Visualization of Immunoassay Based on a Spot Test and Its Application to C-reactive Protein

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A simple and rapid spot test for visual immunoassay based on latex immunoturbidimetry has been developed. A cationic dye, Ethyl Violet, was used as a labeling agent to bind to bovine serum albumin on latex particle surface. When a 10 μL sample solution is set on a hydrophilic polytetrafluoroethylene membrane film, a blue ring-shaped stain is formed on the film surface. This method has been successfully applied to C-reactive protein.

Instrumental techniques for immunoassay, such as time-resolved fluorometry and particle-enhanced nephelometry using laser immunoassay are highly developed. Although ELISA has also been widely used in ultrasensitive protein assays, its application to screening tests is extremely limited because it requires the time-consuming incubation of samples, controls, and calibrators prior to a color reaction in the antibody-coated wells.

The method reported here enables a highly sensitive and cost-effective immunoassay even though it is composed of simple and easy operations, requiring neither complex apparatus nor skilled laboratory support. The proposed method consists of two steps: an antigen–antibody reaction using latex particles labeled with Ethyl Violet cation (EV^+) and the visual detection of the coagulated particles using the spot test on a hydrophilic polytetrafluoroethylene (PTFE) membrane film. The coagulated particles in a sample drop are concentrated into a clearly defined blue ring on the film. Although there have been numerous publications on the conventional spot test since developed by Feigl,[1] there has yet to be a spot test for immunoassay reported.

The proposed method has been successfully applied to the determination of C-reactive protein (CRP). CRP is an extremely sensitive marker of systemic inflammation. The reported values of CRP in the serum of healthy subjects are less than 3 mg L^{-1}.[2] Owing to the speed and magnitude of its response, CRP has been used to detect the outcome of various infectious, inflammatory, and necrotic processes and to assess the efficiency of treatment for those processes. Since MacLeod et al. developed a visual immunoturbidimetry based on the aggregation through antigen–antibody reaction for the detection of CRP in 1941,[3] the analytical methods for this bio-marker have been extensively studied. A semi-quantitative test using a capillary immunoprecipitation was commonly used until the 1970s. Singer et al. developed the latex immune agglutination method using specific antibodies which coat colloidal polystyrene particles in 1956.[4] Typically, the detection limit for CRP using the autoanalyzers in clinical laboratories is 3–5 mg L^{-1}. J. S. Ahn et al. established a fluorescence immunochromatographic assay of CRP in whole blood using a laser fluorescence scanner, which provides a measurable range of 0.13–10 mg L^{-1}.[5] Larsson established a highly sensitive method by increasing the sample volume by a factor of eight for a CRP test kit (Nyco Card, Axis-Shield, Oslo, Norway) based on an immunochromatographic technique using a monoclonal antibody-gold conjugate.[6] The detection range of the test kit (5–150 mg L^{-1}) was improved to 0.6–18.8 mg L^{-1} in their work. Our method has a comparable visual detection limit (0.5 mg L^{-1}) to the immunochromatographic method[6] and uses much smaller sample volumes.

For the densitometric measurement, a flying spot scanning densitometer (Shimadzu, model CS-9300PC) was used. Turbidimetric measurement was made using a JASCO V-560 spectrophotometer. The hydrophilic PTFE membrane film (pore size: 1.0 μm) was purchased from Nihon Millipore Co. Solutions of CRP (Shino-Test Co.) were prepared by dilution in doubly distilled water. The pH buffer solution (pH: 8.0) containing bovine serum albumin (BSA) (Shino-Test Co.) and the antibody-coated latex reagent (Shino-Test Co.) were used. A 20% poly(ethylene glycol) (PEG 10000) solution was prepared by dissolving it in hot water and diluting it with doubly distilled water. A solution (5×10^{-4} mol L^{-1}) of Ethyl Violet (Wako Pure Chemical Industries, Ltd.) was prepared by dissolving the dye in water. All other reagents used were of guaranteed reagent grade.

A typical procedure is as follows: ten microliters of serum was pipetted into a microtube. To the sample the following solutions were added: 310 μL of the pH buffer solution containing

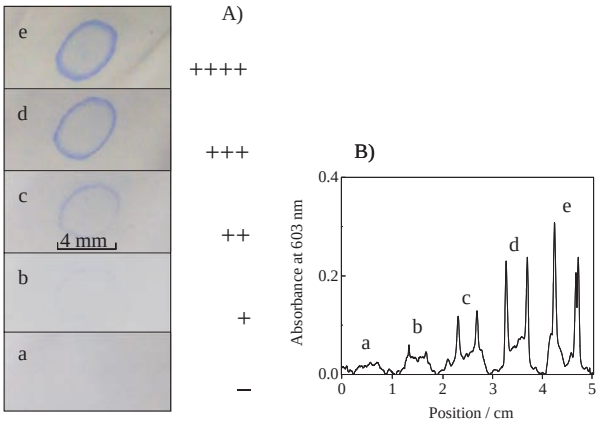

Figure 1. Blue ring for the detection of CRP A) Photographs, B) Densitograms at 603 nm; Beam size 0.4 × 0.4 mm. CRP concentration in 10 μL initial sample solution (mg L^{-1}); (a) 0, (b) 0.5, (c) 2, (d) 10, (e) 60, Spotting volume 10 μL, Substrate hydrophilic PTFE membrane filter (pore size 1.0 μm); [EV^+] 2.6×10^{-6} mol L^{-1}, [PEG 10000] 0.8%, pH buffer 310 μL, Antibody-coated latex reagent 150 μL, Final volume 500 μL, Reaction time 10 min.

BSA, 150 μL of the antibody-coated latex reagent, 20 μL of 20% PEG and 10 μL of EV. The mixture was let stand for 10 min at room temperature for the completion of aggregation caused by the antigen–antibody reaction. Ten microliters of the mixture was set on a hydrophilic PTFE membrane film. Then, CRP concentration was visually determined by comparison of the resulting blue ring with a prepared set of standard rings.

The procedure was optimized with respect to selection of the substrate, the dye and solution compositions. A hydrophilic PTFE membrane film was chosen as a primer among the substrates tested since the blue ring formed on this film is markedly clearly defined. In order to visualize the aggregates formed through the antigen–antibody reaction, dye binding to BSA which is coated on latex surface as a blocking agent was investigated. Although labeling of antibody based on covalent bond has been the subject of a number of investigations, it does not appear to be any reports on dye binding to BSA on latex. The labeling was found not to inhibit the antigen–antibody reaction. EV^+ concentration of 2.6×10^{-6} mol L^{-1} is appropriate for the clear contrast of the rings between blank and sample. The effect of PEG on the turbidity induced by the aggregation of the latex particles was studied, and 0.8% PEG 10000 was adopted. A sample volume of 10 μL was the optimum choice to obtain a clear contrast with sufficient reproducibility.

Figures 1a and 1b show the rings used for the detection of CRP and the corresponding densitograms, respectively. The visual detection limit is 0.5 mg L^{-1} of CRP in serum when a 50× dilution factor is employed. Concentration of CRP can be determined by visual comparison with a standard series of 0, 0.5, 2, 10, and 60 mg L^{-1} of CRP. A prozone effect was observed over the CRP concentrations of 500 mg L^{-1}. The color of the rings remains true for at least three months.

When a 10 μL sample solution containing coagulated particles is set on a hydrophilic PTFE membrane film, the drop retains its semi-spherical shape for an instant due to surface tension and the water repellency of the film. The solution gradually spreads through the capillaries of the membrane film. Then, the aggregate is trapped by the film at the circumference and a blue ring-shaped stain of the coagulated latex is formed on the film surface. This mechanism was directly observed using a high speed camera at 100-exposure per second on a monitor.

In a separate experiment, the binding behaviour of EV^+ was investigated by centrifuge using a mole cut filter with a molecular weight of 30000 followed by the spectrophotometric determination of the filtrate. The results showed that EV^+ binds to both BSA in aqueous solution and to BSA coated on the latex surface, but not to CRP or the antibodies. Both the excess dye bound to free BSA in the solution and the excess latex particles diffuse through the capillary of the membrane film. It should be noted that the diffusion of the excess dye with BSA and free latex reagent labeled with the dye through the film substrate eliminate the reagent blank, thus providing a clear contrast. Because of this no steps for B/F separation are required. Although an aqueous solution of EV forms an obvious blue ring, the ring is not observed in the presence of BSA (Figure 2). The elimination of reagent blank is attributed to the hydrophilic characteristics of the dye–protein conjugates due to the negative charges of BSA at the pH value used (pH 8).

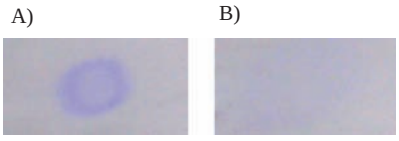

Figure 2. Behavior of Ethyl Violet on the film. $[EV^+] = 5.2 \times 10^{-6}$ mol dm^{-3}. a) BSA free, b) [BSA], 0.7%.

The interferences of foreign substances of biological importance were investigated. There was no interference observed in the blue ring formation with any of the following at 0.5 mg L^{-1} CRP: 200 mg L^{-1} of bilirubin F, 200 mg L^{-1} of biririrubin C, 5000 mg L^{-1} of hemolysis hemoglobin, 20000 mg L^{-1} of chyle, 400 (IU mg L^{-1}) of rheumatoid facter, 10000 mg L^{-1} of sodium fluoride, 2000 mg L^{-1} of EDTA and 20000 mg L^{-1} of trisodium citate dehydrate.

The results by the proposed method for patient's sera were in good agreement with those by the latex turbidimetrty autoanalyzer (Table 1). This method has also been successfully applied to spiked whole blood samples with pretreatment using nonionic surfactants for hemolysis.

Table 1. Analytical results of patient's sera

Patient	This method	Other method[a] mg L^{-1}
1	−	0.2
2	±	0.4
3	+	0.6
4	++	2.4
5	+++	36.0
6	++++	54.0

[a]Using latex turbidimetric autoanalyzer

Our method is simple, cost-effective, and clearly applicable to antigen–antibody reactions in general, and will be useful for mass screening test and also for near-patient testing. Future work will focus on cross reaction of the antigen–antibody as well as the application of the proposed method to other analytes of medical importance.

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References

1 F. Feigl and V. Anger, "Spot Test in Inorganic Analysis," Elsevier, Amsterdam (1972).
2 W. L. Robert, L. Moulton, T. C. Law, and G. Farrow, *Clin. Chem.*, **47**, 418 (2001).
3 C. M. MacLeod and O. T. Avery, *J. Exp. Med.*, **73**, 191 (1941).
4 M. Singer and M. Plotz, *Am. J. Med.*, **21**, 888 (1956).
5 J. S. Ahn, S. Choi, S. H. Jang, H. J. Chang, J. H. Kim, K. B. Nahm, S. W. Oh, and E. Y. Choi, *Clin. Chim. Acta*, **332**, 51 (2003).
6 A. Larsson, *Clin. Chim. Acta*, **334**, 249 (2003).

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