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1,1′-Oxalyldiimidazole chemiluminescent enzyme immunoassay capable of simultaneously sensing multiple markers

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ABSTRACT

In order to rapidly and simultaneously quantify and screen trace levels of multiple biomarkers in a single sample, rapid 1,1′-oxalyldiimidazole chemiluminescence (ODI CL) was applied as a biosensor of immunoassays using various enzymes such as alkaline phosphatase (ALP) and horseradish peroxidise (HRP). (1) Fluorescein was formed from the reaction of fluorescein diphosphate (FDP) and immuno-complex conjugated with ALP. (2) Resorufin was formed from the reaction between Amplex Red and H_2O_2 in the presence of immuno-complex conjugated with HRP. When ODI CL reagents (H_2O_2 in isopropyl alcohol, ODI in ethyl acetate) were injected in a test tube or strip-well containing fluorescein and resorufin formed from above two reactions a bright CL emission spectrum having two peaks (518 nm for fluorescein and 602 nm for resorufin) was observed. The two peaks can be independently quantified with an appropriate statistical tool capable of deconvoluting multiple emission peaks. In conclusion, we expect that ODI chemiluminescent enzyme immunoassays (CLEIAs) using a couple of enzymes conjugated with antigen or antibody and substrates can rapidly and simultaneously quantify and screen multiple biomarkers in a single sample.

1. Introduction

Immunoassays having excellent sensitivity and specificity have been used to quantify trace levels of small molecules such as drug and toxic chemicals as well as large molecules such as proteins. Due to the potential health risks of radioimmunoassays developed in 1959 (Berson and Yalow, 1959), enzyme immunoassays with colorimetric (Asensio et al., 2008; Zhang and Wang, 2009; Yin et al., 2010) or chemiluminescent (Fan et al., 2010; Zhao et al., 2009) detection are most commonly used to diagnose various diseases and to monitor toxic materials. Currently, chemiluminescent enzyme immunoassays (CLEIA) having low background noise are widely applied to diagnose diseases and monitor toxic materials because they are faster and more sensitive than enzyme-linked immunosorbent assays (ELISA) with colorimetric detection.

Horseradish peroxidase (HRP) and alkaline phosphatase (ALP) are widely applied as enzymes in CLEIA (Fan et al., 2010; Zhao et al., 2009). A substrate typically used in CLEIA with HRP is luminol whereas 1,2-dioxetane derivatives are often applied as substrates in CLEIA with ALP. The blue CL emitted from the reaction of luminol and H_2O_2 in the presence of HRP has appreciable overlap with the CL generated by the reaction of 1,2-dioxetane and ALP, the emission maximum for luminol CLEIA (425–450 nm) being only slightly different from 1,2-dioxetane CLEIA (460–480 nm) (Fan et al., 2010; Zhao et al., 2009). Therefore, it is difficult to simultaneously sense two different biomarkers in a single sample using luminol and 1,2-dioxetane CLEIAs.

Recently, we reported that 1,1′-oxalyldiimidazole (ODI) CL detection in CLEIA with HRP for the quantification of prostate specific agent (PSA) (Lee et al., 2010) and melamine (Choi et al., 2010) is more sensitive than conventional EIAs such as ELISA, fluorescent EIA (FEIA), and luminol CLEIA. Resorufin formed from the reaction of Amplex Red and H_2O_2 in the presence of HRP in ODI CLEIA emits red light (602 nm). Also, we developed a new analytical method using ODI CL detection capable of quantifying and screening trace levels of ALP in pasteurized milk (Park et al., 2011a). The

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emission maximum of fluorescein formed from the reaction of fluorescein diphosphate (FDP) and ALP in milk was measured at 518 nm with ODI CL detection. It indicates that ODI CL can be applied as a biosensor of enzyme immunoassay (EIA) using antibody or antigen conjugated with ALP.

As shown in Fig. 1(a), our previous research results (Choi et al., 2010; Lee et al., 2002, 2003, 2010; Park et al., 2011a,b) indicate that ODI CL detection can be utilized to simultaneously sense at least two different biomarkers in a single sample using EIAs with HRP and ALP. The ODI CL spectrum (Fig. 1(b)) of a mixture of fluorescein and resorufin obtained with a charge coupled device (CCD) spectrometer reveals that they can be independently quantified with an appropriate statistical tool capable of deconvoluting multiple emission peaks. We describe in this paper that ODI CLEIA can be developed as an advanced biosensor capable of rapidly and simultaneously quantifying multiple biomarkers in a single sample – conventional CLEIAs can sense only one biomarker. In other words, we expect that results observed using ODI CLEIA reported in this paper can be applied as basic concept and technology to develop diagnosing devices capable of simultaneously quantifying multiple biomarkers in a single sample as well as to develop monitoring system capable of simultaneously and consecutively screening environmental toxic materials and food-borne pathogens in a sample.

2. Experimental

2.1. Chemical and materials

Streptavidin–alkaline phosphatase from *Streptomyces avidinii* and streptavidin–peroxidase from *S. avidinii* were purchased from Sigma (Saint Louis, MO, USA). Biotin Coated Plates (96-well strip) were purchased from Thermo Scientific (Rockford, IL, USA). Alpha-fetoprotein (AFP) ELISA kit was purchased from Immunometrics (UK) Ltd. (Cambridge, UK). AFP standard was purchased from National Institute for Biological Standards and Control, NIBSC (Hertfordshire, UK). Amplex Red was purchased from Cayman Chemical (Ann Arbor, MI, USA). Fluorescein diphosphate tetrammonium salt (FDP) and Lysis buffer were purchased from Anaspec (Fremont, CA, USA). Working solution (pNPP and stop solution) used as a substrate in ELISA for the quantification of streptavidin-conjugated ALP was purchased from Immunometrics (Cambridge, UK). Working solution (TMB, H_2O_2, and stop solution) was purchased from Monobind (Lake Forest, CA, USA). 3.0% hydrogen peroxide and 0.05 M EDTA solutions were from VWR (Bridgeport, NJ, USA). Magnesium chloride ($MgCl_2$) was purchased from EMD (Gibbstown, NJ, USA). 4-Methylimidazole (4MImH, 98%) and zinc chloride ($ZnCl_2$) were purchased from Alfa-Aesar (Ward Hill, MA, USA). Four different 1.0 M Tris–HCl buffers (pH 7.0, 7.5, 8.0, and 8.5) were purchased from Teknova (Hollister, CA, USA). Bis (2,4,6-trichlorophenyl) oxalate (TCPO) was purchased from TCI-America (Portland, OR, USA). Dimethyl sulfoxide (DMSO) was purchased from GI biochem (Boston, MA, USA). Glycerol was purchased from Mallinckrod Baker (Phillipsburg, NJ, USA). Meritus Medical Center (Hagerstown, MD, USA) kindly provided Coded human serums containing AFP.

2.2. Measurement

2.2.1. EIAs using interaction between biotin and streptavidin-conjugated HRP (or ALP)

Streptavidin-conjugated ALP (or HRP) was incubated with biotin coated on the surface of strip-well for 1 h at 36.5 °C using a Luminoskan purchased from Thermo Scientific (Rockford, IL, USA). The strip-well containing immuno-complexes (biotin-bound streptavidin conjugated with ALP or HRP) was washed 3 times Tris–HCl buffer solution (pH 8.0) with 0.05% Tween 20.

2.2.1.1. ODI CLEIAs. 10 μM FDP in working solution (50 mM Tris–HCl, 0.1 mM EDTA, 10 μM $MgCl_2$, 1 μM $ZnCl_2$, and 0.5% glycerol, pH 8.5) was used as a substrate in ODI CLEIA for the quantification of streptavidin-conjugated ALP. The working solution containing (10 μM Amplex Red and 1 mM H_2O_2 in PBS buffer, pH 7.4) was used as a substrate in ODI CLEIA for the quantification of streptavidin-conjugated HRP. As shown in Fig. 2 of the article, 100 μl of FDP (or mixture of Amplex Red and H_2O_2) was inserted into a strip-well containing biotin-bound streptavidin conjugated with ALP (or biotin-bound streptavidin conjugated with HRP) and incubated for a certain time to form fluorescein (or resorufin). After the incubation, 10 μl of fluorescein (or resorufin) added in a 12 mm × 75 mm borosilicate test tube was inserted into a sample holder of Lumat 9507 Luminometer with two dispensers (Berthold, Inc.). When the start button was pressed, the test tube containing fluorescein (or resorufin) moved into the detection area. 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, formed from the reaction of 10 μM TCPO and 50 μM 4MImH, to the test tube through the second dispenser, relative ODI CL intensity was measured immediately for 0.5 s with an interval of 0.1 s.

2.2.1.2. Luminol- and 1,2-dioxetane CLEIAs. 100 μl of PhosphaGLO Reserve AP Substrate (1,2-dioxetane) was inserted into a strip-well containing biotin-bound streptavidin conjugated with ALP. 100 μl of LumiGLO Reserve HRP substrate (the mixture of luminol and H_2O_2) was inserted into a strip-well containing biotin-bound streptavidin conjugated with HRP. After the incubation for 150 s, light emitted in the strip-well was integrated with a Luminoskan for 1 s.

2.2.1.3. FEIAs. 10 μM FDP in working solution (50 mM Tris–HCl, 0.1 mM EDTA, 10 μM $MgCl_2$, 1 μM $ZnCl_2$, and 0.5% glycerol, pH 8.5) was used as a substrate in ODI CLEIA for the quantification of streptavidin-conjugated ALP. The working solution containing (10 μM Amplex Red and 1 mM H_2O_2 in PBS buffer, pH 7.4) was used as a substrate in ODI CLEIA for the quantification of streptavidin-conjugated HRP. 100 μl of FDP (or mixture of Amplex Red and H_2O_2) was inserted into a strip-well containing biotin-bound streptavidin conjugated with ALP (or biotin-bound streptavidin conjugated with HRP). After incubating for 150 s, relative fluorescence (excitation and emission wavelengths: 490 and 520 nm) of fluorescein emitted in each strip-well for the quantification of streptavidin-conjugated ALP was measured with a microplate reader (Infinite M 1000 of Tecan, Inc.). Using Infinite M 1000, relative fluorescence (excitation and emission wavelengths: 570 and 590 nm) of resorufin for the quantification of streptavidin-conjugated HRP was measured after incubating for 150 s.

2.2.1.4. ELISAs. 100 μl of 1-pNPP was inserted into a strip-well containing biotin-bound streptavidin conjugated with ALP. 100 μl of substrate (mixture of TMB and H_2O_2) was inserted into a strip-well containing biotin-bound streptavidin conjugated with HRP. After incubating for 150 s, 100 μl of stop solution was added in each strip-well. Then, the absorbance of chromophore formed in the strip-well in the presence of streptavidin-conjugated ALP was measured at 492 nm. Also, the absorbance of chromophore formed in the strip-well in the presence of streptavidin-conjugated HRP was measured at 450 nm.

2.3. Quantification of AFP using ODI CLEIAs

25 μl of standard (or sample), 50 μl of AFP primary antibody coated on the surface magnetic bead, and 125 μl of AFP antibody

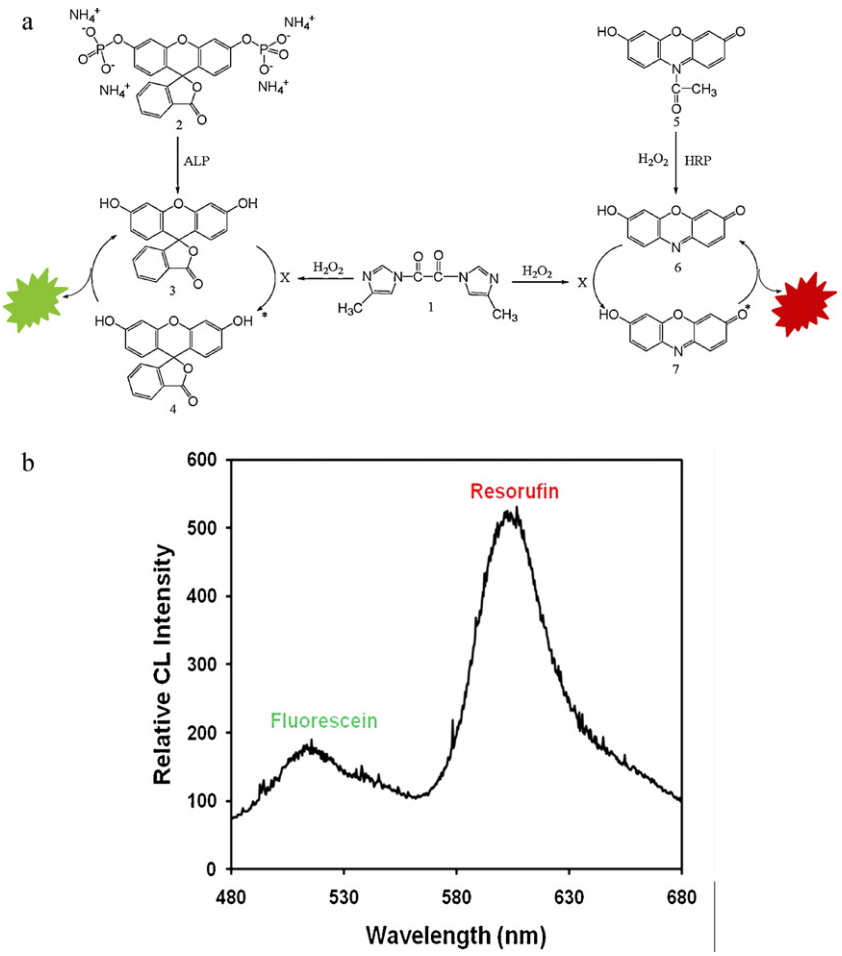

Fig. 1. (a) Reaction mechanism capable of sensing fluorescein and resorufin formed from two different enzyme reactions using ODI CL detection. (1) ODI formed from the reaction of TCPO and 4MImH, (2) FDP, (3) ground state fluorescein, (4) excited state fluorescein, (5) Amplex Red, (6) ground state resorufin, (7) excited state resorufin, (X) high-energy intermediate capable of transferring energy to FDP as well as resorufin. (b) ODI CL spectrum observed from a mixture of fluorescein and resorufin using a CCD spectrometer.

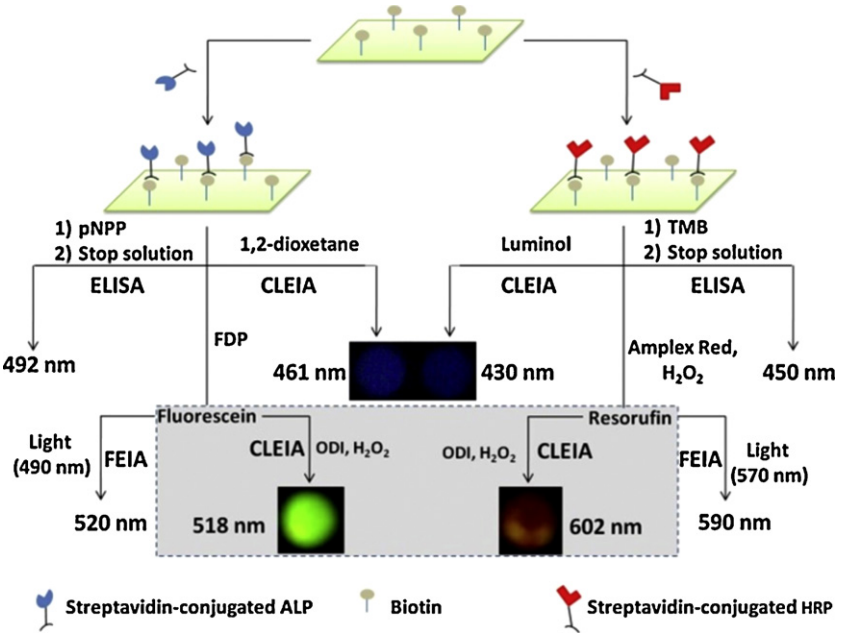

Fig. 2. Differences of ODI CLEIAs and conventional EIAs such as ELISA, FEIA, luminol CLEIA, and 1,2-dioxetane CLEIA.

conjugated with biotin were mixed in a 1.5-ml centrifuge tube and incubated in a refrigerator for 18 h. After decanting supernatant liquid in the centrifuge tube using a magnetic separator at room temperature, 125 μl of streptavidin-conjugated ALP (or HRP) was inserted into the centrifuge tube and incubated at a refrigerator for 30 min. After decanting supernatant liquid in the centrifuge tube using a magnetic separator at room temperature, immuno-complexes in the tube were washed 3 times using AFP EIA washing buffer (0.1 M Tris/HCl buffer, containing magnesium and zinc chloride, a surfactant and 0.05% sodium azide) purchased from Immunometrics. After washing, 250 μl of working solution containing 10 μM FDP was inserted into the centrifuge tube containing immuno-complexes bound with streptavidin-conjugated ALP and incubated for 25 min at 37 °C. Also, 250 μl of working solution containing the mixture (10 μM Amplex Red and 1.0 mM H_2O_2) was inserted into the centrifuge tube containing immuno-complexes bound with streptavidin-conjugated HRP and incubated for 25 min at 37 °C. After the incubation, 10 μl of fluorescein (or resorufin) added in a 12 mm × 75 mm borosilicate test tube was inserted into a sample holder of Lumat 9507 Luminometer with two dispensers. When the start button was pressed, the test tube containing fluorescein (or resorufin) moved into the detection area. 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, formed from the reaction of 10 μM TCPO and 50 μM 4MImH, into the test tube through the second dispenser, relative ODI CL intensity was measured immediately for 0.5 s with an interval of 0.1 s.

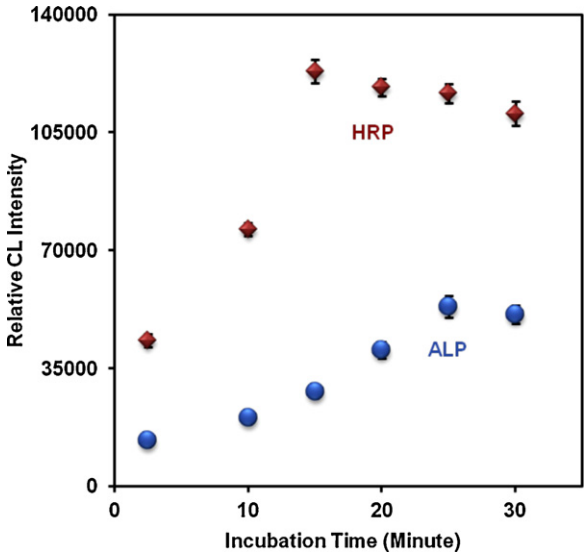

Fig. 3. Kinetics of ODI CLEIAs in the presence of HRP and ALP. Concentrations of streptavidin-conjugated ALP and streptavidin-conjugated HRP were 0.5 μg/ml. The experimental conditions used were the same as those in Table 1.

3. Results and discussion

3.1. Differences between ODI CLEIAs and conventional EIAs using interaction between biotin and streptavidin-conjugated HRP (or ALP)

As shown in Fig. 2, different emitted light from ODI CLEIAs using HRP and ALP can simultaneously quantify and screen two different markers in a single sample even though it is difficult to analyze independently the different markers using conventional CLEIAs (e.g., 1,2-dioxetane CLEIA, luminol CLEIA) which emit only blue light. As shown by the diagram in Fig. 2, 100 μl of streptavidin-conjugated HRP or streptavidin-conjugated ALP was incubated first with biotin coated on the surface of a strip-well for 1 h at 36.5 °C. Each strip-well containing immunocomplexes was washed 3 times with 10 mM Tris–HCl buffer solution (pH 8.5) containing 0.05% Tween 20. An appropriate substrate (100 μl) needed in each EIA was added into the washed strip-well and incubated for 150 s at room temperature (21 ± 2 °C). Finally, each product formed from the reaction of the substrate and HRP (or ALP) in the strip-well was quantified with an appropriate instrument described in Section 2.2.1. As shown in Fig. 2, the emission maxima of fluorescein (518 nm) and resorufin (602 nm) generated by the ODI CLEIAs are different from those (520 and 590 nm) measured in fluorescent enzyme immunoassays (FEIAs) because the property of high-energy intermediate (X), capable of transferring energy to fluorescent dyes formed form the rapid reaction of ODI and H_2O_2, differs from that of a light source such as a laser or Xenon lamp (Park et al., 2011b). In other words, an apparent difference between ODI CL and fluorescence is that fluorescein and resorufin excited by X formed by the ODI CL reaction emit strongly with inherently distinctive emission spectra, even though the excitation wavelength (490 nm) necessary to obtain strong fluorescence of fluorescein at 520 nm is different from that (570 nm) required to generate fluorescence of resorufin at 590 nm. Thus, we expect that a highly sensitive ODI CLEIA system will be more cost-effective and simpler than a FEIA system having relatively high background noise that results from operation of light sources using high-voltage power supplies.

Table 1 shows that the dynamic ranges of ODI CLEIAs are wider than those of other common EIAs. Based on these results and our previous reports (Choi et al., 2010; Lee et al., 2010; Park et al., 2011a), we expect that ODI CLEIAs having wide dynamic ranges will be more sensitive than conventional EIAs. In other words, rapid and simple ODI CLEIAs capable of simultaneously quantifying multiple markers are expected to be more cost-effective, more sensitive, and more selective than conventional EIAs capable of quantifying only a specific marker in a single sample.

The most important factor in developing highly sensitive ODI CLEIAs using HRP and ALP is the incubation time of substrates and immunocomplexes (e.g., biotin-bound streptavidin conjugated with HRP or ALP) for the simultaneous quantifications of two different biomarkers in a single sample. As shown in Fig. 3, relative CL intensities of fluorescein and resorufin depend on the incubation time between the immunocomplexes and substrates. The relative CL intensity of ODI CLEIA in the presence of HRP measured after a 15-min incubation was the highest and then begins to decrease slightly whereas the best incubation time determined in ODI CLEIA in the presence of ALP was 25 min. Relative CL intensity measured after a 30-min incubation in ODI CLEIA in the presence of ALP is similar to that obtained after a 25-min incubation. The optimum incubation time was 25 min when a 5-times lower concentration of H_2O_2 was used in ODI CLEIA in the presence of HRP versus that used in Fig. 3. The relative CL intensity measured after 25 min incubation for this condition was similar to or slightly lower than that obtained after the same incubation time under the conditions of Fig. 3. Also, it was possible to reduce the incubation time of ODI CLEIA in the presence of ALP and enhance relative CL intensity with the increase of FDP concentration. With the increase of FDP concentration, however, background noise measured with this condition was slightly higher than that observed under the conditions of Fig. 3. The results observed with various experimental conditions indicate that an appropriate incubation time between substrates and immunocomplexes in a strip-well can be readily determined to simultaneously quantify multiple markers in a single sample.

Table 1
Comparison between ODI CLEIA and conventional EIAs.

Streptavidin-conjugated ALP			Streptavidin-conjugated HRP		
Methods	Dynamic range[a]	R^2	Methods	Dynamic range[a]	R^2
ODI CLEIA[b]	0–500	0.993	ODI CLEIA[b]	0–1000	0.993
1,2-Dioxetane CLEIA[b]	0–125	0.990	Luminol CLEIA[b]	0–400	0.997
ELISA[b]	0–250	0.994	ELISA[b]	0–250	0.995
FEIA[b]	0–250	0.998	FEIA[b]	0–250	0.999

[a] μg/ml.
[b] Appropriate substrate (100 μl) for each EIA added in a strip-well containing immuno-complexes such as biotin-bound streptavidin conjugated with ALP or HRP was incubated for 150 s.

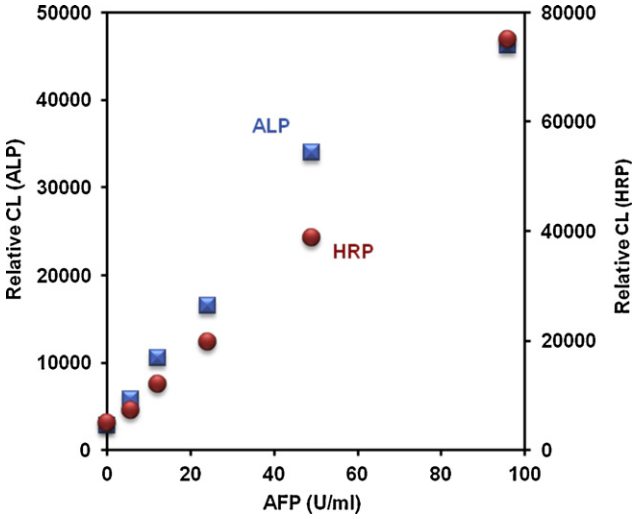

Fig. 4. Calibration curves for the quantification of AFP in human serum using ODI CLEIAs. Relative standard deviations (RSD, $n = 3$) of relative CL intensities measured in the presence of different AFP concentrations were lower than 5.0%.

3.2. Quantification of AFP using ODI CLEIAs with streptavidin-conjugated HRP or AFP

Based on the results shown in Table 1 and Fig. 3, ODI CLEIAs using streptavidin-conjugated ALP and streptavidin-conjugated HRP were developed to quantify alpha-fetoprotein (AFP), a tumor biomarker, in human serum by modifying an ELISA kit purchased from Immunometrics (UK) Ltd. Procedures for these ODI CLEIAs are described in detail in Section 2.2.1.1. As shown in Fig. 4, the dynamic range (0–96 U/ml, $R^2 = 0.9963$) of the calibration curve determined by ODI CLEIA in the presence of HRP for the quantification of AFP was wider than that (0–49 U/ml, $R^2 = 0.9974$) obtained by ODI CLEIA in the presence of ALP. However, the limit of detection (LOD = Background noise + 3σ = 0.84 U/ml) of the former was slightly higher than that (LOD = 0.72 U/ml) of the latter where σ is the standard deviation of average background noise ($n = 20$). In addition, we performed recovery tests for two ODI CLEIAs. Three different concentrations (20, 40, and 80 U/ml) of AFP were prepared by dilution of an AFP standard (National Institute for Biological Standards and Control, NIBSC) in human serum used as "0" calibrator purchased from Monobind. Each AFP diluted was spiked into a human serum containing 5.6 U/ml at a 1:1 volume ratio. Three different mixtures were used to determine recoveries of two different ODI CLEIAs. The range of recovery of ODI CLEIA using HRP was 91–108%. Also, the range of recovery of ODI CLEIA using ALP was 94–105%. These results indicate that two different ODI CLEIAs can be applied to quantify AFP with acceptable accuracy and precision.

4. Conclusion

In conclusion, the results observed in this research indicate that multi-ODI CLEIAs independently occurring in a single sample can be applied as a unique analytical method capable of simultaneously quantifying and screening multiple markers without complicated and expensive separation techniques. Therefore, we expect that ODI CLEIA with a highly sensitive detection system (e.g., cooled CCD spectrometer, multiple photomultiplier tubes having different filters used as a detector for multi-color flow cytometry) can rapidly and simultaneously quantify and screen multiple markers, which will be of great interest to various research fields such as biochemistry, clinical chemistry, environmental toxicology, molecular biology, and pathology.

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