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ORIGINAL PAPER

Determination of Bioactive Matter by Affinity Adsorption Solid Substrate–Room Temperature Phosphorimetry Based on Lectin Labeled with Self-ordered Ring of Eosin Y

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Abstract A new phosphorescent labeling reagent named self-ordered ring (ESOR) of eosin Y (E) was developed. And the application of the determination of bioactive matter by affinity adsorption solid substrate–room temperature phosphorimetry (AA-SS-RTP) based on ESOR labeling lectin was studied. Results showed that pink and homogeneous ESOR could be formed by E on polyamide membrane (PAM) in the presence of cetyltrimethylammonium bromide (CTAB) and ammonia water. ESOR could emit strong and stable room temperature phosphorescence (RTP) signal of E in the presence of heavy atom perturber. Specific affinity adsorption (AA) reactions could be carried out between the products of concanavalin agglutinin (Con A), triticum vulgaris lectin (WGA) labeled with ESOR and alpha-fetoprotein variant (AFP-V), alkaline phosphatase (ALP), glucose (G), respectively. Not only did the products of the affinity adsorption reactions preserve good RTP characteristic of E, but also the ΔI_p ($\Delta I_p = I_{p2} - I_{p1}$, I_{p1} is the RTP intensity of blank reagent, I_{p2} is the RTP intensity of sample) of these products was proportional to the content of AFP-V, ALP and G, respectively. According to the facts above, a new method of AA-SS-RTP for the determination of AFP-V, ALP and G was established, based on ESOR labeling lectin. Detection limits (LD) of this method were 0.040 fg spot^{-1} for AFP-V, 0.045 fg spot^{-1} for ALP and 0.090 fg spot^{-1} for G. And it has been successfully applied to the determination of AFP-V in human serum as well as the survey and forecast of human diseases. This method had high sensitivity, good repeatability, long RTP lifetime and little background interference with λ_{em}^{max} at the long wavelength area. Meanwhile, the mechanism for the determination of trace AFP-V by AA-SS-RTP based on Con A labeled with ESOR was also discussed.

Keywords Bioactive matter · Self-ordered ring of Eosin Y · Lectin · Affinity adsorption solid substrate–room temperature phosphorimetry

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Introduction

Sato et al. [1] have reported that most cirrhosis patients whose AFP-V content increase will be diagnosed as suffering hepatic carcinoma in 3 to 18 months. Bone fracture and rickets will cause the content of ALP to increase [2], but osteoporosis and myeloma etc. will cause the content of ALP to decrease [3]. G is closely related to malignant tumor, diabetes mellitus [4] and so on. Obviously, the content of bioactive matter in living organism is closely related to human diseases. So searching for a high sensitive method for the determination of trace bioactive matters such as AFP-V, ALP and G etc. is of high academic value and significance.

The immunity analysis in the research of life science and clinical supervision has wide application prospect. Although the research and application of the fluorescence immunity analysis (FIA) have made wide and thorough progress both at home and abroad, FIA is disturbed by the strong and large changes of fluorescence background from sample (especially living samples) and scattering influence, etc. The emission wavelength of solid substrate–room temperature phosphorimetry immunity analysis is longer than that of fluorescence, and it can eliminate the interference of the fluorescence background and the scattering light with high selectivity, etc. So it has been developed rapidly. Solid substrate–room temperature phosphorimetry immunity analysis has been successfully realized the determination of bioactive matter in 0.X pg spot^{-1} (a mol) grade, but there are few phosphorescent labeling reagents [5]. In resent years, learners both at home and abroad have developed many labeling reagents (such as a dendrimer molecule containing multiple of luminescence molecules [6], gold nanoparticles [7], fluorescence nanoparticles latex [8], phosphorescence nanoparticles [9], luminescence quantum point [10] and Rhodanme6G–dibromoluciferin–SiO_2 luminescence nanoparticles [11]) and so on. However, the application of nanoparticles and luminescence quantum point, etc. is limited, because they can only be dissolved in water after surface modifying. Therefore, the development of some new phosphorescent labeling reagents has become a research focus of solid substrate–room temperature phosphorimetry immunity analysis.

We suppose that the structure-ordered of self-installed film of luminescent molecules can be formed by luminescent molecules on PAM. The quantitative specific AA reaction can be carried out between Con A, WGA labeled with self-installed film of luminescent molecules and AFP-V, ALP, G. and so on. A new luminescence analysis for the determination of trace bioactive matter was established, based on the relation between the contents of AFP-V, ALP, G and the signal changes of luminescent molecules. Meanwhile, we could also forecast human diseases according to the relation between the contents of AFP-V, ALP, G and human diseases.

The research showed that pink and homogeneous self-installed ring of eosin (ESOR) can be formed by E on PAM in the presence of CTAB and ammonia water. It can emit strong and stable RTP signal in the presence of heavy atom perturber. According to the facts that the specific AA reactions can be carried out between the products of Con A, WGA labeled with ESOR and AFP-V, ALP, G, respectively. The reactions preserved good RTP characteristic of E, and the ΔI_p was proportional to the contents of AFP-V, ALP and G. According to the facts above, a new method of AA-SS-RTP for the determination of AFP-V, ALP and G was established, based on lectin labeled with ESOR. Detection limits (LD) of this method were 0.040 fg spot^{-1} for AFP-V (1.0×10^{-13} g ml^{-1}), 0.045 fg spot^{-1} for ALP (1.1×10^{-15} mol l^{-1}) and 0.090 fg spot^{-1} for G (1.0×10^{-14} mol l^{-1}), respectively. Compared with the methods such as method (LD=9.0×10^{-10} ng ml^{-1}AFP-V) [12], biosensor assay (LD=1.4×10^{-15} mol l^{-1} ALP) [13], catalytic fluorescence method (LD=6.3×10^{-9} mol l^{-1} G) [14], the sensitivity of this method is higher.

The method for the determination of AFP-V content in human serum has been applied to the forecast of human diseases (such as primary hepatic carcinoma, cirrhosis, acute and chronic hepatitis, transfer hepatocellular, etc.), whose result was tallied with enzyme-linked immunosorbent assay (ELISA) method. Using ESOR as phosphorescent labeling reagent can be prepared before experiment with simple operation. ESOR will be a reagent box which can be applied to the rapid clinical detection of AFP-V, ALP, G. and forecast of human diseases. The method is of easy operation, speediness, high sensitivity and good selectivity. There are rarely reports about AA-SS-RTP for the determination of AFP-V, ALP and G based on ESOR labeling lectin.

Materials and methods

Apparatus and reagents

Phosphorescent measurements were carried out on a LS-55 luminescence spectrophotometer with a front-surface attachment (Perkin Elemer Corporation of American). The instrument's main parameters are as follows: delay time 0.1 ms, gate time 2.0 ms, cycle time 20 ms, flash count 1, Ex Slit 10 nm, Em Slit 15 nm, scan speed 1,500 nm min^{-1}. KQ-250B ultrasonic washing machine (Ultrasonic Machine Company Limited of Kunshan); AE240 electronic analytical balance (Mettler-Toledo Instruments Company Limited);and 0.50 μl flat head micro-injector (Medical Laser Instrument Plant, Shanghai, China).

ALP, AFP-V, *N*-acetyl glucosamine, BSA (bovine serum albumin), WGA and Con A are all purchased from Sigma Corporation and stored at 0–4 °C and were diluted to proper concentration before use (1.00 ng ml^{-1} ALP, 1.00 ng ml^{-1} AFP, 1.00 μg ml^{-1} G, 700.0 ng ml^{-1} Con A, 700.0 ng ml^{-1} WGA, 10.00 mg ml^{-1} BSA); 1.0×10^{-5} mol l^{-1}E, Na_2CO_3–$NaHCO_3$ buffer solution, pH=9.12; KH_2PO_4–Na_2HPO_4 buffer solution, pH=7.4; Tris (hydroxymethyl) aminomethane, HCl, NaCl, Tween-20, LiAc, CTAB, ethyl silicate (TEOS), ammonia water and HCl, etc. All reagents were A.R. grade except that G and BSA were of biological reagents. The used water was distilled three times.

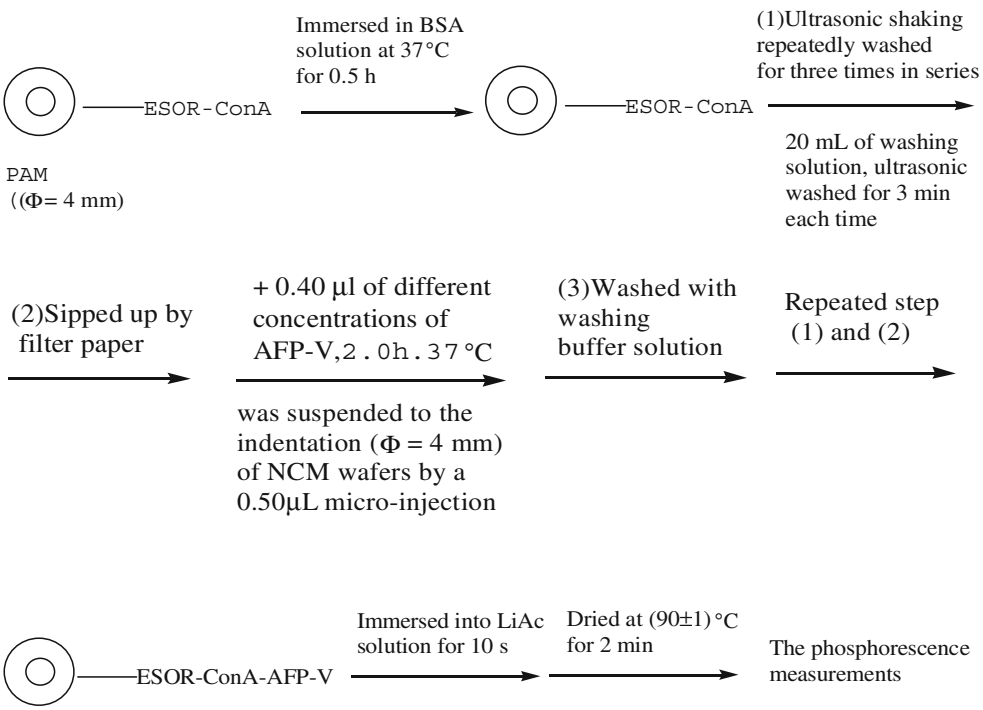


Fig. 1 Direct method (the AA reaction between ESOR-Con A and AFP-V and its RTP detection)

Preparation of Tris–HCl–0.1%Tween-20 washing buffer solution: 1.00 ml of Tween-20, 250 ml of 0.20 mol l^{-1} Tris solution and 400 ml of 0.10 mol l^{-1} HCl containing 150.0 mmol l^{-1}NaCl were mixed together, then diluted to 1,000 ml.

Preparation of 0.10 mol l^{-1} Na_2CO_3–$NaHCO_3$ buffer solution: 200 ml of 0.1 mol $l^{-1}$$Na_2CO_3$ and 800 ml of 0.10 mol l^{-1} $NaHCO_3$ were mixed together. The pH value was 9.40 at 20 °C and 9.12 at 37 °C, respectively.

Preparation of solution containing BSA: BSA was diluted to 10.00 mg ml^{-1} with 0.10 mol l^{-1} Na_2CO_3–$NaHCO_3$ buffer solution and then stored at 4 °C for the following experiments.

PAM, acetyl cellulose membrane (ACM), and nitrocellulose membrane (NCM) were purchased from Luqiaosijia biochemical plastic plant. The paper sheet was pre-cut into wafers (Φ=1.5 cm).

Preparation of ESOR

In a small beaker, PAM was immersed in 5.00 ml of 2.0 mol l^{-1} NaOH and 5.00 ml of H_2O_2 for 24 h. After

Table 1 Optimization of the concentration and volume of reagents

Reagents	Concentrations and volumes	The ΔI_p in AFP-V-Con A-ESOR system	Optimal
E (g)	0.4556, 0.3797, 0.1899, 0.0949, 0.0633, 0.0475	119.3, 119.4, 114.6, 106.5, 98.5, 88.4	0.3797 g
CTAB (g)	0.3, 0.2, 0.1, 0.05, 0.025, 0.012	119.3, 119.5, 108.6, 96.5, 88.5, 78.4	0.2000 g
NH_3 (%, m V^{-1})	5.0, 10, 15, 20, 25	72.6, 89.7, 104.6, 115.3, 119.4	25.0%
NH_3 (ml)	12.0, 15.0, 18.0, 21.0, 24.0	86.6, 102.4, 114.7, 119.6, 119.3	21.00 ml
H_2O_2 (%, V V^{-1})	2.5, 5.0, 7.5, 10.0, 12.5	74.9, 95.7, 109.4, 119.4, 119.1	10.0%
H_2O_2 (ml)	2.00, 3.00, 4.0, 5.00, 6.00	68.7, 89.3, 107.9, 119.5, 119.3	5.00 ml
TEOS (%, m V^{-1})	7.0, 14.0, 28.0, 48.0, 98.0	32.5, 57.4, 78.9, 99.3, 119.2	98.0%
TEOS (ml)	0.50, 0.75, 1.0, 1.25, 1.5	56.5, 74.6, 119.3, 84.7, 69.7	1.00 ml
NaOH (mol l^{-1})	0.50, 1.0, 1.5, 2.0, 2.5	63.5, 84.8, 103.8, 119.5, 119.3	2.0 mol l^{-1}
NaOH (ml)	3.50, 4.00, 4.50, 5.00, 5.50	83.4, 102.7, 113.3, 119.6, 119.0	5.00 ml
Con A (ng ml^{-1})	500.0, 600.0, 700.0, 800.0, 900.0	73, 104.5, 119.4, 119.3, 119.2	700.0 ng ml^{-1}
Con A (ml)	0.10, 0.50, 1.00, 1.50, 2.00	96.4, 112.7, 119.5, 119.29, 119.30	1.00 ml

For 20.0 fg $spot^{-1}$ AFP-V, the optimum concentration and volume of reagents were 0.3797 g E, 0.2000 g CTAB (E/CTAB=1:1), 21.00 ml of 25.0% NH_3, 5.00 ml of 10.0% H_2O_2, 1.00 ml 98% TEOS, 5.00 ml of 2.0 mol l^{-1} NaOH and 1.00 ml of 700.0 ng ml^{-1} Con A.

taken out and washed three times by water, PAM was immersed in water again for 24 h.

After that, took it into a conical flask, and 0.20 g CTAB, 27.00 ml water and 21.00 ml ammonia water were added, then agitated the solution until CTAB was completely dissolved. Added quantitative E (CTAB/E=1:1, molar ratio) until E was dissolved, deposition product was instantly formed after slowly dropping 1.00 ml TEOS. Agitated at 60 °C for 4 h, E could form pink and homogeneous ESOR on PAM. Took ESOR out, washed it with water and ethanol, then dried and stored at room temperature for preparation. During the experiment, we can prepare a certain quantity of ESOR which can be used to label WGA and Con A.

Filtered the deposition product (ESOR), washed with water and ethanol, and then dried it at room temperature for control experiment.

Labeling of Con A and WGA

Different concentrations and volumes of Con A and WGA were prepared with KH_2PO_4–Na_2HPO_4 buffer solution (W V^{-1}, pH=7.4); 0.40 μl of Con A and WGA were suspended onto the indention of ESOR (Φ=4 mm) using 0.50 μl flat head micro-injector and incubated at 37 °C for 2 h. Washed by washing solution, ultrasonic oscillation for three times. The optimal concentration and the volume of Con A was selected according to the results of the AA reaction between Con A and different concentrations of AFP-V, and the results of phosphorescence measurements. It's the same as WGA (the AA reaction between WGA and different concentrations of G).

AA reaction and the measurement of RTP

The type of AA reaction used in this paper is direct method (Fig. 1).

The emission phosphorescence intensity of the blank reagent (ESOR-Con A) signal (I_{p1}) and the sample (ESOR–Con A–AFP-V) signal (I_{p2}) were recorded, respectively. Each sample was measured six times. Then ΔI_p ($=I_{p2}-I_{p1}$) was also calculated.

Con A was replaced by WGA, the content of ALP and G was determined according to the method above, and ΔI_p was calculated ($=I_{p2}-I_{p1}$).

Table 2 Effects of the solid substrate on ΔI_p for the ESOR–Con A–AFP-V system

	ACM	PAM	NCM	Note
ΔI_p	83.3	119.3	70.8	ESOR

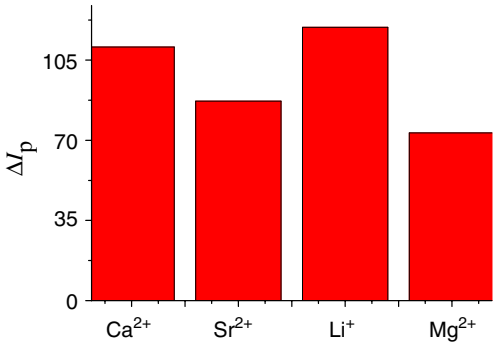


Fig. 2 The effect of ions perturbation on the ΔI_p of the system (when chose 1.00 mol l^{-1} of ion perturbers of Mg^{2+}, Ca^{2+}, Li^+ and Ag^+, the ΔI_p were 110.8, 87.1, 119.3, 73.2, respectively)

Results and discussion

Optimum conditions for determination

Concentration and volume of reagent

For the system containing 20.0 fg AFP-V $spot^{-1}$, the effects of concentrations and volumes of reagents on ΔI_p were studied, respectively. Results showed that when 0.20 g CTBA, 0.3797 g E (CTBA/E=1:1), 21.00 ml of 25.0% NH_3, 5.00 ml of 10.0% H_2O_2, 1.00 ml of 98% TEOS, 5.00 ml of 2.0 mol l^{-1} NaOH and 1.00 ml of 700.0 ng ml^{-1} Con A were selected, the ΔI_p of the system reached maximum and stayed stable (Table 1).

Selection of solid substrate

For the system containing 20.0 fg AFP-V $spot^{-1}$, the effects of different solid substrates such as ACM, NCM and PAM on ΔI_p were studied, respectively (Table 2). Result showed that ΔI_p of the system with PAM was the highest. The membrane of PAM has one bright side, which is easy to

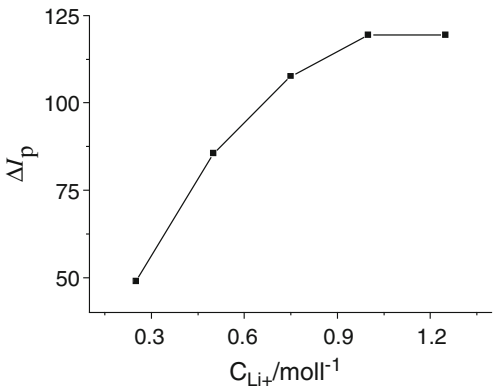


Fig. 3 The effect of concentrations of Li^+ ΔI_p of the system on the ΔI_p of the system (when concentrations of Li^+ were 0.25, 0.50, 0.75, 1.0 and 1.25 mol l^{-1}, the ΔI_p were 48.9, 85.5, 107.6, 119.4, and 119.4, respectively)

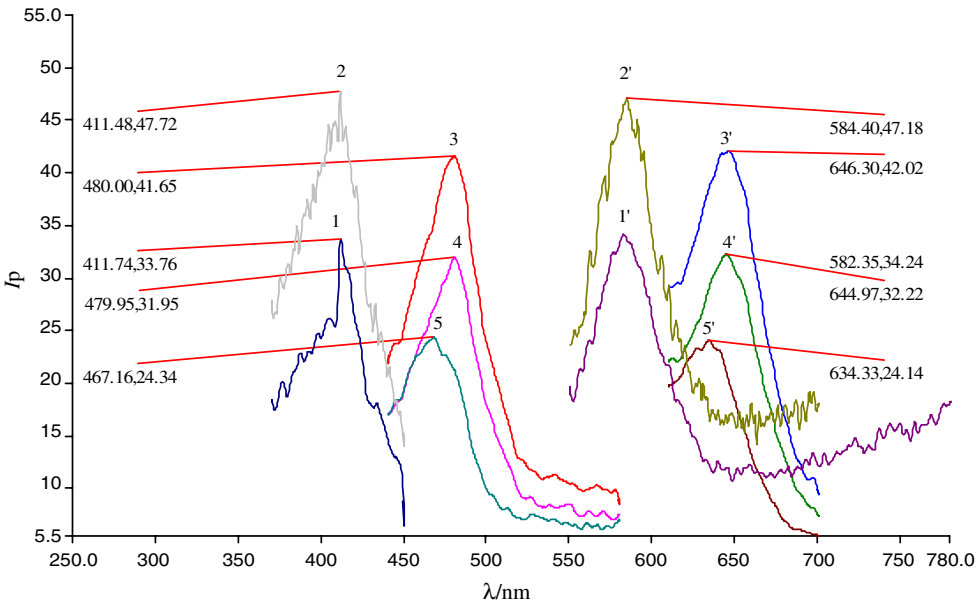


Fig. 4 The RTP spectra for ESOR: *1*, PAM, *1′* PAM, *2* 1 +CTAB, *2′* 1′+CTAB, *3* 1 +ESOR, *3′* 1′+ESOR, *4* 1+E, *4′* 1′+E, *5* 1+ESOR power, *5′* 1′ +ESOR power (*curves 1*, *2*, *3*, *4* and *5* are excitation spectra; *curves 1′*, *2′*, *3′*, *4′* and *5′* are emission spectra)

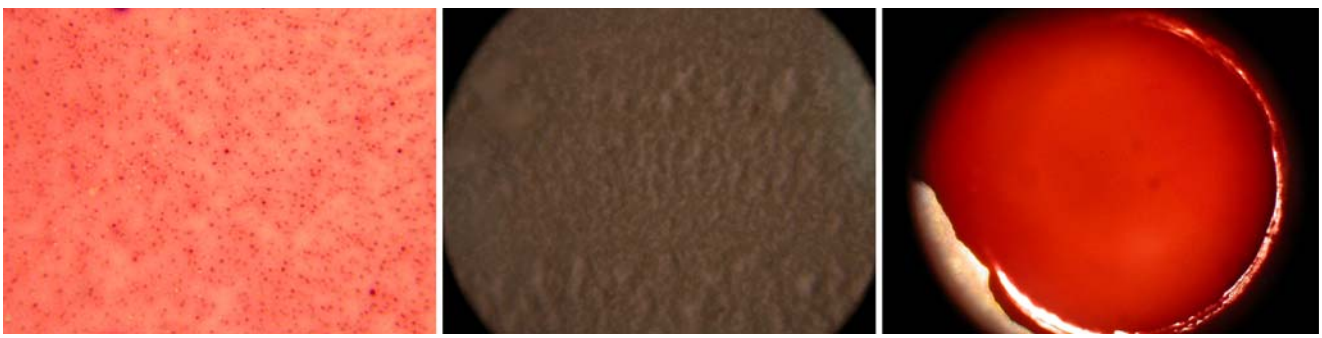
recognize and suspend, the soaking and drying of heavy atom perturbers carry on easily, and it can preserve good rigidity after washed for many times. So PAM was chosen as solid substrate in this experiment.

Ion perturbation

For the system containing 20.0 fg AFP-V spot^{-1}, the effects of ions such as 1.00 mol l^{-1} Mg^{2+}, Ca^{2+}, Li^{+} and Ag^{+} on ΔI_p were examined, respectively (Fig. 2). Results showed that ΔI_p of the system with Li^{+} was the highest, so Li^{+} was selected as ion perturber. Meanwhile, the effects of concentrations of Li^{+} on ΔI_p were also examined (Fig. 3). Results showed that ΔI_p reached the maximum when 1.00 mol l^{-1} Li^{+} was used. For AFP-V, the heavy atom effect of Li^{+} was obviously higher than that of perturbers commonly used such as Mg^{2+}, $Ca^{2+}$and Ag^{+}. So, with the heavy atom effect of Li^{+}, the phosphorescence intensity reached the maximum. This indicated that light element ions had heavy atom effects.

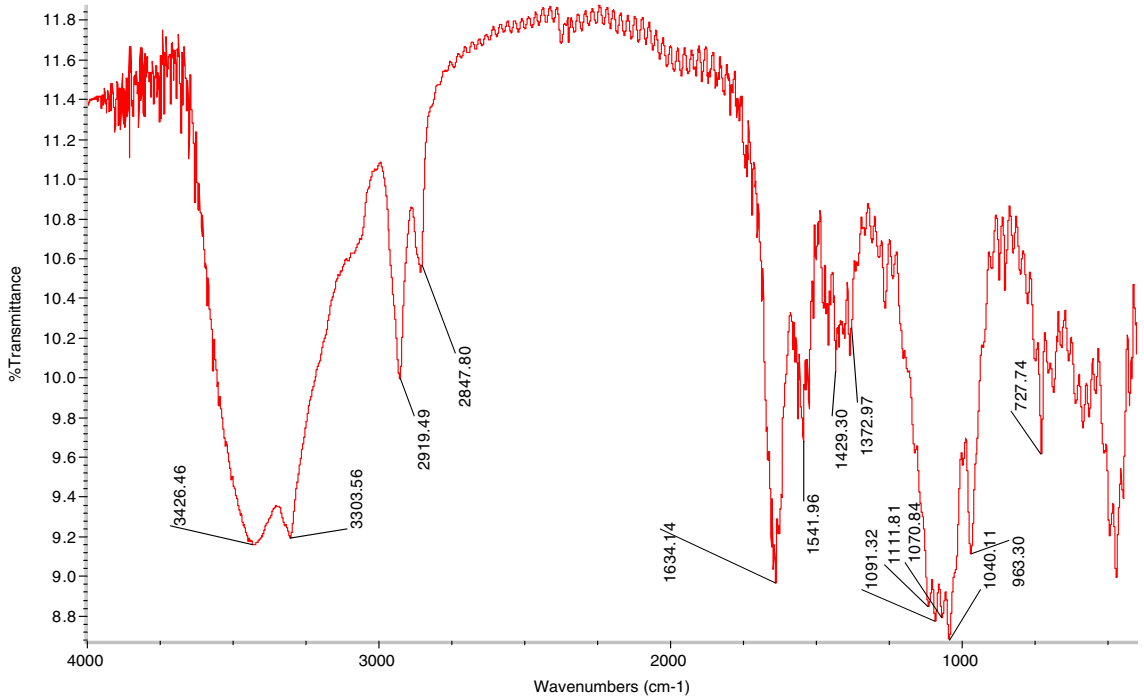
Infusion time

For the system containing 20.0 fg AFP-V spot^{-1}, results showed that the ΔI_p of the system were 24.6, 65.3, 88.2, 108.6, 119.3 and 119.3 when the infusion time were 8, 12, 16, 20, 24 and 28 h, respectively. Result showed that when the infusion time was less than 24 h, the ΔI_p of the system was small, it was mainly because that the reaction was incomplete; however, when the infusion time was between 24 to 28 h, the ΔI_p of the system was the maximum and stable. So, the best infusion time is 24 h.

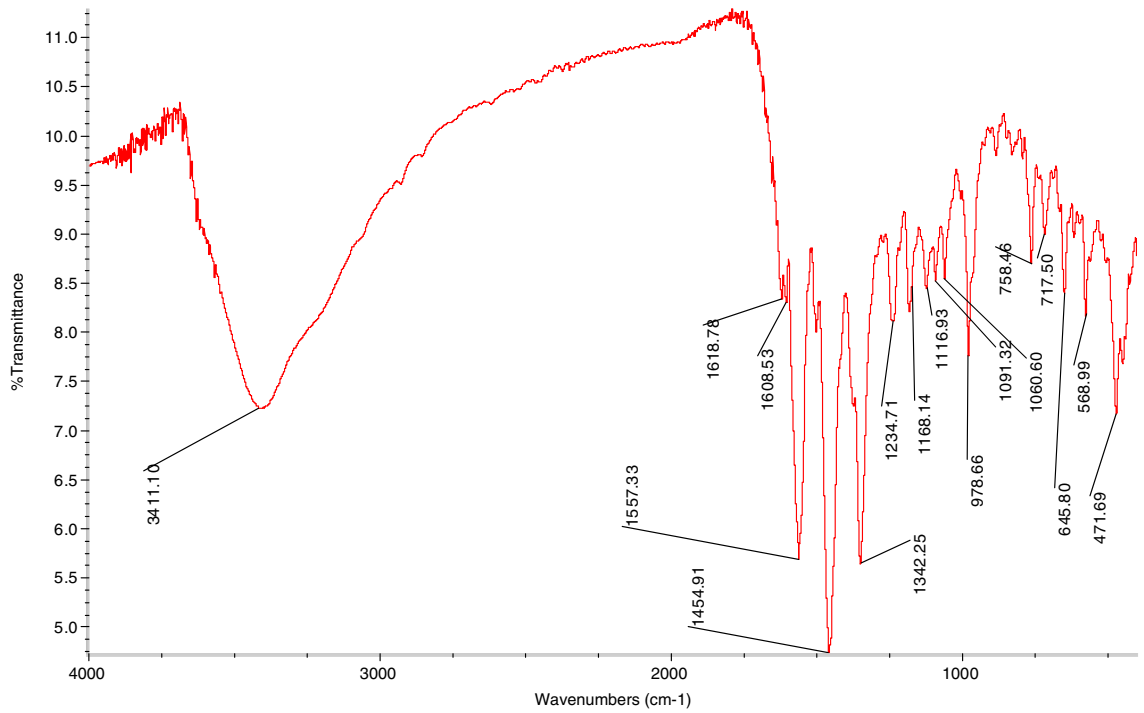


1. E (400) 2. PAM (40 times) 3. ESOR (40 times)

Fig. 5 Photos of biology microscope (LB2 Leica Microsystem wetzlar Gmbh Made in Germany) of E, PAM and ESOR are completely different, which shows that E can form *red* well-proportioned ESOR on PAM. *1* E (×400), *2* PAM (×40), *3* ESOR (×40)



1. ESOR



2. E

Fig. 6 Infrared spectrum (KBr wafer method, Necolet-360 Nicolet Company, USA): there are notable difference in infrared spectrum between ESOR and Eosin Y, which further improved that there were ESOR formed

Time and temperature for agitating

For the system containing 20.0 fg AFP-V spot^{-1}, the effects of temperature and time on ΔI_p were examined. Results showed that the ΔI_p of the system were 24.6, 73.4, 92.2, 105.4, 113.3, 119.5 and 119.3 when the reaction temperatures were 35, 40, 45, 50, 55, 60 and 65 °C, respectively. When the reaction time was 2.0, 2.5, 3.0, 3.5, 4.0 and 4.5, the ΔI_p of the system were 67.6, 86.9, 104.8, 115.4, 119.4 and 119.3, respectively.

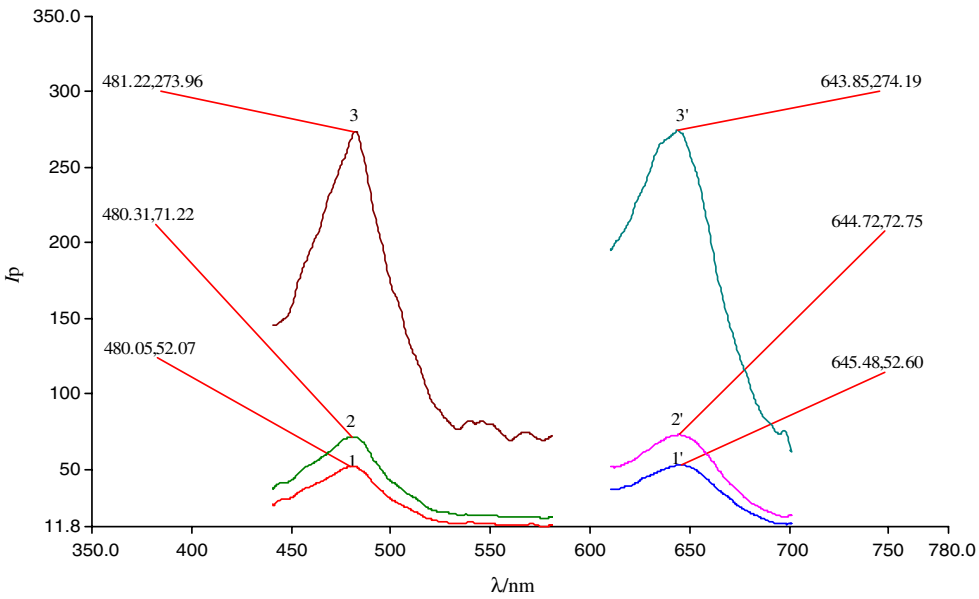


Fig. 7 The RTP spectra for ESOR–Con A–AFP-V method: *1* ESOR+700.0 ng Con A, *1′* ESOR+700.0 ng Con A, *2* ESOR+0.50 pg AFP-V, *2′* ESOR+0.50 pg AFP-V, *3* ESOR +100.0 pg AFP-V, *3′* ESOR +100.0 pg AFP-V (*curves 1*, *2* and *3* are excitation spectra; *curves 1′*, *2′* and *3′* are emission spectra)

Result showed that agitated for 4 h at 60 °C, the ΔI_p of the system was the maximum and stable. If the agitate temperature exceed 60 °C and the agitate time exceed 4 h, PAM will be easy to fall off and the ΔI_p of the system will be decreased. So we chose 60 °C and 4 h as agitate temperature and time.

Oxygen and humidity

For the system containing 2.00 fg spot^{-1}AFP-V, the effects of oxygen and humidity on ΔI_p were examined according to the experiment method mentioned above. Results showed that when desiccated N_2 was passed for 5, 10, 15 and 20 min, the ΔI_p of the system was 119.2, 119.5, 119.3 and 119.4, respectively. When desiccated N_2 was not passed, the ΔI_p of the system was 119.4, 119.1, 119.5 and 119.3, respectively. Results showed that the system was remained stable whether desiccated N_2 was passed or not. Therefore, we may not pass desiccated N_2 for simplifying the experiment.

Stability of system

Under the optimal conditions above, the ΔI_p of the system were 119.6, 119.3, 119.5, 119.4, 119.6 and 119.4 when the replace time of the AA resultant were 1.0, 2.0, 3.0, 4.0, 5.0 and 6.0 h, respectively. It showed that the ΔI_p of the system stayed stable within 6 h.

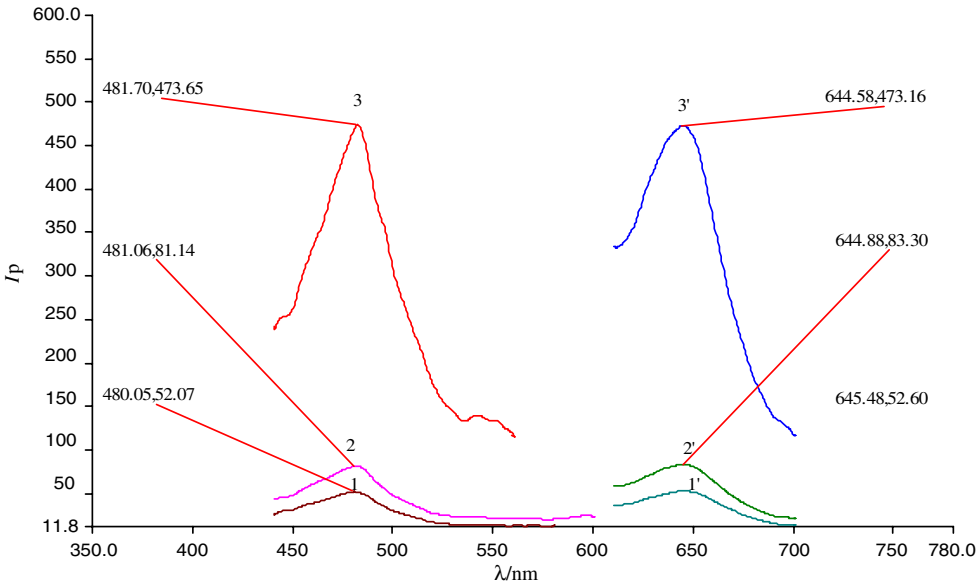


Fig. 8 The RTP spectra for ESOR–WGA–ALP method: *1* ESOR+700.0 ng WGA, *1′* ESOR+700.0 ng WGA, *2* ESOR +1.0 pg ALP, *2′* ESOR+1.0 pg ALP, *3* ESOR+800.0 pg ALP, *3′* ESOR+800.0 pg ALP (*curves 1*, *2* and *3* are excitation spectra; *curves 1′*, *2′* and *3′* are emission spectra)

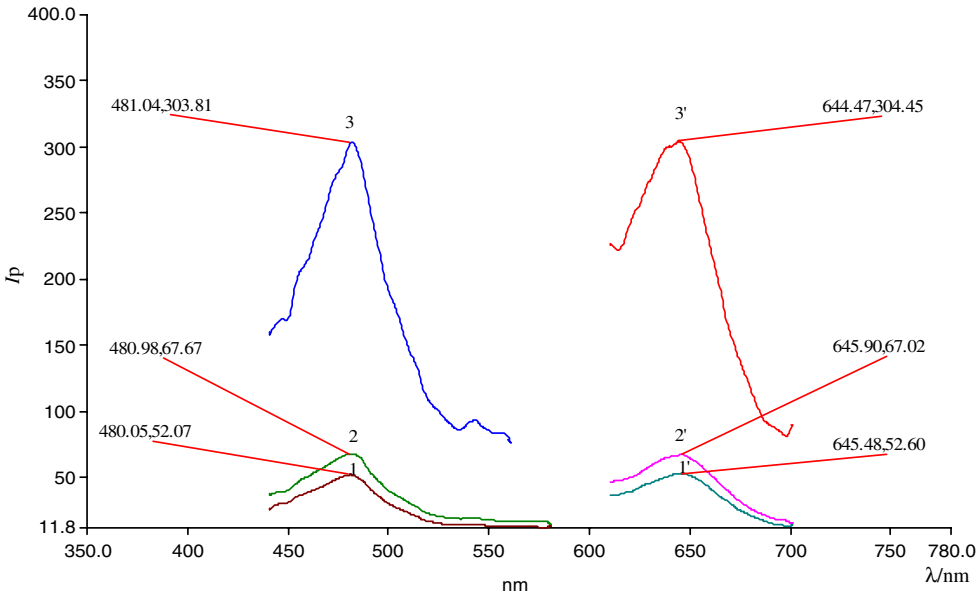


Fig. 9 The RTP spectra for ESOR–WGA–G method: *1* ESOR+700.0 ng WGA, *1′* ESOR+700.0 ng WGA, *2* ESOR +1.0 pg G, *2′* ESOR+1.0 pg G, *3* ESOR+300.0 pg G, *3′* ESOR +300.0 pg G (*curves 1*, *2* and *3* are excitation spectra; *curves 1′*, *2′* and *3′* are emission spectra)

The RTP spectra of ESOR

The phosphorescence spectra for ESOR, ESOR powder, CTAB and E were scanned (Fig. 4). Results showed that CTAB and E could emit RTP on PAM, and the I_p were 47.2, 32.2 when the $\lambda_{ex}^{max}/\lambda_{em}^{max}$ were 411.5/584.4 and 480.0/645.0 nm, respectively (curve 2.2′, 4.4′). ESOR and ESOR powder could also emit RTP on PAM, the I_p were 42.0 and 24.1 when the $\lambda_{ex}^{max}/\lambda_{em}^{max}$ were 480.0/646.3 and 467.2/634.3 nm, respectively(curve 3.3′, 5.5′). The λ_{em}^{max} discrepancy of ESOR and ESOR powder was 12 nm with I_p 9.8 larger than E, which indicated that ESOR still preserved good RTP characteristic. Results proved that the structure of ESOR in order was formed by E on the surface of PAM. ESOR can be used as labeling reagent for its good RTP characteristic.

Comparing the photos of biology microscope of E (×400), PAM (×40) and ESOR (×40) (Fig. 5), the result showed that the pink and homogeneous ESOR was formed on PAM. Meanwhile, there were many great differences between the ESOR and E in infrared spectrum. ESOR [IR (cm^{-1}): 3,426.46, 3,303.56 (NH_2 and OH), 2,919.49, 2,847.80 (CH_2,CH_3), 1,634.14 (CONH), 1,541.96 (Ph), 1,429.30, 1,372.97 (CH_2,CH_3), 1,019.32, 1,111.81, 1,070.84, 1,040.11 (C–O or C–N), 963.30, 727.74 (1, 2 substitution)]; E [IR (cm^{-1}): 3,411.10 (OH), 1,557.33, 1,454.91 (Ph), 1,342.25 (COO^-), 1,234.71, 1,168.14, 1,116.93, 1,091.32, 1,060.60, 978.66, 758.46 (1, 2 substitute C–H)]. The facts above further indicated that ESOR was formed on PAM (Fig. 6).

The RTP of the affinity adsorption resultant

The phosphorescence spectra for the ESOR–Con A–AFP-V system were scanned using experimental method (Fig. 7). Results showed that in the presence of 700 ng Con A ESOR could emit RTP ($\lambda_{ex}^{max}/\lambda_{em}^{max}$=480.0/645.5 nm, $I_p = 52.6$), indicating that the labeled products (ESOR-Con A) could preserve the good RTP property of E. In the presence of 100 pg AFP-V, the RTP of ESOR was increased sharply ($\lambda_{ex}^{max}/\lambda_{em}^{max}$=481.2/643.8 nm, $I_p = 274.2$), ΔI_p= 221.6, while the value of $\lambda_{ex}^{max}/\lambda_{em}^{max}$ still remained the same. So 481/644 nm was chosen as the working wavelength for the determination of trace AFP-V.

Also the phosphorescence spectra for the ESOR–WGA–ALP and ESOR–WGA–G systems were scanned

Table 3 Parameter comparison for the determination of AFP-V, ALP and G

Method	Analytical range, fg $spot^{-1}$ (pg m l^{-1})	Regression equation, fg $spot^{-1}$ (pg m l^{-1})	*r*	LD, fg $spot^{-1}$ (pg m l^{-1}) mol l^{-1}	RSD (n=7) (%)
ESOR–Con A–AFP-V	0.20–40.0 (0.50–100.0)	ΔI_p=12.09+5.165 m_{AFP-V}	0.9982	0.040 (0.10)	3.0–4.7
ESOR–WGA–ALP	0.20–200 (0.50–500)	ΔI_p=15.92+1.986 m_{ALP}	0.9981	0.045 (0.11) 1.1×10^{-15}	1.3–3.6
ESOR–WGA–G	0.40–120.0 (1.0–300.0)	ΔI_p=9.081+1.991 m_G	0.9990	0.09 (0.225) 1.0×10^{-14}	0.70–3.6

Table 4 Phosphorescence lifetimes of products of AA reaction

Sample	The regression equation of the delay curve (ms)	Correlation coefficient (r)	Phosphorescence lifetime (τ) (ms)
20.0 fg AFP-V spot^{-1}	$\ln I_p = 5.8718 - 0.07047t + 0.02643t^2$	−0.9883	14.2
80.0 fg ALP spot^{-1}	$\ln I_p = 5.8449 - 0.0155t + 0.00481t^2$	−0.9884	64.5
80.0 fg G spot^{-1}	$\ln I_p = 5.8350 - 0.01442t + 0.004910t^2$	−0.9954	69.4

(Figs. 8 and 9). Results showed that in the presence of 700.0 ng WGA ESOR could still emit RTP ($\lambda_{ex}^{max}/\lambda_{em}^{max} = 480.0/645.5$ nm, $I_p = 52.6$). Both 800.0 pg ALP and 300.0 pg G could sharply increase the RTP of ESOR (ALP: $\lambda_{ex}^{max}/\lambda_{em}^{max} = 481.7/644.6$, $I_p = 473.2$, $\Delta I_p = 420.6$; G: $\lambda_{ex}^{max}/\lambda_{em}^{max} = 481.0/644.5$, $I_p = 304.4$, $\Delta I_p = 251.8$), which indicated that the affinity adsorption resultant could preserve the good RTP property of E. So 482/645 and 481/644 nm were chosen as the working wavelength for the determination of trace ALP and G, respectively.

Working curve, detection limit and precision

Con A and WGA labeled by ESOR were used to determine AFP-V, ALP and G according to the direct method mentioned above. Results showed that the contents of them were proportional to ΔI_p. The working curve, corresponding coefficient (r), the LD (calculated by $3Sb/k$, n=11) and RSDs (%) were list in Table 3.

Result showed that the sensitivity of this method was higher than that of references [12–14]. This method is suitable for the determination of trace biological sample.

Phosphorescence lifetime

The phosphorescence lifetime of AFP-V, ALP, G could be calculated respectively, according to the phosphorescence decay method in ref. [15] (delay time 0.1–1.9 ms, gate time 2.0 ms). The regression equation of $\ln I_p - t$, correlation coefficient (r), and phosphorescence lifetime (τ) were list in Table 4.

From Table 4, we can see that τ of AFP-V, ALP and G was long, which provide possibility for time resolved spectrometry.

Sample analysis

Took four different person serum samples, A, B, C, D (sample capacity is five, each sample was determined for seven times); 0.50 ml of 0.050 mol l^{-1} Tris–HCl (containing 0.50 mol l^{-1} NaCl) buffer solution was added and mixed homogeneously for 10 min. After centrifugation, the upper clear fluid was deserted. Then 1.0 ml buffer solution was added and mixed homogeneously for 10 min again and stored at 4 °C for preparation. Then adsorbent of Sepharose 4B was added, the upper clear fluid was deserted after centrifugation, then diluted to the pg level with 0.010 mol l^{-1} Tris–HCl buffer solution, and shook in an oscillator (300 rpm) for 20 min. After centrifugation (200 rpm) for 5 min, the content of AFP-V in the upper clear fluid was determined by the method described above, and a standard addition recovery experiment was also conducted. This method was compared with the clinical detection results of ELISA method by Zhangzhou Hospital of Chinese Medicine. The results were list in Table 5.

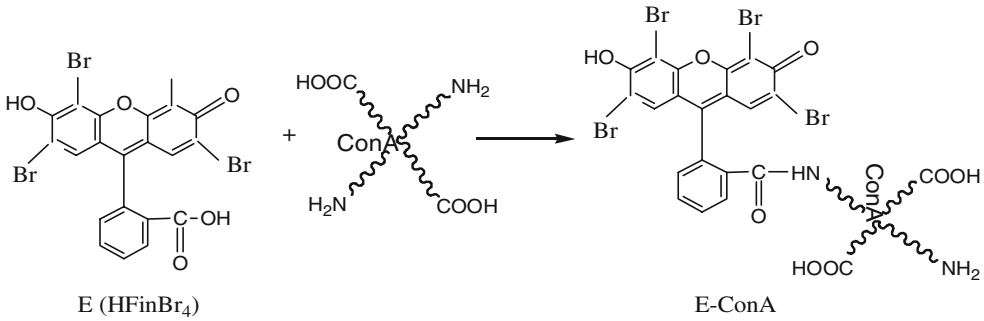
From Table 5, we can see that results of this method were tallied with those of clinical detection and diagnosis using ELISA. Therefore, it can forecast accurately some human diseases such as primary hepatic carcinoma, cirrhosis, acute and chronic hepatitis, transfer hepatocellular carcinoma, etc.

The mechanism of AA-SS-RTP based on ESOR labeling Con A for determination of trace AFP-V

ESOR could emit strong and stable RTP of E ($\lambda_{ex}^{max}/\lambda_{em}^{max} = 639.5/788.8$, $I_p = 45.0$) on PAM (Fig. 4). When Con A was labeled with ESOR, ESOR reacted with Con A to form ESOR-Con A, and it led the I_p (52.2) of E to increase

Table 5 The analytical results of AFP-V in human serum (n=7)

Serum	Found (μg l^{-1})	RSD (%)	Added (μg l^{-1})	Recovery (μg l^{-1})	Percent recovery (%)	ELISA method	Sample number	Age
A. Primary hepatic carcinoma (PHC)	21.8–860.2	4.4–2.8	2.2–86.0	2.2–85.8	100–99.8	22.4–868.2	5	30–70
B. Cirrhosis	22.5–156.3	3.8–4.3	2.3–16.0	2.2–16.2	95.7–101.2	21.6–150.5	5	35–60
C. Acute and chronic hepatitis	24.1–462.4	3.6–3.3	2.4–46.0	2.5–46.7	104–102	25.3–452.7	5	25–35
D. Transfer hepatocellular carcinoma	26.5–35.1	4.8–4.1	2.7–3.5	2.6–3.4	96.3–97.1	25.7–3 6.2	5	45–55



E ($HFinBr_4$) E-ConA

Fig. 10 The reaction of Con A labeled with ESOR (Con A is presented as $(H_2N)_2$-(Con A)-$(COOH)_2$)

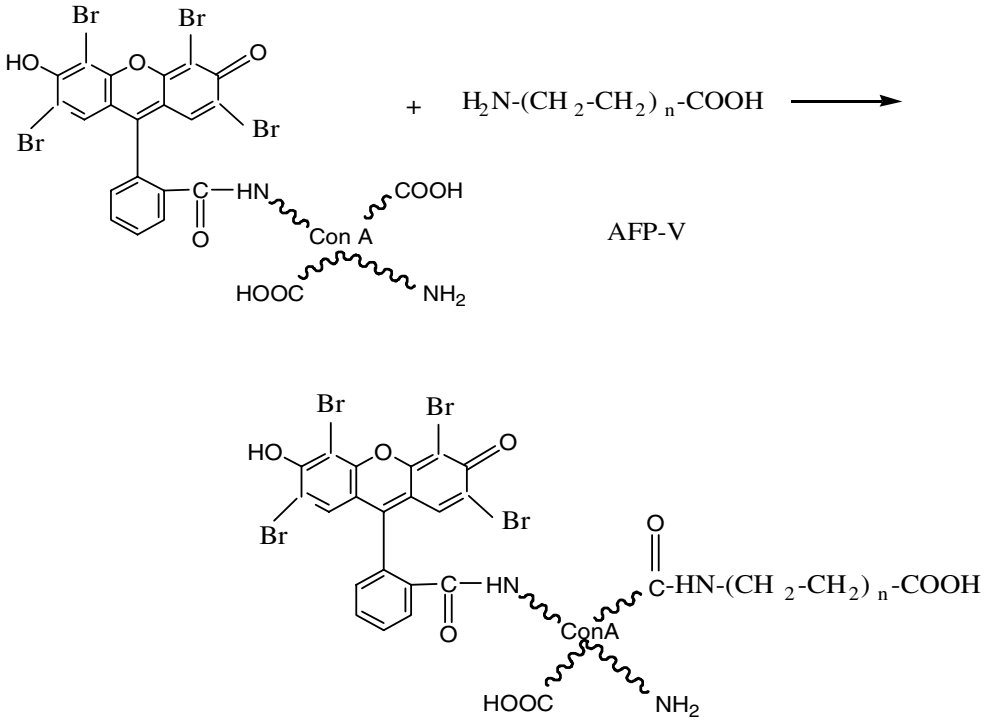
slightly, but $\lambda_{ex}^{max}/\lambda_{em}^{max}$ almost remained the same. The labeling reaction may be expressed as follows (Fig. 10).

Specific AA reaction between AFP-V and Con A-ESOR was carried out to form ESOR–Con A–AFP-V in the presence of AFP-V. The I_p (274.2) was severely enhanced with $\lambda_{ex}^{max}/\lambda_{em}^{max}$ invariable, indicating that ESOR–Con A–AFP-V preserved the good RTP property of E. The determination of AFP-V by AA-SS-RTP based on ESOR labeling Con A was established. The AA reaction was followed (Fig. 11).

Conclusion

ESOR as a new RTP labeling reagent was developed. The labeling product (ESOR-Con A and ESOR-WGA), and the products of AA reaction (ESOR–Con A–AFP-V, ESOR–WGA–ALP and ESOR–WGA–G) all preserved the good RTP property of E, meanwhile, the ΔI_p was proportional to the content of AFP-V, ALP, G, respectively. According to the facts above, a new AA-SS-RTP for determining the contents of bioactive matter (AFP-V, ALP and G) was established, based on lectin labeled with ESOR. This method was tallied with those of clinical detection using ELISA. It can be applied to forecast the human diseases accurately, such as primary hepatic carcinoma, cirrhosis, acute-chronic hepatitis and transfer hepatic carcinoma, etc. The method is of easy operation, speediness, high sensitivity and good selectivity. We can prepare ESOR beforehand and the operation is simple, so ESOR will be a reagent kit which can be applied to the rapid clinical detection of AFP-V, ALP, G. and forecast of human diseases.

The rest may be deduced by analogy, if the labeling product of antibody (Ab) or antigen (Ag) (ESOR-Con A



E-ConA-AFP-V

Fig. 11 The AA reaction between AFP-V and Con A-ESOR (AFP-V is presented as H_2N-(CH_2-CH_2)-COOH)

and ESOR-WGA), and the products of immune response (for example, ESOR-Ab-IgG, ESOR-Ab-IgA, ESOR-Ab-IgM and ESOR-Ab-IgE) all preserved the good RTP property of E, meanwhile, the ΔI_p would be proportional to the content of IgG, IgA, IgM, IgE, respectively. According to the facts above, we can establish new SS-RTP immunoassay to determine IgG, IgA, IgM, and IgE, respectively.

To sum up, this research not only developed a new ESOR phosphorescent labeling reagent, but also established a new method of AA-SS-RTP for the determination of bioactive matter. Meanwhile, this ESOR can be developed into ESOR reagent kit, which can be used to label Ab or Ag; then we can develop many new RTP labeling reagent and SS-RTP, sequentially. The application of these new RTP labeling reagent and these methods, will promote the research progress of SS-RTP, bioanalysis and life science. If E is replaced by other luminescent reagent (L), more LSOR of RTP will be exploited, which can accelerate the research development of self-ordered ring application technique.

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Appendix 1

Table 6 Short list of abbreviations

English	Abbreviations
Eosin Y	E
Self-ordered ring of eosin Y	ESOR
Cetyltrimethylammonium bromide	CTAB
Affinity adsorption solid substrate–room temperature phosphorimetry	AA-SS-RTP
Polyamide membrane	PAM
Stable room temperature phosphorescence	RTP
Concanavalin agglutinin	Con A
Triticum vulgaris lectin	WGA
Alpha-fetoprotein variant	AFP-V
Alkaline phosphatase	ALP
Glucose	G
Affinity adsorption	AA
Phosphorescence intensity of the blank reagent	I_{p1}
Phosphorescence intensity of the sample	I_{p2}
ΔI_p	$\Delta I_p(=I_{p2}-I_{p1})$
Detection limit	LD
Enzyme-linked immunosorbent assay	ELISA
Bovine serum albumin	BSA
(Hydroxymethyl)aminomethane	Tris
Ethyl silicate	TEOS
Acetyl cellulose membrane	ACM
Nitrocellulose membrane	NCM
Antibody	Ab
Antigen	Ag
Immunoglobulin G	IgG
Immunoglobulin A	IgA
Immunoglobulin M	IgM
Immunoglobulin E	IgE
Luminescent reagent	L

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