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ORIGINAL PAPER

A Promising CdSe@CdS-Quantum Dots-Cysteine for the Determination of Trace IgE by Solid Substrate Room Temperature Phosphorescence Immunoassay

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Abstract The labelling reagent CdSe@CdS-QDs-Cys (QDs-Cys) with the grain diameter of 4.5 nm was synthesized by modifying CdSe@CdS quantum dots (QDs) with cysteine (Cys). At the same time, QDs-Cys-Ab_{IgE}, a phosphorescent quantum dot probe, was developed based on the labelling reaction between -COOH of QDs-Cys and -NH_2 of goat anti human IgE antibody (Ab_{IgE}). This probe with excellent biocompatibility and high specificity could not only emit strong and stable room temperature phosphorescence (RTP), but also could carry out specific immunoassay (IA) with immunoglobulin E (IgE), causing the RTP of the system to sharply enhance. Thus, a new solid substrate room temperature phosphorescence immunoassay (SSRTPIA) for the determination of IgE was established. The limit of quantification (LOQ) of the method was 0.12 fg $spot^{-1}$, corresponding concentration was 3.0×10^{-13} g mL^{-1} and sampling quantity was 0.40 μL $spot^{-1}$. This highly selective, sensitive and accurate SSRTPIA has been applied to determine IgE in biological samples and diagnose diseases, and the results agreed well with those obtained by enzyme-link immunoassay (ELISA). Meanwhile, the mechanisms of QDs-Cys labelling Ab_{IgE} and the determination of IgE by SSRTPIA were also discussed.

Keywords CdSe@CdS-quantum dots-cysteine · Phosphorescent quantum dot probe · Immunoglobulin E · Solid substrate room temperature phosphorescence immunoassay · Diagnosis and prediction of human diseases

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Introduction

The content of IgE (μg level, 3×10^{-4} mg mL^{-1}) [1] is much lower than that of IgG (mg level) [2] in human serum. Since the content of IgE is closely related to liver cirrhosis, liver cancer, eczema, asthma and other diseases, it is usually considered as the basis for the clinical diagnosis of diseases [3,4]. Hence, the determination of IgE in human serum is important to diagnose diseases, choose the medicine and research disease mechanisms [3] which have aroused great concern of many researchers. So far, there have been many methods for the determination of IgE, such as radioimmunometric assay (detection limit (LD): 5.0×10^{-11} g mL^{-1} [6] (1 IU is 2.42 ng [1,5] in calculation, and the following ones are calculated in the same way), 4.8×10^{-9} g mL^{-1} [7], 2.4×10^{-9} g mL^{-1}[8]) and ELISA (LD: 3.5×10^{-9} g mL^{-1}) [9]. The sensitivity of radioimmunometric assay is high, while it would cause radioactive pollution. And ELISA is easily affected by many factors [9] with low sensitivity and poor repeatability [6]. Besides, LDs of these methods only reach the ng level. Obviously, only developing a method with safety, high sensitivity and good repeatability can cope with the new challenges for the determination of trace IgE in the biological samples. In the

previous studies, we used the luminescent nanoparticles containing dibromofluorescein [2], rhodamine 6 G [10] and fluorescein isothiocyanate [11] to label Ab_{IgE} for the determination of human IgG, which showed high sensitivity and practical value of SSRTPIA. How to further improve the sensitivity of SSRTPIA to satisfy the need for the determination of trace IgE in biological samples and for the early warning of diseases?

The thought applying fluorescent QDs to biological markers was simultaneously proposed by the groups of Alivisatos and Nie in 1998, and the corresponding results of their studies were published in "Science", which initiated a precedent of research and application of fluorescent QDs in bio-technology. According to the scientific achievements in recent years, we can conclude that the most promising research of QDs concentrates on fluorescent labelling substances in biological systems [12]. The newly synthesized QDs including CdSe@ZnS [13] CdSe@ZnSe [14] CdSe@CdS [15] CdS@ZnS [16] CdS@HgS [17] and CdS@HgS@CdS [18] show such characteristics as durable luminescence, long fluorescence lifetime (over 100 times longer than that of dye molecules [19]) and high quantum yield, which display their potential application prospect in immune biology and clinical examination [20]. At present, QDs, as a fluorescent sensor, have been used in many respects, such as the determination of silver ion [21], protein [22], DNA [23], the one and multiple marker imaging of cells, the tracer of dynamic process in living cells and the target tracer of tumor cells in live animals [24,25]. Phosphorescence sensors of Mn doped ZnS QDs (λ_{em}^{max}=590 nm) [26,27] and ZnSe/ZnMnS/ZnS QDs [28] (λ_{em}^{max}=618 nm) have been used for the determination of enoxacin in biological fluid [26], pentachlorophenol in water sample [27], the stoichiometric sensors for the measurement of energy transfer, as well as biological imaging agents [28] and so on. These outcomes reveal the research and application progress of luminescent sensor.

On the basis of these thoughts, we synthesized QDs-Cys according to the method in Ref. [29] and discovered that QDs-Cys could emit strong and stable RTP on ACM when Pb^{2+} was used as the perturber and its product of labelling Ab_{IgE} (QDs-Cys-Ab_{IgE}) could maintain good RTP property. Thus, this QDs-Cys RTP labelling reagent and QDs-Cys-Ab_{IgE} were developed for the first time, and a new SSRTPIA for the determination of IgE was established. Accordingly, if only the corresponding antibodies are chosen, various QDs-Cys-Ab could be synthesized for the determination of much immunoglobulin (such as IgG, IgA, IgM), showing wide application. This method could not only provide the possibility for the determination of IgE with low content in biological samples and the diagnosis of human diseases, but also open the prelude of the application of QDs in the solid substrate room temperature phosphorimetry (SSRTP) and promote the research progress of SSRTP, RTP labelling reagent and life science.

Experimental

Apparatus and Reagents

Phosphorescent measurements were carried out on a Perkin-Elmer LS-55 luminescence spectrophotometer with a solid surface analysis apparatus (Perkin Element Corporation of U.S.). 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: 1500 nm min^{-1}. KQ-250B ultrasonic cleaner (Kunshan Ultrasonic Machine Company) and an AE240 electronic analytical balance (Mettler-Toledo Instruments Company Limited) were used. A 0.50-μL flat head micro-injector (Shanghai Medical Laser Instrument Plant, China) was used to introduce the solution of μl level.

Human serum IgE was stored at 0–4 °C (Banding Taike Bio-Tech Company). IgE working solution: 1.00 μg IgE mL^{-1} stocking solution was gradually diluted to 100.00, 60.00, 20.00, 10.00 and 0.25 pg mL^{-1} with Na_2CO_3−$NaHCO_3$ buffer of pH 9.12. 1.00 mL 1.00 mg immunoglobulin G (IgG), immunoglobulin A (IgA), immunoglobulin M (IgM) and immunoglobulin D (IgD) stocking solution was gradually diluted to μg mL^{-1} with Na_2CO_3−$NaHCO_3$ buffer of pH 9.12, respectively. Ab_{IgE} was 21 mg mL^{-1}. Goat-anti-human IgG, goat-anti-human IgA, goat-anti-human IgM and goat-anti-human IgD were 20 mg mL^{-1} (Purchased from Xiamen University anti-cancer research center). 2.0×10^{-2} mol L^{-1} $CdCl_2$, 1.0×10^{-2} mol L^{-1} L-cysteine (Cys), 1.0×10^{-2} mol L^{-1} Na_2S, 0.10 mol L^{-1} Na_2CO_3−$NaHCO_3$ buffer (the pH value was 9.12 at 37 °C), 0.050 mol L^{-1} Tris-HCl buffer (the pH value was 7.40 at 25 °C), Tris-HCl-0.10% tween-20 washing buffer, 10.00 mg mL^{-1} of bovine serum albumin (BSA), 1.00 mol L^{-1} $Pb(Ac)_2$ solution, anhydrous Na_2SO_3 and selenium (Se) powder were also used in the experiment. Preparation of EDC-NHS coupling agent solution: the mixture of 5 mmol L^{-1} 1-ethyl-3-3-dimethylaminopropylcarbodiimide hydrochloride (EDC, Alfa Company) and 5 mmol L^{-1} N-hydroxysucci-nimide (NHS, Alfa Company) was prepared with 40% ethanol. All reagents were of analytical reagent grade. All these solutions were prepared with doubly distilled water.

ACM, nitrocellulose membrane (NCM) and polyamide membrane (PAM) were purchased from Luqiaosijia biochemical plastic plant (Hangzhou, China). They were cut into wafers (Diameter was 15 mm.) and a ring indentation (Diameter was 4.0 mm.) was made at the center of each sheet with a standard pinhole plotter for use.

Experimental Method

Synthesis of Water-Soluble Core-Shell QDs-Cys

According to the method in Ref. [29], certain amount of Se powder was added into Na_2SO_3 solution [n (Na_2SO_3): n (Se)=3:1], and the mixture was heated to boil under stirring, refluxing for 5 h to obtain nuclear precursor Na_2SeSO_3 solution. And then 2.0×10^{-2} mol L^{-1} $CdCl_2$ solution and 1.0×10^{-2} mol L^{-1} Cys solution [n ($CdCl_2$): n (Cys)=1:3] were mixed and adjusted pH to 7.0 with NaOH solution, into which Na_2SeSO_3 solution [n ($CdCl_2$): n (Na_2SeSO_3)=1.7:1] was dropped, and reacted at 25 °C for 1 h. At last, a yellow-green transparent CdSe sol was formed, that is CdSe-QDs. $CdCl_2$ and Na_2S solution [n ($CdCl_2$): n (Na_2S)=1:1] were slowly dropped and alternately into the CdSe sol, the mixture reacted at 25 °C for 1 h. With the color of solution becoming deeper, the QDs-Cys was obtained. Repeating the experiment described above for several times, 50 mL CdSe-QDs and 100 mL QDs-Cys were prepared and stored for 7 days without any precipitate formed in the solution. After 10.00 mL of CdSe-QDs and 10.00 mL of QDs-Cys were separately washed with 10.00 mL of acetone for 3 times and then dried in vacuum. The powder obtained was then sent to examine.

Preparation of QDs-Cys-Ab_{IgE}

0.40 μL Ab_{IgE} of different concentrations (V/V, the solution was diluted with Tris-HCl buffer of pH 7.4) and volumes was suspended onto the center indentation of ACM with a 0.50-μL flat head micro-injector. Then, ACM was taken out, on which 0.40 μL labelling reagent (QDs-Cys) and EDC-NHS were dropped, respectively. The sample was kept at 37 °C for 2 h, and washed for 3 times with washing buffer by ultrasonic cleaner to remove the unreacted QDs-Cys on ACM. Then, it was sipped up with the filter paper, and QDs-Cys-Ab_{IgE} was obtained for use. The optimal concentration and volume of Ab_{IgE} were chosen according to the result of immunoreaction between QDs-Cys-Ab_{IgE} and IgE of different concentrations as well as the result of RTP measurement.

Immunoreaction and the Measurement of RTP

The type of immunoreaction used in this paper was sandwich way. To the indentation of the ACM wafers, 0.40 μL Ab_{IgE} was added by a 0.50-μL flat head micro-injector. Then, ACM was stored at 4 °C overnight. Took ACM out and immersed it into BSA solution (10 mg L^{-1}) at 37 °C for 0.5 h in order to inhibit the non specific reactivity, and then washed it for 3 min with 20 mL washing buffer each time in a small beaker with ultrasonic cleaner, repeated 3 times and sipped it up with filter paper. A 0.40 μL of IgE working solution with different concentrations (the IgE was diluted with Na_2CO_3−$NaHCO_3$ buffer of pH 9.12) was respectively suspended on the same indentation center of ACM wafer with a 0.50-μL flat head micro-injector. Then, the membrane was incubated at 37 °C for 2 h. Afterwards, washed it with washing buffer for 3 times in ultrasonic cleaner and sipped it up with filter paper. And then 0.40 μL of Ab_{IgE} was added to the same indentation in the membrane. After being incubated at 37 °C for 2 h, the membrane was washed for 3 times by ultrasonic agitation, and sipped up the water with filter paper. Then, 0.40 μL of QDs-Cys and EDC-NHS were separately added to the same indentation on the membrane, and incubated at 37 °C for 2 h, washing and sipping were carried out in the same way mentioned above. The blank experiment was also conducted.

The ACM containing test solution and blank reagent were respectively immersed in $Pb(Ac)_2$ solution for 10 s, then dried at 90±1 °C for 2 min. And the phosphorescence spectra were measured at 459/625 nm ($\lambda_{ex}^{max}/\lambda_{em}^{max}$). The phosphorescence intensity of blank sample (containing Ab_{IgE} and Ab_{IgE}-Cys-QDs) and the test solution (Ab_{IgE}-IgE-Ab_{IgE}-Cys-QDs) were defined as I_{p1} and I_{p2}, respectively. Each sample was measured in parallel for 7 times. Then, the ΔI_p ($=I_{p2}-I_{p1}$) was calculated.

Preparation of Analytical Sample

Conventionally, each 5.00 mL blood of fasting vein was respectively taken from asthma patients (A, B, C, D), liver disease patients (E, F, G, H) and healthy persons (I, J, K, L), stood for 1 h, centrifugated at the speed of 1500–3000 r min^{-1}, then the human serum was transferred into a colorimetric tube, diluted to 10 mL with Na_2CO_3−$NaHCO_3$ (pH 9.12), and kept at −20 °C for use. Before use, 1.00 mL of A, B, C and D serum was taken for the determination of IgE, respectively. 1.00 mL of E, F, G, H, I, J, K and L serum was also transferred into a volumetric flask, respectively, diluted to 100 mL with Na_2CO_3−$NaHCO_3$, and then 1.00 mL test solution was diluted to 100 mL for the determination of IgE.

Results and Discussion

Synthesis Conditions and TEM Images of QDs-Cys

Synthesis Conditions

The effect of synthesis conditions including the ratio of Na_2SO_3 and Se [n (Na_2SO_3): n (Se)], volume of Cys and CdS, pH value and time for reaction on the ΔF of the

system were examined (Table 1). Results show that when the reaction temperature was 25 °C and the n (Na_2SO_3): n (Se), n (Cys): n (Cd^{2+}), n (Cys): n (Cd^{2+}) were 3:1, 3:1 and 1:1, respectively; the pH value of the system was 7.0; and the reaction time was 1 h, the ΔF of the system reached the maximum. The reason might be that the condition was helpful to the formation of QDs-Cys.

TEM Images of CdSe-QDs and QDs-Cys

In X-ray powder diffraction (XRD) spectra, positions of the strongest diffraction peak (2θ) were 24.2° and 25.4° for CdSe-QDs and QDs-Cys, respectively, which were basically consistent. According to the peak width of XRD, the grain diameters calculated by Scherre formula ($D=k\lambda P\beta\cos\theta$) were 3.2 nm for CdSe-QDs and 4.5 nm for QDs-Cys. The grain diameter of QDs-Cys was larger than that of CdSe-QDs, the reason might be that CdSe-QDs was wrapped by CdS and then modified by Cys. From transmission electron microscopy (TEM) image, we could see that the shape of the grain was close to the roundness, and the grain diameters of CdSe-QDs and QDs-Cys were approximately 3 nm and 5 nm (Fig. 1), respectively, showing that QDs-Cys could be obtained under above conditions.

Fluorescence Spectra and Phosphorescence Spectra of QDs-Cys

In order to further prove the component of the synthesized product, the fluorescence spectra of CdSe-QDs and QDs-Cys were scanned. Results show that the $\lambda_{ex}^{max}/\lambda_{em}^{max}$ of CdSe-QDs and QDs-Cys were 421.7/515.9 nm and 449.1/548.9 nm, and the corresponding fluorescent signals were 274.4 and 852.3, the standard deviations (RSDs) were 1.8% and 1.5%, respectively (Fig. 2). The values of λ_{em}^{max} were basically tallied with those of Ref. [29] (520 nm for CdSe-QDs and 544 nm for QDs-Cys), indicating the synthesized product was QDs-Cys. Thereinto, the emission peak of QDs-Cys was narrower than that of CdSe-QDs, and the Einstein shift of λ_{em}^{max} was 33.0 nm, which further indicated the formation possibility of QDs-Cys in the reaction between CdSe@CdS and Cys. Besides, the fluorescent signal of QDs-Cys was 3.1 times larger than that of CdSe-QDs, maybe the surface defects of CdSe-QDs was eliminated effectively when CdSe-Cys was coated with a certain amount of CdS to form QDs-Cys, resulting in the enhancement of the fluorescent signal of QDs-Cys [29].

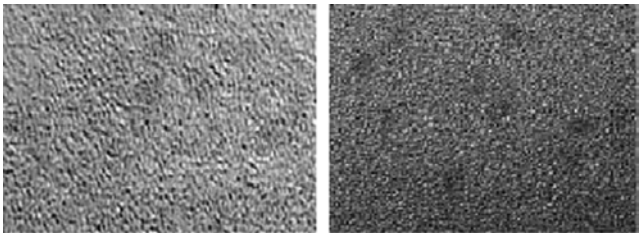


Fig. 1 TEM images of CdSe-QDs and QDs-Cys

The phosphorescence spectra of QDs-Cys were scanned at room temperature when ACM and Pb^{2+} were used as the solid substrate and ion perturber, respectively (Fig. 3). Results show that CdSe-QDs and QDs-Cys only could emit fluorescence in the absence of Pb^{2+} (Fig. 3, curves 2.2′ and 3, 3′), while they could emit strong and stable RTP in the presence of Pb^{2+} on ACM ($\lambda_{ex}^{max}/\lambda_{em}^{max}$=484.0/648.7 nm and I_p=48.1 for CdSe-QDs; $\lambda_{ex}^{max}/\lambda_{em}^{max}$=459.0/625.6 nm and I_p=75.1 for QDs-Cys, Fig. 3, curves 2.2′ and 3, 3′). The reason for this phenomenon was that the perturbation of Pb^{2+} caused the intersystem crossing of CdSe-QDs-Cys and QDs-Cys from the singlet state (S_1) to triplet state (T_1). QDs-Cys was chosen as labelling reagent due to its stronger and more stable phosphorescence signal than that of CdSe-QDs.

Table 1 Effects of synthesis conditions for QDs-Cys ($\Delta F=F_2-F_1$, F_1 and F_2 were the fluorescence signal of the blank reagent (CdSe) and test solution (QDs-Cys, 1: 1), respectively)

Factors	Synthetic condition	ΔF of the system	Optimum
n (Na_2SO_3):*n* (Se)	1:1, 2:1, 3:1, 4:1	512.4, 548.6, 577.8, 553.2	3:1
RSD (%)		1.4, 1.0, 0.80, 0.50	
n (Cys):*n* (Cd^{2+})	2:1, 2.5:1, 3:1, 4:1, 5:1	554.1, 563.7, 571.4, 532.2, 489.9	3:1
RSD (%)		1.1, 0.70, 0.60, 1.3, 1.8	
n (CdS):*n* (CdSe)	0.5:1, 1:1, 2:1, 3:1, 4:1	495.6, 573.1, 544.2, 511.8, 488.5	1:1
RSD (%)		1.6, 0.70, 1.2, 1.4, 1.7	
pH	5.5, 6.3, 7.0, 8.3, 9.0, 10.0, 11.0	557.3, 574.1, 576.4, 544.3, 515.6, 481.4, 436.7	7.0
RSD (%)		1.0, 0.90, 0.70, 1.1, 1.4, 1.8, 2.0	
Time for reaction (h)	0.5, 1.0, 12, 24, 48	544.6, 580.6, 576.3, 566.1, 552.4, 531.8	1.0
RSD (%)		1.2, 0.6, 0.80, 0.90, 1.0, 1.9	

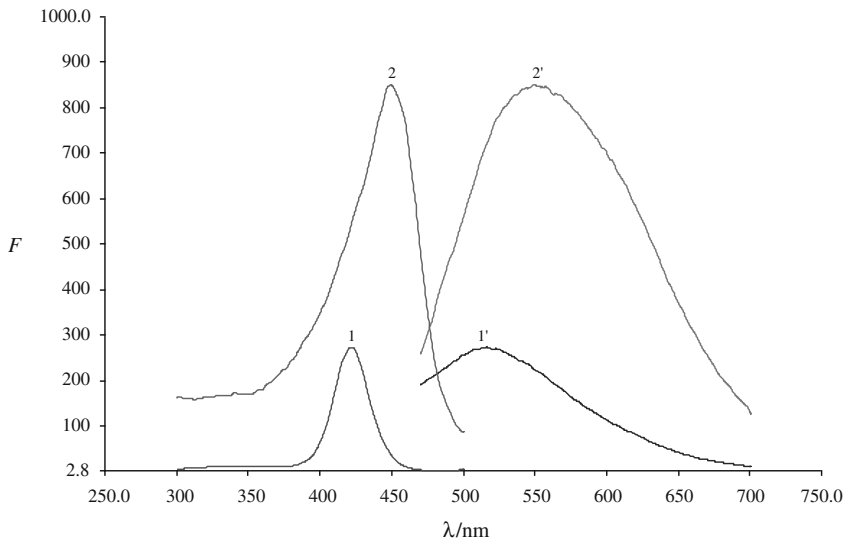


Fig. 2 Fluorescence spectra of CdSe-QDs and QDs-Cys

Phosphorescence Spectra of Immunoreaction Product

The RTP spectra of Ab_{IgE}-IgE-Ab_{IgE}-Cys-QDs system (sandwich way) were scanned in Fig. 4 according to the experimental method. When Ab_{IgE} was added into the system, the RTP of the system was slightly enhanced ($\lambda_{ex}^{max}/\lambda_{em}^{max}$=458.7/625.0 nm, I_p=83.7, ΔI_p=8.6, Fig. 4, curve 1.1′). In the presence of IgE, the RTP signal of the immunoreaction product (Ab_{IgE}-IgE-Ab_{IgE} -Cys-QDs) linearly increased as the content of IgE increased (when the content of IgE were 0.10, 4.0, 8.0, 24.0 and 40.0 fg spot^{-1}, the corresponding ΔI_p were 2.0, 8.0, 12.1, 38.0 and 67.8, respectively). The $\lambda_{ex}^{max}/\lambda_{em}^{max}$ (459/625 nm) was almost constant, providing the possibility to determine the IgE by SSRTPIA in sandwich way, and indicating that QDs-Cys could be used as an excellent RTP labelling reagent to determine IgE. Remarkably, the RTP signal linearly increased with the content of IgE. The reason may be that the output of Ab_{IgE}-IgE-Ab_{IgE}-Cys-QDs increased with the increasing content of IgE, which further resulted in the increasing number of QDs-Cys on biological target. Besides, the perturbation of Pb^{2+} caused the increasing probability of intersystem crossing of QDs-Cys from the singlet state (S_1) to triplet state (T_1) to enhance RTP signal.

At the same time, the RTP spectra of IgE-Ab_{IgE}-Cys-QDs were scanned with direct way, which were similar to RTP spectra of the sandwich way. The RTP signal of the immunoreaction product (IgE-Ab_{IgE}-Cys-QDs) linearly increased as the content of IgE increased (when the content of IgE were 0.10, 4.0, 8.0, 24.0 and 40.0 (fg spot^{-1}), the corresponding ΔI_p were 3.0, 10.0, 16.6, 30.8 and 48.1), the $\lambda_{ex}^{max}/\lambda_{em}^{max}$ (459/625 nm) almost kept unchanged. This

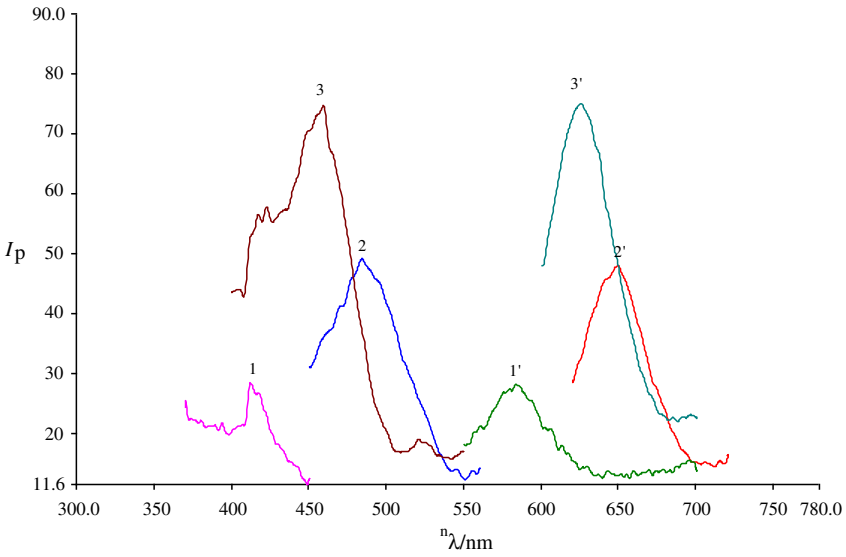


Fig. 3 Phosphorescence spectra of CdSe-QDs and QDs-Cys

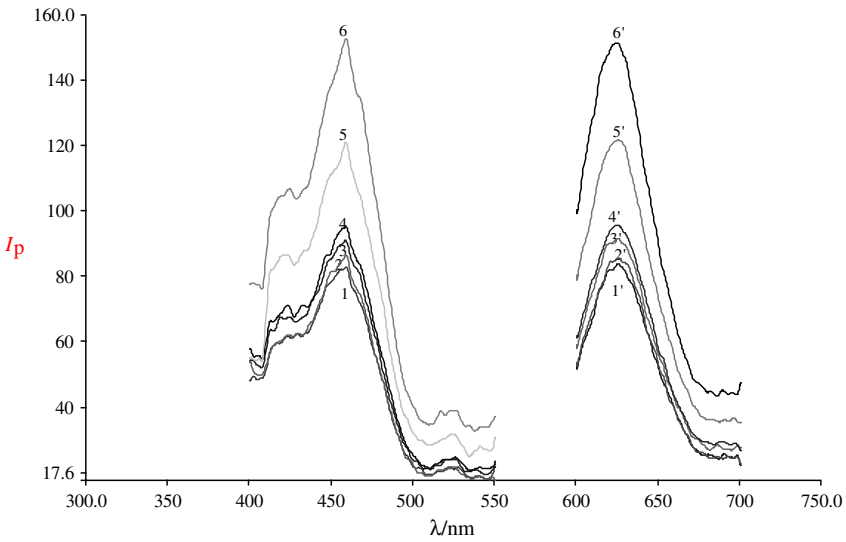


Fig. 4 Phosphorescence spectra of Ab_{IgE}-IgE-Ab_{IgE}-Cys-QDs system (Curves 1–6 were excitation spectra, curves 1′–6′ were emission spectra; the I_p values for 1′, 2′, 3′, 4′, 5′, 6′ were 83.7, 85.7, 91.7, 95.8, 121.8 and 151.5, the corresponding RSDs were 3.4%, 3.2%, 2.9%, 2.5%, 2.1% and 1.7%, respectively)

fact indicated that QDs-Cys was an excellent RTP labelling reagent and it could be used to determine IgE by SSRTPIA in direct way, showing the flexibility of SSRTPIA.

Optimum Conditions for RTP Measurement

In order to optimize the conditions of RTP measurements for the determination of IgE, the influence of solid substrate, ion perturbers, oxygen and humidity, time and temperature for drying and stability of RTP emission on the ΔI_p of the system were investigated in a univariate approach in the measurement process.

Selection of Solid Substrate

For the system containing 8.0 fg IgE $spot^{-1}$, the effects of different substrates including NCM, ACM and PAM on the ΔI_p of the system were studied. Results show that when ACM, PAM and NCM were selected, the ΔI_p of the system were 12.9 (RSD was 4.2%), 10.7 (RSD was 4.5%) and 6.2 (RSD was 4.9%), respectively, the ΔI_p of the system reached the highest when using ACM as substrate. Thus, ACM was selected as the solid substrate in this experiment.

Effect of Ion Perturbation

For the system containing 8.0 fg IgE $spot^{-1}$, the effects of ion perturbers, such as Ca^{2+}, Mg^{2+}, Li^{+}, Pb^{2+} and Ag^{+} with the concentration of 1.00 mol L^{-1} on the ΔI_p of the system were examined. Results show that the corresponding ΔI_p of the system were 7.1 (RSD was 4.8%), 7.4 (RSD was 4.6%), 11.3 (RSD was 4.2%), 12.2 (RSD was 4.0%) and 7.9 (RSD was 4.4%), respectively, and reached the maximum using Pb^{2+} as ion perturber. Meanwhile, the effects of Pb^{2+} with different concentrations (0.10, 0.30, 0.50, 0.70, 1.00 and 1.20 mol L^{-1}) on the ΔI_p of the system were examined. Results show that the corresponding ΔI_p were 8.3 (RSD was 5.0%), 9.9 (RSD was 4.7%), 10.4 (RSD was 4.5%), 11.2 (RSD was 4.3%), 12.4 (RSD was 4.1%) and 9.1 (RSD was 4.8%), respectively, and reached the maximum when 1.00 mol L^{-1} Pb^{2+} was used. The reason might be that the heavy atom effect of Pb^{2+} could greatly enhance the transition probability from S_1 to T_1 of QDs-Cys, which caused the ΔI_p of the system to sharply increase. Similar to Pb^{2+}, Ag^{+} also has heavy atom effect. However, the atomic number and the coupling constant of Ag^{+} are much lower than those of Pb^{2+}, so the effect of Ag^{+} on the ΔI_p of the system is much lower than Pb^{2+}. Besides, though Ca^{2+}, Mg^{2+} and Li^{+} are not heavy atoms, they also can show the heavy atom effect of light element [30,31] with higher ΔI_p of the system. Nevertheless, the ΔI_p of the system with light atoms is still lower than those with Pb^{2+} and Ag^{+}, that is, the ΔI_p of the system was the highest in the presence of Pb^{2+}. Thus, Pb^{2+} was chosen as ion perturber in our experiment.

Oxygen and Humidity

Oxygen and humidity usually lead RTP to quench and must be driven out by passing drying N_2. For the system containing 8.0 fg IgE $spot^{-1}$, when drying N_2 was passed into the system for 10, 15, 20, 25, 30 and 35 min, the ΔI_p of the system were 12.6 (RSD was 3.8%), 12.3 (RSD was 4.1%), 12.4 (RSD was 4.0%), 12.5 (RSD was 3.9%), 12.2 (RSD was 4.2%) and 12.7 (RSD was 3.7%), respectively. While without drying N_2, the ΔI_p were 12.0 (RSD was 4.2%), 11.3 (RSD was 4.5%), 10.4 (RSD was 4.7%), 9.7 (RSD was 5.1%), 9.0 (RSD was 5.3%) and 8.3 (RSD was

5.6%), respectively. Results show that the ΔI_p of the system almost stayed invariable when drying N_2 was passed within 10–30 min.

Time and Temperature for Drying

For the system containing 8.0 fg IgE spot^{-1}, the effects of drying temperature and time on the ΔI_p of the system were examined. Results show that when the drying temperature were 60, 70, 80, 90 and 95 (°C), the ΔI_p of the system were 9.4 (RSD was 4.7%), 10.1 (RSD was 4.4%), 11.3 (RSD was 4.2%), 12.3 (RSD was 3.9%) and 11.7 (RSD was 4.1%), respectively. When the drying time were 0.5, 1.0, 1.5, 2.0 and 2.5 (min), the ΔI_p of the system were 6.4 (RSD was 5.1%), 8.2 (RSD was 4.7%), 9.9 (RSD was 4.5%), 12.4 (RSD was 3.7%) and 10.2 (RSD was 4.0%), respectively. Thus, when the drying temperature and time were 90 °C and 2 min, respectively, the ΔI_p reached maximum and kept stable.

Stability of RTP Emission

Under the optimum conditions above, when the standing time were 10, 20, 30, 40, 50, 60 and 70 min, the ΔI_p of the system were 12.1 (RSD was 4.2%), 12.3 (RSD was 3.8%), 12.4 (RSD was 3.6%), 12.4 (RSD was 3.7%), 12.3 (RSD was 3.9%), 8.3 (RSD was 4.7%) and 6.8 (RSD was 5.0%), respectively. Results show that the ΔI_p of the system almost stayed invariable and had good repeatability within 50 min.

Working Curve, Sensitivity and Precision

The ΔI_p values of the sandwich way and the direct way had linear relationship with the content of IgE in the range of 0.10–40.0 fg spot^{-1} (corresponding concentration was 0.25–100.0 pg mL^{-1}). The regression equation of the working curve of the sandwich way and the direct way were ΔI_p=0.5435+1.646 m_{IgE} (fg spot^{-1}, n=6) and ΔI_p= 5.272+1.080 m_{IgE} (fg spot^{-1}, n=6), the correlation coefficient (r) was 0.9983 and 0.9948, respectively. Compared with Refs. [6–9], RSD (0.10 and 40.0 fg IgE spot^{-1} were determined in parallel for 7 times), LD (calculated by 3Sb/k which referred to the quotient between the standard deviation of 11 blank measurements and the slope of the working curve) and LOQ (calculated by 10Sb/k) are listed in Table 2, respectively.

Results show that both the sandwich way and the direct way had high precision and sensitivity, and the sandwich way had higher sensitivity than direct way. There are two reasons: 1. there was only one binding site for the tested IgE in direct way, while there were two sites in sandwich way; 2. IgE could combine with Ab_{IgE}, Ab_{IgE}-Cys-QDs in sandwich way, respectively, showing higher sensitivity. Though sandwich way had higher sensitivity, simpler operation and more easily to label antibodies than the direct way for the reason that the competitive measurement was used in direct way while non-competitive measurement was used in sandwich way, this method needed once more incubation step which increased the measured time. In this paper, the sandwich way and the direct way were both proposed to meet different requirements in the practical determination, which increases the flexibility of the method and also reflects the potential advantages of the QDs-Cys RTP labelling reagent used in SSRTPIA.

The QDs-Cys could be used as a phosphorescence labelling reagent and QDs-Cys-Ab_{IgE} could be used as an excellent RTP probe to determine IgE after being modified with Cys. Compared with radioimmunometric assay and ELISA (for the determination of human IgE [6–9]), the sensitivity of this new method was much higher. This method not only offered a new technology for the determination of ultra-trace IgE, but also showed that using QDs as labelling reagent and QDs-Cys -Ab_{IgE} as RTP probe was an effective way to improve the sensitivity of SSRTPIA.

Similar to ELISA, this method consumed about 20 h, while only micro level sample solution was needed in the experiment. Additionally, the phosphorescence measurement can be carried out directly after various operations in immune reaction on diverse substrates. The simple operations of this method are similar to those of ELISA, and the method can be applied to detecting IgE in large batch. Thus, SSRTPIA combining SSRTP with IA was established, which showed promising prospect.

Table 2 Linear range, regression equation, r, RSD and LOQ (LOQ in Refs. [6–9] was calculated by 3.3×LD)

Method	RSD (%)	LD (g mL^{-1})	LOQ (g mL^{-1})	Ref.
Ab_{IgE}-IgE-Ab_{IgE}-Cys-QD (Sandwich way)	2.5–3.5	9.0×10^{-14} (k=1.646, Sb=0.020, n=11)	3.0×10^{-13}	
(IgE-Ab_{IgE}-Cys-QDs (direct way)	3.3–4.3	1.8×10^{-13} (k=1.080, Sb=0.026, n=11)	5.9×10^{-13}	
Immunoradiometric assay for determination of human IgE		5.0×10^{-11}	1.6×10^{-10}	Ref. [6]
Immunoradiometric assay for determination of human IgE		4.8×10^{-9}	1.6×10^{-8}	Ref. [7]
Radioimmunometric assay for determination of human IgE	0.7–3.8	2.4×10^{-9}	7.9×10^{-9}	Ref. [8]
ELISA for determination of human IgE	2.1–7.1	3.5×10^{-9}	1.2×10^{-8}	Ref. [9]

Table 3 Effects of coexistent materials on IgE (20.0 pg IgE ml^{-1} and 20.0 pg IgE ml^{-1}-X μg coexistent materials mL^{-1} were parallelly determined by sandwich way (n=6). Meanwhile, its Er and allowed multiple (the quotient obtained between X μg allowed materials and 20.0 pg IgE ml^{-1}) were calculated)

This method					Ref. [9]	
Coexistent materials	I_p	The allowed concentration (μg mL^{-1})	The allowed multiple	Er (%)	The allowed concentration (μg mL^{-1})	The allowed multiple
0	95.6	0	0	0	0	0
IgG	97.5	20.0	1.0×10^6	+2.0	0.020	1.0×10^3
IgA	98.5	15.0	7.5×10^5	+3.0	0.015	7.5×10^2
IgM	100.3	10.0	5.0×10^5	+4.9	0.010	5.0×10^2
IgD	93.2	7.2	3.6×10^5	−2.5	0.0072	3.6×10^2

Interference Experiment

In order to assess the influence of other possible coexistent species on the response of the bioconjugation to IgE, the allowed concentration of coexistent materials were determined by the sandwich way and the method in Ref. [9] in the system containing 20.0 pg IgE ml^{-1}. When the relative error (Er) was±5%, the allowed concentrations of coexistent materials were compared with those in Ref. [9], and the results are listed in Table 3.

Seen from Table 3, different IgG, IgA, IgM and IgD had little interference on the determination of IgE, showing that there was no crossed reaction and the high specificity of this method. Moreover, the maximal allowed concentration of co-existent materials was higher than that in Ref. [9] when Er was less than 5%, which further indicated that less crossed reaction carried out with other immunoglobulin in the sample, showing higher selectivity, accuracy, more suitable to determine the IgE content in bio-sample in the presence of immunoglobulin and wide application prospect.

Phosphorescence Lifetime

The phosphorescence lifetime (τ) of QDs-Cys, QDs-Cys-Ab_{IgE}, IgE-Ab_{IgE}-Cys-QDs and Ab_{IgE}-IgE-Ab_{IgE}-Cys-QDs could be calculated, respectively, according to the phosphorescence decay method in Ref. [32] (delay time: 0.1–1.9 ms, gate time: 2.0 ms). The regression equation of ln I_p-τ, correlation coefficient (r), and phosphorescence lifetime (τ) are listed in Table 4.

From Table 4, we could see that τ of QDs-Cys, QDs-Cys-Ab_{IgE}, IgE-Ab_{IgE}-Cys-QDs and Ab_{IgE}-IgE-Ab_{IgE}-Cys-QDs prolonged with the increase of the ΔI_p, which provided the possibility for the determination of the content of IgE using time resolved spectrometry and showed that the yields of QDs-Cys-Ab_{IgE}, IgE-Ab_{IgE}-Cys-QDs and Ab_{IgE}-IgE-Ab_{IgE}-Cys-QDs were increasing in the process of the labeling reaction and immunoraction, causing the rise of QDs-Cys molecules on the biological target and the corresponding τ. Moreover, the longer τ of Ab_{IgE}-IgE-Ab_{IgE}-Cys-QDs than IgE-Ab_{IgE}-Cys-QDs showed much better specificity of sandwich way than indirect way.

Analysis of Samples

The content of IgE in the test solution was determined according to experimental method (sandwich way). Meanwhile, a standard addition recovery experiment was also conducted. The results of this method were compared with those obtained by ELISA. The results are listed in Table 5.

According to Ref. [33] when the content of IgE is lower than 0.0018 ng mL^{-1} (negative) in human serum, they are non-asthma persons; while more than 0.0018 ng mL^{-1} (positive), they are asthma patients. When the content of IgE is in the range of 60.0–450.0 ng mL^{-1}, they are healthy people [34]. If somebody is hepatopath, the level of human IgE would increase. According to references [33,34] and the analytical results in Table 4, we could diagnose that A, B, C and D suffered from asthma, E, F, G and H were hepatopathes, I, J, K and

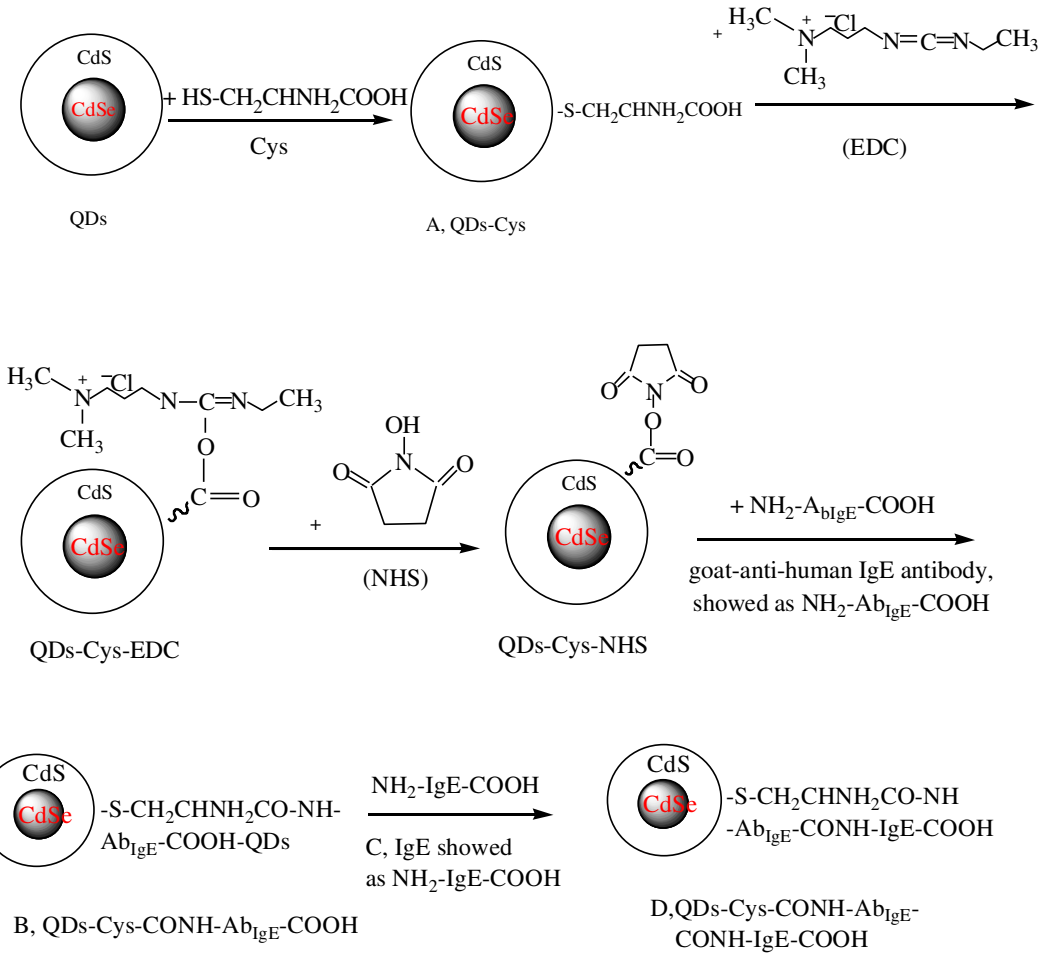
Table 4 Phosphorescence lifetime

Sample	The regression equation of the delay curve (ms)	r	τ (ms)
QDs-Cys	$\ln I_p=4.587-0.01945\ t+0.02436\ t^2$	−0.9962	51.4
QDs-Cys-Ab_{IgE}	$\ln I_p=4.675-0.01769\ t+0.02654\ t^2$	−0.9969	56.5
IgE-Ab_{IgE}-Cys-QDs	$\ln I_p=4.746-0.01457\ t+0.004923\ t^2$	−0.9974	68.6
Ab_{IgE}-IgE-Ab_{IgE}-Cys-QDs	$\ln I_p=4.758-0.01198\ t+0.005061\ t^2$	−0.9987	83.5

Table 5 Analytical results of IgE in human serum (n=8)

Serum IgE	Obtained (ng mL^{-1})	RSD (%)	Added (ng mL^{-1})	Recovered (ng mL^{-1})	Percent recovery (%)	ELISA (ng mL^{-1})	Relative error (%)
Serum A	0.0058	4.5	0.0100	0.0101	101	0.0056	3.5
Serum B	0.0035	4.2	0.0100	0.0099	99.0	0.0034	2.9
Serum C	0.0046	3.4	0.0100	0.0102	102	0.0048	−4.2
Serum D	0.0084	2.2	0.0100	0.0105	105	0.0085	−1.2
Serum E	701.7	4.8	10.00	10.4	104	692.5	1.3
Serum F	611.1	4.6	10.00	9.9	99.0	630.5	3.1
Serum G	588.1	3.5	10.00	10.3	103	561.8	4.7
Serum H	801.6	3.0	10.00	9.8	98.0	829.9	−3.4
Serum I	157.1	3.4	10.00	10.1	101	162.3	−3.2
Serum J	87.0	2.2	10.00	9.7	97.0	89.1	1.1
Serum K	121.4	4.2	10.00	9.8	98.0	115.8	−4.8
Serum L	105.8	2.9	10.00	10.2	102	103.2	2.5

L were healthy people. This method was more sensitive and much directer for the determination of IgE to check exogenous asthma allergen, which increased the accuracy of the original allergy [35]. Therefore, the treatment program could be adjusted by the change of IgE content to evaluate the treatment effect [33]. These facts reflect potential applications of the method in clinical test, disease prediction and diagnosis.



Scheme 1 Labelling reaction and specific reaction

The Mechanism for the Determination of IgE Using the QDs-Cys-Ab_{IgE} Phosphorescent Quantum Dots Probe

Ref. [26] has reported that when Mn^{2+} was dopped, ZnS-QDs could emit phosphorescence (5 mg L^{-1} ZnS-QDs, I_p=1, Ex Slit/Em Slit=10/20 nm), and this phenomenon was attributed to the transition of Mn^{2+} from $^4T_1$ to $^6A_1$. Similar to ZnS-QDs, QDs-Cys (Scheme 1, showed as A [36]) formed by modifying CdSe@CdS-QDs with $HSCH_2CHNH_2COOH$ (Cys) only emitted fluorescence (Fig. 2). However, QDs-Cys could emit strong and stable RTP signal on the ACM when using Pb^{2+} as the perturber (Fig. 3, I_p=75.1). The reason may be that under the perturbed effect of Pb^{2+} ion, the intersystem crossing of QDs-Cys from S_1 to T_1 (the electron of QDs-Cys first reached the high energy level of T_1, and then returned to its lowest energy level through vibrational relaxation, at last returned to ground state S_0 by radiative transition) occurred, which caused QDs-Cys to emit strong and stable RTP signal. Additionally, Yang et al. [37] reported that the silica xerogel doped with Tb_2S_3 nanocrystallites prepared by sol–gel process could emit RTP in silicon xerogel. Moore et al. [38] also reported that $Cd_xZn_{1-x}S$ could emit phosphorescence. Though the luminescence mechanism was not clear, the study suggested the feasibility of QD emitting photoluminescence [39].

In the process of QDs-Cys labelling Ab_{IgE} (showed as NH_2-Ab_{IgE}-COOH), EDC firstly coupled with the -COOH of QDs-Cys which reacted with NHS to generate QDs-Cys-EDC [40] and at last QDs-Cys-NHS reacted with the -NH_2 of NH_2-Ab_{IgE}-COOH to produce the labelling product QDs-Cys-CONH-Ab_{IgE}-COOH (Scheme 1, B). As a result, the number of QDs-Cys molecules on the biological target QDs-Cys-CONH-COOH increased, which led to slight enhancement of RTP signal (I_p=83.7, ΔI_p=8.6, Fig. 3). The specific immunoreaction [41] carried out between -COOH in QDs-Cys-CONH-Ab_{IgE}-COOH probe and -NH_2 in IgE (NH_2-IgE-COOH, expressed as C in Scheme 1), and then the immunoreaction product QDs-Cys-CONH-Ab_{IgE}-CONH-IgE-COOH was obtained, expressed as D in Scheme 1.

The product led the RTP of the system to sharply increase (Fig. 4). And the ΔI_p of the system was directly proportional to the content of IgE. Thus, IgE could be determined by SSRTPIA using QDs-Cys-Ab_{IgE}.

Conclusion

The new QDs-Cys RTP labelling reagent and QDs-Cys-Ab were developed, SSRTPIA for the determination of IgE was established and the mechanism for the determination of IgE was discussed in this paper. This accurate, high selective and sensitive method had been applied to determining of IgE in biological samples, showing potential application prospect in immune biology and clinical examination. Our study not only developed a new immunoassay, QDs, RTP labelling reagent and QDs-Cys-Ab, but also realized the application of QDs and RTP labelling reagent in SSRTP and immunoassay.

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