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Immunodetection by Quartz Crystal Microbalance

A New Approach for Direct Detection of Rabbit IgG and Peroxidase

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Abstract

Biodetection is one of the most important challenges for the twenty-first century: many fields are concerned, mainly environmental and medical. The quartz crystal microbalance (QCM) may offer great possibilities for this purpose: a direct response signal, which characterizes the binding event between a sensitive layer, immobilized onto the surface transducer, and the analyte to be detected, can be obtained. However, for the detection of small biomolecules such as antigens, it is quite difficult to obtain an observable signal that corresponds directly to the binding event. In general, this is owing to the lack of mass sensitivity of the commonly used QCM, with 5- to 10-MHz quartz crystals. For improving this mass sensitivity, a 27-MHz quartz resonator was developed and incorporated in a flow-through microcell. Two biospecies, IgG rabbit and peroxidase enzyme, were studied with this ultrasensitive QCM in terms of specificity, detection limit, and calibration curve.

Index Entries: Quartz crystal microbalance; peroxidase; rabbit IgG; immunodetection; 27-MHz resonators.

Introduction

The development of immunosensors based on piezoelectric transducers is widely studied owing to their attractive potentialities in mass detection *(1–4)*. The quartz crystal microbalance (QCM) adapted to liquid media may give a direct response signal characterizing the binding event between a sensitive layer, e.g., grafted onto the surface transducer, and the analyte to be detected. This idea of direct measurements is important if it is compared with classic colorimetric tests, such as enzyme-linked immunosorbent assay, in which several steps are necessary to obtain an optimal

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signal, which, of course, are time-consuming. However, to detect small biomolecules, such as antigens, it is quite difficult to obtain an observable and direct signal; in general, intermediate steps of amplification are necessary or dip-and-dry techniques are used *(5,6)*. This is mainly owing to the lack of sensitivity of classic QCMs, which are generally built with 5- to 10-MHz quartz resonators *(7,8)*. Moreover, techniques for immobilizing antibodies are often difficult to carry out and sometimes cannot be reproduced easily *(5,9)*, generally owing to involved chemical reactions included in the experimental procedure.

In this article, we propose to increase the mass sensitivity by increasing the resonant frequency of the QCM. To obtain better sensitivity, it is necessary to work at higher frequencies, such as at 9 or 27 MHz. A new cell, incorporating a 27- or 9-MHz QCM was devised to continuously measure immunoreactions in liquids. In a first step, the piezoelectric transducer sensibility was tested by using different resonant frequencies: 6, 9, and 27 MHz. Then, the feasibility of the biosensor was demonstrated by detecting two different biospecies. The performances in terms of specificity, limits of detection, and calibration curves of the biosensor were examined.

Materials and Methods

Basic Principle

The frequency response to an added (or subtracted) mass is described basically by the Sauerbrey *(10)* equation:

$$\Delta f = -\left(\frac{2 \times f_0^2}{S \times n \times \sqrt{\mu \times \rho}}\right) \times \Delta m \qquad (1)$$

in which Δf is the measured frequency shift (hertz); S is the active area of the crystal (square centimeters) defined by the projected overlap of the exciting electrodes; ρ is the quartz density (2.648 g/cm^3); μ is the shear modulus (2.947×10^{11} g/[s^2.cm]); n is the overtone number; and f_0 is the fundamental crystal frequency.

Note that this relation is only valid for thin elastic added layers. In our case, in a first approximation, this hypothesis will be kept. For a 27-MHz (P3) QCM transducer, the theoretical coefficient reaches 54.9×10^7 Hz/(g.cm^2), which gives, in a more concise way, a 364-pg/Hz value for the mass sensitivity (S = 0.2 cm^2). That is, if the QCM resolution is about 1 Hz, the detection limit reaches 364 pg.

Piezoelectric Transducer and Test Cell

AT-cut planar quartz crystals (14 mm in diameter) with a 6- and 9-MHz nominal resonance frequency (CQE, France) were used. Two identical gold electrodes, 2000 Å thick and 5 mm in diameter, were deposited, by evaporation techniques, on both sides of crystals with a chromium

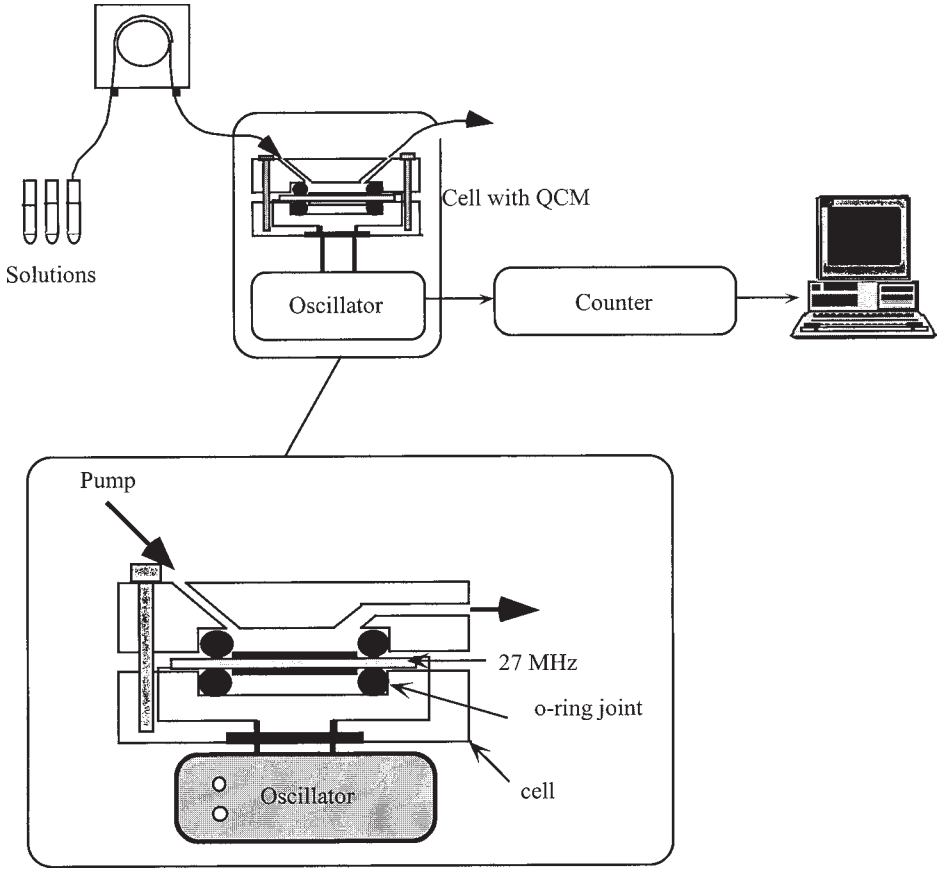

Fig. 1. Experimental setup with the complete system (pump, cell, and QCM).

underlayer. The resonators were carefully cleaned in two solvents, acetone and ethanol (Merck), analytical grade; one after the other, for 2 min in an ultrasonic bath. Then, they were dried under pure nitrogen and connected with a silver conducting paste, through wires, to BNC connectors. Three homemade oscillators were designed to drive the crystal at 6, 9, and 27 MHz (9-MHz crystal used on the third overtone). To improve stability, all the electronic oscillator components were temperature controlled through a heating current monitor (Watlow) with stability better than 0.1 K.

An experimental cell was also developed: the crystal was mounted between two O-ring seals inserted in a Plexiglas cell (Fig. 1). Only one face of the quartz was in contact with the solution. The cell volume was about 50 μL, and the apparatus included a peristaltic micropump (P1; Pharmacia) to ensure a constant flow (60 μL/min) of the solutions on the working quartz crystal. The experimental setup was built by coupling a homemade QCM and a frequency counter (Philips PM 6685) in order to follow the microbalance frequency during the biomolecule binding with the sensitive layer.

Preparation of Transducer

Mouse monoclonal antibodies against peroxidase, purchased from Sigma (St. Louis, MO), were immobilized by direct adsorption onto the surface transducer. The methodology was as follows: One hundred microliters of an antibody solution (100 μg/mL) was deposited onto gold and incubated for 12 h. Then, the quartz was rinsed in phosphate-buffered saline (PBS) (Sigma) and saturated with bovine serum albumin (BSA) (Sigma) solution (1% in mass) for 1 h. At the end, the quartz was rinsed in PBS and then mounted into the cell. The same protocol was kept for the other biospecie:goat antibodies, from Immunotech, against rabbit IgG (Jackson Immunoresearch).

Biological Reagents

The corresponding "antigen" (peroxidase and rabbit IgG) solution circulated over the crystal, which allowed a direct binding of the analyte to the fixed antibodies.

Calibration of Piezoelectric Transducers

The opportunity for increasing the resonant frequency of the piezoelectric transducer was checked through an electrochemical calibration of the mass sensitivity in order to corroborate the Sauerbrey predictions. Indeed, according to Eq. 1, the theoretical mass/frequency sensitivity is proportional to the square of the resonance frequency. Thus, three QCMs with mass sensitivities corresponding to different resonant frequencies—6, 9, and 27 MHz (9-MHz crystal used on the third overtone)—were experimentally compared. The frequency/mass coefficient was obtained by means of a copper electrodeposition, as previously described *(11)*. By following the QCM frequency evolution during this reaction and using the Faraday law, $\Delta f/\Delta m$ was determined with good accuracy; the electrodeposition current was carefully controlled, and therefore the added mass (Δm) was calculated by assuming the reaction yield was equal to 100%. Figure 2 presents the microbalance frequency evolution vs time during copper deposition for the three frequencies. Frequency variations are plotted in Fig. 2A, B, and C, respectively, for 6-, 9-, and 27-MHz (overtone 3) resonant frequencies. At each time, three current densities were chosen and the frequency/mass coefficients were calculated using Eq. 2:

$$k_{exp} = (\Delta f/\Delta t) \times (\Delta t/\Delta m) \times S \quad (2)$$

in which $(\Delta f/\Delta t)$ is the frequency/time evolution recorded with the frequency counter (Fig. 2); and $(\Delta m/\Delta t)$ is the added mass calculated through the Faraday law:

$$(\Delta t/\Delta m) = [(j \times M_{Cu})/(F \times 2)] \times S \quad (3)$$

in which j is the imposed current density; F is the Faraday number; and M_{Cu} is the copper atomic weight.

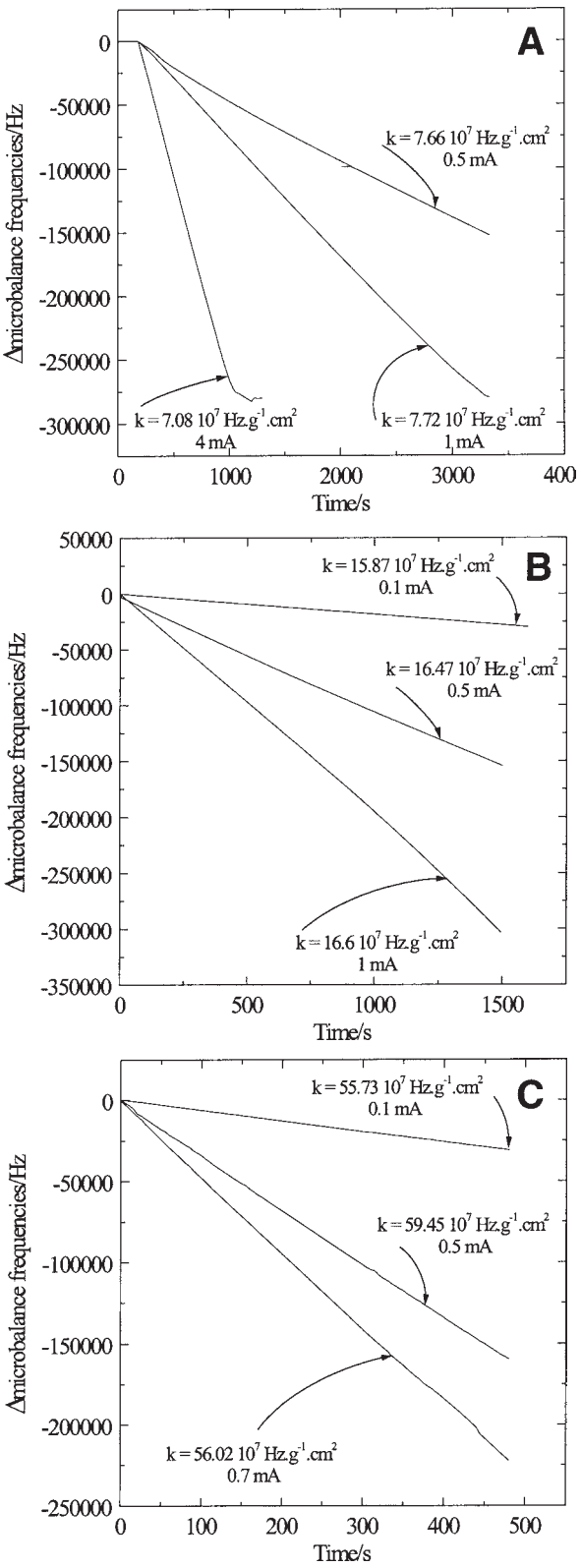

Fig. 2. Calibration of the QCM with copper electrodeposition for various resonant frequencies: **(A)** 6 MHz; **(B)** 9 MHz; **(C)** 27 MHz (P3).

Table 1
Experimental and Theoretical Frequency/
Mass Coefficient for Various Resonant Frequencies

Resonant frequency/ MHz	Experimental coefficient/ Hz g^{-1} $cm^2 \times 10^7$	Theoretical coefficient/ Hz g^{-1} $cm^2 \times 10^7$
6	7.49 ± 0.29	8.15
9	16.31 ± 0.32	18.3
27(3)	57.07 ± 1.69	54.9

Table 2
Comparison Between Added Mass,
Experimental and Theoretical, Corresponding to 1 Hertz Measurements
and 0.2 cm^2 Active Surface for Various Frequencies

Resonant frequency/ MHz	Added mass for 1 Hz measured variations and for 0.2 cm^2		
	Experimental/pg	Theoretical/pg	Difference
6	2670	2454	216
9	1226	1093	133
27(3)	350	364	14

Table 1 presents the experimental results compared with theoretical sensitivity calculated with Eq. 1. For the active surface considered in our case (S) and for a frequency variation of 1 Hz, these values were converted in terms of limits of detected mass as is shown in Table 2. Thus, the most sensitive device was obtained by using the highest frequencies (27 MHz, P3); this result is in good agreement with the Sauerbrey relationship.

Results and Discussion

The biosensor specificity step was checked for each detected antigen, and we consider this part as dominating before any detection tests. Figure 3A presents a preliminary control for rabbit IgG detection in which a pseudo-selective layer was made only by BSA adsorption onto the gold electrode; the rabbit IgG interaction did not occur because no frequency shift was observed (Fig. 3A) even with concentrated solutions of rabbit IgG (25 μg/mL). Thus, in this case, there were no nonspecific interaction. Then, a complementary test was performed as shown in Fig. 3B. The bioselective layer was built as just described; goat IgG was immobilized directly onto the gold electrode and saturated with BSA. Another antigen, alkaline phosphatase, flowed over the QCM. As previously, a steady microbalance frequency signal was observed, which indicated that nonspecific interactions did not occur. This proves the good quality of this layer and the good specificity of the antigen/antibody couple used in the experiment.

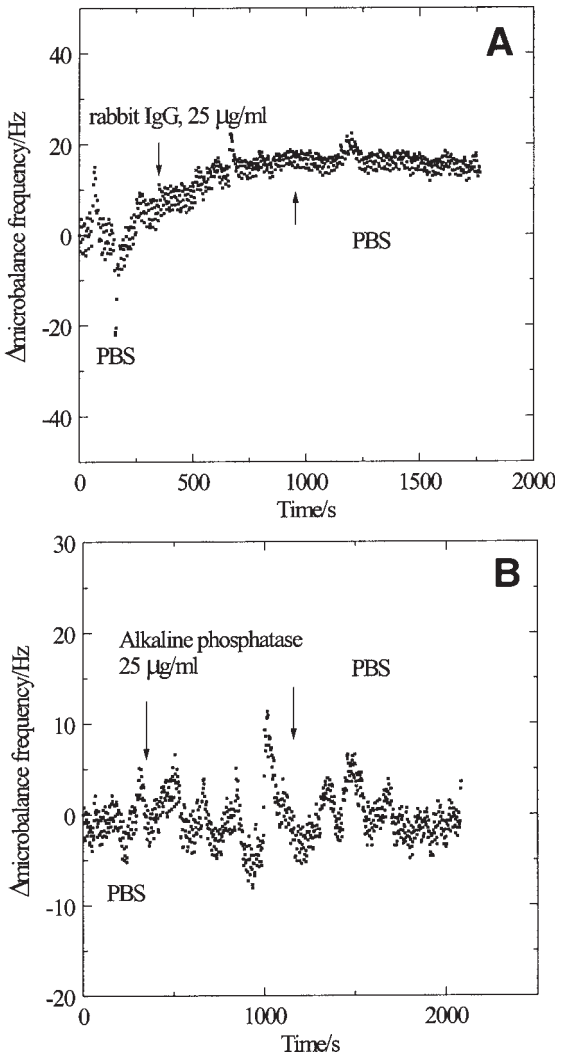

Fig. 3. Specificity test for rabbit IgG detection: **(A)** gold electrode coated with BSA; **(B)** gold electrode coated with goat IgG (antibody) saturated with BSA.

In a second step, an attempt at direct detection was made, as presented in Fig. 4. For a 5 µg/mL rabbit IgG solution injected in the QCM cell, a large decrease in the microbalance frequency, Δf = 150 Hz, was observed. In a second phase, pure PBS solution was injected; the microbalance frequency remained constant. This observation led to two conclusions: (1) the interactions between antibodies (goat IgG) against antigens (rabbit IgG) was strong because the mass was invariable, and (2) changes in solution viscosity/density were negligible because the measurable signal was constant when the nature of the solution was modified. Moreover, 90% of the signal was obtained very quickly, the time response being about a few minutes.

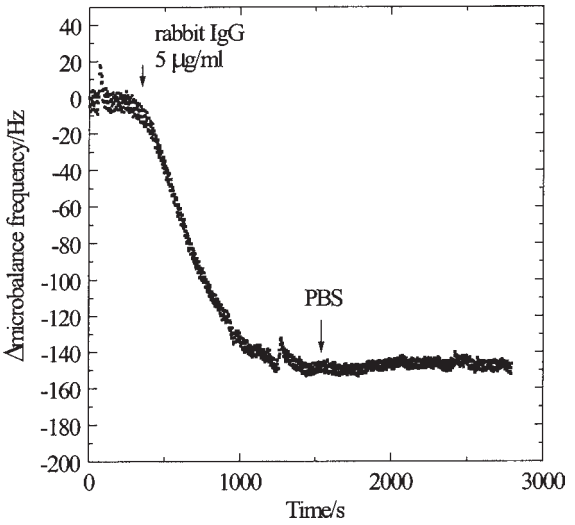

Fig. 4. QCM response to the addition of rabbit IgG ("antigen").

Figure 5A shows the influence of the antigen concentration on the interaction kinetics: the larger the concentration, the faster the rate to reach a similar limiting value. By selecting microbalance frequency variations, 3 min after antigen arrival, for several antigen concentrations, a calibration curve can be drawn (Fig. 5B). Thus, a linear response was observed between 1 µg/mL, which was the detection limit, and 25 µg/mL. To conclude, this immunosensor can be used directly to detect specifically and quickly an unknown solution containing rabbit IgG antigens.

The same approach was used with an another antigen/antibody couple: peroxidase/antiperoxidase. The first step was focused on the biolayer specificity; Figure 6 presents the antigen response for a QCM coated only with adsorbed BSA. The same conclusion can be drawn as for the previous device: this BSA layer is insensitive to the interaction with peroxidase. The second step tested direct detection. A peroxidase solution flowed over the microbalance surface and the frequency decreased consecutively with the interaction. Therefore, antigen detection was feasible because of the high sensitivity of the transducer used. This sensitivity was enough to detect directly the binding event between small biomolecules, such as peroxidase, and the specific antibody layer (Fig. 7A). The 27-MHz microbalance allowed this direct transduction to be carried out without the realization of a sandwich assay.

With a 10 µg/mL peroxidase concentration, the microbalance response reached –80 Hz (Fig. 7A), which was equivalent to 29.1 ng (according to the Eq. 1). Therefore, if the sensitive layer was supposed to be made by an antibody monolayer, and if each IgG interacted with only one antigen (peroxidase), and by taking into account that one antibody was equivalent to a 10-nm-diameter sphere *(12)*, a theoretical mass variation of 14.6 ng is

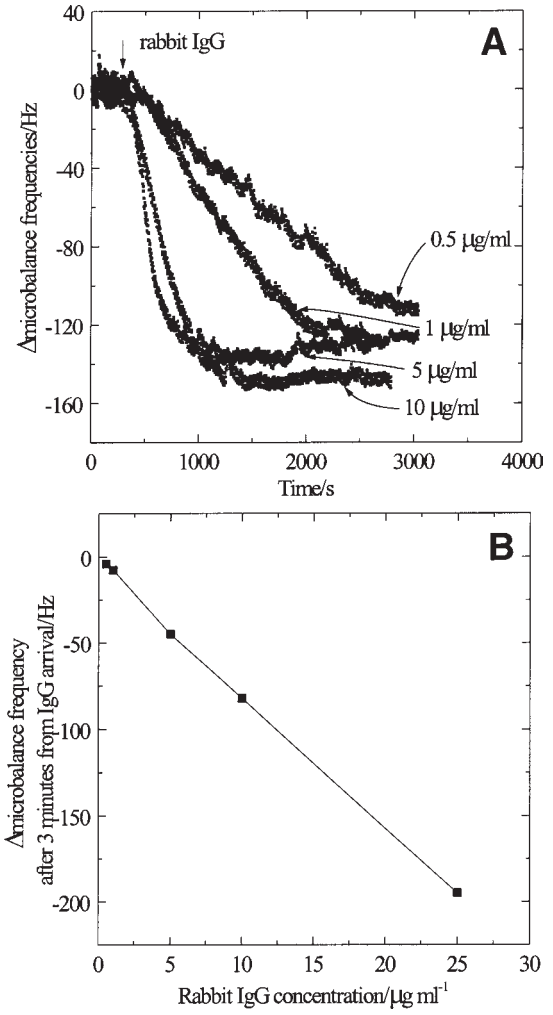

Fig. 5. Influence of rabbit IgG ("antigen") concentration over the QCM response: **(A)** microbalance responses; **(B)** calibration curve calculated from the previous response.

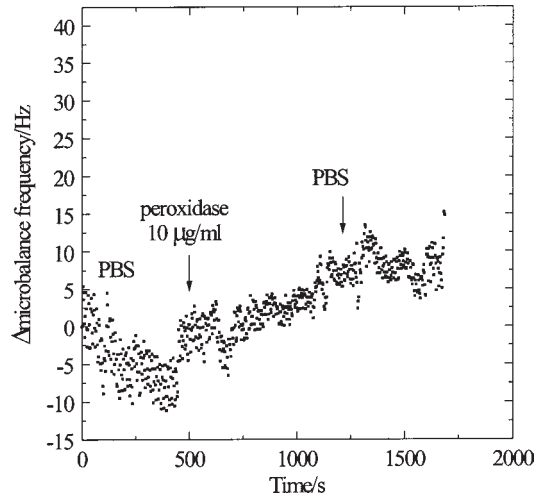

Fig. 6. Specificity test for peroxidase detection; gold electrode is coated only with BSA.

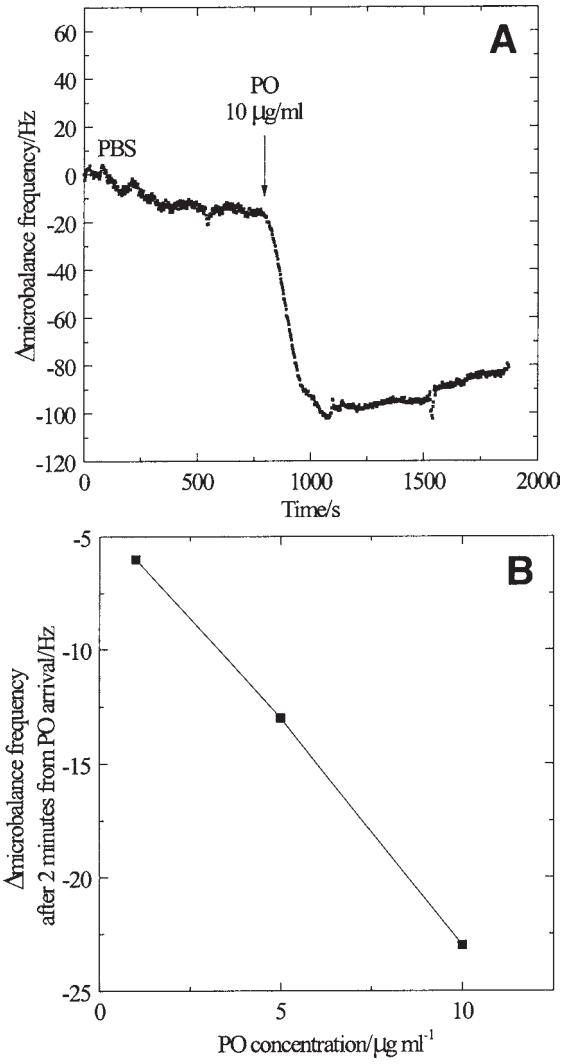

Fig. 7. Detection of peroxidase: **(A)** QCM response; **(B)** calibration curve peroxidase detection.

obtained. Hence, this result is in the same order of magnitude, which can be considered as an ideal case. If one antibody captures two antigens (peroxidase), the mass variation would reach 29.2 ng. Of course, this result must be considered carefully because the actual surface of the gold electrode, deposited onto the microbalance device, is unknown owing to residual roughness. This leads to approximation concerning the number of antibodies and therefore the estimated antigen mass.

Conclusion

This work illustrates the many possibilities given by the QCM in the biosensing field. A new generation of ultrasensitive piezoelectric transduc-

ers was developed for increasing the mass sensivity, compared with the mass sensitivity of a classic QCM, in order to detect directly small amounts of small biomolecules. An electrochemical calibration was realized to select the good resonant frequency and to clearly estimate experimentally the gain in terms of mass vs a classic 6-MHz piezoelectric device. Then, an easy and fast method for preparing biolayers was developed, and the biospecificity was carefully checked with two couples of antigen/antibody. This is a major result for biomedical purposes because a positive response must be given with a small error. Finally, a calibration curve was drawn that allows direct estimation of an unknown concentration of the detected antigen in a reasonable range of concentration from a bioanalytical point of view.

References

1. Barraud, A., Perrot, H., Billard, V., Martelet, C., and Therasse, J. (1993), *Biosens. Electron.* **8,** 39–48.
2. Nakanishi, K., Mugurama, H., and Karube, I. (1996), *Anal. Chem.* **68,** 1695–1700.
3. Sakai, G., Saiki, T., Uda, T., Miura, N., and Yamazoe, N. (1997), *Sensors Actuators* **B42,** 89–94.
4. Harteveld, J. L., Niewenhuizen, M. S., and Wils, E. R. J. (1997), *Biosens. Bioelectron.* **2(7),** 661–667.
5. König, B. and Grätzel, M. (1993), *Anal. Chim. Acta* **276,** 329–333.
6. Sakai, G., Saiki, T., Uda, T., Miura, N., and Yamazoe, N. (1995), *Sensors Actuators* **B24–25,** 134–137.
7. Chu, X., Lin, Z., Shen, G., and Yu, R. (1995), *Analyst* **120,** 2829–2832.
8. Steegborn, C. and Skladal, P. (1997), *Biosens. Bioelectron.* **12,** 19–27.
9. Guilbault, G., Hock, B., and Scmid, R. (1992), *Biosens. Bioelectron.* **7,** 411–419.
10. Sauerbrey, G. (1950), *Z. Phys.* **155,** 206.
11. Gabrielli, C., Keddam, M., and Torresi, R. (1991), *J. Electro. Chem. Soc.* **138(9),** 2657–2660.
12. Nellen, P. M., Tiefenthaler, K., and Lukosz, W. (1988), *Sensors. Actuators* **15,** 285–295.