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Surface plasmon resonance based indirect immunoassay for detection of 17β-estradiol

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ABSTRACT

A surface plasmon resonance (SPR) based immunosensor is presented for highly sensitive and selective detection of 17β-estradiol by the indirect competitive inhibition immuno assay, employing anti-17 β-estradiol antibody as high molecular weight (HMW) interactant. Immobilization of estradiol-BSA conjugate onto the nano thin gold surface was accomplished by covalent amide linkage through self assembled monolayer. The proposed biosensor is simple to fabricate, reproducible and exhibit excellent sensitivity for estrogen (detection limit,1 pg mL^{-1}) without any significant interference from structurally similar steroidal hormone, progesterone and non-steroidal compound bisphenol-A. The proposed surface displayed a high level of stability during repeated regeneration and immunoreaction cycles suitable for biosensor development.

1. Introduction

Monitoring estrogen level in real-time at trace level is of great significance in understanding its role in hormone induced cancer [1]. The assay of ovarian hormone has always been a difficult task although the phenolic characteristic of estrogen provides a means of positive chemical identification and semi-quantitative estimation through Kobler's method [2]. However the small quantities of hormone present in blood have complicated its estimation and requires newer techniques like, radioimmunoassay RIA [3,4], enzyme linked immunosorbent assay (ELISA) [5,6], GC–MS [7], and LC–MS [8,9]. Although these techniques have high detection sensitivity, they are multistep, time-consuming procedures, require radioactive labeled reagents and elaborate instrumental setup. Thus rapid and simple procedures are required to improve the efficiency of measurement. In the present era of point-of-care treatment, the sensors with the ease of fabrication are often preferred for the quick and real time monitoring. As far as sensors for estradiol is concern electrochemical sensor based on measurement of suppression of current for ferricyanide/ferrocyanide redox reaction have been reported employing different strategies for immobilizing the biorecognition element [10–12]. Fluorescence based immuno assay has also been reported using 17α-fluorescein-labeled estradiol as ligand for the estrogen receptor binding studies [13]. Alsager et al. [14] have reported a colorimetric detection of 17 β-estradiol using DNA aptamer as sensing element and coating of gold nanoparticles (AuNPs) as label, where length of aptamer is the deciding factor to discrimination against other steroidal molecules.

Surface Plasmon Resonance (SPR), represents a real time, highly sensitive and full proof technique to study biomolecular interaction in one step. SPR is an optoelectronic phenomenon, where light incident on a plasmonic metal surface (gold or silver) at a given angle, can excite a surface-bound electromagnetic wave, the surface plasmon. Associated with the surface plasmon is an evanescent field that probes local changes in the refractive index of the ambient medium as a result of specific biomolecular binding events. A change in the refractive index shifts the angle of incidence at which SPR excitation occurs. This shift is tracked by monitoring the movement of the intensity minima of the reflected light as a function of time and the binding event is presented as a sensogram. Number of SPR based biomolecular interactions have been reported from our group using variety of biorecognition elements viz. enzymes [15], antibody [16] and receptor [17]. Notable features of SPR based sensing are high sensitivity and selectivity without any label unlike ELISA, fluorescent immunoassay and radioimmunoassay. Moreover, fiber optics based SPR has opened

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Table 1
Biosensor for detection of estrogen.

Biorecognition Element	Targetanalyte	Detection technique and Assay mode	Detection range and Detection limit	Cross reactivity	Ref.
Histidine-tag protein/ thiol-iminodiacetic acid on gold electrode	hER	EC CV /Redox Probe	0.01 – 100 μM –	Endocrine disruptors	Murata et al. [10]
hER-LBD /CdS NPs modified CS peptides on Hg electrode	hER	EC (ASV)	0.1 – 1 nM –	–	Hansen et al. [11]
3-MPA/-hER on gold electrode	E2	EC EIS /Redox Probe	1μM	–	Imet al. [12]
17α-fluorescein-labeled estradiol derivative vs hER	E2	(FIA) (Inhibitive assay)	–	E1, Estriol, DES, Tamoxifen	Ohno et al. [13]
DNA (75-mer) Aptamer on gold NPs	E2	Colorimetric detection	200 pM	BPA, BPF T 33% ; P4 45%	Alsager et al. [14]
Estradiol-BSA conjugate on sensor chip vs hER	E2	SPR (Inhibitive assay)	376 μM (100 μg.mL^{-1})	DES	Pearson et al. [19]
E2-17PeNH /CM5gold sensor chip vs. hrERα	Estrogens	SPR (Inhibitive assay)	0.1 – 1 μM	P4	Usami et al. [20]
E2–BSA on carboxymethylated dextran-coated gold sensor chip vs.Ab-E2	E2	SPR (Inhibitive assay)	0.5 – 20 nM 7nM (200 pg mL^{-1})	DES	Miyashita et al. [21]
DNA element with ERE vs. ERα / Sp1 protein	E2	SPR	– –	–	Neo et al. [22]
Thio ether/3-MPA/E2/ OEG / CM5 sensor chip vs. Secondary Ab-E2	E2	SPR (Inhibitive assay)	– 92 pM (25 pg mL^{-1})	–	Mitchell et al. [23]
11-MUA / E2-BSA conjugate on gold sensor chip vs. Ab-E2	E2	SPR (Inhibitive assay)	0.036– 3.6 pM 0.0036 pM (1 pg mL^{-1})	BPA, P4	Present study

EC: Electrochemical; CV: Cyclic Voltammetry; ASV: Anodic stripping voltammetry; EIS: Electrochemical Impedence Spectroscopy; FIA: Fluorescence Immuno assay; SPR: Surface plasmon resonance; LBD: Ligand Binding Domain; hER: Human Estrogen Receptor; CS: Conformation Specific; hERα : human recombinant Estrogen Receptor-α; transcriptional factor: Sp1; ERE: Estrogen Response Element; BSA: bovine serum albumin; 3-MPA: 3-mercaptopropionic acid; OEG: Oligoethylene glycol; 11-MUA: 11-mercapto undecanoic acid; CM5 sensor chip: Carboxymethylated dextran coated gold chip; E2: Estrogen; E1: Estrone; DES: Diethylstilbestrol; BPA: bisphenol-A; BPF: bis(4-hydroxyphenyl) methane; P4: Progesterone; T: Testosterone.

up huge possibility of miniaturization and point-of-care sensing for biomedical and environmental applications [18]. Towards SPR based screening of estradiol and estrogens, much of the earlier work has been on the estrogen receptor as recognition species [19–21]. SPR based immunoassay has also been used for measurement of estrogen receptor-binding activity [22]. Mitchell et al. [23] have reported anti-estradiol interaction with thioether-linked 3-mercaptopropionic acid derivatives of 17β-estradiol linked to a 15-atom long oligoethylene glycol chin for sensing estradiol in SPR format. Comparative accounts of various estrogen biosensor assay tools found in literature are included in table1.

Herein we report a facile fabrication SPR sensor surface for sensitive and selective detection of 17β-estradiol in competitive inhibition immuno assay format with anti-estradiol as high molecular weight (HMW) interactant.

2. Experimental

2.1. Instrumentation

The SPR system Autolab Model SPRINGLE (Eco-Chemie, Utrecht, Netherlands) equipped with an open cuvette system (20–100 μL of sample volume), optical functioning based on Kretchmann configuration and a flexible software package for controlling the functioning of the instrument is used for present study. BK7 microscopic glass plates (refractive index 1.26–1.38) with gold nano coating (48–50 nm) were used as sensor chips. At each sensor chip, four sites could be made available to study molecular interaction by changing the position. All experiments were carried out at 25 °C.

2.2. Chemical and reagents

11-Mercaptoundecanoic acid (11-MUA), N-hydroxy succinamide, (NHS), N-Ethyl-N′-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), 17-β-estradiol, anti-17β-estradiol (E2885-100TST), progesterone, bisphenol-A, pepsin, glycine and Tris buffer were procured from Sigma Aldrich, USA; sodium dodecyl suplphate from Hi-Media Labs and HCl from QualigenFine Chemicals. Ultra pure deionized water and HPLC grade solvents were used to prepare the solutions. Phosphate buffer saline (PBS), pH 7.2 (0.01 M disodium hydrogen phosphate and 0.01 M potassium dihydrogen phosphate containing 0.8% NaCl and 0.02% KCl) was used as carrier buffer and flowed over sensor surface throughout the SPR experiment. 0.5% sodium dodecyl suplphate (SDS) aqueous solution, 0.1 M HCl and 50 ppm pepsin solution prepared in 0.2 M glycine–HCl buffer (pH 2) were tested as regeneration buffer. 1 mM 11-mercaptoundecanoic acid in ethanol was used for preparing self-assembled monolayer over the sensor chip. A stock solution of BSA (0.2 g /10 mL) was prepared in 0.01 M sodium acetate buffer (pH 4.5). Standard stock E2 solution (200 μg mL^{-1}) was prepared in methanol and further dilutions were made with PBS.

2.3. 17 β-estradiol conjugate

17β-estradiol conjugate was prepared in-house with bovine serum albumin (BSA) as a carrier protein [17]. Briefly, equal quantities of BSA (0.01 gm), NHS (0.01 gm,), and EDC (0.01 gm,) were dissolved in 1 mL Sodium acetate (0.01 M, pH4.5); mixture was sonicated for 2 min in an ultrasonic bath maintained at low temperature. To the above residue, 1 mg 17β-Estradiol (E2) was then added and mixture was again sonicated for 30 s and left incubated at RT for 2 h. The 17β-estradiol-BSA conjugate (E2-BSA) was then dialyzed in PBS buffer followed by distilled water at low temperature (3–5 °C). The product E2–BSA conjugate was lyophilized and stored at −20 °C, and designated as stock conjugate containing 1000 μg mL of E2. Working dilutions were made with 0.01 M sodium acetate. Anti –Estradiol (Ab-E2) as received, was reconstituted in 5 mL of Tris-HCl buffer (0.05 M, pH 8.0), designated as stock Ab-E2, stored at −20 °C; further dilutions were made with PBS buffer.

2.4. Fabrication of biosensor surface

Clean SPR sensor chips were left immersed overnight in 1 mM 11-MUA/ethanol, to allow self assembled monolayer (SAM) formation [15,16], which were then attached to the prism of the SPR instrument using a matching liquid (refractive index = 1.515). A buffer of low salt concentration (0.01 M sodium acetate) was injected over sensor chip to stabilize the self assembled 11-mercaptoundecanoic acid monolayer. The next step was *in-situ* activation of SAM by introducing NHS–EDC reagent (1:1 aqueous solution of 100 mM NHS and 400 mM EDC) for 5 min., followed by introducing E2–BSA conjugate for 15 min. Deactivation of unbound esters was accomplished by introducing BSA protein (500 μg mL^{-1}). All injections were of 50 μL.

2.5. Immunoassay and E2 detection mechanism

Anti-estradiol (Ab-E2) was flown over SPR sensor chip to optimize the SPR conditions for direct immuno-binding interaction between E2 and Ab-E2. The immunoreaction between immobilized estradiol and its antibody (association phase) was studied for different periods. An optimum time period of ~15 to 20 min was found to be satisfactory to get a stable SPR signal for successful interaction and binding. Every association phase was followed by a brief flow of carrier buffer, (PBS at pH 7.2) for 2 min to wash out the unreacted species (dissociation phase). The effective rise at the end of dissociation phase with respect to resonance angle at initiation of association phase is correlated with the analyte concentration in direct immuno assay. The regeneration step comprised a brief flow of pepsin solution for an optimized time of 120 s. Indirect competitive inhibition immunoassay protocol [17] was carried out for detection of E2, wherein, a higher concentration of Ab-E2 optimized by direct immunoassay was mixed with different concentrations standard E2 solution. After incubation at room temperature (25°) for 10 min, mixture was injected over E2–BSA conjugate immobilized sensor surface. Here, estradiol in solution and estradiol–BSA conjugate on the sensor surface will compete with each other for immuno reaction with anti-estradiol, and hence the interaction of anti-estradiol on the sensor surface will reduce with increasing concentration of standard estradiol in solution. Thus decrease in the shift of resonance angle with an increase in the concentration of estradiol was utilized for quantitation.

3. Results and discussion

3.1. Fabrication of biosensor

The gold sensor chip functionalized with 11-MUA provides covalent bonding sites to estradiol-BSA conjugate via primary amine group following *in-situ* carbodiimide activation. The strong affinity of gold for sulphur, results in a perfect orderly monolayer over the bare gold sensor chip and activated carboxylic acid group extending out for amide bonding with amine groups of protein conjugate. Fig. 1 shows the SPR response observed for the preparation of sensor surface, where-in first step is, *in-situ* activation of 11-MUA self assembled monolayer (SAM) modified gold sensor chip by introducing EDC-NHS solution followed by immobilization of estradiol conjugate. A net rise of resonance angle by 85 m° (Fig.1 curve a) signifies a good quality and stable matrix of activated monolayer over SPR sensor surface. Upon introducing 10 μg.mL^{-1} of conjugate onto the activated SAM, a sharp increase of resonance angle (ca. 200 m°) indicated a successful binding of E2-BSA conjugate over 11-MUA functionalized sensor surface (Fig.1 curve b). A brief desorption of the loosely bound conjugate with PBS flow got stabilized which indicates that covalently bound estradiol conjugate is highly stable on the MUA modified sensor surface. Subsequent introduction of E2-BSA conjugate shots revealed a further increase in resonance angle (Fig.1 curve c, d) with a plateau at ~150 μg mL^{-1} (Fig.1 curve e), showing a net rise of ca. ~ 465 m°. Inset, Fig. 1 shows a plot of net rise in resonance angle with increasing concentration of E2-BSA conjugate in the concentration range 10–250 μg.mL^{-1}. The step wise immobilization of E2-BSA conjugate was performed to ascertain the optimum concentration to be used for one-step immobilization. Looking to these results, a single step injection of high concentration of E2-BSA conjugate (200 μg mL^{-1}) over activated 11-MUA immobilized gold sensor chip was attempted. A net rise of ~477 m° was recorded, which suggests that stable immobilization of estradiol conjugate could be accomplished even in single step injection of 200 μg mL^{-1} of E2-BSA conjugated to fully cover the surface area ca. 8.5 mm^2 of sensor chip being used in present experimental set up. To avoid any non-specific interaction at sensor surface, one shot of BSA protein (500 μg mL^{-1}) was introduced to deactivate the unbound esters if any.

E2–BSA conjugate immobilized surface thus fabricated was used as a recognition platform for repetitive analysis of anti-estradiol in a direct immunoassay and for estradiol in an indirect competitive immunoassay.

3.2. Direct immunoassay of Anti-17β-estradiol with 17β-Estradiol-BSA conjugate

The SPR response of the sensor chip with E2-BSA conjugated end-groups to the antibody, anti-17β-Estradiol (Ab-E2) was investigated. The SPR angle transient observed at different dilutions of Ab-E2 are shown in Fig. 2 as overlay curves. Shift in resonance angle with increasing concentration of anti-17β-Estradiol (inset, Fig. 2) revealed a linear response (R^2 = 0.992).

Shift in SPR angle upon introducing the Ab-E2 indicates specific immunoreaction with E2 heptens present on the sensor surface. When the flow was switched back to the PBS buffer, a small dip in the SPR angle was observed due to the removal of nonspecifically bound Ab-E2 from the sensor surface. Further, flow of PBS did not bring any change the SPR angle, indicating stability of the binding of Ab-E2 with the E2 present at sensor chip. The antibody bound to the sensor chip was removed to regenerate the biosensor surface for multiple and cost-effective analyses. Different regeneration solution including 0.1 M HCl, 0.5% SDS and 50 ppm pepsin solution were evaluated for their ability to regenerate the conjugate surface. Among them pepsin solution prepared in glycin-HCl buffer (pH 2.0) was shown to be effective in dissociating the antigen-antibody immunocomplex without disturbing the conjugated antigen at sensor surface. A brief flow of regeneration buffer (pepsin solution) for optimized time of 120 s was sufficient to liberate the surface-bound Ab-E2, leaving the sensor surface active for next injection. The conjugate immobilized assay format used in present study had a major advantage that it could easily be regenerated to dissociate the antibody without disturbing the E2-BSA conjugate on sensor surface. Thus, the sensor chip could be reused for multiple analyses.

3.3. Estradiol detection by indirect competitive inhibition

For quantitation of 17β-Estradiol, principle of indirect competitive inhibition was employed wherein 17β-Estradiol-BSA conjugate served as a ligand/recognition element and *Anti*-17β-Estradiol estrogen as high molecular weight (HMW) interactant. Equal volumes of antibody (at an optimized concentration,1:10 dilution) and different concentration of 17β-Estradiol (0.001 ng mL^{-1}–10 μg mL^{-1}) were incubated for 15 min at room temperature and were introduced over the 17β-Estradiol-BSA immobilized gold surface. Fig. 3 shows SPR response observed for the interaction of immobilized 17β-Estradiol-BSA conjugate and antibody

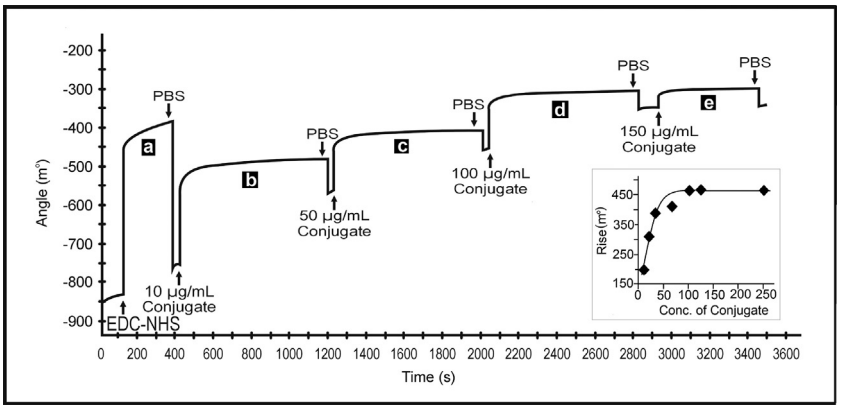

Fig. 1. SPR response showing activation of 11-MUA modified gold surface followed by.
stepwise immobilization of E2-BSA conjugate ; Inset shows plot of net rise in resonance angle with increasing concentration of E2-BSA conjugate in the concentration range 10–250 µg mL^{-1}.

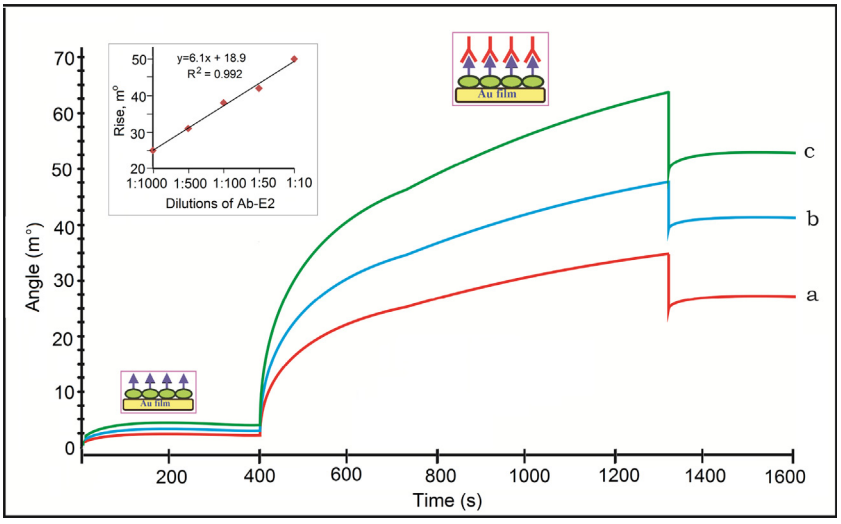

Fig. 2. Direct immunoassay: SPR response of Ab-E2 with E2-BSA conjugate interaction at different dilutions of antibody, 1:1000 Ab-E2 (a); 1:100 Ab-E2 (b); 1:10 Ab-E2 (c). Inset shows plot of rise in resonance angle with increasing concentration of anti-17 β-estradiol.

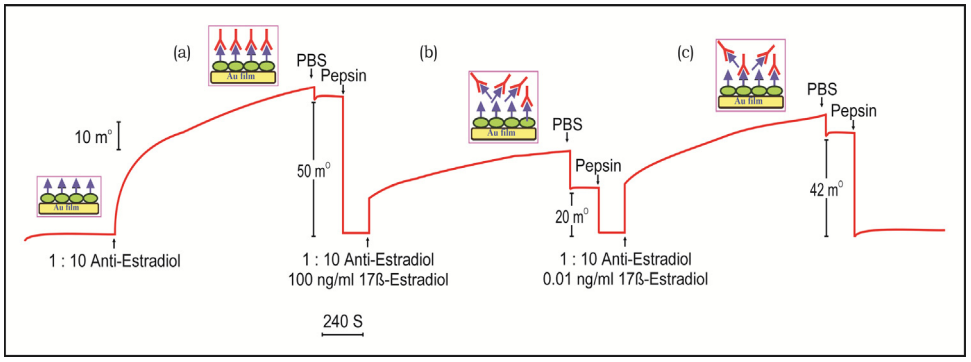

Fig. 3. Indirect competitive inhibition immunoassay: SPR response of E2-BSA binding interaction with 1:10 Ab-E2 (a); and incubated with different concentration of E2 (b,c).

in absence and presence of free 17β-Estradiol. After each injection, regeneration of conjugate surface was achieved by introducing pepsin solution over sensor surface for a brief interaction time of 3–5 min. The shift in resonance angle decreased with an increase in the concentration of free 17β-Estradiol in solution. This is in accordance with the principle of indirect competitive inhibition, where in the 17β-Estradiol in free solution inhibit the binding interaction of anti-17β-Estradiol with 17β-Estradiol-BSA conjugate. All measurements were made in triplicate and mean of each was used for curve fitting. The four parameter logistic function [24] is:

$$f(x) = \{[1\text{-}a]/[1 + (x/c)b]\} + d$$

where, parameters a and d are the asymptotic maximum and minimum values respectively; c is the value at the inflection point (IC_{50}) and b is the slope. The calibration curve was plotted between the concentration of standard E2 solution (0–10000 ng mL^{-1}) and

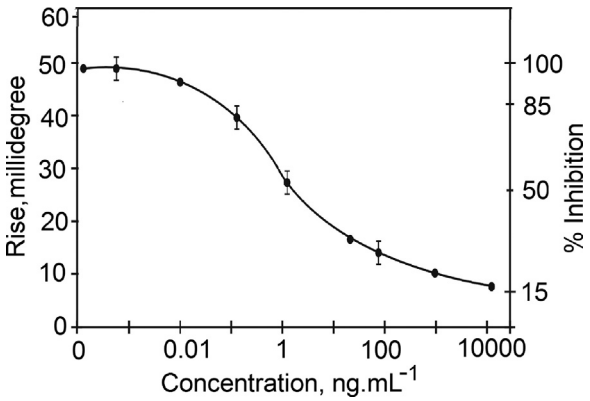

Fig. 4. Calibration plot for detection of E2 by indirect competitive inhibition (R^2 =0.991; 4-parameters logistic curve: a = 54, b = 0.2, c = 34, d= - 4).

change in resonance angle (Fig. 4) following the 4-parameters logistic curve. The sigmoidal calibration curve clearly indicates the inhibition of the binding interaction between AbE2 and E2-BSA in presence of E2. The percentage of inhibition with respect to the E2 concentration has also been shown in Fig. 4. The extent of inhibition depends linearly with the concentration of free E2 mixed with Ab–E2. Under the optimal condition, the biosensor revealed a linear detection range from 0.01 ng mL^{-1} to 1000 ng mL^{-1} corresponding to 85%–15% inhibition with a lower detection limit of 1 pg mL^{-1} (S/N = 3). This sensitivity is significantly higher than most of the biosensors reported for detection of E2 (Table1). Further, considering the normal range of estrogen in blood as 2–40 pg mL^{-1} [9], proposed biosensor could be used for monitoring E2 concentration in clinical samples. The high sensitivity observed with present biosensor is possibly due to the combined advantages of SPR format and the inherent specific immunoreaction between Ab-E2 and E2, the covalent bonding of immobilized E2–BSA conjugate with self assembled thioic acid monolayer on nanothin gold surface and indirect competitive inhibition assay principle.

3.4. Specificity and cross reactivity studies

The biggest challenge for a biosensor of clinical application is the specificity of detection and cross reactivity. In order to evaluate the specificity of present biosensor the effect of structurally similar non-steroidal compound bisphenol-A (BPA) and steroidal hormone, progesterone (P4) were examined. The SPR response of the binding interaction of E2-BSA conjugate with antibody 1:10 Ab-E2 spiked with 100 ng.mL^{-1} of BPA, E2 and P4 is shown in Fig. 5. As expected, a decrease in resonance angle shift was observed in case of E2, whereas no appreciable change in resonance angle was observed in presence of a bisphenol and progesterone. The results clearly indicate that 17β-Estradiol-BSA conjugate has high selectivity towards 17β-Estradiol.

4. Conclusion

During present study, the SPR based immunosensor has been developed wherein 17β-Estradiol -BSA conjugate served as a ligand/recognition element and *Anti*-17β-Estradiol estrogen as high molecular weight (HMW) interactant, employing the indirect competitive inhibition immuno assay. A linear dynamic range was 0.01 ng/mL–1000 ng/mL, where inhibition ranged from 85% to 15% with lowest detectable concentration as 10 pg/mL. The IC_{50} i.e. concentration of analyte causing 50% inhibition of SPR signal was found to be 1 ng/mL. No interference from potent cross reactant like progesterone and bisphenol-A confirms present immunosensor format to be highly specific and selective for estradiol. Moreover, sensor surface fabrication supports the regeneration of the sensor surface for multiple, cost-effective analyses.

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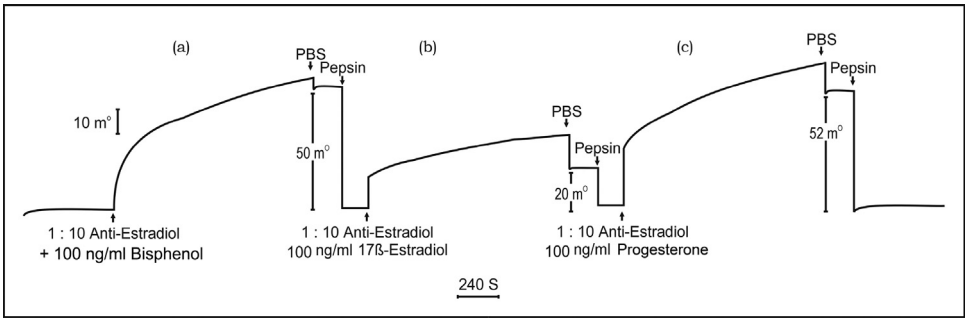

Fig. 5. Cross reactivity of estrogen immune sensor: SPR response for binding interaction of E2–BSA with 1:10 Ab-E2 incubated with 100 ng.mL^{-1} Bisphenol (a); 1:10 Ab-E2 (b); 1:10 Ab-E2 incubated with 100 ng.mL^{-1} progesterone (c).

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