Pergamon

Talanta, Vol 41, No 3, pp 401–406, 1994

0039-9140/94 $6 00 + 0 00

IMPROVEMENT OF LATEX PIEZOELECTRIC IMMUNOASSAY: DETECTION OF RHEUMATOID FACTOR

HEDAYAT O. GHOURCHIAN and NAOKI KAMO*

Department of Biophysical Chemistry, Faculty of Pharmaceutical Sciences, Hokkaido University, North 12, West 6, Kita-ku, Sapporo 060, Japan

TOSHIAKI HOSOKAWA and TATSUO AKITAYA

Pharmaceutical Research Laboratory, Hitachi Chemical Co , Ltd , 13-1, 4-chome, Higashi-cho, Hitachi-shi, Ibaraki 317, Japan

(*Received* 6 *July* 1993 *Revised* 7 *September* 1993 *Accepted* 7 *September* 1993)

Summary—Latex piezoelectric immunoassay is a method for detection of agglutination of antibody- or antigen-bearing latex by immunoreaction using a piezoelectric quartz crystal, the agglutination decreases the oscillation frequency of the crystal. This is advantageous in that coating the surface of the crystal followed by fixation of antibody or antigen is unnecessary There is, however, a drawback, and to improve this, we designed a micro-cell in which only one side of the crystal is exposed to the solution A method for regenerating the crystal was also devised Measurement was carried out using a calibration curve of the frequency change against rheumatoid factor activity The improvement made it possible to use one crystal repeatedly and reproducibility was satisfactory. The calibration curve became almost independent of the crystal used

Piezoelectricity was discovered in 1880[1] and Sauerbrey[2] applied piezoelectricity of a quartz crystal to a mass sensor in 1959. Application of an alternative electric field to the crystal raises the vibration wave within the crystal and resonance occurs when the inherent vibration frequency of the crystal matches the frequency of the external electric field. This phenomenon is utilized in an electric oscillator circuit whose oscillation frequency is largely determined by the physical property of the crystal. Adsorption on the surface of the crystal changes the oscillation frequency, and an adsorption of about 1 ng of material decreases the frequency by 1 Hz when a 9 MHz AT-cut quartz is used. Due to its high sensitivity, this mass sensing device is often referred to as the quartz crystal microbalance (QCM).

Many attempts[3] have been made to use this very sensitive and convenient device for immunoassay. The general approach to utilizing the device in an immunosensor is to coat the crystal with a certain film to which either antigen or antibody is fixed by a chemical treatment. Immunoreaction increases the mass, which is detected by the QCM. The problem of such a piezoelectric immunosensor is often a lack of reversibility.[4,5]

Kurosawa *et al.*[6] developed a new method of piezoelectric immunosensor and called it the Latex Piezoelectric Immunoassay (LPEIA); they found that agglutination of antibody-bearing latex by immuno-reaction in the solution caused a frequency change of the quartz crystal dipped into the solution. With this method, they succeeded in assaying C-reactive protein and later Muratsugu *et al.*[7] used it to detect anti-streptolysin O. antibody. In contrast to usual methods, this technique does not require the formation of a thin film on the crystal followed by fixation of the antibody or antigen. However, there are some points requiring improvement. In order to oscillate stably in a phosphate-buffered saline solution, one side of the crystal should be sealed with a silicon sealant. This treatment is time-consuming and moreover, changes the sensitivity between crystals. After its use, the sensitivity of a crystal was changed, due presumably to alteration of the surface. This made necessary a time-consuming calibration process prior to each use to improve the poor reproducibility. In the present work the

*To whom all correspondence should be addressed

LPEIA was modified and an effort made to improve the above mentioned problems. We applied the improved method to the detection of rheumatoid factors.

EXPERIMENTAL

Apparatus and materials

A 9 MHz AT-cut quartz crystal (8 × 8 mm) with silver electrodes (diameter 5 mm) on both sides was used in all experiments, and was obtained from Yakumo Tsushin Kogyo Co. (Tokyo). In order to expose only one side of the crystal to the assay solution, a new detection cell was designed; the crystal was mounted in the cell between two sheets of silicon rubber and held tightly by two screws (Fig. 1(B)). The electrode was in contact with the inside cell solution through a hole whose diameter was the same as that of the electrodes. The total volume of the cell was adjusted to 400 μl, and can be reduced to less than 100 μl when necessary. To keep the temperature constant, the detection cell was placed inside a water-jacket vessel, and the space between the cell and the vessel was filled with sand (sea sand B, 20–90 mesh, Nacalai Tesque Inc., Japan). Thermostat water (Taiyo Thermo Unit C-600, Japan) was circulated through the water jacket vessel (25 ± 0.2°C except during the examination of temperature effect). This vessel was accommodated in an isolated chamber whose temperature was regulated.

A laboratory-made oscillating circuit (see Fig. 1(A)) was used to measure the oscillation frequency, and the frequency was counted with a universal frequency counter (Model SC7201; Iwatsu, Tokyo) which was controlled by a micro-computer (PC9801, NEC, Tokyo) with a GP-IB interface. Every 20 sec the value of the oscillating frequency was stored in the computer. The entire assembly of the assay system is shown in Fig. 1(C).

Antibody-bearing latex (0.11 μm in diameter) and rheumatoid factor serum (polyclonal rheumatoid factor, anti IgG, 72 IU/ml) was obtained from Hitachi Chemical Co. (Hitachi, Japan) or Behring (Tokyo, Japan). The latex concentration was 0.25% w/v Human serum albumin (HSA) and bovine serum albumin (BSA) were

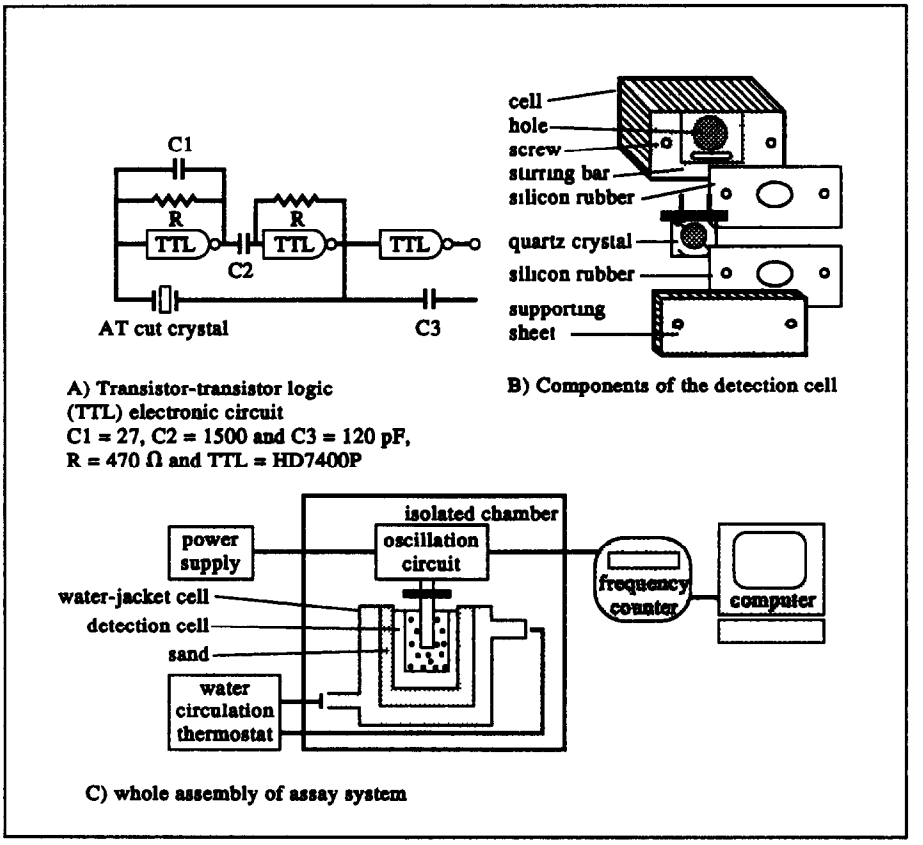

Fig 1 Schematic diagram of (A) electronic circuit, (B) detection cell, and (C) entire assembly of the assay system

purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.). Poly(vinyl sulfonic acid, sodium salt, 25% wt in water solution) (PVS) was obtained from Aldrich Chemical Company Inc (Milwaukee, WI). Polyethylene glycol (PEG) whose MW was 6000 and other chemicals were obtained from Wako Pure Chemical Co. (Osaka, Japan). Water with 18.3 MΩ · cm was prepared with a Millipore Milli-Q apparatus (Tokyo, Japan).

Buffer solution

To optimize the medium composition, frequency changes under a variety of conditions were measured. The optimized buffer solution was 10 m*M* phosphate buffer (pH 7.4) containing 3 5 g/l sodium chloride and 15 g/l PEG as a reaction enhancer.[8,9] The ionic strength of the buffer solution is important to avoid non-specific precipitation of labile substances in the samples.[8]

The C1q protein contained in human serum may bind to the Fc portion of IgG, possibly causing positive interference. To prevent this binding, PVS was added to the phosphate buffer solution (final conc. 6 g/l).[9] PVS does not reduce rheumatoid factor activity. The phosphate buffer prepared in this way can be stored in a refrigerator

Procedure

A 9 MHz quartz crystal was placed in water for about 72 hr. After washing with milli-Q water it was dried in air and fastened to the detection cell, into which 360 μl of the phosphate buffer described above was poured. The solution was gently agitated with a small stirring bar (2 × 2 mm). When a frequency change was no longer observed 10 μl of the antibody-coated latex solution (2.5 g/l) was added. After stabilization of the frequency, 1–30 μl of the standard serum containing rheumatoid factor (72 IU/ml) was added. A decrease in the frequency was observed and the rate of this frequency change gradually lessened; when the rate reached a value of less than 2 Hz/min, the total shift was recorded and denoted as a frequency change (ΔF) (see Fig. 2). From the frequency change and the amount of rheumatoid factor, a calibration curve was plotted. The curve was prepared by successive addition of a standard sera containing active rheumatoid factor.

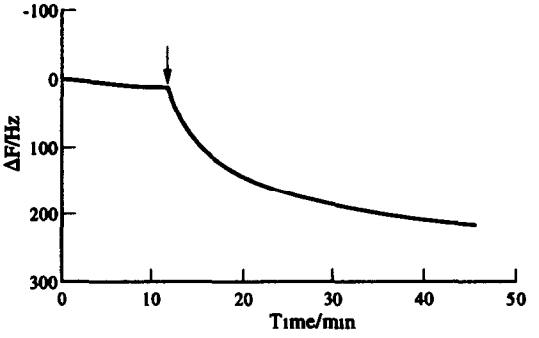

Fig 2. Typical time-course of the frequency change Ten microlitres of latex solution (2 5 g/l) was added to 360 μl of buffer solution After frequency stabilization, 12 μl of rheumatoid factor (72 IU/ml) was added to the buffer solution, and the frequency decreased (The arrow shows the addition moment of the rheumatoid factor in the solution) The final concentration of latex and rheumatoid factor was 0 0654 mg/ml and 2 26 IU/ml Temperature was 25°C The magnitude of the response ΔF is defined hereafter as the frequency difference between that before the addition of rheumatoid factor and that when the rate of change is less than 2 Hz/min

RESULTS AND DISCUSSION

Crystal surface regeneration

As described above, the original LPEIA has some drawbacks: one is the necessity for sealing that prevents re-use of the crystal, and, second, due to this sealing the calibration curve depends on the crystal used (see Ref. 5). To avoid sealing, we developed a cell so that one of the crystal surfaces is open to the assay solution. After a crystal was used for assay, its sensitivity was decreased by 40–50%, implying that the electrode surface becomes dirty, perhaps due to the adsorption of the latex particles or proteins This also suggests that the surface of the electrode must be clean for reproducible results

Accordingly, to remove the adsorbed latex and regenerate the surface of the electrode, at the end of each assay the crystal was washed carefully with water followed by ethanol. It was then placed in a petri dish to be heated at *ca* 200°C for 5 min and then was cooled down to room temperature. The resonator had a thick, wide plug so that the crystal stuck out into the air. Care was taken that the crystal surface did not come in contact with the petri dish during the heating process. The optimized condition was that the cooled-down crystal was allowed to stand overnight at room temperature and was again heated for 5 min before use.

Table 1 shows the effect of the regeneration process: after use in the immunoassay, the crystal was subjected to the regeneration process

Table 1 Reversibility of the quartz crystal after regeneration of the surface

Number of regeneration	Frequency of the quartz crystals (*Hz*) (Number of crystal) #1	#2	#3	#4	#5	#6
1	8,990,568	8,992,138	8,992,301	8,991,800	8,992,045	8,992,523
2	8,990,447	9,919,498	8,992,402	8,992,203	8,991,915	8,992,723
3	8,990,312	8,992,012	8,992,339	8,991,903	8,991,725	8,992,568
4	8,990,247	8,992,071	8,992,058	8,992,218	8,991,806	8,992,751
5	8,990,270	8,992,145	8,992,403	8,992,138	8,992,022	8,992,951
6	8,990,440	8,991,968	8,992,333	8,992,244	8,991,753	8,992,842
Mean value	8,990,370	8,992,035	8,992,306	8,992,084	8,991,877	8,992,726
Spread	321	196	345	418	300	428
σ	125	85	128	187	137	126

*The values show the stabilized frequency in the buffer solution after the regeneration process of crystals designated from #1 to #6 For details of the process, see text Here, spread means the difference between maximum and minimum of stabilized frequencies, and σ standard deviation

and the oscillating frequency was measured in the phosphate buffer solution. Six separate crystals were tested. The maximum spread of the frequency (difference between maximum and minimum) was 428 Hz for crystal number 6 and the maximum standard deviation was ± 187 for crystal number 4; these values are negligible compared with the fundamental frequency of the crystal (9 MHz). Figure 3 shows the ability for re-use and reproducibility for LPEIA: after 38 uses and the subsequent regeneration process, the frequency was still stable and the response (ΔF) was reproducible

Pre-conditioning of a new crystal in water for about 72 hr increases the frequency change remarkably· for example, a response of about 60 Hz was obtained when pre-conditioning was omitted, while responses of 125, 150 and 170 Hz were obtained, respectively, after 24, 40 and 72 hr pre-conditioning of a quartz crystal. It is worth noting that a regenerated (heated) crystal showed *ca* 20 Hz higher response than a pre-conditioned crystal that had not been subjected to the regeneration process. Exposing the used and washed crystal to the hot air possibly facilitates the evaporation of adsorbates and prepares a clean surface for the next experiment. The regeneration process, therefore, not only allows repeated use but also increases sensitivity.

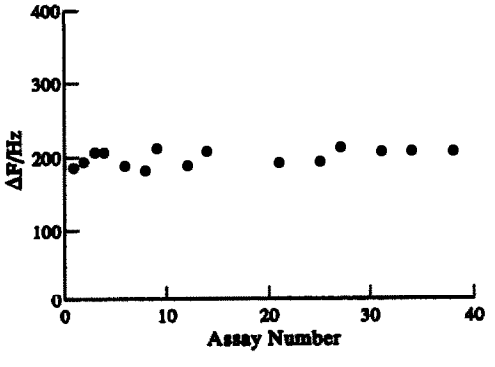

Fig 3 The regeneration procedure gives reproducible ΔF. After 38 uses of the same crystal and the subsequent regeneration process, values of ΔF were almost the same For details of the procedure, see text Experimental conditions were the same as in Fig. 2.

Measurement method

The rheumatoid factor activity was evaluated from ΔF. To determine optimal experimental conditions, ΔF was measured under a variety of conditions. In these experiments, the volume ratio of buffer solution, antibody-bearing latex and standard serum was, respectively, kept constant at 360/10/12 μl. (For all of the experiments the rheumatoid factor serum and the antibody-bearing latex suspension were taken directly from the stock solutions of 72 IU/ml and 2.5 g/l, respectively.) Figure 4 shows the effect of temperature on ΔF; the response decreases with the increase in temperature and the decrease is manifest above 37°C. It is unlikely that at higher temperature the activity of IgG

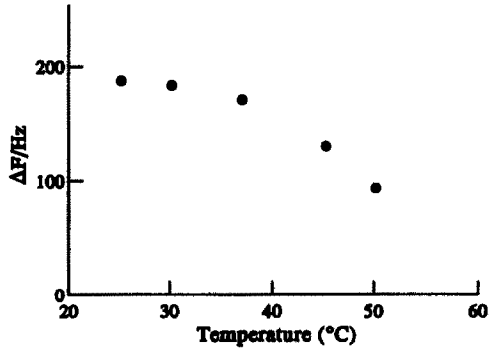

Fig 4 Temperature dependence on ΔF Experimental conditions was the same as in Fig. 2 except for the temperature

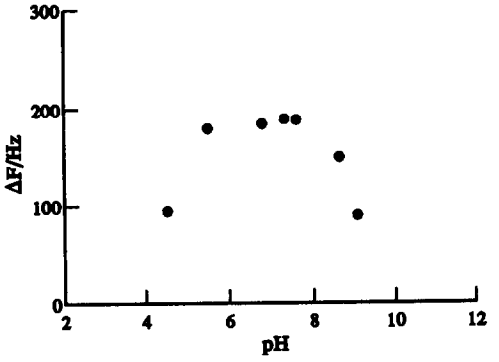

Fig 5 pH dependence on ΔF Experimental conditions were the same as in Fig 2 except for the pH in the assay medium

decreases, since after 50°C-treatment to inactivate complements, IgG is usually still active. This decrease therefore seems to come from the efficiency that the agglutination of latexes induces the frequency decrease, not from the decrease in the antigen–antibody reaction. The plot of ΔF against pH in the experimental medium (Fig. 5) shows that the optimum pH ranges from 5.5 to 8.0. It is most probable that outside the optimum pH ranges, the reactivity may decrease, which in turn decreases the frequency changes.

Figure 6 represents a calibration curve resulting from four independent measurements. The mean value and standard deviation at different concentrations are shown. The values of ΔF were almost the same even though four independent crystals were used, a point in sharp contrast to that of the original LPEIA (cf. Ref. 6); we should stress that this is due to the regeneration process developed in this paper. We are able to measure the concentration of rheumatoid factor of even less than 5 IU/ml, making the

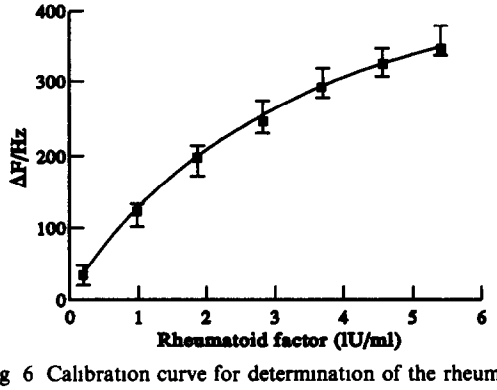

Fig 6 Calibration curve for determination of the rheumatoid factor The optimized experimental conditions were employed (for details, see text). Vertical bars stand for the standard deviation

present system adaptable for clinical use from the view point of its sensitivity.

Note on mechanism of the frequency change

When BSA (bovine serum albumin) or HSA (human serum albumin, main components of serum proteins) was added to the buffer solution (final concentration, 5 mg/ml) containing the antibody-bearing latex, there was a change of about 50 Hz. This is due to the adsorption of the protein to the surface, which reduces the oscillating frequency. As shown in Fig. 6, the addition of serum containing rheumatoid factor induced even a greater change, depending on the activity of the factor, indicating that the frequency change comes from the agglutination of the latex caused by immunoreaction.

When the crystal is dipped into a solution, the origin of the frequency change is that of $(\rho\eta)^{1/2}$,[10,11] as well as due to substances adsorbed on the surface. Here, ρ and η stand for the density and viscosity of the solution, respectively. Formation of agglutinated latex complex may change $(\rho\eta)^{1/2}$ which, in turn, changes the oscillation frequency. It seems, however, that the change of $(\rho\eta)^{1/2}$ is not the only factor that induces the frequency change. When the used crystal was rinsed with water and/or ethanol, a frequency change of about 95 Hz was observed. This indicates that antibody-bearing latex is adsorbed onto the electrode surface and forms a thin coating layer at the interface so that, during the first assay, the antibody-latex layer is selectively attached to the antigen through the antibody–antigen complex formation. The importance of the interfacial structure in solutions which affects the frequency change was pointed out.[12] The result mentioned above also indicates that the complex cannot be removed by rinsing alone, however, the regeneration process can remove it

CONCLUSION

The designed cell with a minimum volume of about 100 μl can be used in the LPEIA and can replace the time-consuming and complex procedure for one side sealing of the crystal employed previously. Use of the surface regeneration procedure developed in the present paper allows cleaning of the electrode surface and repeated use of a crystal. The process also increases the frequency change and the calibration curve becomes less dependent on the crystal used. The exact mechanism of the

frequency change is still not known, however, and further study on this is required.

Acknowledgements—The authors wish to thank Dr Makoto Muratsugu of Asahikawa Medical College for his useful advice and Mrs Toshi Honna of Hokkaido University Hospital who kindly prepared human sera containing the rheumatoid factor This work was supported financially in part by the Suzuken Memorial Foundation

REFERENCES

1 J Curie and P Curie, *Comp Rend*, 1988, **91**, 294
2 G Z Sauerbrey, *Z Phys*, 1959, **155**, 206
3 J H T Luong and G G Guilbault, *Bioprocess Technol*, 1991, **15**; (*Biosens Princ Appl.*), 107 Dekker, New York
4 G G Guilbault, J H T Luong and E P Sochazewski, *Biotechnology*, 1989, **7**, 349
5 J Janata, *Anal Chem*, 1992, **64**, 197 R
6 S Kurosawa, E Tawara, N Kamo, F Ohta and T Hosokawa, *Chem Pharm Bull*, 1990, **38**, 1117
7 M Muratsugu, S Kurosawa and N Kamo, *Anal Chem*, 1992, **64**, 2483
8 P Tuengler, E Metzmann, H E Pauly and W Beker, *Behring Inst Mitt*, 1988, **82**, 282
9 L Borque, A Rus and R Ruiz, *Eur J Clin Chem Clin. Biochem*, 1991, **29**, 521
10 S Bruckenstein and M Shay, *Electrochim Acta*, 1985, **30**, 1295
11 K K Kanazawa and J G Gordon II, *Anal Chim Acta, 1985*, **175**, 99
12 M Thompson, A L Kipling, W C Duncan-Hewitt, L V Rajakovic and B A Cavic-Vlasak, *Analyst*, 1991, **116**, 881