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Chemiluminescent immunoassay of thyroxine enhanced by microchip electrophoresis

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ABSTRACT

A homogeneous 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 HPR-catalyzed luminol–H_2O_2 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 s. The linear range for T4 was 5–250 nM with a detection limit of 2.2 nM (signal/noise ratio = 3). The current method was successfully applied for the quantification of T4 in human serum samples. It was demonstrated that the current MCE–CL-enhanced competitive immunoassay was quick, sensitive, and highly selective. It may serve as a tool for clinical analysis of T4 to assist in the diagnosis of thyroid gland functions.

Thyroxine (T4,[1] 3,5,3′,5′-tetraiodo-L-thyronine) is the primary active hormone synthesized within the follicular cells of the thyroid gland [1]. It affects metabolic activity in many tissues, leading to increased consumption of oxygen and stimulation of mitochondrial respiration. Measurement of the 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, and time-resolved fluorescence immunoassay (TRFIA) [2]. All of them involve immunoreactions of T4 with anti-T4 antibodies (Abs). 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 Ab 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 developed recently [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], and DNA [13,14]. 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 immunoglobulin G (IgG) [18], insulin [19,20], and inflammatory cytokines [21] has been reported. However, in most of these works, laser-induced fluorescence (LIF) detection was deployed for detecting the separated

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[1] *Abbreviations used:* T4, thyroxine; RIA, radioimmunoassay; TRFIA, time-resolved fluorescence immunoassay; Ab, antibody; MCE, microchip electrophoresis; CE, capillary electrophoresis; IgG, immunoglobulin G; LIF, laser-induced fluorescence; CL, chemiluminescence; MCE–CL, MCE with CL detection; HSA, human serum albumin; IAP, immunosuppressive acidic protein; HRP, horseradish peroxidase; HRP–T4, HRP-labeled T4; PIP, para-iodophenol; H_2O_2, hydrogen peroxide; $NaHCO_3$, sodium hydrogen carbonate; PMT, photomultiplier tube; PDMS, polydimethylsiloxane; S, sample reservoir; SW, sample waste reservoir; B, buffer reservoir; BW, buffer waste reservoir; R, oxidizer solution reservoir; HPLC, high-performance liquid chromatography; S/N, signal/noise ratio; RSD, relative standard deviation.

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species. LIF detection requires 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 devices. Methods based on MCE with CL detection (MCE–CL) have been developed for analysis of amino acids [22], biogenic amines [23], peptides [24], proteins [25], and the like. MCE–CL-enhanced immunoassay for the determination of rat IgG [26], as well as human serum albumin (HSA) and immunosuppressive acidic protein (IAP) [27], has also been reported.

In this work, we report on the development of an MCE–CL-enhanced homogeneous immunoassay of T4. It is 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 Ab. 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.

Materials and methods

Chemicals and reagents

Luminol was purchased from Fluka (Buchs, Switzerland). Paraiodophenol (PIP), hydrogen peroxide (H_2O_2), Na_3PO_4, and sodium hydrogen carbonate ($NaHCO_3$) were obtained from Taopu Chemicals (Shanghai, China). A T4 assay kit (lot 08280), which consisted of T4 standards containing 0, 5, 15, 50, 150, and 500 μg/L T4 in human serum, T4 controls (containing low and high concentrations of T4 in human serum), T4 enzyme conjugate (HRP–T4), and anti-T4 mouse monoclonal Ab, was obtained from Diagnostics Systems Laboratories (Webster, TX, USA). All other chemicals were of analytical grade. Ultrapure water (18.2 MΩ) 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 $NaHCO_3$ (pH 8.6) containing 100 mM H_2O_2 and 1.2 mM PIP. All solutions were filtered through 0.22-μm membrane filters prior to use.

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 for CL detection. CL signal was collected by means of a microscope objective and detected by a photomultiplier tube (PMT, Hamamatsu R105). Signals from the PMT were recorded and processed with a computer using a Chromatography Data System (Zhejiang University Star Information Technology, Hangzhou, China). A multiterminal 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/polydimethylsiloxane (PDMS) microchip was described previously [23,24]. Its schematic layout is illustrated in Fig. 1. The width of all microchannels except the oxidizer introduction channel (250 μm) was 70 μm, the depth of all microchannels was 25 μm, and the length of double T was 60 μm. All reservoirs were 4.0 mm in diameter and 1.5 mm deep. The channel between the sample reservoir (S) and the sample waste reservoir (SW) was used for sampling, the channel between the buffer reservoir (B) and the buffer waste reservoir (BW) was used for separation, and the channel between the oxidizer solution reservoir (R) and BW was used for oxidizer introduction.

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 votexed and left to stand for 5 min at room temperature to release free T4 from protein-conjugated T4 [29,30]. The solution was centrifuged (12,000*g* for 10 min). The supernatant was transferred into a centrifuge tube and diluted to 2 ml. The pH of the solution was adjusted to approximately 7.4. The obtained solution was kept at −20 °C before analysis.

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 Ab in a 0.5-ml microcentrifuge tube. The solution was incubated for 15 min at 37 °C before an MCE–CL run.

MCE–CL operation

All of the microchannels on the microchip were sequentially washed with 0.1 M NaOH, water, and electrophoresis buffer for 1 min each before each run. The microchannels were filled with electrophoresis. The sample was transferred into S. SW, B, and

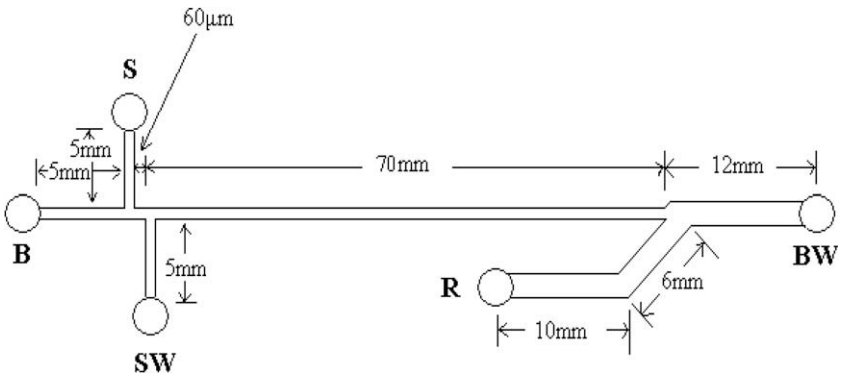

Fig. 1. Layout and dimension of the glass/PDMS hybrid microchip. S, sample reservoir; B, buffer reservoir; SW, sample waste reservoir; BW, buffer waste reservoir; R, oxidizer solution reservoir.