Mimetic-enzyme fluorescence immunoassay using a thermal phase separating polymer

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Poly-*N*-isopropylacrylamide (PNIP), a water-soluble, thermally precipitated synthetic polymer, has been conjugated together with a monoclonal antibody and utilized as a novel separation method for an immunoassay. PNIP precipitates out of water above a critical temperature of 31 °C, enabling a polymer-bound immune complex to be separated from the solution. These characteristics were used to develop a novel polymer–mimetic enzyme immunoassay method for determination of α-1-fetoprotein (AFP) with hemin as a labeling reagent to catalyze the reaction of *p*-hydroxyphenyl acetic acid (HPA) and hydrogen peroxide in alkaline medium. After a one-step competitive immunoreaction, the polymer–antibody–antigen–hemin conjugate moiety was determined by coupling the fluorogenic reaction of HPA and hydrogen peroxide. The calibration graph for AFP was linear over the range of 0–380 ng cm^{-3} with a detection limit of 1.0 ng cm^{-3}. This method combines some advantages of both homogeneous and heterogeneous immunoassays, and has been applied to determine AFP in human blood serum with satisfactory results.

Keywords: *Poly-*N*-isopropylacrylamide; thermal phase separating technique; hemin; α-1-fetoprotein; mimetic enzyme immunoassay; fluorescence detection*

Application of immunoassay techniques is now very widespread playing an important role in a variety of fields, for example, diagnostic medicine, forensics, drugs and diagnosis of infectious diseases. Generally, immunoassays can be divided into two categories, homogeneous and heterogeneous. Homogeneous immunoassays have the advantages of being rapid, easy to perform, and readily amenable to automation. However, the drawbacks of these methods such as poor sensitivity, and being prone to interference, have limited their application. On the other hand, heterogeneous assays are generally more sensitive than homogeneous assays, less prone to interference, and can be used for both low and high molecular weight analytes. However, they tend to be time consuming and labor-intensive because of the requirement of multiple wash steps.

The problems mentioned above for both homogeneous and heterogeneous immunoassays can be solved by using certain water-soluble polymers to carry out a fast homogeneous immune reaction and a simple heterogeneous separation process. The polymers are known to precipitate when the temperature is raised above the lower critical solution temperature (LCST) at which demixing occurs.[1,2] Among the polymers, poly-*N*-isopropylacrylamide (PNIP) is known to have an LCST of about 31 °C throughout a wide concentration range (up to *ca.* 5%). We have utilized this demixing behavior of PNIP above the LCST as the separation technique in our new immunoassay.

Enzymes such as horseradish peroxidase (HRP) and alkaline phosphatase (ALP) are the most common labels for enzyme immunoassays. However, enzymes are relatively expensive and their solutions are not very stable; in addition, enzyme labels inevitably lead to steric hindrance against the immunoreaction of antigen and antibody due to their large molecular weight. Therefore, the study of small molecular mimetic enzymes as catalysts for fluorogenic reactions has increased markedly in recent years.[3–6] Hemin, a natural low molecular weight Fe–porphine complex, is an inexpensive product from bovine blood and very stable in solution, and has been used as a catalyst in chemiluminescence immunoassays.[7,8] Recently, we reported that hemin exhibited very significant catalytic activity for the peroxidation reaction of thiamine and hydrogen peroxide,[9] and we developed a new sandwich mimetic enzyme immunoassay for the hepatitis B surface antigen.[10] A search of the *Chemical Abstracts* database in November 1997 indicated that using hemin as a labeling reagent in a competitive mimetic enzyme fluorescence immunoassay had not previously been reported. In the present work, hemin was used as a substitute for HRP to label AFP antigen, and it was found that the conjugated hemin retained high catalytic activity for the reaction between HPA and hydrogen peroxide in alkaline media. Therefore, we describe here a novel polymer–mimetic enzyme immunoassay method. This method can be employed for sensitive detection of AFP by a competitive immunoassay technique. The standard AFP and AFP–hemin first reacted with PNIP–antibody (PNIP–Ab), then the temperature of the solution was raised above the polymer's LCST to cause the polymer–immune complex to precipitate, and the bound hemin-labeled antigen was quantified by fluorescence measurement (Fig. 1). The use of PNIP as a carrier and hemin as labeling reagent is expected to provide not only a simple immunoassay procedure but also long-term stability. In addition, since the polymer–immune complex may be reversibly redissolved by cooling, the method may be used to concentrate and isolate the analyte.

Experiment

Materials

Standard AFP and monoclonal mouse anti-AFP antibody were obtained from Xiamen Advanced Scientific Inc. (Xiamen, China). Water-soluble carbodiimine (EDC) and Sephadex G-50 were purchased from SABC (Shanghai, China). Bovine serum albumin (BSA) was obtained from Shanghai Biochemicals (Shanghai, China). All the chemical reagents were of analytical-reagent grade. Hemin was obtained from Dongfen Biochemical Reagent Co. (Shanghai, China). *p*-Hydroxyphenyl acetic acid was purchased from TCI-EP (Tokyo Kasei, Japan). Hydrogen peroxide (30%) was from Shanghai Taopu Chemical Factory (Shanghai, China). *N*-Isopropylacrylamide (NIP), *N*-hydroxysuccinimidyl acrylate (NAS) were synthesized as described.[11,12] Ammonium persulfate and *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED) were purchased from TCI-EP

(Tokyo, Kasei) and used without further purification. Phosphate buffered saline (PBS) solutions at pH 7.4 and 8.0 at a concentration of 0.1 mol cm^{-3} were used. Distilled, deionized water was used throughout.

Instruments

A Hitachi 650-10S spectrofluorimeter (Tokyo, Japan) equipped with a plotter unit and a 1 cm quartz cell was used for recording and making fluorescence measurements. The absorption spectrum was performed on a Shimadzu UV-240 UV/VIS spectrophotometer (Kyoto, Japan). All the pH measurements were made with a digital pH and temperature meter 631 (Extech, Boston, USA). A TGL-16G supercentrifuge (Shanghai, China) and a THE-82 water-bath vibrator (Shenzhen, China) were used.

Methods

Synthesis of PNIP–Ab

A 4 cm^3 portion of PBS, pH 8.0, containing 200 μg of antibody and 30 μl of 0.1% (m/v) NAS were mixed and vibrated in a 37 °C water bath for 60 min in order to form the monomer conjugate, then the mixture was dialyzed three times against PBS (pH 8.0) at 4 °C and concentrated to 2 cm^3. The solution was poured into 8 cm^3 PBS (pH 7.4), and then 150 mg of NIP was added to this solution. The polymerization was carried out with 200 μl of 1% (m/v) ammonium persulfate and 10 μl of TEMED at 25 °C for 2 h. After finishing the polymerization reaction, the solution was centrifuged at 37 °C for 10 min (10 000 rpm) to precipitate and separate the PNIP–Ab conjugate. The supernatant was removed, and the precipitate dissolved in 10 cm^3 of cold PBS, pH 7.4. The procedure was repeated three times. Finally, the PNIP–Ab conjugate was dissolved in 10 cm^3 of cold PBS buffer solution, pH 7.4, and stored at −10 °C.

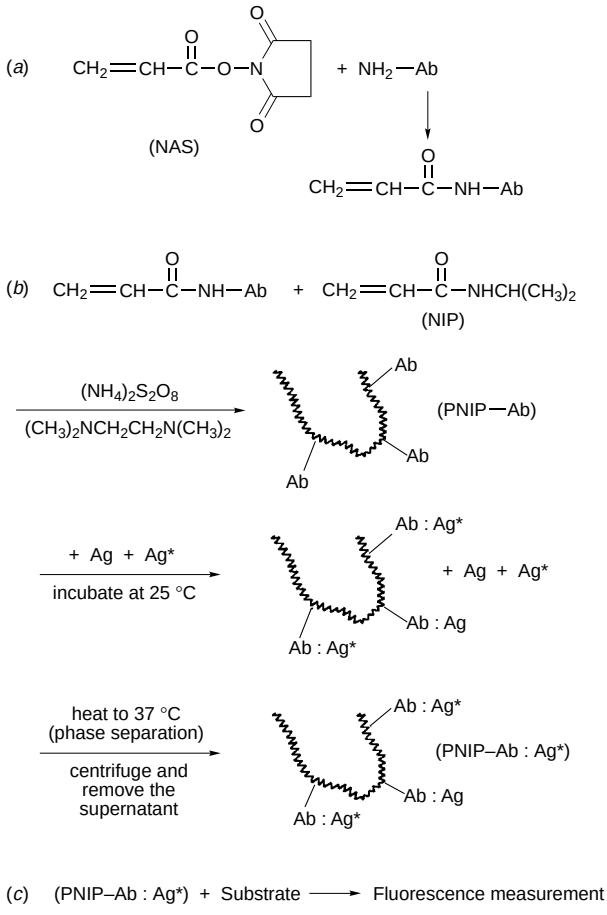

Fig. 1 Protocol for the polymer–mimetic enzyme immunoassay method.

Preparation and characteristics of hemin-labeled AFP conjugate

Hemin was bound to AFP with EDC as a coupling agent. A portion (15 mg) of hemin was dissolved in 1 cm^3 of dimethylsulfoxide, and 10 mg of the EDC was added. The mixture was incubated for 30 min at 25 °C with magnetic stirring. Then 2 cm^3 of AFP solution (the AFP solution was dialyzed three times against 1% (m/v) sodium chloride solution at 4 °C and then concentrated to 2 cm^3) was added. The mixture was stirred for 3 h at 25 °C. The pH of the solution was maintained at 5.0 ± 0.5 by the addition of 0.1 mol dm^{-3} HCl and NaOH during the coupling reaction. The mixture was centrifuged at 4 °C and the centrifugate was collected, then 5 cm^3 of 0.1 mol dm^{-3} phosphate buffer of pH 7.0 was added to the precipitate, followed by centrifugation at 4 °C, to retrieve the soluble product. All the centrifugates were mixed and saturated by adding enough ammonium sulfate and then left at 4 °C overnight. The precipitate was dialyzed three times against 0.1 mol dm^{-3} phosphate buffer solution (pH 7.0) at 4 °C (each time for 1 h against 1.0 l of phosphate buffer solution). The final product, a pale brown solution, was concentrated and passed through a 50 × 1.5 cm column packed with Sephadex G-50. The eluent was 0.1 mol dm^{-3} phosphate buffer solution (pH 7.0).

The conjugate gave a broad absorption band with a peak at 390 nm and a shoulder at 280 nm. The concentrations of AFP and hemin were determined by the measurements of the corresponding absorbance at 260, 280 and 390 nm based on the assumption that the molar absorptivities of hemin at 260, 280 and 390 nm were 3.1×10^4, 3×10^4 and 9.3×10^4 dm^3 mol^{-1} cm^{-1}, respectively. The concentrations of hemin and AFP were 1.77×10^{-5} mol dm^{-3} and 9.04×10^{-6} mol dm^{-3}, respectively. The approximate molar ratio was 2.

Immunoassay for AFP

Standard AFP solution (50 μl) or patient serum sample (or 500 μl of normal human blood serum) and 25 μl of hemin-labeled AFP (1 : 10 dilution) were added to the mixed solution of 50 μl of PNIP–Ab and 350 μl of PBS, pH 7.4, containing 3% BSA and 0.1% Tween-20. The mixture was diluted to 1.0 cm^3 with 0.1 mol dm^{-3} PBS (pH 7.4) and vibrated for 10 min at 25 °C to allow the specific binding to occur. The polymer was then precipitated by incubating for 10 min at 37 °C. The resultant precipitate was centrifuged at 10 000 rpm for 10 min at 37 °C, the supernatant was withdrawn, and the precipitate redissolved in 1 cm^3 of ice-cold PBS (pH 7.4). This procedure was repeated three times, and the precipitate finally gained was mixed with 500 μl of 0.01 mol dm^{-3} NaOH, 300 μl of 6×10^{-4} mol dm^{-3} H_2O_2 and 200 μl of 0.01 mol dm^{-3} HPA. The mixture was allowed to stand at 25 °C for 15 min and the fluorescence intensity was measured at 410 nm with excitation at 320 nm. A calibration curve of fluorescence intensity *vs.* AFP concentration was plotted.

Results and discussion

Fluorescence spectrum

HPA is an excellent fluorescent substrate for horseradish peroxidase.[13,14] Similarly, in the presence of conjugated hemin, it can also be rapidly oxidized by hydrogen peroxide to produce fluorescent bi-*p*, *p*′-hydroxyphenylacetic acid which shows an excitation maximum at 320 nm and a fluorescence emission maximum at 410 nm.

Optimization of catalytic reaction conditions

The fluorescence reaction catalyzed by the conjugated hemin was studied. The results indicated that the maximum fluorescence intensity was reached when the HPA concentration was in the range of 1.6×10^{-3}–4.0×10^{-3} mol dm^{-3}. A HPA concentration of 2.0×10^{-3} mol dm^{-3} is recommended. Similarly, the fluorescence intensity was the highest and constant when the concentration of H_2O_2 was varied from 1.2×10^{-4} mol dm^{-3} to 2.0×10^{-4} mol dm^{-3}; when the H_2O_2 concentration exceeded 4.0×10^{-4} mol dm^{-3}, the fluorescence intensity was decreased remarkably. In this work, 1.8×10^{-4} mol dm^{-3} H_2O_2 is recommended for use.

The effect of pH on the catalytic reaction was examined; the results showed that the fluorescence intensity dramatically increased with the increased of pH. In this work, NaOH was chosen to control the pH of the system, and the maximum fluorescence occurred when the final concentration of NaOH was in the range of 4.0×10^{-3}–8.0×10^{-3} mol dm^{-3}. Therefore, 5.0×10^{-3} mol dm^{-3} NaOH (final pH 11.7), is recommended. The influence of temperature and reaction time was also studied. The fluorescence intensity was constant over the temperature range of 20–30 °C; 25 °C is recommended. The fluorescence intensity of the system approximated to constant after reacting for 13 min at 25 °C (Fig. 2). In this paper, a reaction time of 15 min was chosen, and the equilibrium method was used.

Catalytic activity of hemin–AFP conjugate

Conjugated hemin was assayed for its catalytic activity for the reaction of HPA with hydrogen peroxide under the optimum conditions described above. The results showed that hemin retained its catalytic activity when it was covalently bound to AFP, and there was good linearity between the fluorescence intensity and the concentration of hemin–antigen conjugate in the range of 0–600 ng cm^{-3} AFP (higher concentrations were not tested) (Fig. 3). Thus the conjugate can be quantified in this concentration range. This indicated that hemin can be used as a sensitive label in the immunoassay due to its excellent amplification in the HPA–hydrogen peroxide system. Hemin–AFP conjugate was reacted with PNIP–anti-AFP antibody in a solution, and the catalytic activity in PNIP–antibody–antigen–hemin form was tested. There was no noticeable difference in catalytic activity between hemin–AFP and PNIP–antibody–antigen–hemin conjugate.

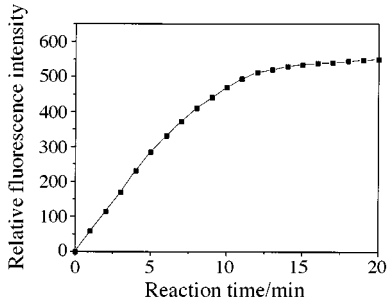

Fig. 2 Kinetic profile for the fluorescence development with the time. Temperature, 25 °C; HPA, 2.0×10^{-3} mol dm^{-3}; H_2O_2, 1.8×10^{-4} mol dm^{-3}; conjugated hemin, 1.8×10^{-8} mol dm^{-3}.

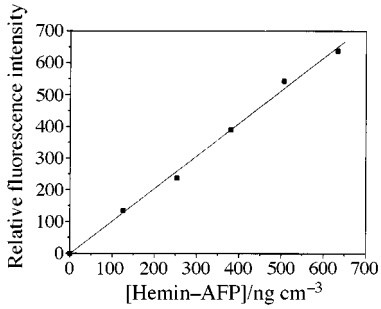

Fig. 3 Catalytic activity of hemin–AFP conjugate. HPA, 2.0×10^{-3} mol dm^{-3}; H_2O_2, 1.8×10^{-4} mol dm^{-3}; temperature, 25 °C; reaction time, 15 min.

Optimization of immunoassay conditions

The effect of the concentration of hemin-labeled AFP conjugate was examined; the experiement results showed that the optimal concentration for the conjugate was in the range of 0.63–1.89 μg cm^{-3}. A hemin–AFP conjugate concentration of 1.62 μg cm^{-3} (25 μl of 1 : 10 dilution of the original solution) is recommended. The influence of the volume of PNIP–anti-AFP solution was studied, and 50 μl of PNIP–anti-AFP in the system was optimal.

Calibration graph for AFP

The calibration curve for the determination of AFP was constructed under the optimal conditions aforementioned. A good linear relationship was observed between the fluorescence intensity and AFP concentration, and AFP can be determined in the range of 0–380 ng cm^{-3}. The linear regression equation was $y = -2.2x + 915.2$, with a correlation coefficient of 0.996 (n = 8), where x (AFP) is in ng cm^{-3}. The detection limit (3σ) for AFP was calculated from the standard deviation of the blank (n = 6) as 1.0 ng cm^{-3}

Our thermally induced phase-separation immunoassay method offers several advantages over conventional heterogeneous methods, such as second antibody precipitation, antibody-coated magnetic particles and antibody-coated test tubes, *etc*. First, the specific binding reaction occurs in solution rather than on a solid surface. Hence, the immune reaction is more rapid, and the incubation time can be reduced significantly. Second, nonspecific bonding is low. This is probably related to the fact that the polymer used in our method is hydrophilic, unlike typical solid-phase systems which utilize polystyrene or other hydrophobic materials. Finally, since the polymer–immune complex can be reversibly redissolved by cooling, the signal (or analyte) may be concentrated.

Immunoassay of AFP in blood sera

The AFP levels in healthy human serum and colon carcinoma patient serum were determined by the proposed assay. These samples were also analyzed in an anti-epidemic station laboratory by the conventional ELISA method. Results obtained by these two methods are summarized in Table 1. The

Table 1 Determination of AFP in human serum

	AFP levels				
Sample No.	This method*/ ng cm^{-3}	ELISA*†/ ng cm^{-3}	RSD (n = 6) (%)	Added/ ng	Recovery (%)
Healthy serum					
1	25.2	—	6.3	—	—
2	28.9	—	6.7	—	—
Patient serum					
3	477	> 400	8.0	20	90
4	421	> 400	7.3	20	85
5	513	> 400	5.2	20	99

* Mean of six determinations. † ELISA assay for AFP detection was performed with a commercial kit (Beijing North TZ-Biotech. Development Co.), and the results of detection were supplied by Xiamen Municipal Health and Anti-epidemic Station.

relative standard deviation within a batch was below 8% (n = 6) for the determination of AFP in human blood serum. The possibility of using the proposed method for the analysis of samples was further confirmed by determining the recovery of known amounts of AFP added to the sample. The results in Table 1 show that the recoveries are satisfactory, and the reproducibility of the determination is good.

Conclusion

By using PNIP to replace surface in ELISA, the immune reaction can be carried out in the homogeneous phase because of the solubility of PNIP in water, and the polymer–antibody–antigen immune complex can be separated by precipitation and centrifugation above 31 °C. Hemin–AFP conjugate shows a high catalytic activity for the fluorescent reaction of HPA and hydrogen peroxide. The use of hemin as a labelled catalyst leads to a paticularly stable, inexpensive, simple and sensitive immunoassay for AFP. The proposed method has been applied to determine AFP in healthy and patient human blood serum with satisfactory results.

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