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A novel bifunctional europium chelate applied in quantitative determination of human immunoglobin G using time-resolved fluoroimmunoassay

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ABSTRACT

The authors demonstrate herein a novel time-resolved fluoroimmunoassay (TRFIA) protocol for quantification of human IgG with the new bifunctional chelate Eu(TTA)$_3$(5-NH$_2$-phen) (ETNP) labeling the goat anti-human IgG. The immunoassay was conducted by following the typical procedure for sandwich-type immunoreactions. Goat anti-human IgG was immobilized on aldehyde-modified glass slides. The human IgG analyte was first captured by the primary antibody and then sandwiched by a secondary antibody labeled with the chelate ETNP. The experimental procedure was simple to follow and gave desirable levels of sensitivity and low limits of detection. To the best of our knowledge, this is the first application of the new chelate, ETNP, in an immunoassay. In comparison to typical organic, fluorescent compounds and other lanthanide fluorescent chelates used in immunoassay, the detection sensitivity of our method using ETNP chelate in the solid phase was greatly improved and a concentration of human IgG about 5 μg/L could be detected under optimal conditions. The main result of this work shows that the new chelate ETNP can be applied as a powerful fluorescent labeling material for constructing ultrasensitive TRFIAs. The detection of human IgG, using ETNP as the chelate, is a model example of the effectiveness of this immunoassay. Many other types of antigen–antibody immunoassays should be possible using the protocol described herein.

In recent years there has been increased interest in the development of new, simple, sensitive, and specific immunoassays for the quantification and detection of analytes of clinical and biological importance. Labeled immunoassays for biological detection have been extensively developed and include fluorescence immunoassays [1,2], radioimmunoassays (RIA)[1] [3,4], enzymatic immunoassays [5,6], chemiluminescent immunoassays [7], electrical chemiluminescent immunoassays [8,9], and time-resolved fluoroimmunoassays (TRFIA) [10–15]. Among them, TRFIA methodology has developed rapidly and has become a new milestone in the development of labeled immunoassays and clinical medicine [14,15]. TRFIA holds great promise for immunoassays, owing to its unique advantages, such as high sensitivity, easy automation, less susceptibility to matrix interference, and simultaneous multilabeling.

At present, many different types of TRFIA-based bioanalytical assays have been studied, for instance, the dissociation-enhancement lanthanide fluoroimmunoassay (DELFIA) [16], the directly luminescent lanthanide chelate label-based assay [17], the enzyme-amplified lanthanide luminescence assay [18], and others [19,20]. Among all these methods, the assays using lanthanide chelate labels have received the greatest attention.

TRFIA takes full advantage of the unique fluorescent characteristics of trivalent rare-earth ions and their chelates [21]. The chelates act as and replace the fluorescent substance, enzyme, isotope, or chemiluminescent substances that play important roles in labeling proteins and many other biological materials [18,21]. Up to now, only a few efficient labels used in TRFIAs have been reported in the literature [17,22–24]. Therefore, great interest in developing new chelates with strong fluorescent intensity and bioactivity groups has arisen owing to their great importance in bioanalysis.

In this study, a novel bifunctional chelate Eu(TTA)$_3$(5-NH$_2$-phen) (ETNP) with a long fluorescence lifetime and intense luminescence was used to label the goat anti-human IgG as a biomarker, and a new TRFIA protocol for quantification of human IgG has been established. To explore the feasibility of this protocol, the immunoassay was performed by following the typical procedure for sandwich-type immunoreactions on glass slides. The human IgG was first captured by the primary antibody covalently immobilized on the aldehyde-modified glass slides, and consecutively sandwiched by the secondary antibody labeled with ETNP. Time-resolved fluorescence intensity could be directly measured

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[1] *Abbreviations used:* APTES, 3-aminopropyltrimethoxysilane; BSA, bovine serum albumin; ETNP, Eu(TTA)$_3$(5-NH$_2$-phen); 5-NH$_2$-phen, 5-amino-1,10-phenanthroline; PBS, phosphate-buffered saline; RIA, radioimmunoassay; TRFIA, time-resolved fluoroimmunoassay; TTA, thenoyltrifluoroacetone.

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on a solid support after several washing steps. A minimum of 5 μg/L could be detected which was greatly improved in sensitivity, compared with RIA of 1000 μg/L [25] and ELISA of 200 μg/L [26] in human IgG detection. To the best of our knowledge, this was the first time that a sensitive and specific immunofluorometric method with antigen detection was established by using the novel europium chelate ETNP as the fluorescence label. Different from traditional TRFIAs, no enhancement solution was needed to dissociate the free Eu^{3+} from the labeled compound, and no biotin-streptavidin was used for signal amplification to improve the sensitivity of the target materials. The establishment of a new protocol using ETNP as the label material has greatly improved the quantitative detection of human IgG. The authors also contend that the method has enormous promise for further applications to antigen and antibody detection.

Materials and methods

Instrumentation

All fluorescence measurements were conducted on a Perkin–Elmer LS-55 spectrofluorimeter with both excitation and emission slits set at 10 nm and controlled by a personal computer data processing unit. A modified home-made poly(tetrafluoroethylene) detection cell and two arms of the bifurcated optical fiber were fixed in the detecting chamber of the spectrofluorimeter to transact the excitation and emission light. The excitation light was carried outside the spectrofluorimeter to the sensor fixed in the detection cell through one arm of the fiber and the emission light was collected inside the spectrofluorimeter through the other. A foursquare glass slide (side length 13 mm, thickness 1.5 mm) covered with the antigen–antibody complex was fixed on the top of the flow chamber by a mounting screw nut. All measurements were performed at room temperature and atmospheric pressure.

Materials and reagents

The chelate ETNP was synthesized by our group. Bovine serum albumin (BSA), Tween 20, and Tween 80 were obtained from Sinopbarm Chemical Reagents Co. Ltd. (Tianjin, China). Glutaraldehyde, glacial acetic acid, aminomethane, and 3-aminopropyltrimethoxysilane (APTES) were purchased from Shanghai Shi Yi Chemicals Reagent Co. Ltd. (Shanghai, China). Purified goat anti-human IgG and human IgG were obtained from Institute of Biology of Chinese Academy of Sciences (Chengdu, China). Sephadex G-50 (1.5 × 20 cm) was purchased from Pharmacia Co. (England). All other chemicals of analytical reagent grade were commercially obtained. Double-distilled water was used for the preparation of all aqueous solutions.

Buffers

The wash buffer PBST was 0.1 M (pH 7.4) phosphate-buffered saline (PBS) containing 0.05% (v/v) Tween 20. The assay buffer was 0.05 M Tris–HCl (pH 7.8) containing 0.9% (m/v) NaCl, 0.05% (m/v) NaN_3, and 0.05% (v/v) Tween 80. The coating buffer (pH 9.6) was 0.05 M Na_2CO_3–$NaHCO_3$ solution. The blocking buffer (pH 9.6) was 20 g/L BSA in 0.05 M Na_2CO_3–$NaHCO_3$. Washing solution A was 0.05 M Tris–HCl (pH 7.8), containing 0.9% (m/v) NaCl and 0.05% (v/v) Tween 20. Washing solution B was 0.05 M Tris-HCl (pH 9.1), containing 0.9% (m/v) NaCl and 0.05% (v/v) Tween 20.

Characteristics of the europium chelate Eu(TTA)$_3$(5-NH$_2$-phen)

The europium chelate ETNP was synthesized by using thenoyltrifluoroacetone (TTA) and 5-amino-1,10-phenanthroline (5-NH_2-phen) as the ligands [27]. The structure of the complex is shown in Fig. 1. The mass spectral analysis of the chelate verified a m/z of 1032 ($M^+ + H_2O$). The complex has the advantage of a long fluorescence lifetime of 0.688 ms, intense luminescence, high fluorescence quantum yield of 0.62, a large Stokes' shift, and a narrow emission spectrum, all of which contributed to the lower background and improved detection sensitivity when the immunoassay was quantified using time-resolved fluorescence.

Preparation of ETNP-IgG conjugate

Goat anti-human IgG-Eu(TTA)$_3$(NH_2-phen) conjugate was prepared as described in Ref. [28] with slight modifications. A solution of 4 mg of ETNP in 200 μL of ethanol was added into 4 mL 2.5% (v/v) glutaraldehyde in PBS buffer for 3 h at room temperature. The solution was heated to 40 °C to evaporate off the ethanol. After centrifuging at 18,500 rpm for 5 min, the soft sediment was washed with PBS and distilled water in turn and resuspended in PBS solution. Goat anti-human IgG (2 mL of 1 mg/mL) was added and the mixture was gently shaken in the incubator for 2 h at room temperature. The labeled IgG was separated from the free chelate and aggregated antibodies through gel filtration with a Sephadex G-50 column and eluted with 0.05 M Tris-HCl buffer (pH 7.3). The effluent liquid was collected by the standard of 1 mL per tube.

Aldehyde modification of the glass slides

The glass slide's surface was modified as described in the literature with some minor modifications [29]. The foursquare glass slides were submerged in chromosulfuric acid overnight. After that, the glass slides were immersed in 25% (v/v) ammonium hydroxide overnight and then washed with double-distilled water. A solution (about 10 mL) of 2.0% (v/v) APTES in 95% (v/v) ethanol was prepared for the slides and adjusted to pH 4.5 with glacial acetic acid. The glass slides were soaked in this solution for 30 min and then ultrasonically washed by ethanol and double-distilled water in turn. Reactive amino groups were then introduced onto the glass surface using APTES. The amine-modified glass slides were then immersed in a solution of 2.5% glutaraldehyde in PBS buffer and stirred for 2 h before being washed with PBS buffer and double-distilled water in turn and dried at room temperature.

Immobilization of capture antibody on the solid surface

Capture antibodies of purified goat anti-human IgG were diluted in coating solution to 5 mg/L, and 50 μL of the solution was added onto each slide for incubation overnight at 4 °C. The slides were washed four times with PBST and immersed in the blocking solution for 1 h at room temperature. After discarding the blocking

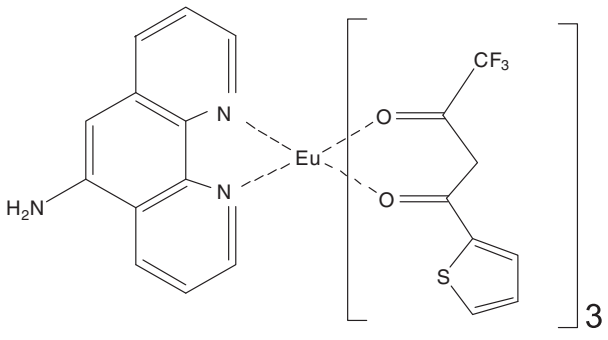

Fig.1. The structure of ETNP.

solution, the slides were dried in vacuum at room temperature, sealed, and preserved at −20 °C for ready-use.

Time-resolved fluoroimmunoassay protocol

The immunoassay was conducted by following the typical procedure for a sandwich immmunoreaction which is shown in Fig. 2. Human IgG samples were diluted with the assay buffer to 1000, 900, 800, 700, 600, 500, 400, 300, 200, 150, 100, 75, 50, 25, and 0 μg/L. A 50-μL aliquot of the standard human IgG solution was added to the capture antibody-coated slides and then incubated for 75 min at 25 °C. Blank control sample was prepared with double-distilled water instead of the human IgG sample in this step and all the assays were performed in triplicate (unless otherwise stated, blank control sample followed the same experimental procedure as the human IgG sample). After the slides were washed four times using washing buffer A, 50 μL of ETNP-IgG solution was pipetted onto the slide and incubated for 90 min at 25 °C. Then the slides were washed six times using washing buffer B and dried at room temperature. The time-resolved fluorescence intensity was measured by detecting the antigen–antibody complex left on the glass slides. All measurements were carried out at λ_{ex} = 375 nm, λ_{em} = 611 nm, delay time 0.1 ms, gate time 1.0 ms, and slitwidth at 10 nm.

Results and discussion

Human IgG detection

It is desirable to use time-resolved fluoroimmunoassays of lanthanide chelate-labeled IgG in antigen and antibody assays as a means of improving the signal-to-noise ratio. Time-resolved fluorescence intensity of different concentrations of human IgG was detected from glass surfaces. As shown clearly in Fig. 3, the fluorescence intensity increases as a function of the concentration of human IgG (from 3.23 of blank to 103.10 at 600 μg/L). These results demonstrated that the chelate ETNP could be used as a highly efficient labeling reagent for antigen and antibody detection with high sensitivity.

Optimization of a sandwich-style immunoassay conditions

In a sandwich-style immunoassay, the binding affinities of both the antigen human IgG to the capture antibody and the labeled antibody to the antigen were significantly affected by incubation temperatures, incubation times, and relative molar ratios of each reactant. As such, several experimental parameters were optimized when the authors performed experiments using 500 μg/L of human IgG as the analyte. Fig. 4A shows the effect of goat anti-human IgG concentration used as capture antibody on the immune response.

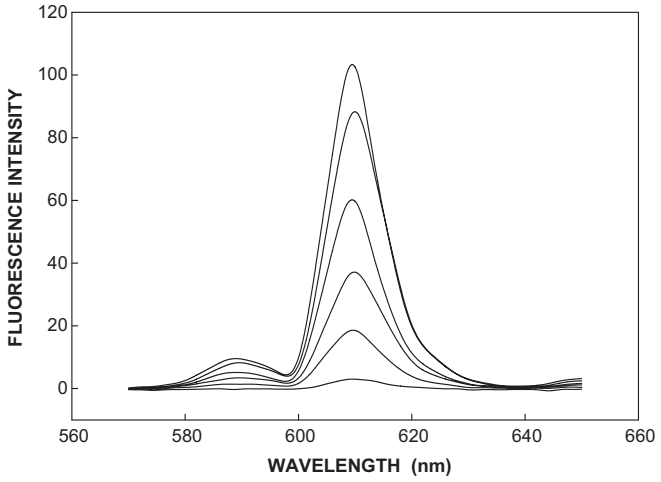

Fig.3. Time-resolved fluorescence emission spectra for different concentrations of human IgG (from top to bottom: 600, 500, 300, 150, 75, and 0 μg/L, respectively) for time-resolved spectra, λ_{ex} = 375 nm, λ_{em} = 611 nm; delay time, 0.1 ms; gate time, 1.0 ms.

One can see that fluorescence intensities increase gradually with increasing concentrations of goat anti-human IgG in the range of 1–5 mg/L, but intensities decreased at higher concentrations up to 15 mg/L. Therefore, a goat anti-human IgG concentration of 5 mg/L was applied as capture antibody throughout all experiments.

Both stages in the assay that required incubation times were investigated. The first stage is when the capture antibody mixes with the antigen IgG, and the second stage is when the captured human IgG reacts with the ETNP-labeled antibody. When the capture antibody reacted with the detected human IgG, the time of the immunoreaction was set from 15 to 105 min, and the corresponding fluorescence intensities are shown in the Fig. 4B. When the initial stage incubation time is less than 75 min, the fluorescence intensity increased, and then reached a stable state at an incubation time longer than 75 min. According to this, all the experiments were carried out with the first incubation time at 75 min.

Similarly, the second incubation time of captured human IgG with labeled antibody was also examined. As shown in Fig. 4C, after 90 min the fluorescent signal is stable and strong. The signal plateaued after 90 min. Consequently, 90 min was chosen as the appropriate incubation time for the second stage and was used in all the following experiments.

For immunoassays, the appropriate incubation temperature has a great effect on the outcome of the experiments. Therefore, incubation temperatures for both stages, the first stage of capture antibody with the antigen IgG and the second stage of captured IgG with the ETNP-labeled IgG, had to be optimized. The incubation

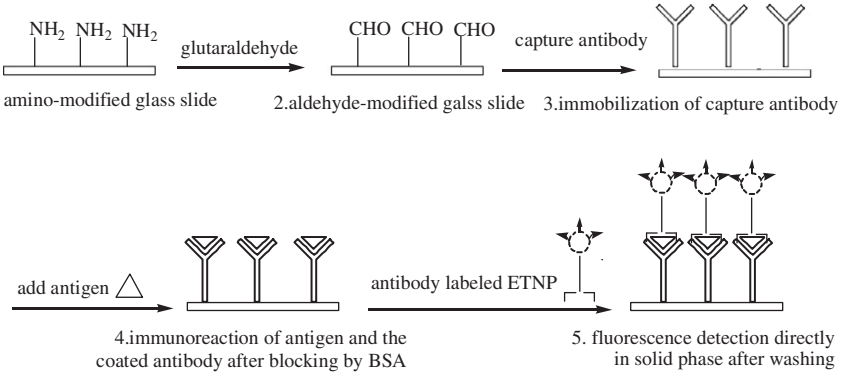

Fig.2. Schematic illustration for immunoassay protocol using the proposed method.

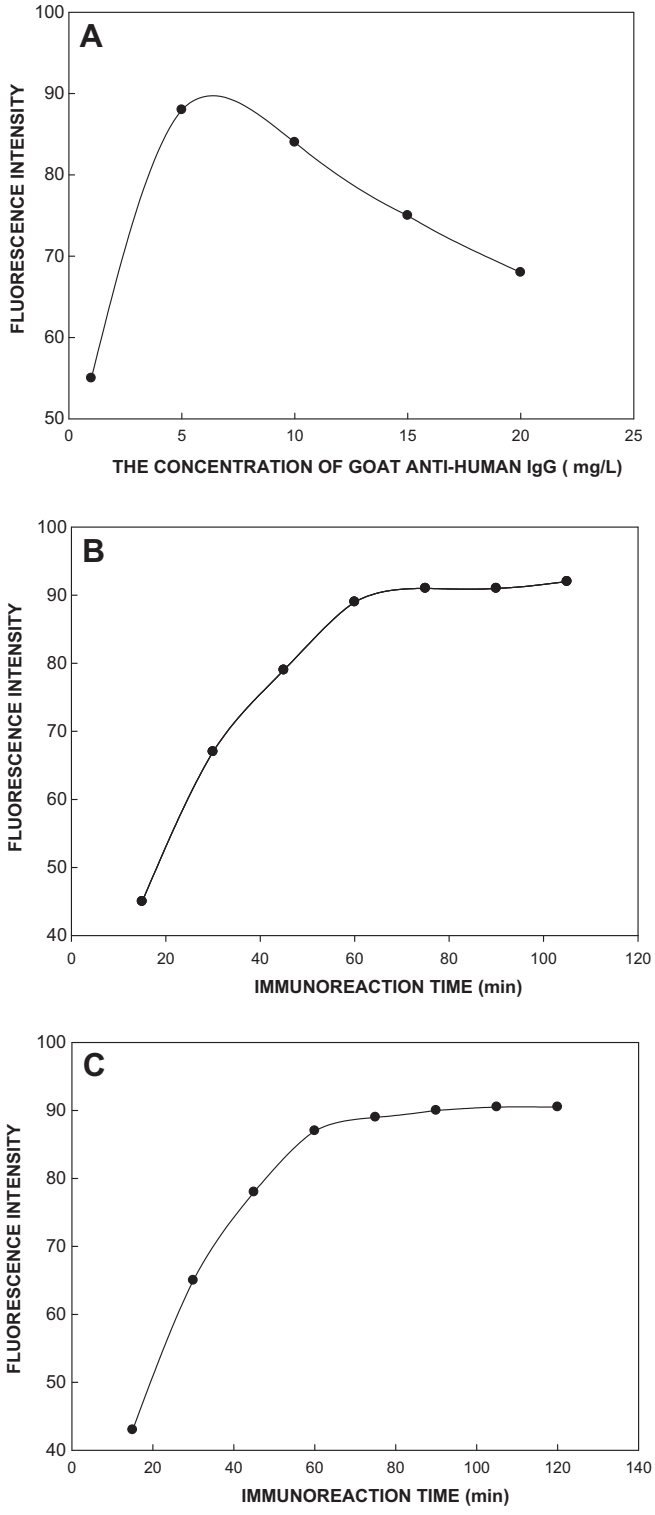

Fig.4. (A) Effect of the concentration of goat anti-human IgG used as capture antibody. The amount of 500 μg/L human IgG was used. (B) Fluorescence intensities of first stage under different incubation times. (C) Fluorescence intensities of second stage under different incubation times.

temperature of the first stage varied from 15 to 35 °C, respectively. The fluorescence intensity increases when the temperature changes from 15 to 25 °C and then reaches a maximum at 25 °C. The optimization of the second stage was similar to the first stage and a satisfactory result was obtained at 25 °C. These results indicate that incubation temperatures for both stages should be set at 25 °C for the rest of the experiments.

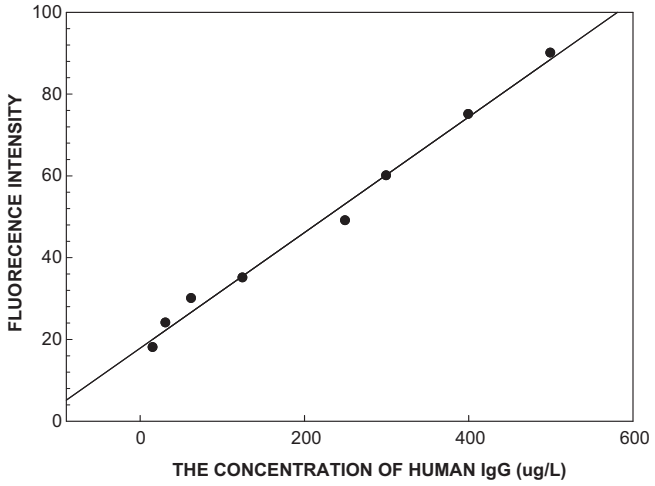

Fig.5. The standard curve for human IgG by TRFIA under optimized conditions.

Calibration curve for determination of human IgG

The time-resolved fluorescence intensity of the ETNP is directly proportional to the concentration of human IgG in the range of 16–500 μg/L which is shown in Fig. 5. The standard curve of fluorescent intensity vs. human IgG was found to be linear over this concentration range with a correlation coefficient of 0.99. The line equation for the calibration curve of human IgG was $y = 1.4333x + 17.8655$ [x is concentration (μg/L); y is fluorescence intensity]. The detection limit as defined by 3σ rule is 5 μg/L.

Conclusion

This work reports a new protocol for the determination of human IgG by using a new lanthanide chelate-labeled IgG on the surface of the solid glass. Because of the high fluorescence intensity and the strong covalent binding capacity with the protein, the authors can measure the signal from the solid phase directly with low limits of detection and high sensitivity. The chelate ETNP exhibited a long lifetime and excellent signaling ability even with a trace amount of human IgG. To the best of our knowledge, this is the first time an immunoassay has been developed using the chelate ETNP and the glass slides solid-phase detection protocol. The authors believe the method has enormous promise in many other applications pertaining to immunoassays and DNA detection. The chelate ETNP could be employed as a powerful labeling material for constructing ultrasensitive TRFIAs.

Acknowledgments

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