Talanta 129 (2014) 106–112

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

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

Julie Kim [1,a,b], Jennie Kim [1,a,c], Tae Ho D. Rho [a], Ji Hoon Lee [a,*]

[a] Luminescent MD, LLC, Hagerstown, MD 21742, USA
[b] Thomas Jefferson High School for Science and Technology, Alexandria, VA 22312, USA
[c] Langley High School, McLean, VA 22101, USA

ARTICLE INFO

Article history:
Received 1 April 2014
Received in revised form
14 May 2014
Accepted 15 May 2014
Available online 22 May 2014

Keywords:
1,1′-oxalyldiimidazole (ODI)
chemiluminescence
Chemiluminescent enzyme immunoassays
p-iodophenol
Enhanced chemiluminescence
Tumor markers

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

* Corresponding author. Tel.: +1 301 393 9091.
E-mail address: jhlee@luminescentmd.com (J.H. Lee).
[1] These authors contributed equally in this research.

http://dx.doi.org/10.1016/j.talanta.2014.05.020
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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

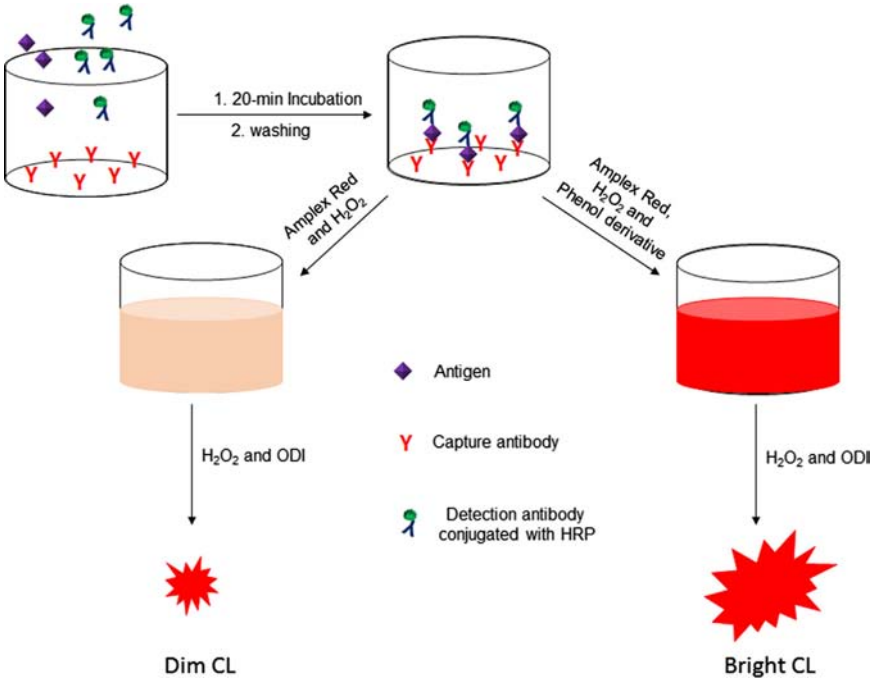

Fig. 1. ODI CLEIAs in the absence and presence of phenol derivatives.

200, medium binding, Greiner Bio-One). Luminescence emitted in each strip-well was measured with Luminoskan Ascent (Thermo-Scientific Inc.) with two dispensers. In other words, 0.2 M H_2O_2 (25 μl) in isopropyl alcohol was dispensed into each strip-well using the first dispenser. ODI (50 μl), formed from the reaction of 5 μM TCPO and 20 μM 4-methylimidazole in ethyl acetate [21,22], was dispensed with the second dispenser. Then, relative CL intensity emitted in each well was immediately integrated for 0.5 s.

2.3. ODI CLEIAs in the presence of p-iodophenol

We used a strip-well, provided from Monobind (http://www.monobind.com/), capable of capturing AFP, CEA, and PSA using AFP, CEA, and PSA primary antibodies immobilized on the surface of the strip-well. As shown in Fig. 1, human serum (25 μl) containing AFP, CEA, and PSA (or patient sample) was added into the strip-well. Then, 100 μl detection antibody conjugated with HRP capable of specifically binding with one of multiple tumor markers in a sample was added immediately. The mixture of each strip-well was incubated for 20 min at 37 °C. After the incubation, the strip-well was washed 4 times with 300 μl TBS buffer (pH 7.5) containing 0.05% Tween 20. The mixture (500 μl) containing 30 μM Amplex Red and 40 μM H_2O_2 was mixed with 450 μM *p*-iodophenol (500 μl) in a 1.5-ml centrifuge tube. The mixture (100 μl) was injected into the strip-well. It was incubated for 7 min at RT. After the incubation, 10 μl of resorufin solution formed from the enzyme reaction in the strip-well was transferred to a borosilicate test tube (12 mm × 75 mm). Each test tube was placed in the sample holder of Lumat LB 9507 Luminometer with two dispensers (Berthold, Germany). 25.0 μl of H_2O_2 (0.05 M in isopropyl alcohol) was injected into the test tube through the first dispenser. After injecting 25.0 μl of ODI into the test tube through the second dispenser, relative ODI CL intensity was integrated immediately for 0.5 s.

3. Results and discussion

3.1. HRP assay in the presence of p-iodophenol

3.1.1. Determination of phenol derivative to optimize HRP assay with ODI-CL detection

As shown in Fig. 2(a), relative CL intensity with the addition of phenol derivatives (e.g., *p*-iodophenol, 4-(1,2,4-Triazol-1-yl) phenol, 4-aminopyridine) in HRP assay with ODI-CL detection was higher than that in the absence of phenol derivatives. Also, the enhancement of CL intensity was dependent on the properties of phenol derivative [17–20] as well as the concentration of each phenol derivative. Fig. 2(a) indicates that the relative CL intensity in the presence of 100 μM *p*-iodophenol was the highest under the experimental condition. Relative CL intensity with the addition of *p*-iodophenol was about 9-fold higher than that in the absence of *p*-iodophenol.

Fig. 2(b) shows that relative CL intensity was enhanced with the increase of *p*-iodophenol up to 80 μM. However, relative CL intensity in the presence of 160 μM was similar to that with the addition of 80 μM. This is because the concentration of resorufin formed with the addition of 160 μM *p*-iodophenol was too high to emit light from all resorufin molecules due to the self-quenching observed in the presence of excess luminescent molecules in ODI-CL reaction [23,24].

The results shown in Fig. 2 indicate that *p*-iodophenol acts as a reagent to rapidly produce resorufin from Amplex Red before adding ODI CL reagents (e.g., H_2O_2, ODI) to enhance ODI-CL as shown in Scheme 1. Reactions (1) to (3) were already confirmed in luminol chemiluminescence reaction in the presence of

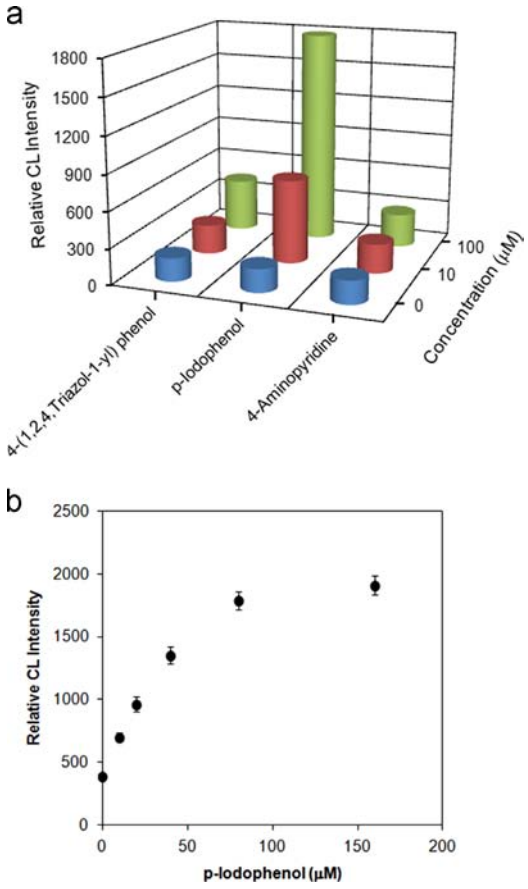

Fig. 2. (a) Effect of phenol derivatives in HRP assay with 2-min incubation in PBS (pH 7.4) at RT. (b) Effect of *p*-iodophenol in HRP assay with 4-min incubation in PBS at RT. Experimental conditions were described in detail in Section 2.2. The error range of each value (i.e., the average of triplicated measurements, $N=3$) determined under different conditions was 3–7%.

$$\text{HRP} + H_2O_2 \longrightarrow \text{Compound I} + H_2O \quad (1)$$

$$\text{Compound I} + \text{PIP} \longrightarrow \text{Compound II} + \text{PIP}^* \quad (2)$$

$$\text{Compound II} + \text{PIP} \longrightarrow \text{HRP} + \text{PIP}^* \quad (3)$$

$$\text{Amplex Red} + \text{PIP}^* \longrightarrow \text{Resorufin} + \text{PIP} \quad (4)$$

$$\text{Resorufin} \xrightarrow[\text{ODI}]{H_2O_2} \text{Resorufin}^* + \text{phenol} + H_2O \quad (5)$$

$$\text{Resorufin}^* \longrightarrow \text{Resorufin} + h\nu \quad (6)$$

Scheme 1. Possible reaction mechanism in enzyme assay with the addition of *p*-iodophenol (IP).

p-iodophenol as an enhancer [17]. HRP converts to compound I (HRP-intermediate) in the presence of H_2O_2 as shown in reaction (1). *p*-iodophenol (PIP) is oxidized in the presence of compound I like PIP* with compound II as shown in reaction (2). Then, as shown in reaction (3), the rest of the PIP is also oxidized by compound II formed from reaction (2). Amplex Red reacts with PIP* formed from reactions (2) and (3) to rapidly produce resorufin, as shown in reaction (4). Resorufin formed in the presence of PIP was excited by high-energy intermediate formed from the reaction of ODI and H_2O_2, as shown in reaction (5) based on the principle of chemically initiated electron exchange luminescence (CIEEL) mechanism [21,22]. Finally, resorufin excited from reaction (5) emits strong light as shown in reaction (6). Based on the reaction mechanism of Scheme 1, we expect that the interaction between Amplex Red and compound I formed from

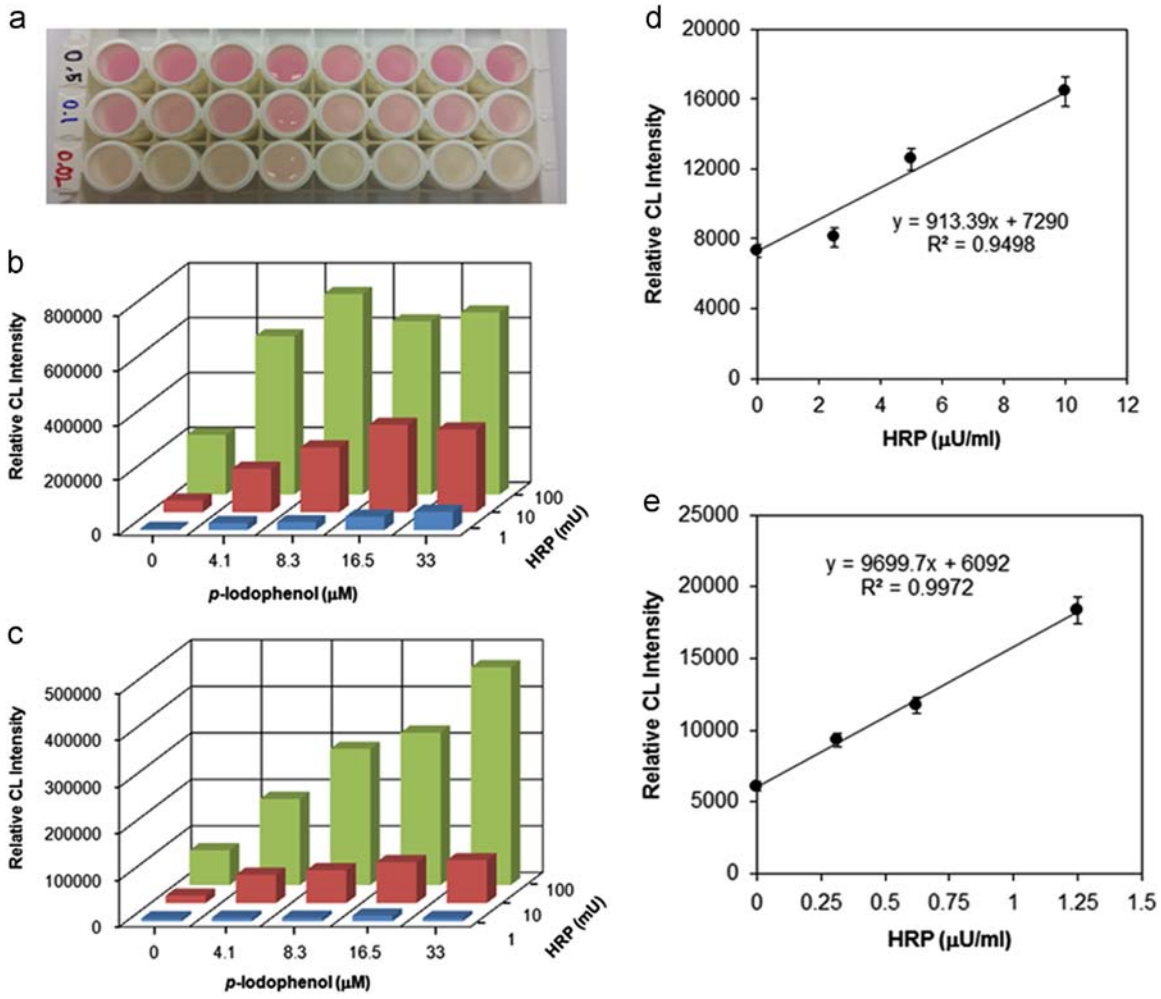

Fig. 3. (a) Effect of buffer in enzyme assay in the presence of 20 μM *p*-iodophenol. Incubation time: 20 s, from left to right: Tris–HCl (pH 7), Tris–HCl (pH 7.5), Tris–HCl (pH 8), Tris–HCl (pH 8.5), TBST (pH 7.5), sodium phosphate (pH 7.0), sodium phosphate (pH 8.5), and PBS (pH 7.4). (b) Effect of *p*-iodophenol in TBST buffer (pH 7.5). (c) Effect of *p*-iodophenol in Tris–HCl (pH 7). (d) Calibration curve to assay HRP with ODI-CL detection in the absence of *p*-iodophenol in TBST. (e) Calibration curve to assay HRP with ODI-CL detection in the presence of *p*-iodophenol in TBST. The error range of each value ($N=3$) determined under different conditions was 4–6%. (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.)

reaction (1) will be much slower than that between PIP and compound I. Also, we expect that PIP* oxidized from reactions (2) and (3) will be a strong reactant that rapidly produces resorufin from the reaction of Amplex Red and PIP*.

3.1.2. *Quantification of HRP using ODI-CL detection in the presence of p-iodophenol*

Fig. 3(a) shows the effect of buffer composition in HRP assay in the presence of 20 μM *p*-iodophenol. All buffer solutions containing 0.5 mU/ml HRP were immediately changed from no color to pink. All buffer solutions containing 0.1 mU/ml HRP changed color during 20-s incubation. In the case of 0.02 mU/ml HRP, we were able to observe effect of buffer solution in HRP assay. Enzyme reaction in TBST (pH 7.5) was the fastest. Enzyme reaction in Tris–HCl (pH 7) was also faster than in other buffer solutions, except for TBST (pH 7.5).

Based on the results shown in Fig. 3(a), thus, we studied the concentration effect of *p*-iodophenol in ODI-CL enzyme assay with samples prepared in TBST (see Fig. 3(b)) and Tris–HCl at pH 7 (see Fig. 3(c)). Relative CL intensity in TBST was higher than that in Tris–HCl. Also, 8.25 or 17 μM *p*-iodophenol in TBST was appropriate for quantifying HRP using ODI-CL detection, whereas the optimum concentration of *p*-iodophenol in Tris–HCl was 33 μM. Based on the results shown in Fig. 3(a)–(c), we confirmed that the best buffer solution for the quantification of HRP with ODI-CL detection is TBST at pH 7.5.

As shown in Fig. 3(d) and (e), HRP assay with ODI-CL detection in the presence of 17 μM *p*-iodophenol was more sensitive than that in the absence of *p*-iodophenol. HRP assay with ODI-CL detection in the presence of *p*-iodophenol can quantify lower concentration than 0.3 μU/ml HRP, whereas ODI-CL enzyme assay in the absence of *p*-iodophenol can quantify higher concentration than 5 μU/ml HRP. Also, the limit of detection (LOD = background + 3 standard deviation, 0.08 μU/ml, 1.4 pM [25]) determined in ODI-CL enzyme assay with *p*-iodophenol was about 30-fold lower than that (2.34 μU/ml, 42 pM) without *p*-iodophenol. Background is the average of values ($N=20$) measured in the absence of HRP. Standard deviation is the error range computed with values measured in the absence of HRP. LOD using ODI-CL detection in the presence of *p*-iodophenol was as good as that using luminol chemiluminescence in the presence of *p*-iodophenol [18].

The effect of *p*-iodophenol observed in HRP assay with ODI-CL detection implies that ODI CLEIAs in the presence of *p*-iodophenol can rapidly quantify trace levels of tumor markers.

3.2. Optimization of variables in ODI CLEIAs in the presence of p-iodophenol

3.2.1. Determination of p-iodophenol concentration in ODI CLEIA

In order to study the effect of *p*-iodophenol in ODI CLEIAs, first, sandwich complex-conjugated HRPs were formed the interaction between tumor marker and two antibodies (e.g., capture antibody, detection antibody-conjugated HRP) in a strip-well at 37 °C based on the procedure shown in Fig. 1. After washing the strip-well, mixture of Amplex Red, H_2O_2, and *p*-iodophenol was inserted into the strip-well. The mixture was incubated for a certain time to produce resorufin from the reaction between Amplex Red and H_2O_2 in the presence of sandwich complex-conjugated HRP and *p*-iodophenol.

The ratios of signal to background (S/B) measured in the absence and presence of *p*-iodophenol shown in Fig. 4(a)–(c) indicate that the mixture of Amplex Red, H_2O_2, and *p*-iodophenol in the strip well needs to be incubated for at least 3 min at room temperature to form resorufin even though the concentration (50 μM) of *p*-iodophenol in ODI CLEIAs was higher than that (8.3 μM) optimized in HRP assay with ODI-CL detection shown in Fig. 3(b).

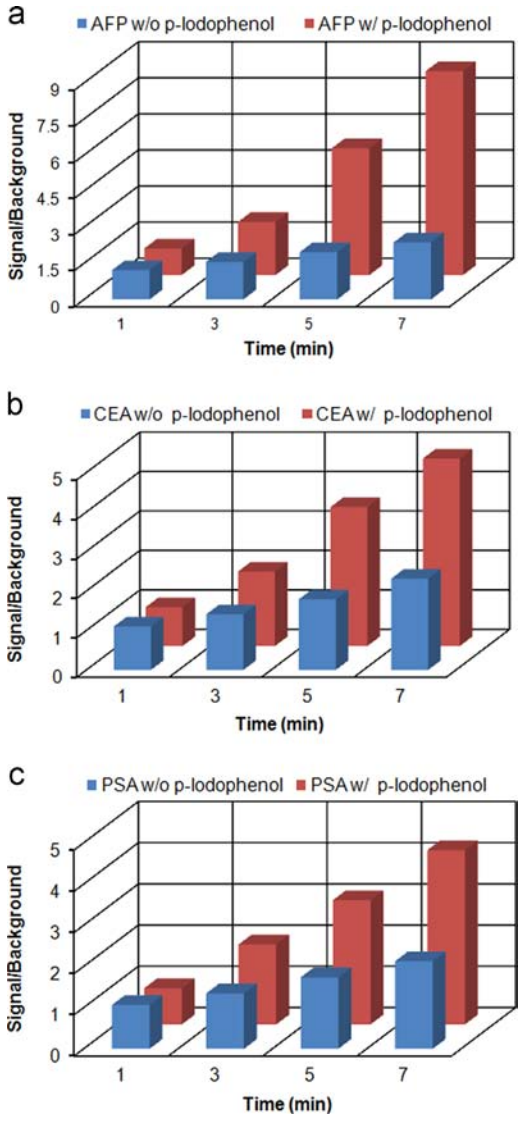

Fig. 4. Effect of incubation time in ODI CLEIAs to quantify tumor markers in the absence and presence of *p*-iodophenol. (Tumor markers: (a) AFP (5 ng/ml), (b) CEA (5 ng/ml), and (c) PSA (2 ng/ml). The error range of each value ($N=3$) determined under different condition was 3–7%.

Figs. 3(b) and 4 indicate that the concentration of *p*-iodophenol necessary to rapidly react with HRP immobilized on the surface of strip-well in EIAs with ODI-CL reaction is higher than 50 μM, whereas relatively lower concentration of *p*-iodophenol (as low as 8.3 μM) can rapidly react with HRP freely moving in TBST for HRP assay with ODI-CL detection. Fig. 4 indicates that S/B in the presence of *p*-iodophenol determined after the incubation for 5 min was apparently higher than that in the absence of *p*-iodophenol. Based on the results shown in Fig. 4, we selected the 7-min incubation for the quantification of trace levels of tumor markers, which are lower than or as low as the cut off values (e.g., AFP: 20 ng/ml, CEA: 5 ng/ml, PSA: 4 ng/ml) used to diagnose cancers [26], in ODI CLEIAs in the presence of *p*-iodophenol.

3.2.2. Determination of p-iodophenol concentration in EIAs in ODI-CL detection

We optimized the concentration effect of *p*-iodophenol in ODI CLEIAs to rapidly quantify tumor markers. In order to study the concentration effect of *p*-iodophenol, tumor marker and specific detection antibody-conjugated HRP were mixed with capture antibody coated on the surface of strip-well. Then the mixture was incubated for 20 min at 37 °C. After the incubation, each strip-well was washed 4 times with TBST (pH 7.5). Then, the mixture of Amplex Red, H_2O_2 and *p*-iodophenol in TBST was inserted in the strip-well and incubated for 7 min. As shown in Fig. 5, the optimum concentration of *p*-iodophenol was 0.45 mM in ODI CLEIAs. The concentration of *p*-iodophenol used in ODI CLEIAs is about 54-fold higher than that (8.3 μM) applied in HRP assay with ODI-CL detection. This is because sandwich complex-conjugated with HRP in ODI CLEIAs is immobilized on the surface of strip-well, whereas HRP in ODI-CL enzyme assay is in TBST. However, relative CL intensities in the presence of higher concentration of 0.45 mM *p*-iodophenol were lower than that in the presence of 0.45 mM *p*-iodophenol because the concentration of resorufin formed under the former conditions was so high that all resorufin molecules excited based on CIEEL mechanism cannot emit light due to the self-quenching. Based on the results shown in Fig. 5, we selected 0.45 mM *p*-iodophenol to develop ODI CLEIAs to rapidly and consecutively quantify multiple tumor markers in a sample.

3.2.3. Determination of incubation time to form complexes from the interaction between tumor marker and antibodies in ODI CLEIAs

Figs. 4 and 5 indicate that ODI CLEIAs in the presence of *p*-iodophenol are more sensitive than that in the absence of *p*-iodophenol. Thus, by enhancing the sensitivity of ODI CLEIAs in the presence of *p*-iodophenol, it is possible to more rapidly quantify tumor markers with the reduction of incubation time to capture them using capture and detection antibody-conjugated HRP (see the procedure shown in Fig. 1). Based on the hypothesis, we

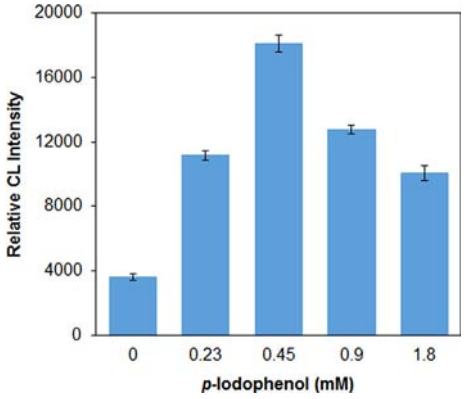

Fig. 5. Effect of *p*-iodophenol in ODI CLEIAs.

investigated the effect of incubation time to form sandwich complex-conjugated HRP from the interaction between tumor marker and two antibodies in ODI CLEIAs in the presence of *p*-iodophenol (0.45 mM). As shown in Fig. 6, relative CL intensity measured with ODI CLEIAs for the quantification of PSA (10 ng/ml) was enhanced with the increase of incubation time from 20 to 40 min. However, the results shown in Fig. 6 indicate that 20-min incubation is enough to quantify trace levels of PSA in human serum using ODI CLEIAs in the presence of *p*-iodophenol because relative CL intensity in the presence of PSA was about 22 fold higher than the background (i.e., relative CL intensity in the absence of PSA). Thus, the mixture of tumor marker and antibodies in a strip-well was incubated for 20 min to develop highly sensitive ODI CLEIAs operated in the presence of *p*-iodophenol.

3.3. Quantification of tumor markers using ODI-CLEIA in the presence of p-iodophenol

Based on the procedure shown in Fig. 1 and experimental results shown in Figs. 4–6, we were able to consecutively quantify three different tumor markers in a human serum sample. As shown in Fig. 4 and Table 1, ODI CLEIAs in the presence of *p*-iodophenol can rapidly quantify trace levels of tumor markers with wide linear calibration curves shown in Fig. 7. Also, LODs of ODI CLEIAs in the presence of *p*-iodophenol were lower than those in the absence of *p*-iodophenol. In addition, ODI CLEIAs in the presence of *p*-iodophenol were faster than commercially available ELISA we used in this research. This is because that total incubation time (27 min) for ODI CLEIAs in the presence of *p*-iodophenol was at least 3-fold faster than that for the ELISA.

In order to study the recovery of ODI CLEIAs in the presence of *p*-iodophenol, 50 μl of sample 1 prepared in human serum was mixed with 50 μl of sample 2. The results of recovery presented in

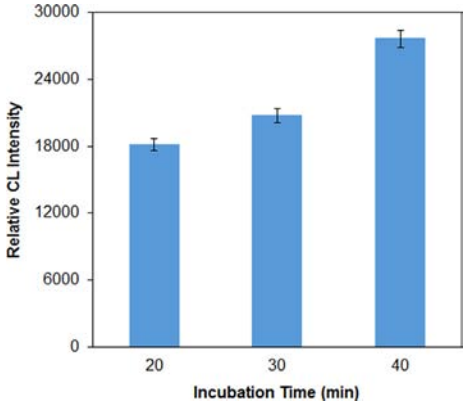

Fig. 6. Effect of incubation time in ODI CLEIAs.

Table 1
Quantification of tumor markers using ODI-CLEIAs in the absence or presence of *p*-iodophenol.

Tumor marker	*p*-iodophenol (PIP)	Dynamic range[a]	R^2	LOD[a,b]
AFP (20 ng/ml)[c]	Without PIP	1.0–500	0.998	0.59
	With PIP	0.1–500	0.995	0.08
CEA (5 ng/ml)[c]	Without PIP	2.0–250	0.998	0.72
	With PIP	0.4–250	0.999	0.21
PSA (4 ng/ml)[c]	Without PIP	1.0–50	0.996	0.47
	With PIP	0.3–50	0.997	0.11

[a] ng/ml.
[b] Limit of detection.
[c] Cut-off value to diagnose cancer.

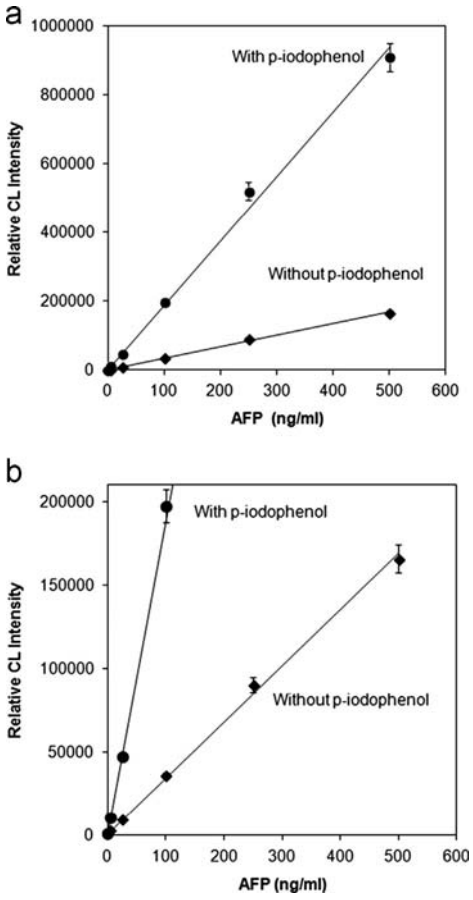

Fig. 7. Calibration curves to quantify AFP using ODI CLEIAs in the absence or presence of *p*-iodophenol. (a) Scale of relative CL intensity: 0–1,000,000, (b) Scale of relative CL intensity: 0–200,000.

Table 2
Recovery test of ODI-CLEIAs in the presence of *p*-iodophenol ($n=5$).

Tumor Maker	Sample 1	Sample 2	Calculated	Experimental	Recovery (%)
AFP	5	25	15	14.2	94.6
(ng/ml)	25	100	62.5	64.7	103.5
CEA (ng/ml)	5	10	7.5	7.9	105.3
	5	25	15	15.6	104.0
PSA (ng/ml)	2	5	3.5	3.2	91.4
	5	25	15	14.5	96.7

Table 2 indicate that tumor markers in a sample could be quantified using the advanced ODI CLEIAs with acceptable accuracy. Also, the range of precision computed with recovery test of ODI CLEIAs in the presence of *p*-iodophenol was as good as 3∼5%.

4. Conclusions

In summary, HRP in a sample was rapidly quantified using ODI-CL detection with understanding of *p*-iodophenol's role added in the mixture of Amplex Red, H_2O_2, and HRP. Also, we confirmed that highly sensitive ODI CLEIAs in the presence of *p*-iodophenol can be consecutively and rapidly quantified multiple tumor markers in a sample.

In conclusion, we expect that ODI CLEIAs in the presence of *p*-iodophenol can be applied as a convenient and simple method for the rapid quantifications of multiple biomarkers, which is necessary for the diagnosis of a specific disease in a sample. Also, scientists of various research fields such as biochemistry, food

safety, homeland security, and toxicology will be able to apply the advanced EIAs with ODI-CL detection as a method to rapidly monitor various targets in a sample.

References

[1] P. Anand, A.B. Kunnumakkara, C. Sundaram, K.B. Harikumar, S.T. Tharakan, O. S. Lai, B. Sung, B.B. Aggarwal, Pharm. Res. 25 (2008) 2097–2116.
[2] W.J. Tao, J. Lagergren, Nat. Rev. Clin. Oncol. 10 (2013) 519–533.
[3] A. Gomez-Hens, J.M. Fernandez, M.P. Aguilar-Caballos, Mini-Rev, Med. Chem. 9 (2009) 1064–1074.
[4] Y.M. Yin, Y. Cao, Y.Y. Xu, G.X. Li, Int. J. Mol. Sci. 11 (2010) 5078–5095.
[5] S.A. Berson, R.S. Yalow, Nature 184 (1959) 1648–1649.
[6] I. Surugiu, J. Svitel, L. Ye, K. Haupt, B. Danielsson, Anal. Chem. 73 (2001) 4388–4394.
[7] A. Ambrosi, M.T. Castaneda, A.J. Killard, M.R. Smyth, S. Alegret, A. Merkoci, Anal. Chem. 79 (2007) 5232–5240.
[8] T. Kashiwakuma, A. Hasegawa, T. Kajita, A. Takata, H. Mori, Y. Ohta, E. Tanaka, K. Kiyosawa, T. Tanaka, S. Tanaka, N. Hattori, M.M. Kohara, J. Immunol. Methods 190 (1996) 79–89.
[9] J.H. Lee, J.Y.R. Rho, T.H.D. Rho, J.G. Newby, Biosens. Bioelectron. 26 (2010) 377–382.
[10] R. Chong, J.E.R. Rho, H.J. Yoon, T.H.D. Rho, P.S. Park, Y.H. Kim, J.H. Lee, Biosens. Bioelectron. 32 (2012) 19–23.
[11] L.J. Kricka, Anal. Chim. Acta 500 (2003) 279–286.
[12] J.H. Lin, P.F. Chu, Z.J. Wu, Anal. Sci. 28 (2012) 21–25.
[13] R. Chong, J.E.R. Rho, H.J. Yoon, P.S. Park, T.H.D. Rho, J.Y. Park, L. Park, Y.H. Kim, J. H. Lee, Talanta 116 (2013) 403–408.
[14] M. Tsunoda, K. Imai, Anal. Chim. Acta 541 (2005) 13–23.
[15] L. Park, J. Kim, J.H. Lee, Talanta 116 (2013) 736–742.
[16] L. Park, H. Bae, Y.T. Kim, J.H. Lee, Anal. Methods 3 (2011) 156–160.
[17] T. Kamidate, M. Maruya, H. Tani, A. Ishida, Anal. Sci. 25 (2009) 1163–1166.
[18] I.S. Alpeeva, I.Y. Sakharov, J. Agric. Food Chem. 53 (2005) 5784–5788.
[19] I.Y. Sakharov, A.S. Demiyanova, A.V. Gribas, N.A. Uskova, E.E. Efremov, M. M. Vdovenko, Talanta 115 (2013) 414–417.
[20] M.M. Vdovenko, C.-T. Hung, I.Y. Sakharov, F.-Y. Yu, Talanta 116 (2013) 343–346.
[21] J.H. Lee, J.C. Rock, S.B. Park, M.A. Schlautman, E.R. Carraway, J. Chem. Soc. Perkin Trans. 1 (2002) 802–809.
[22] P.S. Park, T.H.D. Rho, Y.T. Kim, So. O. Ko, M.A. Schlautman, E.R. Carraway, J. H. Lee, Chem. Commun. 47 (2011) 5542–5544.
[23] H. Cho, S. Lee, J.H. Lee, Analyst 137 (2012) 5368–5373.
[24] J. Kwun, S. Yun, L. Park, J.H. Lee, Talanta 119 (2014) 262–267.
[25] Specific activity (Unit) of HRP was converted to M based on the Data sheet (⟨http://www.sigmaaldrich.com/content/dam/sigma-Aldrich/docs/Sigma/Datasheet/ 1/p8125dat.pdf⟩) provided from Sigma-Aldrich.
[26] C.D. Nicoll, in: S.J. McPhee, M.A. Papadakis, L.M. Tierney Jr. (Eds.), Current Medical Diagnosis & Treatment, vol. 46, McGraw Hill, New York, 2007, pp. 1767–1777.