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Rapid chemiluminescent sandwich enzyme immunoassay capable of consecutively quantifying multiple tumor markers in a sample

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ABSTRACT

Using the role of *p*-iodophenol in enzyme assay, enhanced 1,1′-oxalyldiimidazole chemiluminescent enzyme immunoassays (ODI-CLEIAs) were developed to consecutively quantify trace levels of triple tumor markers, such as alpha fetoprotein (AFP), carcinoembryonic antigen (CEA), and prostate specific antigen (PSA) in a sample. Due to the high sensitivity of enhanced ODI-CLEIAs, it was possible to fix the incubation times (1) to capture a tumor marker with two antibodies, which are primary antibody immobilized on the surface of polystyrene strip-well and detection antibody-conjugated horseradish peroxidase (HRP), and (2) to form resorufin with the addition of substrates (e.g., Amplex Red, H_2O_2) in order to quantify triple markers in human serum. Enhanced ODI-CLEIAs capable of consecutively and rapidly quantifying triple markers with the same incubation time were more sensitive than conventional enzyme-linked immunosorbent assay (ELISA) capable of separately and slowly quantifying them with different incubation times. In addition, accuracy, precision, and recovery of enhanced ODI CLEIAs in the presence of *p*-iodophenol were acceptable within statistical error range.

1. Introduction

One of the critical human diseases occurring in industrial countries is cancer, which is a broad group of diseases involving unregulated cell growth. Main causes of cancer are tobacco use, dietary factors, infection, exposure to carcinogenic compounds and radiation, and obesity even though 5–10% cancers come from inherited genetic defects [1]. Fortunately, the rate of deaths by cancer has been rapidly reduced with various in-vivo or in-vitro diagnosing methods for early diagnosis of human cancers [2].

It is well-known that in-vitro immunoassay using human blood collected from patients is an excellent method for early diagnosis of cancers. This is because in-vitro immunoassays capable of detecting a specific tumor marker in human serum or plasma are cost-effective, selective and sensitive [3,4]. Radioimmunoassays (RIAs) developed since 1959 [5] have been applied to quantify trace levels of tumor markers in human samples even though the method has several problems such as stability of labeled tumor markers or antibodies, safety of laboratory personnel, waste, and the requirement of building special laboratory facilities [6]. In order to solve the critical problems of RIAs, enzyme immunoassays (EIAs) using horseradish peroxidase (HRP), alkaline phosphatase (ALP) labeled with detection antibody or antigen, instead of radioisotopes conjugated with detection antibody or antigen, were developed. With the appearance of various optical sensors using colorimetry [7], fluorescence [8], and chemiluminescence [9–13] detections, the accuracy, precision and sensitivity of EIAs operated without the safety problems were as good as those of RIAs [7–13].

The time necessary for the quantification of a biomarker using EIAs is dependent on the sensitivity of optical sensor. This is because the incubation time necessary to capture the biomarker with antibodies in EIAs with a highly sensitive optical sensor is not as long as that in EIAs with a relatively non-sensitive optical sensor [9–10]. It is well-known that chemiluminescence detection is more sensitive than other optical sensors such as colorimeter and fluorescence detection because chemiluminescence detection operated without light source (e.g., laser, Xenon and mercury lamps) generated with high-voltage power supply has lower background noise [14,15].

Both 1,2-dioxetane [11] and luminol [12] chemiluminescent EIAs have been widely applied to diagnose various diseases, whereas 1,1′-oxalyldiimidazole chemiluminescent enzyme immunoassays (ODI CLEIAs) were recently developed as an advanced and new method capable of rapidly quantifying trace levels of biomarkers [9,10,13]. Recent research papers reported that ODI CLEIAs are more cost-effective and sensitive than the conventional 1,2-dioxetane and luminol EIAs. Also, ODI CLEIAs using two

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different enzymes (e.g., ALP, HRP) can simultaneously quantify two biomarkers in a sample [10,16], whereas conventional CLEIAs operated with a specific enzyme (e.g., ALP in 1,2-dioxetane CLEIAs, HRP in luminol CLEIAs) can only sense a specific biomarker in a sample.

Using the advantages of ODI CL detection, it is possible to develop a more advanced ODI CLEIAs capable of rapidly and consecutively (or simultaneously) quantifying trace levels of biomarkers in a sample if the time necessary for the quantification of a biomarker in a sample is the same as that for the analyses of other biomarkers in the sample. However, based on research results reported so far, it is difficult to consecutively quantify multiple biomarkers in a sample using current ODI CLEIAs because the incubation time necessary for the binding between biomarker and antibodies (e.g., capture antibody and detection antibody labeled with HRP or ALP) to quantify low concentration of the biomarker is apparently different from those to sense trace levels of other biomarkers existing in a sample. For example, ODI CLEIAs could not consecutively quantify three different biomarkers (e.g., unconjugated estriol (uE3), alphafetoprotein (AFP), and human chorionic gonadotropin (hCG)) used to early diagnose genetic disorders such Down Syndrome because the incubation time necessary for the quantification of each biomarker in human serum was apparently different from those of other biomarkers [13].

Various phenol derivatives can enhance relative intensity of luminol CL because phenol derivatives act as an enhancer in luminol CL reaction [17–20]. EIAs with luminol CL detection using the role of phenol derivatives were able to rapidly quantify biomarkers with the reduction of incubation time between biomarker and antibodies. These reports imply that phenol derivatives can be applied to ODI-CL reaction to quantify HRP in a sample if phenol derivatives act as a reagent to rapidly produce resorufin from Amplex Red in the presence of H_2O_2 and HRP. Based on the hypothesis, we first investigated the role of phenol derivatives in HRP assays with ODI-CL detection in this research. Based on the role of phenol derivatives observed in HRP assay with ODI-CL detection, it was possible to develop more advanced ODI CLEIAs capable of consecutively quantifying three different tumor markers (e.g., alpha fetoprotein (AFP), carcinoembryonic antigen (CEA), and prostate specific antigen (PSA)) for the first time.

2. Experimental

2.1. Chemicals and materials

p-iodophenol and horseradish peroxidase (HRP, Type 1, 5KU) were purchased from Sigma (Saint Louis, MO, USA). 4-(1,2,4-Triazol-1-yl) phenol (98%), 4-methylimidazole and dimethyl sulfoxide were purchased from Alfa Aesar (Ward Hill, MA, USA). 4-aminopyridine was purchased from MP Biomedicals, Inc. (Solon, OH, USA). Amplex Red (10-Acetyl-3,7-dihydroxy phenoxazine) was purchased from Cayman Chemical Company. 3% hydrogen peroxide (H_2O_2) was purchased from VWR (Radnor, PA, USA). 0.01 M buffers (Tris–HCl, pH 7.0, 7.5, 8.0, 8.5; TBST, pH 7.4; Sodium Phosphate Buffer, pH 7.0, 8.5; PBS, pH 7.4) were purchased from Teknova (Hollister, CA). Bis(2,4,6-trichlophenyl) oxalate (TCPO) was purchased from TCI-America (Portland, OR). AFP, CEA and PSA AccuBind VAST ELISA kits were purchased from Monobind Inc. (Lake Forest, CA, USA).

2.2. HRP assay in the presence of phenol derivatives

20 μU/ml HRP was prepared in deionized water with the stock (1000 U/ml). 10 and 100 μM of 4-(1,2,4-Triazol-1-yl) phenol, *p*-iodophenol, and 4-aminopyridine were prepared in Tris–HCl buffer (pH 7.0, 10 mM). The stock solution of Amplex Red (15 mM) in DMSO was stored at −20 °C. The stock solution of H_2O_2 (20 mM) was prepared in deionized water. The stocks solutions of Amplex Red and H_2O_2 were mixed in water to prepare a working solution at 30 μM and 40 μM respectively. The mixture (100 μl) containing Amplex Red and H_2O_2 was mixed with 4-(1,2,4-Triazol-1-yl) phenol, *p*-iodophenol, or 4-aminopyridine (10 or 100 μM, 50 μl). 50 μl of this solution was mixed with the same volume of HRP. Resorufin (25 μl) formed with 5-min incubation of each mixture at RT (21 ± 2 °C) was transferred into a white strip-well (LUMITRACTM

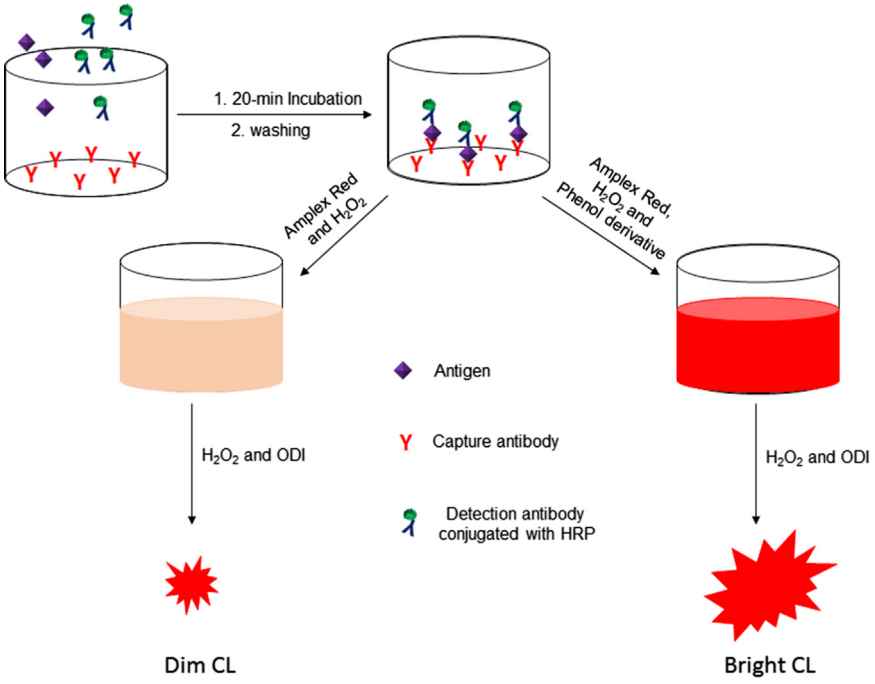

Fig. 1. ODI CLEIAs in the absence and presence of phenol derivatives.