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## Label free biosensor for screening estrogenic activity

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Estrogens and estrogen mimics prevalent in aquatic environment are of great environmental concern because of their endocrine disrupting and carcinogenic activities. Looking to the wide variety of natural as well as structurally different synthetic estrogen mimics, a reliable *in-vitro* assay is required for screening the estrogenic activity of endocrine disrupting compounds (EDCs). Surface plasmon resonance (SPR) is one of the most promising analytical tools to monitor the high-performance biomolecular interactions in a label free, real time format. Present paper demonstrates a facile SPR based affinity bioassay employing estrogen receptor- $\alpha$ , human (hER $\alpha$ ) functionalized self assembled monolayer covalently bound onto the gold sensor chip as recognition species. A successful interaction of potential estrogen mimics with estrogen receptor is evidenced by net rise in SPR angle. The assay has been validated in terms of optimum experimental conditions and specificity with estrogen as a positive control showing maximum estrogenic activity. As a proof of concept, proposed affinity assay is tested for screening the estrogenic activity of progesterone, pregnenolone, tamoxifen, and bisphenol-A as representative examples of potential EDCs of different classes.

**Keywords:** Surface plasmon resonance, Label-free affinity, Biosensor, Estrogen receptor, Estrogenic activity, Estrogen mimics, Endocrine disrupting chemicals

Endocrine-disrupting compounds (EDCs) are chemical substances of natural or synthetic origin prevalent in surface and waste-waters, are shown to block/ interfere with the production, metabolism and/or action of endogenous hormones posing threat to aquatic life, wild life and human health<sup>1,2</sup>. Naturally occurring estrogens and estrogen mimics (chemicals that antagonize the actions of estrogen) typically referred as having estrogenic activity, represent the most significant class of EDCs. Natural estrogens (e.g. estrone, estradiol, estriol and estetrol), natural androgens (e.g. testosterone, dihydrotestosterone and

androstenedione), phytoestrogen (e.g. coumestrol, genistein), pharmaceuticals (e.g. 17 $\alpha$ -ethynyl estradiol, contraceptive pill formulations), pesticides and industrial chemicals (e.g. Atrazine, 2,4-dichlorophenoxyacetic acid), polyaromatic hydrocarbons (e.g. benzo[a]pyrene), poly chlorinated compounds (e.g. PCBs, dioxins, furans) and alkylphenols (e.g. 4-nonylphenol) are some of the major contributors of estrogenic activity in the aquatic environment. Fluorescence immunoassay (FIA) and radio-immunoassay (RIA), liquid chromatography, gas chromatography-mass spectrometry (GC-MS) are conventional standard analytical techniques<sup>3,4</sup> for detecting EDCs, however multistep sample preparation and elaborate instrumental setup pose them as unsuitable for rapid screening of estrogenic activity. *In-vivo* biological assays for EDCs<sup>5</sup> involving cell lines, rodent uterotrophic test and vitellogenin assay using medaka fish also require expertise in handling highly specialized laboratory protocols, long assay time and ethical clearance. In present era of point-of-care testing (POCT), sensors and biosensors are the most convenient *in-vitro* assay tool for field monitoring at or near point of care.

The third/fourth generation sensors are self-contained integrated devices, capable of providing specific quantitative or semi-quantitative analytical information using a biological recognition element. Inclusion of biological element (e.g. enzyme, antibody, receptors etc) to sensor surface adds inherent specificity whereas nano thin sensor film combined with advanced transducers and the processing power of modern microelectronics offer highly sensitive and specific analytical tools as nano-biosensor. Mozaz *et al.*<sup>6</sup> have reviewed biosensor strategies for EDCs monitoring. The electrochemical biosensors<sup>7</sup> are based on depletion in voltammetric current of mediator redox probe upon binding; whereas most optical biosensors employ fluorescent and /or nanoparticles as label<sup>8</sup>. Surface plasmon resonance (SPR) based biosensors represents label free, real time, highly sensitive device to monitor organics and bioorganic analytes involving single step biomolecular interaction using a variety of biorecognition elements viz. enzymes<sup>9</sup>, antibody<sup>10</sup> and receptor<sup>11</sup>.

The concept of SPR introduced by Ritchie<sup>12</sup> has led to the emergence of a versatile optical phenomenon of SPR. Briefly, SPR occurs when a plane-polarized light strikes a plasmonic metal film interface, under the conditions of total internal reflection. In thin (ca. 50 nm) plasmonic metal (ca. Au) layers, electrons behave as free electron gas, the plasmon and the irradiated light can excite them as surface plasmon. Associated with the surface plasmon is an evanescent wave that probes local changes in the refractive index (RI) of the ambient medium as a result of specific biomolecular binding events as shown in Fig 1. A glass prism coupling configuration or recently introduced fibre-optic configuration can be used to obtain evanescent wave to excite the surface plasmon<sup>13</sup>.

Review of literature revealed number of SPR based biosensor assay protocol for estrogens and estrogen mimics, Table 1 includes significant parameters. Most of the SPR based studies have employed EDC specific antibodies<sup>14-20</sup> for their sensitive detection.

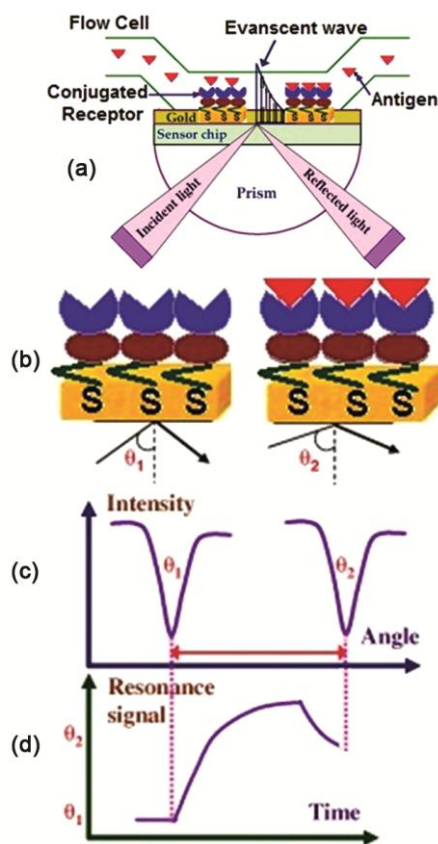


Fig. 1 — Schematic illustrations: (a) SPR at metal-dielectric interface in a prism based Kretschmann configuration, (b) functionalized sensor surface before and after affinity binding, (c) SPR response as reflectivity curve and (d) sensogram.

Estrogen receptors (ER), estrogen response element (ERE), and DNA element with ERE has also been reported as recognition species under varied immobilization strategies for biosensor application in inhibitive assay detection format<sup>21-25</sup>. Estrogen, E2 show strong binding to antibody, Ab-E2<sup>22,26</sup> as well as to receptor, ER whereas, the estrogen mimics e.g diethylstilbestrol and bisphenol-A (BPA) do not bind to Ab-E2 instead bind to ER<sup>21,22</sup> and antagonize the actions of estrogen in human body. Therefore, it is pertinent to look for affinity based detection for screening the estrogenic activity.

Herein, we report a facile SPR based direct affinity assay employing estrogen receptor as recognition species for screening the estrogenic activity of some of the potential EDCs of different categories (Fig. 2). Progesterone (P4) and pregnenolone (P5) are endogenous steroidal hormones structurally similar to estrogen. Progesterone is a progestogen sex hormone and pregnenolone is a precursor/metabolic intermediate in the biosynthesis of most of the steroid hormones. Tamoxifen (Tm) and BPA are non-steroidal estrogen mimics. Tamoxifen is a pharmaceutical drug, act as estrogen receptor agonist and BPA is an estrogenic chemical of industrial origin. The assay has been validated in terms of optimizing the experimental conditions for direct affinity assay with estradiol as positive control standard with maximum estrogenic activity.

## Materials and Method

### Instrumentation

A glass prism based SPR system in Kretschmann optical configuration (Autolab Model SPRINGLE (Eco-Chemie, Utrecht, Netherlands) and circulator (Julabo, GmbH) was used for present study. Microscopic glass chips (refractive index 1.26 -1.38) with gold coating (48-50 nm) were used as sensor chips. A matching liquid (refractive index = 1.515) was used to fix the chip onto the prism. All experiments were carried out at 25 °C and sample tubes were kept in mini fridge cooled 4-5 °C during assay.

### Chemicals, reagents and bio-reagents

17- $\beta$ -estradiol (E2), estrogen receptor- $\alpha$ , human (hER $\alpha$ ) (E1528), progesterone (P4), pregnenolone (P5), bisphenol-A (BSA), tamoxifen (Tm), 11-mercaptoundecanoic acid (11-MUA), N-hydroxyl succinamide, (NHS), N-Ethyl-N'-(3-dimethylamino-propyl) carbodiimide hydrochloride (EDC), bovine

Table — 1 SPR based biosensors assay protocol for estrogens and estrogen mimics

| Recognition element for SPR sensor surface   | Target analyte                       | SPR Assay mode               | Detection range   | References                            |
|--|--------------------------------------|------------------------------|---|---------------------------------------|
| Normorphine-BSA / Au vs. Ab-morphine   | Morphine                             | Inhibitive Immuno assay      | 0.1 - 10 ng.mL <sup>-1</sup>                                | Miura <i>et al.</i> <sup>14</sup>     |
| MA-BSA / Au vs. Ab-MA  | MA                                   | Inhibition Immuno assay      | 0.1–1000 ng.mL <sup>-1</sup>                                | Sakai <i>et al.</i> <sup>15</sup>     |
| E3G-ovalbumin / Au vs. AbE3G   | E3G                                  | Inhibitive Immuno assay      | 10 to 150 ng.mL <sup>-1</sup>                               | Sesay <i>et al.</i> <sup>16</sup>     |
| BaP-BSA/ Au vs. Ab-BaP   | BaP                                  | Inhibitive Immuno assay      | 0.01 -2 ng.mL <sup>-1</sup>                                 | Miura <i>et al.</i> <sup>17</sup>     |
| CA and 2-MEA mix SAM/ BaP/ Au vs. Ab-BaP   | BaP                                  | Inhibitive Immunoassay       | 50 pg.mL <sup>-1</sup> to 100 ng.mL <sup>-1</sup>           | Gobi <i>et al.</i> <sup>18</sup>      |
| C <sub>11</sub> - and C <sub>16</sub> -alkanethiols mix SAM/ analyte/ Au vs. respective antibodies | Atr, BaP, 2,4-D, 4-NP                | Inhibitive Immunoassay       | 0.05 - 0.26 ng.mL <sup>-1</sup>                             | Dostalek <i>et al.</i> <sup>19</sup>  |
| C <sub>11</sub> - and C <sub>16</sub> - alkanethiols mixed SAM / Au vs. Ab-BPA                     | BPA                                  | Inhibitive Immunoassay       | 0.14 ng.mL <sup>-1</sup>                                    | Hegnerová <i>et al.</i> <sup>20</sup> |
| E2-BSA conjugate/ CM5/Au vs. hER   | E2, DES                              | Inhibitive Affinity assay    | 100 µg.mL <sup>-1</sup>                                     | Pearson <i>et al.</i> <sup>21</sup>   |
| E2-BSA conjugate/ CM5/Au vs. Ab-E2 / hER   | E2, DES                              | Inhibitive affinity assay    | 0.2 ng.mL <sup>-1</sup> E2                                  | Miyashita <i>et al.</i> <sup>22</sup> |
| ERα <sup>LBD</sup> - αβ/1 biotinylated peptide / CM5/Au vs. peptides                               | E2, EE2, 4-NP, Tm                    | Inhibitive affinity bioassay | 20 ng.L <sup>-1</sup> E2                                    | Spina <i>et al.</i> <sup>23</sup>     |
| E2-17PeNH/CM5/Au vs. hrERα   | E3, E2, DES, E1, Tm, BPA, 4NP, T, P4 | Inhibitive affinity bioassay | 0.1– 1 µM<br>No response for P4                             | Usami <i>et al.</i> <sup>24</sup>     |
| SA dextran /DNA /biotinylated ERE/ ERα vs. E2  | EDCs, E1, DHS, E2                    | Inhibitive affinity bioassay | 1nM for low responder<br>10 nM to 100 nM for high responder | Asano <i>et al.</i> <sup>25</sup>     |
| hERα- BSA/ 11-MUA/ gold surface  | E2, P5, Tm, BPA, P4                  | Direct affinity bioassay     | 0.1 -10 µg.mL <sup>-1</sup><br>No response to P4            | Present study                         |

SPR: Surface plasmon resonance; hERα: Estrogen Receptor-α, human; Sp1: transcriptional factor; BSA: bovine serum albumin; OG: oligoethylene glycol; CA: Cysteamine; 2-MEA: 2-mercaptoethanol CMD: Carboxymethylated Dextran; 11-MUA: 11-mercaptoundecanoic acid; ERα<sup>LBD</sup>: Engineered Estrogen Receptor-α; ERE: estrogen response element; E1: Estrone; E2: Estradiol; E3: Estriol, EE2: ethinyl-estradiol; MA: Methamphetamine; E3G: estrone-3-glucuronide; DES: Diethylstilbestrol; BPA: bisphenol-A; Tm: Tamoxifen; BPF: bis(4-hydroxyphenyl) methane; Atr: Atrazine; BaP: benzo[a]pyrene; 2,4-D: 2,4-dichlorophenoxyacetic acid; MA: Methamphetamine; 4NP: 4-nonylphenol; P4: Progesterone; ; P5: pregnenolone; T: Testosterone; E2-17PeNH: aminated estradiol with a spacer molecule; estrogen response element (ERE), Streptavidine (SA), endocrine disrupting compounds (EDCs), LBD: ligand binding domain

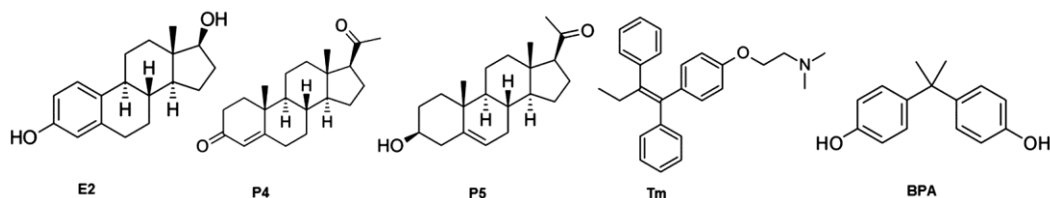


Fig. 2 — Structure of Estradiol (E2), Progesterone (P4), Pregnenolone (P5), Tamoxifen (Tm) and Bisphenol-A (BPA)

serum albumin (BSA), ethanol amine (EA), pepsin, glycine and tris-buffer were procured from Sigma Aldrich, USA. All other reagents were of AR grade from Merck. Deionized water and HPLC grade solvents were used to prepare 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) in deionized water, was used as carrier

buffer. Pepsin solution (0.2 M) prepared in glycine–HCl buffer (pH 2) was used as regeneration buffer. A stock solution of BSA (0.2 g /10 ml) was prepared in 0.01 M sodium acetate buffer (pH 4.5).

#### ER-BSA conjugate

Estrogen receptor-α human (ER) conjugate with bovine serum albumin (BSA) as a carrier protein was prepared in-house. Briefly, equal quantities of BSA

(0.01 g), NHS (0.01 g), and EDC (0.01 g) were dissolved in 1 mL sodium acetate (0.01 M, pH 4.5); mixture was sonicated for 2 min in an ultrasonic bath maintained at low temperature. To the above residue, estrogen receptor (ER) as received was then added and mixture was again sonicated for 30 s and left incubated at RT for 2 h. The estrogen receptor-BSA conjugate (ER-BSA) was then dialyzed in PBS buffer followed by distilled water at low temperature (3–5 °C). Finally, the product was lyophilized, designated as stock ER-BSA conjugate and stored at –20 °C. Dilutions of stock ER-BSA conjugate were made with 0.01 M sodium acetate.

#### Functionalization of SPR chip and assay procedure

Clean SPR sensor chips were immersed in 1 mM 11-MUA/ethanol for minimum 3 h to allow self assembled monolayer (SAM) formation, however best results were obtained with overnight immersion<sup>10</sup>. SAM immobilized gold chip was placed carefully onto the prism of the SPR instrument using a matching liquid. First, a buffer of low salt concentration (0.01 M sodium acetate) was injected over sensor chip to stabilize the SAM. The next step was *in-situ* activation of SAM by introducing 50 µL NHS–EDC reagent (1:1 aqueous solution of 100 mM NHS and 400 mM EDC) with interaction time of 5 min, followed by functionalization of sensor surface by introducing 50 µL ER-BSA conjugate (100 µg.mL<sup>-1</sup>) and allowing interaction for 15 min. Next, blocking step was performed by introducing one shot of ethanolamine (1 M, pH 8). All injections of over sensor surface were of 50 µL and carrier buffer (PBS, pH 7.2) was flowed over sensor surface after each interaction throughout the SPR experiment.

Estradiol (E2) was flown over SPR sensor chip to optimize the SPR conditions for direct affinity binding interaction of ER and E2. The affinity binding interaction of immobilized ER-BSA conjugate and antigen, E2 (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 for 120 s to wash out the un-reacted species (dissociation phase). The effective rise at the end of dissociation phase with respect to the resonance angle at the initiation of association phase is correlated with the analyte concentration in direct affinity assay. The regeneration step, comprised a

brief flow of 0.2 M pepsin solution (in glycine–HCl buffer, pH 2).

#### Results and Discussion

The estrogenic activity of EDCs depends upon their binding with estrogen receptor which may pose a disrupting effect on gene expression. For screening the estrogenic activity, it is envisaged to establish the extent of affinity of estrogen receptor with potential EDCs, therefore efforts are made to establish a SPR based direct affinity assay employing sensor chip functionalized with estrogen receptor as sensing element.

To avoid non-specific adsorption/binding at the sensor and to provide covalent bonding sites to the ligand, a self-assembled monolayer of 11-mercaptopundecanoic acid (11-MUA) acted as matrix for immobilization the sensing element. In SPR mode, a net rise of resonance angle by ~ 200 m° (milli degree) as compared to the bare gold chip signifies a good quality and stable matrix of SAM over the gold surface<sup>10</sup>. The strong affinity of gold for sulphur, results in a perfect orderly monolayer over the bare gold sensor chip. The *in-situ* activation of free carboxylic groups of SAM was achieved through carbodiimide chemistry by introducing EDC-NHS reagent. Homemade estrogen receptor-BSA (ER-BSA) conjugate as ligand was immobilized onto the gold sensor chip through strong covalent amide bond via primary amine group of BSA and activated carboxylic group of long chain thioic acid.

Fig. 3a shows SPR response of different steps of estrogen receptor functionalized biosensor surface fabrication. The *in-situ* activation of SAM is evident with a net rise of resonance angle by ~120 m° (Fig. 3a, Inj 2) SPR sensor surface immobilized with SAM. Step-wise ligand immobilization of ER-BSA conjugate was performed to ascertain optimum concentration to be used for one-step immobilization. For *in-situ* ligand immobilization, an optimized ER-BSA conjugate concentration as 100 µg.mL<sup>-1</sup> over the activated SAM and interaction time for 15 min was found appropriate for fabrication of biosensor in present study. A sharp rise of resonance angle ca. 200 m° (Fig. 3a, Inj 4) indicates a stable fabrication of ER-BSA conjugate functionalized SPR sensor surface. Brief desorption of the loosely bound conjugate got stabilized with PBS flow (Fig. 3a, Inj 5), indicates that covalently bound estrogen receptor conjugate is highly stable on the MUA

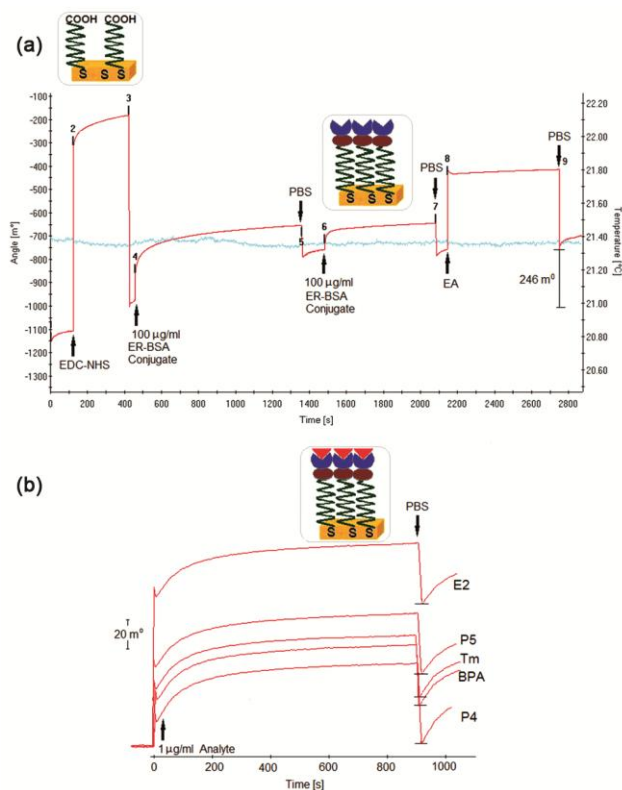


Fig. 3 — (a) SPR response of *in-situ* fabrication of affinity biosensor (ER-BSA/11-MUA/Au) [steps 2- activation by EDC-NHS solution; step 4- immobilization of ER-BSA conjugate; step 6- repeat of step 4; step 8- ethanolamine (EA); steps 3-, 5-, 7-, 9- PBS flow] and (b) Sensogram showing direct affinity interaction of  $1 \mu\text{g.mL}^{-1}$  each of estradiol (E2), progesterone (P4), pregnenolone (P5), tamoxifen (Tm) and bisphenol-A (BPA).

modified sensor surface. Subsequent introduction of ER-BSA conjugate shot revealed a rise of  $10 \text{ m}^\circ$  only, which shows that sensor surface is fully covered with conjugated ligand. Deactivation of unbound esters at sensor surface was accomplished by introducing 1 M ethanolamine (pH 8).

To evaluate the affinity of estradiol and to monitor the estrogenic activity potential estrogen mimics towards estrogen receptor,  $1 \mu\text{g.mL}^{-1}$  each of estradiol, progesterone, pregnenolone, tamoxifen and bisphenol-A was introduced over SPR sensor chip (ER-BSA/11-MUA/Au) for an optimized time of 15 min to undergo association phase. A brief flow of carrier buffer for 2 min was important to wash out the un-reacted species (dissociation phase). The net rise at the end of dissociation phase with respect to the resonance angle at initiation of association phase is an affirmative sign for affinity with ER. The regeneration step comprised a brief flow of pepsin solution to liberate the surface-bound Ab-E2, leaving

the sensor surface active for next injection. The receptor conjugate bound to the sensor chip was removed to regenerate the biosensor surface for multiple and cost-effective analyses.

Fig. 3b shows association phase showing affinity binding interaction of proposed biosensor with estradiol as positive control and potential EDCs chosen for present study. The strong affinity interaction of E2 for ER-BSA is clearly marked as rise of  $\sim 100 \text{ m}^\circ$  of SPR signal (Fig. 3b). The rise in SPR signal for E2 interaction at proposed sensor surface served as a mark of highest estrogenic activity (i.e. positive control). The progesterone (P4) introduction to biosensor surface did not bring any net rise in SPR angle (Fig. 3b) which shows that P4 did not bind effectively with estrogen receptor at sensor surface and gets washed away with the flow of PBS. The binding interaction of with pregnenolone, tamoxifen and bisphenol-A is evidenced in sensogram with a net rise of SPR angle  $\sim 55 \text{ m}^\circ$ ,  $\sim 35 \text{ m}^\circ$  and  $\sim 30 \text{ m}^\circ$ , respectively (Fig. 3b). On comparing the estrogenic activity of estrogen mimics at concentration level of  $1 \mu\text{g.mL}^{-1}$  towards estrogen receptor with respect to estradiol as positive control, pregnenolone, tamoxifen and bisphenol-A are EDCs with high estrogenic activity whereas progesterone shows no estrogenic activity. Results are in concordance with reported estrogenic status<sup>24,25</sup>. The regeneration capability of the sensor platform and its adaptability to a portable SPR device makes this assay promising for screening the estrogenic activity for field applications.

## Conclusions

The proposed SPR based affinity assay employing homemade estrogen receptor-BSA conjugate covalently bonded to a long chain thioic acid monolayer immobilized over gold sensor chip (ER-BSA/11-MUA/Au) is suitable for label free, *in-vitro* screening of estrogen activity of hormone mimics and potential endocrine disrupting chemicals. An optimum time period of 15 min was found to be satisfactory for interaction between immobilized receptor and estrogens (association phase) to get a stable SPR signal showing successful interaction and binding and 2 min to wash out the un-reacted species (dissociation phase). The presence of chemical with estrogenic activity is evidenced by the effective rise in SPR angle at the end of dissociation phase with respect to resonance angle at initiation of association phase. Further on comparing the SPR signal of



hormone mimics with that of estradiol as positive control standard, degree of estrogen activity could be ascertained in a much simple, single step, real time, label free direct affinity assay protocol. The simplicity of proposed SPR based direct affinity interaction protocols for detection of estrogen and estrogen mimics has potential for possible transformation into futuristic fiber optics based SPR probes for on-site environmental monitoring.

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### References

- Soto A M & Sonnenschein C, *Nat Rev Endocrinol*, 6 (2010) 363.
- Kabir E R, Rahman M S & Rahman I, *Environ Toxicol Pharm*, 40 (2015) 241.
- Snyder S A, Keith T L, Verbrugge D A, Snyder E M, Gross T S, Kannan K & Giesy J P, *Environ Sci Technol*, 33 (1999) 2814.
- Omar T F T, Ahmad A, Aris A Z & Yusoff F M, *Trends Anal Chem*, 85 (2016) 241.
- Inoue T, *Methods of the Biological Assays of the Endocrine Disrupting Chemicals*. Springer, Verlag, Tokyo, 2000.
- Rodriguez-Mozaz S, Marco M P, Lopez de Alda M & Barcelo D, *Anal Bioanal Chem*, 378 (2004) 588.
- Hansen J A, Sumbayev V V & Gothelf K V, *Nano Lett*, 7 (2007) 2831.
- Ohno K, Fukushima T, Santa T, Waizumi N, Tokuyama H, Maeda M & Imai K, *Anal Chem*, 74 (2002) 4391.
- Gehlot R, Sharma K, Mathew K & Kumbhat S, *Indian J Chem*, 47A (2008) 1804.
- Kumbhat S, Sharma K, Gehlot R, Solanki A & Joshi V, *J Pharm Biomed Anal*, 52 (2010) 255.
- Kumbhat S, Shankaran D R, Kim S J, Gobi K V, Joshi V & Miura N, *Biosens Bioelectron*, 23 (2007) 421.
- Ritchie R H, *Phys Rev*, 106 (1957) 874.
- Bhandari S, Agrawal V V & Birader A M, *Ind J Pure Appl Phys*, 55 (2017) 349.
- Miura N, Ogata K, Sakai G, Uda T & Yamazoe N, *Chem Lett* (1997) 713.
- Sakai G, Nakata S, Uda T, Miura N & Yamazoe N, *Electrochim Acta*, 44 (1999) 3849.
- Sesay A M & Cullen D C, *Environ. Monit Assess*, 70 (2001) 83.
- Miura N, Sasaki M, Gobi K V, Kataoka C & Shoyama Y, *Biosens Bioelectron*, 18 (2003) 953.
- Gobi K V, Kataoka C & Miura N, *Sens Actuators B*, 108 (2005) 784.
- Dostálek J, Přibyl J, Homola J & Skládal P, *Anal Bioanal Chem*, 389 (2007) 1841.
- Hegnerová K, Piliarik M, Štejnachová M, Flegelová Z, Černohorská H & Homola J, *Anal Bioanal Chem*, 398 (2010) 1963.
- Pearson J, Gilla A, Margison G P, Vadgama G & Povey A C, *Sens Actuators B*, 76 (2001) 1.
- Miyashita M, Shimada T, Miyagawa H & Akamatsu M, *Anal Bioanal Chem*, 381 (2005) 667.
- Spina R L, Ferrero E V, Aiello V, Pedotti M, Varani L, Lettieri T, Calzolari L, Haasnoot W & Colpo P L, *Biosensors*, 8 (2018) 1.
- Usami M, Katsuyoshi M & Ohno Y, *J Steroid Biochem Mol Biol*, 81 (2002) 47.
- Asano K, Ono A, Hashimoto S, Inoue T & Kanno J, *Anal Sci*, 20 (2004) 611.
- Kumbhat S, Gehlot R, Sharma K, Singh U & Joshi V, *J Pharm Biomed Anal*, 163 (2019) 211.