

Piezoelectric Immunosensor for SARS-Associated Coronavirus in Sputum

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A piezoelectric immunosensor was developed for the detection of SARS-associated coronavirus (SARS-CoV) in sputum in the gas phase. Horse polyclonal antibody against SARS-CoV was bound onto the PZ crystal surface in an ordered orientation through protein A. The antigen sample was atomized into aerosol by an ultrasonicator, by which the antibody on the crystal could specifically adsorb SARS antigen and the changed mass of crystal would lead a frequency shift. A frequency counter was employed to record the admittance frequency, and the plot of changed frequency was displayed on the computer. Under the optimized conditions, the frequency shifts were linearly dependent on antigen concentration in the range of 0.6–4 $\mu\text{g/mL}$. The device has good reproducibility (could be reused 100 times without detectable loss of activity), stability (the immunosensor was stable for more than two months when stored over silica gel blue at 4–6 $^{\circ}\text{C}$), short analyzing time (less than 2 min), and specificity.

Severe acute respiratory syndrome¹ (SARS) is a viral respiratory illness caused by a coronavirus, called SARS-associated coronavirus (SARS-CoV). SARS was first reported in November 2002. Over the next few months, the illness spread to more than 30 countries. According to the World Health Organization (WHO), to present, more than 8000 people worldwide became sick with SARS; The SARS global outbreak of 2003 was contained; however, it is possible that the disease could reemerge when the temperature is low enough.

The main way that SARS seems to spread is by close person-to-person contact. The virus that causes SARS is thought to be transmitted most readily by respiratory droplets (droplet spread) produced when an infected person coughs or sneezes. On the other hand, oral–fecal transmission might also be an important route of transmission of the disease.²

To cope with the challenge of SARS, many organizations have begun to study how to detect and diagnose it and have had some success. At present, the methods of SARS detection are mainly based on two different theories. One theory is by examining virus RNA by molecular biology, such as reverse transcription-poly-

merase chain reaction (RT-PCR)^{3–5} and fluorescent polymerase chain reaction.^{6,7} They can identify the SARS-CoV in the first part. They require a high specificity between primers and templates but do not have a very high sensitivity so that false negative results are likely. On the other hand, these methods need specific equipment, costly reagents, and complicated technique. The other one is based on SARS antibody analysis in patient sputum, such as the enzyme-linked immunosorbent assay method^{8,9} and immunofluorescence assay.¹⁰ But methods require more time (10–20 days). In this period, the virus would have been transmitted to others

The piezoelectric (PZ) immunosensor is an important biosensor. In 1972, Shons¹¹ made the first PZ immunosensor to detect cow serum IgG antibody. Since then, this method has been important in biodetection for sensitivity, specificity, simplicity, and swiftness. The PZ immunosensor consists of a PZ crystal with an antigen or antibody immobilized on its surface. The biospecific reaction between the two interactive molecules, one immobilized on the surface and the other free in solution or gas phase, can be followed in real time. The PZ crystal is a mass device, which means that any surface mass change reflects on its resonant frequency. The increase of mass at the sensor results in a decrease of the frequency as shown by Sauerbrey, who first introduced the quartz crystal microbalance principle.¹² This relationship of frequency to mass has been outlined by the following equation:¹³

$$\Delta f = - (F^2 \Delta m / NA \rho) \quad (1)$$

where Δf is the change in resonance frequency of the coated

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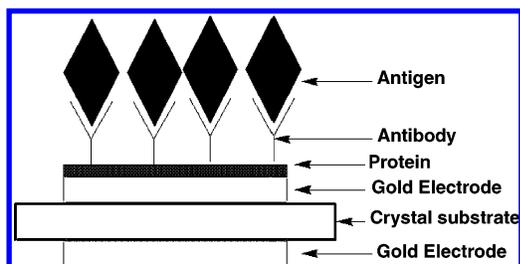


Figure 1. Schematic of adsorption.

crystal, F is the resonance frequency of the crystal, and ρ is the density of the crystal. For the AT-cut quartz crystal ($F = 9$ MHz, $N = 167$ kHz, $\rho = 2.65$ g/cm³), numerical substitution in eq 1 yields

$$\Delta f = -0.183 \Delta M^4 \quad (2)$$

where $\Delta M = \Delta m/A$ and the units of Δf and ΔM are hertz and nanogram per square centimeter, respectively. In this study, A is 0.2826 cm². Thus, if a substance with the weight of 1 ng is homogeneously attached to the active area of crystal, a frequency change of 0.6476 Hz.

In this study, PZ crystals were coated with horse polyclonal antibody induced by SARS-CoV (BJ-01). A protein A layer was chosen to immobilize the antibody.¹⁵ SARS-CoV aerosol was generated by an ultrasonicator. When the coated antibody binds with its antigen by a direct reaction in the gas phase, the mass change on the crystal generates a frequency shift that has a linearity relation with the concentration of the antigen within a certain range. Compared to other SARS detection techniques, this method can rapidly test SARS-CoV at low cost. The adsorption schematic is shown in Figure 1.

The current status of research in PZ immunosensors is considerable, but most research concerns detection in solution. Studies of detection in gas are few in number.^{16–17} In this paper, we investigated a novel method to detect biomacromolecules in the gas phase by ultrasonic atomization.

EXPERIMENTAL SECTION

Instrumentation. All the PZ crystals were AT-cut with a basic resonance frequency of 9 MHz. The crystal consisted of a 12.5 × 0.2 mm quartz wafer, which was placed between two 6-mm gold electrodes, mounted in ceramic holder with a plug. They were purchased from 707 factory (Beijing, China). A low-frequency transistor oscillator, made by our laboratory, was used to drive the crystal. Power for the oscillator was provided by a dual dc power supply DPS-3015, set at 6 V (model 5131A Frequency C & Counter, Agilent Co.), which was used to record the frequency of the vibrating crystal. It collected the current frequency every 6 s. A data collection and processing system, studied by our

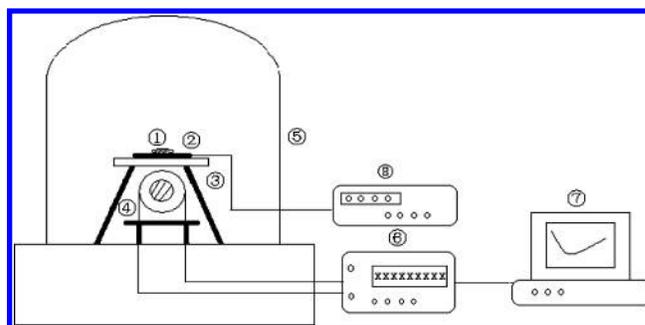


Figure 2. Schematic of experimental setup.

laboratory, could accept the data from the counter and real time display a plot of frequency shift on computer. An ultrasonic logical oscillator with a piezoelectric ceramic wafer (20 × 1 mm), which was used to convert solution to aerosol, was purchased from Yadu Co. (Beijing, China). A BO-2 centrifugal machine, which was used to deal with sputum, was made by Jintan Medical Instrument Factory (Jangsu, China). The schematic of the setup is shown in Figure 2.

Reagents. SARS-CoV (BJ-01) was obtained from the Academy of Military Medical Sciences (AMMS). The SARS-CoV had been inactivated by being dipped in β -propiolactone at 4 °C overnight and at 37 °C for 2 h, but the structure that could especially combine with antigen was still preserved. Horse polyclonal antibody against SARS-CoV was a gift of the AMMS also. Antibody and antigen were both in phosphate-buffered saline (PBS, pH 7). Flu-associated coronavirus (SH-A1) in PBS was purchased from Chinese Center for Disease Control and Prevention. Protein A was obtained from Sigma Co. (St. Louis, MO). All other reagents and solvents were analytical reagent grade or better.

Immobilization Procedure. The PZ crystal was dipped in 1.2 M NaOH for 5 min and in 1.2 M HCl for 5 min and then washed with dual-distilled water and ethanol twice. It was then dipped in ethanol for 15 min and dried in air at room temperature. During treatment, care care was taken to keeping the electrode welding points out of the solution. Its basic frequency F_1 was measured. Then protein A (0.5 μ L, 3 mg/mL) was evenly spread on both sides of the PZ crystal by a 5- μ L syringe, which covered only gold electrode area. (If the reagents adhered to the ambient crystal, the vibration would be interfered with.) It was dried at room temperature, and frequency F_2 was recorded. At last it was coated with 0.5- μ L of antibody of a certain concentration on the protein A layer with the same method. The modified crystal was incubated over silica gel blue at 4–6 °C for 1 h, measuring frequency F_3 .

Measurements. Antigen powder, dried by lyophilization at –70 °C, was dissolved in the sputum of a healthy person. Then the solution was centrifuged at 3000 rpm for 15 min. The top clear liquid was drawn out by syringe and dripped on the wafer of ultrasonic logical oscillator. The SARS-CoV antigen solution was sprayed into the aerosol by ultrasonic oscillation. There was a baffle between the flake and the PZ crystal in order to prevent aerosol from being sprayed into crystal directly. After the antigen solution was sprayed into aerosol, specific adsorption on immunosensor happened so quickly that the decrease of frequency

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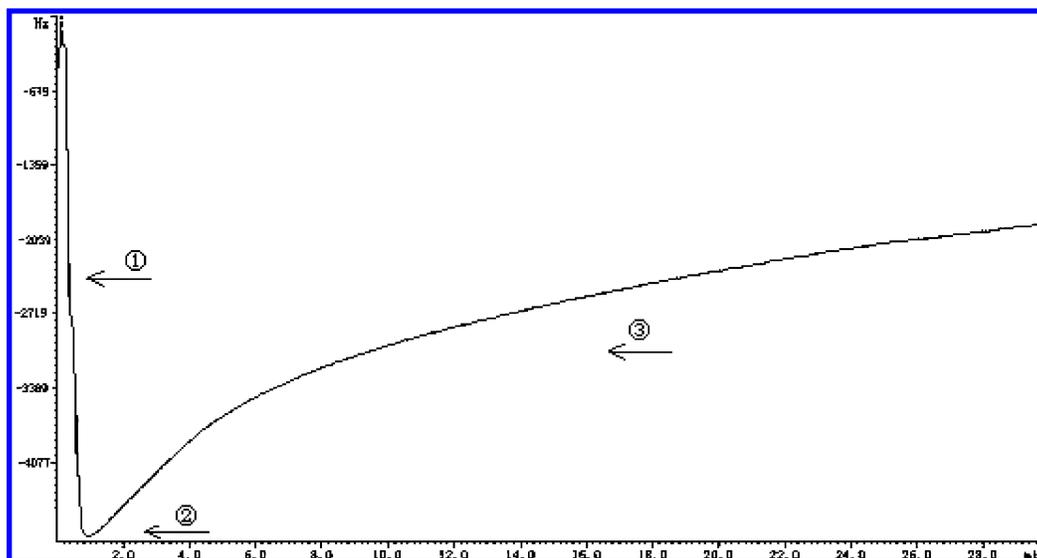


Figure 3. Typical PZ crystal response to antigen gas. The vertical coordinate was the frequency shift and the horizontal coordinate was the elapsed time. (1) was the course of adsorption, (2) was the maximum of frequency shift, and (3) was the course of desorption.

Table 1. Responses of Sensors with Different Antibody Concentrations to 1 $\mu\text{g}/\mu\text{L}$ Antigen Sputum Areasol and Reference Sputum at 25 $^{\circ}\text{C}$ ^a

concn of antibody (mg/mL)	37.50	17.75	15.00	12.50
Δf_1 (ΔHz)	2732	2220	1981	
Δf_2 (ΔHz)	2987	2805	2354	
$\Delta f_2 - \Delta f_1$ (ΔHz)	255	585	373	

^a The volumes of antibody were 0.5 μL . The volumes of antigen solutions were 1 μL and atomized in a 500-mL closed gas cell.

reached maximum within 2 min. The results in Figure 3 depict a typical crystal response. The whole frequency decrease was caused by the antigen aerosol and another (mainly water) gas. So the centrifuged sputum without antigen was measured under the same conditions as a reference (figure not shown).

RESULTS AND DISCUSSION

Atomization. Because the test results were influenced by the status of atomization, a series of experiments was done to optimize the atomizing conditions of the ultrasonic logical oscillator. We determined the peak oscillation conditions as current, 440 mA, time, 0.09 s in a interval of 0.20 s, and the number of oscillations, 15. Under these conditions, the solution (1 μL) could be sprayed into aerosol entirely and the temperature of the surface of the ultrasonic flake would not harm the SARS antigen.

Influence of Antibody Concentration. The 0.5- μL antibody solutions of various concentrations were coated on a protein A layer on crystal and the frequency response to SARS antigen sputum (Δf_2) and reference sputum (Δf_1) was measured. Table 1 shows the different frequency changes.

In the experiment, we found that, when the concentration of antibody was 37.50 mg/mL, the mass of adsorption was large.

Table 2. Differences of 10 Responses of One Sensor to Reference Sputum and Antigen Sputum (1 $\mu\text{g}/\mu\text{L}$) at 25 $^{\circ}\text{C}$ ^a

	times										\bar{X}	δ	RSD
	1	2	3	4	5	6	7	8	9	10			
Δf_1 (Hz)	1825	2231	2143	2160	2253	2057	2233	2109	1827	1870	2071	161	0.0779
Δf_2 (Hz)	2539	2821	2751	2700	2845	2539	2812	2879	2522	2422	2683	155	0.0578
$\Delta f_2 - \Delta f_1$ (Hz)	714	590	608	540	592	482	579	770	695	552	612	84	0.1366

^a The volumes of solutions were 1 μL and were atomized in a 500-mL closed gas cell.

Table 3. Responses of Ten Sensors to Reference Sputum and Antigen Sputum (1 $\mu\text{g}/\mu\text{L}$) at 25 $^{\circ}\text{C}$ ^a

	serial number										\bar{X}	δ	RSD
	1	2	3	4	5	6	7	8	9	10			
$F_2 - F_3$ (Hz)	20513	23806	24278	25160	25308	27136	27581	27652	28251	28679	25836	2399	0.0929
$\Delta f_2 - \Delta f_1$ (Hz)	655	762	884	539	635	490	734	600	697	661	666	107	0.1610

^a The antigen solution and reference were 1 μL , atomized in a 500-mL gas cell.

Table 4. Comparison of SRAS-CoV and Flu-Associated Coronavirus^a

	SARS-CoV	flu-associated coronavirus	reference sputum
$\Delta\bar{f}$ (Hz)	2953	2240	2234

^a Antigen solution concentration was 1 $\mu\text{g}/\mu\text{L}$, and all the liquid volumes were 1 μL , atomized in a 500-mL gas cell.

But the change of frequency often was in disorder (figure not shown). This was because the antigen concentration was too high to be a uniform layer on the crystal. That perhaps would influence the normal vibration of the crystal. When the concentration was 17.75 mg/mL, the response to antigen sputum was 2987 Hz, but to the reference sputum was 2732 Hz. What we needed was the biggest difference between antigen sputum and reference sputum. So the best concentration of antibody was 15.00 mg/mL, and the difference between antigen sputum and reference was as large as 585 Hz.

Precision. Under the same conditions, we measured responses of one 15.00 mg/mL SARS antibody layer sensor to reference sputum and antigen sputum (1 $\mu\text{g}/\mu\text{L}$) for 10 times. To get complete desorption, before the next measurement, the sensor was placed in air for ~ 20 min until the frequency of the sensor recovered. The differences are shown in Table 2.

The average of 10 times was 612 Hz and the relative standard deviation was 0.137. From eq 2, the mass of adsorption was 0.945 μg . Theoretically speaking, if 1 μg of antigen was absorbed on the PZ sensor, the difference of frequency shift would be 647.6 Hz. But, from the above table, some differences were more than this theoretic value. The reason was perhaps that the antigen was much larger than the antibody (the diameter¹ of SARS-CoV was 60–140 nm), so the adsorbed antigens could make a further adsorption of water. We can call that a amplification effect, which improved the immunosensor's sensitivity. From above discussion, we think the average weight of adsorbed antigens was not as high as 0.945 μg .

Parallelism. With the same method, 10 immunosensors were coated. We measured the frequency shifts of crystals with antibody layers (F_3) and without antibody layers (F_2). The differences between F_3 and F_2 related to the thickness of antibody layers as the coating areas were the same. Then, the responses of the 10 immunosensors to the reference sputum and antigen sputum (1 $\mu\text{g}/\mu\text{L}$) were measured. Table 3 shows the differences of the results.

The average of response of frequency was 666 Hz. The relative standard deviation was 0.1610. From the above table, we could

Table 5. Stability of PZ Immunosensor^a

$\Delta\bar{f}_2 - \Delta\bar{f}_1$ (Hz)	stored time (days)												
	0	5	10	15	20	25	30	35	40	45	50	55	60
	2456	2423	2547	2504	2249	2448	2315	2485	2304	2254	2168	2356	2237

^a The concentration of SARS-CoV was 3 $\mu\text{g}/\mu\text{L}$. Before measurement, the sensor would be placed in air at room temperature for 1 h in order to be stable.

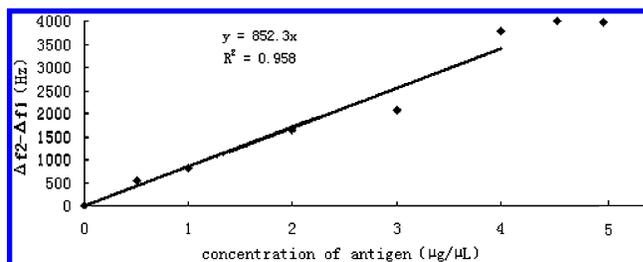


Figure 4. Relationship between antigen concentration and difference of frequency change between responses of antigen and reference.

not find the relation between the resonant frequency and thickness of the antibody layer, for on sensor surface of the same size, the amount of antibody was similar. So the thickness was not an important factor that would influence the frequency shift.

Response to Different Concentrations of Antigen. The 1- μL aliquots of antigen sputum solution containing various concentrations from 1 to 5 $\mu\text{g}/\mu\text{L}$ and the reference sputum were measured to find the relation between resonance frequency and concentration of antigen. The result is depicted in Figure 4, which has good linearity in the range of 1–4 $\mu\text{g}/\mu\text{L}$. The correlation coefficient was 0.958. According to $S/N = 2$, the detection limit was 0.60 mg/mL. Beyond 4 $\mu\text{g}/\mu\text{L}$, The adsorption of surface antibody reached saturation, and the linearity could not be determined.

Interference. Antigen/antibody reactions are theoretically very selective and specific for certain substances even in the presence of larger amounts of similarly structured molecules. To test the SARS-CoV immunosensor's ability against interference from other similar antigens, the coated crystal was exposed to 1 $\mu\text{g}/\mu\text{L}$ flu-associated coronavirus (SH-A1), which is a kind of non-SARS human coronaviruses. The average of five measurements is shown in Table 4.

From the above table, we can see the response of an interference virus was almost negligible.

Reproducibility and Stability. We have tested the immunosensor's reproducibility for more than 100 times, and did not find that the sensitivity had obvious loss (figure not shown). A PZ immunosensor was placed over silica gel blue at 4–6 °C and its response to antigen was measured every 5 days. Table 5 shows the results.

We can see that the immobilized antibody had a wonderful stability. In two months, the responses to antigen did not have a detectable reduction. This result was close to other report¹⁵ that immobilized antibody could keep its activity for a long time in dry conditions. The active limit of the sensor should be further researched.

Conclusion. The result of the experiment is of great importance. It proves the feasibility and simplicity of the piezoelectric immunosensor in detecting SARS-associated coronavirus in sputum in the gas phase. It also paves the way for further research. On the other hand, the method of atomizing antigen aerosol by ultrasonicator and use in detection by a PZ immunosensor has not, to the best of our knowledge, been reported.

In our study, the difference between sputum with SARS-CoV and without SARS-CoV is so obvious that it could be used in

detection of SARS, but it is necessary to investigate further to increase the stability and precision.

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