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Method of Spectrophotometric Microanalysis Based on HRP/PET Self-assembly Film

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Abstract: Horseradish peroxidase monolayer was assembled on the surface of PET-CO_2^- substrate. The reaction kinetics of HRP/PET film and H_2O_2 in micro reactor was studied using improved spectrophotometer. The relative activity of self-assembly HRP/PET film still remains above 80% after storing for 150 days at 4℃. When applied to determination of H_2O_2 in sample, the recoveries of H_2O_2 are 96.5%~101.1%.

Keywords: Electrostatic self-assembly film, horseradish peroxidase (HRP), spectrophotometry, atomic force microscope (AFM).

Electrostatic self-assembly is an effective method making organized ultrathin ordered film of enzymes. Enzyme molecules on the film prepared by electrostatic assembly can keep the conformation in the solution and have high biologic activity[1-7]. Electrostatic self-assembly on the surface of thin solid film (for example Au and Si) has been applied to preparing selective electrochemistry or biology sensors[8-10]. But it is rarely applied to spectrophotometric analysis, because the thin solid film can not be made into enzyme tube or column and can not determine the color reaction of enzyme on the film and substrate by using conventional flow injection analysis (FIA)[11,12]. We set up a spectrophotome- tric microanalysis method and apply the electrostatic self-assembly monolayer of Horseradish peroxidase (HRP)/ negative ionized poly(ethylene terephthalate) (PET) to spectrophotometric analysis. HRP/PET film coupling with other enzyme system (such as glucose-oxidase) may be applied to the determination of compound in micro scale of biologic and medicinal samples.

Experimental

Substrate preparation and HRP self-assembly

The method of negative ionization on the surface of PET film is the same as that in the reference[13] and the PET-CO_2^- substrate was obtained. Then the PET-CO_2^- substrate was

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submerged into the HRP enzyme solution (0.5 mg•mL^{-1} in pH 6.8 phosphate buffer solution, PBS) at 25℃ and the HRP monolayer is assembled. After about 30 min, the PET film was taken from solution, rinsed with PBS twice. The HRP/PET film was stored in refrigerator at 4℃.

Color reaction of self-assembly HRP/PET film and H_2O_2 in micro cuvette

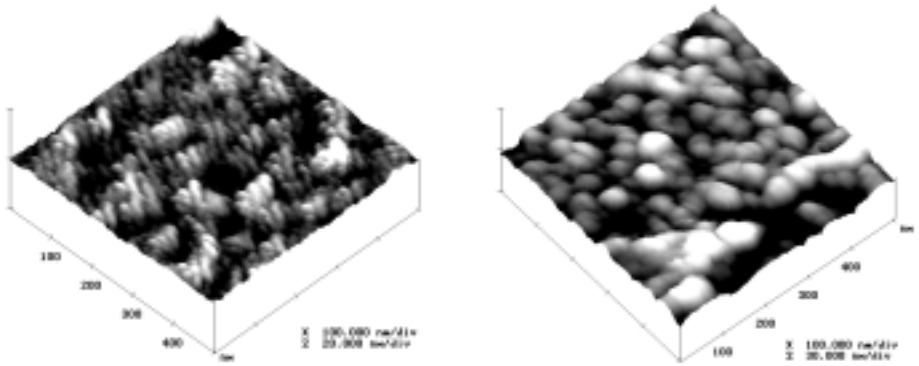
A shutter was placed on the light path of spectrophotometer to make the width of light beam less than 1 mm. Accurately added about 0.2 mL fresh H_2O_2 solution and 0.2 mL color reagent (consisting of 0.5 mg•mL^{-1} 4-amino antipyrine and 16 mg•mL^{-1} phenol in pH 7.0 PBS) into a micro cuvette. Warmed up 5 min in water bath at 37℃. Then inserted a 0.9 cm width of self-assembly HRP/PET film, stirred at 37℃. After 10 min, took out the film and determined the absorbance at 510 nm using the improved spectro- photometer.

Results and Discussion

The HRP self-assembly monolayer and AFM analysis

In pH 6.8 buffer solution, the HRP molecules (isoelectric point pI=7.2) were introduced to be positively charged and deposited on the surface of PET-CO2$^-$ by electrostatic interaction. The AFM images (Figure 1) of PET and HRP/PET film indicated that monolayer film of HRP was assembled on the surface of PET-CO2$^-$ substrate. The root-mean-square roughness of PET film was 0.645 nm. After assembling the root-mean-square roughness of HRP/PET film increased to 2.580 nm.

Figure 1 AFM image of PET-CO_2^- substrate and HRP/PET film at surface



The stability of HRP/PET enzyme film

The activity of HRP/PET enzyme film stored at 4℃ was determined using Worthington method[14]. The results are list in **Table 1**. The relative activity of HRP film on PET decreased to about 85% in the first week and then almost kept constant. After 150 days, the relative activity still remained above 80%. The activity stability of HRP in storing at 4℃ was very good.

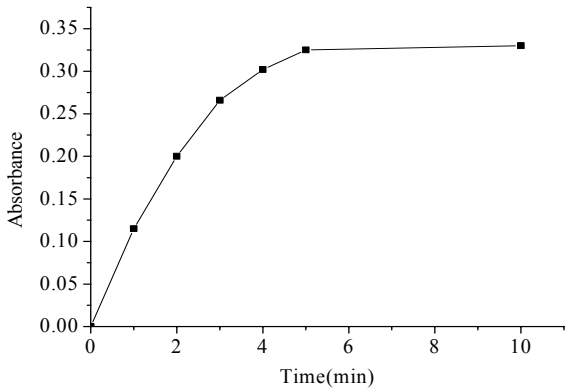
Table 1 Activity of HRP/PET film in storing

Number of days	Relative activity%	Number of days	Relative activity%	Number of days	Relative activity%
1	100	15	84.4	90	82.8
3	89.3	30	83.6	120	83.5
7	86.5	60	82.3	150	82.4

Color reaction of self-assembly HRP/PET film and H_2O_2

In this paper, the application of self-assembled HRP/PET in spectrophotometric analysis was studied by the reaction system of H_2O_2 and 4- amino antipyrine. This system was catalyzed by HRP. Because the weight of enzyme assembled on the surface of PET film is very small, the volume of reaction solution reacted to the enzyme on the surface of HRP/PET film must be small enough to finishing the color reaction quickly. So the micro cuvette with inner width of 2 mm and volume of 0.8 mL was used as reactor. The reaction progress was recorded (**Figure 2**) and the color reaction finished in 5 min. The Michaelis constant(K_m^{app}) calculated by Henri-Michaelis-Menten equation[15] was 3.2×10^{-5} mol•L^{-1}(for the substrate H_2O_2).

Figure 2 Kinetic curve of the reaction of HRP/PET and H_2O_2($C_{H_2O_2}=4.20\times10^{-5}$ mol · L^{-1})



When the absorbance was measured by using micro cuvette, the width of light beam was larger than 2 mm and the inner surface of cuvette reflected the light beam. The measurement error was very large. Therefore a shutter was placed on the light path and made the width of light beam smaller than 1 mm. The precision of determination of H_2O_2 indicated that this method was effective (**Table 2**, Average: A=0.326, relative standard deviation: RSD=1.9%).

Table 2 Determination results of H_2O_2 using micro cuvette as reactior

Number n	1	2	3	4	5	6	7	8	9
Absorbance A	0.324	0.319	0.339	0.328	0.320	0.322	0.322	0.326	0.330

The sample determination

To research the practicability of self-assembly HRP/PET enzyme film, the calibration curve of color reaction and the recovery of H_2O_2 in sample were determined. The relationship between absorbance of color solution and concentration of H_2O_2 was very good and the recoveries of H_2O_2 were in the range of 96.5%~101.1%.

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