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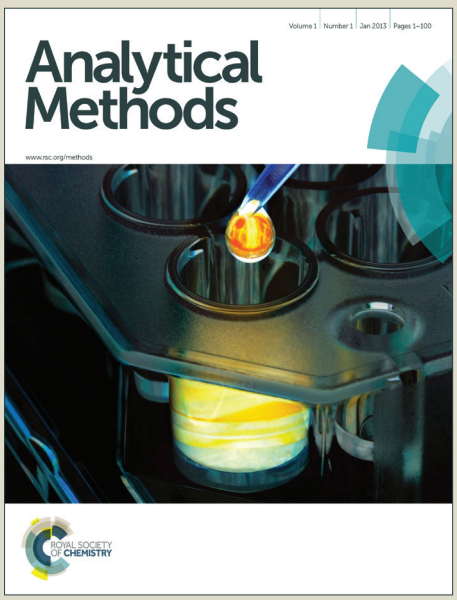

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A novel flow injection chemiluminescence method for the determination of indole-3-acetic acid in biological sample by using trivalent silver

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Abstract

In this paper, a novel flow-injection chemiluminescence method was developed for the determination of indole-3-acetic acid (IAA). The chemiluminescence signal from the reaction of Ag(III) complex and sulfuric acid system was enhanced in presence of IAA. The conditions of the CL system were investigated and optimized. Under the optimal conditions, the relative chemiluminescence intensity was linear with the IAA concentration in the range of 1.0×10^{-10} g·mL^{-1}-1×10^{-8} g·mL^{-1}. The detection limit for IAA was 7.7×10^{-11} g·mL^{-1}, and the relative standard deviation (n = 11) was 0.5%. The proposed method was applied to the analysis of IAA in human urine, mung bean sprouts and soil samples with the recoveries of103.5%-117.1%, 87.0%-98.4% and 94.1%-118.8%, respectively, and the relative standard deviations was0.6-2.7%. The results obtained by the proposed method agreed well with those obtained from HPLC method. The chemiluminescence mechanism was discussed by comparison of fluorescence spectra and the UV-vis absorption spectra.

Keywords: Chemiluminescence; Flow injection, Indole-3-acetic acid; Trivalent silver; biological sample.

1. Introduction

Indole-3-acetic acid is released by the terminal bud of a shoot and acts as a true chemical messenger regulating some important biological processes of plants, e.g., division, elongation and differentiation of cells.[1] And the effects of using IAA in agriculture on environment and human health have aroused widespread concern.[2] However, it is difficult for determination of IAA because the concentration in plants is at very low level(at the ng/g level) .[3] Therefore, it is significant to search for a method with good sensitivity and high accuracy for IAA detection.

So far, some sensitive methods have been reported for determination of IAA in biological samples, such as immunoassay,[4] capillary electrophoresis(CE) ,[5, 6] electrochemical methods[1, 7], colorimetry[8], fluorometry,[9, 10] chromatography methods(LC or GC) with different detection strategies ,[3, 11-15] and LC/GC-MS.[16-20] However, there are some disadvantages exist, for example, enzyme-linked immunosorbent assay suffers from antibodies' cross-reactivity with other compounds of similar structure present in the same sample;[11] chromatography and MS need special instruments and strict experimental conditions. The application of CE and electrochemical methods in analysis of complex real sample is limited for the relatively poor reproducibility.[16]

Chemiluminescence method (CL) has received much more attention for the analysis of organic and inorganic species owing to its low detection limit, high sensitivity, wide linear dynamic range, short response time and relatively simple instrumentation.[21] The flow injection technique (FIA) is based on the injection of a sample into a non-segmented carrier stream targeting to the reaction system. The FIA-CL method is characteristic of well

sensitivity and reproducibility. Diperiodatoargentate (III) (DPA) is widely used as oxidizing agent[22] in inorganic chemistry and may also be used as polymerization initiator.[23] In recent years, there are some reports about using DPA in chemiluminescence,[24] however, to our best knowledge, no work in literature have reported using DPA to detect IAA without any luminescence reagent such as luminol involved.

The weak chemiluminescence in acid conditoins is result from the DPA as oxidant. It can be enhanced in presence of IAA. Based on the CL reaction, a novel chemiluminescence method has been established for the determination of IAA by combining with flow-injection technique. The experimental conditions have been optimized. It has been proved to be a simple, fast and precise method for the determination of IAA with low detection limit. The proposed method has been successfully applied for the determination of IAA in human urine, mung bean sprouts and soil samples. The chemiluminescence mechanism also has been studied by using of the ultraviolet visible absorption spectra and fluorescence spectra.

2. Experimental

2.1. Materials

IAA was purchased from Sigma-Aldrich (St. Louis, MO, USA). The used reagents were listed as follow: Potassium periodate (KIO_4)，potassium persulfate ($Na_2S_2O_8$) and argentum nitricum ($AgNO_3$) was purchased from Guangzhou Guanghua Chemical Factory Co. Ltd. (Guangzhou, China). Potassium hydroxide (KOH) and concentrated sulfuric acid (H_2SO_4) were purchased from Guangzhou Chemical Reagent Factory (Guangzhou, China). All of the chemicals were of analytical reagent grade, and were used without further purification. Doubly distilled water was used throughout the work.

The DPA complex was prepared by Ag(I) compound oxidized in an alkaline medium according the known method.[25] In brief, KIO_4 (3.24 g), $AgNO_3$ (1.36 g), $Na_2S_2O_8$ (3.0 g) and KOH (8.0 g) were added in 200 mL of deionized water. The mixture was heated reflux to boiling for about 40 min on a hot plate with constant stirring. The boiling mixture turned intensely red and the boiling was continued for another 20 min until the completion of the reaction. The mixture was then cooled and filtrated. The obtained DPA complex was stored under refrigeration in dark place, and was found to be fairly stable for several months. DPA solutions were freshly prepared before use. The complex was characterized by the UV/Visible spectrum, which exhibited a brood band at 361 nm with the molar absorptive (ε) of 1.26×10^4 $L \cdot mol^{-1} \cdot cm^{-1}$. The IAA stock solution was 1.6 $mg \cdot mL^{-1}$, it was prepared by dissolving approprite amount of standard substance with a small volume of methanol and then diluted into brown volumetric flask with double-distilled water. The IAA working standards solutions were prepared from the stock solution by appropriate dilutions.

2.2. Apparatus

The CL-FIA system used in this work was shown in **Fig. 1**. Two peristaltic pumps (BT100-1J, Henan Baoding Lange, China) were used to deliver all chemicals at a flow rate of 4.0 $mL \cdot min^{-1}$. PTFE flow tubes (0.8 mm i.d.) were used to connect all components in the system. Injection was made by using a sixteen-port injection valve (Hanzhou, China) equipped with a loop of 100 μL. The CL signal was monitored by a BPCL ultra-weak luminescence analyzer (Institute of Biophysics, Chinese Academy of Science, Beijing, China) consisting of a flat coil glass flow cell facing the window of the photomultiplier tube (PMT). Data acquisition and treatment were performed with BPCL software. The UV-absorbance was

detected with the Cary 300 spectrophotometer (Varian Ltd, American). The CL spectrum was obtained with the RF-5301PC fluorospectrophotometer (Shimadzu Ltd, Japan). MAS-I Microwave extraction response instrument (Shanghai new instrument microwave chemical technology Co., LTD).

2.3. Procedures

FI-CL method: The flow-injection system was easy to operate. As shown in the **Fig. 1**, the peristaltic pump propelled the DPA solution, H_2SO_4 solution and analyte (a standard IAA solution or a sample containing IAA) through the system at 4.0 mL min^{-1}, respectively. When the injection valve was set to the load position, the H_2SO_4 was merged with DPA solution at "M" point before the DPA ran through the whole system until a stable baseline was recorded. When the injection valve was switched to the inject position, the H_2SO_4 carried the sample solution in the reagent loop (100 μL IAA solution), and ran directly through the flow cell, producing CL emission. Also the CL signal was then recorded simultaneity. The reagent IAA injection mode was chosen in the FIA system for the lower baseline was recorder in the absence of IAA solution. The PMT operated at -1200 V. The relative CL intensity, ΔI (defined as the difference of CL intensity between in present and in absence of IAA solution, respectively), was proportional to the corresponding concentration of IAA.

2.4 Sample preparation

Urine, bean sprout and soil were collected for analysis. Urine sample was taken from a healthy male person. In order to remove the reducing substances such as ascorbic acid and glutathione, the 50 mL urine sample (contain 0.1 g Na_2CO_3) was heated at 60℃ for 10 min. An appropriate amount of standard IAA solution was added into the 10 mL treated urine

sample. The spiked samples were extracted with CH_2Cl_2 for three times after stayed for 30 min. Then the distilled water was added for analysis after the organic phases were collected and drying. The spiked urine sample with concentration of 10.0, 20.0, 40.0 $ng \cdot mL^{-1}$ were directly analyzed. The experimental were approved by the Ethics Committee of Sun Yat-sen University, and all the procedures were performed in accordance with the Regulations for the Administration of Affairs Concerning Experimental. Informed consent was obtained for the experimentation with human urine.

The bean sprout was purchased from the market. 500 g bean sprout were grinded and centrifuged. The obtained liquid was filtered by using filter paper and stored in refrigerator overnight. A suitable volume filtrate was set into a flask and mixed with IAA standard solutions. It was mixed thoroughly and kept for 0.5 h. The extraction was performed in 25 mL CH_2Cl_2 for three times after stayed for 0.5 h. The distilled water was added for analysis after the organic phases were collected and evaporate to dryness. The beans sprout without IAA standard solutions was regarded as blank sample. Three different concentration levels of IAA were spiked into the samples with the concentration of 0.050, 0.100 and 0.200 $mg \cdot Kg^{-1}$.

The soil sample was collected from the lawn in the campus. And allowed it to air dry. The dry soil was ground into powder and sieving. An appropriate amount of standard IAA solution was added into the 2.5 g soil sample. The spiked samples were oscillation extracted with CH_2Cl_2 for three times after stayed for 30 min. The extract liquor endured vacuum distillation and ultrasonic pretreatment. Then the distilled water was added for analysis after the organic phases were removed. The spiked urine sample with concentration of 0.50, 1.25, 2.50 $mg \cdot Kg^{-1}$ were directly analyzed.

3. Results and Discussion

3.1 Kinetics curve of the CL reaction of DPA-H_2SO_4-IAA

In the primary experiment, the emitted CL was based on the oxidation reaction of IAA by DPA in the acid medium. In the batch mode, a typical intensity versus times response curvewas used to describe the CL reaction. The response curve depended on the experimental factors such as reagent concentrations. So the experimental parameters were kept constant, the intensity time curve of IAA-H_2SO_4-DPA was recorded to study the kinetic characteristic of the CL reaction in **Fig.2**. The CL intensity peak appeared within 0.8 s since the DPA was injected into the mixture solution of H_2SO_4 and IAA (or blank). The CL signals would decrease to baseline within 32 s. As seen from the **Fig. 2**, the CL reaction of IAA-DPA-H_2SO_4 was a quick reaction obviously. The kinetic curve indicated the CL system was rapid and sensitive enough and suitable for the analysis of IAA.

3.2 Optimization of the experiment procedure

3.2.1 The effect of DPA solution

The CL was emitted from the oxidation reaction of IAA by DPA in acid medium. As the only oxidant, the concentration of DPA was important. To test the effect of DPA solution, a range of concentrations of DPA (5×10^{-5} mol·L^{-1} to 6×10^{-4} mol·L^{-1}) was investigated. As shown in **Fig.3**, the CL intensity was increased along with the increase of the DPA concentration in a low-concentration range, and reached the maximum at 4.5×10^{-4} mol·L^{-1}. When the concentration was over 4.5×10^{-4} mol·L^{-1}, the CL intensity decreased probably because higher concentration of DPA caused self-absorption. Hence 4.5×10^{-4} mol·L^{-1} was optimal concentration and was selected in the subsequent work.

3.2.2 The effect of acid medium

In the prime experiment, the CL intensity involved DPA in acid medium could be enhanced in presence of IAA. The influence of the common acid such nitric acid, hydrochloric acid, poly phosphoric acid, sulfuric acid and phosphoric acid were investigated. The results show that the CL intensity of DPA-IAA in the sulfuric acid was maximum and stable. When the other conditions were fixed, the effect of H_2SO_4 concentration was test over the range 0.1 mol·L^{-1} to 4.0 mol·L^{-1}. As the result in **Fig. 4** demonstrated, the relative CL intensity was increased alone with an increase of the H_2SO_4 concentration in a concentration range (<1.0 mol·L^{-1}), and reached a maximum; when the concentration was over 1.0 mol·L^{-1}, the CL intensity decreased obviously with an increase of the H_2SO_4 concentration. One possible explanation for our observation may be that enhanced baseline noise resulted from released heat when the H_2SO_4 was at higher concentrations. Consequently, the optimal H_2SO_4 concentration was 1.0 mol·L^{-1}.

3.2.3 The effect of flow rate

Within a given variable range of 2.0-6.0 mL·min^{-1}, the CL system was investigated. The result showed that the relative CL intensity increased with the increasing of flow rate in the range of 2.0-4.0 mL·min^{-1}, no obvious increasing of the relative CL intensity was observed when further increase flow rate to 6.0 mL·min^{-1}. And high flow rate may lead to high background, decrease of signal-to-noise ratio and poor reproducibility. Based on the points above, we used 4.0 mL·min^{-1} in the whole experiment.

3.3 Analytical characteristic of the FI-CL method

Under the optimum experimental conditions, the calibration graph of change of CL intensity, ΔI, against IAA concentration was linear in the range of 1.0×10^{-10} g·mL^{-1} to 1.0×10^{-8} g·mL^{-1} with a detection limit of 7.7×10^{-11} g·mL^{-1} (3σ). The regression equation was ΔI =66.69+411.15c (c being the IAA concentration (ng·mL^{-1})). The relative standard deviation was 0.5% for 1.0×10^{-9} g·mL^{-1} IAA (n=11). The result was comparable and even better than most of those obtained from the other methods reported in literatures, as shown in **Table 1.** The proposed method displayed higher sensitivity and a wider linear range.

3.4 Influence of coexisting foreign species

Under the optimum experimental conditions, the influence on CL intensity of some foreign coexisting component in human urine, bean sprouts and soil samples was examined. The experiments were carried out by comparing with the intensities obtained with and without the potentially interfering substances added (**Table 2**). The results indicated that under the enperimental conditions of 1 ng·mL^{-1} IAA and a given relative error (less than 5%)，the interference from common component and ions can not be ignored, especially uric acid and Cl$^-$, that is probably because they affected the formation of luminescence intermediates. So it was necessary to do pretreatment work before the detection of urine sample.

3.5 Analytical applications in the determination of real sample

According to the procedure detailed in the experimental section, the proposed method was applied to the determination of IAA in human urine, bean sprouts and soil. And recovery studies were carried out on real samples for the evaluation of the validity of the proposed method. The No.1 results of each matrix in **Table 3** were further compared with that obtained

by HPLC method. As shown in the table **Table 3**, the recovery values for the three samples were in the range of 87.0-118.1% with the RSDs of 0.6 – 2.7%. The HPLC value for the three metrix were 42.8 ± 0.3, 0.102 ± 0.006 and N.D. respectively, which agreed well with the results obtained by the proposed method.

3.6 Mechanism studies of the CL reaction

To explore the mechanism of the CL reaction, the ultraviolet visible absorption spectra and fluorescence spectra of the proposed system were recorded.

The ultraviolet visible absorption spectra of IAA-$[Ag(HIO6)_2]^{5-}$-H_2SO_4 is shown in **Fig. 5**. DPA had a characteristic absorption peak at 357 nm. After adding sulfuric acid to DPA solution, the color of DPA gradually faded away，and intensity of the characteristic absorption at 357 nm decreased，which demonstrated reactions took place between sulfuric acid and DPA. When IAA mixed with DPA or DPA-sulfuric acid，the spectra of IAA is obviously different from that obtained from simple superpose the spectra of IAA and DPA or DPA-sulfuric acid. It was obvious that reactions have taken place between IAA and DPA.

The fluorescence spectra of IAA-$[Ag(HIO_6)_2]_5$-H_2SO_4 is shown in **Fig. 6**. Both sulfuric acid and DPA were non-fluorescent in the wavelength range of 300-450 nm, but the matrix spectral peak of DPA existed. However, after the two compounds were mixed, the matrix spectral peak of DPA was greatly changed. Based on these spectra, we could conclude that DPA had reacted with sulfuric acid. IAA could generate characteristic fluorescence emission under ultraviolet excitation and the maximum excitation and emission wavelength were observed at 289 nm and 361 nm respectively. The fluorescence spectra of

the mixture containing IAA and sulfuric acid showed a decline in fluorescence emission intensity, but no significant shift was observed. It suggested that there was no chemical reaction took place between sulfuric acid and IAA. When IAA mixed with DPA, the fluorescence emission intensity of IAA decreased and significant shifts were observed, it can be inferred that a chemical reaction occurred between sulfuric acid and IAA. When sulfuric acid, IAA and DPA were mixed together, the emission peak at 361 nm disappeared and a new fluorescence emission with much lower intensity arised at wavelength of 385 nm, which proved that intensive reaction had take place among the three compounds. Based on the discussion above, the possible chemiluminescence mechanism of IAA-$[Ag(HIO_6)_2]^{5-}$-H_2SO_4 system could be described as follows(**Fig. 7**).

4. Conclusion

In this study, a novel FI-CL method for the determination of IAA was reported based on its luminescence enhancement of diperiodatoargentate (III) in sulfuric acid. The enhanced CL systems proposed has been applied for the determination of IAA at the 10 $ng \cdot mL^{-1}$ level in human urine, mung bean sprouts and soil samples with satisfactory results. With rapidity, low detection limit, high sensitivity, good linearity, this approach showed great potential for the trace determination of IAA in biological samples in the routine use. The CL reaction mechanism was discussed briefly.

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Figure Captions

Fig. 1 Schematic diagram of flow injection CL analysis system

P, peristaltic pump; V, injection valve; M, mixtured position; F, spiral glass flow cell; PMT, photomultiplier tube; W, waste; PC, personal computer.

a, IAA solution or blank solution; b, H_2SO_4 solution; c, DPA solution.

Fig. 2 Kinetic curves of IAA-$[Ag(HIO_6)_2]^{5-}$-H_2SO_4

$[Ag(HIO_6)_2]^{5-}$: 4.5×10^{-4} mol·L^{-1}; IAA: 1.0×10^{-8} g·mL^{-1}; H_2SO_4: 1.0 mol·L^{-1}.

Fig. 3 Effect of DPA concentration on chemiluminescence intensity

H_2SO_4: 0.01mol·L^{-1}; IAA: 5.0×10^{-8} g·mL^{-1}.

Fig. 4 Effect of H_2SO_4 concentration on chemiluminescence intensity

DPA: 4.5×10^{-4} mol·L^{-1}, IAA: 5.0×10^{-8} g·mL^{-1}.

Fig. 5 UV-vis spectra of different systems

(1)H_2SO_4 ; (2) $[Ag(HIO_6)_2]^{5-}$; (3)H_2SO_4+ $[Ag(HIO_6)_2]^{5-}$;(4)$[Ag(HIO_6)_2]^{5-}$ + IAA ;

(5)H_2SO_4+ IAA ;(6)IAA ;(7)H_2SO_4 + $[Ag(HIO_6)_2]^{5-}$+ IAA.

H_2SO_4: 0.05 mol·L^{-1}； IAA : 1×10^{-5} g·mL^{-1}； $[Ag(HIO_6)_2]^{5-}$: 5.0×10^{-5} mol·L^{-1}.

Fig. 6 Fluorescence emission spectra of IAA-$[Ag(HIO_6)_2]^{5-}$-H_2SO_4 system

(1)H_2SO_4; (2)IAA ;(3)$[Ag(HIO_6)_2]^{5-}$; (4)H_2SO_4+IAA; (5)$[Ag(HIO_6)_2]^{5-}$+IAA;

(6)H_2SO_4+$[Ag(HIO_6)_2]^{5-}$; (7)H_2SO_4+ $[Ag(HIO_6)_2]^{5-}$+ IAA.

Fig. 7 Possible mechanism of IAA-$[Ag(HIO_6)_2]^{5-}$-H_2SO_4 system

Tables

Table 1 Comparison of analytical method reported in literatures

Method	Matrix	Linear range ($ng \cdot mL^{-1}$)	Detection Limit ($ng \cdot mL^{-1}$)	Reference
ED	cinnamomum camphora, prunus yedoensis Mats, firmiana simplex.	17.52-1226.4	8.75	1
CE-UV	banana, cabbage, cucumber.	2–500	0.67	5
FL	fruit juice.	45-640	12	10
HPLC-UV	coconut juice.	10-2000	1.3	15
HPLC-FL	cucumber, tomato.	0.18-35.08	0.063	11
LC-MS/MS	pyropia haitanensis, laminaria japonica.	5-500	0.105	18
MIM-SPR	peach, rosa, crape myrtle.	$(1.75\text{-}87.60)\times10^{-4}$	0.35×10^{-4} (Peach)	26
FI-CL	human urine, mung bean sprouts, soil.	0.10-10	0.077	This work

ED: electrochemical detection

FL: fluorescence

CL: chemiluminescence

MIM-SPR: Molecular Imprinting Monolayer Techniques on a Surface Plasmon Resonance Sensor.

Table 2 The interference of some common foreign species on the determination of 1.0×10^{-9} g·mL^{-1} IAA

Foreign species	Tolerance limits	Foreign species	Tolerance limits
Cl^-	0.1	uric acid	0.1
CO_3	100	glucose	1
NO_3	1	starch	10
Ac^-	1000	urea	100
EDTA	1	vitamin C	0.05
Co^{2+}	0.1	Ni^{2+}	100
Na^+	200	Zn^{2+}	100
NH_4^+	1	Cu^{2+}	10000
Fe^{3+}	0.5	Fe^{2+}	0.1
Mg^{2+}	1	Ni^{2+}	1

341 Table 3 The results of IAA in human urine, mung bean sprouts and soil samples(n = 3)

Samples	No.	Detected	Added	Found	Recovery (%)	RSD (%)
human urine ($ng \cdot mL^{-1}$)	1	44.1 ± 0.9	10.0	55.8	117.1	1.5
			20.0	65.6	107.8	2.5
			40.0	85.9	104.5	1.5
	2	92.2±1.1	10.0	102.6	103.5	1.3
			20.0	115.5	116.4	2.6
			40.0	134.1	104.7	2.0
Mung bean sprouts ($mg \cdot Kg^{-1}$)	1	0.101± 0.003	0.050	0.148	94.6	2.4
			0.100	0.199	97.6	2.2
			0.200	0.298	98.4	1.2
	2	0.114±0.004	0.050	0.160	92.4	2.7
			0.100	0.210	95.7	1.3
			0.200	0.288	87.0	2.1
soil ($mg \cdot Kg^{-1}$)	1	N.D.	0.50	0.59	118.8	1.1
			1.25	1.45	115.9	1.0
			2.50	2.54	101.8	0.7
	2	N.D.	0.50	0.57	113.1	1.4
			1.25	1.27	101.3	0.6
			2.50	2.35	94.1	1.2

342 N.D. not detected

343

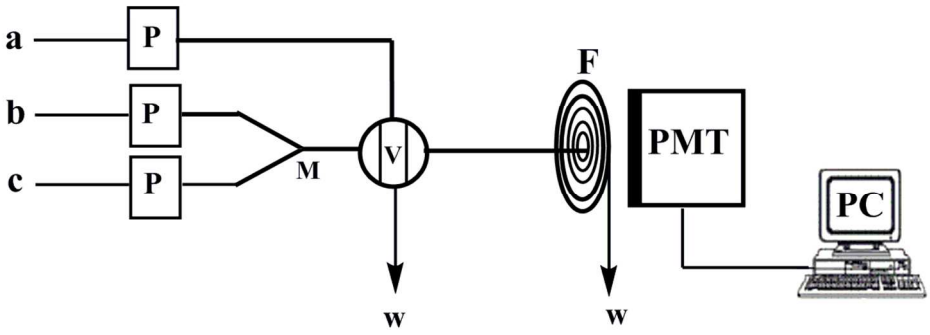

Fig.1
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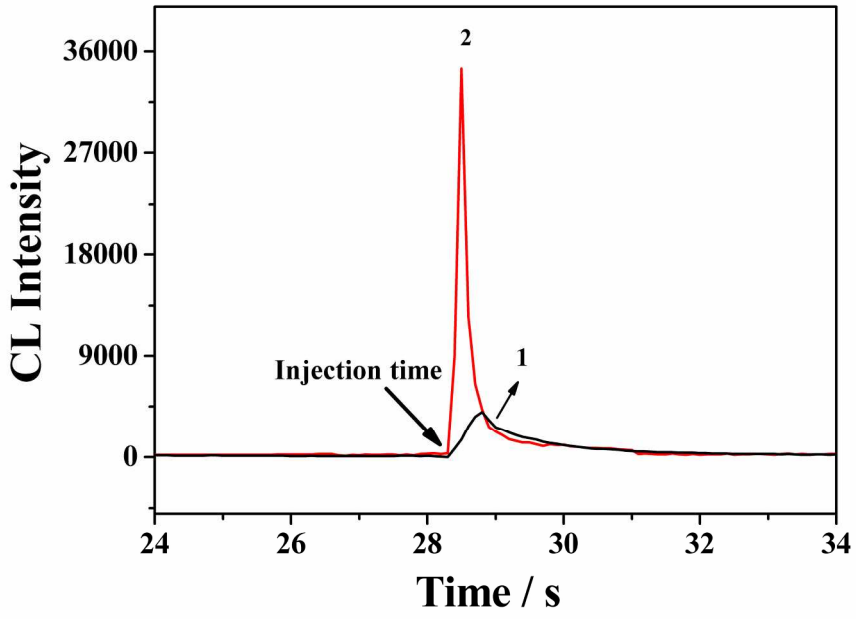

Fig.2
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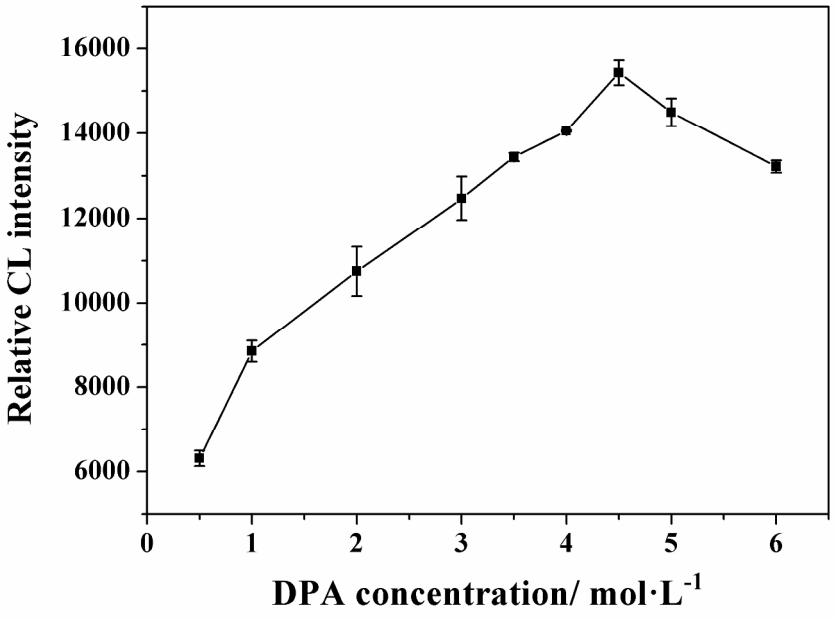

Fig.3
508x359mm (150 x 150 DPI)

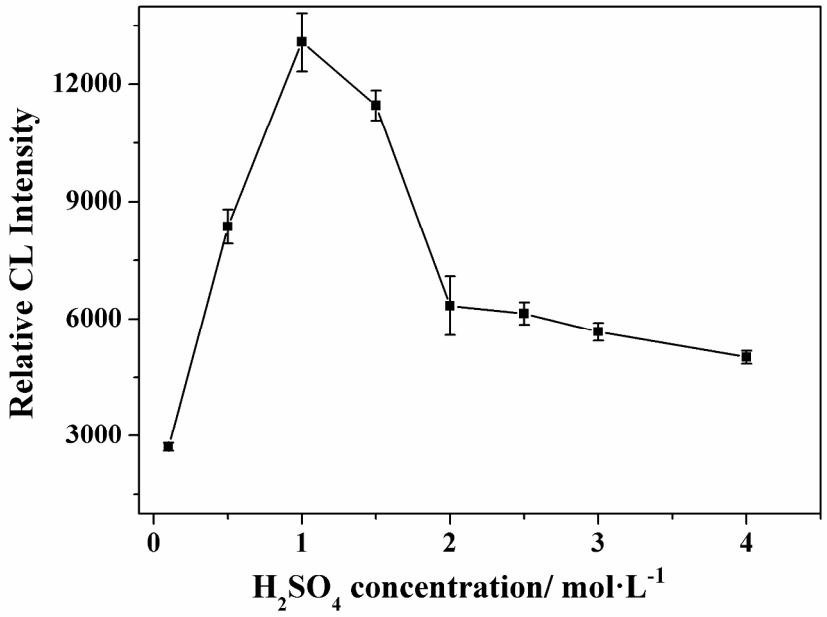

Fig.4
508x359mm (150 x 150 DPI)

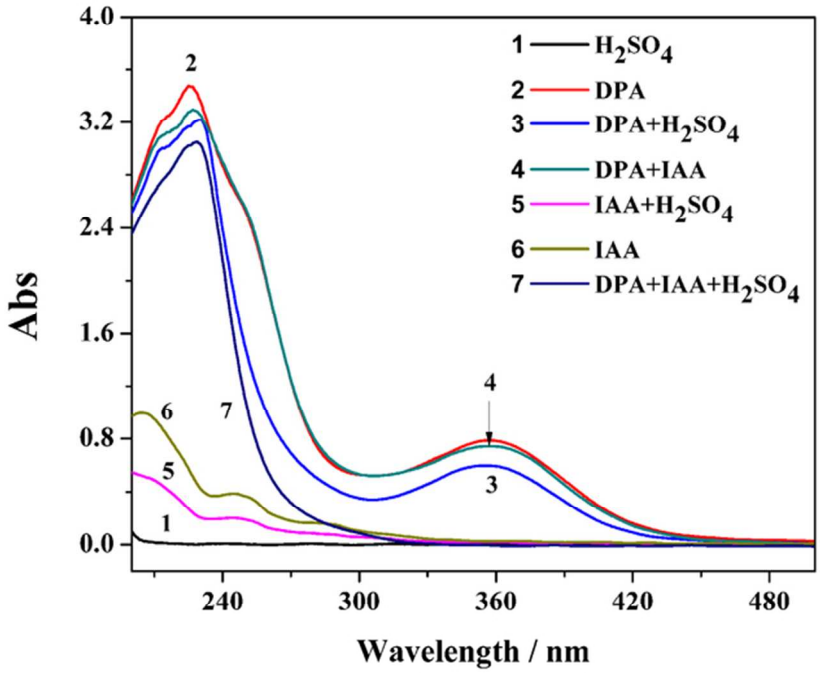

Fig.5
65x50mm (300 x 300 DPI)

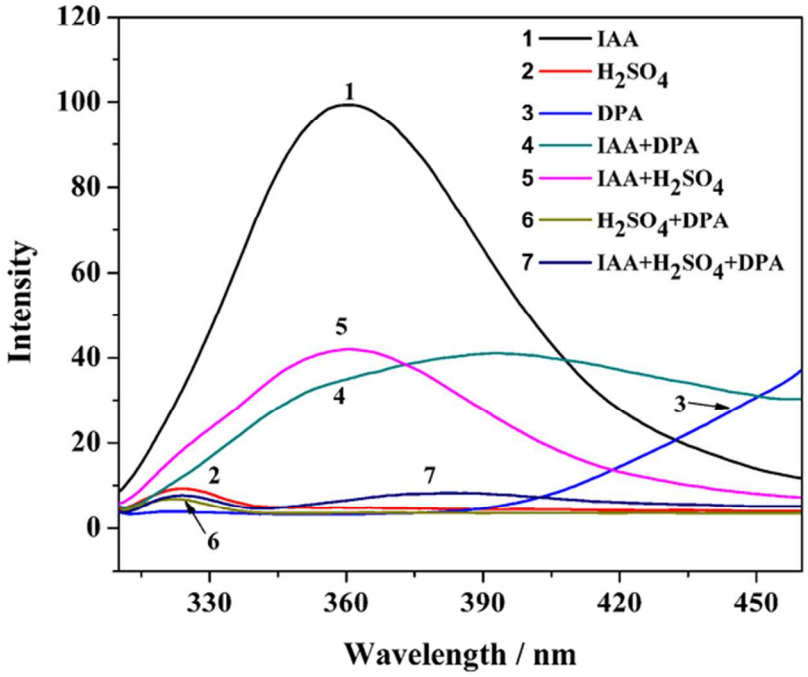

Fig.6
65x50mm (300 x 300 DPI)

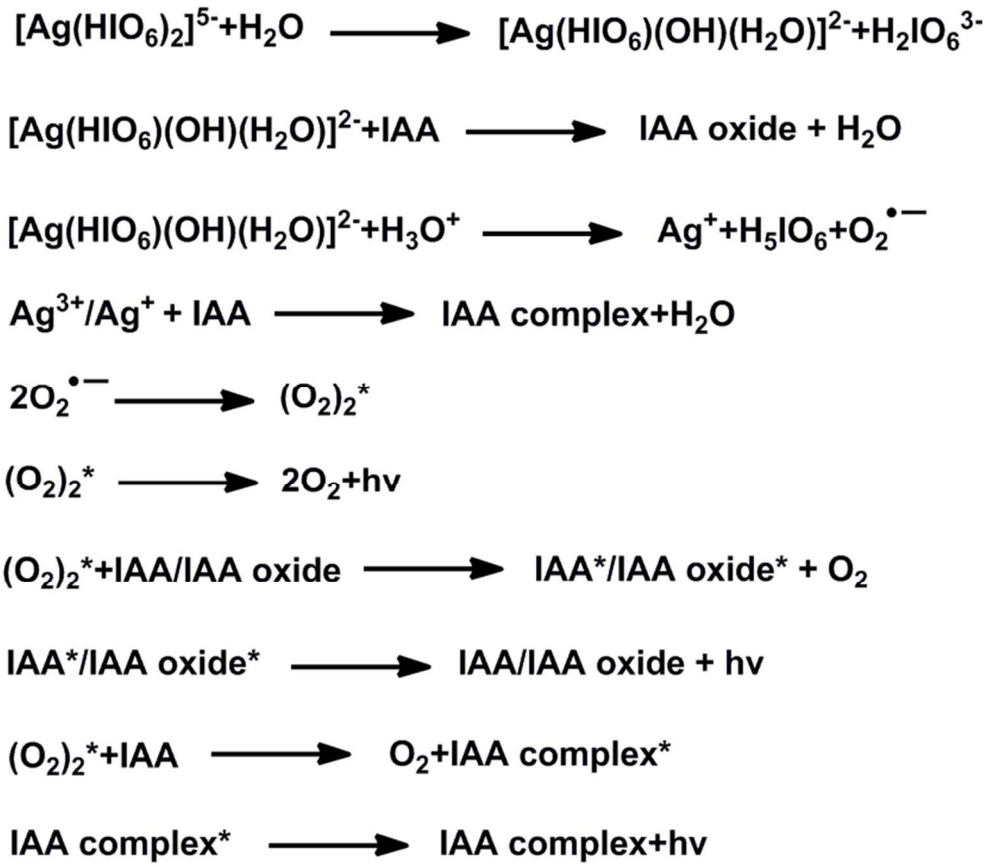

Fig.7
71x62mm (300 x 300 DPI)