Chapter 11

Using a Quartz Crystal Microbalance Biosensor for the Study of Metastasis Markers on Intact Cells

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Abstract

The use of biosensors has become a standard method to characterize biomolecular interactions. Data obtained from biosensor studies are widely used to evaluate drug candidates, particularly in relation to their binding properties towards a selected target. The importance of measuring such interactions in a biologically relevant environment has become the new challenge for the biosensor technologies. In this chapter we describe a method for studying interactions between proteins and targets at the surface of intact cells using a quartz crystal microbalance (QCM) biosensor.

Key words: QCM biosensor, Lectins, Intact cells, Cell immobilization, Interaction rate constants, Kinetics

1. Introduction

1.1. Biosensors

The analysis of biomolecular interactions to determine the kinetics and affinity of such interactions has become essential for the development of new pharmaceutical substances. A biosensor is defined as a device that enables the measurement of interactions between one binding partner (ligand) immobilized on the surface of a transducer and a second binding partner in solution (analyte). The essential aspect of the biosensor, the transducer, senses a change in the mass associated with the interaction of interest and converts this physical modification into a measureable electric signal. Various transducers may be used and are of two main types, optical and acoustic. The quartz crystal microbalance (QCM) system is the most widespread acoustic transducer. Biomolecular interactions, especially protein–protein, protein–carbohydrate, and protein–DNA binding events are involved in key aspects of development

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and metabolism and have been implicated in cancer metastasis. Continuous flow biosensors provide a valuable insight into the mechanism of a specific interaction by permitting the determination of the kinetic parameters; including the association rate constant (k_a), the dissociation rate constant (k_d), and the affinity of the interaction. Several other techniques, including isothermal titration calorimetry (ITC), radioimmunoassay (RIA), or enzyme linked immunosorbent assay (ELISA), may also be used to determine the affinity of a given interaction. However, these approaches determine the affinity by equilibrium analysis using reporter labels, whereas biosensors provide label-free and kinetic real-time analysis of biomolecular interactions and provide more information and enable further understanding of the molecular interactions.

There are several biosensors available on the market for studies of biomolecular interactions. These are based on either optical detection principles, for example the Biacore (GE Healthcare), ProteOn (Biorad), Octet (Fortebio) systems, or acoustic devices based on the quartz crystal microbalance technology (QCM) for instance Q sense E4 (Qsense) or Attana 200 (Attana AB). In this chapter we will focus on the use of a QCM biosensor for studies of molecular interactions at the surface of intact cells.

QCM technology is based on the piezoelectric property of quartz crystals. The piezoelectric effect was first discovered in quartz crystals by Jacques and Pierre Curie in 1880 (1). By applying an alternating voltage to an AT-cut crystal briefly, with a frequency close to the resonance frequency of the crystal, the crystal will start to oscillate at its specific resonance frequency. The key feature of the QCM technology was discovered by Sauerbrey (2) who established a proportional relationship between a change in frequency with mass added to the crystal surface.

QCM biosensors act as very sensitive balances where the mass added to, or removed from, the sensor surface (corresponding respectively to the association and dissociation of the binding partners) is monitored in real time, providing information about the kinetic rate constants, as well as the affinity that characterizes a specific interaction. Analysis using a biosensor system may provide insights into the mechanism of the interaction between binding partners.

1.2. Cell Analysis Using a QCM Biosensor

The QCM biosensor has mainly been used to study bimolecular interactions, where one partner (ligand) is immobilized on the crystal surface using covalent amine coupling, physisorption, or capturing on streptavidin-coated crystals (if the ligand is biotinylated). The second partner (the analyte) is then injected over the sensor surface and the interaction is monitored in real time by measuring the change in resonance frequency of the quartz crystal. The main applications include: off-rate screening, affinity measurement, kinetics, active concentration measurement, binding assays as well as epitope mapping.

Biosensors based on QCM technology have proven to be powerful and efficient tools for studies of molecular interactions, mainly antigen–antibody interactions (3), and also lectin–carbohydrate recognition (4). Almost all biosensor studies are performed using isolated and purified molecules. However, it has become essential for the development of new therapeutics to provide a tool for screening and characterization of drug candidates in a more biologically relevant environment, for example, at the surface of intact cells. Previous work using QCM biosensors and mammalian cells have been focused on monitoring morphological changes in response to drugs in static systems (5). More recently, a QCM biosensor was developed for studies of real-time biomolecular interactions at the cell surface. The applications include lectin–carbohydrate and antibody–receptor interactions. This approach enables kinetic evaluation of the interactions between lectins and glycoproteins, or antibodies and over-expressed receptors, in a biological context. This flow-through system provides access to real-time measurement of affinity constants in a biologically relevant context.

In this chapter we will provide a guide for generating real-time, qualitative, and quantitative analysis of biomolecular interactions at the surface of cancer cells.

2. Materials

2.1. Equipment

1. Attana Cell 200 QCM biosensor (Attana AB).
2. Upright fluorescence microscope.
3. Cell culture compatible polystyrene-coated quartz crystal sensor chips (Attana AB, product number: 3621-3033).
4. Cell culture equipment and plasticware.
5. Cell counting device (Hemocytometer).

2.2. Reagents

1. Trypsin/EDTA 0.25% v/v (store at –20°C). Heat the solution to 37°C before use.
2. Phosphate buffered saline (PBS). Dissolve in distilled water and check that the pH is 7.2. Autoclave for 20 min at 121°C.
3. DAPI-dihydrochloride nucleic acid stain (stock solution: 14.3 μM in PBS), stored at 4°C in an opaque vial.
4. Cell culture medium appropriate for the cell type used.
5. Foetal calf serum (FCS).
6. Formaldehyde solution, 3.7% v/v in PBS.
7. 70% v/v ethanol or industrial methylated spirits (IMS).

3. Methods

3.1. Preparation of the Cell-Sensor Surface

Different sensor surfaces may be used for cell immobilization these include gold or polystyrene. Typically, cell culture compatible polystyrene-coated crystals are used. Briefly, the whole quartz crystal sensor is incubated with an appropriate cell suspension for about 24 h, in order to allow the cells to attach, grow, and proliferate on the surface. The concentration of cells used may vary but it is normally adequate if about 60–80% of the sensor surface is covered. After about 24 h the cells are chemically fixed on the sensor crystals washed and subsequently placed in a flow of running buffer in the QCM biosensor. After a period of stabilization of up to several hours (typically overnight), interactions of biomolecules such as antibodies or lectin with the cells can then be evaluated.

In order to generate high-quality data, the preparation of a suitable cell-covered sensor surface is essential. For this system adherent cells that grow as monolayers are typically used. The following steps provide detailed guidelines to allow the preparation of such surfaces using the MCF7 breast cancer cell line but adjustments might be necessary depending on the cell type used and/ or the type of experiment to be performed.

1. Remove cell medium from cultivation flask (we typically use a 75-cm^2 flask).
2. Add an appropriate amount of trypsin/EDTA (for example, 2.5 ml trypsin/ 0.25% w/v EDTA) and incubate at 37°C until cells have started to lift from dish if needed.
3. Add sufficient cell medium to the detached cells to re-suspend them. This step neutralizes the trypsin activity.
4. Centrifuge the cells to pellet them (5 min at 1,500 × *g*) and re-suspend the cells in fresh medium. A cell count should now be performed.
5. Adjust the cell concentration to 10^6 cells/ml.
6. A cell culture compatible polystyrene-coated quartz crystal sensor is placed on the bottom of the well of a 24-well plate.
7. Prepare an appropriate dilution of the cell suspension to obtain between 20,000 and 70,000 cells (depending on experiment) on the sensor surface. This represents between 125,000 and 450,000 cells/cm^2. In this example, 250,000 breast cancer cells/cm^2 were incubated with the sensor crystal in a 24-well plate and then placed in a tissue culture incubator at 37°C for 24 h. The duration of this phase of the experiment can be adjusted according to the growth and proliferative properties of the cells to be studied.
8. After incubation, the cells are chemically fixed to the surface using standard fixation procedures. As an example, 10 min

incubation in 1 ml of ice cold formaldehyde solution, 3.7% v/v in PBS, may be used. After the fixation step several washes in PBS must be performed in order to remove the formaldehyde solution (typically we use five washes each 1 ml of PBS). The cell-coated crystal may then be stored in PBS at 4°C for several weeks.

9. In order to evaluate the cells on the sensor surface, the nuclei of fixed cells are stained using, for example, the fluorescent nucleic acid dye DAPI hydrochloride (2.9 μM) which is directly loaded onto the sensor surface in 50 μl volume and left to incubate for 5 min in the dark. After removal of the DAPI solution, wash three times with PBS wash. Visualize the sensor surface using an upright fluorescence microscope.
10. If the cell coverage on the sensor surface is adequate, the quartz crystal may be placed into the sensor chip housing, and introduced into the QCM biosensor. A continuous flow of an appropriate running buffer, for example PBS, supplemented with non-ionic detergent such as Tween 20 at 0.05% v/v is commenced at a flow rate of 25 μl/min.
11. Prior to undertaking measurements, the sensor needs to equilibrate for several hours in running buffer (typically an overnight equilibration is used) with a flow rate of 25–50 μl/min (see Note 1).

3.2. Interaction Measurements

A typical experiment designed to measure the interaction between a lectin/antibody and the surface of cancer cells is described.

1. After equilibration of the sensor, the crystal should oscillate at a stable frequency with a drift <0.2 Hz/min.
2. An injection of an appropriate reference sample is performed by loading the injection loop connected to the valve. The valve is switched to reference and is introduced into the line of flow allowing it to be transferred to the sensor surface. When an appropriate volume of the reference sample has been injected (usually 70% of the injection loop volume) the valve is switched to the original position (see Note 2).
3. The sample to be analyzed is injected by following step 2, above. Appropriate concentrations of analyte (lectin or antibody) should be prepared in running buffer. In this example, several concentrations of lectin are prepared (ranging from 12.5 to 100 μg/ml). During the injection step, the frequency is monitored, and increases in response to the interaction between the lectin and the cell surface, adding mass on the sensor surface. After injection of the sample, the dissociation of the lectin from the cell glycans, may be monitored for at least 200 s. The dissociation time needed is related to the stability of the interaction, i.e. longer dissociation times are required for

slowly dissociating interactions, and are needed in order to acquire enough data for reliable rate determinations.

4. The sensor surface needs to be regenerated in order to enable other interactions to be evaluated using the same sensor, therefore any remaining bound lectin (or antibody) should be desorbed. Typically, the regeneration solution consists of a 10 mM glycine solution at low pH (pH 1.0–3.0). In the example presented (Fig. 1), the high degree of avidity of binding required potent regeneration conditions. 10 mM glycine pH 1.0, supplemented with 500 mM NaCl was injected three times in order to remove all of the bound lectin from the surface of the cells. The regeneration efficiency is evaluated to ensure that the frequency recorded after the regeneration is similar to the frequency monitored before injection of the sample (see Note 3).
5. Sensor surfaces can typically be regenerated between 10 and 20 times; however, this will depend on the cell type under investigation and therefore should be determined for each cell type in turn.

3.3. Data Analysis

The binding response curves obtained for the interaction of the binding partners of interest must be processed and analyzed to extract the rate constants for the reaction.

1. The signal obtained using the reference sample needs to be subtracted from the interaction curves obtained with the sample of interest (Fig. 1a) in order to obtain referenced interaction curves (Fig. 1b).
2. This operation needs to be repeated for every interaction analyzed.
3. To determine the kinetic parameters of an interaction, three or four binding curves obtained with various concentrations of analyte should be analyzed simultaneously. In the example shown in Fig. 1b, the binding curves obtained with the lectin at 12.5, 25, 50, and 100 µg/ml were analyzed using the biosensor kinetic data analysis software ClampXP developed by Myszka and Morton (6). In this software the kinetics of the reaction are determined by fitting theoretical models, for the interaction, to the experimental data. Data-sets fitted with a 1:1 interaction model (with or without adjustment for mass transport limitations) enables evaluation of the accuracy by which the model describes the interaction of interest. Global fitting using the 1:1-model to the experimental data sets may be used to extract kinetic rate constants, such association rate constant (k_a) and dissociation rate constant (k_d). From these, the dissociation constant (K_D), characterizing the affinity of the interaction of interest, can be calculated (see Note 4).

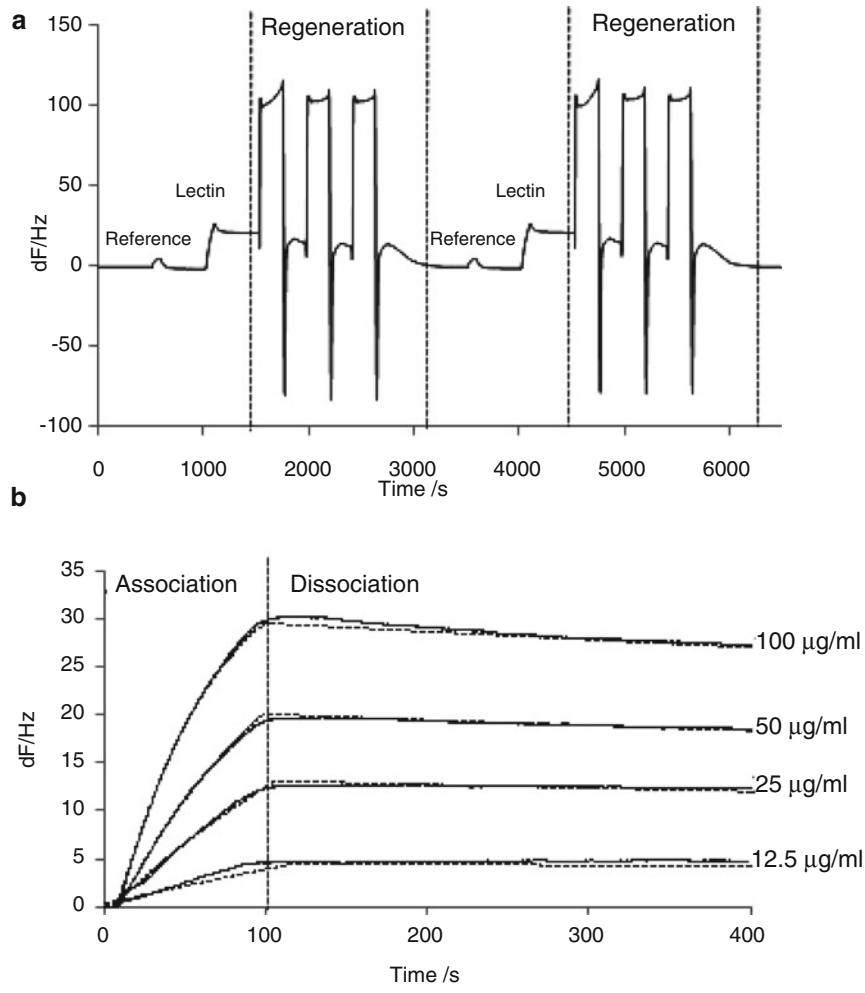

Fig. 1. (**a**) Two interaction cycles consisting of a reference injection, followed by a lectin injection and subsequent regeneration of the surface. (**b**) Kinetic evaluation of the interaction between a lectin and breast cancer cells. Referenced interaction curves at increasing concentrations (*solid dark lines*), overlaid with curves resulting from global fit using a theoretical 1:1 model (*dashed lines*). Rate constants were extracted using the Clamp XP software, ($k_a = 2.0 \times 10^4$ M^{-1}/s, $k_d = 3.0 \times 10^{-4}$ s^{-1}). The affinity of the interaction was also determined, $K_D = 15$ nm.

4. Notes

1. Preparation of crystals: The preparation step is essential in order to generate high-quality data using the QCM biosensor technology. A polystyrene-coated quartz crystal sensor must be covered with enough cells, evenly distributed over the surface, to generate a sufficient signal when the interaction of interest is monitored. However, the surface should not be saturated as the heavy load of cells may challenge the mechanical

performance of the biosensor and, hence, may decrease the quality of the data. The empirical approach adopted is to define conditions enabling cell coverage of 60–80% (as an indication) of the sensor surface. A visual evaluation using a fluorescent nuclei dye using an upright fluorescence microscope is important to ensure that cells evenly cover the sensor surface.

2. Referencing: QCM biosensors are sensitive to physico-chemical parameters such as viscosity and/or conductivity of the buffers. Hence, differences in salt composition and concentration between the running buffer and the sample injected over the surface may produce non-specific signal (typically <2–3 Hz) that may interfere with the analysis. This is especially important for weak responses, as the low density of binding sites on the sensor surface, are by definition more sensitive to interference. Appropriate reference solutions must be chosen and allowed to interact with the surface using the same injection parameters as for the sample injections. This approach makes it possible to subtract non-specific binding of the analyte interaction responses and generate high-quality data.
3. Surface regeneration: Surface regeneration is performed to remove remaining analyte bound to the cells before a subsequent analysis cycle can begin. The regeneration must desorb bound analyte, but should not affect the cells on the surface, for example it must leave the surface intact and active to enable further binding events to be reproducibly monitored. Empirical determination of appropriate regeneration conditions must be performed, starting with low concentration solution followed by higher concentration and acidic pH if needed. Examples of regeneration solutions to be used: weak 10 mM glycine/HCl pH >2.5; 10 mM HEPES/NaOH pH <9; 1 M NaCl, intermediate (10 mM glycine/HCl pH 2–2.5; 10 mM glycine/NaOH pH 9–10, strong 10 mM glycine/HCl pH <1.5 supplemented with 0.5 M NaCl.
4. Kinetic analysis of interaction data: QCM biosensors enable measurements of interactions occurring between two biomolecules, e.g. an analyte (A) and a surface bound ligand (B), to form a complex (AB), in a biological context. A single-step bimolecular interaction can be described by A+B AB and is characterized by interaction-specific association and dissociation rate constants that can be obtained by kinetic evaluation of the experimental data against a theoretical model using a global curve-fitting software (for example, Clamp XP). Rate constants relate the rate of reaction to the concentration of the binding partners. The association rate constant (several abbreviations may be found such as k_1, k_{on}, k_{fwd} or k_a) is expressed in M^{-1}/s and the dissociation rate constant (k_{-1}, k_{off}, k_{rev} or k_d) is expressed in s^{-1}. The affinity of the reaction is often referred to

as the dissociation constant, $K_D = k_d/k_a$, and is expressed in molar (M). The smaller the value for k the higher the affinity of the interaction. The use of the association constant, $K_A = 1/K_D$ expressed in M^{-1} may also be observed in some studies.

Several considerations must be taken into account when performing a kinetic study, particularly for an interaction occurring at the cell surface. Sometimes the transport/diffusion rate of the analyte from the bulk flow to the vicinity of the surface may restrict the association of the complex. In these cases, the concentration close to the interaction site will be lower than in the bulk flow due to an association rate faster than the dissociation rate, and the association rate will be determined by the diffusion rate to the surface. This is often referred to as mass transport limitation, and will result in biased results if this is not compensated for. One way to reveal such mass transport limitations is to assess the shape of the binding curve. Instead of a typical (exponential) curved response in the association phase (at least at higher concentrations), the response curves are more or less linear, with little or no curvature. If this is the case, the best solution is to increase the transport of the analyte to the surface by increasing the flow rate, or to reduce the association rate at the surface by reducing the surface density of the immobilized ligand. If this is not possible, an appropriate curve fitting model, taking the diffusion rate of the analyte into account, must be used to enable correct evaluation of the kinetic parameters of the reaction of interest. In addition, avidity of the bio molecules in the system might need to be taken into account, this is very important in the case of multivalent analytes, such as lectins. Avidity is defined as the combined strength of several bond interactions as opposed to affinity, which is determined according to the 1:1 interaction model, describing the strength of a single bond interaction. Hence, it is difficult to determine the affinities for multivalent analytes other than in relative terms.

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