Time-Resolved Luminescent Analysis of Thyroid Stimulating Hormone

Liu Ying*

ABSTRACT

In this study, the experimental conditions for the determination of thyroid stimulating hormone by time-resolved fluorescence immunoassay were optimized to further expand the accuracy of the detection method, especially for the detection of hyperthyroidism. In this study, europium fluorescent nano-particles were linked with TSH monoclonal antibody. Different commercial detection kits, nanoparticles size, antibody dosage, bovine serum albumin, and the effects of serum on detection were studied in detail and optimized. We detected the fluorescence values by referring to the experimental procedures in the diagnostic kits of North Biotechnology Institute and XinBo Biotechnology Co., LTD., and determined the optimal experimental conditions according to the fluorescence values. The results show that the sensitivity of TSH detection is improved obviously after the condition optimization. This study provides more accurate and reliable data for clinical detection of hyperthyroidism or further scientific research.

Keywords: Time-Resolved Fluorescence Analysis; Thyroid Stimulating Hormone; Europium particle na-noprobe; Hyperthyroidism; High sensitivity detection

Citation: Liu Ying. Time-Resolved Luminescent Analysis of Thyroid Stimulating Hormone. Int Case Rep Jour. 2022;2(2):1-11.

Received Date: 04 February, 2022; Accepted Date: 08 February, 2022; Published Date: 10 February, 2022

*Corresponding author: Liu Ying, Kowloon, Hong Kong, China

Copyright: © Liu Ying. Open Access 2022. This article, published in Int Case Rep Jour (ICRJ) (Attribution 4.0 International), as described by http://creativecommons.org/licenses/by/4.0/.

1 INTRODUCTION

Thyroid Stimulating Hormone (TSH) is a glycoprotein hormone secreted by adenohypophyses basophils, which is composed of 211 amino acids with a relative molecular weight of 25,000-28,000. It is composed of α and β two subunits connected by non-covalent bonds. Its α subunit and adenohypophysis secrete Follicle Stimulating Hormone (FSH), Luteinizing Hormone (LH) and Human Chori-onic Gonadotropin(hCG) secreted by placenta are heterodimer glycoprotein hormones. The non-specific α subunit and the specific β subunit are connected by non-covalent bonds. The main physiological role of TSH is to control thyroid secretion of Thyroxine (T4) and Triiodothyronine (T3).[1]

The secretion of TSH is regulated by the hypothalamic-pituitary-thyroid axis, as well as the negative feedback of T3 and T4. Thyroid Stimulating Hormone (TSH) secreted by the anterior pituitary gland (also known as adenohypophysis) regulates the thyroid gland by reaching the thyroid gland through blood, binding with the
corresponding receptors on the membrane of the thyroid acinar, and promoting the synthesis and release of thyroid hormones T3 and T4 through the intracellular cAMP-protein kinase pathway. TSH first promotes the hydrolysis of thyroglobulin, releasing T3 and T4, then enhances the activity of iodine pump, promotes polyiodine in acinar epithelial cells, and enhances the activation of iodine, tyrosine iodization, and hormone synthesis. The secretion of TSH by anterior pituitary is controlled by hypothalamus and regulated by the feedback of target gland hormones. The neuroendocrine cells of hypothalamus produce TRH, which enters the anterior pituitary with blood through the pituitary portal system to promote the synthesis and release of TSH. The production of hypothalamic TRH is affected by the central nervous system. For example, ambient stimuli (cold, excessive stress, etc.) direct the release of monoamine transmitters from descending nerve fibers through the brain's central nervous system, thus affecting the release of TRH in the hypothalamus. Hypothalamus releases TRH to promote pituitary TSH secretion. At the same time, somatostatin produced by hypothalamus can inhibit the response of TSH secreting cells to TRH. It can be seen that pituitary TSH secretion is regulated by both hypothalamic TRH and somatostatin, but TRH plays a major role. When the concentration of free T3 and T4 in blood increases, it will bind to adenohypophysial thyrotropin-specific receptor, thereby promoting the production of inhibitory protein and reducing the release and synthesis of TSH. Meanwhile, the response of anterior pituitary to TRH was also weakened. When T3 and T4 in the blood are too high, TSH secretion can be inhibited by negative feedback; when T3 and T4 in the blood are too low, TSH secretion can be promoted, thus promoting the release of thyroid hormones T3 and T4. Through this feedback regulation mechanism, the concentration of thyroid hormone in the blood can be kept within the normal range.\[2\]

TSH plays an important role in promoting the uptake of thyroid iodine and the release of thyroid hormone, as well as in glucose oxidation, phospholipid and protein synthesis of thyroid. When thyroid function changes, the change of fluctuations are more rapidly and obvious of TSH than thyroid hormone. Activation of secretory neurons in the hypothalamus results in rapid release of Thyroid Stimulating Hormone releasing hormone (TRH), and soon reached the target cells of pituitary, within a few minutes by increasing the release of TSH. However, it takes several hours for TSH to react to the secretion of T3 and T4. Metabolic reactions to T3 and T4 also take several hours to be visible. So clinical detection of blood TSH level can mainly be used to evaluate the functional status of hypothalamic-pituitary-thyroid axis. Clinical laboratory diagnosis of hypothyroidism, hyperthyroidism, and cretinism includes TSH tests. Therefore, TSH in blood is an important indicator to diagnose thyroid diseases. The normal range of the internationally recognized TSH reference index is 0.3-5.5 mIU•L\(^{-1}\). Hypothyroidism was diagnosed when the blood TSH concentration was higher than 6.5 mIU•L\(^{-1}\). Hyperthyroidism was diagnosed when the blood TSH concentration was lower than 0.15 mIU•L\(^{-1}\). At present, TSH testing is widely used in screening, diagnosis, evaluation of therapeutic effect and prognosis of thyroid diseases.

Radioimmunoassay (RIA) was the earliest established TSH assay in clinic.\[3\] The minimum value of this assay was 1.2 mIU•L\(^{-1}\), but the TSH level in normal people was 0.3-5.5 mIU•L\(^{-1}\), which was lower than 1 mIU•L\(^{-1}\). The TSH level can only be detected in the range above the median of normal thyroid function, and the difference between
hyperthyroidism and normal thyroid function cannot be distinguished. TSH Immunoradiometric Assay (IRMA),\(^4\) due to the use of excessive labeled anti-bodies, can fully reflect the amount of antigen in the sample to be tested, thus improving the detection sensitivity. The sensitivity of the method can be improved to 0.1-0.2 mIU•L\(^{-1}\). Can be used to detect normal thyroid function, hyperthyroidism and hypothyroidism. However, it is difficult to distinguish TSH concentrations in treated hyperthyroidism patients (TSH concentrations range from 0.1-0.01 mIU•L\(^{-1}\)).

Time Resolved Fluorometric Assay (TRIFA) is a new ultrafine fluorescence Assay.\(^5\) Fluorescence time resolution technology is a fluorescence spectrum technology that separates the fluorescence of the emitter by using the difference of fluorescence lifetime. The time-delay method can eliminate the background fluorescence of the sample, thus greatly improving the detection sensitivity.

Time-resolved fluorescence analysis (TRFIA) is characterized by high sensitivity, high degree of automation, short time required, and no radioactive contamination. It has been applied from laboratory research into routine clinical diagnosis. Trace detection is a key index to judge the relationship between hypothalamic-pituitary-thyroid axis and evaluate its functional status, and has guiding significance for clinical treatment. To assess the degree of thyroid inhibition, the concentration of TSH will be 10 times the level of basal TSH concentration after TRH stimulation in people with normal hypothalamic-pituitary-thyroid function. For these patients, if the basal concentration of TSH can be accurately measured, harmful TRH stimulation tests can be avoided. TSH measurement is not only a sensitive indicator of thyroid function, but also the only diagnostic indicator of subclinical hyperthyroidism. Time-resolved fluorescence analysis can provide significant information for diagnosis and management of patients with subclinical hypothyroidism and hyperthyroidism. Transient low TSH concentrations (0.01 to 0.4 mIU•L\(^{-1}\)) are sometimes present in patients with non-thyroid disease, and time-resolved fluorescence analysis can distinguish moderate TSH inhibition in patients with non-thyroid disease from patients with typical thyrotoxicosis (TSH levels less than 0.02 mIU•L\(^{-1}\)). In patients with normal pituitary function, TSH levels in the blood can be suppressed to less than 10% of the control value 48 h after taking thyroid hormones (L-T3, L-T4). Time-resolved fluorescence analysis can be used to diagnose patients with thyroid hormone resistance and TSH-secreting pituitary tumors, as well as to detect serum samples with low TSH levels.\(^6\)

At present, a series of highly sensitive TSH analysis kits have been established by exploring and improving the conditions of the kits. A high sensitivity human thyroid stimulating hormone biotin-avidin ELISA kit\(^7\) was established by using horseradish peroxidase labeled streptomycin avidin as probe and tetramethyl benzidine as substrate. The sensitivity was up to 0.02 mIU•L\(^{-1}\). The high sensitivity kit was established using anti-hTSH monoclonal antibody 8E7 coated with polystyrene 40-well plate as solid phase antibody, rabbit anti-hTSH polyclonal antibody with high titer as liquid phase anti-body, horseradish peroxidase labeled sheep anti-rabbit IgG as labeled second antibody as reaction dis-play system, and o-phenylenediamine as substrate.\(^8\) The sensitivity was 0.03 mIU•L\(^{-1}\). Anti-TSH \(\beta\) subunit specific monoclonal antibody labeled with horseradish peroxidase, paired with another mAb of solid-phase-coated anti-TSH \(\alpha\) subunit, and use luminol as the substrate. A chemiluminescence
immunoquantitative assay of serum TSH with double locus sandwich was established by two-step method. The sensitivity was 0.0788 mIU•L⁻¹. Optimized TSH detection will exclude patients with subclinical thyroid disease previously classified as normal and provide more accurate and reliable clinical data.

2 EXPERIMENT

2.1 Instruments and Reagents
Time-resolved fluorescence analyzer (Synergy H4, Biotek); Shaker (Eppendorf, Germany); Centrifuge (Z36HK, Germany); 96-well coated reactive plate (North Biotechnology Institute, Xinbo Biotechnology Co., LTD.); 50 KD ultrafiltration centrifugal tube (Millipore, USA); TSH mono-clonal antigen (North Biotechnology Institute); EDC (Purity ≥99.9%, Japan); NHS (Purity ≥98%, Bellingway Technology Co., LTD)

2.2 Reference standard preparation
The standard protein solution of low concentration was prepared using the standard protein in the kit of North Biotechnology Institute. After the TSH antigen was calibrated, the standard protein solution of 0-0.35 mIU•L⁻¹ was successively diluted for use.

2.3 Preparation of europium nanoparticles
1 mg of hydrophobic europium luminescent complex was dissolved in 15 mL acetone solution. Dissolve 10 mg styrene-maleic anhydride copolymer in the same volume of acetone solution. The two solutions were dissolved in 30 mL deionized water under decompression conditions. The solution was then evaporated at 35°C for 1 hour to remove the acetone in the mixed solution. The prepared europium nanoparticles were stored in a refrigerator at 4°C for later use.

2.4 Europium nanoparticles link to TSH monoclonal antibody
Remove EDC, NHS from fridge and return to room temperature. EDC/NHS (mole ratio 2:5) was added to a solution of europium fluorescent nanoparticles and reacted on a shaker at 350 R for 15 min at room temperature. Activated europium fluorescent nanoparticles were precipitated by precipitation centrifugation. The precipitation was transferred to a beaker and europium fluorescence nanoparticles were fully dispersed by ultrasound. An appropriate amount of TSH monoclonal antibody (0.92 mIU•L⁻¹) was added and the reaction was carried out at room temperature 350 r for 2 h on a shaker. The obtained europium fluorescent nanoparticles-TSH was repeatedly cleaned with ultrafiltration tubes until the free monoclonal antibodies of TSH were washed away. The obtained europium nanoparticles-TSH solution was placed in the refrigerator for later use.

2.5 Experimental testing
Remove the coated slats and restore them to normal temperature. Put the remaining slats back into the packaging bag and seal them. Use the removed slats within 30 minutes. Shake all reagents well be-fore adding sample, and
balance to room temperature, add 100 μL well\(^{-1}\) TSH standard protein solution to coated plate, double well parallel control, respectively. The connected europium fluorescent nano particle TSH probe or the probe in the original kit (the markers should be diluted with experimental buffer 1:150 within 1 hour prior to use according to the required amount of markers) was added to the coated plate at the rate of 100μL•hole\(^{-1}\), and the pipette tip should not touch the liquid in the coated plate wall or hole when adding each hole. Shake at room temperature for 1.5 hours ± 10 minutes.

The coated lath fully reacts with europium fluorescent nanoparticle probe. Pour 40 ml of the con-centrated washing solution into a clean container, add 960 ml of distilled water for 1:25 dilution, the final pH of the washing solution is 7.8. Add 200μL washing solution to each well, wash the strip 6 times. The fluorescence value of each hole was measured by time-resolved fluorescence analyzer after the reaction coating was dried. If using the probe, quickly add 200μL of reinforcement solution to each well, be careful not to touch the coated panel. In order to avoid the degradation of the fluorescence signal caused by some external factors, the results should be measured immediately on the time-resolved fluorescence analyzer within one hour after the shaking in room temperature for 10 minutes. Plot with known standard TSH content and corresponding fluorescence value.

3 RESULTS AND DISCUSSION

3.1 Properties and Characterization of Europium Nanoparticles and Europium nanoprobes

Small europium nanoparticles can be synthesized by decompression reaction. The results show that europium nanoparticles have a diameter of about 11 nm and a Zeta potential of -21 mV. After attaching the antibody, the diameter of europium nanoprobe was about 28 nm and the Zeta potential was close to 0 mV. Electron microscope results showed that europium nanoparticles could be well dispersed in aqueous solution without visible aggregation. Europium nanoparticles have very good spectral properties. The emission spectrum is sharp and the half peak width is less than 10 nm. The absorption spectrum is broad and the maximum absorption peak is about 420 nm.

![Figure 1: Properties and characterization of Europium nanoparticles and Europium nanoprobes](image-url)
A: Electron microscopic images of Europium nanoparticles; B: Absorption spectra of Europium nano-particles dissolved in aqueous solution (solid line) and acetone (dotted line); C: Excitation spectrum (dotted line) and emission spectrum (solid line) of Europium nanoparticles; D: Dynamic light scattering results of Europium nanoparticles; E: Dynamic light scattering results of Europium nanoprobes.

3.2 Particles were compared with the diagnostic kit of North Biotechnology Institute

Using Europium luminescent nanoparticle-TSH probe, the luminescence intensity increases with the concentration of TSH standard antigen measured by time-resolved fluorescence analyzer. However, the EU-anti-TSH labeled probe used in the diagnostic kit of the Northern Biotechnology Institute showed a large deviation at each point of low concentration, and it was almost impossible to clearly distinguish the fluorescence values of each point below 0.3 mIU•L⁻¹ concentration. A highly sensitive TSH kit that meets clinical requirements should have a sensitivity of 0.05 mIU•L⁻¹ or less. At present, the sensitivity of most highly sensitive TSH kits on the market is 0.03 mIU•L⁻¹. The TSH content of 20 zero standards was determined simultaneously, and the sensitivity of the fitting curve calculated by x+2s was 0.0237 mIU•L⁻¹. The experimental data show that our probe can meet the international standard of TSH hypersensitivity determination.

Figure 2: Comparison results of Europium nanoprobes with the diagnostic kit of the Northern Institute of Biotechnology

A: The result of time-resolved fluorescence determination of the reagent of the North Institute of Bio-technology; B: Time-resolved fluorescence measurements using Europium nanoprobes.

3.3 Particles were compared with the diagnostic kit of XinBo Biotechnology Co., LTD

In order to determine the performance of our synthetic luminescent probe is superior to commercial detection kits, we used Eu-anti-TSH and europium fluorescent nanoparticle-TSH in the diagnostic kit of XinBo Biotechnology Co., Ltd. in the same kit under the same conditions, as shown in the figure. At low concentration, the detection sensitivity of Europium fluorescent nanoparticle-TSH was significantly higher than that of the fluorescent labeling probe of XinBo Biotechnology Co., LTD. A monoclonal antibody against TSH was coated in a coated plate by solid phase double antibody sandwich immunoassay, and an antigen-antibody conjugate was formed with TSH in the standard
sample. Eu3+ is labeled on another monoclonal antibody against TSH, which binds to another binding site of TSH to form an antibody-antibody-EU complex on the coated plate. In this procedure, Eu-anti-TSH probes bind to the standard TSH antigen to be tested, and an enhancer solution is added to the 96-well plate to dissociate the Eu3+ bound to the antibody and chelate with the components in the enhancer solution to form new chelates. And it needs to be detected in the liquid state, so the solution is inevitably contaminated by the environment. Complex operation procedures and environmental pollution lead to inaccurate measurement results.

![Figure 3](image.png)

**Figure 3:** Comparison results of Europium nanoprobe and XinBo Diagnostic kit with time-resolved fluorescence.
A: Time resolved fluorescence determination results of the diagnostic kit of XinBo Biotechnology Co., LTD. B: Time-resolved fluorescence measurements using Europium nanoprobes

### 3.4 The influence of Europium fluorescent nanoparticles of different sizes and the amount of linked antibodies on the detection results

We used 11 nm and 16 nm Europium fluorescent nanoparticle probes and the North Institute of Biotechnology diagnostic kit slats to detect the effect of different sizes of Europium fluorescent nanoparticle on the detection results. The reaction process and conditions are described above. As shown in the figure, the deviation of results using Europium fluorescent nanoparticle probe at 11 nm is significantly less than that of Europium fluorescent nanoparticle probe at 16 nm. This is mainly due to the large specific surface area of the nanoparticles and therefore the non-specific adsorption to the slat substrate is particularly large. Moreover, nanoparticles themselves tend to agglomerate and become embedded in irregular spaces. To solve the non-specific binding problem of Europium fluorescent nanoparticle probe on the substrate, we will attempt to modify the substrate surface to avoid the close contact and mosaic of nanoparticles with the substrate, or reduce the concentration of europium fluorescent nanoparticle probe solution used. We found that the non-specific binding of the diluted antibody solution was significantly lower than that of the high concentration antibody solution. Usually, the antibody solution was used after dilution on the basis of the original concentration. When the concentration was high, there would be strong non-specific binding, and this was caused by the non-specific interaction between the antibody protein and the substrate. Since the size of the antibody protein molecule is the same order of magnitude as the europium fluorescent nanoparticle probe, we hypothesized that the non-specific binding effect of the europium fluorescent nanoparticle probe could be reduced and the accuracy of detection could be improved if the europium fluorescent nanoparticle probe...
probe was diluted. The pH value of buffer solution is also a factor affecting the surface charge of solid phase carrier. The removal of agglomeration particles after europium fluorescent nanoparticles probe surface active group coupling with TSH antibody is also a problem that we should pay attention to in future experiments.

Figure 4: Influence of europium fluorescent nanoparticles of different sizes and the amount of linked antibodies on the detection results.

A: Time-resolved fluorescence determination using 11 nm europium fluorescent nanoparticles; B: Time-resolved fluorescence determination using 16 nm europium fluorescent nanoparticles; C: Time-resolved fluorescence determination after halving the linked antibody using 16 nm europium fluorescent nanoparticles.

3.5 Seal the slats with a large amount of bovine serum albumin

Bovine Serum Albumin (BSA) is a Serum Albumin extracted from Bovine blood. It is often used as a standard protein concentration. The full length of the BSA precursor protein is 607 amino acids, and the N-terminal signal peptide with 18 amino acid residues is cut off during secretion, so the initial protein product contains 589 amino acid residues. The other four amino acids were sheared to form mature bovine serum albumin containing 585 amino acids. Bovine serum albumin has many biochemical applications, including Enzyme-Linked Immunosorbent Assay (ELISA), western blot and immunohistochemistry. It also serves as a nutrient for cultured cells and microorganisms. BSA is also used to stabilize some enzymes during DNA digestion and prevent the adsorption of enzymes with reaction tubes and pipette tips. BSA did not affect the stability of other enzymes. By comparing the quantity of the unknown protein with the known BSA, which is also commonly used to determine the quality of other proteins. BSA can be combined with many cations, anions and small molecules, and is an important carrier of many compounds in the blood. It has strong stability and good biocompatibility, and does not affect the biochemical reaction. Therefore, it is widely used as a carrier of hydrophobic drugs.\textsuperscript{[10]}

Europium fluorescent nanoparticle probes previously used BSA as a protective agent to block the active sites on europium fluorescent nanoparticle probes that had been activated and were not linked to TSH antibodies. In order to prove the influence of BSA on experimental detection, a large amount of BSA was first added to the reaction strip, and then europium fluorescent nanoparticle probe was added. Experimental results showed that europium fluorescent nanoparticle probe could hardly bind to the corresponding antigen after adding BSA, indicating that BSA had a strong influence on the experiment. Therefore, in our next experiment, BSA will be replaced with PEG,
striving to achieve the purpose of not only sealing the unreacted active site, but also not interfering with the experimental results.

**Figure 5:** Seal the slats with large amounts of bovine serum albumin.

### 3.6 Normal experimental results of bovine serum and human serum simulation

Since the kit will be used for the detection of serum TSH in clinical patients, we added an equal amount of serum to the standard protein to simulate the measurement environment of normal human serum TSH. From the experiment, it is found that if the serum is bovine serum, the experimental results are more accurate. However, if human serum was used, the experimental results showed a decreasing trend with the increase of standard protein addition. This may be due to experimental errors caused by higher TSH content in human serum itself.

**Figure 6:** Normal experimental results of bovine serum and human serum simulation.
A: Normal experimental results of bovine serum simulation; B: Human serum mimics normal experimental results

### 3.7 Influence of reaction mode on detection results
Due to the interference of serum to the detection results, we adopted a two-step method. Step 1: After adding standard TSH antigen, the reaction system was incubated at 37°C for 40 minutes. Step 2: europium fluorescent nanoparticle TSH probe was added, and the reaction system was incubated at 37°C for 50 minutes. The structure shows that europium nanoparticles have high sensitivity for TSH diagnosis.

Figure 7: Influence of reaction mode on detection results.

4 CONCLUSION
In this paper, the experimental conditions for the determination of thyroid stimulating hormone by time resolved fluorescence immunoassay were optimized on the basis of commercial detection kits, and the accuracy of the detection method was expanded, especially for the detection of hyperthyroidism at low concentrations of TSH. In this experiment, europium fluorescent nanoparticles connected by TSH monoclonal antibody were used as detection probes, and europium ion fluorescent probes in diagnostic kits of North Biotechnology Institute and XinBo Biotechnology Co., LTD were used as detection and comparison results, as well as europium fluorescent nanoparticles of different sizes. The influence of the amount of junction antibody and bovine serum albumin on the detection results was studied in detail. Finally, in order to simulate the real detection environment, we added bovine serum and human serum respectively. In order to avoid the interference of serum, we adopted a two-step measurement method. We detected the fluorescence values by referring to the experimental procedures in the diagnostic kits of North Biotechnology Institute and XinBo Biotechnology Co., LTD., and determined the optimal experimental conditions according to the fluorescence values. The results showed that the sensitivity of the
determination of thyroid stimulating hormone was improved and more accurate and reliable data were provided for clinical detection or scientific research.

REFERENCES