Chapter 35

Biosensor-Based Evaluation of Liposomal Binding Behavior

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Abstract

Biosensors can be regarded as analytical devices that transform biologically given facts, such as the appearance of physiological substrates, or biological recognition processes of ligands and receptors into detectable signals without the need of further labeling. This chapter introduces acoustic wave sensors as mass-sensitive tools to investigate the liposomal binding behavior onto simulated biological surfaces. These sensors do not only allow for quantification of the liposomal binding intensity, but further analytical readings give insight into the liposomal appearance at the binding site, e.g., deformation or fusion. Since the liposomal behavior at the target binding site might have strong impact on therapeutic effects, a prediction of liposomal appearance and a controlled modulation thereof appear possible with the help of biosensors.

Here, the function of a quartz crystal microbalance (QCM) and the bio-functionalization of quartz sensors are reported for a series of liposomal binding experiments. Liposomes containing biotin as model ligands were selected to evaluate their binding to avidin-modified sensors. The data, representing binding intensity and liposome deformation, are explained with respect to the role of binding strength and lipid composition for liposomal behavior.

Key words: Biosensors, Quartz crystal microbalance (QCM), Liposome binding, Fusion, Liposome deformation

1. Introduction

Successful liposomal binding and accumulation at the target site is the basis for therapeutic targeting approaches. However, therapeutic effects will result finally from the mode of liposomal interaction with the target cells. With respect to the liposomal drug load or the pharmacological approach, it might be anticipated that liposomes will either be taken up by cells for generating intracellular effects, or stay at the target cells and disrupt the release of the drug load locally outside the target cells. Both options will

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overlap in practice and can partly be directed by cellular epitopes, such as targeting internalizing receptors, or by the liposomal composition. Although the prediction of the liposome–cell interactions would greatly increase therapeutic benefit, most analytical techniques can neither detect the liposome–cell interactions directly in the physiological situation nor in adequate model systems.

The development of biosensor analytics during the last decade and successful biosensor application in a wide variety of analytical approaches might be an option to get further insight into the liposomal binding characteristics. Biosensors are analytical devices of different analytical principles, design, and application, which allow label-free detection of biological substrates or processes by transforming the information into detectable readouts. Biosensors can be classified according to the mode of signal transduction.

An early established and the most popular biosensor in biosciences is an optical sensors using surface plasmon resonance (SPR) based technique (1, 2), known as BIAcore systems.

We refer to acoustic wave sensors that are based on piezoelectric properties of the sensor materials, in most cases quartz crystals. It was in 1959 when Sauerbrey showed the mathematical correlation that the shift in resonance frequency of a thickness-shear mode resonator is proportional to the mass load on its surface (3), which given in modified form in Eq. (1), where Δf is the change in frequency, f_0 the oscillation frequency, A the sensing area, ρ_q the density of the resonator, μ_q the shear-modulus, and Δm the bound mass on the surface.

$$\Delta f = -\frac{2 f_0^2}{A\sqrt{\mu_q \rho_q}} \Delta m = -C_f \Delta m \qquad (1)$$

This was the basis for the development of piezoelectric, mass-sensitive devices (4). Besides surface acoustic wave (SAW) sensors (5), quartz crystal microbalance (QCM) is the most popular piezoelectric sensor (6). In principle, an electrical excitation of a thin quartz sensor results in an oscillation of the resonance frequency (most commonly between 5 and 10 MHz), which indirectly correlates with the mass load on its surface. Consequently, changes in mass can be detected in real time by monitoring frequency shifts. To apply the QCM as a biosensor, the quartz electrodes have to be functionalized with the relevant bio-component. QCM has successfully been applied in a wide spectrum of analytical approaches, such as polymer surface characterization, ligand–receptor recognitions, protein adsorption onto membranes, or DNA/RNA interaction with complementary strands.

Biosensors have also been applied in the field of liposomes. In most cases, liposome adhesion and the formation of supported

planar bilayers have been studied extensively as a model for cellular membranes in biophysical studies and biomedical application. In combination with other techniques, spreading or vesicle fusion onto different surface materials was investigated (7) and interpreted with respect to the lipid species (8). The binding of biotinylated liposomes to surface bound avidin was investigated under static conditions, and the liposomal deformation was confirmed finally by atomic force microscopy (AFM) (9).

Several studies have used the liposomal presentation of ligands to amplify the ligand binding signals onto the immobilized receptors at the QCM sensors (10, 11), and others immobilized intact liposomes at the quartz surface to investigate release kinetics (12) or protein–liposome interactions (13).

Besides a quantification of liposome binding by detecting the frequency shifts, QCM devices can have further analytical readings, namely monitoring of the dissipation energy (QCM-D) or alternatively damping analysis (Fig. 1). Both provide information on the flexibility of surface-bound layers. These parameters are most useful when given as a ratio with the frequency drop (*D/f* ratio). Thus, differences between intact (higher damping) or flattened liposomes (lower damping) can be detected. On a biophysical background, liposome fusion onto different materials was investigated with respect to liposomal size and concentration (14, 15). Patel and Frank illustrated rapid formation of dense surface bilayers using osmotically stressed vesicles in a QCM dissipation study (16).

In a recent report (17), a QCM damping study was performed to investigate the liposomal behavior under simulated target binding conditions. It was probed whether the liposomal behavior at the target site is predictable. Liposomes that differed

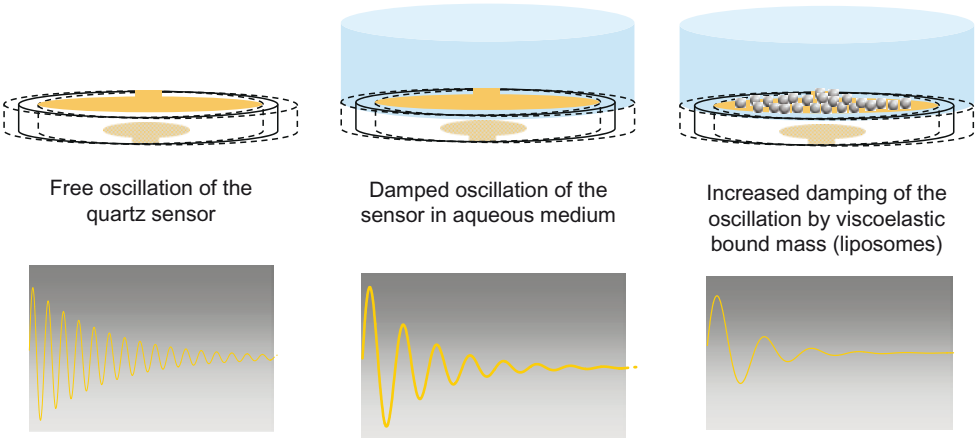

Fig. 1. Schematic illustration of oscillating quartz sensors and the damping increase by viscoelastic masses at the sensor surface (*upper cartoons*), and the corresponding oscillation amplitudes (*lower cartoons*)

in lipid composition, amount, and affinity of the coupled homing device were analyzed with respect to binding intensity and deformation onto immobilized target molecules. Damping analysis, e.g., the *D–f* plot, appeared as meaningful parameter. Biotinylated liposomes deformed upon binding to avidin and the deformation correlated with biotin concentration. Liposomal deformation can trigger fusion and content release when liposomes contain dominant fractions of unsaturated phosphatidylethanolamine (PE). In contrast, PEGylated liposomes displayed high damping, indicating conformational stability. It was concluded that liposome behavior can be controlled by certain parameters and be predicted with therapeutic relevance.

This chapter refers to the QCM device used in (17). The following instructions assume the application of a QCM biosensor with damping analysis, such as a Liqui Lab21 model (*ifak* e.V. Barleben, Germany), to investigate the liposomal binding under simulated shear force conditions given in a flow chamber system, as illustrated in Fig. 2.

Although the principle of measurement appears to be comparable to similar QCM devices, the absolute changes in frequency and damping frequency are related to this model and may deviate in quantity and dimensions using other QCM biosensors.

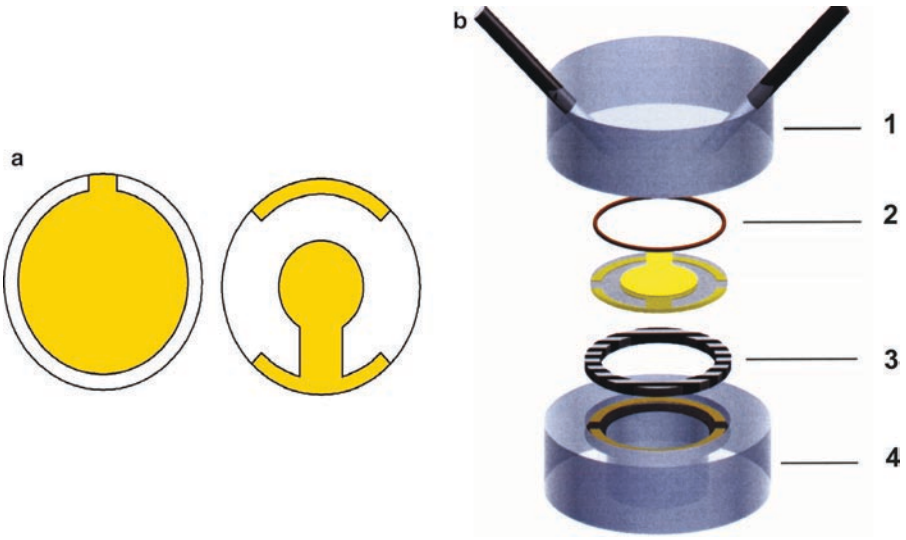

Fig. 2. (**a**) Schematic illustration of the gold-covered quartz sensors (diameter 14 mm, the resulting sensitive area A 0.282 cm^2, f_0 10 MHz, μ_q the shear-modulus 2.947×10^{11} g/cm s^2, density ρ_q 2.648 g/cm^3. According to Eq. (1), these parameters result in a constant C_f of 1.24 ng/Hz. (**b**) The quartz sensor is integrated into the schematically depicted flow chamber, where 1 is top of chamber with tube connections for medium flow, 2 is a rubber gasket, 3 is a conductive gasket and 4 the bottom with contacts to the oscillator. The chamber has a volume of about 100 μL

2. Materials

2.1. Liposome Preparation

The following lipids were dissolved as 10 mM chloroform (Riedel de-Haen, Seelze, Germany) stem solutions, kept at –20°C, and equilibrated to room temperature for pipetting and use.

1. Soy phosphatidylcholine (SPC) from Lipoid AG Ludwigshafen (Germany).
2. Methoxy-polyethyleneglycol-phosphatidylethanolamine (PEG-PE 2000) from Avanti Polar Lipids (Alabaster, AL, USA).
3. *N*-biotinyl-dipalmitoyl-phosphatidylethanolamine (Avanti Polar Lipids).
4. *N*-[biotinyl(polyethylenglycol)-2000]-dipalmitoyl-phosphatidylethanolamine (Avanti Polar Lipids).
5. Phosphate buffer (150 mM), pH 7.4 prepared according to standard protocols and kept at 7°C until use.

2.2. Biosensor Activation

1. 6-Mercaptohexan-1-ol (Fluka, Neu-Ulm, Germany) was dissolved in chloroform to obtain a 1 mM solution, which was kept at –20°C before use.
2. Cyanuric chloride (Sigma, Deisenhofen, Germany) was kept dry at –20°C before use and freshly dissolved in chloroform at 1% (mass/volume).
3. Borate buffer (10 mM), pH 8.8 was prepared according to standard protocols and kept at 7°C until use.
4. Avidin (Sigma, Deisenhofen) was freshly dissolved in borate buffer at 0.2 mg/mL for immediate use.

3. Methods

The following instructions refer to the investigation of large unilamellar vesicles (LUVs) (diameter about 100 nm), PEGylated or non-PEGylated with different concentrations of biotin–PE or biotin–PEG–PE as ligand for binding sensor-immobilized avidin. Taking biotin–avidin interaction as model for ligand–receptor recognition, the impact of binding affinity and ligand concentration on liposome binding intensity and liposomal deformation should be evaluated. Detailed results can be found in (17). In principle, these instructions can be transferred to other liposomal targeting approaches, e.g., immunoliposomes, taking the immobilization of the corresponding biological recognition structures as prerequisite.

3.1. Liposome Preparation

1. Suitable aliquots of the chloroform lipid stem solutions (10 μmol) were pipetted into a 25-mL round bottom flask.
2. Chloroform was removed by a rotary evaporator for 30 min in a 35°C water bath, followed by drying the resulting lipid film for 16 h in a vacuum exsciccator.
3. The lipid film was hydrated by adding 1 mL phosphate buffered saline (PBS, pH 7.4) to obtain 10 mM multilamellar liposome vesicle (MLV) dispersion after vortexing for about 10 min, followed by mechanical shaking for about 16 h.
4. Unilamellar liposomes were prepared with a mini-extruder (Avanti Polar Lipids) from multilamellar vesicles extruded 19 times through a 200-nm polycarbonate membrane, 19 times through a 100-nm polycarbonate membrane, and 10 times through a 50-nm polycarbonate membrane (Whatman Nuclepore 18 mm polycarbonate membrane, Richmond, USA).
5. The liposome size has to be controlled by photon correlation spectroscopy (PCS).

3.2. Activation of the Sensor Surface

1. For preparing the cleaning solution of the sensors (piranha solution), conc. H_2O_2 and conc. H_2SO_4 were mixed at a volume ratio of 1:3 in a cooling water bath (see Note 1).
2. When the mixture is cooled down to room temperature (RT), quartz sensors can be dipped in the solution with the help of a pair of tweezers (or similar) for about 10 s followed by rinsing the sensor with demineralized water.
3. The cleaning procedure is repeated six times to remove any remaining bound residues at the gold surface.
4. The sensor has to be rinsed with absolute ethanol and dried under a stream of nitrogen.
5. The cleaned quartz sensors were immersed into a 1 mM chloroform solution of 6-mercaptohexan-1-ol for about 12 h at RT. Due to the thiol–gold reaction, a kind of monolayer is formed with terminal hydroxyl groups.
6. After rinsing the quartz crystals briefly with ethanol and drying them under a stream of nitrogen, the sensors were put into a chloroform solution of cyanuric chloride (see Note 2) for 2 h at RT, followed by ethanol washing and drying. Cyanuric chloride is a reactive linker with three binding sites (with successive reactivity). Attached to the mercaptohexanol, it is able to react with nucleophils, resulting in protein immobilization. Reactivity can be obtained in aqueous media under mild basic conditions.
7. A solution of 20 μL avidin (1 mg/mL in 10 mM borate buffer, pH 8.8) was placed on the upper gold surface of the quartz

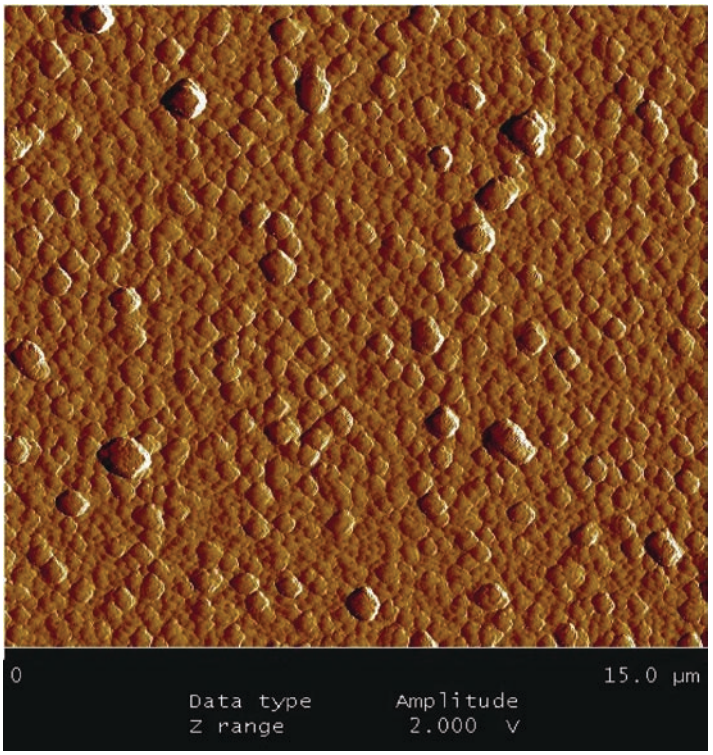

Fig. 3. AFM image of an avidin-coated quartz sensor. Avidin was coupled to 6-mercaptohexan-1-ol via cyanuric chloride; dimension of the image is 15 μm

sensors. After 2 h at RT, the quartz crystals were rinsed once again with demineralized water and dried under a stream of nitrogen. The resulting dense layer of immobilized avidin is illustrated in Fig. 3, as seen by AFM.

8. To control binding specificity, the coupling step in 7 can be performed with a bovine serum albumin (BSA) solution of identical concentration.

3.3. Biosensor Measurements of the Liposomal Binding Behavior

1. The functionalized biosensor is inserted into the flow chamber system as illustrated in Fig. 2, taking care of sealing the chamber by proper screwing down.
2. The flow chamber is connected to the oscillator by pins and attached with tubes to a peristaltic pump (via other flow chambers, since up to four flow chambers can be detected simultaneously) (Fig. 4). Tubes can be arranged to allow a closed circuit flow system or (as used here) flow from a buffer reservoir for a single liposome passage along the sensors.
3. The transparent cover of the biosensor has to be closed to allow thermostatic conditions (see Note 3). A constant flow of PBS (volume flow rate of 270 μL/min) is started to equilibrate the quartz crystals under flow conditions, and the

Fig. 4. The QCM biosensor device used for this study. Four flow chamber systems, each connected to an oscillator, are combined within a peristaltic pump flow system and inserted into a thermostatic device. Data were collected and evaluated by a computerized system

frequency is monitored in the corresponding computerized system.

4. When equilibration has occurred, and no trend in frequency is evident, liposomal solution can be introduced into the flowing medium by a three-way valve, resulting in a final liposomal concentration of 0.5 mM (see Note 4).
5. Liposome binding to the quartz surface is evident by a significant drop in frequency, while the damping frequency is increased. The binding of the biotinylated liposomes reaches saturation within 4 min. The data are monitored in real time (Fig. 5) and stored for subsequent evaluation.
6. For comparison of the binding quantity, the maximum frequency drop can be used and calculated in bound mass by Eq. (1). Insight into the liposomal binding behavior can be obtained by analyzing damping changes. Whereas intact liposomes induce a higher damping by viscoelasticity (Fig. 5a),

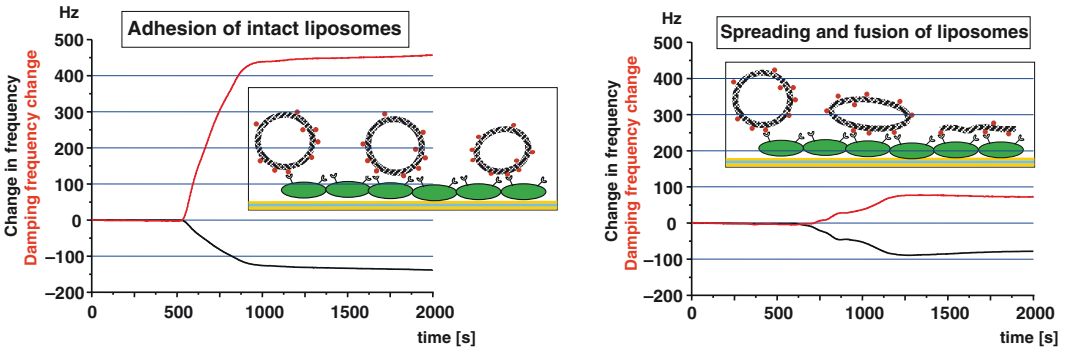

Fig. 5. Schematic illustration of a frequency and damping frequency course of liposome binding onto the quartz sensor surface. Although liposome binding might induce a similar drop in frequency in (**a**) and (**b**), indicating similar mass increase at the sensor surface, the damping change refers to the binding of intact liposomes in (**a**) or the deformation and flattening in (**b**)

flattening and fusion of liposomes is evident by lower damping (Fig. 5b).

7. The data obtained with the QCM device described here can be used for the evaluation of the liposomal appearance. *D/f* ratios of 4–3 indicate nondeformed or less deformed, e.g., PEGylated, liposomes and further decrease in the *D/f* ratio indicates stronger deformation, while *D/f* ratios of 1 and lower refer to fused liposomes and bilayer formation. In case of the biotinylated liposomes, liposomes with up to 5 mol% biotin–PE bind with low deformation, higher biotin–PE concentrations (up to 20 mol%) induce much stronger flattening, while 30 mol% induced liposomal disruption and bilayer formation (17). Liposomes with biotin coupled onto the PEG terminus tend also to be deformed.

4. Notes

1. When preparing the cleaning solution (Subheading 3.2, step 1), take proper care when mixing the reagents, because the solution is very corrosive and gets hot and has to be cooled. Do not clean the sensors in the hot solution. The cleaning of the sensor surface is often accompanied with the appearance of small pinholes. Long-term contact with the sensors can destroy the gold electrodes.
2. For successful biosensor activation (Subheading 3.2, step 5.), the use of dried chloroform is essential, since saturation of chloroform with air humidity might impede the coupling reaction of cyanuric chloride. A protocol for drying chloroform can be taken from chemical textbooks. For example,

phosphorus pentoxide is added to commercial chloroform for about 12 h followed by a distillation procedure. The freshly distilled chloroform should be kept in closely stoppered flasks.

3. Oscillation frequency is highly sensitive to temperature changes within the QCM device or the flow chambers. Although the QCM device is equipped with a thermostatic system, direct sunlight, airflow or non-thermostatic solutions can impede the detection.
4. When inserting the liposomal dispersion into the flow medium, air bubbles have to be avoided.

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