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Chemiluminescent immunoassay of thyroxine enhanced by microchip electrophoresis

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Abstract

A homogenous chemiluminescent immunoassay of thyroxine (T₄) enhanced by microchip electrophoresis separation has been developed. The method deployed the competitive immunoreaction of T₄ and horseradish peroxidase (HRP)-labeled T₄ (HRP-T₄) with anti-T₄ mouse monoclonal antibody (Ab). HRP-T₄ and the HRP-T₄-Ab complex were separated and quantified by using microchip electrophoresis (MCE) with chemiluminescence (CL) detection. Highly sensitive CL detection was achieved by means of HRP-catalyzed luminol-H₂O₂ reaction. Due to the effective MCE separation, the CL analytical signal was less prone to sample matrix interference. Under the selected assay conditions, the MCE separation was accomplished within 60 sec. The linear range for T₄ were 5–250 nM with a detection limit of 2.2 nM (S/N = 3). The present method was successfully applied for the quantification of T₄ in human serum samples. It was demonstrated that the present MCE-CL enhanced competitive immunoassay was quick, sensitive, and highly selective. It may serve as a tool for clinical analysis of T₄ to assist diagnosis of thyroid gland functions.

Keywords

Thyroxine; Homogeneous competitive immunoassay; Microchip electrophoresis; Chemiluminescence detection; Thyroid gland function diagnosis

1 Introduction

Thyroxine (3,5,3',5'-tetraiodo-L-thyronine, T₄) is the primary active hormone synthesized within the follicular cells of the thyroid gland [1]. It affects metabolic activity in many tissues leading to an increased consumption of oxygen and stimulation of mitochondrial respiration. Measurement of serum T₄ level is commonly used for diagnosis of thyroid gland diseases, such as hypothyroid, hyperthyroid, thyroidectomy, and thyroiditis. Assays currently used in clinical practice include radioimmunoassay (RIA), chemiluminescent enzyme immunoassay,

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time-resolved fluorescence immunoassay (TRFIA), etc. [2]. They all involve immunoreactions of T4 with anti-T4 antibodies. However, due to differences in reagent specificity the concentration of free T4 in a given specimen determined with assays from different manufacturers can vary. In addition, heterophile antibody interference with T4 quantification that caused clinical confusions has been reported [3,4]. To improve the reliability of assay results, mass spectrometry –based analytical protocols have been recently developed [5,6].

Microchip electrophoresis (MCE), regarded as a miniaturized version of capillary electrophoresis (CE), has become a very attractive separation technique [7]. It offers many advantages such as miniaturized apparatus, extremely small sample size, high separation speed and efficiency, short analysis time, and ease of integration and automatization that make it unequally suitable for biological and clinical analysis. The technique has been successfully applied to separation of chemical species of biomedical interest including amino acids [8,9], biogenic amines [10], proteins [11,12], DNA [13,14], etc. Immunoassay is known as one of the most important and widely used analytical techniques in clinical diagnoses and biochemical studies. Performing immunoassays by means of microfluidic devices is currently gaining research interest. Incorporation of a microfluidic system in an immunoassay significantly simplifies the procedure and offers advantages including high separation and reaction efficiency, shortened assay time, and lower sample, reagent, and energy consumption. Over the past decade, MCE enhanced immunoassay of cortisol [15], theophylline [16], 2,4,6-thrinitrotoluene [17], rat IgG [18], insulin [19,20], and inflammatory cytokines [21] have been reported. However, in most of these works, laser induced fluorescence (LIF) detection was deployed for detecting the separated species. LIF detection requires relatively large and expensive apparatus systems. Chemiluminescence detection (CL) offers advantages such as simplicity in instrumentation, high sensitivity, and wide linear range, and is particularly suitable for integration on microfluidic device. Methods based on MCE with CL detection (MCE-CL) were developed for analysis of amino acids [22], biogenic amines [23], peptides [24], proteins [25], etc. MCE-CL enhanced immunoassay for the determination of rat IgG [26], and human serum albumin (HSA) and immunosuppressive acidic protein (IAP) [27] were also reported.

In this work, we report on the development of an MCE-CL enhanced homogeneous immunoassay of thyroxine. It's well known that horseradish peroxidase (HRP) catalyzes luminol- H_2O_2 CL reaction and greatly enhances the CL emission. Therefore, HRP-labeled T4 (HRP-T4) was selected as the competing reagent of T4 in the sample for anti-T4 antibody. Both HRP-T4 and the HRP-T4-Ab complex were sensitively detected by CL after MCE separation. The use of MCE separation might also improve the assay selectivity by isolating HRP-T4 from other potentially chemiluminescent species. The MCE-CL enhanced competitive immunoassay was preliminarily validated by quantifying T4 in serum samples taken from patients suffering from various thyroid diseases.

2 Materials and methods

2.1 Chemicals and reagents

Luminol was purchased from Fluka (Buchs, Switzerland). Para-iodophenol (PIP), hydrogen peroxide (H_2O_2), Na_3PO_4 , and sodium hydrogen carbonate (NaHCO_3) was from Taopu Chemicals (Shanghai, China). T4 assay kit (Lot 08280) which consisted of T4 standards containing 0, 5, 15, 50, 150, and 500 $\mu\text{g/L}$ controls (containing low and high concentrations of T4 in human serum), T4 enzyme conjugate (HRP-T4), and anti-T4 mouse monoclonal antibody was obtained from Diagnostics Systems Laboratories (Webster, TX, USA). All other chemicals were of analytical grade. Ultra pure water (18.2 M Ω) prepared from double-distilled water with Millipore Simplicity was used throughout the work. The electrophoresis buffer was 10 mM Na_3PO_4 (pH = 10.2) containing 1.2 mM luminol and 0.005% (w/v) Brij 35, and the

oxidizer solution was 35 mM NaHCO₃ (pH = 8.6) containing 100 mM H₂O₂ and 1.2 mM PIP. All solutions were filtered through 0.22 μm membrane filters prior to use.

2.2 MCE-CL system

The lab-built MCE-CL system was described previously [28]. Briefly, the microchip assembly was mounted on the X-Y translational stage of an inverted microscope (Olympus CKX41) that also served as a platform of CL detection. CL signal was collected by means of a microscope objective, and detected by a photomultiplier (PMT, Hamamatsu R105). Signals from the PMT was recorded and processed with a computer using a Chromatography Data System (Zhejiang University Star Information Technology, Hangzhou, China). A multi-terminal high voltage power supply, variable in the range of 0–8000 V (Shandong Normal University, Jinan, China), was used for sample injection and MCE separation. The inverted microscope was placed in a black box.

The fabrication of the glass / PDMS microchip was described previously [23,24]. Its schematic layout is illustrated in Fig. 1. The width of all microchannels except oxidizer introduction channel (250 μm) is 70 μm, the depth of all microchannels is 25 μm, and the length of double T is 60 μm. All reservoirs were 4.0 mm in diameter and 1.5 mm deep. The channel between reservoir S and SW was used for sampling, the channel between B and BW was used for the separation and the channel between R and BW was used for the oxidizer introduction.

2.3 Pretreatment of human serum samples

Human serum samples were kindly provided by The No. 5 People's Hospital, Guilin, China. To 500 μL of a serum sample, 0.5 mL of a sulfosalicylic acid solution (5 mg/mL) was added. The mixture was vortexed and left stand for 5 min at room temperature to release free T4 from protein conjugated T4 [29-30]. The solution was centrifuged (12000 × g for 10 min). The supernatant was transferred into a centrifuge tube and diluted to 2 mL. pH of the solution was adjusted to around 7.4. The obtained solution was kept at -20°C before analysis.

2.4 Immunoreaction

To carry out the immunoreaction, 20 μL of T4 standards or serum samples was mixed with 20 μL of 6.0×10^{-7} M HRP-T4 and 20 μL of 4.0×10^{-7} M mouse anti-T4 monoclonal antibody in a 0.5-mL microcentrifuge tube. The solution was incubated for 15 min at 37°C before MCE-CL run.

2.5 MCE-CL operation

All the microchannels on the microchip were sequentially washed with 0.1M NaOH, water, and electrophoresis buffer for 1 min each before each run. The microchannels were filled with electrophoresis buffer. The sample was transferred into reservoir S. Reservoir SW, B and BW were filled with electrophoresis buffer, and reservoir R with the oxidizer solution (H₂O₂). Platinum wires as electrodes were inserted into these reservoirs. Sample injection was performed by applying 800V to the sample reservoir for 15 s with the sample waste reservoir grounded, while B was set at 250 V, BW at 500 V, and R was left floating. To carry out MCE separation, 2800V was applied to B, 2500V to both S and SW with BW grounded. At the same time, 550 V was applied to R. The analyte was transported into the separation channel toward BW, and then mixed with the oxidizer solution at the junction of oxidizer introduction channel and separation channel producing CL emission which was collected through a microscope objective and then detected by a photomultiplier.

3 Results and discussion

3.1 Optimization of MCE-CL conditions

Effective separation of Ag^* and $\text{Ag}^*\text{-Ab}$ is a key step for success in all competitive immunoassays. In this work, MCE was used to accomplish the separation. However, it was found that adsorption of proteins by the glass channel surface deteriorated significantly the separation of HRP-T4 from the HRP-T4-Ab complex. To overcome this difficulty, a surface active agent, Brij 35, was added to the electrophoresis buffer. The effect of Brij 35 concentration in a range of 0.002% to 0.006% (w/v) was investigated. The results are shown in Fig. 2. It can be seen that the resolution of HRP-T4 and the HRP-T4-Ab complex increased with increasing Brij 35 concentration. However, at high Brij 35 concentrations the migration times of both HRP-T4 and HRP-T4-Ab were increased significantly. A concentration of 0.005% Brij 35 was selected for an acceptable resolution and a favorable separation time. Other MCE separation conditions such as buffer pH and separation voltage were also studied. pH of the electrophoresis buffer was tested in a range from pH 9.0 to 10.5. HRP-T4 and HRP-T4-Ab were best resolved at 10.2. The separation voltage directly affected the migration rate and peak shape of analytes. After a careful study, 2800 V was selected to achieve a good separation within a short separation time (< 60 sec.).

Like in all conventional chemiluminescent immunoassays, enhancing the sensitivity of CL detection improves assay sensitivity. In order to maximize the sensitivity of CL detection following MCE separation, the conditions such as concentrations of luminol, H_2O_2 , PIP, and the pH of the oxidizer solution were optimized. In this MCE-CL system, HRP-T4 was used as the competitive reagent and, therefore, it was used as test compound in the optimization studies. As shown in Fig. 3, the CL intensity from HRP-T4 rapidly increased with increase in the pH of the oxidizer solution, reaching the maximum at pH 8.6 and then dramatically decreased. It was found that the concentrations of H_2O_2 and luminol also significantly affected the CL intensity. The optimal concentrations were 1.2 mM for luminol and 100 mM for H_2O_2 , respectively. In order to further improve the detection sensitivity, PIP was added as a CL enhancer [31]. Effects of PIP concentration on the CL intensity is illustrated in Fig.4. The optimum PIP concentration was 1.2 mM. In the presence of PIP, CL intensity increased about eight times.

3.2 Competitive immunoassay for T4 by MCE-CL

In this work, a competitive format was adopted to determine free T4. The format can be described as following:

where Ab is anti-T4 antibody added at a limited amount, Ag^* is HRP-T4 added at a fixed amount, and Ag is free T4. HRP-T4 competes with free T4 in the sample for binding to a limited amount of anti-T4 antibody. The concentration of T4 in the sample is directly proportional to that of free HRP-T4, but inversely proportional to that of the HRP-T4-Ab complex. Thus, the concentration of T4 in the sample can be determined by measuring the CL signal from HRP-T4 after being separated from the HRP-T4-Ab complex.

The incubation time for the immunoreaction was studied. The HRP-T4 and Ab mixture was incubated for 4 to 20 min and then was injected into the MCE-CL system to determine the optimal incubation time when the maximum binding yield was obtained. The results are summarized in Fig 5. As can be seen, the CL signal from the HRP-T4-Ab complex increased rapidly with increasing the incubation time till 15 min at 37°C, and then remained constant till 20 min. These results indicated that the binding of HRP-T4 to anti-T4 antibody reached an

equilibrium at 15 min., Therefore, an incubation time of 15 min was selected for further experiments.

Fig. 6 shows typical electropherograms of the competitive immunoassay of T4. Trace “a” was obtained from a solution containing HRP-T4 only. Trace “b” was obtained from a solution containing HRP-T4 and anti-T4 antibody. A new peak at a longer migration time was observed that was from the HRP-T4-Ab complex. Trace “c” was obtained from a solution containing T4, HRP-T4, and anti-T4 antibody. Compared with those in Trace “b”, the CL signal (peak height) from HRP-T4 increased and that from HRP-T4-Ab complex decreased. These results indicated the competitive binding of T4 to the antibody. It is worth noting that the HRP-T4-Ab complex was well separated from free HRP-T4, which made the present assay very selective and useful for clinical sample analysis.

An advantage of the present assay is the short analysis time of ~20 min. Since the Ag+Ag*+Ab incubation is carried out in a homogenous solution in this assay, the binding equilibrium can be reached within 15 min. However, an incubation time of 45 min or longer is normally required in the widely used heterogeneous immunoassays based on Ab-coated micro-plates [2] or magnetic particles [32] due to the limited interaction among the analyte, the reagents and the immobilized antibody. Further, the MCE separation of Ag*-Ab from Ag* in this assay is very quick (< 1 min), which represents a distinct advantage of MCE over other separation techniques such as HPLC and capillary electrophoresis (CE). In a study previously reported, CE was deployed in a homogeneous immunoassay of T4. The CE separation time used was 20 min [33].

3.3 Analytical figures of merit

The method was evaluated in terms of response linearity, limit of detection, and reproducibility. The CL signal (peak height) from free HRP-T4 was used for quantification of T4. Under the optimized conditions, seven standard T4 solutions at various concentrations from 5 to 250 nM T4 were analyzed. Linear regression analysis of the results yielded the following equations:

$$H=6.898C+0.624 \quad r^2=0.9927$$

Where H is the relative CL intensity (μV) from HRP-T4, and C is the concentration of T4 (nM). The calibration curves showed excellent linearity with a correlation coefficient of 0.993. Based on signal /noise ratio [S/N] = 3, the detection limit for T4 were estimated to be 2.2 nM. Assay reproducibility was investigated by analyzing a standard solution 11 times. The results showed that RSDs of peak heights and migration times were 3.1% and 4.8% for HRP-T4, and 3.2% and 4.1% for HRP-T4-Ab, respectively.

3.4 Quantification of T4 in human serum

Human serum samples taken from 5 healthy volunteers (euthyroid) and 10 patients suffering from different thyroid diseases were analyzed to demonstrate the feasibility of the present MCE-CL enhanced immunoassay. Electropherograms obtained from analyzing three serum samples are shown in Fig. 7. As can be seen, HRP-T4 peak heights varied from sample to sample indicating the variation of free T4 content in these samples. It's also worth noting that the peak heights from HRP-T4 and the HRP-T4-Ab complex changed accordingly, that is when HRP-T4 peak became higher the HRP-T4-Ab complex peak appeared lower. This was the expected result of a competitive immunoreaction. From our analysis, the serum level of T4 in healthy subjects was in the range from 8.2×10^{-8} to 15.3×10^{-8} M. This result was consistent with that reported in literature ($6.4 - 15.4 \times 10^{-8}$ M) [34]. Serum T4 results measured in this work for healthy subjects and thyroid patients are shown in Table 1. Serum samples taken from

patients having hypothyroid, hyperthyroid, thyroidectomy, goitre, and thyroiditis were analyzed. The highest level of T4 was detected for the thyroidectomy patients at 3.56×10^{-7} M ($n = 1$), and the lowest level was for the hypothyroid group with an average of 4.33×10^{-8} M ($n = 3$). To evaluate the assay reliability, recovery of T4 from the serum sample matrix was determined. The results are summarized also in Table 1. Recovery was from 94.0 to 105.0% with RSD less than 4.8% ($n = 7$) for all the samples analyzed.

4 Concluding remarks

In this work, an MCE-CL enhanced immunoassay was developed for quantifying T4 in serum samples. The immunoreaction involved was based on the competitive format, where free T4 competed with HRP-labeled T4 for binding anti-T4 antibody. After MCE separation, both HRP-T4 and the HRP-T4-Ab complex were sensitively detected by measuring the CL emission from HRP catalyzed luminol- H_2O_2 reaction. Due to the high emission efficiency of the CL reaction, the sensitivity of the assay was very high with a detection limit of 2.2 nM T4 ($S/N=3$). More importantly, separating HRP-T4 from other potentially chemiluminescent species by MCE made the CL analytical signal much less prone to the influence from the sample matrix, thus likely improving the assay selectivity. The present MCE-CL enhanced immunoassay was preliminarily validated by analyzing human serum samples taken from both healthy volunteers and patients suffering from different thyroid diseases. The serum level of T4 in healthy subjects was $1.22 \pm 0.27 \times 10^{-7}$ M ($n = 5$) while T4 level was found to be 3.56×10^{-7} M in thyroidectomy patients ($n = 1$) and 4.33×10^{-8} M ($n = 3$) in hypothyroid patients, respectively. As demonstrated in this work, combining commercially available immunoassay kits containing an HRP-labeled antigen and an antibody with an MCE-CL platform promises to develop immunoassays offering not only improved sensitivity and selectivity, but also advantages such as simplified procedure, shortened analysis time, and very low consumption of expensive reagents for clinical analysis.

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Abbreviations used

MCE	microchip electrophoresis
CL	chemiluminescence
HRP	horseradish peroxidase
PIP	para-iodophenol
T4	thyroxine
Ab	antibody

Fig. 1. Layout and dimension of the glass /PDMS hybrid microchip. S: sample reservoir; B: buffer reservoir; SW: sample waste reservoir; BW: buffer waste reservoir; R: oxidizer solution reservoir.

Fig. 2.

Effects of Brij 35 concentration on resolving HRP-T4 and HRP-T4-Ab complex.

Electrophoresis buffer was 10 mM phosphate buffer (pH = 10.2) containing Brij 35 at varying concentrations. The oxidizer solution was 35 mM NaHCO_3 (pH = 8.6) containing 100 mM H_2O_2 and 1.2 mM PIP.

Fig. 3. pH Effects of the oxidizer solution on CL intensity. Electrophoresis buffer was 10 phosphate buffer (pH = 10.2) containing 0.005% (w/v) Brij 35. The oxidizer solution was 35 mM NaHCO₃ containing 100 mM H₂O₂ and 1.2 mM PIP at varying pH values. The concentration of HRP-T4 was 3.0×10^{-7} M.

Fig. 4. Effects of PIP concentration on CL intensity. The oxidizer solution was 35 mM NaHCO_3 (pH = 8.6) containing 100 mM H_2O_2 and PIP at different concentrations. Other conditions were as in Fig. 3.

Fig. 5. Incubation time and the immunoreaction yield. A mixture prepared from 20 μL of 6.0×10^{-7} M HRP-thyroxine and 20 μL of 4.0×10^{-7} M mouse anti-T4 monoclonal antibody was incubated at 37°C and analyzed at different time intervals from 4 min to 20 min. Electrophoresis buffer was 10 mM phosphate buffer (pH = 10.2) containing 0.055% Brij 35. The oxidizer solution was 35 mM NaHCO_3 (pH = 8.6) containing 100 mM H_2O_2 and 1.2 mM PIP. See text (Section 2.5) for other conditions.

Fig. 6. Electropherograms from separating the competitive immunoreaction solutions: (a) 6.0×10^{-7} M HRP-T4 solution; (b) a mixture of 6.0×10^{-7} M HRP-T4 and 3.0×10^{-7} M anti-T4 antibody; and (c) a mixture of 8.0×10^{-8} M T4, 6.0×10^{-7} M HRP-T4, and 4.0×10^{-7} M anti-T4 antibody. MCE-CL conditions were as in Fig. 5.

Fig. 7. Electropherograms from analyzing three human serum samples: (a) from a healthy subject; (b) from a thyroidectomy patient; and (c) from a hypothyroid patient. MCE-CL conditions were as in Fig. 5.

Table 1

Analytical results of T4 content in healthy and patient serum samples

Thyroid condition	T4 found (10^{-8} M)	RSD (% n=7)	Added (10^{-8} M)	Total found (10^{-8} M)	Recovery (%)
Euthyroid	11.5	3.4	10.0	21.8	103.1
Euthyroid	8.2	2.3	10.0	17.8	96.4
Euthyroid	15.3	2.7	10.0	25.3	100.2
Euthyroid	13.9	3.6	10.0	23.7	98.1
Euthyroid	12.3	1.9	10.0	22.8	105.0
Hypothyroid	3.8	4.8	5.0	8.5	94.0
Hypothyroid	4.2	3.1	5.0	9.1	98.0
Hypothyroid	5.0	2.2	5.0	9.7	94.0
Hyperthyroid	20.1	3.7	20.0	39.0	94.5
Hyperthyroid	18.8	3.5	20.0	38.1	96.6
Thyroidectomy	35.6	2.5	40.0	74.7	97.6
Goitre	14.6	2.0	20.0	33.8	96.0
Goitre	13.5	3.9	20.0	23.8	102.8
Thyroiditis	9.4	2.4	10.0	19.1	97.0
Thyroiditis	11.2	3.0	10.0	21.1	99.0