Latex Piezoelectric Immunoassay: Detection of Agglutination of Antibody-Bearing Latex Using a Piezoelectric Quartz Crystal

Shigeru Kurosawa, Emiko Tawara, Naoki Kamo, Fumihiko Ohta, and Toshiaki Hosokawa

Department of Biophysics and Physical Chemistry Faculty of Pharmaceutical Sciences, Hokkaido University. North 12, West 6, Kita-ku, Sapporo 060, Japan and Ibaraki Research Laboratory, Hitachi Chemical Co., Ltd. 13-1, 4-chome, Higashi-cho, Hitachi-shi, Ibaraki 317, Japan.

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A method for immunoassay of CRP (C-reactive protein) was developed using a piezoelectric quartz crystal. Previous immunoassays using a piezoelectric crystal have required the formation of a thin film on the crystal, to which an antibody is affixed. The occurrence of antigen–antibody reaction increases the weight attached to the crystal surface, which causes a reduction in the oscillation frequency. In our method, the frequency reduction was observed using antibody-bearing latex without any film. One possible mechanism of the frequency change is that the crystal acts as a sensing apparatus for viscosity or density change in the solution due to aggregation of latex particles. The detection limit was almost the same as that for latex photometric immunoassay (LPIA). The present method has been designated as latex piezoelectric immunoassay (LPIA).

Keywords antibody-bearing latex; CRP (C-reactive protein); piezoelectric quartz crystal; immunoassay

An increase in C-reactive protein (CRP) has been found in the blood during the course of virtually all diseases involving active inflammation or tissue destruction, sometimes to a level one thousand-fold above normal. Estimating the blood concentration of this protein is hence a good indicator for the diagnosis of many inflammatory diseases.1–3

CRP has been measured by immunoassay: the methods employed are the capillary precipitation test, single radial immuno-diffusion (SRID) and latex photometric immunoassay (LPIA).4,5 LPIA involves an agglutination reaction of anti-CRP-bearing latex when the assay medium contains CRP. The reaction is measured in terms of light scattering or absorbance change. This method is very sensitive, highly precise and reproducible, and is easy to use in automated serum tests.

A piezoelectric quartz crystal has been used as a microbalance. Adsorption on the surface of the crystal changes the oscillation frequency, and the adsorption of about 1 ng of material decreases the frequency by 1 Hz when a 9 MHz AT-cut quartz is used.6 Many attempts have been made to use this very sensitive and convenient apparatus for immunoassay7–9; in those studies, a thin film was formed on the quartz surface to which antibody was affixed, usually by chemical treatments. Binding of antigen to the antibody increases the mass at the surface, resulting in a reduction in the frequency of oscillation. In some cases,10 latex was used to increase the frequency change by about 2 times, because the use of antibody immobilized on latex increases the mass attached to the crystal, compared with antibody only. Here, we should stress that the fixation of antibodies is essential in that method; the frequency change is thought to be due only to the change in the weight attached to the crystal, and the latex plays only an auxiliary role in the detection. Formation of a thin membrane and fixing of antibodies is generally not easy.

Bruckenstein and Shay11 and Kanazawa and Gordon12 derived a theoretical equation concerning the oscillation frequency change in the quartz in solution, \( \Delta F = -K \sqrt{\rho \eta} \), where \( \Delta F \) represents the frequency change in solution, \( K \) a proportionality coefficient, \( \rho \) the density of the solution and \( \eta \) the viscosity of the solution. Our previous paper13 experimentally demonstrated the validity of this equation. Utilizing the characteristic that the frequency change of the crystal is very sensitive to viscosity change, Muramatsu et al.14 observed the frequency change due to the gelation of fibrinogen.

Antigen induces an agglutination reaction of the antibody-bearing latex. The occurrence of agglutination may change the viscosity and/or density in the assay solution and should be detectable as a change of the oscillation frequency of the quartz crystal. This expectation led us to carry out the present experiment using anti-CRP-bearing latex, and we found the frequency decrease to be dose-dependent. Serum containing no CRP did not induce any frequency change. This method, termed “latex piezoelectric immunoassay” (LPIA) has the advantage that the formation of a thin film on the crystal and attachment of the antibody by elaborate chemical treatments are unnecessary. The method can also easily be automated for use in clinical tests.

Method and Materials

Chemicals used were guaranteed reagents purchased from Wako Pure Chemical Co. (Osaka). Water was prepared with a Milli-Q (Millipore Ltd.) and its specific resistance was more than 18 MΩ/cm. CRP standard (CRP concentration 5.1 mg/dl, Seratestam S, Hitachi Chemical Co., Ltd.) and anti-CRP coated latex (size ca. 0.1 μm, 0.05% Seratestam CRP-H, Hitachi Chemical Co., Ltd.) were used. Normal human serum containing no CRP (whose total protein concentration was 5.2 g/dl) was obtained from I.I.C. Japan and diluted 6-fold before use with 10 ms phosphate-buffered saline (PBS), pH 6.5, (formed by mixing Na₂HPO₄ and KH₂PO₄ solutions, which contained 5% bovine serum albumin (BSA) (fraction V from Sigma), 16.5 mmNa₃S, and 135 mmNaCl. To examine the selectivity, anti-streptolysin O-coated latex (serastersam ASO-6, Hitachi Chemical Co., Ltd.) was used instead of anti-CRP-coated latex.

AT cut piezoelectric quartz crystals (9 MHz resonance frequency) were purchased from Yukumo Tushin Co. (Tokyo). One side of each crystal was sealed with a quartz plate and silicon sealant-45 (Shin-Etsu Kagaku); as described,15 this treatment stabilizes the oscillation frequency in solution.

Figure 1 shows a schematic illustration of an experimental setup. An electronic circuit with TTL was the same as that described previously.12,15 The oscillation signal was fed to a universal counter (Model 7202, Iwatsu, Tokyo) and the frequency change was stored in a microcomputer (NEC PC9801). The latex solution was circulated over the crystal by means of a peristaltic pump (model MP-3, Tokyo Rika Kikai); the total volume of the circulated solution was 9.0 ml. The chamber into which the quartz crystal
was placed had a volume of 2.0 ml. The buffer solution used to suspend the suspension of latex was 10 mM phosphate buffer (prepared by mixing Na₂HPO₄ and KH₂PO₄) at pH 6.50; the rate of circulation was 5.7 ml/min. Silicon tubing (inside diameter, 2 mm) was used. The entire apparatus (except the microcomputer) was accommodated in a chamber maintained at 37 ± 1 °C, unless otherwise noted.

As described above, in solution the following equation holds: \( \Delta F = -KJ \sqrt{\eta} \). The procedure of sealing one side of the crystal altered the K value to different extents from one crystal to another depending on the amount of sealant, but \( \Delta F \) remained proportional to the change in \( \sqrt{\eta} \). The value of K could be easily determined with 5% and 10% sucrose solutions. In order to calibrate the frequency change, data were expressed in terms of \( -\Delta F/K \).

The procedure was as follows. First, pure water was applied and the oscillation frequency was measured. A sucrose solution of 5 wt.% was circulated and the frequency change from that in pure water was recorded, followed by the same procedure with a sucrose solution of 10 wt.% These two values of frequency change allowed us to calculate K of the quartz crystal, and the values determined agreed within 3%. Then, the crystal was rinsed with pure water and a latex suspension (9 ml) was circulated. For most experiments, 1 ml of the stock latex suspension (0.05%) was added to 8 ml of 10 mM phosphate buffer at pH 6.5. The effect of the latex concentration on the frequency decrease is described later. After the frequency had stabilized (ca. 30 min), CRP standard serum solution (25–150 μl) was added.

The agglutination reaction was traced in terms of the change in turbidity. The spectrophotometer used was a Shimadzu UV-300 (Shimadzu, Kyoto). The cuvette was stirred (360 rpm) by a magnetic stirrer and the absorbance change at 570 nm was monitored.

Results and Discussion

Figure 2 shows typical data on frequency change during the addition of CRP. At \( t = 0 \), 100 μl of CRP stock solution (final concentration, 56 μg/ml) was added and a reduction in frequency was observed. About 60 min after the addition, the frequency became constant. The time course of the change seemed to have two components, but their origin is not clear at present. When the temperature was decreased to 25 °C, the rate of change as well as the extent was decreased (data not shown). Table I shows the reproducibility of the present method (LPEIA) using nine crystals. The value of \( \Delta F \) is the frequency change of the crystal upon addition of CRP (56 μg/ml final CRP concentration and 0.00556% final latex concentration). The mean value and the standard deviation are 105.1 and 20.3 Hz, respectively and the variation coefficient is 19.3%. The fourth column lists values of \( (-\Delta F/K) \times 10^3 \) and the second column gives the K values of the crystals after being sealed on one side. The mean value and standard deviation of \( -\Delta F/K \) are \( 3.82 \times 10^{-3} \) and \( 0.354 \times 10^{-3} \), respectively. The variation coefficient is 9.27%, which is much smaller than that of \( \Delta F \) (19.3%), implying the validity of the treatment of reduced frequency changes (\( -\Delta F/K \)).

When the same volume (100 μl) of normal human serum as a control was added, only a 20-Hz change (which is equivalent to \( -\Delta F/K \) of 1.60 × 10⁻²) was observed. At 50 μl of normal human serum, the change was only 15 Hz (\( -\Delta F/K \) of 0.98 × 10⁻³). Another control experiment employed latex bearing an antibody other than CRP; we used anti-streptolysin O-coated latex. The same experimental conditions as in Fig. 2 were employed, and application of CRP and normal human serum caused a change of only 27 Hz (\( -\Delta F/K \) of 0.99 × 10⁻³). These results show that the present system is specific to CRP, whose antibody is immobi- lized on the latex. Another control experiment was performed without antibody-bearing latex; addition of CRP (final concentration of 56 μg/ml) and normal human serum of the same concentration gave almost the same frequency change of 250–300 Hz (12–13 × 10⁻³ of \( -\Delta F/K \)). Possible reasons for the frequency change are adsorption of proteins on the crystal surface and the change in the viscosity and density of the solution upon addition of proteins.
that the sensitivity is almost the same for the two methods. A comparison of Figs. 3 and 4 also supports this conclusion. Thus, LPEIA should be applicable for clinical use. A possible way to reduce the assay volume is discussed later.

The agglutination reaction was followed in terms of the turbidity change at 570 nm. The cuvette contains 0.21 ml of latex suspension, 1.5 ml of 10 mM phosphate buffer (pH = 6.5) and various amounts of standard CRP serum solutions. The results are shown in Fig. 4, where the absorbance changes at final CRP concentrations of 44.3, 88.0 and 145 µg/dl are plotted against time. The magnitude of absorbance change was almost the same at 145 and 88 µg/dl, indicating that the response is saturated at about 88 µg/dl. Figure 3 indicates that the present method has a wider working range of concentration. When the CRP concentration was above 88 µg/dl, the absorbance change after 10 min was found to be decreased, perhaps due to the precipitation of bridged particles. Comparison of the time courses in Figs. 2 and 4 shows that they are not the same, indicating that the factor giving rise to the frequency decrease is different from that causing the turbidity change; the reason for this is not clear at present. We cannot rule out the possibility that aggregates formed between antigen and antibody-bearing latex are adsorbed on the crystal surface, leading to a weight increase and thus the frequency decrease.

Umezawa developed an ion-selective immuno-electrode for CRP determination. His method involves liposome immune lysis: Liposomes are lysed by an antigen–antibody reaction in the presence of complement. The degree of lysis is dependent on the concentration of antigen and is measured in terms of the leakage of membrane-impermeable markers entrapped within the liposomes. He used tetrapentylammonium (TPA+) as a marker and the concentration of leaked TPA+ was measured with an ion-selective electrode. According to his data on potential change versus CRP concentration curve, the most suitable CRP concentration was around 10000 µg/dl (100 µg/ml), which is much larger than that optimal for the present method.

A number of papers have been published concerning immunoassay using piezoelectric quartz crystal. In all cases, the antibody on the film was affixed on the crystal surface. Reaction of antigen with the fixed antibody increased the mass, which, in turn, changed the frequency; a typical frequency change was about 250 Hz. Although the magnitude of the frequency change in LPEIA is less than that, it is sufficient and offers the added advantage of not requiring the troublesome fixation procedure. Moreover, the previous method requires an exact quantity of antibody to be affixed if a constant dose–response curve is to be obtained for every crystal, or requires a method for dissociation of the antigen from the antibody–antibody complex without detachment of the antibody from the crystal surface in order to make a calibration curve. Table I shows the reproducibility of our method with nine independent crystals; the results imply that the present method does not require such a process.

The latex-immunoassay method described here is simple, but several modifications should be made for actual clinical application.
Fig. 5. Relationship between Latex Concentration and Magnitude of Frequency Change Caused by the Agglutination Reaction

The latex concentrations tested were 0.00556, 0.0111, and 0.0167%, and CRP concentration was maintained at 25 μg/dl.

**Reduction of Assay Volume** For this purpose, we should develop a small cuvette into which the crystal can be dipped, or a small chamber on one of the crystal surfaces where the immuno-reaction can be performed.

**Reduction of Assay Time** The turbidity method showed that the agglutination reaction took about 10 min to complete. Since the crystal and electronic equipment are not expensive and since the reproducibility with independent crystals is good (see Table 1), a device allowing several parallel assays could be employed to increase efficiency. Another possibility may be the use of initial rate analysis. Unfortunately, the present experimental system cannot afford suitable data for initial rate analysis. The poor reproducibility of initial rates may be due to the influence of the peristaltic pump, and further development or modification is needed to improve this.

**Increase of the Magnitude of Frequency Change** There are several possible ways of achieving this. As shown previously, the magnitude of the frequency change increases with increase of the resonance frequency of the crystal, and we may therefore use a crystal of higher resonance frequency. Figure 5 shows the dependence on the latex concentration. The data described above were obtained at 0.00556% (1 ml of the stock latex suspension was diluted to a final 9 ml). As shown in this figure, the magnitude of response increases with increase of the latex concentration. Although the present experiment was done at a relatively low concentration of the latex to minimize consumption of the expensive antibody-bearing latex, this figure shows clearly that a higher concentration of latex would be more effective. Therefore, some means of reduc-

ing the assay volume is necessary to reduce the amounts of latex required. The adsorption on the crystal surface induces changes of the resonance frequency together with √(ρ/η). If the surface of the crystal is covered with a thin film which binds antigen or antibody, an increase in the magnitude of frequency change can be anticipated. We found that the amount of antibody adsorption is increased 2- or 3-fold when a plasma-polymerized film of amine compounds is employed. Thus, covering the crystal surface by this means may increase the response magnitude. Umezawa and his colleagues discovered that CRP bound specifically at the site of phosphoryl choline residues on the lipid membrane. Another possibility, therefore, is to coat the crystal surface with phospholipids. Further studies are in progress.

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**References**