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A novel mimetic enzymatic fluorescence immunoassay for hepatitis B surface antigen by using a thermal phase separating polymer

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Iron tetrasulfonatophthalocyanine (FeTSPc), a peroxidase mimic, was used as a labeling reagent and poly(*N*-isopropylacrylamide) (PNIP) as the separation support of the immune complex for the mimetic-enzymatic immunoassay of hepatitis B surface antigen (HBsAg). PNIP was precipitated from aqueous solution when the ambient temperature was higher than its lower critical solution temperature of 31 °C. In a sandwich immunoassay, the antigen (HBsAg) first reacted with mouse anti-human HBsAg antibody immobilized on PNIP (PNIP–antibody) and then further reacted with FeTSPc-labeled mouse anti-HBsAg antibody (antibody–FeTSPc) at room temperature in a homogeneous format. After changing the temperature to separate the PNIP–antibody–HBsAg–antibody–FeTSPc conjugate moiety, it was re-dissolved and determined by coupling with the fluorogenic reaction of hydrogen peroxide and *p*-hydroxyphenylpropionic acid. The sensitivity of this method (3 ng mL^{-1}) was close to that of the traditional ELISA using the same reactants. However, the assay was much faster (the assay time decreased from 100–120 to 45 min). This method was applied to determine HBsAg in human serum with satisfactory results.

1 Introduction

Enzyme immunoassays based on a selective antigen–antibody binding and an enzyme label have gained increasing importance in recent years. Among the enzymes used, horseradish peroxidase (HRP) and alkaline phosphatase are the most widely used enzyme labels. However, owing to the instability and the steric hindrance of the natural enzymes in the immune reaction, the search for alternatives to them has gradually been an area of interest. For HRP mimesis, previous exploration was based on the substances containing a porphine skeleton, such as hemin, hematin and some synthetic metal porphyrin compounds.[1–4] Recently, these porphyrin compounds have been used as labeling reagents in enzymatic immunoassays and the results showed that the mimetic enzymatic immunoassay had the advantages of easy labeling, cheapness and high reproducibility.[5–7] In addition to the porphyrins, metal phthalocyanine compounds, which possess an active center similar in structure to that of porphine, have been used as a mimetic peroxidase in the catalytic reactions of fluorogenic substrates and hydrogen peroxide.[8–10] The results showed that the metal phthalocyanines exhibited comparable catalytic activities to those of porphyrins and, furthermore, the water soluble metal phthalocyanines with labeling groups are easy to synthesize and are stable in various media, and may therefore be proposed as promising labeling mimetic-enzymes for fluorogenic immunoassay.

Over the past 10 years, biomedicine and biotechnology have benefitted considerably from the emergence of 'intelligent' polymers. Poly(*N*-isopropylacrylamide) (PNIP), a thermally sensitive polymer, has the property of molecular transition from a hydrophilic to a hydrophobic structure when the ambient temperature exceeds its lower critical solution temperature (LCST), 31 °C. Monji and Hoffman[11] took advantage of the precipitation of PNIP at elevated temperature to develop an alternative approach for immunoassay technology. Recently, we reported the use of PNIP in mimetic-enzymatic immunoassay and developed a hemin-based polymer immunoassay for AFP.[6,7]

In this work, iron tetrasulfonatophthalocyanine (FeTSPc) (see Fig. 1), a water soluble phthalocyanine compound with four sulfonic groups, was synthesized and used as a labeling catalyst for the fluorogenic reaction of *p*-hydroxyphenylpropionic acid (*p*-HPPA) and hydrogen peroxide in a mimetic immunoassay for HBsAg based on a thermal phase separating technique. With the use of PNIP as a support, the immunoassay process including the homogeneous immune reaction of the PNIP-conjugated antibody and antigen, heterogeneous separation of the PNIP-bound immune complex and homogeneous fluorescence detection of the final product can be carried out by control of the temperature.

The scheme for the FeTSPc-based polymer–enzymatic immunoassay is shown in Fig. 2. In the sandwich immunoassay, the HBsAg first reacted with antibody immobilized on PNIP and then further reacted with FeTSPc-labeled antibody. After the separation process, the PNIP–antibody–HBsAg–antibody–

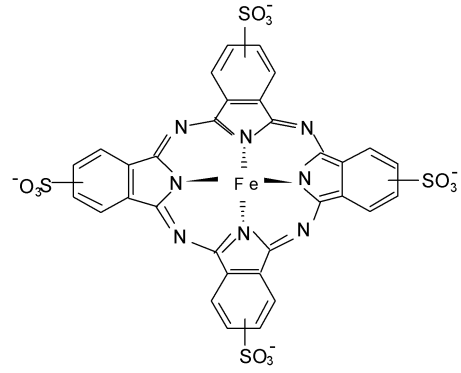

Fig. 1 Molecular structure of FeTSPc.

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FeTSPc conjugate moiety served as the catalyst for the reaction of hydrogen peroxide and *p*-HPPA and thus the HBsAg can be indirectly determined. The proposed method offers several advantages over the conventional solid-phase immunoassay. First, the specific binding of the antibody and antigen occurs in solution rather than on a solid phase and therefore a shorter incubation time is needed. Second, the hydrophilic structure of PNIP greatly reduces the non-specific binding and thus simplifies the procedure. Third, using a metal phthalocyanine compound as a labeling catalyst in the immunoassay may lead to a simpler, cheaper and more stable method compared with those with HRP as a catalyst.

2 Experimental

2.1 Materials

All the reagents were of analytical grade or better and distilled, de-ionized water was used throughout.

FeTSPc was synthesized and purified according to Weber and Busch.[12] Its stock solution (1.0×10^{-3} mol L^{-1}) was prepared by dissolving 0.0478 g of FeTSPc in 50 mL of water. It was stable at room temperature for several months. A *p*-HPPA stock standard solution of 1.0×10^{-2} mol L^{-1} was obtained by dissolving the appropriate weight of *p*-HPPA (Sigma) in water, and working standard solutions were obtained by further dilution with water. H_2O_2 solution for analysis was freshly prepared by appropriate dilution of a stock standard solution standardized with potassium permanganate. Ammonium peroxodisulfate, PCl_5 and polyethylene glycol 20000 were obtained from Shanghai Reagent (Shanghai, China). Sephadex G-50 was purchased from SABC (China). HBsAg and mouse anti-HBsAg antibody was kindly offered by the Cancer Research Center of Xiamen University. *N*-hydroxysuccinimidyl acrylate (NAS) was synthesized as described.[13] *N*-Isopropylacrylamide (NIP) and *N*,*N*,*N*′,*N*′-tetramethylethylendiamine (TEMED) were purchased from TCI-EP (Tokyo Kasei, Japan) and used without further purification. Na_2CO_3–$NaHCO_3$ buffer (0.1 mol L^{-1}, pH 9.5) was made by mixing 30 mL of 0.1 mol L^{-1} Na_2CO_3 solution and 70 mL of $NaHCO_3$ solution (0.1 mol L^{-1}). A pH 7.4 phosphate-buffered saline (PBS) solution (0.1 mol L^{-1}) was prepared by mixing 202.5 mL of Na_2HPO_4 (0.2 mol L^{-1}) and 47.5 mL of NaH_2PO_4 (0.2 mol L^{-1}) solutions and 4.25 g NaCl, then diluting the mixture with water to 500 mL.

2.2 Apparatus

The fluorescence spectra and relative fluorescence intensity were measured with a Hitachi 650-10s spectrofluorimeter with a 10 mm quartz cell. The absorption spectra were measured on a Beckman DU-7400 ultraviolet–visible spectrophotometer. A TGL-16G centrifuge (Anting Scientific Instrument Factory, Shanghai, China) and a SHZ-88 water-bath vibrator (Taichang Experimental Instrument Factory, Jiangshu, China) were used.

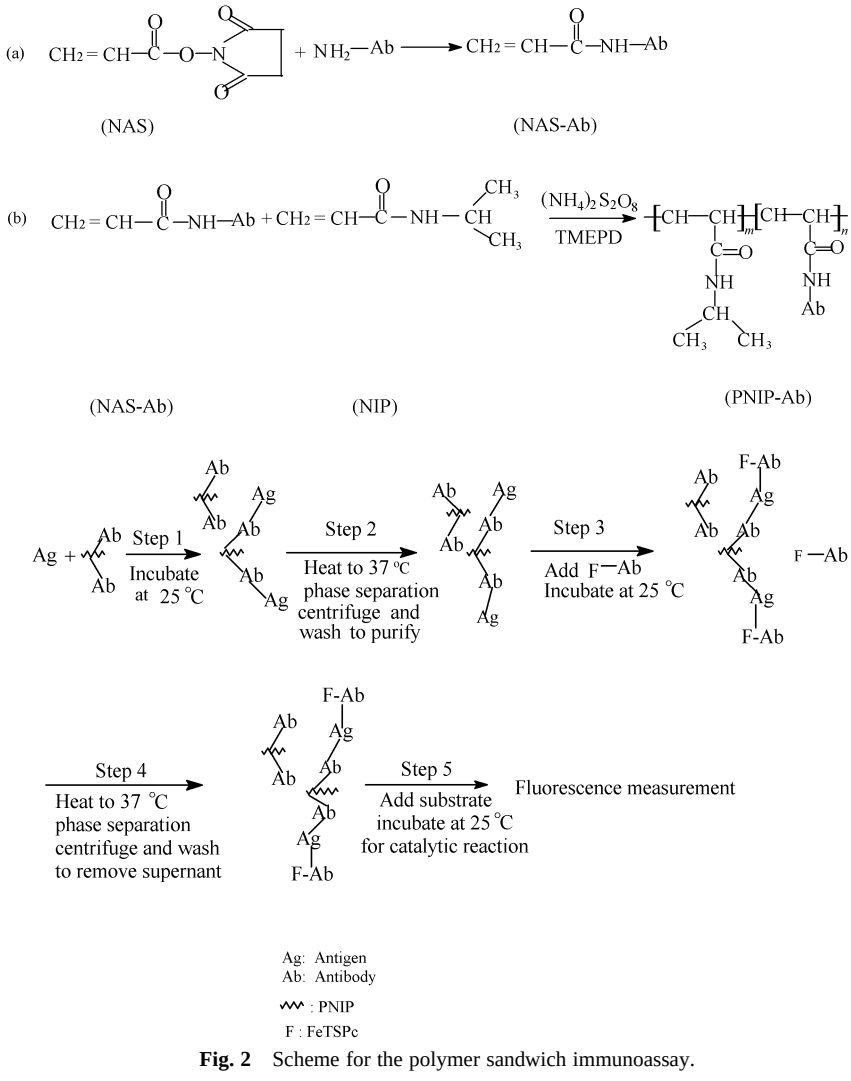

Fig. 2 Scheme for the polymer sandwich immunoassay.

2.3 Preparation of PNIP–antibody conjugate

0.2 mL of NAS (10% v/v) was added to 2.0 mL of PBS solution (pH 7.4) containing 1.5 mg mouse anti-HBsAg antibody. The mixture was vibrated in a 37 °C water-bath for 1 h to form the monomer conjugate and dialyzed against 0.1 mol L^{-1} PBS (pH 7.4) three times at 4 °C. The dialyzed product of NAS–antibody was diluted to 4 mL with PBS (pH 7.4), then 50 mg NIP, 10 mg of ammonium peroxodisulfate and 20 μL of TEMED were added and the mixture was allowed to stand for 2 h in a 25 °C water-bath to carry out the polymerization reaction. The PNIP–antibody conjugate was precipitated and separated by centrifugation at 37 °C for 10 min (10 000 rpm); after removing the supernatant, the precipitate was dissolved in cold PBS (pH 7.4). The procedure was repeated three times. Finally, the PNIP–antibody conjugate was dissolved in 10 mL of PBS (pH 7.4) and stored at 4 °C.

2.4 Preparation of FeTSPc–antibody conjugate

0.3 g of FeTSPc was mixed with 0.5 g of PCl_5 in a mortar and the mixture was ground for 5 min in a ventilated cabinet. 5.0 mL of anhydrous acetone was added and the mixture was stirred for 5 min before rapid filtration. 0.1 mL of the filtrate was added dropwise to 5.0 mL of mouse anti-HBsAg antibody solution (a pH 9.5 Na_2CO_3–$NaHCO_3$ solution containing 1.0 mg of the antibody) with continuous stirring at room temperature for 30 min. The mixture was kept overnight at 4 °C and then dialyzed three times against 0.01 mol L^{-1} PBS solution (pH 7.4) at 4 °C. The final product was concentrated to 2.0 mL with polyethylene glycol 20000 and passed through a 50 × 1.5 cm id column packed with Sephadex G-50 with PBS (0.01 mol L^{-1}, pH 7.4) as the eluent.

The concentrations of antibody and FeTSPc label in the conjugate were determined by measuring the corresponding absorbance at 280 nm and 632 nm on the assumption that the value of $A^{1\%}_{280\,nm}$ for antibody was 12.8, and the absorbance of FeTSPc at 280 nm and 632 nm remained unchanged before and after labeling. From the calibration graphs for FeTSPc, the concentrations of FeTSPc and antibody were calculated to be 1.43×10^{-5} and 2.64×10^{-6} mol L^{-1}, respectively. The approximate molar ratio of FeTSPc to antibody was 5.4.

2.5 Immunoassay for HBsAg

In a 1.5 mL plastic centrifuge tube, various volumes of standard HBsAg solution or patients' serum samples (or normal human blood serum), 20 μL of PNIP–antibody and 50 μL of BSA (3%) were placed and diluted to 1.0 mL with PBS (pH 7.4). The mixture was vibrated at 25 °C for 10 min for the specific binding to occur, then incubated at 37 °C for 10 min to precipitate the PNIP–antibody–HBsAg complex. The precipitate was separated by centrifugation at 10 000 rpm for 10 min at 37 °C and dissolved in 1.0 mL of cold PBS (pH 7.4). The above procedure was repeated three times. The resultant precipitate was mixed with 20 μL of FeTSPc–antibody and diluted with cold PBS to 1.0 mL. The solution was vibrated at 25 °C for 10 min, and then the final immune complex, PNIP–antibody–HBsAg–antibody–FeTSPc, was separated by using the same procedure for the PNIP–antibody–HBsAg complex described above. Finally, the precipitate obtained was mixed with 0.20 mL of 0.01 mol L^{-1} of *p*-HPPA solution and 0.05 mL of 0.001 mol L^{-1} H_2O_2 solution, and the mixture was diluted to 1.0 mL with water. The mixture was allowed to stand for 30 min at room temperature, and then 10 μL of 1.00 mol L^{-1} NaOH was added. The fluorescence intensity of the solution was measured at 410 nm with excitation at 325 nm. A calibration graph of fluorescence intensity *versus* HBsAg concentration was plotted.

3 3 Results and discussion

3.1 Spectral characteristics

p-HPPA is a non-fluorescent substrate, but di-*p*,*p*′-hydroxyphenylpropionic acid, the product of the reaction of *p*-HPPA and H_2O_2 catalyzed by HRP[14] or FeTSPc,[8] is strongly fluorescent. Similarly, in the presence of conjugated FeTSPc, *p*-HPPA can also be rapidly oxidized by hydrogen peroxide to the fluorescent product, showing an excitation maximum at 325 nm and a fluorescence emission maximum at 410 nm.

3.2 Optimization of experimental parameters

The influence of pH on the oxidation reaction of *p*-HPPA and H_2O_2 catalyzed by FeTSPc and PNIP–antibody–HBsAg–antibody–FeTSPc immune complex was similar. The optimum pHs for both the catalytic reaction and final fluorescence measurement exhibited a wide range, pH 1.0–7.0 and 9.5–13.0, respectively. In addition, the control of the final pH for the fluorescence measurement did not need specific buffer systems, *e.g.*, Na_2CO_3–$NaHCO_3$, NH_3–NH_4Cl or NaOH solutions could all offer similar fluorescence intensity of the product. In our work, 0.01 ml of 1.00 mol L^{-1} NaOH was chosen for the control of the final pH. The influence of reaction time and temperature was studied, and the results showed that the fluorescence intensity of the system became approximately maximum and constant after reaction for 30 min, and the fluorescence enhancement was constant over the temperature range 15–30 °C. Therefore, the equilibrium method was chosen and for the reaction temperature, room temperature was adopted.

The optimum amounts of *p*-HPPA and H_2O_2 were also studied. The results showed that both the fluorescence of the product and the background fluorescence increased with increasing amount of *p*-HPPA. The fluorescence enhancement was maximum and constant when the final concentration of *p*-HPPA was in the range 8.0×10^{-4}–5.0×10^{-3} mol L^{-1}. Therefore, a *p*-HPPA concentration of 2.0×10^{-3} mol L^{-1} was adopted. Similarly, the fluorescence intensity was maximum and constant when the concentration of H_2O_2 was varied from 4.0×10^{-5} to 1.0×10^{-4} mol L^{-1}. In this work, 5.0×10^{-5} mol L^{-1} H_2O_2 was used.

3.3 Catalytic activity of FeTSPc–antibody conjugate

The catalytic activities of FeTSPc and FeTSPc–antibody in the reaction of *p*-HPPA and H_2O_2 were studied by the initial rate method with the steady-state assumption where the concentration of H_2O_2 was saturated in the test system. The Michaelis–Menten constant, K_m, and the transformation constant, K_{cat}, were obtained from the equation $V_{max} = K_{cat}[E_0]$, where $[E_0]$ is the initial concentration of the enzyme and the K_{cat} value represents the catalytic activity.

A comparison of the catalytic parameters between free and bound FeTSPc is given in Table 1. The catalytic activity of FeTSPc increased more than threefold when it was labeled with antibody. Further results also indicated that the PNIP–antibody–HBsAg–antibody–FeTSPc conjugate retained more than 80% of the catalytic activity of FeTSPc–antibody.

Table 1 Catalytic parameters with free FeTSPc and antibody–FeTSPc as catalysts and p-HPPA as substrate

Mimetic enzyme	K_m/ 10^{-4} mol L^{-1}	V_{max}/ 10^{-6} mol L^{-1} s^{-1}	$[E_0]$/ 10^{-7} mol L^{-1}	K_{cat}/ s^{-1}
FeTSPc	2.27	0.65	5.0	1.3
Antibody–FeTSPc	1.38	2.01	5.0	4.0

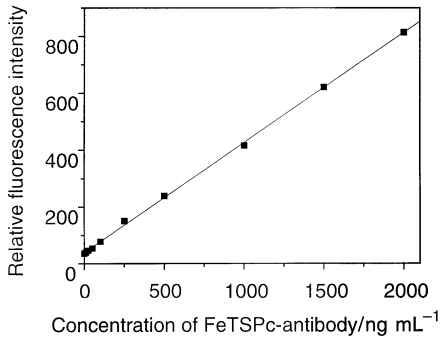

Fig. 3 Catalytic activity of FeTSPc–antibody conjugate. *p*-HPPA, 2.0×10^{-3} mol L^{-1}; H_2O_2, 5.0×10^{-5} mol L^{-1}; temperature, 25 °C; reaction time, 30 min.

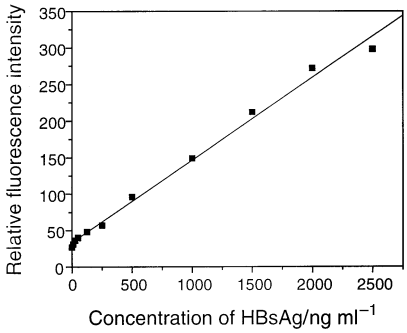

Fig. 4 Calibration graph for the determination of HBsAg.

Further results showed that there was good linearity between the fluorescence intensity and the concentration of FeTSPc–antibody conjugate in the range 0–2000 ng mL^{-1} (higher concentrations were not tested) (Fig. 3). Thus, the conjugate can be quantified in this concentration range. This indicates that FeTSPc can be used as a sensitive label in the immunoassay owing to its excellent amplification in the *p*-HPPA–hydrogen peroxide system.

3.4 Calibration graph for HBsAg

The calibration graph for HBsAg was constructed under the optimum conditions. The result is shown in Fig. 4. A good linear relationship was obtained between the fluorescence intensity and the concentration of PNIP–antibody–HBsAg–antibody–FeTSPc conjugate, that is, the concentration of HBsAg. The calibration graph for HBsAg was linear in the range 0.0–2000 ng mL^{-1}, with the linear regression equation $F = 30.44 + 0.12$[HBsAg], where HBsAg concentration was in ng mL^{-1}. The detection limit (3σ) calculated from the standard deviation of the blank determination ($n = 9$) was found to be 3.0 ng mL^{-1} HBsAg. The correlation coefficient was 0.993.

3.5 Immunoassay of HBsAg in blood samples

The HBsAg levels in healthy human serum and colon carcinoma patients' serum were determined by the proposed assay. These samples were also analyzed in the hospital of Xiamen University by the conventional ELISA method. The results obtained by these two methods are summarized in Table 2. The relative standard deviation within a batch was $< 8\%$ ($n = 6$) for the determination of HBsAg in human serum. The possibility of using the proposed method for the analysis of samples was further confirmed by determining the recovery of known amounts of HBsAg added to the sample. The results in Table 2 show that the recoveries are satisfactory, and the reproducibility of the determination is good.

Table 2 Determination of HBsAg in human serum

Sample No.	HBsAg level[a]/ng mL^{-1}				Recovery (%)
	This method	ELISA[b]	RSD (%)	Added/ng	
Healthy serum[c]—					
1	0	0	—	—	—
2	0	0	—	—	—
Patients' serum[d]—					
3	2160	2000	7.8	500	104
4	1920	2100	5.4	500	106
5	1020	1200	6.1	500	103

[a] Mean of six determinations. [b] Results supplied by the Hospital of Xiamen University. [c] The sample was not diluted. [d] The original sera were diluted 10-fold.

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