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A convenient homogeneous enzyme immunoassay for estradiol detection

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Abstract.

A convenient homogeneous enzyme immunoassay for estradiol is described. Unlike heterogeneous immunoassays, which require time-consuming separation steps or expensive automated systems, homogeneous immunoassays, wherein all reagents are freely suspended in bulk solution, can be simple and fast without costly instrumentation. The key component of this assay system, an estradiol–reporter enzyme conjugate, was prepared by covalently binding β-estradiol-6-(O-carboxymethyl)oxime to glucose-6-phosphate dehydrogenase (G6PDH) by an *N*-hydroxysuccinimide-enhanced, carbodiimide-mediated coupling reaction. The estradiol–G6PDH activity can be repressed up to 46% upon anti-estradiol antibody binding. The lower detection limit of the assay is 1 nM estradiol in aqueous solution, and the standard curve is linear on logit-log scale-up to 6.7 μM estradiol. A detection limit of 11.5 nM in estradiol-spiked human serum samples suggests the feasibility of applying this assay to monitor estradiol levels for the prediction and prevention of ovarian hyperstimulation syndrome.

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1. Introduction

The increase in infertility and the rising demand of infertility services have stimulated the rapid development of assisted reproductive techniques (ARTs) in the past few decades [1]. Most of the ART methods involve ovarian stimulation, which could cause ovarian hyperstimulation syndrome (OHSS), an iatrogenic complication characterized by obvious multiple follicular enlargement, hemoconcentration, and intravascular volume depletion to various degrees [2],[3]. Severe cases of OHSS, though rare, can be life threatening [4],[5]. Estradiol level is a commonly used marker when evaluating patient risk for developing OHSS. It has been observed that nearly all the at-risk patients have elevated estradiol levels [6],[7]. One study further showed that 85% of the patients who developed OHSS had an estradiol level above 11 nM (within this group, a few patients had over 20 nM) as compared with 6 nM for normal patients on the 11th day of ovarian stimulation [8]. It has also been suggested that reducing the estradiol level by coasting (discontinuing the stimulating drug until the estradiol level drops) helps to prevent OHSS [9]. Therefore, an economic and convenient estradiol assay with a suitable detection range is valuable for the prediction and prevention of OHSS [10].

Currently, radioimmunoassay (RIA) is the principle method for estradiol detection [11]. Although RIA provides high sensitivity and specificity, it requires handling of radioactive materials. Other heterogeneous immunoassays such as enzyme-linked immunosorbent assays [12],[13], fluoroimmunoassays [14],[15], chemiluminescent immunoassays [16–18], and electrochemiluminescent immunoassays [19] have also been developed as alternative methods. However, these heterogeneous assays require tedious and time-consuming washing and separation steps [13]. Automated systems have been developed to make assays more convenient, but the required instruments are costly. In contrast, the enzyme-multiplied immunoassay technique (EMIT®; Siemens, New York City, NY, USA), a homogeneous enzyme immunoassay (EIA) method [20], is simple and fast and does not require expensive instrumentation [21].

Similar to many immunoassay approaches, a key component of this homogeneous EIA technique is a reporter enzyme that is responsible for signal generation. However, the homogeneous EIA method used in this work, which is based on EMIT®, is distinguished by its reliance on antibody (Ab)-induced inhibition of the reporter enzyme. The working principle of this EIA is illustrated in Fig. 1. When Ab binds to analyte conjugated to the reporter enzyme, a physical blockage and/or a conformational change of the enzyme active site occurs, hindering the substrate from accessing the active site or reducing intrinsic catalytic

Abbreviations: EC, Enzyme Commission; HRP, Horseradish peroxidase; LDS, Lithium dodecyl sulfate; MOPS, 3-(N-morpholino)propanesulfonic acid; SDS, Sodium dodecyl sulfate.

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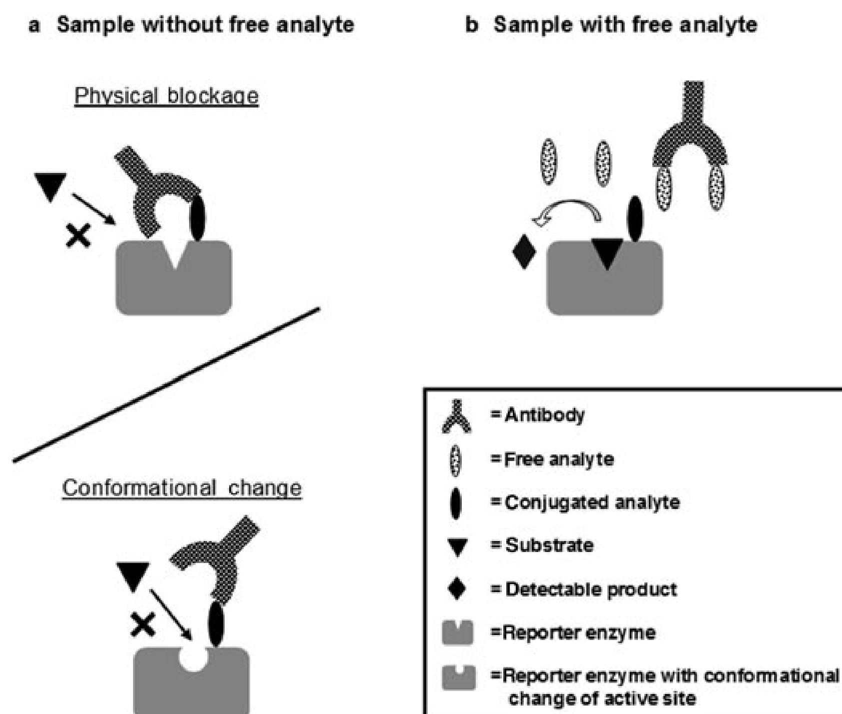

Fig. 1. A general schematic of the homogeneous EIA working principle. (a) When the antibody (Ab) binds to the analyte conjugated to the reporter enzyme, a physical blockage and/or a conformational change of the enzyme active site occurs, thereby repressing enzyme activity. (b) When introduced, free analyte competes for Ab binding sites and therefore partially prevents this repression.

activity. As a result, the reporter enzyme activity is repressed. When introduced with Ab, free analyte competes for Ab binding, thereby partially preventing repression. Because the number of Ab sites available to inhibit the enzyme depends on the concentration of free analyte, the measurable reporter enzyme activity is related to the concentration of the free analyte [20].

Although homogeneous EIA methods for estradiol detection using lysozyme [22] and β-galactosidase (β-gal) [23] have been developed, systems using these reporter enzymes are not suitable for serum samples. Lysozyme is a poor choice because bacterial cells serve as substrates that agglutinate serum [24]. The drawback to β-gal is that the enzyme is subject to binding by inhibitory anti-β-gal Ab present in human serum. As a result, systems with either of these reporter enzymes are susceptible to interference and should not be used for serum samples [25],[26]. A reporter enzyme that was found to be compatible with serum sample is glucose-6-phosphate dehydrogenase (G6PDH; Enzyme Commission (EC), 1.1.1.49) from *Leuconostoc mesenteroides* [24]. The cofactor of this bacterial enzyme can be β-nicotinamide adenine dinucleotide (NAD^+) or nicotinamide adenine dinucleotide phosphate ($NADP^+$) [27]; however, the human G6PDH is specific only for $NADP^+$ [28]. Therefore, the use of NAD^+ as the cofactor in the G6PDH-based homogeneous EIA can prevent interference from the endogenous G6PDH in human serum. The 109 kDa homodimer [29] is the most commonly used homogeneous EIA reporter enzyme. Automated homogeneous EIAs utilizing G6PDH have been developed and commercialized for over 30 different analytes [30–32]. Two earlier reports proposed creating a homogeneous estradiol-EIA using G6PDH [22],[32]. However, these reports either described unsuccessful results or presented few experimental details and little actual data. Therefore, there remains a need for a homogeneous EIA system for estradiol detection based on the convenient standard reporter enzyme, G6PDH.

Human serum is a complex body fluid that contains abundant proteins (*e.g.*, albumin, glycoproteins, and enzymes) [33] and biochemicals (*e.g.*, amino acids, metabolites, and carbohydrates) [34]. The interference from these serum constituents may cause significant problems in EIAs. Several separation methods (*e.g.*, extraction, dialysis, and ultrafiltration) [35],[36] have been proposed to purify serum samples prior to the performance of the immunoassay; however, these separation methods require specialized equipment and tedious and time-consuming procedures. When EIAs based on dehydrogenase enzymes are employed, the interference from the endogenous dehydrogenases can lead to false-positive responses. In human serum, lactate dehydrogenase (LDH) is the major interfering endogenous enzyme [37] when G6PDH is used as the reporter enzyme, as both LDH and G6PDH use NAD^+ as the coenzyme. In this study, we incorporated an endogenous LDH inhibitor, oxalic acid (OxA) [38],[39], to the assay reagents along with simple filtration steps to eliminate the interference.

This work describes the successful construction of a homogeneous estradiol-EIA using G6PDH as reporter enzyme. The assay has a short analysis time (13 Min), and requires only mixing and measuring (without washing and separation). These

attributes make the homogeneous estradiol-EIA an attractive estradiol monitoring method for the prediction and prevention of OHSS.

2. Materials

β-Estradiol-6-(O-carboxymethyl)oxime (E2–6-CMO), β-estradiol, anti-17β-estradiol Ab (anti-estradiol Ab) produced from rabbit (whole antiserum, lyophilized powder), G6PDH from *L. mesenteroides* (lyophilized powder), *N*,*N*-dimethylformamide (DMF; anhydrous, 99.8%), D-glucose-6-phosphate sodium salt (G6P), NAD^+, magnesium chloride hexahydrate ($MgCl_2$), gelatin from porcine skin (type A), sodium phosphate monobasic salt, sodium phosphate dibasic salt, Trizma Preset crystals (pH 8.0), sinapinic acid (SA), OxA, α-cyclodextrin, lactic acid (LA), and bovine serum albumin (BSA) were purchased from Sigma–Aldrich (St. Louis, MO, USA). 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS), and Luminol Enhancer Solutions were purchased from Thermo Fisher Scientific (Rockford, IL, USA). Amersham Biosciences PD-10 desalting columns and nitrocellulose membranes were obtained from GE Healthcare (Piscataway, NJ, USA). Microcon YM-30 centrifugal filters were obtained from Millipore (Billerica, MA, USA). Methanol, acetonitrile (ACN), trifluoroacetic acid (TFA), micro ultraviolet (UV) cuvettes, Whatman Anotop™ sterile 0.1 μm disposable syringe filters, luer-slip 5 mL disposable syringes, and Tween 20 were obtained from Fisher Scientific (Pittsburgh, PA, USA). NuPAGE® (Invitrogen, Carlsbad, CA, USA) Novex 4%–12% Bis–Tris gel, 3-(N-morpholino)propanesulfonic acid (MOPS) sodium dodecyl sulfate (SDS) running buffer (20x), NuPAGE® (Invitrogen Corporation, Carlsbad, CA, USA) lithium dodecyl sulfate (LDS) Sample Buffer (4x), and NuPAGE transfer buffer (20x) were purchased from Invitrogen (Carlsbad, CA, USA). Goat anti-rabbit Ab–horseradish peroxidase (HRP) was obtained from Bio-Rad (Hercules, CA, USA). Spectroscopic analyses were performed with an UV-visible spectrophotometer (model DT-1000-BT-CE; Analytical Instrument Systems, Flemington, NJ, USA).

3. Methods

3.1. Estradiol–G6PDH conjugation

The carboxyl group of E2–6-CMO at 10 mM was first activated by 20 mM EDC and 40 mM NHS in DMF for 2 H. The activated E2–6-CMO was added to G6PDH in 100 mM sodium phosphate buffer, pH 7.3, to give a final concentration of 1.1 μM [100 units per milliliter (U/mL)] G6PDH and 20% DMF. Three different E2–6-CMO concentrations (0.26, 0.52, and 0.78 mM) were used to prepare three different conjugate samples. The mixtures were allowed to react for 1–4 H to form the estradiol–G6PDH conjugate. After the reaction, the excess crosslinkers and E2–6-CMO were separated from protein by gel filtration with PD-10 (GE Healthcare) columns and three rounds of centrifugal filtration with Microcon YM-30 centrifuge filters (which were pretreated with 1% milk to minimize nonspecific adsorption). The conjugation and purification steps were conducted under ambient conditions.

3.2. Western blot analysis

Western blot analysis was performed according to the NuPAGE® (Invitrogen) technical guide. Briefly, protein samples were denatured in NuPAGE® (Invitrogen) LDS Sample Buffer at 70 °C for 10 Min. The denatured samples were loaded onto a Bis–Tris gel. Electrophoresis was conducted at 200 V for 55 Min in 3-(N-morpholino)propanesulfonic acid (MOPS) sodium dodecyl sulfate (SDS) running buffer. Then, the proteins were transferred to a nitrocellulose membrane in NuPAGE® (Invitrogen) transfer buffer by applying 30 V for 90 Min. Subsequently, the membrane was first incubated in 5% milk in Tris-buffered saline with 0.5% Tween (TBST) for 1 H at room temperature, then in rabbit anti-estradiol Ab solution (50 × diluted in TBST) for 2 H, and finally, in a goat anti-rabbit IgG–HRP solution (2,000 × diluted in TBST) for 1 H. Between each incubation step, the membrane was washed three times with TBST. The HRP signal was amplified using Luminol Enhancer Solutions.

3.3. Matrix-assisted laser desorption/ionization–time of flight mass spectrometry

The matrix-assisted laser desorption/ionization–time of flight mass spectrometry (MALDI–TOF MS) spectra were obtained using an Applied Biosystems (Life Technology, Foster City, CA, USA) Voyager DE-STR MALDI–TOF mass spectrometer. The estradiol–G6PDH sample was desalted using a Microcon YM-30 centrifugal filter to obtain a final salt concentration of less than 1 mM, and a final protein concentration of approximately 5 μM. The Microcon filter was passivated with 1% milk to minimize protein adsorption to the filter membrane. An equal volume of 0.5 mg/mL BSA was added to the estradiol–G6PDH sample as an internal standard. SA dissolved in 70:30 ACN/water, 0.1% TFA at a concentration of 10 mg/mL was used as the matrix. The sample (1.0 μL) and the matrix (1.2 μL) were spotted on the target using the sandwich spotting method [40].

3.4. G6PDH activity photometric assay (general procedure)

A substrate solution containing 50 mM G6P and 30 mM NAD^+ was added to an assay buffer, which contained 55 mM Tris–HCl buffer with 3.3 mM $MgCl_2$ at pH 7.8. The mixture was equilibrated at 37 °C for 6 Min. Subsequently, the testing enzyme solution was added and mixed into the warmed mixture by repeated micropipetting. Immediately after the addition of the testing enzyme solution, the optical density at 340 nm (OD_{340}) was recorded at 37 °C for approximately 3 Min. The change in the OD at 340 nm per minute (ΔOD_{340}/Min) was obtained from the slope of the initial linear portion of the curve. From the ΔOD_{340}/Min, the activity in U/mL was calculated using Beer's law where 6.22 $cm^2/\mu mol$ was used as the nicotinamide adenine dinucleotide (NADH) extinction coefficient.

3.5. Repression of estradiol–G6PDH activity upon antibody binding

The purified estradiol–G6PDH was diluted to 25 nM in the dilution buffer, which contained 50 mM Tris–HCl buffer at pH 8.0, 0.1 M NaCl, and 0.1% gelatin. Anti-17β-estradiol Ab was reconstituted in 1.5 mL of dilution buffer (0.5 mL of this 33-fold

diluted and reconstituted antiserum can bind at least 40% of 5–10 pg of tritiated 17β-estradiol with a specific activity of approximately 100 ci/mmol based on the product information from Sigma). Two enzyme testing solutions were prepared to obtain the enzyme activity of the repressed and unrepressed states. The solution in the repressed state consisted of 30 μL of anti-estradiol Ab and 15 μL of estradiol–G6PDH, whereas the solution in the unrepressed state contained 30 μL of dilution buffer without Ab and 15 μL of estradiol–G6PDH. The amount of Ab (30 μL) used was determined to be sufficient for the system to achieve maximum repression in an optimization study (data not shown). The solutions were incubated at room temperature for 3 H. Subsequently, the enzyme activity was measured according to the general procedure described above using 135 μL of assay buffer (55 mM Tris–HCl buffer with 3.3 mM $MgCl_2$ at pH 7.8) and 30 μL of substrate solution (50 mM G6P and 30 mM NAD^+). The repression of the estradiol–G6PDH conjugate was calculated using Eq. (1).

$$\%\text{Repression} = \frac{\text{Activity}_{\text{unrep}} - \text{Activity}_{\text{rep}}}{\text{Activity}_{\text{unrep}}} \times 100 \qquad (1)$$

where $\text{Activity}_{\text{rep}}$ and $\text{Activity}_{\text{unrep}}$ are the estradiol–G6PDH conjugate activities measured using the solutions in the repressed and unrepressed states, respectively.

3.6. Homogeneous estradiol–EIA calibration curve

Estradiol–G6PDH activity was measured at different concentrations of estradiol in the presence of anti-estradiol Ab. A set of stock assay buffer solutions with different concentrations of β-estradiol (0–100 μM) was prepared in DMF. The stock solutions were further diluted 100-fold in the actual assay buffer (55 mM Tris–HCl buffer with 3.3 mM $MgCl_2$ at pH 7.8). Enzyme activity was measured according to the general procedure described above using 100 μL of assay buffer with estradiol, 20 μL of substrate solution (50 mM of G6P and 30 mM of NAD^+), and 10 μL of 25 nM estradiol–G6PDH. In addition, 20 μL of anti-estradiol Ab was also added to the substrate and estradiol–assay buffer for pre-assay equilibration (37 °C, 6 Min). The calibration curve was generated by plotting estradiol–G6PDH activity versus estradiol concentration. Basic calibration data were also fitted further using the four-parameter logistic (4-PL) model [Eq. (2)]

$$\text{Activity} = \frac{a - d}{1 + \left(\frac{[\text{E2}]}{c}\right)^{b}} + d \qquad (2)$$

where, a, b, c, and d are curve-fitting parameters. Activity and [E2] represent the estradiol–G6PDH activity and free estradiol concentration, respectively. The parameters were obtained by nonlinear least squares curve fitting using KaleidaGraph (Reading, PA, USA).

3.7. Homogeneous estradiol–EIA calibration curve in serum

Normal human serum samples were purchased from Innovative Research (Novi, MI, USA). The serum samples were shipped on dry ice and stored at −20 °C immediately upon arrival. Thawing of a frozen serum sample was done rapidly in the water bath at 37 °C before use. To prepare serum calibrators, β-estradiol in DMF was first spiked into a serum stock. Serum samples with different concentrations of β-estradiol (0–30 μM) were prepared by serial dilution starting from the spiked stock. Each sample was then filtered with a syringe (0.1 μm pore size). The filtrate (or calibrator) was collected and temporarily stored at 4 °C until analysis. Fifty microliters of assay buffer (55 mM Tris–HCl buffer with 3.3 mM $MgCl_2$ at pH 7.8) containing OxA (3.125 mM) and α-cyclodextrin [2% (w/v)], 50 μL of serum calibrator, and 20 μL of anti-estradiol Ab were mixed for pre-assay equilibration (37 °C, 6 Min). OxA and α-cyclodextrin were used to inhibit the activity of the endogenous LDH [38],[39] and to reduce the nonspecific binding of estradiol to serum proteins [41], respectively. Enzyme activity was monitored continuously after adding 20 μL of substrate solution (50 mM of G6P and 30 mM of NAD^+) and 10 μL of 25 nM estradiol–G6PDH to the pre-assay mixture. The calibration curve was generated and fit using methods described above.

4. Results and discussion

4.1. Estradiol–G6PDH conjugation

Estradiol was conjugated to G6PDH using NHS-enhanced carbodiimide-mediated coupling [42]. The carboxyl group of E2–6-CMO, an estradiol derivative, was first activated by EDC and NHS, forming an NHS ester. The NHS ester was then reacted with a primary amine ($-NH_2$) group of the lysines or N-terminus of the G6PDH, forming an amide bond. Although coupling could also occur between the NHS ester and the hydroxyl (—OH) groups from tyrosine [43], the majority of the labeling is believed to occur at the $-NH_2$ groups because $-NH_2$ is substantially more nucleophilic than —OH at pH 7.3 (the pH level at which conjugation took place). Studies on other analyte–G6PDH conjugates that were prepared using similar reaction conditions have also shown that the coupling to —OH groups is negligible [43]. Up to approximately 90% enzyme deactivation was observed after this step. Subsequent to the conjugation reactions, Western blot analysis and MALDI–TOF MS were employed to characterize the estradiol–G6PDH conjugate.

4.2. Western blot analysis

Western blot analysis was utilized to confirm the covalent coupling of estradiol and G6PDH. All three estradiol–G6PDH conjugate samples bound anti-estradiol Abs as indicated by the band that appears at approximately the molecular weight (MW) of a single G6PDH monomer (54 kDa) [29] (Fig. 2a). This verifies that the G6PDH monomers were conjugated covalently to estradiol molecules. The absence of a signal on the G6PDH control indicates that the binding of the anti-estradiol Ab was specific.

4.3. Matrix-assisted laser desorption/ionization–time of flight mass spectrometry

Matrix-assisted laser desorption/ionization–time of flight mass spectrometry was used to estimate the mass increase corresponding to the covalent attachment of estradiol molecules to G6PDH, and thereby determine the degree of conjugation. The attachment of each estradiol molecule was expected to con-

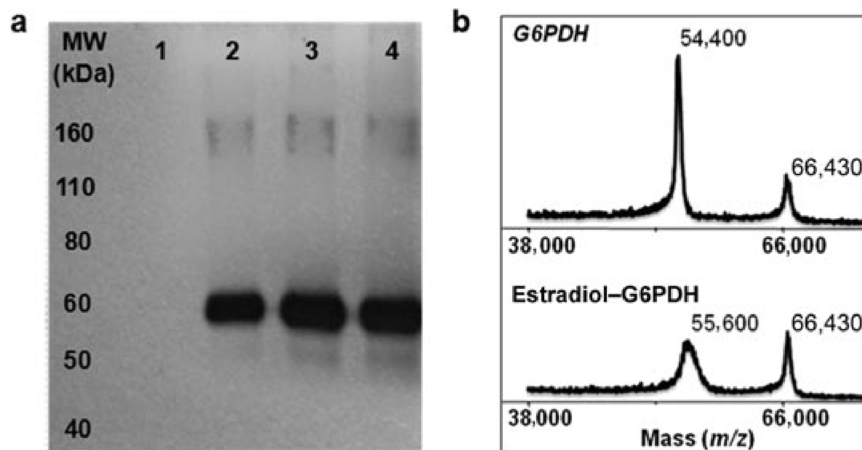

Fig. 2. (a) Western blot analysis of G6PDH (lane 1) and estradiol–G6PDH conjugates prepared with 0.26 mM (lane 2), 0.52 mM (lane 3), and 0.78 mM (lane 4) E2–6-CMO. (b) MALDI–TOF MS spectra of G6PDH (top) and estradiol–G6PDH conjugate using 0.78 mM E2–6-CMO (bottom) with BSA (66,430 Da) as an internal standard.

Table 1
Degree of conjugation of estradiol–G6PDH prepared with different concentrations of E2–6-CMO estimated using MALDI–TOF MS

E2–6-CMO concentration in conjugation (mM)	m/z	Average degree of conjugation (per monomer)
0.26	54,800 ± 50	1.1 ± 0.1
0.52	55,300 ± 200	3 ± 1
0.78	55,610 ± 30	3.5 ± 0.3
G6PDH control	54,420 ± 30	–

The ± values are standard errors ($n = 4$–9).

tribute a mass increase of 341.40 Da, given that the MW of E2–6-CMO is 359.42 Da and the formation of the amide bond between the carboxyl group of the E2–6-CMO and the $-NH_2$ group of the G6PDH results in the release of one water molecule (MW = 18.02 Da). Figure 2b shows the MALDI–TOF MS spectra of G6PDH and estradiol–G6PDH that was prepared using 0.78 mM E2–6-CMO and 1.1 μM G6PDH. BSA (MW = 66,430 Da) was added to the samples as an internal standard. The G6PDH sample gave a peak at m/z 54,420 ± 30, which corresponds to the expected MW of a G6PDH monomer. The MALDI–TOF MS spectrum of the estradiol–G6PDH conjugate included a peak at m/z 55,610 ± 30, which indicated an average of 3.5 ± 0.3 estradiol molecules attached to each G6PDH monomer. Estradiol–G6PDH conjugates were prepared using three different E2–6-CMO concentrations, and the degree of conjugation is summarized in Table 1. As expected, an increase in E2–6-CMO concentration resulted in an increase in the degree of conjugation, which therefore also affected the homogeneous estradiol–EIA performance. Further details on this topic are discussed in a later section.

Each G6PDH monomer contains 37 $-NH_2$ groups [29], thus approximately 3%–9% of the $-NH_2$ groups were labeled with estradiol molecules, depending on the E2–6-CMO concentration used during conjugation. Although a higher degree of conjugation can be achieved by increasing the E2–6-CMO concentration, it may not be beneficial because increased E2–6-CMO conjugation also caused increased enzyme activity loss during conjugation. Because the estradiol–G6PDH with a degree of conjugation of 3.5 is sufficient to create a useful homogeneous estradiol–EIA (see below), we did not prepare an estradiol–G6PDH conjugate using a higher E2–6-CMO concentration.

4.4. Repression of estradiol–G6PDH activity upon antibody binding

Figure 3a contrasts estradiol–G6PDH conjugate activities in the presence and absence of anti-estradiol Ab. The increase of OD_{340} with time was a result of the accumulation of NADH, one of the products of G6PDH-catalyzed oxidation, which therefore can be correlated to the G6PDH activity. The enzyme activity in the presence of Ab was lower than that in the absence of Ab. This result is consistent with an important feature of this homogeneous estradiol–EIA method, namely that Ab bound to the estradiol of the enzyme conjugate represses reversibly the reporter enzyme activity.

With the enzyme activity measured in the presence of the Ab (the repressed state) and in the absence of the Ab (the unrepressed state), the percent repression can be calculated by Eq. (1). The maximal repression level is an important homogeneous estradiol–EIA characteristic because a higher achievable level of repression can provide a higher assay sensitivity (less background). Figure 3b summarizes the repression that was obtained by estradiol–G6PDH conjugates prepared at different E2–6-CMO concentrations. The estradiol–G6PDH conjugates prepared with 0.26, 0.52, and 0.78 mM E2–6-CMO gave 23 ± 1%, 41.5 ± 0.4%, and 46 ± 2% repression, respectively, upon Ab binding. A negligible difference in enzyme activity in

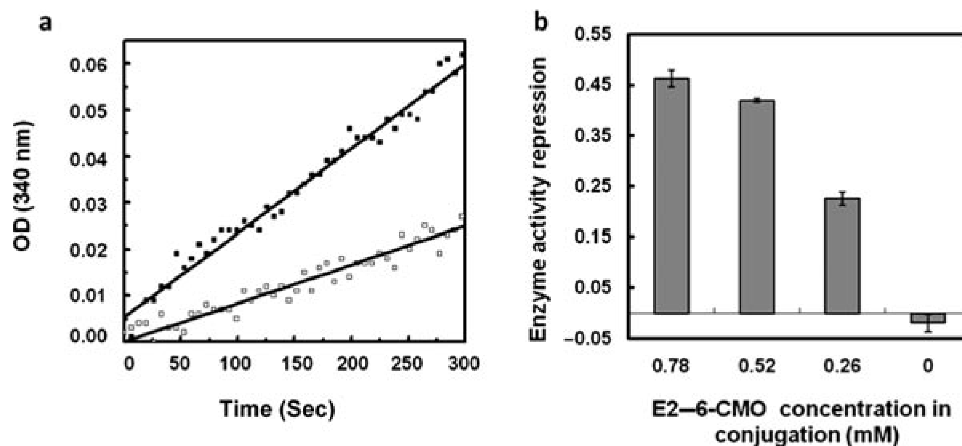

Fig. 3. (a) Enzyme activity repression of the estradiol–G6PDH conjugate (prepared with 0.78 mM E2–6-CMO) upon anti-estradiol antibody (Ab) binding. Repressed sample: estradiol–G6PDH in the presence of Ab (□). Unrepressed sample: estradiol–G6PDH in the absence of Ab (■). (b) Enzyme activity repression of estradiol–G6PDH conjugates prepared with 0.26, 0.52 and 0.78 mM E2–6-CMO after 3 H incubation with anti-estradiol Ab. Unmodified G6PDH was used as a control. Error bars represent standard errors ($n = 3$–4).

unmodified G6PDH in the presence and absence of Ab was observed ($-2 \pm 2\%$ repression), thereby showing as expected that covalently bound estradiol was necessary in addition to Ab to repress reporter enzyme kinetics.

The observed trend of increasing reporter enzyme activity repression with increasing E2–6-CMO concentrations during conjugation is expected because a higher E2–6-CMO concentration gives a higher degree of estradiol attachment as shown by the MALDI–TOF-MS data (Table 1). A higher degree of conjugation increases the probability of estradiol attachment to sites that would mediate activity repression upon Ab binding. Because the estradiol–G6PDH conjugate prepared with 0.78 mM E2–6-CMO gave the highest level of repression, which is comparable to that of existing commercial homogeneous estradiol–EIA systems (typically 40% to above 90%) [32],[44],[45], this conjugate was used further in this homogeneous estradiol–EIA study.

4.5. Homogeneous estradiol–EIA calibration curve

Figure 4a shows a typical homogeneous estradiol–EIA calibration curve. It relates free estradiol concentration, ranging from 0 to 6.7 μM, to estradiol–G6PDH activity (in the presence of anti-estradiol Ab). The data show that estradiol–G6PDH activity increases with increasing free estradiol and approaches a saturation response corresponding to virtually all Abs bound to free analyte (the completely unrepressed state). Like other immunoassay data, these calibration data could be linearized by using the logit transformation [46] and fitting the resultant data using the 4-PL model [Eq. (2)] [47]. The logit function can

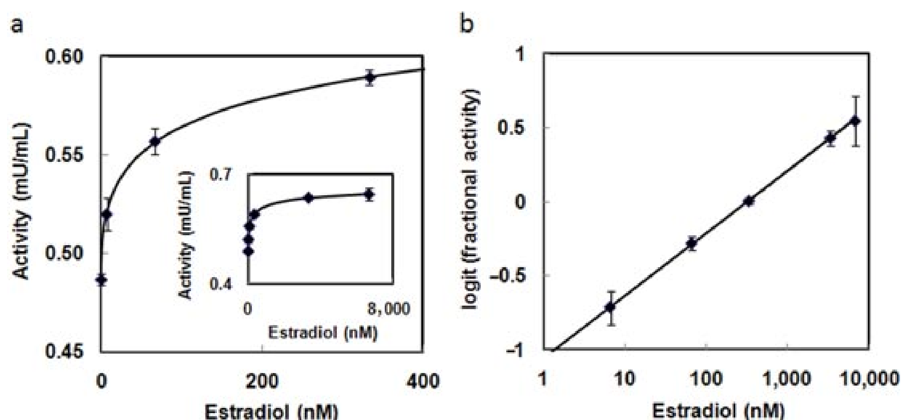

Fig. 4. Homogeneous estradiol–EIA calibration curve in aqueous buffer generated using estradiol–G6PDH conjugated with 0.78 mM E2–6-CMO: estradiol–G6PDH activity versus free estradiol concentration. The experimental data (◆) and the 4-PL model fit (——) are shown in (a) linear scale and (b) logit–log scale. Error bars represent standard errors ($n = 3$).

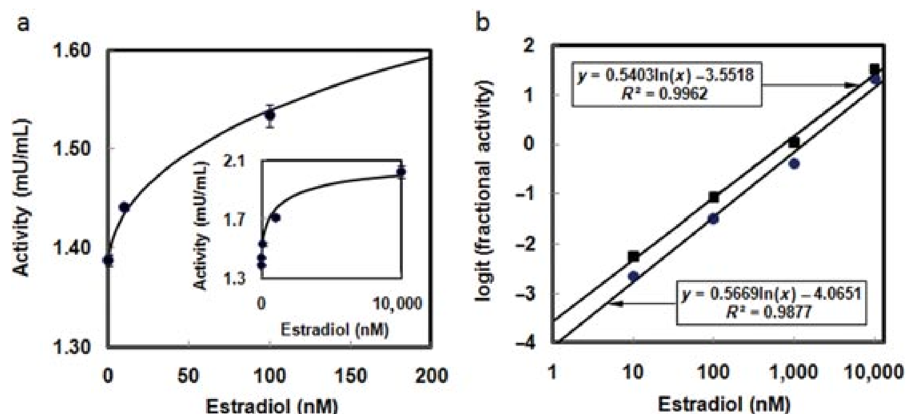

Fig. 5. Homogeneous estradiol–EIA calibration curve in human serum generated using estradiol–G6PDH conjugated with 0.78 mM E2–6-CMO: estradiol–G6PDH activity versus free estradiol concentration. The experimental data in serum (•) and the 4-PL model fit (—) are shown in linear scale (a). The experimental data obtained in serum (•) and in buffer (■), along with the 4-PL model fit (—), are shown in in logit–log scale (b). Error bars represent standard errors ($n = 3$–6).

be expressed as

$$\text{logit}\, Y = \ln\left[Y/(1-Y)\right] \quad (3)$$

where Y is the fractional activity, which can be defined as

$$Y = (R_N - R_0)/(R_\infty - R_0) \quad (4)$$

where R_N is the activity/response corresponding to a certain dose of estradiol, R_0 is the activity/response at zero concentration, and R_∞ is the maximum activity/response corresponding to a saturating dose. When the logit of fractional activity was plotted against the log of the estradiol concentration, a linear trend was obtained (Fig. 4b) up to 6.7 μM, the highest concentration tested. Using the 4-PL model, the detection limit (which is the estradiol concentration corresponding to two standard deviations above the repressed state activity) was determined to be 1 nM.

In order to reduce light scattering from highly concentrated proteins and colloidal particles in serum, which caused high background noise, samples were filtered prior to the assays. The background signals of serum samples at 340 nm were reduced 2–3-fold after syringe filtration (0.1 μm filter). To eliminate the interference from the endogenous LDH, OxA was employed as an enzyme inhibitor. The activity of LDH was significantly repressed by OxA, but the activity of G6PDH, the reporter enzyme, was unaffected. At 1 mM OxA, the activity of endogenous LDH (~17 mU/mL) was more than 96% repressed in the presence of 0.6 mM LA and was approximately 88% repressed in presence of 15 mM LA. OxA and LA compete for access to the enzyme's active site [48] and therefore relatively high concentrations of OxA are able to outcompete the substrate, LA.

Serum samples were also used to establish a homogeneous estradiol–EIA calibration curve (Fig. 5a). Using the 4-PL model, the detection limit in serum was determined, by a method similar to that used for estradiol in simple aqueous solution, to be 11.5 nM and the logit–log calibration curve was linear up to 30 μM. Similar assay sensitivities in buffer and in serum using the same estradiol–G6PDH conjugates (Fig. 5b) indicated that the enzyme conjugates retained their level of repression in serum samples. Compared with the result in buffer, the detection limit in serum was approximately 10 times higher. Although serum samples were filtered and diluted to reduce the light scattering, the background noise still was larger compared with that in buffer, resulting in the higher detection limit. Given the demonstrated detection range and sensitivity, the homogeneous estradiol–EIA presented in this report may be useful for identifying patients who are at risk for developing OHSS. Further work is required to optimize the system, which could lead to a lower detection limit, and to determine the precision of the assay.

5. Conclusions

The present study describes the construction and characterization of an appealing homogeneous EIA for estradiol. The homogeneous assay was conducted by straightforward mixing of two prepared solutions and monitoring of estradiol–G6PDH activity spectrophotometrically. The detection limits of the homogeneous estradiol–EIA were estimated at 1 nM estradiol in aqueous solution and 11.5 nM in human serum, respectively, whereas the respective logit–log calibration curves were linear to more than 6.7 and 30 μM estradiol. The convenient, economical, and sensitive nature of this assay makes it attractive for applications such as OHSS prediction and prevention.

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