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A homogeneous immunoassay of thyroxine based on microchip electrophoresis and chemiluminescence detection

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Summary

A homogenous chemiluminescent immunoassay of thyroxine (T4) present in serum samples is described. The proposed 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. The MCE separation was accomplished within 60 sec. Highly sensitive CL detection was achieved by means of HRP-catalyzed luminol-H₂O₂ reaction. The linear range for T4 were 5 –250 nM with a detection limit of 2.2 nM (S/N = 3).

Keywords

Thyroxine; Immunoassay; Microchip electrophoresis; Chemiluminescence detection

1. Introduction

Measurement of serum thyroxine 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, time-resolved fluorescence immunoassay (TRFIA), etc. (1). They all involve immunoreactions of thyroxine with anti- thyroxine antibodies. However, due to differences in reagent specificity the concentration of free thyroxine in a given specimen determined with assays from different manufacturers can vary. In addition, heterophile antibody interference with thyroxine quantification that caused clinical confusions has been reported (2,3). To improve the reliability of assay results, mass spectrometry –based analytical protocols have been recently developed (4,5). Microchip electrophoresis (MCE), regarded as a miniaturized version of capillary electrophoresis (CE), has become a very attractive separation technique (6). 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. 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 (7), theophylline (8), 2,4,6-trinitrotoluene (9), rat IgG (10), insulin (11,12), and inflammatory cytokines (13) have been reported. However, in most of these works, laser induced fluorescence (LIF) detection was deployed for detecting the separated species. Although LIF detection is highly sensitive, it requires pre-column tagging of the analytes with a fluorophore in many cases, and relatively large and expensive apparatus systems. Chemiluminescence (CL) detection offers advantages such as simplicity in instrumentation, high sensitivity, and wide linear range, and is particularly suitable for integration on microfluidic device. Here, we demonstrate an MCE-CL immunoassay of thyroxine present in biological sample. Human serum is analyzed as the proof-of-concept samples. Thyroxine is detected at 10^{-7} M. Compared with conventional immunoassay methods, the present MCE-CL method offer 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.

2. Materials

1. Prepare all solutions using Milli-Q ultrapure water (18 M Ω cm at 25°C) and analytical grade reagents.
2. All solutions used in MCE are filtered through a 0.22 μ m nylon membrane (see Note 1).
3. MCE running buffer: 10 mM Na_3PO_4 (pH 10.2) containing 1.2 mM luminol and 0.005% (w/v) Brij 35.
4. CL reaction buffer: 35 mM NaHCO_3 (pH 8.6) containing 100 mM H_2O_2 and 1.2 mM PIP.
5. Human serum sample: To 500 μ L 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 thyroxine from protein conjugated thyroxine. The solution was centrifuged ($12000 \times g$ 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.
6. Microchip: The design of the glass / PDMS microchip (95 mm $\times$ 25 mm) is illustrated in Figure 1. The glass layer with microchannels is fabricated by using a standard photolithography and wet chemical etching technique (14). The PDMS surface for bonding to the etched glass slide is prepared from Sylgard 184 (PDMS) silicone elastomer mixed with its curing agent at 10:1 (w/w).
7. *Set-up for MCE-CL assay*: Figure 2 illustrates the set-up. The microchip is mounted on the *X-Y* translational stage of an inverted microscope that also serves as a platform of CL detection. CL signal was collected by means of a microscope objective, and detected by a photomultiplier (PMT, Hamamatsu R105). The PMT is mounted in an integrated detection module including HV power supply, voltage divider, and amplifier. The output signal of PMT is recorded and processed with a computer. A multi-terminal high voltage power supply, variable in the range of 0–8000 V is used for sample loading and MCE separation. The inverted microscope is placed in a black box.

¹Filtering all solutions before use in MCE is very important since channels in the microchip are very small in size and they are easily blocked by minute particles in solutions.

3. Method

3.1. Immunoreaction

Twenty microlitre of thyroxine 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.

3.2. Calibration of the MCE-CL system

1. Rinse the microchannels sequentially with 0.1 M NaOH, water, and MCE running buffer for 2 min each. (*see Note 2*)
2. Fill reservoirs B, S, and SW with the MCE running buffer.
3. Fill reservoir R with the CL reaction buffer.
4. Apply vacuum to reservoir BW. (*see Note 3*)
5. Replace the MCE running buffer solution in reservoir S with a thyroxine standard solution.
6. Apply a set of electrical potentials to reservoirs as following: reservoir S at 800 V, reservoir B at 250 V, reservoir BW at 500 V, reservoir SW at grounded, and reservoir R at 0 V to inject the sample. Duration: 15 seconds. (*see Note 4*)
7. Change the potentials applied as following: reservoir B at 2800 V, reservoir S at 2500 V, reservoir SW at 2500 V, reservoir R at 550 V and reservoir BW at ground. At the same time, start to record the MCE-CL electropherogram (as shown in Figure 3a). Duration: 1.5 min.
8. Plot peak height from free HRP-T4 against thyroxine concentrations to obtain a calibration curve and the equation from linear regression for thyroxine.

3.3. Quantification of thyroxine in human serum

1. Do steps 1 ~ 4 described in 3.2.
2. Replace the MCE running buffer solution in reservoir S with a sample solution.
3. Do steps 6~7 described in 3.2.
4. Record the MCE-CL electropherogram (as shown in Figure 3b and 3c), and determine the concentration from the peak height from free HRP-T4 using the calibration equation obtained above for thyroxine.

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²Rinse the microchannels with 1 M NaOH for 30 min for the first use of the microchip.

³Apply a vacuum to reservoir BW to fill the channels with the electrophoresis running buffer. Check to make sure there are no air bubbles in the channels.

⁴Under the potential conditions applied, the thyroxine standard solution is transported from reservoir S to SW. That is, the sampling channel that involves a small section of the separation channel is filled with the thyroxine standard solution.

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Figure 1. Layout and dimensions of the glass /PDMS microfluidic chip used in this work. S: Sample reservoir; B: buffer reservoir; SW: sample waste reservoir; BW: buffer waste reservoir; and R: oxidizer solution reservoir.

Figure 2.
Schematic of the integrated MCE-CL system for single cell analysis.

Figure 3. Electropherograms from analyzing three human serum samples: (a) from a healthy subject; (b) from a thyroidectomy patient; and (c) from a hypothyroid patient.