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Magnetic particle-based chemiluminescence enzyme immunoassay for free thyroxine in human serum

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ABSTRACT

A magnetic particles-based chemiluminescence enzyme immunoassay with high sensitivity, specificity, rapidity, and reproducibility was developed for the determination of free thyroxine in human serum. A competitive assay has been proposed with horseradish peroxidase labeled thyroxine analog. The immunomagnetic particles coated with anti-fluorescein isothiocyanate antibody was used as dispersed solid phase and separation means for the immunoassay. Experimental conditions, such as temperature, the volume of magnetic particles and substrate, incubation time, dilution ratio and other relevant variables upon the immunoassay have been examined and optimized. The proposed method exhibited high performance which the linear range was 1.59–122 pmol L^{-1} and the detection limit was 0.25 pmol L^{-1}. A coefficient of variance of less than 15% was obtained for both intra-assay and inter-assay precision. The present method has been successfully applied to the analysis of free thyroxine in human serum. The diagnostic accordance rate of the method for normal serum, hyperthyroidism and hypothyroidism are satisfactory. Good correlations were obtained between the results by the proposed method and the commercial radioimmunoassay kit. The present method exhibits good potential in the fabrication of FT_4 diagnostic kits which could be used in the clinical analysis and facilitated the development of automated operation systems in the clinical practice.

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1. Introduction

Thyroid disease is a hackneyed disease of internal secretion in clinical diagnosis, with more than 300 million people suffering from this disease in the world. In general, the concentration of hormones that secreted by the thyroid gland was determined to evaluate the thyroid function of the patient and meanwhile, propose therapy scheme for the disease. Thyroxine (3,5,3′,5′-tetraiodothyronine, T_4), with molecular weight of 777 Da, is one of the important hormones that regulates many biological functions. T_4 has been proved necessary for normal neural development and normal cellular metabolism. For example, children born with thyroid hormone deficiency will not grow well and the brain development can be severely impaired. Normally, 99.97% of T_4 exists as protein-binding compounds, among which 60% binding to thyroxine binding globulin (TBG), 30% binding to thyroid binding prealbumin (TBPA), and 9.97% binding to albumin (Alb). Only 0.03% were free [1]. For most of the serum T_4 is bound to these transport proteins leaving only about 0.03% free to exert its effect on cells [2]. It is the free T_4 (FT_4) that represents the metabolically active fraction; for this reason the measurement of FT_4 concentration is considered to be an indicator of patient thyroid function.

There were various methods that have been employed to the determination of FT4 in human serum, such as equilibrium dialysis [3,4], ultrafiltration [5], chromatography on polyacrylamide gel [6] or sephadex [7], radioimmunoassay (RIA) [8–10], enzyme immunoassay [11], time-resolved fluorescence [12], bioluminescent immunoassay [13], fluoroimmunoassay (FIA) [14,15], chemiluminescence immunoassay (CLIA) [16,17], electrochemiluminescence immunoassay [18], mass spectrometry [19], and micro-plate CLEIA [20]. However, there are several problems to overcome for a simple, highly sensitive, and reliable immunoassay for FT_4 detection. As we know, direct methods are complex and time consuming. The radioactive labels of RIA are harmful to the operators, and the radioactive waste needs to be treated. Moreover, the half-life of the radioelement is a restricted factor for the storage life. Electrochemical method requires preparation of working electrodes, needs expensive instrumentation and complicated operation. The lanthanide labels in FIA are susceptible to outside interference, and the sensitivity of FIA is also limited. The detection limit of micro-plate CLEIA is relatively high and its linear range is relatively narrow.

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In the recent years, magnetic particles are widely used in the biochemical fields, including immunoassay, genetic engineering, and cell separation [21–26]. MPs modified with biological ligands possess their unique property of superparamagnetism, enabling their stability and wide dispersion within the solution upon removal of the magnetic fields [27,28]. In such a homogenous system, the mass transfer distance of analytes and reagents to the immobilized antibody is greatly reduced and consequently, the antibody–antigen binding equilibrium can be achieved more rapidly than when antibodies are immobilized on the planar surface, such as micro-plate wells. Therefore, magnetic particles (MPs)-based immunoassay has been widely used [29–31].

In this study, we reported an approach for FT_4 detection based on competitive reaction using magnetic microparticles. FT_4 presenting in a patient's serum competes with enzyme-T_4 analog conjugate for FITC-labeled anti-T_4 antibodies. The anti-FITC-labeled magnetic particles were served as both the solid support and the means of separation. When MPs dispersed evenly into the reaction mixture, rapid reaction kinetics and easily automated operation were achieved. When added a magnetic field, selective separation of immobilized enzymes or other proteins from a reaction mixture could be accomplished. Due to such advantages as no radioactive waste, relatively simple and inexpensive instrumentation, and larger linear range, the methodological potential shown here might be available for the analysis of FT_4 in the clinical diagnosis.

2. Experimental

2.1. Apparatus and chemicals

A universal luminometer reader (chain-based flash in glow device from Berthold Technologies GmbH & Co. KG, Germany) with test tubes (10-mm diameter × 60-mm length) that could be placed into the luminometer was used for the particle-based chemiluminescence detection. The incubation and shaking procedures at 37 °C were carried out at a thermostatic culture oscillator (ZHWY-100, Shanghai Zhicheng Analytical Instrument Manufacturing Co. Ltd., Shanghai, China). The magnetic separator was obtained from Beijing ChemClin Biotechnology Co. Ltd. (Beijing, China). Data acquisition and treatment were performed with an integrated 16-bit microprocessor system. In using the commercial radioimmunoassay kit for sample analysis, a Gamma counter (GC-400, USTC Chuangxin Co. Ltd., Zonkia Branch, Hefei, China) was used.

FT_4 standard was obtained from Sigma (St. Louis, USA). Goat anti-FT_4 polyclonal antibody was purchased from Sichuan Zheng Long Company (Sichuan, China). HRP-T_4 analog conjugate was obtained from Beijing Atom High Tech Co. Ltd. (Beijing, China). Fluorescein isothiocyanate (FITC) was from Sigma (USA). The immunomagnetic particles (2 μm, 0.1% w/v) coated with anti-FITC antibody and suspended in solution were purchased from Adaltis (Italy). Human sera samples were purchased from Beijing 301 Hospital (Beijing, China). The commercial RIA kits for FT_4 were from Beijing Chemclin. Biotech. Co. Ltd. (Beijing, China).

2.2. Buffers and calibrators

Washing buffer was 0.05 mol L^{-1} phosphate solution with 0.05% Tween-20 (PBST). Dilution solution for HRP-labeled T_4 analog was 0.1 mol L^{-1} Tris–HCl buffer, pH 6.5. Dilution solution for FITC-labeled anti-T_4 antibody was 0.05 mol L^{-1} phosphate buffered saline (PBS) containing 1.5% (w/v) bovine serum albumin (BSA), pH 7.4. The chemiluminescence substrate were luminol in 0.1 mol L^{-1} Tris–HCl of pH 8.5 and H_2O_2 in citrate buffer of pH 4.5.

For calibration, FT_4 standard was prepared in freshly free hormone human serum according to the following procedures. A serial quantity of pure FT_4 was dissolved in fixed volume of free hormone human serum. The obtained calibrators were equilibrated at 4 °C and the equilibrium concentration was monitored by a commercial RIA kit until a stable linear stand curve was obtained with correlation coefficient bigger than 0.99 (n = 3). Finally, the serial concentration of calibrators were determined as 0, 1.59, 3.45, 15.6, 37.5, and 122 pmol L^{-1}, assigned to S_0, S_1, S_2, S_3, S_4, and S_5, respectively. The prepared calibrators were stored at 4 °C for further use.

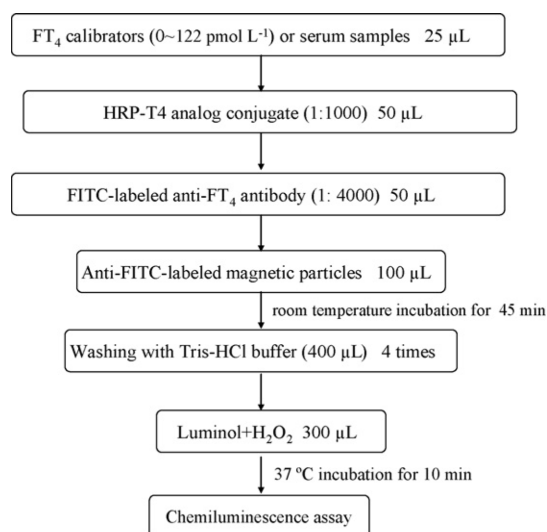

Fig. 1. Schematic illustration of the MPs-CLEIA procedures.

2.3. FITC-labeled anti-FT_4 antibody

The FITC-labeled anti-FT4 antibody was prepared based on our previous work [32]. Anti-FT_4 antibody was dialyzed overnight at 4 °C against 0.05 mol L^{-1} $NaHCO_3$ buffer, pH 9.3. FITC was applied to react with primary amines of the protein. 25 μL FITC dissolved in 0.05 mol L^{-1} carbonate/bicarbonate buffer, pH 9.5 (2 mg mL^{-1}) was added to 200 μL anti-FT_4 antibody (3 mg mL^{-1}). After reaction with stirring for 12–16 h at 4 °C, the mixture was dialyzed for 3 days at 4 °C against phosphate buffered saline (PBS), pH 7.4. The dialyzed antibody was centrifuged at 3000 rpm for 30 min to discard any precipitates. Aliquots of the antibody-FITC conjugate were stored at −20 °C until further use.

2.4. Immunoassay procedures

2.4.1. Magnetic particles-based CLEIA (MPs-CLEIA)

The immunoassay procedures of the proposed MPs-CLIA are as follows: first, 25 μL FT_4 calibrators or serum samples were added into the test tubes. Then 50 μL HRP-T_4 analog conjugate (dilution ratio of 1:1000) was added. After that, 50 μL FITC-labeled FT_4 antibody (dilution ratio of 1:4000) and 100 μL anti-FITC antibody coated MPs were added. Afterwards, the mixture was incubated at room temperature for 45 min (immunoreaction time). The samarium–cobalt magnet was inserted under the test tube rack for separation (5 min). The antibody coated MPs and any specific captured substances were attracted by the magnets to the bottom of the test tubes, and free substances removed by gently tapping the test tubes against tissue paper. During the washing steps, 400 μL washing solution was added into the test tubes by placing them outside the magnet, so the MPs were resuspended in the washing solution. Finally, 300 μL CL substrate solution was added, the mixture was incubated for 10 min at 37 °C and the emitted photons were measured. The detailed reaction process was shown in Fig. 1.

2.4.2. Radioimmunoassay (RIA) [33]

The immunoassay procedures followed the details on the product label. First, 50 μL calibrators or serum samples were added into the assigned test tubes which had been coated with anti-FT_4 antibody. Then, 1000 μL ^{125}I tracer solution was added to each tube and mixed well. After that, incubation at 37 °C for 1.5 h was carried out. Then the solution was discarded and 1000 μL distilled water was added. After the solution was aspirated, the tubes were transferred to a Gamma counter to measure the radioactive counts for 1 min.

2.5. Data analysis

Standards and samples were measured in double tubes, and CL intensity values were integrated. Standard curves were obtained by plotting CL intensity against the logarithm of analyte concentration and fitted to the equation of logit Y–log X, in which the value of logit Y was calculated according to the formula as follows:

$$\text{logit}\,Y = \ln\left[\frac{y}{1-y}\right]\left(y = \frac{\text{RLUS1}\sim\text{S5}}{\text{RLUS0}}\right)$$

2.6. Samples

The ultimate purpose of this work was applied in the clinical diagnosis. Therefore, serum samples from hyperthyroidism, hypothyroidism, and normal people were collected from Beijing 301 Hospital (Beijing, China). After vein blood sampling, the blood was centrifugated. After that, the obtained sera were aspirated, subpackaged, and stored at −20 °C for further use.

3. Results and discussion

3.1. Optimization of immunoreaction reagents

3.1.1. Influence of dilution ratio of HRP-T_4 conjugate and FITC-labeled anti-FT_4 antibody

Generally speaking, the appropriate dilution ratios of HRP-T_4 conjugate and FITC-labeled anti-FT_4 antibody are important for obtaining an accurate standard curve. Moreover, the sensitivity and specificity of a competitive immunoreaction is also influenced by the dilution ratio of reagents. The influence of dilution ratio of HRP-T_4 conjugate was shown in Fig. 2. As can be seen, in the standard FT_4 concentration range of 0.59–122 pmol L^{-1}, when the dilution ratio of HRP-T_4 conjugate was increased from 1:500 to 1:4000 (lower dilution ratio corresponds to higher concentration), the relative light unit (RLU) was decreased. The inhibition ratio (Table 1), namely RLU_{S1}/RLU_{S0} (82.6%) and RLU_{S5}/RLU_{S0} (15.4%), was appropriate, indicating a low sensitivity and wide linear range. Empiristicly, in a competitive reaction, the inhibition ratios of RLU_{S1}/RLU_{S0} and RLU_{S5}/RLU_{S0} at about 85 and 15% mean not only a good separation and even distribution of calibrator points on the dose–response curve, but also a proper detection sensitivity. As can be seen from Table 1, dilution ratio of 1:1000 was selected finally.

The influence of dilution ratio of FITC-labeled anti-FT_4 antibody was shown in Fig. 3. As can be found in Fig. 3, dilution ratio of 1:4000 corresponds to an inhibition ratio of 87.4%, which was appropriate for a competitive immunoreaction. So the dilution ratio of 1:4000 was selected for further investigation.

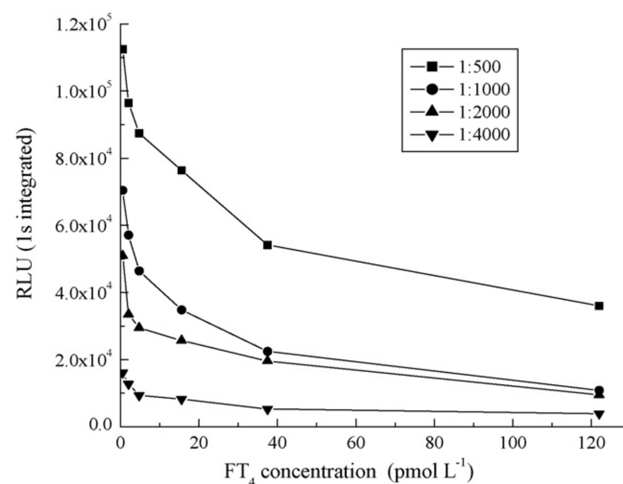

Fig. 2. Influence of dilution ratios of HRP-FT_4 conjugate. The four curves correspond to a series of dilution ratios (i.e., 1:500, 1:1000, 1:2000, and 1:4000). Detection conditions: dilution ratio of FITC-labeled anti-FT_4 antibody: 1:2000; volume of MPs: 100 μL; volume of substrate: 300 μL; 37 °C incubation for 60 min.

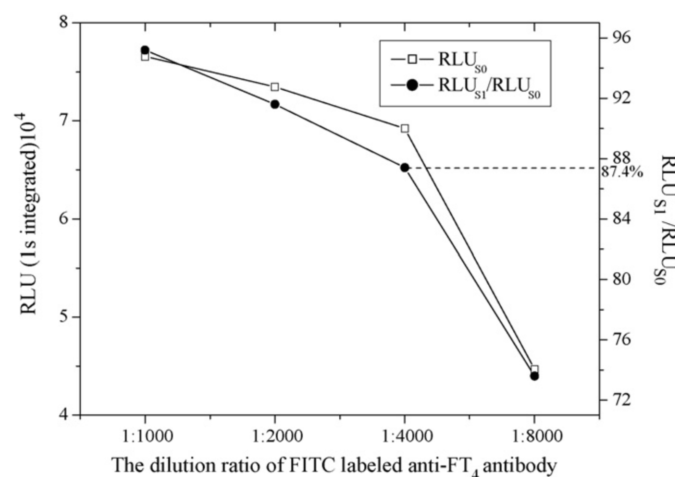

Fig. 3. Influence of dilution ratios of FITC-labeled anti-FT_4 antibody. Detection conditions: dilution ratio of HRP-T_4 conjugate: 1:1000; volume of MPs: 100 μL; volume of substrate: 300 μL; 37 °C incubation for 60 min.

3.1.2. Optimization of volume of magnetic particle and substrate solution

The effect of volume of magnetic particles and substrates solution were studied and the results were shown in Table 2. From Table 2, it can be concluded that 100 μL of magnetic particle and 300 μL of substrate solution were the appropriate values, corresponding to inhibition ratios of 87.8 and 14.6, and 87.6 and 14.9%, respectively. In addition, the correlation coefficients were the best in case of 100 μL of magnetic particle and 300 μL of substrate.

Table 1
Influence of dilution ratio of HRP-T_4 conjugate (n = 3).

Dilution ratios	RLU_{S0}	RLU_{S1}	RLU_{S5}	Inhibition ratio	
				RLU_{S1}/RLU_{S0} (%)	RLU_{S5}/RLU_{S0} (%)
1:500	1.12×10^6	9.65×10^5	3.60×10^5	86.2	32.1
1:1000	7.04×10^5	5.82×10^5	1.09×10^6	82.6	15.5
1:2000	5.10×10^5	3.35×10^5	9.48×10^4	65.7	18.6
1:4000	1.61×10^5	1.27×10^5	3.95×10^4	78.9	24.5

Table 2
The influence of volume of MPs and substrate solution ($n = 3$).

	Volume of MPs (μL)					Volume of substrate solution (μL)				
	50	100	150	200	250	150	200	250	300	350
RLU_{S0}	2.56×10^5	4.51×10^5	4.66×10^5	4.78×10^5	4.66×10^5	2.15×10^5	3.57×10^5	4.32×10^5	4.99×10^5	5.45×10^5
RLU_{S1}	1.66×10^5	3.96×10^5	4.22×10^5	4.25×10^5	4.13×10^5	1.36×10^5	2.95×10^5	3.46×10^5	4.37×10^5	3.97×10^5
RLU_{S5}	4.53×10^4	6.57×10^4	6.14×10^4	5.93×10^4	5.51×10^4	2.57×10^4	3.55×10^4	4.39×10^4	7.43×10^4	8.82×10^4
RLU_{S1}/RLU_{S0} (%)	64.8	87.8	90.6	88.9	88.6	63.3	82.6	80.1	87.6	72.8
RLU_{S5}/RLU_{S0} (%)	17.7	14.6	13.2	12.4	11.8	12.0	9.94	10.2	14.9	16.2
r	0.9712	0.9957	0.9924	0.9905	0.9946	0.9852	0.9707	0.9914	0.9969	0.9846

3.2. Optimization of immunoreaction conditions

3.2.1. Influence of incubation conditions

Two incubation conditions, including 37 °C incubation for 45 min and room temperature incubation for 45 min, were investigated. The results showed that there was no obvious difference between the two incubation conditions. Although higher temperature could accelerate the diffusion process and increase the interaction probability, room temperature incubation for 45 min was selected finally because of its simple operation.

3.2.2. Influence of immunoreaction time

The effect of incubation time at room temperature was shown in Fig. 4. It was found that at each FT_4 concentration, the immunoreaction almost reached an equilibrium when the incubation time achieved 45 min. After that, little change of RLU was observed with longer incubation time. Therefore, 45 min was chosen as the incubation time to improve the efficiency of the assay.

3.2.3. Influence of chemiluminescence reaction conditions

The influence of chemiluminescence reaction time was investigated by recording RLU_{S0} in 5-min interval after adding the chemiluminescence substrate. It was observed that the platform of the chemiluminescence reaction could not be achieved even after 80 min reaction at room temperature. Therefore, the reaction system was first incubated at 37 °C for different time, then its chemiluminescence response was recorded. The results were shown in Fig. 5. As can be seen in Fig. 5, at each tested incubation time, RLUs increased in the time range of 0–30 min, and then reached a platform from 30 to 70 min. Among these, incubation time of 10 min showed higher RLUs and faster platform arrival. Therefore, after adding the substrate solution, incubation for 10 min at 37 °C was selected before detection.

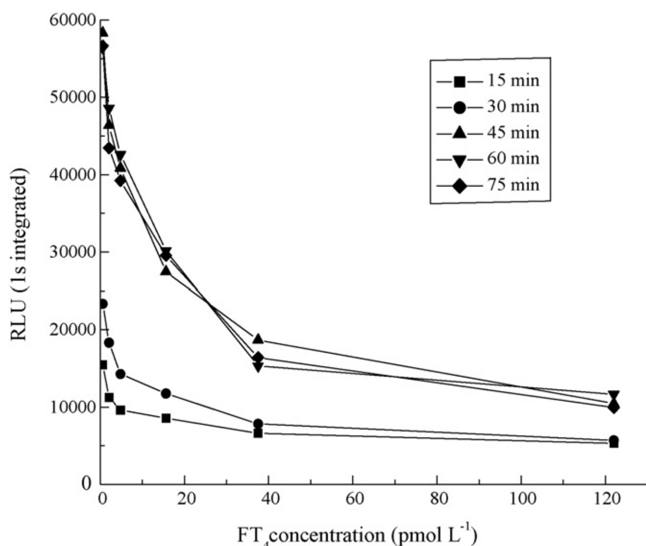

Fig. 4. Influence of immunoreaction time. The five curves correspond to a series of incubation time (i.e., 15, 30, 45, 60, and 75 min). Detection conditions: dilution ratio of HRP-T_4 conjugate: 1:1000; dilution ratio of FITC-labeled anti-FT_4 antibody: 1:4000; volume of MPs: 100 μL; volume of substrate: 300 μL; room temperature.

3.3. Methodology evaluation

3.3.1. Standard curve

Under the optimal reaction conditions, a standard curve of logit $Y = -2.374 \log X + 2.633$ ($r = -0.9967$) was obtained.

3.3.2. Detection limit

The detection limit was determined by calculating the minimum amount of FT_4 that could be markedly distinguished from S_0 (mean LOD = $S_0 - 2$S.D., 10 replicates). The detection limit was repeated five times, and calculated from the experimental results was 0.25 pmol L^{-1}.

3.3.3. Precision

Three different concentration of sera were measured ten times within one assay to obtain the intra-assay precision. Inter-assay precision was calculated by measuring the sera in three times assays. Good precisions were obtained. The intra- and inter-assay coefficients of variation (CV) varied from 2.3 to 14.6% (Table 3).

3.3.4. Specificity

To estimate the specificity of the anti-FT_4 antibody, some molecules, such as 3,5,3′-triiodothyronine (T_3) and 3,3′,5′-triiodothyronine (reverse T_3, rT_3), which have analogous structure to T_4, were tested. The structural formula of T_4, T_3, and rT_3 was shown in Fig. 6. The cross-reactivity (CR%) of anti-FT_4 anti-

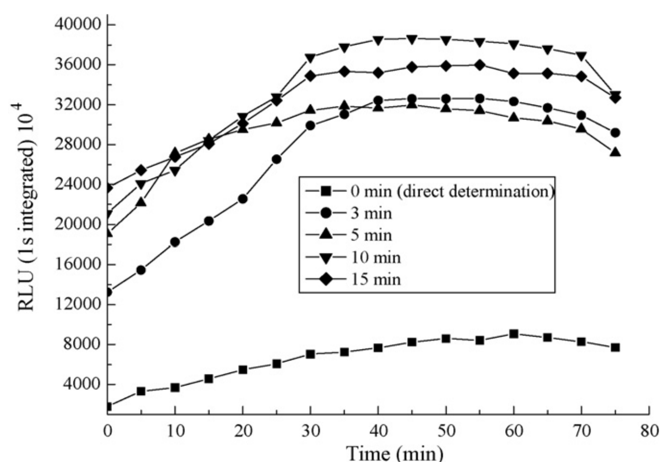

Fig. 5. The chemiluminescent kinetics of different reaciton time at 37 °C after adding the substrate solution. The five curves correspond to a series of different chemiluminescence reaction time, i.e., 0, 3, 5, 10, and 15 min. Detection conditions: dilution ratio of HRP-T_4 conjugate: 1:1000; dilution ratio of FITC-labeled anti-FT_4 antibody: 1:4000; volume of MPs: 100 μL; volume of substrate: 300 μL; room temperature incubation for 45 min.

Table 3
The precision of the proposed method.

Samples	Number	Intra-assay		Inter-assay	
		S.D. (pmol/L)	CV (%)	S.D. (pmol/L)	CV (%)
1	1	0.23	13.1	0.24	12.9
	2	0.27	14.6		
	3	0.21	11.3		
2	1	0.45	7.62	0.47	7.81
	2	0.52	8.60		
	3	0.47	7.90		
3	1	0.59	2.30	0.93	3.58
	2	0.72	2.70		
	3	0.78	3.10		

S.D.: standard deviation.

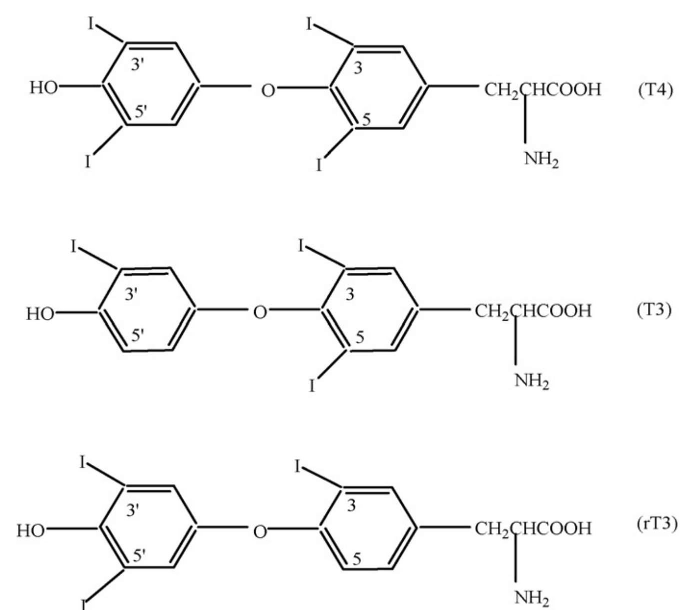

Fig. 6. Structural formula of T4, T3, and rT3.

Table 4
The specificity estimation of anti-FT_4 antibody.

Cross-reactant	Added cross-reactant ($pmol L^{-1}$)	Determined concentration ($pmol L^{-1}$)	Cross-reactivity (%)
rT_3	10	0.02	0.002
T_3	60	0.78	0.013

body was calculated by the formula as follows: cross-reactivity (%) = (concentration of FT_4 at IC_{50})/(concentration of cross-reactant at IC_{50}). IC_{50} means the concentration which causing 50% inhibition of RLU_{max}. Results in Table 4 showed that there was no cross-reactivity with T_3 and rT_3.

3.3.5. Stability

Calibrators, FITC-labeled anti-FT_4 antibody, HRP-T_4 conjugate, anti-FITC-labeled MPs, buffers, and chemiluminescence substrate were stored at 4 and 37 °C for 3 and 7 days, respectively. After that, they were used to perform the assay. As shown in Table 5, there was no obvious effect on the assay results.

Table 5
Stability of the reagents ($n = 3$).

Time (day)	Temperature (°C)	$RLU_{S1/S0}$	r	CV (%)
3	4	86.7%	0.9951	5.6
	37	85.3%	0.9976	6.2
7	4	86.3%	0.9924	5.1
	37	84.6%	0.9943	7.8

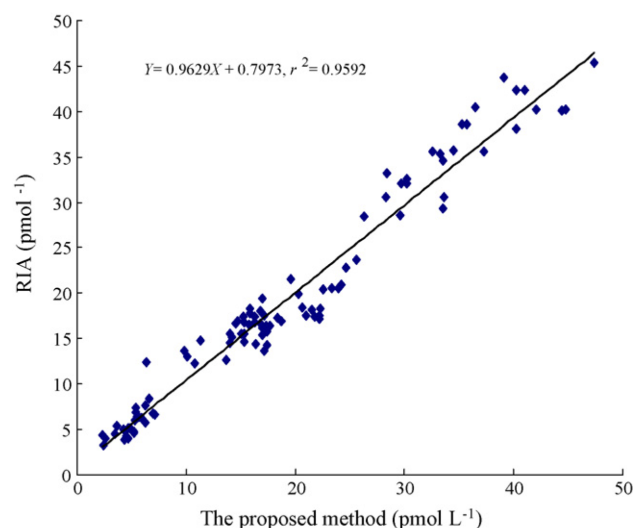

Fig. 7. Correlation between results measured by the proposed method and RIA.

3.4. Serum samples analysis

FT_4 in 100 serum samples, including 25 hyperthyroidism samples, 25 hypothyroidism samples and 50 normal samples were determined with the proposed method and the results obtained were compared with that from a commercial RIA kit [33]. A good correlation was obtained with a satisfied r^2 of 0.9592, as shown in Fig. 7. The positive ratios for 25 hyperthyroidism samples and 25 hypothyroidism samples were 100 and 92%, respectively. The normal serum samples were all in the normal range (8.6–26 $pmol L^{-1}$).

3.5. The comparison between the proposed method and other methods

In this paper, we compared the analytical parameters among RIA, ELISA, CLEIA, and the proposed method, which was shown in Table 6. From Table 6, we can conclude that although the total assay time of the present method is the same as that of micro-plate CLEIA, the present method also shows advantages in terms of wider linear range and lower detection limit.

Table 6
The comparison between the proposed method and other methods.

Methods	Linear range ($pmol L^{-1}$)	Detection limit ($pmol L^{-1}$) (LOD = $S_0 - 2S.D.$)	Limit of quantification ($pmol L^{-1}$)	Total assay time (min)
RIA [33]	0.30–93.0	0.58	2.26	>90
ELISA [34]	1.29–129	1.29	4.52	100
Micro-plate CLEIA [20]	5.80–96.8	1.16	4.87	60
MPs-CLEIA	1.59–122	0.25	1.01	60

4. Conclusions

Through optimizing the dosage of immunoreagents and the parameters of the competitive immunoreaction, a highly sensitive and specific MPs-CLEIA using luminol–H_2O_2–HRP system was applied to the determination of FT_4 in human serum. The results showed that the positive ratios for 25 hyperthyroidism samples and 25 hypothyroidism samples were 100 and 92%, respectively. The normal serum samples were all in the normal range. A good correlation was obtained with a satisfied r^2 of 0.9592. The proposed method exhibits good application potential in the analysis of FT_4 in the clinical practice.

Acknowledgements

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