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Determination of bovine haemoglobin by a piezoelectric crystal immunosensor

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Summary. A piezoelectric crystal immunosensor for the detection of bovine haemoglobin has been developed. The immobilizing process was monitored by determining the frequency shift of a quartz crystal microbalance (QCM). Bovine haemoglobin concentrations in the range of 0.001 to 0.1 mg/ml could be detected by the system. The influence of the liquid depth on the frequency of QCM was discussed. The interference of bovine serum albumin was also studied.

Directly measuring sensors are of importance for the analyst. Increasing attention is paid to the development of biosensors, especially of those that can be used to determine clinically important molecules. These sensors are so small that they can be carried easily. They can be used without expensive instruments and additional chemicals. The determining procedure is simple and the user does not need special training. The determining speed is fast and is suitable for practical detection. The piezoelectric immunosensor is such a kind of sensor.

Immunoassay methods for the diagnostic determination of biological analytes such as drugs, proteins and hormones have proven valuable for biological analysis, because of the highly specific binding between analytes and antibodies elicited for these analytes. Although immunoassay methods based on labelling techniques such as radioimmunoassay (RIA), fluorescence immunoassay (FIA) and enzyme-linked immunoassay (ELIA) have been extensively used, the cost and time-consumption of these procedures and the safety hazard of RIA have stimulated investigations of new methods. Much attention has been paid to immunosensors in which an immunological reaction, that occurs at the interface of a transducer, results in the output of an electrical signal. Recently, a piezoelectric immunosensor employing the quartz crystal microbalance (QCM) has attracted great interest. The inherent high sensitivity of QCM combined with the selectivity provided by biological coatings could result in a new class of biosensors.

The first application of piezoelectric sensors in immunoassay was made by Shons et al. [1]. They used an antigen-coated crystal exposed to specific antisera and demonstrated a mass increase after washing and drying. In the 1980s, intensive research was made towards developing new piezoelectric biosensors in liquid. The first immunoassay in solution was reported by Bastiaans [2]. A piezoelectric quartz surface acoustic wave (SAW) device was developed to detect human IgG in solution. The sensitivity of the technique was found to be 13 μg and the calibration curve was linear over an IgG concentration range of 0.0225–2.25 mg/ml. Recently, much attention is being paid to the development of new biological coatings, new film-forming techniques and new reuseable biosensors [3–7].

The detection of haemoglobin (Hb) in blood is of importance in clinical diagnosis. Some methods have been developed for the detection of Hb [8, 9]. A spectrophotometric method was employed by Nyyssonen et al. [8] to make an indirect detection of Hb. The linear range of Hb is 0.004–0.5 mg/ml. He Zhike et al. [9] developed a very sensitive chemiluminescence analytical method for the determination of Hb in the presence of KOH, KIO_4 and H_2O_2 with a linear range of $1.0\times10^{-2}-1.6\times10^{-7}$ mg/ml. But the luminescence effect is influenced by the way and time of mixing.

In this paper, we first used a piezoelectric immunosensor to determine bovine haemoglobin (BHb). The antibody of bovine haemoglobin (anti-BHb) was immobilized on one side of the piezoelectric crystal (PC). The interface reaction of BHb and anti-BHb caused the oscillating frequency shift of PC. This shift is proportional to the amount of BHb in solution. Compared with the methods mentioned above, this method is direct, simple and sensitive. We discuss the sensitivity and selectivity of the immunosensor. Some factors influencing the frequency of PC are also discussed.

Experimental

Apparatus

A quartz piezoelectric crystal (AT-cut) with a fundamental frequency of 9 MHz was purchased from Beijing 707 Plant with gold electrodes (about 0.22 cm^2) on either side. The crystal was housed in a perspex cell and clamped between two O-rings. Only one side of the electrodes was exposed to the liquid (Fig. 1). Connection to the external circuit was made by a silver foil glued to the electrode surfaces. An oscillator circuit was constructed from a transistor-transistor logic integrated circuit (TTL-IC), and the crystal frequency was monitored with a frequency counter (CN3165, The Forth Radio Plant, Shijiazhuang, China) (Fig. 2).

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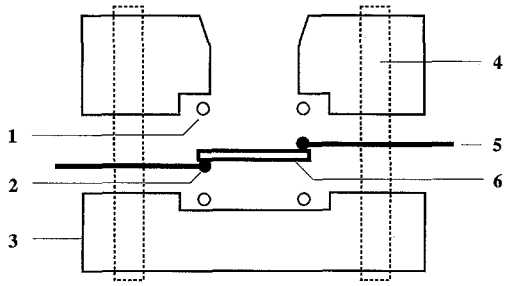

Fig. 1. Structure of the perspex cell. *1* = O-ring; *2* = electric conduction glue; *3* = perspex; *4* = spiral coil; *5* = silver wire; *6* = crystal

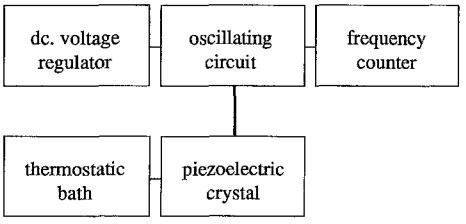

Fig. 2. Block scheme of experimental device

Table 1. Observation of the immobilizing process frequency (MHz)

Crystal	1	2	3
Unmodified electrode	9.017341	9.015490	9.022560
Polyethyleneimine	9.016800	9.015309	–
Glutaraldehyde	9.016512	9.015107	9.021115
Antibody	9.016418	9.015012	9.020904

Table 2. Influence of liquid depth on PC frequency

Volume of PB (ml)	Frequency of UME (MHz)	Frequency of ME (MHz)
0.1	9.017620	9.019713
0.2	9.017810	9.019937
0.3	9.017949	9.020057
0.4	9.018061	9.020145
0.5	9.018142	9.020195
0.6	9.018182	9.020212
0.7	9.018192	ST

PB = phosphate buffer; UME = unmodified electrode; ME = modified electrode; ST = stop oscillating

Materials

Preparation of antibody for BHb. Antigen and Freund's complete adjuvant were injected into male rabbits of 2.6 – 3 kg. The amount of injected antigen was 3 mg for the first time and 6 mg for the others. Subcutaneous muscular injection was used here. The titer was determined after 5 – 7 days for each injection with the double immunodiffusion method. The amount of antigen used in titer determination was 150, 75 and 37.5 μg/ml. 75 or 37.5 μg/ml was optimum. The antiserum with the highest titer was used.

Bovine haemoglobin (BHb) and bovine serum albumin (BSA) were produced by the Shanghai Institute of Biochemistry. Polyethyleneimine, glutaraldehyde and other chemicals were analytically pure; deionized water was used.

Immobilizing method

2 μl methanol solution containing 0.2% polyethyleneimine was dispersed on the surface of the electrode (only one face) and the crystal was dried with an electric dryer. After volatilization of methanol, a thin film of polyethyleneimine formed on the electrode. Then 0.5 – 0.8 ml 2.5% glutaraldehyde solution was added into the perspex cell mounted with the crystal. After 30 min, the unreacted solution was discarded and the electrode was washed with water; then the crystal was exposed to 10 μl anti-BHb and incubated at 4° C for 2 – 3 h. After washing with water, the crystal was exposed to 0.02 mol/l phosphate buffer containing 0.1 mol/l glycine and 0.17 mol/l NaCl (pH 7.2) to block the unreacted aldehyde group. Finally, the electrode was rinsed with HCl-NaCl (1.5 mol/l) solution (pH 3), water and 0.005 mol/l phosphate buffer (pH 7.0) in proper order. The frequency change in the immobilizing process is listed in Table 1. It can be seen from this table that the frequency of PC decreased after every immobilizing step, indicating a mass increase after each step.

Determination procedure

After the quartz PC had been modified as above, 0.3 ml 0.005 mol/l phosphate buffer (pH 7.0) was added into the cell, and the stable frequency was measured. Then the buffer solution was discarded, 0.3 ml BHb solution was added into the cell and reacted for 5 min. After washing the electrode several times with the phosphate buffer, 0.3 ml of the latter was added and the stable frequency was measured. To obtain the frequency shift (Δf) of each BHb concentration, BHb solution was added into the cell from lower to higher concentration and the stable frequency under each concentration was recorded successively. The frequency shift was the difference before and after BHb was added. BHb was added until the immobilized antibody reacted completely with antigen. The measurement was performed at 15 ± 0.1° C.

Results and discussion

Influence of liquid depth on the oscillating frequency

We found that the frequency of PC in the liquid phase changed with the liquid depth above the PC crystal (Table 2). When the depth of the liquid increased, the frequency also increased for both modified crystal and unmodified crystal. The modified crystal ceased oscillating when a certain depth was reached. This may be a result of the influence of liquid pressure on the PC surface. Therefore we must fix the volume of the phosphate buffer solution when recording the stable frequency in our experiment.

Response of the modified PC for BHb

When anti-BHb modified PC was exposed to the solution of BHb, the frequency of PC decreased. For a BHb concentration of 0.001 – 0.1 mg/ml, the frequency shifts (Δf) responded proportionally to the BHb concentration. The linear relation between Δf and BHb concentration could be described as:

$$C = 5.655 \times 10^{-4} \times \Delta f - 0.047$$

where C is the BHb concentration (mg/ml), Δf is the frequency shift (Hz). The linear relation coefficient was 0.989.

The detection limit was 1×10^{-4} mg/ml. No visible frequency change was observed below this concentration.

In the liquid phase it is clear that the resonant frequency is affected by the density, viscosity and conductivity of the liquid. Several equations have been derived to relate the Δf to these factors [10–13].

But each of these equations cannot predict all these factors. In order to solve this problem, some authors employed a reference crystal to compensate the influence of these factors. As the surface of the working electrode is much different from that of the reference electrode, the solution does not have the same influence on them. Our method is to determine the frequency in the same solution under the same temperature. In each frequency measurement the solution remained the same, and it can diminish the influence of non-mass factors.

Selectivity

The response of the modified PC for BSA was also studied. With the same method as for studying the response for BHb, the frequency determined after BSA was added did not decrease compared with that determined before. This suggests that BSA did not react with the anti-BHb immobilized on the crystal. It implies that the linear response is caused by the special binding between BHb and its antibody.

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