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Enzyme-free chemiluminescence immunoassay for the determination of thyroid stimulating hormone

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ABSTRACT

Based on the chemiluminescent resonance energy transfer (CRET) from high-energy intermediate formed in 1,1′-oxalyldiimidazole chemiluminescence (ODI-CL) reaction to fluorescent microsphere-conjugated detection antibody, a cost-effective and highly sensitive enzyme-free sandwich immunoassay with chemiluminescence detection was developed for the early diagnosis of thyroid cancer. Thyroid stimulating hormone (TSH) primary antibody immobilized on paramagnetic bead captures rapidly TSH in human serum within 15 min. Also, TSH detection antibody-conjugated yellow-green microsphere, capable of emitting green light, rapidly interacted with TSH-bound TSH primary antibody immobilized on paramagnetic bead during the 30-min incubation at room temperature. The sandwich complexes immobilized on paramagnetic in PBS buffer (pH 7.4) emit bright green light with the addition of ODI-CL reagents (e.g., H_2O_2, ODI). The dynamic range of enzyme-free sandwich immunoassay was 0.037–18 μIU ml^{-1} with 3–5% coefficient of variation. The limit of detection (LOD=background+3σ) of the enzyme free sandwich immunoassay was as low as 0.011 μIU ml^{-1}. We expect that the enzyme free sandwich immunoassay with ODI-CL detection can be a new assay method for the early diagnosis of thyroid cancer with statistically acceptable accuracy, precision, and reproducibility.

1. Introduction

Thyroid stimulating hormone (TSH) in human serum is commonly used as a biomarker applied for the early diagnosis of thyroid cancer [1,2]. The normal TSH level (0.4–4.2 μIU ml^{-1}) in adults (http://www.webmd.com/women/thyroid-stimulating-hormone-tsh) is so low that it is difficult to develop a biosensor capable of accurately, precisely, and reproducibly quantifying and rapidly monitoring TSH in human serum. Furthermore, the level of TSH monitored with the appearance of a pituitary gland tumor is much higher than the normal TSH level [3]. Thus, the requirement of biosensor for the early diagnosis of thyroid is that the dynamic range of biosensor should be wide to rapidly determine TSH levels without diluting human serum of patients.

In order to quantify trace levels of TSH in human serum, sandwich immunoassays using capture and detection antibody conjugated with an appropriate enzyme such as alkaline phosphatase (ALP) and horseradish peroxidase (HRP) have been developed [4–6]. Also, colorimetry [7], chemiluminescence [5,6,8], and fluorescence [9,10] were applied as optical sensors to detect signal generated from the sandwich enzyme immunoassay. Many research groups reported that the sandwich enzyme immunoassay with chemiluminescence detection is more sensitive than those with absorbance and fluorescence detections operated with a light source such as halogen, Xenon, and laser [11–15]. Currently, 1,1′-oxalyldiimidazole (ODI), luminol, and 1,2-dioxetane chemiluminescence detection systems are widely used to develop a highly sensitive sandwich enzyme immunoassay [15–18]. Recently, it was reported that ODI chemiluminescence detection is more sensitive than luminol and 1,2-dioxetane chemiluminescence detection [15]. Also, the dynamic range of the former was wider than those of the latter [15,19].

ODI chemiluminescence (ODI-CL) detection is applied in sandwich immunoassays using ALP as well as HRP [19]. This is because bright chemiluminescence is generated from the energy transfer between a high-energy intermediate, formed from the reaction of ODI and H_2O_2, and fluorescent dye, formed from the reaction of enzyme (e.g., ALP, HRP) and substrate (e.g., fluorescein diphosphate, Amplex Red). This report [19] indicates that a detection antibody conjugated with fluorescent dye, instead of ALP or HRP, can be applied to develop a rapid and simple enzyme-free immunoassay.

Currently, flow cytometry system using a specific detection antibody conjugated with fluorescent microspheres is popularly applied to diagnose various human diseases [20–22]. This is because fluorescence

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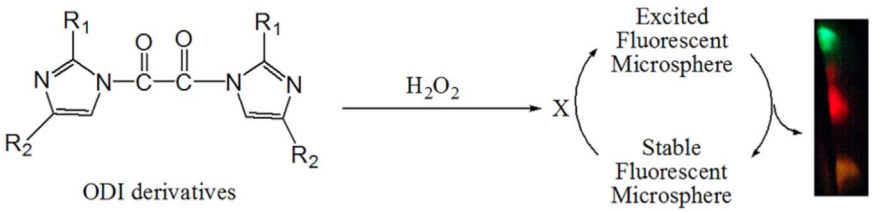

Scheme 1. Chemiluminescent resonance energy transfer (CRET) between fluorescent microsphere and high-energy intermediate formed in ODI-CL reaction. X: high-energy intermediate formed from the reaction of ODI and H_2O_2. If R_1 is H R_2 is H or CH_3. R_2 is H R_1 is H or CH_3.

quantum efficiency of fluorescent microsphere is much higher than that of individual fluorescent dye. These reports imply that fluorescent microspheres can be applied as an emitter in ODI-CL detection system. Scheme 1 shows that fluorescent microsphere can emit bright light due to the chemiluminesent resonance energy transfer (CRET) between fluorescent microsphere and high-energy intermediate formed from the reaction of ODI and H_2O_2. Furthermore, the efficiency and color of light emitted in ODI-CL reaction will be dependent on the chemical and physical properties of fluorescent microspheres excited by high-energy intermediate formed from reaction if ODI and H_2O_2 [23–26].

Using the advantages of fluorescent microspheres and ODI-CL detection, we developed for the first time an innovative enzyme-free chemiluminescent immunoassay for the early diagnosis of thyroid cancer with the rapid quantification of TSH in human serum. Details are described in this report.

2. Experimental

2.1. Chemicals and materials

TSH enzyme immunoassay kit containing TSH standards, capture antibody coated on paramagnetic bead and detection antibody conjugated with ALP, monoclonal TSH capture antibody (BIS6-P6), and monoclonal TSH detection antibody (MIX. 11–071008) were purchased from Immunometrics (UK), Ltd. TSH (> 95%), follicle stimulating hormone (FSH, > 95%,), luteinizing hormone (LH, > 98%), and human chorionic gonadotropin (hcG, > 96%) were purchased from Lee Biosolutions. Bis(2,4,6-trichlorophenyl)oxalate (TCPO) and 4-methylimidazole (4MImH) were purchased from TCI America. Three different types of carboxylate-modified fluorescent (yellow-green, orange, and red) microspheres (2 μm, 2.5%), bovine serum albumin (BSA) were purchased from Sigma-Aldrich. N-hydroxysuccinimide (NHS), 2-(4-morpholino) ethanesulfonic acid (MES, 0.5 M, pH 5.5) buffer solution, and fluorescein 5(6)-isothiocyanate (FITC) were purchased from Alfa Aesar. Streptavidin-FITC (1 mg/ml) was purchased form Protein MODS. Carboxyl-modified paramagnetic bead (2.5%) was purchased from Spheror, Inc. 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) was purchased from Fluka. Deionized water (HPLC grade), ethyl acetate (HPLC grade), isopropyl alcohol (HPLC grade), and 10×PBS (pH 7.4) were purchased from VWR. Meritus Health (Washington County Hospital) located in Hagerstown, MD, USA, provided the patient serum samples.

2.2. Conjugation of fluorescent microsphere and TSH detection antibody

Carboxylate-modified yellow-green microsphere (100 μl) was centrifuged for 10 min at 2000 rpm. After the centrifugation, supernatant in the 1.5-ml centrifuge tube were removed. Then 0.05 M MES buffer (pH 5.5, 200 μl) was added in the centrifuge. Carboxylate-modified yellow-green microspheres in the centrifuge tube was dispersed with a Vortex Genie 2 (Fisher Scientific, Inc.). 200 mg/ml EDC (20 μl) and 100 mg/ml NHS (20 μl) were added in the centrifuge tube. In order to activate carboxyl group of yellow-green microsphere, the mixture in the centrifuge tube was incubated for 30 min at room temperature (21 ± 2 °C). After the incubation, monoclonal TSH detection antibody (200 μg) was added in the centrifuge tube. The final mixture in the centrifuge tube was incubated for 1 h at room temperature. After the incubation, the final product was centrifuged for 10 min at 2000 rpm. Then, supernatant in the centrifuge tube was removed. 1×PBS buffer containing 0.05% BSA (1 ml) was added in the centrifuge tube. After vortexing the centrifuge tube, it was centrifuged for 10 min at 2000 rpm. After the centrifugation, supernatant in the centrifuge tube was removed. After the two more washing procedure, 1×PBS buffer containing 0.05% BSA (1 ml) were added in the centrifuge tube. Then TSH detection antibody-conjugated yellow-green microsphere in 1×PBS buffer containing 0.05% BSA (1 ml) was stored at a refrigerator (2–8 °C).

2.3. Preparation of TSH capture antibody immobilized on the surface of white strip-well

Monoclonal TSH capture antibody (5 μg/ml) was prepared in 1×PBS buffer. The capture antibody (100 μl) added in a polystyrene white strip-well (Greiner Bio-one, Inc.) was incubated for 18 h at a refrigerator. Then, each strip-well was four-time washed using 1×PBST buffer containing Tween 20. Then, TSH capture antibody coated on the white strip-well and a desiccate wrapped in a foil bag was stored at a refrigerator.

2.4. Comparison of fluorescence and ODI-CL spectra of yellow-green microspheres

Yellow-green microspheres (0.25%, 200 μl) in 1×PBS buffer (pH 7.4) was mixed with isopropyl alcohol (100 μl) and ethyl acetate (100 μl). Then, the fluorescence spectrum of yellow-green microspheres in the mixed solvent was obtained with a CCD spectrometer (USB2000, Ocean Optics) with Xenon lamp (PX-2, Ocean Optics). Also, yellow-green microsphere (200 μl) in 1×PBS buffer was mixed with H_2O_2 (0.05 M, 100 μl) in isopropyl alcohol in a micro-cuvette. In order to generate ODI-CL in the micro-cuvette, ODI (100 μl) formed from the reaction of TCPO (0.1 mM) and 4MImH (0.4 mM) in ethyl acetate was added in the micro-cuvette. ODI-CL emitted in the micro-cuvette was observed with the CCD spectrometer without any light source.

2.5. ODI-CL sandwich immunoassay with TSH capture antibody bound on the white strip-well

As shown in Fig. 1(A), a certain concentration of TSH (50 μl) in human serum was added in the white strip-well to bind with TSH capture antibody for 15 min at room temperature. After the incubation, each strip-well was washed with 1×PBST buffer containing Tween 20. After the washing, TSH detection antibody-conjugated yellow-green microsphere (100 μl) was added in the white strip-well. The strip-well was incubated for 30 min at room temperature. After the incubation, each strip-well was four-time washed with 1×PBST buffer containing Tween 20. After the washing, strip-wells containing different concentration of TSH were inserted into a microplate reader with two dispensers (Luminoskan Ascent, Thermo Scientific, Inc.). H_2O_2 (25 μl) in isopropyl alcohol was injected into each strip-well using