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Sensitive determination of estriol-16-glucuronide using surface plasmon resonance sensing

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ABSTRACT

For the quantitative evaluation of low levels of an estriol metabolite of estriol (estriol-16-glucuronide (E3-16G)) in liquid media, we developed a simple and highly sensitive immunoassay using a surface plasmon resonance (SPR) biosensor which did not require any time-consuming sample pretreatment steps. E3-16G was conjugated to ovalbumin (OVA) through an oligoethylene glycol (OEG) linker to form protein conjugates (E3-16G-OEG-OVA), which were then immobilized on a carboxymethyl dextran-coated sensor chip via amine coupling to develop inhibition immunoassays. A limit of detection (LOD) of 76 pg/mL was achieved using a rabbit anti-sheep primary antibody as a binding agent. The detection limit was further improved by using synthesized gold colloids (15 nm) as high mass labels conjugated to the primary antibody. In this Au nanoparticle-enhanced assay, the concentration of E3-16G in aqueous samples could be determined in 7.5 min at a level as low as 14 pg/mL. In addition, the high stability of the E3-16G-OEG-OVA surface gave no obvious drop in antibody-binding capability after more than 1000 binding/regeneration cycles which significantly lowered the research cost.

1. Introduction

Estriol (E3) is one of the major urinary metabolites of ovarian estradiol, produced throughout a woman's reproductive years and increases to high levels during pregnancy. E3 plays an important role in the monitoring of foetal well-being and development during pregnancy [1], but it has also been found to be associated with breast cancer [2]. E3 is converted to a number of conjugates in the maternal circulation and then excreted into the environment by the passage of urine. Four of the major estriol conjugates are estriol-3-glucuronide (E3-3G), estriol-3-sulphate (E3-3S), estriol-16-glucuronide (E3-16G), and estriol-3-sulphate-16-glucuronide (E3-SG) [3]. These derivatives and their hydrolysis products have been detected in environmental waters at levels down to 5 ng/mL [4]. Pharmaceutical estriol compounds are believed to be harmful to animals and the environment since they have high biological activity and are not readily degraded [5]. Due to their potential adverse effects in both the environment and human health, including their important association with normal and abnormal pregnancies and breast cancer, there is an increasing need for the development of rapid, sensitive, quantitative techniques for measuring trace levels of E3 metabolites.

A number of techniques have been reported for estimating E3 metabolite concentration, such as, radioimmunoassay (RIA) for E3-16G using [^{3}H] [6] or [^{125}I] [7] labeled radio ligands, high pressure liquid chromatography (HPLC) [8], and liquid chromatography (LC) coupled with fluorescence measurement [9], mass spectrometry (MS) [10] or UV spectrophotometry [11]. In all these methods however, either the sample preparation is time-consuming (RIA, HPLC, and LC/MS assay) or the limit of detection (LOD) is insufficient (RIA assay) to detect environmental concentrations [4].

In this present study, we report a rapid and quantitative method for determination of E3-16G in solution using surface plasmon resonance (SPR) immunoassay. The method is both rapid and sensitive. It is also simple to apply and does not require any sample pretreatment, such as hydrolysis, extraction, or derivatization. Quantification of E3-16G was performed in aqueous samples by means of a competition between injected free E3-16G (pre-mixed with anti-E3-16G-antibody) and E3-16G conjugate (E3-16G-OEG-OVA) immobilized on a CM5 BIAcore biosensor chip. Ultimate sensitivity was achieved by using anti-E3-16G-antibody coated gold nanoparticles (NPs) as high molecular weight labels. This resulted in a maximum sensitivity of 14 pg/mL which was several orders of magnitude more sensitive than the alternate literature methods [4–11].

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2. Experimental

2.1. Chemicals and instruments

Trisodium citrate dihydrate, tetrachloroauric acid trihydrate, trehalose, ovalbumin (OVA), and bovine serum albumin (BSA) were all purchased from Sigma–Aldrich (USA). Glacial acetic acid, octanoic acid, sucrose, sulphuric acid, and di-sodium hydrogen orthophosphate were obtained from BDH Laboratory Supplies (England) and ammonium sulphate was from Merck Chemicals (Darmstadt, Germany). Triton-X 100 was purchased from Pure Science Limited (Wellington, New Zealand), sodium azide was from Serva Electrphoresis (Heidelberg, Germany), and the 0.22 μm cellulose acetate filters were from ADVANTEC MFS, Inc. (Dublin, USA).

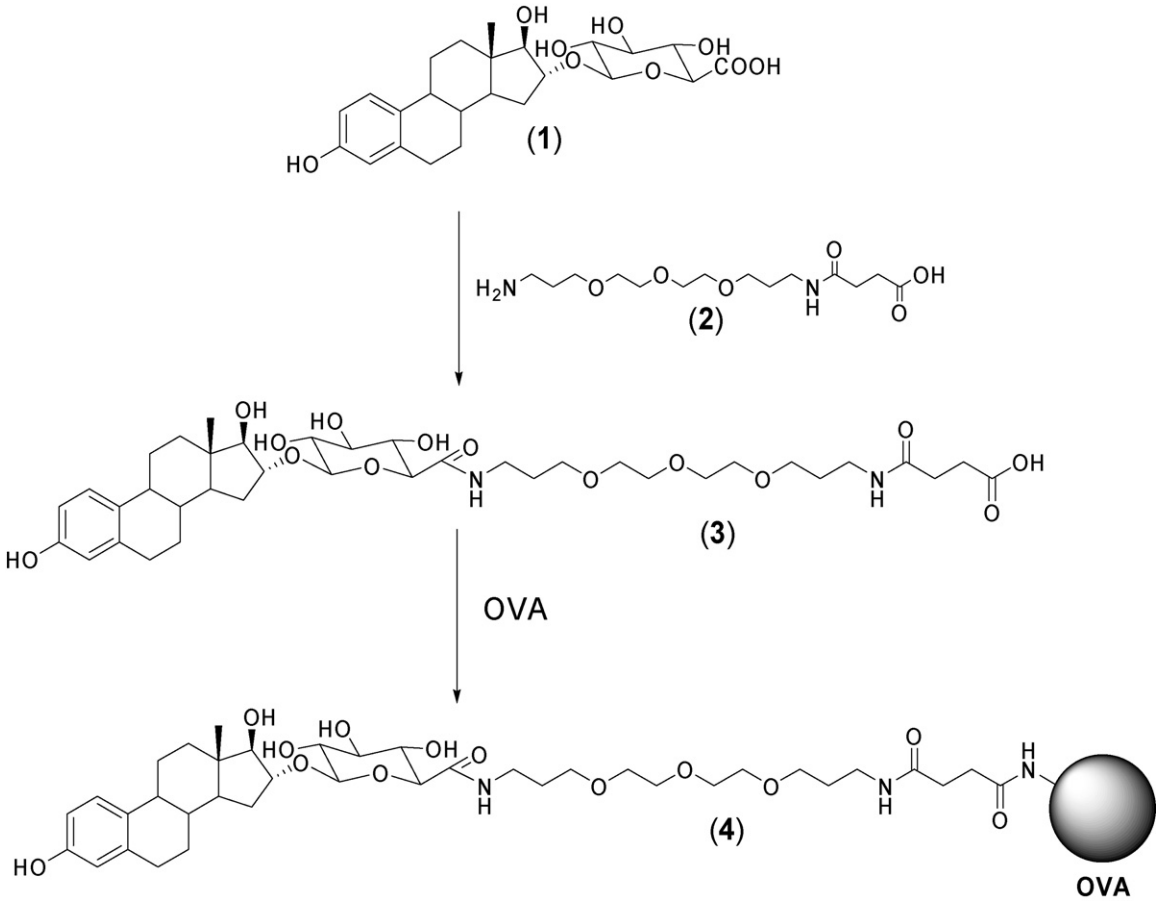
All absorbance spectra were acquired with a UV–vis-NIR spectrophotometer (UV-3101PC SHIMADZU, Japan). Gold colloid images were taken with a Philips CM10 transmission electron microscope (TEM). ^{1}H and ^{13}C NMR spectra were obtained using either a Bruker Avance 400 or 500 MHz spectrometer. Chemical shifts for spectra in $CDCl_3$ are given in parts per million (ppm) down field from tetramethylsilane as internal standard (^{1}H) or relative to residual solvent (^{13}C). High resolution mass spectra were recorded using a VG7070 mass spectrometer operating at a normal accelerating voltage of 70 eV. A BIAcore X100 system (GE Healthcare, Uppsala, Sweden) was used to monitor the antibody-binding performance on an E3-16G-OEG-OVA surface in real time. In addition, the CM5 chip, amine coupling kit (0.1 M NHS, 0.4 M EDC, and 1 M ethanolamine), and HBS-EP running buffer (0.01 M HEPES, pH = 7.4, 0.15 M NaCl, 3 mM EDTA, and 0.005% surfactant P20) all were purchased from GE healthcare.

For statistical analysis, all assay standard curves were fitted to four-parameter logistic equation using Graph Pad Prism 5 (La Jolla, CA, USA). All IC_{50} values (defined as the concentration that gives half-maximal effect) were given as a parameter with the curve fitting using prism. All LOD values were computed using OriginPro 7.0 (OriginLab, Northampton, MA, USA).

2.2. Synthesis of E3-16G-OEG-OVA conjugate (Scheme 1)

E3-16G (**1**) and a heterobifunctional oligoethylene glycol (OEG) linker (**2**) were prepared separately according to two previous methods [12,13]. A solution of DCC (0.16 mmol) and NHS (0.16 mmol) in DMF (0.5 mL) was added to a solution of E3-16G (25 mg, 0.0538 mmol) in DMF (0.5 mL) under an atmosphere of nitrogen. The reaction mixture was stirred for 4 h, followed by the addition of the OEG linker (0.3 mmol) in $CHCl_3$ (1 mL) and triethylamine (0.5 mL), and subsequently stirred for further 12 h. Water (50 mL) was added and the reaction mixture extracted with dichloromethane (DCM) (20 mL 3×). After removing the DCM layer from the aqueous phase the solution was passed through an XAD-2 column and eluted with MeOH/water (1:1). After evaporating the solvent, the residue was purified on by flash column chromatography eluting with CH_2Cl_2/MeOH/HOAc (10:1:0.1) to afford the product N-(4,7,10-trioxa-1,13-tridecanyl-13-butanamidoicacid)-3,17b-dihydroxy-1,3,5(10)-estratriene-16a-yl-b-D-glucopyranosamide (**3**) as a colourless solid (18 mg, 44%) (Scheme 1).

^{1}H NMR (500 MHz, $CDCl_3$): 0.82 (s, 3H), 1.31–1.49 (m, 4H), 1.57 (q, J = 9.4 Hz, 1H), 1.75–1.99 (m, 7H), 2.17–2.23 (m, 1H), 2.29–2.35 (m, 1H), 2.48 (t, J = 7.2 Hz, 2H), 2.61 (t, J = 7.2 Hz, 2H), 2.78–2.82 (m, 2H), 3.25–3.35 (m, 9H), 3.44 (t, J = 8.9 Hz, 1H), 3.49 (t, J = 7.9 Hz, 1H), 3.53–3.70 (m, 10H), 3.75 (d, J = 9.4 Hz, 1H), 4.17 (q, J = 5.2 Hz, 1H),



Scheme 1. Synthesis of estriol-16-glucuronide-olbumin conjugate (E3-16G-OEG-OVA).

4.39 (d, J = 7.9 Hz, 1H), 6.50 (d, J = 4.3 Hz, 1H), 6.55 (dd, J = 2.6, 6.8 Hz, 1H), 7.10 (d, J = 8.5 Hz, 1H) ppm.

^{13}C NMR (125 MHz, $CDCl_3$): 1.5, 25.8, 27.2, 28.9, 29.3, 31.0, 36.2, 36.4, 36.6, 38.6, 43.5, 43.9, 68.4, 48.5, 68.8, 70.1, 72.2, 73.3, 73.7, 76.3, 85.4, 87.3, 102.2, 112.4, 114.7, 125.8, 131.0, 154.6, 170.8, 173.1 and 174.9 ppm; HRMS (EAB$^+$): MH$^+$, found 767.39630. $C_{38}H_{58}N_2O_{14}$ requires 767.39663.

N-(4,7,10-trioxa-1,13-tridecanyl-13-butanamidoicacid)-3,17b-dihydroxy-1,3,5(10)-estratriene-16a-yl-b-D-glucopyranosamide (**3**) (10 mg, 0.013 mmol) was dissolved in a mixture of DCC (0.044 mmol) and NHS (0.044 mmol) in DMF (0.26 mL), and the reaction was stirred at room temperature for 3 h until white precipitates were formed. The solution was added to OVA (0.65 mol) in phosphate buffer (2.6 mL, 0.2 M, pH = 7.4) and stirred at 4 °C overnight. After dialysis against deionized water for 2 days (3 changes per day) and PBS/T for 1 day (2 changes) at 4 °C, the conjugate (**4**) (2.5 mL) was further purified by passing it through a PD-10 column using PBS/T as the eluent, and the purified conjugate (**4**) (3.5 mL) was collected. The protein concentration of E3-16G-OEG-OVA (**4**) was determined by a bicinchonimic acid protein assay.

2.3. Purification of the polyclonal primary antibody (pAb)

The antibody purification started with serum harvested from the fifth bleed of a sheep inoculated with estriol-16-glucuronide–thyroglobulin conjugate. The sheep serum (5 mL) was added to acetate buffer (10 mL of 0.06 M, pH = 6.0) and the pH was adjusted to 4.8 using 0.1 M HCl. Octanoic acid (330 μL) was added dropwise to the solution (15 mL) with vigorous stirring to remove non-globulin material the mixture was stirred on an ice bath for 30 min and centrifuged at 3345 × g for 60 min. The resulting supernatant was transferred to a fresh tube for a second centrifugation for 35 min under the same conditions and dialysed against phosphate buffer (10 mM, pH = 7) overnight. Two further buffer changes were carried out after 1 and 2 h, respectively.

The dialysed sample was centrifuged at 3345 × g for 40 min, and the supernatant was mixed with an equal volume of saturated ammonium sulphate solution (76 g ammonium sulphate dissolved in 100 mL MilliQ water and pH adjusted to 7.0 using sulphuric acid) in a fresh tube. The solution was left stirring overnight at 4 °C for complete precipitation of the immunoglobulins. The antibodies were spun down (2× 30 min at 3345 g) and washed with phosphate buffer (4.5 mL of 10 mM, pH = 7). The pellet was resuspended in the phosphate buffer and dialysed for 40 h with 7× 1 L changes of phosphate buffer (10 mM, pH = 7). Finally the antibodies were spun down at 23,036 g for 30 min and stored in 10 mM phosphate buffer.

2.4. Synthesis of antibody–Au (pAb–Au) conjugates

Gold nanoparticles (NPs) were synthesized via citrate reduction according to the strategy of Frens [14] with some modification. $NaC_6H_5O_7{\cdot}2H_2O$ (1.47 mL, 170 mM) and $HAuCl_4{\cdot}3H_2O$ (50 mL, 1 mM) were mixed and left to stand in a warm water bath (75–80 °C) for 15 min. The colloidal Au solution was filtered through a 0.22 μm cellulose membrane and then characterized by UV–vis spectrophotometry and TEM. All samples were stored at 4 °C.

The gold NPs were diluted 1:2 with MilliQ water, and the conjugates formed by adding 78.2 μL pAb (4.8 mg/mL) to the Au colloids (20 mL, pH = 8, adjusted with 0.2 M K_2CO_3) followed by 10% BSA (2 mL, w/v) to block unreacted sites on the surface of the NPs. The conjugates were spun down (9750 g for 60 min) using a SS-34 rotor and the pellets were resuspended in buffer (2 mL) containing 0.25% Triton-X (v/v), 0.06 M trehalose, 0.175 M sucrose, 0.154 M sodium azide, 0.05 M Na_2HPO_4, and 1% BSA (w/v).

2.5. Immobilization of E3-16G-OEG-OVA onto CM5 surface

Both flow cell 1 (FC1) and flow cell 2 (FC2) of a BIAcore CM5 chip were activated by mounting the chip within the SPR instrument and exposing each cell to 90 μL of a 1:1 (v/v) mixture of EDC (0.4 M) and NHS (0.1 M) for 18 min. This was followed by injection in FC2 of E3-16G-OEG-OVA (50 μL of 0.33 mg/mL) in sodium acetate (10 mM, pH = 4.0) at a flow rate of 5 μL/min, and then ethanolamine hydrochloride (90 μL of 1.0 M) was injected and the solution stood for 18 min. This resulted in an antibody-binding response around 8000 RUs (response units). For the reference flow cell (FC1), following activation of the surface, OVA (50 μL, 0.05% (w/v)) in sodium acetate (10 mM, pH = 4) was coupled to the chip surface in 10 min to give a response in the range 8000–9000 RU. In the binding experiments, samples were passed over both FC1and FC2 and responses measured by subtracting the reference response (binding at FC1) from the binding response collected from reaction surface (FC2). Thus, the results represented only the specific binding to the OVA-OEG-E3-16G molecule.

2.6. Assays developed with SPR

2.6.1. Binding performance of pAb and pAb–Au conjugates

For analysis of the binding affinity of the pAb on the chip surface, samples (20 μL) containing 6 different concentrations of pAb (0, 0.1, 0.5, 1.0, 5.0, and 10.0 μg/mL) were injected for 2 min at a flow rate of 10 μL/min. The surface was regenerated by injecting a mixture of 100 mM NaOH and 10% acetonitrile for 40 s at a flow rate of 10 μL/min. For evaluating the affinity of the pAb–Au conjugates to the surface, the conjugates were diluted with running buffer to form 6 samples in which the pAb concentration was 0.156, 0.312, 0.623, 1.250, 2.490, and 4.990 μg/mL, respectively. These samples were also injected at 10 μL/min for 2 min. Regeneration was achieved using 10 μL of 200 mM NaOH and 20% acetonitrile for 1 min.

2.6.2. Inhibition assay using pAb

A series of E3-16G standards (in the range of 0–300 ng/mL) were prepared using HBS-EP buffer as diluent. Samples were prepared by mixing Tris (TM) buffer (10 mM), E3-16G standards, and a constant concentration of pAb according to a volume ratio of 1:1:1 and incubating for 5 min at room temperature. Samples containing pAb (15 μL of 5 μg/mL) were injected over the chip surface in 1.5 min while for the samples containing pAb (1 μg/mL) 75 μL was injected in 7.5 min. Regeneration was performed with a solution of 100 mM NaOH and 10% acetonitrile in MilliQ water (5 μL for 30 s).

2.6.3. Inhibition assay using pAb–Au conjugates

The pAb–Au NP conjugates were firstly diluted with the buffer (dilution factors were 12.5, 25, 100, and 200) and mixed with equivalent volumes of E3-16G standards and TM buffer. The mixtures were injected at 10 μL/min for 1, 1.5, 4.5, and 7.5 min according to the dilution factors. The chip surface was regenerated with a solution of 200 mM NaOH and 20% acetonitrile in MilliQ water (10 μL for 60 s).

All SPR experiments were performed in triplicate with a total time of 100 s between cycles.

3. Results and discussion

3.1. Synthesis of OEG-OVA-E3-16G conjugates

Due to the relatively small size of E3-16G molecule (464.51 Da) it was chemically linked to OVA via a 23-atom polyethylene oxide spacer to reduce steric effects on the chip surface and to promote extension away from the surface thus facilitating antibody

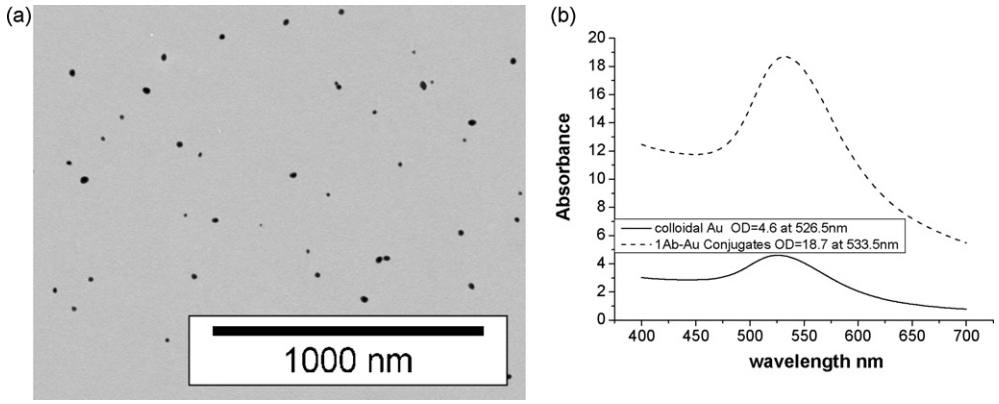


Fig. 1. (a) (left) TEM images of spherical Au NPs. The black dots are the Au NPs. The image scale is 1000 nm. (b) (right) Absorbance spectra of naked Au NPs (solid) and pAb–Au conjugates (dashed). Maximum absorbance (4.6) occurs at 526.5 nm for naked Au NPs and at 533.5 nm (18.7) for the conjugates.

binding. OVA was chosen as the carrier molecule because it contains multiple amino acid residues for linking with E3-16G and it also allows for efficient immobilization of the conjugate (E3-16G-OEG-OVA) onto the chip surface through amine coupling. The E3-16G density on the chip surface was estimated to be approximately 11 steroid moieties per OVA molecule [15]. The extended 23-atom long OEG as a spacer molecule lowered non-specific binding of the antibodies or antibody–Au conjugates to the dextran surface [13,15]. An advantage of binding the E3-16G-OEG-OVA conjugate to the sensor surface, as opposed to E3-16G-OEG, was the high stability and reproducibility of the sensor response [16]. In a typical example, a 1 μg/mL sample of pAb (75 μL injected over 450 s) exposed to a freshly functionalized chip gave an initial sensor response of 129 RU, which decreased slightly to 123.6 RU after 1012 repeat binding/regeneration cycles.

3.2. Preparation of pAb–Au conjugates

The average size of the colloidal gold NPs was 15.8 ± 2.66 nm based on TEM images (Fig. 1a) with a coefficient of variation of 17% (N = 66). It can be seen that most of the colloidal particles were monodispersed and they were essentially spherical in shape. In the preparation of the gold NP conjugate with the estriol-16-glucuronide antibody there was a single plasmon resonance peak situated at 526.5 nm with an optical density (OD) of 4.6 AU (absorbance unit) (Fig. 1b). The Plasmon resonance peak of the pAb–Au conjugates was red-shifted 7 nm (about 1.3%) presumably as a result of the binding of the pAb and the BSA used for blocking of the colloidal Au surface. The absorbance of the final conjugate solution was 18.7 AU. Since the volume of the conjugate solution (1.2 mL) was about 1/8.3 of the initial volume of the colloids (10 mL 0.5 mM Au NPs were added for synthesis) and the absorbance of used colloids was 2.3 AU the absorbance of 1 mM Au NPs was 4.6), it was estimated that only 6% of the colloids were lost during the synthesis of the conjugates.

3.3. pAb/pAb–Au conjugates binding performance on chip surface

To compare the binding performance of the pAb and pAb–Au conjugates on the chip surface (Fig. 2), the binding response was plotted versus the pAb concentration in the samples. In both formats, the binding response was linear with increasing concentration of antibody in the samples. The higher slope of calibration curve in the NP enhanced format suggests that the response was improved by the presence of the Au NPs, and therefore required less pAb for the same response. The ratio of the two linear regressions (enhanced assay/non-enhanced assay) suggests that for samples containing the same concentration of antibody the binding response could be theoretically enhanced 1.37-fold by conjugating the antibody to Au NPs. This enhancement has the potential to give a more sensitive assay by reducing the amount of antibody in the system.

3.4. Competitive assay developed with only pAb

Firstly we developed a SPR assay using the biosensor response on binding of just the pAb. An advantage of this format was that the result could be obtained in <10 min. In addition, since only the pAb molecule was bound to the biosensor surface the distance from the chip surface to the analyte was minimized with a corresponding increase in the SPR signal. Two standard curves were performed with solutions containing concentrations of either 5 or 1 μg/mL pAb, as shown in Fig. 3. As expected the IC_{50}, and the LOD were both improved when using solutions containing the lower concentration of pAb. A LOD value of 76 pg/mL was obtained with the solutions containing 1 μg/mL pAb whereas it was 969 pg/mL for the 5 μg/mL pAb solutions. The intra-assay coefficient of variation (CV) of the triplicate measurements for each sample was lower than 3.90% for the 1 μg/mL curve and 4.35% for the 5 μg/mL curve. The inter-assay CV (obtained from 3 separate assays) of the LOD was 12.29% (1 μg/mL) and 9.64% (5 μg/mL). The inter-assay CV at the IC_{50} was 0.84% for the 1 μg/mL antibody standard curves and 5.86% for the 5 μg/mL antibody standard curves.

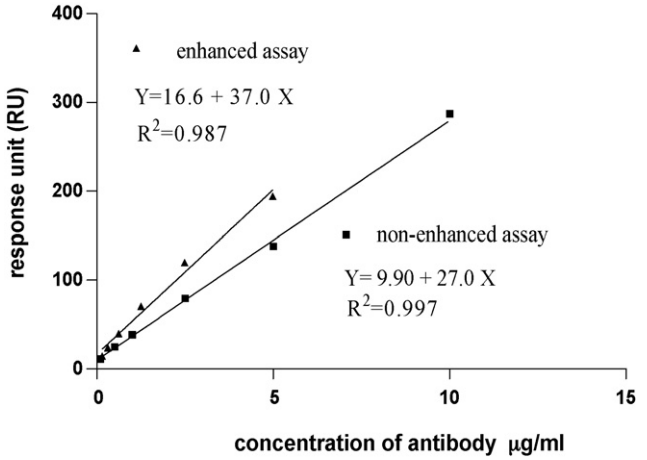


Fig. 2. Correlation between the concentration of pAb in samples and the response. The regression line of antibody (■) was calculated by injecting 6 different concentrations of pAb. The regression line for antibody–Au (▲) was obtained with measurements of 6 samples containing different concentrations of pAb. Each data represents mean and stand deviation (SD) of three measurements.

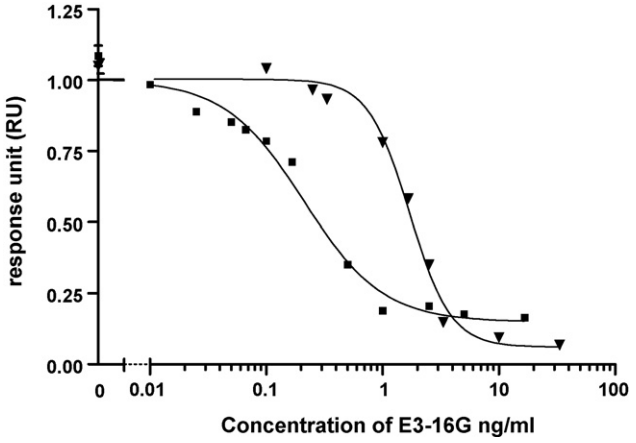


Fig. 3. Standard curves developed with samples containing 1 μg/mL (▼) and 5 μg/mL (■) pAb. Values are mean ± SD from triplicate injections of each sample.

3.5. Enhanced competitive assay developed with pAb–Au conjugates

In an attempt to develop an even more sensitive assay the effect of binding the antibody to the gold NPs was investigated. The main difference from many published methods [17,18], was that in our approach the Au NPs as the high mass label were directly conjugated to primary antibodies instead of to secondary antibodies. Thus the non-specific binding caused by the secondary antibody (to polyclonal primary antibody) was obviously lowered. Our system brought the Au NPs closer to the chip surface and provided a greater SPR effect since there were no secondary antibodies involved. In addition, results were obtained in one step, therefore the time to achieve a result and the cost were also reduced.

In the present study, colloidal gold NPs of 15.8 ± 2.66 nm diameter were employed. The regressions shown in Fig. 4 demonstrated that both the sensitivity (IC_{50}) and LOD were improved in a linear fashion with a lowering of the concentration of antibody in the samples. Based on these results, a sensitive standard curve was established (Fig. 5) using a high dilution of the conjugates (1:200). The concentration of pAb in the sample was 0.31 μg/mL. The LOD was reduced from 76 to 14 pg/mL and the sensitivity was increased by lowering the IC_{50} by 6.38-fold (0.211/0.033 ng/mL). These results clearly show that gold NP labeling of the anti-estriol-16-glucuronide antibody lowered the values for both the LOD and the IC50 significantly. The present assay was nearly 10 times more sensitive than the similar nanoparticle-enhanced primary antibody conjugate reported by Mitchell et al. [19]. Furthermore, the presence of the gold colloids reduced the amount of pAb required, thus it was more economical than a non-enhanced assay.

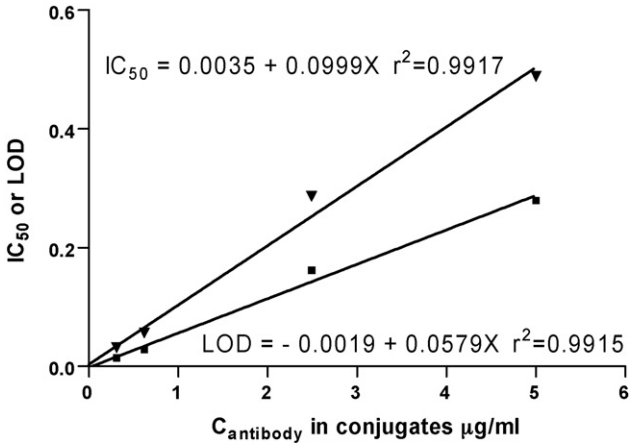


Fig. 4. Sensitivity (■) or LOD (▼) as a function of antibody concentration in conjugates. Values are mean ± SD from triplicate measurements of each sample. Data for both regression lines were calculated for 3 separate standard curves. Error bars represent SD of three replicates.

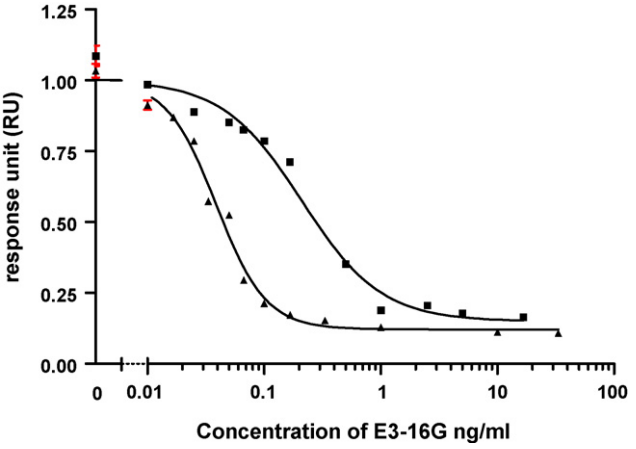


Fig. 5. Standard curves obtained from enhanced assay (▲) and non-enhanced assay (■). Values are mean ± SD from triplicate measurements of each sample. For the enhanced curve, intra-assay CV of 3 measurements is lower than 4.46%; inter-assay CV (from 4 separate curves) is 9.75% (LOD) and 7.3% (IC_{50}). For non-enhanced assay, inter-assay CV is 12.29% (LOD) and 0.84% (IC_{50}).

4. Conclusion

The results demonstrate an ultra-sensitive SPR assay (several tens of pg/mL) suitable for the detection of E3-16G in liquid samples established by using rabbit anti-sheep pAb and pAb–Au conjugates. The sensitivity of the assay indicates its potential applicability in monitoring E3-16G in applications including, the menstrual cycle, foetal well-being, detecting high-risk pregnancy, and to monitor environmental liquid, waste waters, and drinking water. These applications are the focus of on-going investigations.

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