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Analysis of Protein Interactions Using a Quartz Crystal Microbalance Biosensor

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INTRODUCTION

ELISA, calorimetry, and fluorescence spectroscopy are well-established techniques for the investigation of protein–protein interactions. In addition, biosensor-based approaches have gained increasing importance due to the advantages of easy handling and high-throughput capability. Biosensors consist of a biologically active component and a transducer integrated into a single system for reagent-free measurements (Hall 1990). The most prominent biosensor systems for the study of protein–protein interactions are based on surface plasmon resonance (SPR) (Chapter 14). Recently, the quartz crystal microbalance (QCM) has been established as an alternative to SPR. A comparison of the two methods is given by Kösslinger et al. (1995, 1998).

The QCM is an acoustic sensor based on a piezoelectric crystal. Mass changes can be detected on the sensor surface in the nanogram range. The relation is expressed in the Sauerbrey formula (Eq. 1) (Sauerbrey 1959):

$$\Delta F = S \cdot \Delta m = \frac{-2F^2}{Zp} \cdot \Delta m \quad (1)$$

where ΔF , frequency shift [Hz]; Δm , change of mass per area [ng cm^{-2}]; S , mass sensitivity [$\text{Hz ng}^{-1} \text{cm}^{-2}$]; F , resonant frequency [Hz]; Zp , acoustical impedance [Hz ng cm^{-2}].

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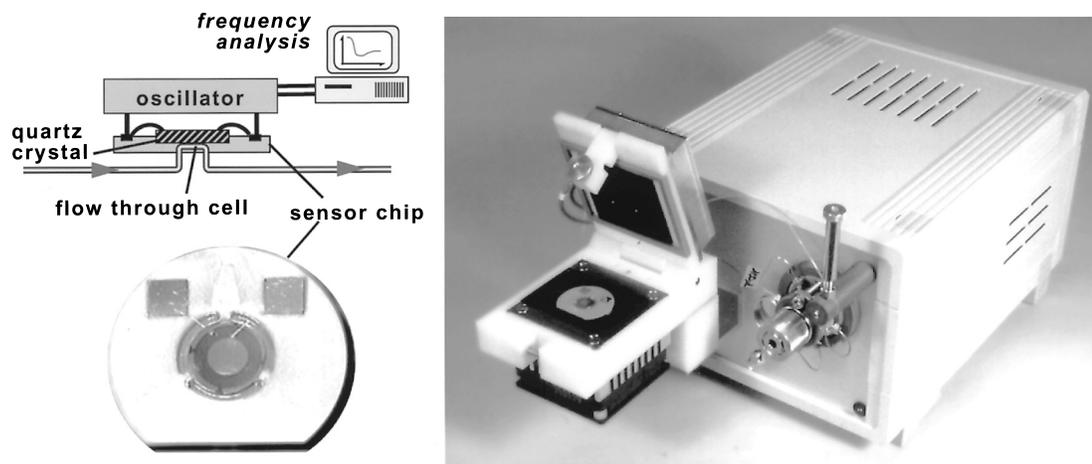


FIGURE 1. The QCM biosensor. (A) Scheme of the measurement setup. (B) The AFFCo2000 QCM system. The samples are transported to the oscillator-driven QCM sensor chip mounted in a flowthrough cell by a flow injection analysis (FIA) system. The frequency signal is detected by a frequency counter and processed electronically in a separate computer.

The sensor can be used for the direct, marker-free measurement of specific interactions between immobilized molecules and analytes in solution. Binding of a soluble analyte to the immobilized ligand causes a shift in the resonance frequency, and this signal can be recorded using a frequency counter with high resolution. This method, despite its existence for four decades, has only recently been developed for immunological measurements in a flowthrough system (Kösslinger et al. 1992), as shown in Figures 1 and 2. The resulting frequency versus time curve is called a “sensorgram.” As real-time measurements are performed, the sensorgrams are capable of deducing not only the equilibrium binding constants (Sladal et al. 1994), but also the affinity rate constants. This methodology was recently applied to describe the binding kinetics of various phage-presented proteins (Hengerer et al. 1999a; Decker et al. 2000), including recombinant antibodies (Hengerer et al. 1999b).

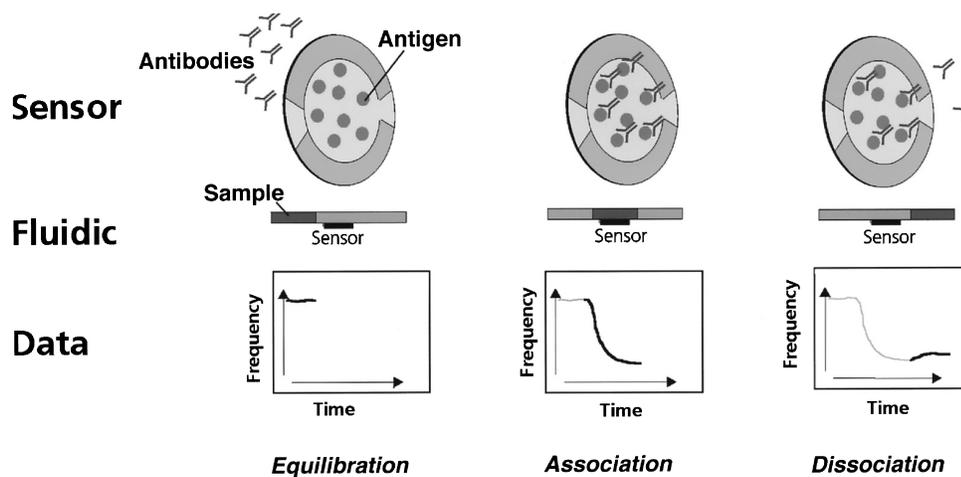


FIGURE 2. Scheme of the relation between action on sensor surface (“sensor”), status in FIA system (“fluidics”), and resulting frequency versus time curve (“data”), showing an antibody–antigen reaction as an example.

OUTLINE OF PROCEDURE

In QCM, the ligand is immobilized as a monolayer on a flat gold surface. Immobilization can be achieved using various methods. The easiest way is absorption of the ligand on the gold surface. This method, however, does not allow regeneration of the ligand layer (Uttenthaler et al. 1998). As the gold reacts with thiols, yielding a stable, semi-covalent bond, proteins can be immobilized by the thiol groups of their cysteine residues. Alternatively, the sensor surface can be activated by using a thiol-containing bifunctional linker. Reproducible results were obtained with dithiobis-succinimidyl-propionate (DSP) (Hermannson 1992). DSP forms disulfide bonds to the gold surface and provides *N*-hydroxysuccinimide (NHS) groups that can react with the free amino groups on the ligand. If streptavidin is immobilized using DSP, biotinylated ligands can be conveniently coated. The availability of *in vivo* (Weiss et al. 1994) or *in vitro* systems (Saviranta et al. 1998) for sequence-specific biotinylation should allow the generation of highly ordered ligand monolayers.

Samples are applied to the immobilized ligand on the sensor surface by a continuous constant flow. A constant analyte concentration at any part of the flowthrough cell is thus provided, and diffusion effects can be neglected. Moreover, an optimized cell design helps to avoid mass transport effects. Viscosity effects, however, can lead to frequency shifts interfering with the specific signal. Therefore, the biosensor should be calibrated with a viscous solution to determine the time in which the viscosity change generates a signal. This time interval should be excluded from the sensorgram. A sample sensorgram is given in Figure 3.

After a measurement, the sensor has to be regenerated to remove bound analyte from the sensor surface. For this regeneration step, elution buffers as used in affinity chromatography can be applied as long as they do not destroy the native structure of the ligand. A good overview regarding ligand stability is given in Harlow and Lane (1988).

It should be noted that the QCM method does not allow the measurement of true affinities. Because the ligand is immobilized, one degree of freedom is lost. Therefore, the measured affinities could be influenced by decreased mobility and sterical hindrance. Furthermore, avidity effects

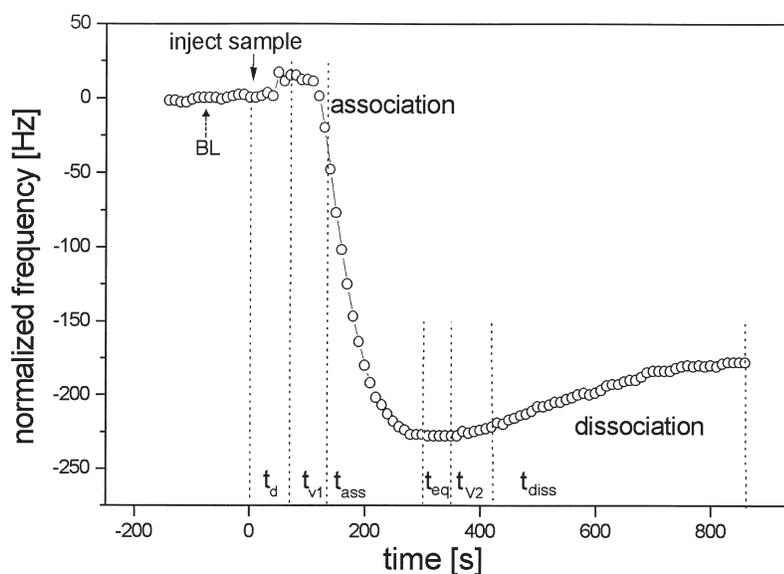


FIGURE 3. Evaluation scheme for the sensorgram. The sample is injected into the system at t_0 . After the delay time t_d , the binding analyte generates a signal, which is interfered with by the viscosity effect during t_{v1} . The segment t_{ass} shows the association yielding a steady-state t_{eq} . When the sample buffer runs over the surface again, it induces a viscosity effect t_{v2} and the dissociation phase t_{diss} . (BL) Baseline.

resulting from multivalent binding might affect the apparent affinity. On the other hand, partial denaturation of the immobilized ligand may decrease the apparent binding affinity. In the case of phage-presented derivatives, as tested by Hengerer et al. (1999b), the affinity data obtained are only apparent, because the fraction of total phage carrying functional single-chain fragments of an antibody (scFv fragments) may not be 100%, and a fraction of surface-expressed scFv antibodies could be shed by proteolysis (Hengerer et al. 1999b). In some phage-display systems, soluble scFv fragments may be present in the phage supernatants due to the existence of an amber codon between scFv and the virus coat protein. Finally, more than one fragment could be presented per phage. Thus, for the determination of true affinities, an accurate determination of true molarities of scFv fragments would be required. Nevertheless, this method allows the ranking of a set of antibody clones from a phage-screening experiment according to their apparent affinities.

Protocol 1

Quartz Crystal Microbalance Analysis

A QCM analysis in this protocol is broken down into the following steps: sensor cleaning, immobilization of the ligand on the sensor, sample preparation, system calibration, measurement procedure, optimization of regeneration buffer, and evaluation of sensorgram.

Sensor cleaning and immobilization of ligand are performed before the sensor chip is mounted onto the AFFCo 2000 device. Sensor cleaning should be performed directly before immobilization. The sensor chip with the immobilized ligand can be stored for several days at 4°C in a wet chamber (storage conditions depend on the immobilized protein).

MATERIALS

CAUTION: See Appendix for appropriate handling of materials marked with <!.>.

Buffers and Solutions

Acetone <!.>

Activation solution

0.4 mg/ml of dithiobissuccinimidyl-propionate (DSP) dissolved in water-free dimethylformamide (DMF)<!.>

This solution must be prepared directly before use.

Blocking reagent

0.1 mg/ml bovine serum albumin (BSA) in PBS

Can be stored in aliquots of 0.5 ml for ~1 month at -20°C.

Calibration solution

2% (w/v) glucose

1 M hydrochloric acid (HCl) <!.>

1 M sodium hydroxide (NaOH) <!.>

PBS buffer

2.6 mM potassium chloride (KCl) (200 mg/liter if anhydrous salt is used)

138 mM sodium chloride (NaCl) (8 g/liter if anhydrous salt is used)

10 mM sodium hydrogen phosphate (Na_2HPO_4) (1.44 g/liter if anhydrous salt is used)

1.8 mM potassium hydrogen phosphate (KH_2PO_4) (240 mg/liter if anhydrous salt is used)

Adjust to pH 7.4. Can be stored for ~4 weeks at room temperature.

Piranha solution

3 parts (3 ml) of 98% sulfuric acid (H_2SO_4) <!.>

1 part (1 ml) of 30% hydrogen peroxide (H_2O_2) <!.>

This solution should be prepared directly before use. The solution heats up quickly upon mixing; therefore, wait ~2 minutes for it to cool down.

Regeneration buffer (glycine buffer)

100 mM glycine (0.7507 mg/100 ml if anhydrous salt is used)

100 mM NaCl (0.5855 mg/100 ml if anhydrous salt is used)

Adjust pH to 2.5 or 11.5 with 100 mM HCl <!.> or 100 mM NaOH <!.>. Can be stored in aliquots of 1 ml for ~1 year at -20°C.

Regeneration buffer (borate buffer)

borate buffer solution (pH 11) (Merck, Darmstadt, Germany)

Store at 4°C. Check pH before use. No stability data are available. Adjust pH to 11.5 or up to 12.5 with NaOH

Regeneration buffer (sodium isothiocyanate)

4 M sodium isothiocyanate (NaSCN)

Dissolve in distilled H₂O. Adjust pH to 11.0 with NaOH, if necessary. Store at room temperature, and check pH before use. No stability data are available.

Streptavidin (1 mg/ml) in PBS

Can be stored in aliquots of 25 ml for ~1 year at -20°C.

Ligands

Ligands with free amino groups (for immobilization procedure 1) or biotinylated ligands (for immobilization procedure 2).

Negative Control

Nonbinding protein, nonspecific antibody, helper phage, or unspecific phage-displayed protein.

The control should be a molecule very similar to the sample, but showing no binding properties to the immobilized ligand. Use the control in a concentration similar to that of the highest sample concentration.

Biosensor Equipment

AFFCo 2000 Quartz Crystal Microbalance Device, developed at the Fraunhofer-Institute of Microelectronic Circuits and Systems, Munich, Germany (FhG-IMS)

A scheme of the device is depicted in Figure 1. The system consists of a mount for the exchangeable sensor chips (see below), an injection valve with, e.g., a 0.1-ml loop (range 0.050–0.250 ml) for the sample application, and a pump to maintain a continuous buffer flow of ~0.03 ml/minute (range 0.020–0.050 ml/minute). The sensors, produced at the FhG-IMS, are round-shaped 20-MHz quartz crystals with gold electrodes and are mounted on a flowthrough cell. The quick exchange mount for the chip provides temperature control of the buffer and sample solution. The measurement time depends on sample loop size and buffer flow rate. Optimal conditions should be determined by the user.

Additional Equipment

Gaseous N₂ or clean low-pressure air

Hamilton syringe (0.250 ml) for sample application

Wet chamber and dry chamber (blue silica gel)

METHOD

Sensor Cleaning

Procedure 1

1. Rinse the sensor with ~0.2 ml of acetone.
2. Dry the sensor carefully with N₂.
If the gas pressure is too strong, the sensor can be pressed out of the chip.

Alternative: Procedure 2

1. Rinse the sensor with ~0.2 ml of piranha solution.
2. Rinse the sensor with ~0.2 ml of H₂O.
3. Rinse sensor with ~0.2 ml of ethanol.
4. Dry the sensor carefully with N₂.
If the gas pressure is too strong, the sensor can be pressed out of the chip.

Alternative: Procedure 3

1. Rinse the sensor with 1 M NaOH (20 minutes of flowthrough, flow rate of 0.05 ml/minute).
2. Rinse the sensor with 1 M HCl (20 minutes, flow rate of 0.05 ml/minute).
3. Rinse the sensor with ~0.2 ml of ethanol.
4. Dry the sensor carefully with N₂.
If the gas pressure is too strong, the sensor can be pressed out of the chip.
Optimal results depend on properties of individual molecules. Flow rate is not critical except in steps where it is specifically mentioned.

Immobilization of the Ligand on the Sensor

Procedure 1: Ligands with Free Amino Groups

1. Rinse the sensor with ~0.2 ml of activation solution.
2. Activate the sensor surface for 20 minutes with 0.01 ml of activation solution at room temperature in a dry chamber.
3. Rinse the sensor with ~0.2 ml of PBS.
4. Incubate the sensor with 0.01 ml of ligand solution (1 mg/ml) for 16 hours at 4°C in a wet chamber.

Procedure 2: Alternative

1. Rinse the sensor with ~0.2 ml of activation solution.
2. Activate the sensor surface for 20 minutes with 0.01 ml of activation solution at room temperature in a dry chamber.

3. Rinse the sensor with ~0.2 ml of PBS.
4. Incubate the sensor with 0.01 ml of streptavidin solution (1 mg/ml) for 16 hours at 4°C in a wet chamber.
5. Rinse the sensor with ~0.2 ml of PBS.
6. Incubate the sensor with 0.01 ml of biotinylated ligand solution (1 mg/ml) for at least 1 hour at room temperature in a wet chamber.

Sample Preparation

1. Dilute the sample (containing the analyte) in PBS. The resulting concentrations should be as follows:

Protein <100 kD	100–10,000 ng/ml
Antibody	10 to 1000 ng/ml
Phage	10^{11} to 10^{13} pfu/ml
2. Approximately five different concentrations should be prepared and tested. One sample, consisting only of diluent, should be prepared as a negative control. At least 0.5 ml of every concentration (corresponding to two sample volumes) is required.

All sample dilutions should be done in flow buffer to avoid matrix effects.

System Calibration

1. Inject the calibration solution.
2. Evaluate the sensorgram according to the Evaluation of Sensorgram protocol (see p. 281) to determine the time of a measurement, the delay time t_d , and time of viscosity effects t_{v1} and t_{v2} .

Measurement Procedure

1. Rinse the sensor with ~0.2 ml of PBS.
2. Dry the sensor carefully with gaseous N_2 .
3. Insert the sensor in the exchange mount of the AFFCo 2000 device.
4. Start the pump at a rate of 0.030 ml/minute.
5. Wait until the baseline drift is less than 2 Hz/10 minutes and the baseline noise is less than 5 Hz (BL).
6. Rinse and fill the loop with 0.25 ml of blocking reagent.
7. Inject the blocking reagent into the buffer stream. Note the time.
8. A starting “peak” should be seen after ~2 minutes.
9. Wait until the new stable baseline is reached.
10. If the resulting frequency shift is more than 30 Hz when compared to the baseline before blocking reagent injection, repeat blocking.

This may indicate incomplete immobilization.
11. Wait until a new stable baseline is reached.

12. Rinse and fill loop with 0.25 ml of negative control.
13. Inject the negative control into the buffer stream. Note the time.
14. No decrease of the baseline should be seen after ~2 minutes.
15. Wait until the measurement time, as determined in system calibration, has passed.
16. Rinse and fill the loop with 0.25 ml of sample.
17. Inject the sample into the buffer stream. Note the time.
18. An initial drop should be seen after ~2 minutes.
19. With continuous flow, a decreasing frequency, which indicates the association phase, is followed by an increasing frequency, which indicates the dissociation of the analyte after the sample has completely passed through the cell.
20. Rinse and fill the loop with 0.25 ml of the appropriate regeneration buffer.
21. Inject the regeneration buffer into the buffer stream. Note the time.
22. An increase or decrease of the frequency should be seen (viscosity change) after ~2 minutes. After regeneration, the frequency should have adjusted to the same level observed before the sample measurement.
23. Wait until a new stable baseline is reached.
24. Inject the next sample and repeat the procedure from steps 16 to 23.
 Start measurements with a negative control sample and continue from low to higher concentrations.

Optimization of Regeneration

1. Note the frequency before the first sample injection.
2. Inject the regeneration buffer. Start with a mild buffer (for example, glycine at pH 2.5).
3. Note the frequency of the new stable baseline after regeneration. It should approximately equal the baseline before sample injection.
4. If the frequency is higher than before sample injection (more than 50 Hz), the ligand layer is partially lost. In this case, use less stringent conditions (more neutral pH or other regeneration buffer).
5. If the frequency is lower than before sample injection, bound analyte is not completely removed. In this case, use more stringent conditions (lower pH down to 2.0, try alkaline pH [9–11], or 1–3 M NaSCN).
6. Inject the first sample again. The frequency shift should be similar to the first measurement. If this is not the case, try another regeneration buffer. The amount of deviation may be up to 20–30%; the deviation limit should be determined by the required accuracy of the individual experiment.

Evaluation of Sensorgram

1. Analyze sensorgram according to the following scheme. An example curve is given in Figure 3.
 - a. BL, stable frequency before injection (drift less than 2 Hz/10 minutes).
 - b. t_d , delay time. Time between injection and start of signal.

- c. t_{v1} , viscosity effect. During this time, the viscosity effects overlay the association effect.
 - d. t_{ass} , association. Time during which the sample flows over the sensor and association can take place.
 - e. t_{eq} , steady state is reached.
 - f. t_{v2} , viscosity effect. Buffer reaches the sensor again. During this time, the viscosity effects overlay the dissociation effect.
 - g. t_{diss} , dissociation. Time during which buffer flows over the sensor and dissociation takes place. This curve segment should contain at least 50 data points.
2. Fit curve segment of t_{diss} according to Equation 2. This curve segment should consist of at least 50 data points.

$$F_{(t)} = F_0 \cdot e^{k_{diss}(t-t_0)} \quad (2)$$

Where $F_{(t)}$, Frequency at time t [Hz]; t_0 , start of dissociation; F_0 , frequency at t_0 [Hz]; k_{diss} , dissociation rate constant [s^{-1}].

3. F_0 is the frequency at the start of t_{diss} , t_0 is the time at the start of t_{diss} . Insert F_0 in Hz and t_0 in s. The dissociation rate constant k_{diss} [s^{-1}] is obtained.
4. Fit curve segment t_{ass} according to Equation 3. This curve segment should contain at least 50 data points.

$$F_{(t)} = \frac{k_{ass} \cdot C \cdot \Delta F_{max}}{k_{ass} \cdot C + k_{diss}} \cdot \left(e^{-(k_{ass} \cdot C + k_{diss})(t-t_0)} - 1 \right) + F_0 \quad (3)$$

where k_{ass} , association rate constant [$M^{-1}s^{-1}$]; C , concentration of analyte [M]; ΔF_{max} , frequency obtained for a complete analyte monolayer [Hz].

5. F_{max} is the frequency shift for a completely occupied surface, C is the concentration of the analyte, t_0 is time at start of t_{ass} , k_{diss} is determined from step 3. Insert assumed or experimentally determined values for F_{max} in Hz, C in mol/liter, t_0 in s, and k_{diss} in s^{-1} . The association rate constant k_{ass} is obtained.
6. Calculate the dissociation constant K_D using Equation 4.

$$K_D = \frac{k_{diss}}{k_{ass}} \quad (4)$$

where K , dissociation constant [M^{-1}].

7. Repeat for each sample concentration.

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