surface are detectable by a change in the oscillation of the surface acoustic wave. In combination with model membranes, transferred to the sensor surface, the analytical value of SAW biosensors has strongly been increased and extended to different topics of biomedical investigations, including antibiotic research. The interaction with the bacterial membrane or certain target structures therein is the essential mode of action for various antibacterial compounds. Beside targeted interaction, an unspecific membrane binding or membrane insertion of drugs can contribute to the antibacterial activity by changing the lateral order of membrane constituents or by interfering with the membrane barrier function. Those pleiotropic effects are hardly to illustrate in the bacterial systems and need a detailed view at the in vitro level. Here, we illustrate the usefulness of a SAW biosensor in combination with model membranes to investigate the mode of membrane interaction of antibiotic active peptides. Using two different peptides we exemplary describe the interaction analysis in a two-step gain of information: (1) a binding intensity or affinity by analyzing the phase changes of oscillation, and (2) mode of membrane interaction, i.e., surface binding or internalization of the peptide by following the amplitude of oscillation.

Key words Biosensors, Surface acoustic wave (SAW), Model membranes

1 Introduction

Biosensors have attracted much attention during the last two decades in biosciences in light of their potential to obtain a label-free detection of biological recognition processes. Biosensors can be classified according to their principles to transform a biological event into detectable readouts, e.g., optical or electrical signals. The most established biosensor technique in biomedical research utilizes the optical phenomenon of surface plasmon resonance

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(SPR). These sensors are commonly used for kinetic analysis of versatile compound-target interaction [1]. Here, we use another type of mass-sensitive biosensor, namely surface acoustic wave (SAW) sensors. SAW sensors have been developed during the last years as powerful and promising systems for detecting various biological recognition events, e.g., protein-protein, protein-nucleic acid, or cell-virus interactions [2–7]. This technique is based on piezoelectric properties of quartz sensors. Applying an electrical field to gold coated ST-cut quartz crystal slides, a Love-shear wave is generated at a thin (5 μm) guiding layer directly deposited at the sensor surface [2]. Consequently, binding events can be detected by changes of the physical properties of the shear wave in two different ways: (1) attachment of components equivalent to an increased mass leads to an angular phase shift, and (2) the viscoelastic properties of the bound analytes were reflected by changes in the oscillation amplitude and thus, give insights into the mode of analyte attachment.

Biological membranes possess not only essential barrier functions to compartmentalize cellular and subcellular components, they are also crucial elements of cellular recognition, communication, or transport [8]. In the field of antibiotic research, bacterial membranes are the most important point of attack for antibiotics, (1) either indirectly by an unspecific attachment for initial contacts and subsequently internalization, (2) by affecting the barrier properties, or (3) directly by targeting certain membrane components to interfere with essential cellular activities, such as cell wall biosynthesis [9, 10].

However, in light of the complex nature of natural cell membranes, the simplification of bacterial membranes by the use of model membranes appears as a promising strategy. To combine model membrane approaches with the above-mentioned SAW biosensor technology, we have recently developed a drying and conservation technique of model membranes at SAW sensor chips [11]. This allows, e.g., for kinetic binding investigations of different components within a simulated membrane compartment [12].

Furthermore, a well-defined model membrane at the sensor surface appears as a suitable screening tool to investigate the intrinsic capacity for membrane interactions of various compounds. Referring to antibiotic agents, the intensity and probably the mechanisms of membrane interaction should be illustrated by those investigations as a helpful contribution to interpret the mode of action. This strategy is presented here using two linear peptide structures of comparable molecular weight (~300 Da) and a net negative charge at neutral pH, both of them have been examined as experimental antibiotic active components. We made both compounds anonymous referred as “Compound A” and “Compound B” to focus the view solely on the way of membrane interaction. The SAW sensor device, used here is a sam® 5 Blue, SAW

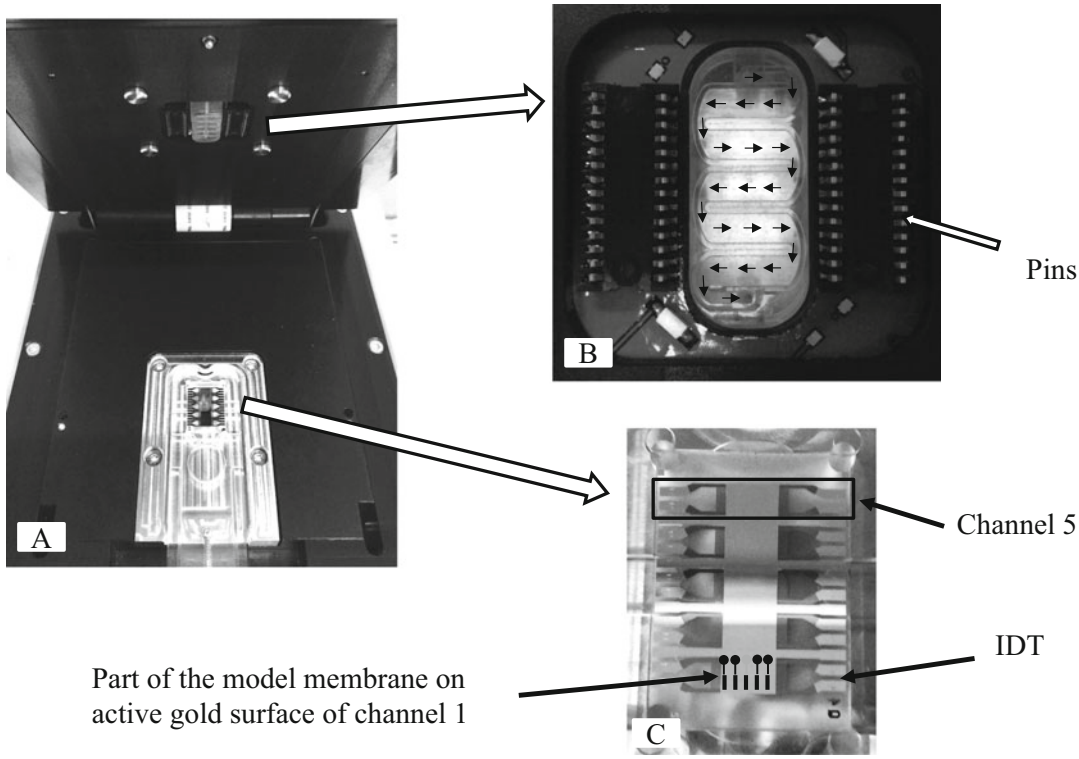

Fig. 1 (**a**) SAW-sensor chip mounted on the flow cell compartment of the sensor device (overview). (**b**) Detail of the flow cell with the electronic interface. Direction of the buffer flow is indicated by *arrows*. Due to the barriers of the flow cell, five compartments are formed. (**c**) Detail of the mounted chip and active surface in the center. During measurement, the flow cell is pressed on the sensor chip and their compartments establish five individual channels. Oscillation is initiated and the signals were collected with the help of interdigital transducers (IDT)

Instruments GmbH Bonn, now part of NanoTemper Technologies, Munich. The essential parts of the device, the sensor quartz, and the flow chamber with embedded sensor quartz are illustrated in Fig. 1.

2 Materials

2.1 Model Membrane Preparation

1. 1 mM 1,2-di-(9Z-octadecenoyl)-sn-glycero-3-phospho-(1′-RAC-glycerol) (sodium salt) (DOPG) (Avanti Polar Lipids Inc., Alabaster, AL, USA) stock solution in chloroform.
2. 1.66 μM D-(+)-trehalose dihydrate stock solution in ultrapure water.
3. 10 mM 1-hexadecanethiol dissolved in anhydrous chloroform.
4. 10 mM 1-hexadecanoyl-2-(9Z-octadecenoyl)-sn-glycero-3-phosphocholine (POPC) (Avanti Polar Lipids Inc., Alabaster, AL, USA) stock solution in chloroform.

5. Chloroform, anhydrous.
6. Compressed air.
7. Ethanol.
8. Langmuir-Blodgett trough (trough and damper made of Teflon®).
9. SAW-sensor quartzes (NanoTemper Technologies, Munich, Germany).
10. Teflon® tweezers.
11. Ultrapure water ("Type 1").

2.2 Running Buffer

1. 200 mM MOPS (3-morpholinopropanesulfonic acid) buffer grade, pH 7.0 (adjusted with 10 mM NaOH) (*see* **Note 1**).
2. Calcium chloride dihydrate.

2.3 Cleaning of Quartz Sensors

1. 35 % hydrogen peroxide.
2. Compressed air.
3. Conc. sulfuric acid.
4. Piranha solution (30 % hydrogen peroxide and concentrated sulfuric acid, ratio 1–3 as described in Subheading 3.2).

2.4 Membrane Interaction of Two Peptides Detected by Biosensor Measurement

1. 1 mM stock of antibiotic peptides in DMSO.
2. Dimethyl sulfoxide (DMSO), analytical grade.

3 Methods

3.1 Model Membrane Preparation (Fig. 2)

1. A new or cleaned (*see* **Note 2**) sensor quartz was put in a solution of 10 mM 1-hexadecanethiol in chloroform for at least 12 h. During this period, a self-assembled monolayer (SAM) is formed on the sensor quartz by a coupling reaction of the thiol groups to the gold surface. The extended lipophilic moieties represent the "inner monolayer" (Step 1) for the subsequently formed membrane bilayer that serves as a model of the biological membrane.
2. The quartz sensor was removed from the thiol solution after the given interval, rinsed with ethanol and dried with compressed air.
3. The second part of the lipid bilayer was established by the Langmuir-Blodgett technique [11, 13]. An illustration of this procedure is given in Fig. 2 (Steps 2–4). The teflon® trough is filled with ultrapure water and the lipid mixture is carefully

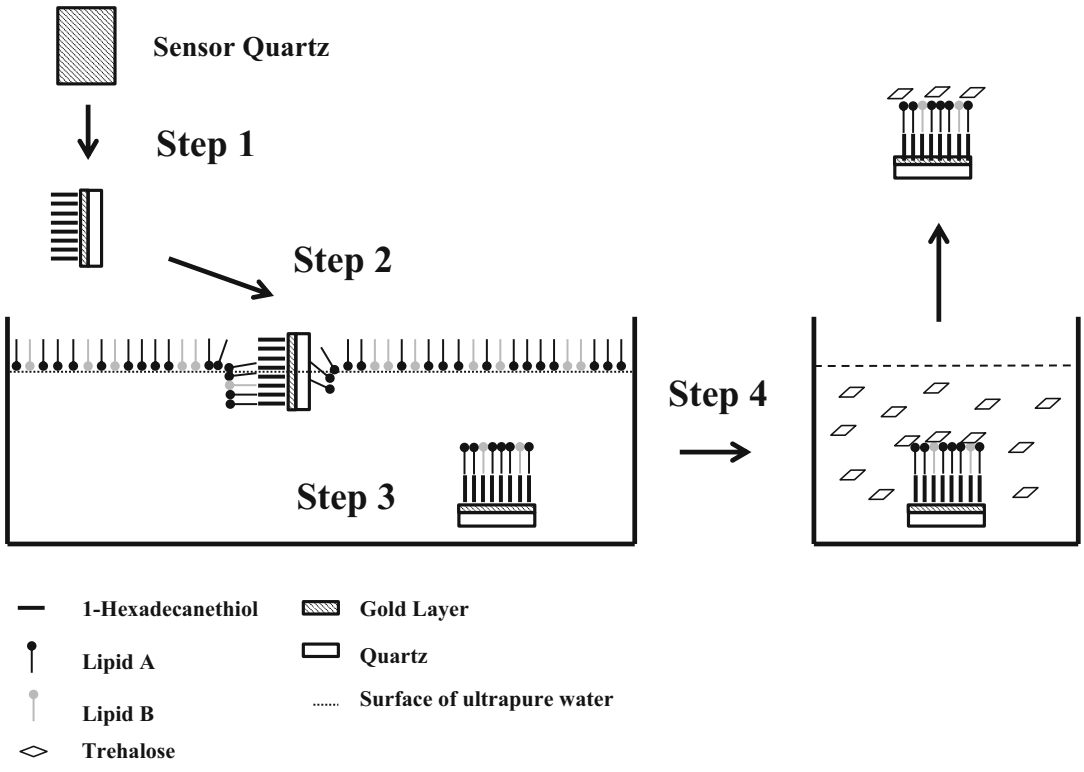

Fig. 2 Schematic illustration of the coating procedure to achieve an artificial lipid bilayer on the sensor surface. The membrane composition can be varied easily by changing the number and/or amount of individual lipids, leading to versatile available membrane functionalization

dropped (*see* **Note 3**) onto the surface. Depending on the composition of the lipid mixture, the characteristics of the membrane can be modified according to the analytical problem. Here, we applied a mixture of 5 µl POPC (10 mM in chloroform) and 5 µl of DOPG (1 mM in chloroform). For a randomly spreading of the lipids at the water surface they were kept for at least 10 min unaffected before the resulting lipid layer was laterally compressed by a teflon® damper until a value of 5 mN/m below the individual collapse pressure. The collapse pressure, a characteristic of the lipid mixture, has to be evaluated before performing the experiment. The procedure results in a lipid monolayer with clear orientation of lipophilic tail to the air and hydrophilic head to the aqueous compartment.

4. The sensor quartz (prepared as described in **1** and **2**) was dipped through the established monolayer (in **step 3**) resulting in completing the surface-supported lipid bilayer. The dipping procedure is done by a lifting device ensuring a very low speed not disrupting the monolayer on the water surface. The

lipid density of the surface is maintained through tracking the damper according to the difference of desired and actual pressure.

5. Figure 2 illustrates the formation of the lipid bilayer which depends in its intactness on an aqueous environment. For this reason the chip cannot easily be taken out of the water basin.
6. To avoid a disintegration of the bilayer, the sensor chip has to be kept under exclusion of air, primarily achieved by receiving the coated chip in a reservoir put into a cavity of the teflon® trough designated for this purpose before starting the coating procedure.
7. Superfluous lipids at the water surface were aspirated by a water-jet vacuum pump before the reservoir was taken out of the cavity. The reservoir contains the coated quartz and supernatant with some lipids that were collected when passing the water surface. The water is aspirated until only a small liquid portion protects the sensor surface from air.
8. To mount the sensor chip on the biosensor's contacts, one has to assure that the chip is dry and that the bilayer is not disrupted by air. Therefore, according to a protocol established by Reder-Christ et al. [11], the supernatant liquid is stepwise (to avoid dilution effects) exchanged by 1.66 μM trehalose. After 10 min equilibration in the pure trehalose solution, the sensors can be exposed to air and were wept with an additional amount of trehalose (1.66 μM) and dried overnight at 2–8 °C. Trehalose protects the bilayer from disruption when handled under exposure of air and enables a dry mounting on the instrument. Later, first amounts of running buffer will wash away the trehalose and the native model membrane solely remains and is ready for use in the binding experiment.

3.2 Cleaning of SAW-Sensor Quartzes

At the end of the experiment quartz sensors are demounted from the sensor and can be prepared for reuse by applying the following cleaning procedure. To surely detach all remnants of membranes or even proteins bound to the surface during the earlier measurement, harsh conditions have to be applied. Piranha solution was found to be a reliable agent for this purpose. Caution has to be taken while handling Piranha solution as it is very aggressive to tissues and even disrupting the gold surface (*see* **Note 4**) of the sensor quartz.

1. Piranha solution is always prepared fresh from 30% hydrogen peroxide and concentrated sulfuric acid in a ratio of 1–3 (caution hot!).
2. The sensor quartzes are covered (use a pasteur pipette!) with piranha solution (cooled down to room temperature) for reaction taking place 2 min.

3. The reaction is stopped by dipping (use teflon® tweezers!) the quartzes in a basin with ultrapure water. The quartzes were additionally rinsed with ultrapure water and dried with compressed air.
4. **Step 3** is repeated with the variation that after drying the quartzes, they are dipped short time in an acetone containing beaker, followed by dipping in ethanol. Then the quartzes are again dried by air stream.
5. Quartzes are covered another time with piranha solution, which is washed away after 2 min. They are rinsed with ultrapure water and dried by air stream before doing the same with ethanol.
6. Quartzes prepared that way can be stored in the refrigerator until use.

3.3 Membrane Interaction of Two Peptides Detected by Biosensor Measurement

Here, we describe the measurement of membrane interaction of two different peptides (Compounds A and B) with the biosensor. The membrane preparation according to the Langmuir-Blodgett technique [13] allows for a plenty of different assay conditions due to the major influence and easy variation of the lipid composition. In both formats we used a POPC membrane containing 10 mol% DOPG, and a 200 mM MOPS pH 7.0 flow buffer supplemented with 2.5 mM $CaCl_2$. During measurement, the membrane bearing sensor chip is embedded in the flow chamber compartment of the sensor device and rinsed by degassed (*see* **Note 5**) running buffer at a flow rate of 40 µl/min equilibrated at 22 °C (Fig. 1). The resulting sensorgrams, obtained for a single channel, are presented in Figs. 3 and 4.

1. A frequency spectrum for the individual quartz is automatically recorded for optimization of the operating frequency.
2. Before starting the measurement procedure one has to wait about 20 min until trehalose is completely washed away and the membrane is equilibrated with running buffer resulting in a stable baseline. With the start of the measurement the phase shift and the amplitude signals were recorded and can be observed in real time as a sensorgram.
3. The samples are prepared in microvials at the desired concentration (a volume of 200 µl is needed for each vial, of which 160 µl is injected). The injections were conducted by an autosampler and characteristics like injection volume and waiting time between different injections can be defined manually.
4. Here, we applied Compound A by injections with the buffer stream over the membrane in the following concentration series, beginning with the lowest: 0.075 µM, 0.1 µM, 0.25 µM, 0.5 µM, 0.75 µM, 1 µM, 2.5 µM, 5 µM, 7.5 µM, 10 µM. The same concentration series was prepared for Compound B (*see* **Note 6**).

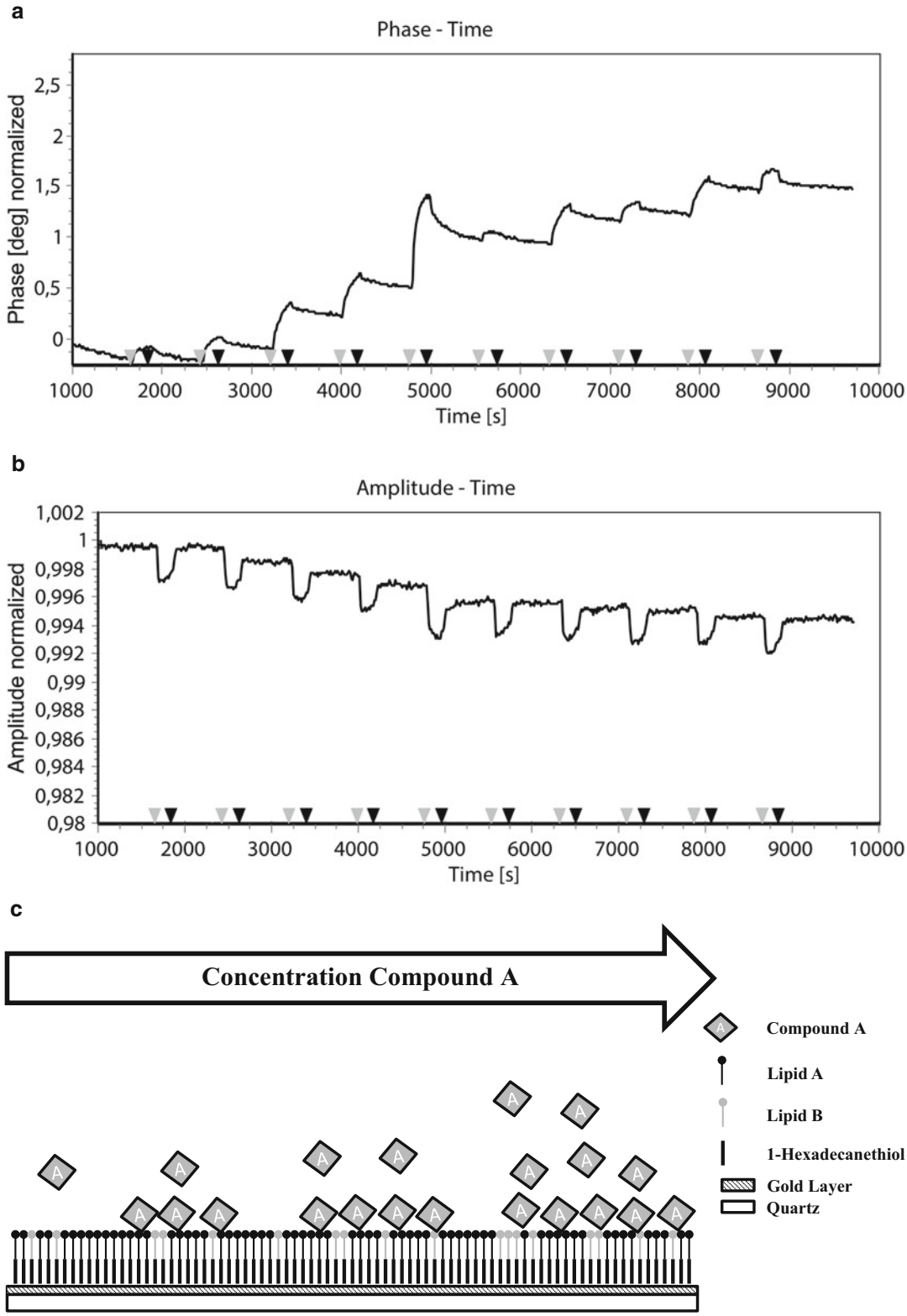

Fig. 3 (**a**) Sensorgram of the phase signal derived from membrane binding of Compound A. Binding to the membrane and corresponding deposition of mass on the sensor is accompanied by a shift in phase. Bound molecules were rarely washed away by flow buffer, indicated by the continuously increasing signal. (*Gray triangles*: Starting injection of an individual concentration of Compound A. *Black triangles*: End of injection.)

The above assigned concentrations were reached by dilution of 1 mM DMSO stock with running buffer instead of DMSO (*see* **Note 7**).

5. The injections (gray triangles in Figs. 3 and 4) start with the lowest concentration and a binding event can be identified by a phase shift establishing a higher baseline level, explained by a permanently bound mass. It has to be mentioned that this is not the case for most dynamic binding processes; where at the end of injection (black triangles in Figs. 3 and 4) dissociation of the attached analyte is the dominant process, leading to a decrease in phase shift.
6. The steeper the phase shift increases at an individual concentration, the more total mass is immobilized on the sensor surface respectively the model membrane. The reversible peaks at higher concentrations were mainly induced by viscosity changes as a result of, e.g., solvent residues and should not be considered for evaluation.
7. Considering the amplitude signals, viscosity changes occurring at the sensor surface can be monitored in real time. Binding of a flexible ligand to a pure sensor surface leads to a decrease in amplitude, while the rare event of increasing the amplitude would represent a rigidification, e.g., a membrane condensing effect.

A pure POPC membrane provides an uncharged surface to an analyte at pH 7.0. Since both Compounds A and B represent peptides with a net negative charge, we simulated the physiological conditions by incorporating a negative charge into the membrane by DOPG and supplementation of divalent cations (2.5 mM Ca^{2+}). This leads to an acceleration of the interaction with the model membrane at pH 7.0, driven by ionic interaction.

Comparing the sensorgrams of the phase signals obtained for Compound A (Fig. 3a) and Compound B (Fig. 4a), there is no major difference detectable. Both compounds are able to bind the membrane indicated by a step-wise increase in the phase shift, confirming a simultaneous mass deposition at the membrane. Considering the single steps, only small amounts of bound compound appear to be washed away, indicating a tight binding.

Fig. 3 (continued) (**b**) Sensorgram of the amplitude signal influenced by Compound A. The binding of Compound A to the membrane leads to an agglomerate that is more flexible than the pure membrane. These viscoelastic changes are recorded with the amplitude signal and the more viscous state is represented by an overall decrease. (*Gray triangles*: Starting injection of an individual concentration of Compound A. *Black triangles*: End of injection.) (**c**) Schematic illustration of the sensor surface during application of increasing concentrations of Compound A. The amount of Compound A deposited to the membrane increases, which is reflected in the increase in phase shift. Compound A only binds to the outer area of the model membrane and therefore the additional layer on the membrane allows for more flexibility of the whole material on the sensor surface. The more viscous properties are reflected in the decreasing amplitude

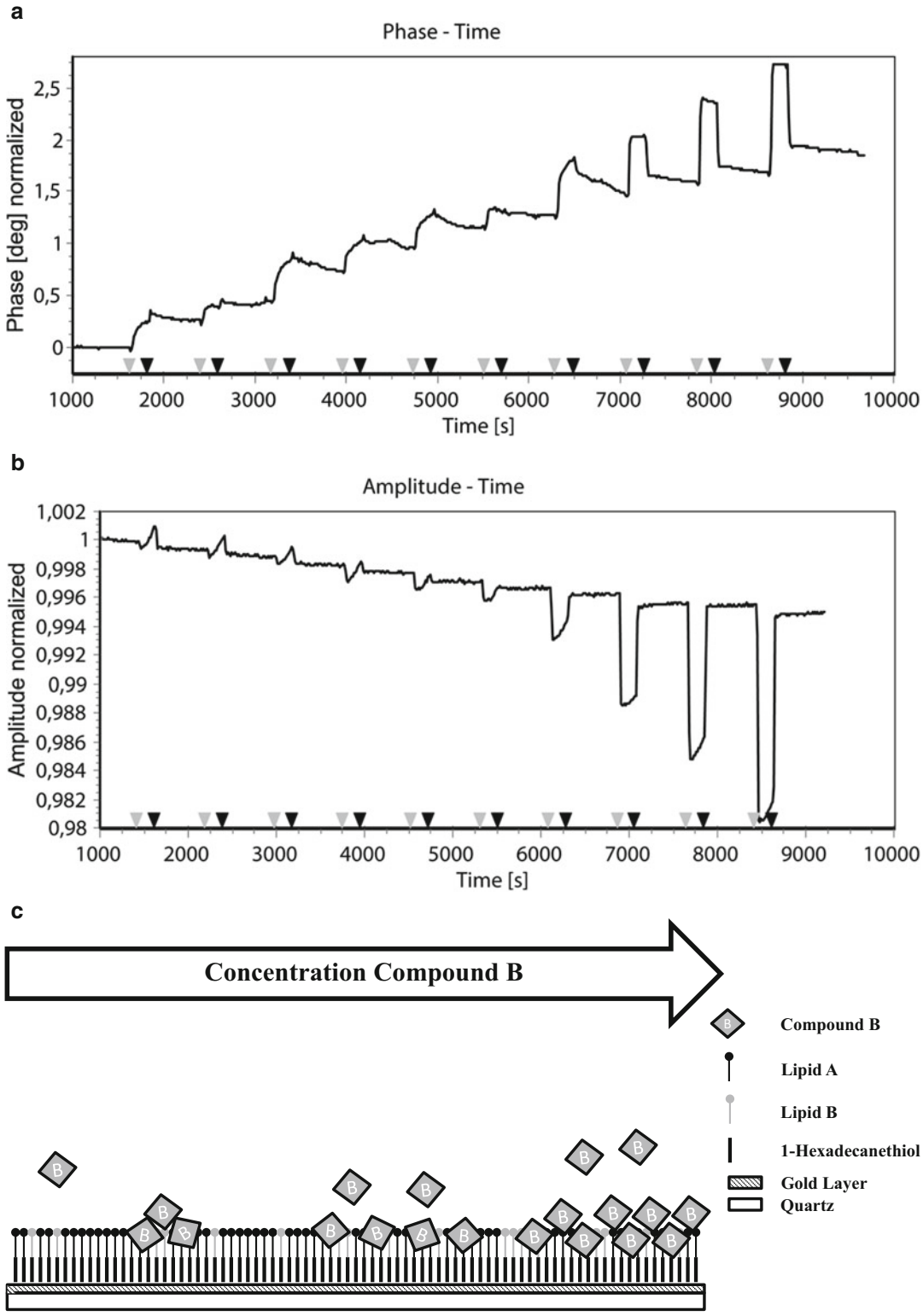

Fig. 4 (**a**) Sensorgram of the phase signal influenced by Compound B. In comparison to Compound A there is virtually no difference in the binding behavior of Compound B, regarding only the deposited mass. (*Gray triangles*: Starting injection of an individual concentration of Compound B. *Black triangles*: End of injection.) (**b**) Sensorgram of the amplitude signal influenced by Compound B. Regarding injections one to three, the amplitude

Furthermore, with increasing concentrations, the steps become smaller referring to an obvious saturation of the membrane interaction capacity (*see* also **Note 8**).

The amplitude signal allows deriving further information. Compound A (Fig. 3b) influences the amplitude in the expected manner as binding of the compound to the membrane surface leads to a decrease in amplitude, which results from a higher overall flexibility of the whole construct on the sensor surface. This molecular mode of action is depicted in Fig. 3c.

In case of Compound B the amplitude signal has a different appearance (Fig. 4b). During the first three injections of Compound B the amplitude increases. Increasing amplitudes can be observed if a modification turns the whole bound mass on the sensor surface more rigid. Starting with the fourth injection, a decrease in amplitude is evident which is similar to the shape resulting from Compound A. Figure 4c provides a schematic model for this behavior. The initially injected small amounts of Compound B are able to incorporate into the membrane and thus change the viscoelastic membrane properties to a more rigid nature. With the buffer flow adding more and more Compound B, the process is saturated and the molecules are thenceforward solely attached to the membrane surface.

In conclusion, these two examples of peptide interaction with model membranes illustrate that despite the obvious identical tendency for membrane binding, the SAW-biosensor approach is able to discriminate different modes of membrane interaction that justifies this technology as an appropriate tool for an initial and rapid characterization of compounds, like antibiotic drug candidates.

4 Notes

1. MOPS buffer was used instead of the common DPBS (Dulbecco's Phosphate-Buffered Saline) buffer based on optimization studies showing a better reproducibility of the binding data.

Fig. 4 (continued) signal increases upon application of Compound B to the model membrane. Afterwards the signal decreases and later shows a similar appearance as seen with Compound A. The initial increase of the amplitude indicates a more rigid nature of the construct on the sensor surface in response to compound application and is explained in Fig. 4c, (*Gray triangles*: Starting injection of an individual concentration of Compound B. *Black triangles*: End of injection.) (**c**) Schematic illustration of the sensor surface during application of increasing concentrations of Compound B. Compound B interacts besides an inherent binding ability and therefore overall mass deposition (Compound A) in an additional manner with the membrane. Compound B is able to integrate in the membrane and as a result changes their viscoelastic properties. At small concentrations (Injections 1–3) the integration leads to a more rigid construct and the monitored increasing amplitude signal. The ability of the membrane for integration of Compound B is limited and with increasing compound concentrations in the flow buffer, the mass attached to the surface predominates the viscoelastic properties, which leads in sum to a decrease in amplitude

2. The quality of the gold surface is directly related to the quality of the established model membrane, as it is true for the physical properties being reflected in the sensorgram. Therefore, special care has to be taken to an intact and homogenous gold film. Best results are obtained using new sensor quartzes, but the regeneration procedure with piranha solution can be executed 1–2 times without influencing data in an inappropriate manner.
3. The lipid mixture can easily be prepared by aspirating the indicated volume from the stock solution with a glass microsyringe (e.g., Hamilton®) and mixing with the syringe's piston. Avoid contact of the lipid containing chloroform solution with plastic parts and execute aspiration and mixing procedures with glass equipment.
4. If the quartz surface is obviously damaged (e.g., black pinholes or scratches) one should discard it.
5. Liquid serving as flow buffer should always be properly degassed because otherwise the chip surface (which is due to the membrane that in general is rather lipophilic!) tends to adsorb gas. The measurement is disturbed by these air-bubbles because the true active surface is reduced and also leads to falsified phase signal. If only one or two of the five channels of the sensor are disturbed by air, the remaining channels suffice to interpret the phase shift in the sensorgram. No significant influence on the buffer flow rate assumed as a precondition.
6. The concentration series being most suitable to be applied on the sensor has to be determined empirically in preliminary tests until binding occurs with appropriate signal intensities.
7. As the SAW technique is very sensitive to viscosity changes in the measurement medium, one has to take special care when performing dilution series. The ready diluted samples should be comprised to most possible extent of flow buffer to avoid artifacts in the sensorgram resulting from viscosity changes in the running buffer.
8. Regarding the phase changes in the original sensorgrams, indicating the bound mass, they seem not to follow a linear principle according to the concentration series. As this is not an uncommon finding, one should bear in mind that the concentration range in this kind of assay is very low and the detection principle is rather sensitive even to small deviations in sample preparation.

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