Talanta 116 (2013) 403–408

Contents lists available at ScienceDirect

Talanta

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

Role of Triton X-100 in chemiluminescent enzyme immunoassays capable of diagnosing genetic disorders

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ARTICLE INFO

Article history:
Received 24 April 2013
Received in revised form
6 June 2013
Accepted 7 June 2013
Available online 15 June 2013

Keywords:
Chemiluminescent enzyme immunoassay
Triton X surfactants
Gender disorders
1,1′-Oxalyldiimidazole chemilumescence
Peroxyoxalate

ABSTRACT

The use of Triton X surfactants in developing 1,1′-oxalylimidazole chemiluminescent enzyme immunoassays (ODI CEIs) with extended linear response range for the quantification of unconjugated estriol (uE3), alpha-fetoprotein (AFP), and human chorionic gonadotropin (hCG) is reported for the first time. The wider linear dynamic range in ODI CLEIA results from Triton X series (e.g., Triton X-100, -114, -405, -705) acting as an inhibitor in the interaction between Amplex Red (hydrophobic substrate) and horseradish peroxidase (hydrophilic enzyme) to produce resorufin (hydrophobic fluorescent dye). Triton X-100 acts as the appropriate inhibitor in ODI CLEIA. The maximum concentrations of AFP and hCG quantified with sandwich ODI CLEIA in the presence of Triton X-100 were 8 times higher than when analyzed with the same system in the absence of Triton X-100. In addition, the lowest concentration of uE3 determined using competitive ODI CLEIA in the presence of Triton X-100 was 20 times lower than that measured with competitive ODI CLEIA in the absence of Triton X-100. These results indicate that rapid quantification of AFP, uE3, and hCG using cost effective and highly sensitive ODI CLEIAs in the presence of Triton X-100 can be applied as an accurate, precise, and reproducible method to diagnose genetic disorders (e.g., trisomy 18 and trisomy 21) in fetuses.

1. Introduction

Enzyme immunoassays (EIAs), developed to solve critical problems of radioimmunoassays related to safety and time-consuming analysis, have been used widely to screen and quantify specific markers in samples containing various components without complicated, expensive, or time-consuming procedures for marker separation [1–4]. Using the sensitivity and reproducibility of EIAs enhanced with developing various substrates capable of interacting with enzyme-conjugated immuno-complexes and detection methods (e.g., chemiluminescence, colorimeter, electrochemical, fluorescence), it is possible to early diagnose various diseases and rapidly monitor trace levels of toxic materials in the environment [1–4].

Unfortunately, however, the linear dynamic range obtained with highly sensitive sandwich EIAs often is too narrow to directly quantify the actual concentration of target marker (antigen) present in a sample without diluting the sample first with appropriate working solutions such as buffer and serum. For example, to quantify human serum albumin collected from a patient using a commercially available sandwich enzyme linked immunosorbent assay (ELISA) kit (e.g., Human Albumin ELISA Kit, Immunology Consultants Lab, Inc.), the collected sample needs to be diluted 10^5 times with appropriate working solution. Thus, there is a possibility of misdiagnosing a disease due to the inaccuracy and poor reproducibility of EIAs resulting from excessive dilution of the sample.

The response of competitive EIAs used when dual antibodies are not available is inversely proportional to the concentration of antigen, whereas the response of sandwich EIAs operated with a capture and detection-conjugated enzyme is proportionally enhanced with increasing antigen [5,6]. Therefore, the sensitivity and reproducibility of competitive EIAs generally are not as good as those of sandwich EIAs.

Concentrations of alpha-fetoprotein (AFP), unconjugated estriol (uE3), and human chorionic gonadotropin (hCG) tend to increase in the blood of women pregnant with a healthy fetus [7,8]. In particular, the level of hCG rises rapidly during the first trimester of pregnancy and then decreases slightly. For example, hCG levels (13,300–254,000 mIU/ml) during a 13–16 weeks gestation period are much higher than those in non-pregnant women (<5 mIU/ml). In general, AFP, uE3, and hCG concentrations in blood collected after 15–17 weeks gestation are quantified using EIAs to screen genetic disorders (e.g., trisomy 18, trisomy 21) of the fetus [7,8]. When the fetus is carrying

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http://dx.doi.org/10.1016/j.talanta.2013.06.008

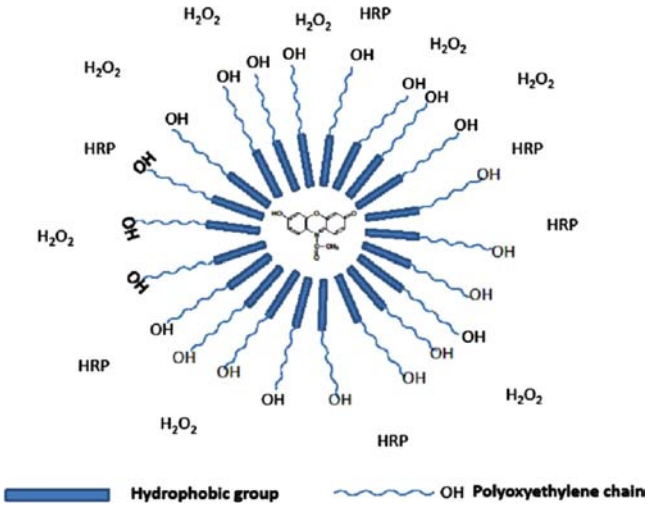


Fig. 1. Role of Triton X-series surfactant micelles in the interaction between hydrophobic molecules (e.g., Amplex Red) and hydrophilic molecules (e.g., HRP, H_2O_2).

the chromosomal defect that results in trisomy 21 (Down Syndrome), the levels of AFP and uE3 tend to be lower than for a normal fetus. 7 In contrast, the opposite result is observed with levels of hCG in trisomy 21. When a fetus has trisomy 18, however, uE3 and hCG levels are lower than those with a normal fetus but AFP levels can vary [8]. To accurately and precisely diagnose genetic disorders of fetuses, it is necessary to develop highly sensitive EIAs having wide linear response ranges that can rapidly quantify high concentrations of AFP and hCG as well as low concentration of uE3 in undiluted or limited-time diluted samples and thus replace current EIAs that have only narrow linear ranges and require multiple-time diluted samples to quantify the three markers.

Triton X-series (e.g., Triton X-100, -114, -405, -705), nonionic surfactants having a hydrophilic polyethylene oxide group and a hydrophobic group, are used widely to enhance the solubility of hydrophobic materials in aqueous solution or extract hydrophobic bimolecular from aqueous samples [9–11]. Substrates commonly used in EIAs (e.g., Luminol, Amplex Red, 1,2-Dioxetane, Fluorescein Diphosphate (FDP), *p*-Nitrophenyl Phosphate, 3,3′,5,5′-Tetramethylbenzidine) are hydrophobic compounds whereas alkaline phosphatase (ALP) and horseradish peroxidase (HRP) commonly used as enzymes in EIAs are hydrophilic biomolecules. Therefore, it can be expected that Triton X surfactants will interfere with the interactions between substrates and enzymes in aqueous solution because they can sequester hydrophobic molecules in their micelles and prevent them from reacting with hydrophilic molecules as shown in Fig. 1.

Based on the present hypothesis, we have studied for the first time the role of Triton X-series surfactants in enzyme assays to quantify HRP and in 1,1′-oxalyldiimidazole chemiluminescent enzyme immunoassays (ODI CLEIAs) to analyze the three markers (AFP, hCG, uE3) used to diagnose genetic disorders in fetuses. Recently, we reported that cost effective and rapid ODI CLEIAs, having a relatively wide linear dynamic range, is more accurate, precise, sensitive, and reproducible than other EIAs such as ELISA, fluorescent enzyme immunoassays (FEIA), luminol CLEIA and 1,2-dioxetane CLEIA [12–15].

2. Experimental

2.1. Chemicals and materials

Triton X-100 (> 99%), Troton X-114 (> 99%), Triton X-405 (70% in H_2O), Triton X-705 (70% in H_2O), 30% H_2O_2, HRP, phosphate buffered saline (PBS, pH 7.4) solution, sodium azide and Tween 20 were purchased from Sigma (Saint Louis, MO, USA). hCG (Standard grade, > 3000 IU/ml) was purchased from EMD (Darmstadt, Germany). AFP standard (100.000 IU/ample, 1 IU = 1.21 ng) was purchased from the National Institute for Biological Standards and Control (Hertfordshire, UK). AFP-hCG-uE3 AccuBind VAST ELISA Kits and 0 calibrator for diluting standards and samples were purchased from Monobind (Lake Forest, CA, USA). Amplex Red was purchased from Cayman Chemical (Ann Arbor, MI, USA). 4-Methylimidazole (4MImH, 98%) was purchased from Alfa-Aesar (Ward Hill, MA, 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).

2.2. Measurement

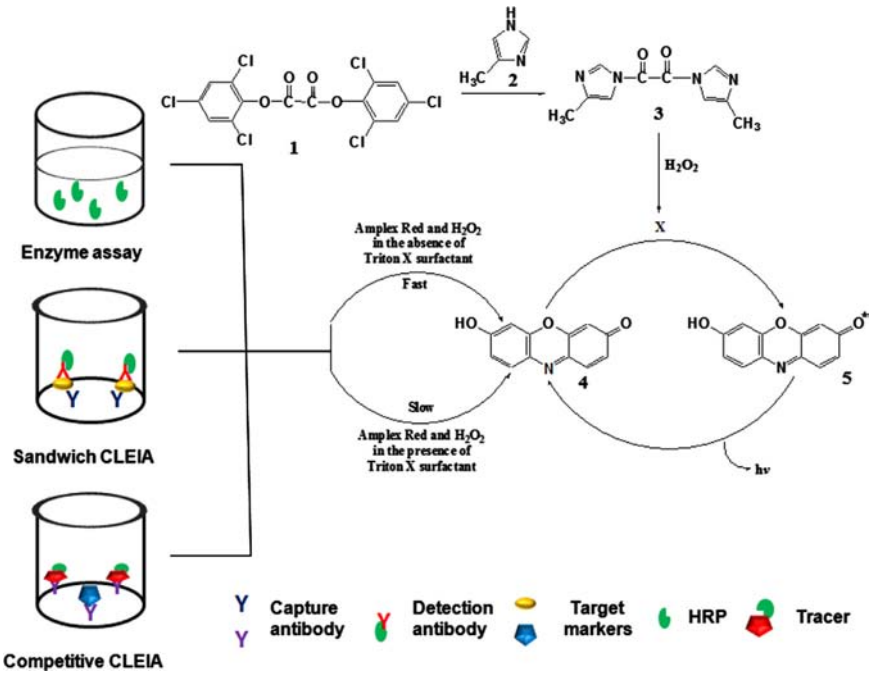
As shown in Scheme 1, ODI CL from enzyme assays used to quantify HRP and from ODI CLEIAs used to quantify AFP, hCG, and uE3 was generated based on the mechanism shown in Scheme 1 [12–14,16,17]. ODI, one of two ODI CL reagents, was prepared daily by reacting 10 μM TCPO and 50 μM 4 MImH in ethyl acetate. ODI reagents was stable for at least 8 h. The rest reagent (0.05 M H_2O_2) in ODI CL was prepared in isopropyl alcohol. The mixture of Amplex Red (10 μM) and H_2O_2 (1 mM) in PBS buffer (pH 7.4) in the absence or presence of Triton X-series was prepared as working solution in both enzyme assays and ODI CLEIAs (see Scheme 1). Resorufin, chemiluminescent dye, was formed from the reaction of Amplex Red and H_2O_2 in the presence of HRP as shown in Scheme 1. Resorufin excited by X formed from the reaction between ODI and H_2O_2, based on the concept of chemically-induced electron exchange luminescence mechanism, [17–19] emitted bright light.

2.2.1. Enzyme assay

Seven different standards (0, 0.01, 0.02, 0.04, 0.06, 0.08, 0.1 IU/ml) of HRP in PBS buffer (pH 7.4, 10 mM) were prepared with 10 IU/ml HRP stock solution. Each standard (0.5 ml) was added to a 1.5 ml centrifuge tube containing 0.5 ml of the mixture of Amplex Red and H_2O_2 in the absence or presence of a single Triton X surfactant. After mixing each centrifuge tube with a vortex mixer for 1 s, it was incubated for 5 min. After incubation, 10 μl of resorufin formed in each centrifuge tube was added to a 12×75 mm^2 borosilicate test tube. Each test tube was inserted into a sample holder of a Lumat 9507 Luminometer with two dispensers (Berthold, Inc.). When the start button was pressed, the test tube moved into the detection area. A volume (25.0 μl) of H_2O_2 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, the relative ODI CL intensity was measured immediately for 0.5 s with an interval of 0.1 s.

2.2.2. ODI CLEIAs

AFP-hCG-uE3 AccuBind VAST ELISA Kits were used to study the effect of Triton X-100 in ODI CLEIAs. Primary antibodies of AFP, hCG, and uE3 were coated on the surface of a strip-well. AFP and hCG in human serum were quantified using sandwich ODI CLEIAs in the absence or presence of Triton X-100. Solution volumes of 25 μl of AFP (or hCG) and 100 μl of AFP (or hCG) detection antibody-conjugated HRP were added to a strip-well and incubated for 30 min at room temperature (21 ± 2 °C). After washing the strip-well with PBS buffer solution containing 0.5% Tween 20, 100 μl of the mixture of Amplex Red and H_2O_2 in the absence or presence of Triton-X 100 was added to the strip-well and incubated for 15 or 20 min at room temperature. A volume (10 μl) of



Scheme 1. ODI CL enzyme assay and sandwich and competitive ODI CLEIAs in the absence and presence of Triton X series. **1**. TCPO, **2**. 4-Methylimidazole, **3**. ODI, **4**. Resorufin under the ground state, **5**. Resorufin under the excited state, **X**. high-energy intermediate capable of transferring energy to resorufin.

the resorufin formed in the strip-well was added to a 12×75 mm^2 borosilicate test tube and then ODI CL was measured using the same procedure described above for the enzyme assay.

uE3 in human serum was quantified using competitive ODI CLEIA in the absence or presence of Triton X-100. Solution volumes of 25 μl of uE3 and 100 μl of Tracer were added to the strip-well and incubated for 60 min at room temperature. After washing the strip-well with washing solution, 100 μl of the mixture of Amplex Red and H_2O_2 in the absence or presence of Triton-X 100 was added to the strip-well and incubated for 10 min at room temperature. Relative CL intensity of resorufin (10 μl) formed in the strip-well was measured using the same method described above.

2.2.3. *Comparison between conventional ELISA and ODI CLEIAs in the presence of Triton X-100*

In order to study the correlation between ELISA and ODI CLEIAs in the presence of 0.1 wt% Triton X-100 using AFP-hCG-uE3 AccuBind VAST ELISA Kits, various concentrations of samples containing hCG, AFP, or uE3 were prepared in human serum (0 calibrator purchased from Monobind, Inc.). Dynamic ranges of calibration curves obtained using ODI CLEIAs in the presence of 0.1 wt% Triton X-100 were different from those determined using conventional ELISAs. In order to solve the problems, for example, high concentrations of hCG and AFP, which can be quantified using sandwich ODI CLEIAs in the presence of 0.1 wt% Triton X-100, were diluted to be quantified using conventional sandwich ELISA. Also, relatively high concentration of uE3 prepared to be quantified using conventional competitive ELISA was diluted to be quantified using competitive ODI CLEIA in the presence of 0.1 wt% Triton X-100. Quantifications of hCG, AFP, and uE3 using conventional ELISAs and a microplate reader (InfiniteM 1000 of Tecan, Inc.) were performed based on the instructions provided from Monobind, Inc. In addition, the methods for quantifying hCG, AFP, and uE3 using ODI CLEIAs in the presence of 0.1 wt% Triton X-100 are described in 2.2.2.

3. Results and discussion

3.1. *Effect of Triton X-series in enzyme assay using ODI CL detection*

In order to develop sandwich CLEIAs with ODI CL detection capable of quantifying high concentration (activity) of AFP and hCG and competitive CLEIA with ODI CL detection capable of quantifying low concentration of uE3, first, we studied the effect of Triton X-series in enzyme assay with ODI CL detection capable of quantifying HRP based on the experimental procedure shown in Scheme 1. In other words, we studied whether enzyme assay in the presence of Triton X-series can quantify high activity of HRP, which cannot be quantified using enzyme assay in the absence of Triton-X surfactants.

As shown in Fig. 2(a), enzyme assay with ODI CL detection in the absence of Triton X surfactants can quantify lower activity of HRP than 0.01 IU/ml. This is because the concentration of resorufin, formed from the reaction of Amplex Red, H_2O_2, and HRP for a certain incubation time, is higher when no micelles exist. Thus, relative CL intensities measured in the absence of surfactant sharply increase by increasing HRP activity up to 0.02 IU/ml. Above 0.02 IU/ml, however, the relative CL intensity plateaus and then ultimately decreases due to self-quenching of the excess resorufin formed in the presence of higher HRP activity.

Fig. 2(a) also demonstrates that the linear range for analysis depends on the properties of the Triton X surfactants. For example, the linear dynamic range when using Triton X-100 (0.01–0.1 IU/ml, $R^2 = 0.9943$) is wider than when using the other Triton X surfactants, although the slope of its linear range is smaller than the slopes obtained with the other surfactants. These results indicate that the ability of Triton X-100 to interfere with the interaction of the hydrophobic reactant molecule (e.g., Amplex Red) and the hydrophilic reactant molecules (e.g., HRP, H_2O_2) is stronger than those of the other Triton X surfactants. The reasons that the ability of Triton X-100 is better than other Triton X-series may be because the number of micells formed in the presence of 1.0 wt% Triton X-100 are bigger than those formed in the presence of 1.0 wt% Triton

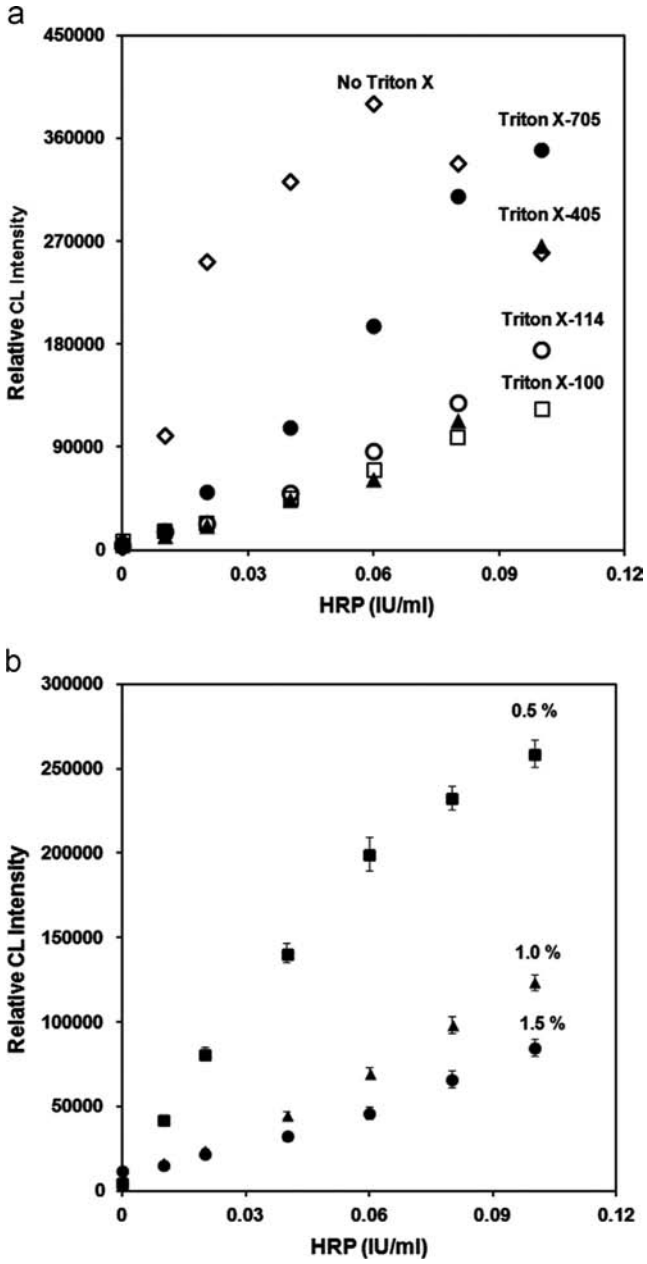


Fig. 2. (a) Effects of Triton X-series surfactants (1 wt%) in enzyme assay with ODI CL detection for the quantification of HRP. The error of each value was lower than 5.0%. (b) Concentration effect of Triton X-100 in enzyme assay with ODI CL detection for the quantification of HRP.

X-405 (or Triton X-705) in addition to the fact that polyoxyethylene chain ($n=9.5$) of Triton X-100 is longer than that ($n=7.5$) of Triton X-114 (see technical data sheet (http://www.dow.com/surfactants/products/octyl.htm) provided by DOW Chemical Company).

Fig. 2(b) shows that the linear range obtained when using Triton X-100 depends on its concentration, or more specifically, the concentrations of its micelles. For 0.5 wt% Triton X-100, the maximum HRP activity of the linear range (0–0.06 IU/ml, $R^2=0.9937$) is lower than those obtained with 1.0 and 1.5 wt% Triton X-100 because the lower concentration leads to fewer micelles being formed. The slope of the linear range (0.04–0.1 IU/ml, $R^2=0.9942$) for 1.5 wt% Triton X-100 is smaller than for the two lower concentrations. In addition, background noise in the presence of 1.5 wt% Triton X-100 was about three times higher than when no Triton X-100 was present. This is because background noise (self emission of X formed from the reaction of ODI and H_2O_2) in ODI CL is enhanced in the presence of surfactants such as Triton X-100. The results shown in Fig. 2 (b) is consistent with those reported that Nagashima research group [20] even though they did not use Triton X series. They described that the enhancement or reduction of peroxyoxalate chemiluminescence is dependent on chemical and physical properties as well as concentration of a surfactant based on the results with 15 different surfactants (e.g., 6 anionic-, 2 cationic-, 2 Zwitterionic-, 5 nonionic-type surfactants) [20]. Therefore, the reproducibility obtained with 1.5 wt% Triton X-100 was worse than when using the lower 1.0 wt% surfactant concentration. Also, we determined that the recovery of 0.02 IU/ml HRP when added to an aqueous solution containing 0.06 IU/ml was diminished in the presence of 1.5 wt% Triton X-100 (90.8%, $N=8$) versus its higher recovery (102.4%, $N=5$) when using 1.0% surfactant concentration. Thus, 1.0 wt% Triton X-100 was the optimum concentration to use to quantify relatively high concentrations of HRP using ODI CL detection.

3.2. *Effect of Triton X-100 in sandwich ODI CLEIAs*

The optimum condition of sandwich ODI CLEIA in the absence of Triton X-100 for the quantification of AFP (or hGC) was determined based on previous research (e.g., incubation time, temperature) related to sandwich ODI CLEIAs that we reported [13,14]. Then, a certain concentration of Triton X-100 was added when HRP bound with target on the surface of strip-well interacted with Amplex Red in the presence of H_2O_2 (see Scheme 1).

Fig. 3(a) shows that 1.0 wt% Triton X-100 is too high a surfactant concentration to use for detecting hCG with ODI CLEIA, because the HRP-conjugated immuno-complex coated on the surface of the strip-well has difficulty interacting with Amplex Red and H_2O_2 dissolved in aqueous solution. In other words, the interaction between Amplex Red in aqueous solution and HRP immobilized on the surface of the strip-well is not as active as that between Amplex Red and HRP both freely moving in aqueous solution. At the much lower Triton X-100 concentration of 0.1 wt%, however, the linear dynamic range (0–1000 ng/ml, $R^2=0.9989$) for quantifying hCG using sandwich ODI CLIEA was eight times wider than when no Triton X-100 was used (0–125 ng/ml, $R^2=0.9999$; Fig. 3(a)). This finding suggests that it is possible to quantify hCG concentrations higher than 3000 mIU/ml using sandwich ODI CLEIA in the presence of 0.1 wt% Triton X-100 because we prepared our standards with hCG (> 3000 IU/mg) purchased from EMD. Finally, with 0.025 wt % Triton X-100 the linear dynamic range (0–500 ng/ml, $R^2=0.9976$) in sandwich ODI CLEIA was narrower than when using a 0.1 wt% concentration. Based on these results, 0.1 wt% Triton X-100 was used to obtain wide linear calibration curves to quantify AFP and uE3.

To study the accuracy and precision of ODI CLEIA using 0.1% Triton X-100 to quantify high concentrations of hCG, two serums containing different concentrations of hCG were mixed together at a 1:1 volume ratio. The good recoveries (e.g., within $\pm 6\%$, Table 1) indicate that ODI CLEIA using 0.1 wt% Triton X-100 can quantify high hCG concentrations with excellent reproducibility. Table 2 shows that sandwich ODI CLEIA in the presence of 0.1 wt% Triton X-100 can be applied as a new immunoassay method for quantifying hCG in human serum. This is because results obtained using sandwich ODI CLEIA in the presence of 0.1 wt% Triton X-100 were consistent with those determined using conventional ELISA with acceptable error ranges. These results indicate that hCG samples collected during a 15–17 week gestation period do not need to be diluted as much as 1000 times if ODI CLEIA in the presence of 0.1 wt% Triton X-100 is applied to quantify hCG in human serum.

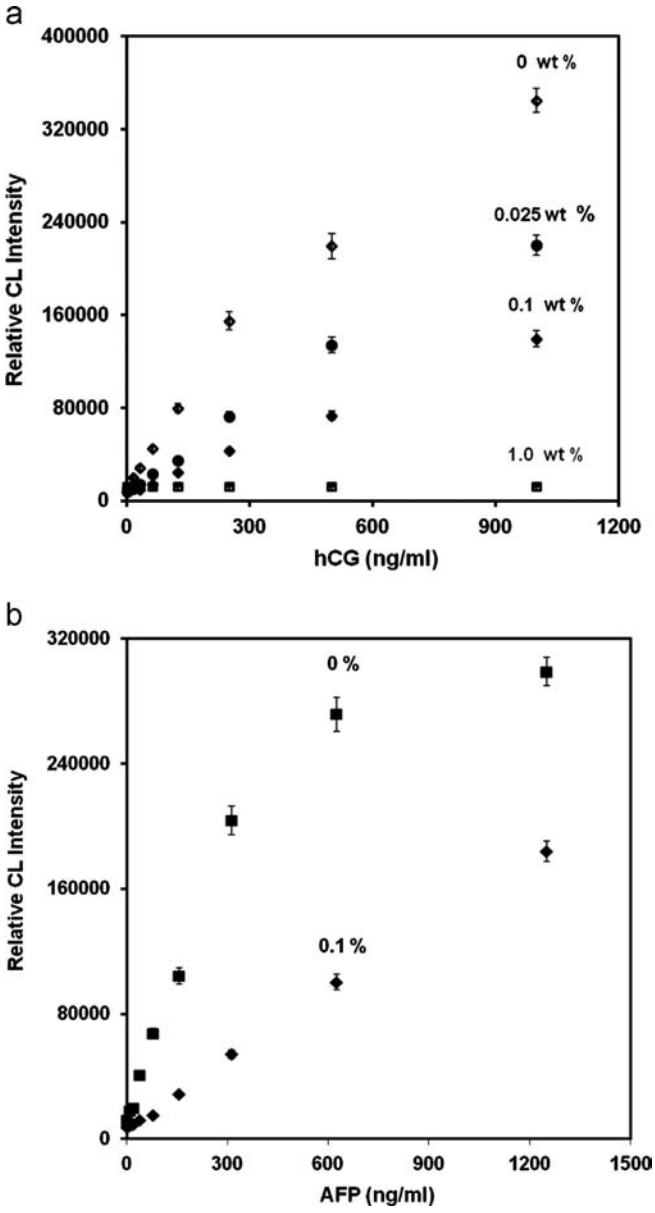


Fig. 3. (a) Concentration effect of Triton X-100 in sandwich ODI CLEIA for the quantification of hCG. (b) Concentration effect of Triton X-100 in sandwich ODI CLEIA for the quantification of AFP.

Table 1
Accuracy, precision, and recovery test ($N=8$) of ODI CLEIA in the presence of 0.1% Triton X-100.

Sample number	hCG (ng/ml)	hCG added (ng/ml)	Expected (ng/ml)	Results (ng/ml)	Accuracy (%)	Precision (%)	Recovery (%)
1	250	100	175	168.6	3.7	4.9	96.3
2	500	100	300	289.8	3.4	4.4	96.6
3	500	200	350	368.9	5.4	5.7	105.4
4	1000	400	700	687.0	1.9	4.2	98.1

As shown in Fig. 3(b), the linear dynamic range (0–1250 ng/ml, 0–1033 IU/ml), $R^2=0.9987$) for quantifying AFP in human serum using ODI CLEIA and 0.1 wt% Triton X-100 was four times wider than when no surfactant was used (0–312.5 ng/ml (0–258 IU/ml), $R^2=0.9962$), even though the incubation time (20 min) of the former was longer than that of the latter (15 min). The highest AFP concentration quantified with the linear calibration curve obtained after the 15 min incubation of Amplex Red, H_2O_2, and HRP in the presence of 0.1 wt% Triton X-100 was extended up to 2500 ng/ml (2066 IU/ml). Therefore, this finding suggests that very high concentrations of AFP in undiluted samples can be quantified with a linear calibration curve using sandwich ODI CLEIA in the presence of 0.1 wt% Triton X-100 upon reducing the incubation time of Amplex Red, H_2O_2, and HRP. We confirmed that ODI CLEIA in the presence of 0.1 wt% Triton X 100 can quantify AFP with acceptable recovery (e.g., within $\pm$7%). In addition, Table 2 shows the good correlation between conventional ELISA and sandwich CLEIA in the presence of 0.1 wt% Triton X-100 for the quantification of AFP in human serum.

3.3. *Effect of Triton X-100 in competitive ODI CLEIAs*

The optimum condition of competitive ODI CLEIA in the absence of Triton X-100 for the quantification of uE3 was determined based previous research (e.g., incubation time, temperature) related to competitive ODI CLEIAs we reported in the past [12]. Then, a certain concentration of Triton X-100 was added when HRP bound with tracer on the surface of strip-well interacted with Amplex Red in the presence of H_2O_2.

uE3 in human blood is quantified using competitive ODI CLEIA instead of sandwich ODI CLEIA (see Scheme 1). Thus, as shown in Fig. 4, the sensitivity of ODI CLEIA in the presence of 0.1 wt% Triton X-100 for quantifying uE3 is at least 10 times better than when no surfactant is present because HRP conjugated with tracer coated on the surface of the strip-well has difficulty interacting with Amplex Red and H_2O_2 in the presence of 0.1 wt% Triton X-100. In other words, competitive ODI CLEIA (0.125–2.5 ng/ml) using 0.1 wt % Triton X-100 can quantify 20 times lower concentrations of uE3 than that (2.5–20 ng/ml) with no surfactant present, whereas sandwich ODI CLEIAs in the presence of 0.1 wt% Triton X-100 can quantify eight times higher concentrations of APF and hCG than that with no surfactant as shown in Fig. 3. Also, in quantifying trace levels of uE3 in human serum, the incubation time of the mixture (HRP-conjugated tracer, Amplex Red, and H_2O_2) was as short as 10 min because the uE3 is quantified with excess HRP-conjugated tracer (uE3) using competitive ODI CLEIA. The dynamic range and sensitivity of competitive ODI CLEIA in the presence of 0.1 wt% Triton X-100 were dependent on the incubation time of Amplex Red and H_2O_2 in the presence of HRP-conjugated tracer. For example, 2.5 ng/ml uE3 was not detected with 5-minute incubation because the concentration of HRP-conjugated tracer was too low to produce enough resorufin from Amplex Red and H_2O_2. As another example, 0.125 ng/ml uE3 after 15 min incubation was not quantified because Amplex Red formed in the reaction was so high that relative CL intensity was as high as that measured in the absence of uE3. Table 2 indicates that competitive ODI CLEIA in the presence of 0.1 wt% Triton X-100, having good recovery (e.g., within $\pm$7%), can be applied as an appropriate immunoassay method for the quantification of trace levels of uE3 based on the good correlation between conventional ELISA and ODI CLEIA in the presence of 0.1 wt% Triton X-100 (see Table 2). Therefore, quantification of uE3 with competitive ODI CLEIA and 0.1% Triton X-100 will be useful for screening genetic disorders in fetuses, because uE3 levels in fetuses with trisomy 18 or trisomy 21 are lower than in normal fetuses.

4. Conclusion

The effects of Triton X surfactants on the interactions between enzyme-conjugated immuno-complexes and substrates in ODI CLEIAs were studied for the first time. Due to the shielding/

Table 2
Correlation between conventional ELISA and ODI CLEIA in the presence of Triton X-100.

Biomarker	Method	Samples (ng/ml)				
		1	2	3	4	5
hCG	ELISA[a]	19.5 ± 0.7	45.4 ± 2.3	105 ± 5.2	375.3 ± 20.4	765 ± 32.5
	ODI CLEIA[b]	20.2 ± 0.5	44.9 ± 1.9	102 ± 4.5	380.4 ± 14.2	752.2 ± 18.4
AFP	ELISA[c]	15.4 ± 0.6	60.5 ± 2.2	128 ± 7.5	422 ± 22.9	780.4 ± 27.5
	ODI CLEIA[d]	15.2 ± 0.6	59.4 ± 2.6	124 ± 4.7	409.8 ± 17.6	809.9 ± 19.5
uE3	ELISA[e]	3.2 ± 0.1	4.8 ± 0.3	6.5 ± 0.4	12.5 ± 0.7	17.2 ± 0.8
	ODI CLEIA[f]	3.0 ± 0.2	4.9 ± 0.2	6.2 ± 0.3	12.9 ± 0.6	16.6 ± 0.6

[a] Samples (2–5) were diluted with 0 calibrator due to the narrow dynamic range (0–40 ng/ml).
[b] Dynamic range: 0–1000 ng/ml).
[c] Samples (3–5) were diluted with 0 calibrator due to the narrow dynamic range (0–100 ng/ml).
[d] Dynamic range: 0–1250 ng/ml.
[e] Dyanmic range: 2.5–20 ng/ml.
[f] Samples (1–5) were diluted with 0 calibrator because dynamic range of highly sensitive competitive ODI CLEIA was 0.125–2.5 ng/ml. Each experiment was repeated three times ($n=3$).

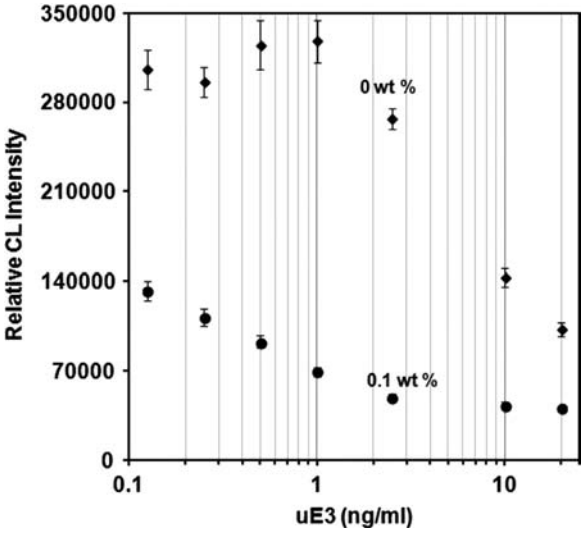


Fig. 4. Concentration effect of Triton X-100 in competitive ODI CLEIA for the quantification of uE3.

sequestering effect of Triton X-100 micelles on the reactions between hydrophobic molecules (e.g., Amplex Red) and hydrophilic molecules (e.g., HRP) in aqueous solution, we obtained wider linear dynamic ranges that are then capable of quantifying very high concentrations of target markers (e.g., AFP, hCG) in sandwich ODI CLEIAs as well as trace levels of target markers (e.g., uE3) in competitive ODI CLEIAs. Based on our results, we expect that sandwich ODI CLEIAs developed using Triton X-100 can be applied to rapidly quantify higher concentrations of target markers in samples without time-consuming sample preparation such as the dilution of sample with working buffer (or serum). In addition, competitive ODI CLEIA using Triton X-100 can rapidly quantify lower concentrations of target markers than without the surfactant.

In colusion, we expect that high concentrations of target markers, which cannot be quantified with conventional sandwich EIAs in samples collected from living organisms or the environment, can be quantified without sample dilution using sandwich ODI CLEIA in the presence of 0.1 wt% Triton X-100. Also, trace levels of target markers, which cannot be quantified with conventional competitive EIAs, in samples can be quantified with higly sensitive competitive ODI CLEIA in the presence of 0.1 wt% Triton X-100. Therefore, ODI CLEIA in the presence of Triton X-100 can be applied to various research fields such as biochemistry, clinical chemistry, food safety, molecular biology, and toxicology. In addition, we expect that Triton X-100 can be applied to other EIAs (e.g., ELISA, FEIA, luminol CLEIA, 1,2-dioxetane CLEIA) because substrates (e.g., Luminol, Amplex Red, 1,2-Dioxetane, Fluorescein Diphosphate (FDP), *p*-Nitrophenyl Phosphate, 3,3′,5,5′-Tetramethylbenzidine) used in other EIAs are also hydrophobic compounds.

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