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A NEW BISPHENOL A DERIVATIVE FOR ESTROGEN RECEPTOR BINDING STUDIES WITH SURFACE PLASMON RESONANCE

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Abstract: A new bisphenol A (BPA) derivative was synthesized and immobilized onto the surface of a CM5 sensor chip for a binding study with orphan estrogen-related receptor γ using the surface plasmon resonance technique. The kinetic parameters, including dissociation and association constants and dissociation and association rate constants, were investigated. Moreover, a competitive inhibition assay was conducted to understand the competition relationship between the free BPA molecules in solution and the immobilized BPA molecules on the sensor chip surface. The CM5 sensor chip immobilized with the BPA derivative can be regenerated by using a high-concentration running buffer for repeated use more than 150 times without any adverse effect on its performance in the binding studies. The results indicate that the system has potential for further development as a sensitive surface plasmon resonance–based biosensor for endocrine-disrupting chemicals. *Environ Toxicol Chem* 2015;34:1390–1396. © 2015 SETAC

Keywords: Bisphenol A CM5 sensor chip Estrogen-related receptor Surface plasmon resonance Biosensor

INTRODUCTION

Estrogen is a C_{18}-steroid hormone and plays an important role in both the development and normal physiological function of various tissue systems [1,2]. Estrogen also acts as an endocrine signal from the granulosa cells of the ovary and is involved in the development of secondary sexual characteristics, regulation of gonadotropin secretion for ovulation, maintenance of bone mass, and regulation of insulin responsiveness. The biological action of estrogen is mediated through interaction with estrogen receptors [3,4]. When estrogen receptors bind to the endogenous ligand (estrogen), their conformation of is changed. Therefore, estrogen binds to a specific sequence of DNA (estrogen response elements) in the target gene, thus activating the transcription of the gene [5–7]. Estrogen receptors are classified as the nuclear receptor subfamily 3. Both estrogen receptor α and estrogen receptor β are members of this subfamily. Orphan estrogen–related receptors (estrogen-related receptor α, estrogen-related receptor β, and estrogen-related receptor γ [ERRγ]) also are classified as members of the nuclear receptor 3 subfamily and are closely related to estrogen receptors [8]. The amino acids involved in ligand binding, receptor dimerization, and nuclear localization are conserved in estrogen receptors and estrogen-related receptors. Their amino acid sequences show a great similarity and identity [2,9,10].

Bisphenol A (BPA) is a typical endocrine-disrupting chemical [11]. Bisphenol A alters the functions of endocrine and reproductive systems by directly contacting the hormone receptor. Therefore, BPA mimics or antagonizes the actions of endogenous hormones in an organism [10]. Moreover, BPA can interact with the human estrogen receptor, thus hindering the functions of estrogen [9–11]. Several studies have shown that BPA can act as an antagonist for the human androgen receptor [12,13]. Both the hormonal activity and binding affinity of BPA to estrogen receptors are very weak, approximately 10 000-fold and 100 000-fold less potent and weaker than those of estradiol, respectively; however, BPA has a very low dose effect [14]. Recent studies also have suggested that BPA exerts its effects not only through binding to estrogen receptors but also by stimulating cellular responses through different pathways even at a very low concentration. The cell functions could change at a dose as low as 1 pM of BPA by nongenomic mechanisms and by involving membrane-associated forms of estrogen receptors [14].

Bisphenol A is commonly used as a stabilizer or antioxidant in the production of polyvinyl chloride [15], polycarbonate plastics, and epoxy resins present in metal cans, toys, water pipes, and drinking containers [10,14]. Thus, BPA is released to the environment as a pollutant during manufacturing processes and leached from food and beverage containers. Therefore, wastewater, river water, sediments, foods, and drinking water are contaminated by BPA [15]. The European Community announced that the tolerance daily intake of BPA is 0.01 mg/kg/d [16,17].

To better understand the interactions between BPA and estrogen-related receptors, investigation techniques for the binding processes on the molecular level should be pursued. Several methods for the binding studies of estrogen-related receptors with BPA have been reported. The radioactive labeling technique is a traditional approach. This method involves the competition of BPA with radiolabeled 17β-estradiol or ^{125}I-labeled BPA for the binding site of estrogen receptors or the antibody conjugate, respectively [18,19]. However, this method cannot be performed in conventional laboratories because radioisotope facilities and the precautions against exposure to radiation are required and the experimental procedures usually are complicated [4]. In some other methodologies recently developed based on the surface plasmon resonance technique, a large protein moiety is immobilized onto the surface of a sensor chip instead of a small molecule. For example, the conjugate of BPA with bovine serum albumin

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protein is immobilized on the surface of a sensor chip for the competition assay [11] or polyclonal rabbit-antimouse antibodies are coated on the surface of a sensor chip for the direct immunoassay [15] for interaction with and detection of BPA. In these methods, small molecules (analytes) in a solution interact with large protein molecules immobilized on the sensor surface during the assay and, therefore, only a slight change in the molecular mass is induced (response signal) because of the large molecular mass of the immobilized protein. To obtain a significant change in the response signal, significant amounts of analytes are required to bind onto the sensor surface. Therefore, the sensitivity, limit of detection, and applications of the assay are limited. In the present study, we aimed to develop a small molecule, a charged and water-soluble BPA derivative, for immobilization onto CM5 sensor chips and to investigate its interactions and kinetic properties with ERRγ. The binding sensitivity toward BPA in a competitive binding assay was also determined. The working principle of the assay is shown in Figure 1.

MATERIALS AND METHODS

Materials

Bisphenol A, 1,8-dibromooctane, 1-bromohexane, sodium hydroxide (NaOH), *N*,*N*,*N*′,*N*′-tetramethylethylenediamine, *N*,*N*-dimethylethylenediamine, 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (endocrine-disrupting chemical), *N*-hydroxysuccinimide, ethanolamine hydrochloride, sodium acetate, and sodium carbonate were purchased from Sigma-Aldrich. Research-grade CM5 sensor chips were supplied by Biacore. The human recombinant protein (ERRγ) was purchased from Abnova. All solvents were of analytical grade and used as received without further purification.

Instrumentation

Nuclear magnetic resonance (NMR) spectra were obtained using a Bruker 400-MHz DPX-400 spectrometer. Mass spectra were recorded using a Finnigan MAT 95S mass spectrometer. Surface plasmon resonance analyses were performed using a Biacore 3,000 instrument.

Experimental procedures

Preparation of compound 1. First, BPA (0.23 g, 1 mmol) was dissolved in 5 mL of ethanol in a flask. Then, NaOH (0.044 g, 1.1 mmol) was added to the flask, and the reaction mixture was refluxed at 80 °C for 30 min. Finally, 1,8-dibromooctane (0.54 g, 2 mmol) was added to the reaction mixture, and the resulting solution was refluxed at 80 °C overnight. Deionized H_2O was added dropwise to the reaction mixture, and the solution was neutralized with dilute HCl solution. The product was extracted with ethyl acetate and then purified by flash chromatography (ethyl acetate/petroleum ether = 1:7, v/v). The removal of solvent under vacuum afforded compound 1: yield, 0.21 g (50.0%); ^{1}H NMR (400 MHz, CD_3CN), δ 1.14–1.49 (m, 9H), 1.58–1.70 (m, 3H), 1.71–1.80 (m, 2H), 1.84–1.90 (m, 4H),

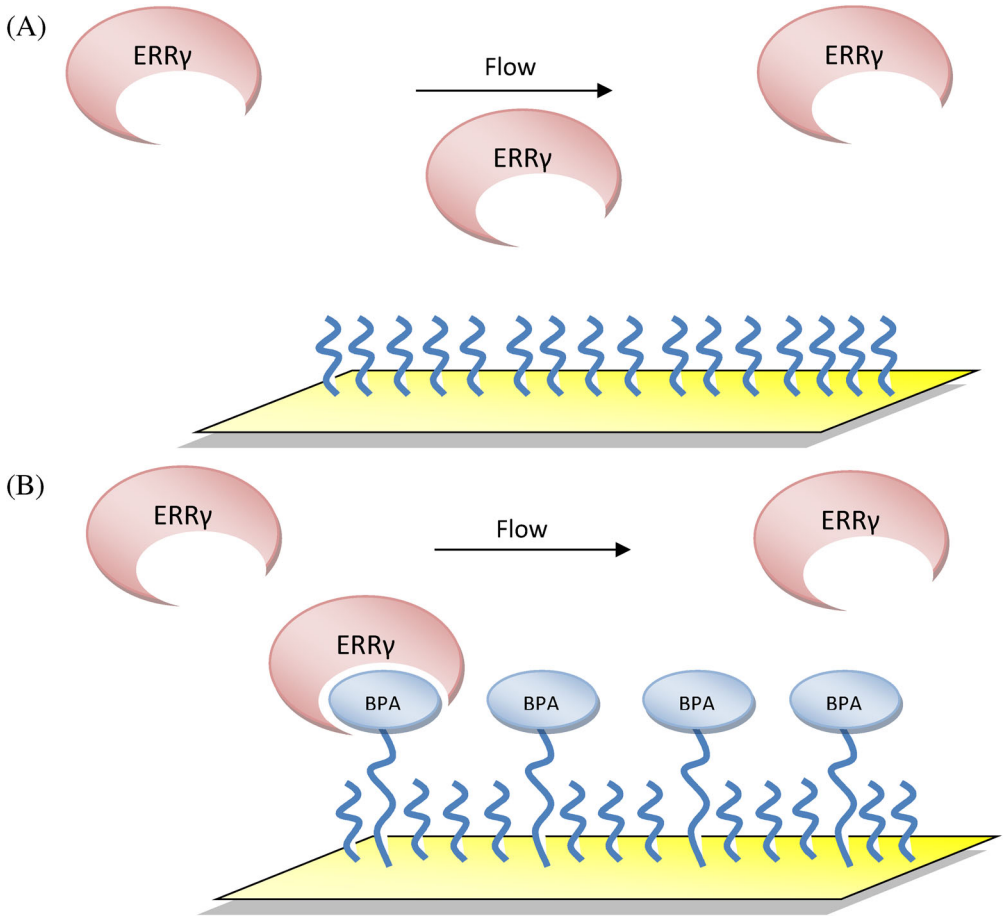

Figure 1. Scheme of the estrogen-related receptor γ (ERRγ)–bisphenol A (BPA) binding assay: (**A**) Reference flow cell, no specific interactions; (**B**) ERRγ binding with the immobilized BPA derivative and detachment in the flow cell.

3.35–3.39 (t, $J = 13.6$ Hz, 2H), 3.81–3.85 (t, $J = 13.2$ Hz, 2H), 6.59–6.70 (dd, $J = 6.0$ Hz, 4H), 6.95–7.04 (dd, $J = 6.0$ Hz, 4H); electrospray ionization mass spectrometry (ESI-MS) mass-to-charge ratio (*m/z*) $[M]^+$, 419.0; $[M - OH]^+$, 402.0.

Preparation of compound 2. A solution of compound 1 (0.42 g, 1 mmol) in 3 mL of ethanol was added to a solution of *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (0.35 g, 3 mmol) in 3 mL of ethanol in a flask. The reaction mixture was stirred at 60 °C overnight, and then the solution was concentrated using a rotary evaporator. The liquid residue was washed with diethyl ether/acetone (10:1, v/v) repeatedly to remove all of the unreacted starting materials. After removal of solvent under vacuum, compound 2 was obtained: yield, 0.35 g (64.8%); ^{1}H NMR (400 MHz, MeOD), δ1.10–1.20 (m, 2H), 1.35–1.55 (m, 9H), 1.59 (s, 6H), 1.72–1.85 (m, 5H), 2.29 (s, 6H), 2.72–2.75 (t, $J = 12.0$ Hz, 2H), 3.34 (s, 2H), 3.38–3.40 (m, 2H), 3.40–3.49 (m, 2H), 3.92–3.95 (t, $J = 12.0$ Hz, 2H), 6.65–6.77 (dd, $J = 8.0$ Hz, 4H), 7.00–7.11 (dd, $J = 8.0$ Hz, 4H); ESI-MS *m/z* $[M]^+$, 455.07, 457.15; $[M]^{2+}$, 244.99.

Preparation of compound 3. To a solution of compound 2 (0.54 g, 1 mmol) in 6 mL of ethanol in a flask, 1,8-dibromooctane (0.82 g, 3 mmol) was added, and the resulting mixture was refluxed at approximately 80 °C overnight. Then, the mixture was concentrated using a rotary evaporator. The liquid residue was washed with diethyl ether/acetone (10:1, v/v) until all of the unreacted starting materials were removed. The removal of solvent under vacuum afforded compound 3: yield, 0.30 g (37.0%); ^{1}H NMR (400 MHz, MeOD), δ 1.19–1.23 (t, $J = 7.0$ Hz, 2H), 1.49 (s, broad, 12H), 1.62–1.64 (m, 7H), 1.78–1.82 (m, 3H), 1.88–1.89 (m, 4H), 3.26 (s, 3H), 3.30 (s, 3H), 3.31–3.45 (m, 9H), 3.46–3.60 (m, 5H), 3.74-3.85 (m, 1H), 3.91–4.00 (m, 4H), 4.09 (s, 1H), 6.69–6.82 (dd, $J = 7.0$ Hz, 4H), 7.05–7.15 (dd, $J = 7.0$ Hz, 4H); ESI-MS *m/z* $[M - Br^-]^{2+}$, 322.97, 323.93.

Preparation of BPA-O-C8-NN-C8-NNH$_2$. *N*,*N*-dimethylethylenediamine (0.88 g, 10 mmol) was added to a solution of compound 3 (0.81 g, 1 mmol) in 3 mL of ethanol/acetone (1:1, v/v) in a flask and stirred at 60 °C overnight. Then, the mixture was concentrated using a rotary evaporator. The liquid residue was washed with acetonitrile and diethyl ether until all of the unreacted starting materials were removed. The removal of solvent under vacuum afforded BPA-O-C8-NN-C8-NNH$_2$: yield, 0.30 g (33.3%); ^{1}H NMR (400 MHz, MeOD), δ 1.30–1.57 (m, broad signal, 16H), 1.58 (s, 6H), 1.65–1.82 (m, broad signal, 4H), 1.82–1.95 (m, broad signal, 4H), 3.26 (s, 6H), 3.27–3.30 (m, 11H), 3.31 (s, 6H), 3.45–3.58 (m, 5H), 3.88–3.95 (m, 2H), 4.01–4.15 (m, 4H), 6.64–6.78 (dd, $J = 8.0$ Hz, 4H), 7.02–7.11 (dd, $J = 8.0$ Hz, 4H); ESI-MS *m/z* $[M - Br^-]^{2+}$, 366.95, 367.96.

Preparation of C6-NNH$_2$. A solution of 1-bromohexane (1.32 g, 8 mmol) in 3 mL of acetonitrile was added to a solution of *N*,*N*-dimethylethylenediamine (0.88 g, 10 mmol) in 4 mL of acetonitrile in a flask. The reaction mixture was stirred at room temperature overnight. Then, the mixture was concentrated using a rotary evaporator. The liquid residue was washed with acetonitrile and diethyl ether until all of the unreacted starting materials were removed. A white powder (C6-NNH$_2$) was obtained after drying under vacuum: yield, 1.20 g (59.1%); ^{1}H NMR (400 MHz, D_2O), δ 0.86–0.95 (t, $J = 7.0$ Hz, 3H), 1.32–1.48 (m, 6H), 1.76–1.85 (m, 2H), 3.16 (s, 6H), 3.16–3.20 (m, 2H), 3.26–3.30 (m, 2H), 3.35–3.41 (m, 2H); ESI-MS *m/z* $[M - Br^-]^+$, 172.89.

Procedures and conditions for immobilization on surface plasmon resonance sensor chip surface. The prepared BPA-O-C8-NN-C8-NNH$_2$ and C6-NNH$_2$ (1.2 equivalent, used as the spacer) were immobilized onto the sensor chip surface in a flow cell by the general amine coupling method at 25 °C. The detailed procedures are described as follows. First, a CM5 sensor chip was docked in a Biacore 3,000 instrument and primed with a potassium phosphate buffer (pH 7.4), which is the running buffer for the immobilization of the BPA derivative. The running buffer was passed in the flow cell at rate of 5 μL/min for 5 min. Then, the surface was activated by injecting 0.4 M 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (endocrine-disrupting chemical)/0.1 M *N*-hydroxysuccinimide (1:1, v/v) at a flow rate of 5 μL/min for 7 min. The flow cell again was allowed to stabilize with the running buffer at a flow rate of 5 μL/min for 5 min. After decreasing the flow rate to 4 μL/min, the running buffer was passed in the flow cell for another 5 min. A mixture of BPA-O-C8-NN-C8-NNH$_2$ (30 mM, 0.01 mmol) and C6-NNH$_2$ (33 mM, 0.012 mmol) was prepared in 10 mM sodium carbonate (pH 8.5) with a total volume of 360 μL and a final concentration of 4% dimethyl sulfoxide. The mixture was then injected to the flow cell with a flow rate of 4 μL/min for 80 min. The flow in the flow cell was continued at a flow rate of 4 μL/min for 5 min, followed by 5 μL/min for 5 min. Finally, the unreacted CM5 sensor chip surface was deactivated with 1 M ethanolamine hydrochloride at a flow rate of 5 μL/min for 7 min. The running buffer was passed in the flow cell at a flow rate of 5 μL/min overnight. The same procedures and conditions were applied to the reference cell with a C6-NNH$_2$ spacer (0.1 M, 0.036 mmol). The unreacted sensor surface was finally deactivated with a C6-NNH$_2$ solution (1.0 M).

Procedures and conditions for ERRγ binding assay. The binding studies of ERRγ were investigated by the Wizard program at 25 °C. The immobilized sensor chip was primed with a sodium acetate solution (5 mM, pH 7.4), which also acts as the running buffer during the binding assay. Then, the running buffer was passed through the sensor chip at a flow rate of 10 μL/min until the baseline became steady before starting the binding assay. Solutions of ERRγ with different concentrations were prepared by diluting the stock solution (1.21 μM) with the running buffer. A series of ERRγ solutions with the concentration ranging from 0.05 nM to 2 nM were prepared. All the ERRγ solutions were then mixed thoroughly and centrifuged to remove air bubbles. First, the immobilized flow channels were stabilized with the running buffer at a flow rate of 10 μL/min for 2 min. Then, 30 μL of the ERRγ solution was injected into the flow channels at a flow rate of 10 μL/min for 3 min. After an additional 3 min for dissociation, 30 μL of a sodium acetate solution (100 mM, pH 7.4), which acts as the regeneration buffer, was injected at a flow rate of 10 μL/min for 3 min. The running buffer was again passed in the flow cells at a flow rate of 10 μL/min until the baseline became steady before the next assay. The ERRγ solution was then injected to the reference flow cell for the reference assay. The binding sensorgrams were fitted using the BIAevaluation software (Ver 4.1.1) after subtracting those of the reference assay to determine the maximum response unit (R_{max}) and parameters of binding kinetics and equilibrium binding constants. Finally, the association and dissociation rate constants (k_a and k_d) and the association and dissociation constants (K_A and K_D) were derived. The molecular mass of the ERRγ used in the present study was approximately 74 kDa.

Procedures and conditions for the study of the binding relationship between ERRγ and BPA-O-C8-NN-C8-NNH$_2$ in a competitive binding assay. Different concentrations of BPA solutions were prepared by diluting the stock solution (10 mg/mL in 35% acetone) with the running buffer. A series

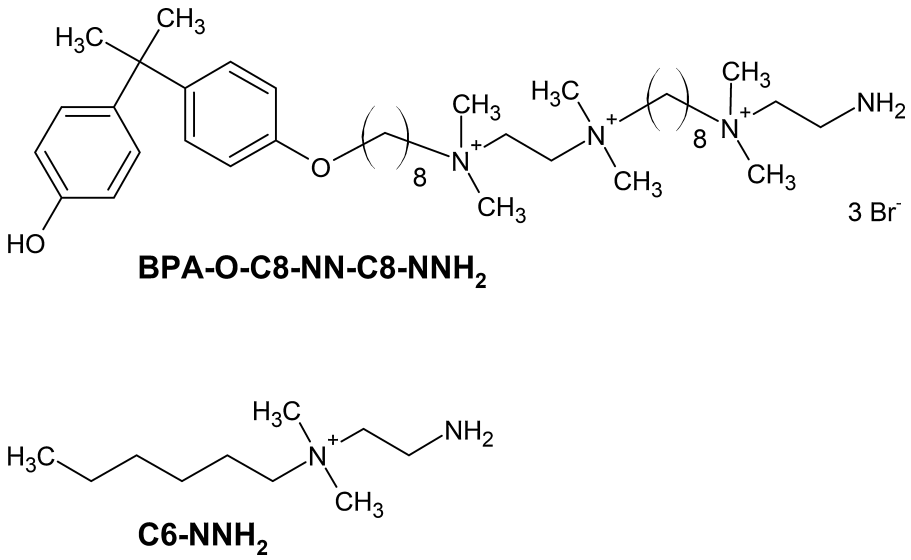

Figure 2. The new compounds synthesized and used in the present study. BPA = bisphenol A.

of BPA solutions in the concentration range 0.01 ng/mL to 1.0 ng/mL were prepared by diluting the stock solution. The ERRγ solution was added to the BPA solutions, and the final concentration of ERRγ in the binding assay was fixed at 1 nM. The mixture was incubated for 30 min at room temperature. After incubation, the solutions were injected to both the reference flow cell and the flow cell with immobilized BPA. The sensorgrams were obtained, and the changes in the signals corresponding to the concentrations of BPA solutions were investigated.

RESULTS AND DISCUSSION

Synthesis of water-soluble BPA derivative and spacer

The small molecule, BPA-O-C8-NN-C8-NNH_2, shown in Figure 2, was synthesized in several steps under simple reaction conditions. The synthetic processes are outlined in Supplemental Data, Scheme S1. First, BPA was reacted with 1,8-dibromooctane to obtain compound 1 with a flexible alkyl chain and a reactive bromo functional group at the terminal position. Then, the terminal bromo group was readily reacted with *N*,*N*,*N*′,*N*′-tetramethylethylenediamine to afford the positively charged compound 2, with a reactive tertiary amino group at the terminal position of the molecule. Compound 2 was then reacted with 1,8-dibromooctane to afford compound 3, with 2 positively charged ammonium groups and a reactive bromo group at the terminal position. The bromo group was further reacted with *N*,*N*-dimethylethylenediamine to afford the target compound (BPA-O-C8-NN-C8-NNH_2) with a long carbon chain, a BPA molecule tagged at 1 end, and a reactive NH_2 functional group at the other terminal for immobilization onto the surface of a CM5 sensor chip for surface plasmon resonance studies.

Figure 3 shows the key features of the BPA derivative for surface plasmon resonance applications: the long aliphatic carbon chain is positively multicharged, making it readily water-soluble; the molecule contains a terminal NH_2 group for coupling and immobilization; the long aliphatic carbon chain is flexible and renders it easy for the BPA to interact and bind with ERRγ; and the terminal NH_2 group is a reactive functional group and is readily coupled with the activated dextran layer of the sensor chip surface.

Immobilization of BPA-O-C8-NN-C8-NNH_2 and C6-NNH_2 onto the sensor chip surface

The surface of the CM5 sensor chips is coated with a layer of a matrix of carboxymethylated dextran functionalized with carboxylic acid groups. This layer facilitates the covalent immobilization of NH_2-functionalized molecules onto the surface of the sensor chip. Reactive succinimide ester groups are generated after activating the dextran layer using endocrine-disrupting chemical/*N*-hydroxysuccinimide. The reactive ester groups can form covalent bonds with the NH_2 groups of the injected compound by amine coupling. In the present study, compounds BPA-O-C8-NN-C8-NNH_2 and C6-NNH_2 were successfully immobilized onto the sensor chip surface through their primary NH_2 terminals by following the standard amine

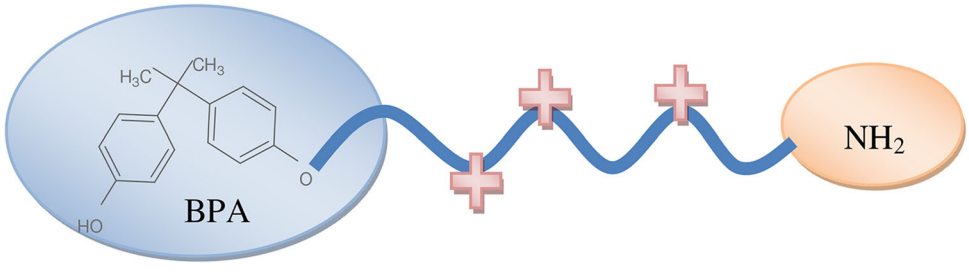

Figure 3. Key features of the small molecule BPA-O-C8-NN-C8-NNH_2. BPA = bisphenol A.

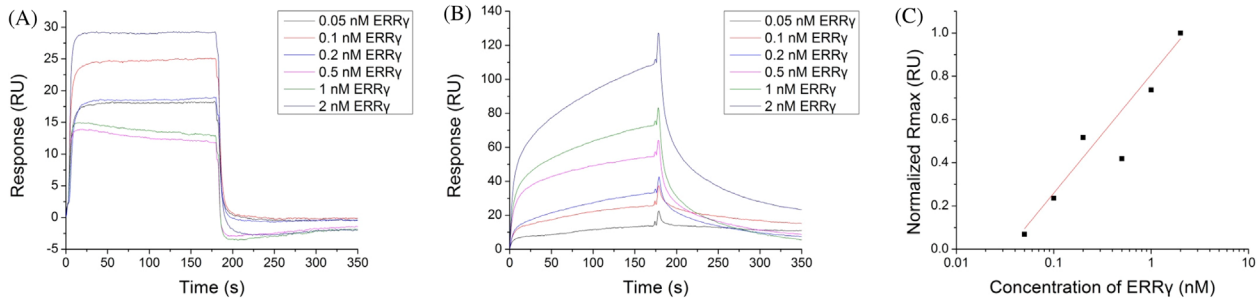

Figure 4. (**A**) Sensorgrams of different concentrations of estrogen-related receptor γ (ERRγ) injected to the C6-NNH_2–coated reference sensor chip surface. (**B**) Sensorgrams of different concentrations of ERRγ injected to the BPA-O-C8-NN-C8-NNH_2–coated sensor chip surface corrected by the reference flow cell. (**C**) Dose-dependent response curve from maximum response unit (R_{max}) obtained after fitting using the BIAevaluation software and normalized with an R_{max} of 2.0 nM ERRγ injected to the flow cell ($r^2 = 0.96$). (All results are corrected with the reference channel.) RU = response unit.

coupling procedure. A large volume of high-concentration immobilizing agent was used because surface saturation and maximum immobilization can usually be ensured with a large excess of immobilizing solution with a slow flow rate during the coupling. The small molecule (BPA-O-C8-NN-C8-NNH_2) possesses 3 positive charges, and thus, these charged molecules repel each other. Therefore, BPA-O-C8-NN-C8-NNH_2 molecules were immobilized not too close to each other with possibly a few hundreds of angstroms distance even under high concentrations. The approach is beneficial to reduce the hindering effects caused by adjacent ERRγ proteins (bulky size) during the interaction with the immobilized BPA molecules on the surface, thus probably increasing the effectiveness of ERRγ binding. Moreover, during the immobilization of BPA-O-C8-NN-C8-NNH_2 on the sensor chips, a spacer (C6-NNH_2) was also introduced to provide an adequate space and increase the density of BPA-O-C8-NN-C8-NNH_2 molecules on the surface.

ERRγ binding studies on immobilized flow cells

To investigate the interactions between ERRγ and the immobilized BPA molecules on the sensor chip surface (coated with BPA-O-C8-NN-C8-NNH_2), the binding behavior of ERRγ with various concentrations was studied. The same series of ERRγ solutions also were injected into the reference flow cell as a control. Figure 1 shows the binding of ERRγ with BPA molecules under different conditions. Figure 4A shows the sensorgrams of the association and dissociation processes with different concentrations of ERRγ injected to the reference flow cell. The results clearly indicate that ERRγ did not bind to the control sensor chip surface coated with C6-NNH_2 because BPA molecules were not tagged. The increase in the response signal is mainly the result of the buffering effect and nonspecific interactions.

To eliminate the possible error sources induced from a buffering effect and nonspecific interactions, the ERRγ binding results shown later are thus corrected by subtraction of the signal obtained from the reference flow cell. The binding signal (response) of the flow cell immobilized with BPA-O-C8-NN-C8-NNH_2 molecules increased with the increase in the concentrations of ERRγ. Figure 4B shows the sensorgrams of the association and dissociation processes with different concentrations of ERRγ. In the range 0.05 nM to 2.0 nM ERRγ, the response signals show a reasonable linear relationship with the concentrations of ERRγ. Figure 4C shows the dose-dependent response curve. When the concentration of ERRγ was less than 0.05 nM or higher than 2.0 nM, however, the response signal did not lie in the linear region. This is probably because the number of ERRγ proteins significantly decreased at a low protein concentration due to the adsorption onto the contact surfaces of vials, pipette tips, and other surfaces, whereas at a higher concentration, >2.0 nM, the surface of the sensor chip was saturated with ERRγ. However, the addition of excess ERRγ did not further increase the response signal. We thus assumed that 2.0 nM ERRγ is sufficient to saturate the sensor chip surface. The R_{max} of 2.0 nM ERRγ was then used to normalize the R_{max} values of other assays. The dose-dependent response curve plotted with R_{max} was thus established with $R^2 = 0.96$ based on the above assumptions (Figure 4C).

After the completion of each binding assay, the sensor chip surface was regenerated before starting the second cycle of binding experiment with ERRγ. The ERRγ proteins bound on the immobilized BPA were readily removed using a high-concentration sodium acetate solution (100 mM, pH 7.4) as the regeneration buffer for the regeneration process. Figure 5 shows the typical complete cycles of the binding assay with the addition of various concentrations of ERRγ conducted in a flow cell immobilized with BPA-O-C8-NN-C8-NNH_2 molecules. After the regeneration, the new baseline almost completely reached the starting point of the assay. This indicates that all of the bound ERRγ proteins dissociated from the sensor chip surface. To demonstrate the robustness and stability of the coating, a sensor chip (surface immobilized with BPA-O-C8-NN-C8-NNH_2) was repeatedly used more than 150 times

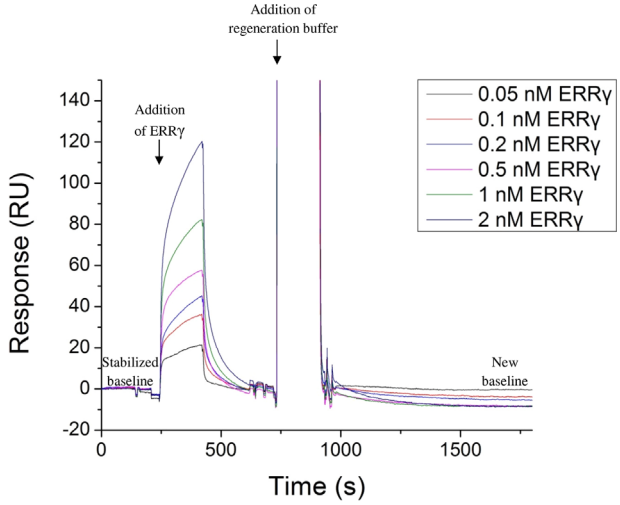

Figure 5. Entire picture of an assay cycle with the addition of estrogen-related receptor γ (ERRγ) onto the BPA-O-C8-NN-C8-NNH_2–coated sensor chip surface. RU = response unit.

Table 1. Kinetic parameters for the binding of estrogen-related receptor γ (ERRγ) to immobilized bisphenol A[a]

Concentration of ERRγ (nM)	Dissociation rate constant ($\times 10^{-3}$ s^{-1})	Association rate constant ($\times 10^{5}$ $M^{-1}s^{-1}$)	Dissociation constant ($\times 10^{-8}$ M)	Association constant ($\times 10^{7}$ M^{-1})
0.05	5.23 ± 1.78	7.12 ± 5.87	2.39 ± 3.00	6.68 ± 4.04
0.1	4.38 ± 0.88	3.17 ± 1.06	1.46 ± 0.36	7.22 ± 1.84
0.2	7.01 ± 1.91	2.07 ± 0.73	3.43 ± 1.81	2.74 ± 1.76
0.5	13.20 ± 6.27	1.61 ± 0.73	8.22 ± 1.53	1.26 ± 0.29
1.0	2.84 ± 4.75	1.22 ± 1.33	7.82 ± 5.81	2.42 ± 2.22
2.0	6.39 ± 1.59	1.43 ± 0.78	8.04 ± 6.15	2.12 ± 1.48

[a]Binding experiments were repeated 5 times under the same conditions.

without showing any decrease in the response signals under the same binding assay conditions.

By fitting the data obtained from the sensorgrams with the 1:1 (Langmuir) association/dissociation fitting model provided by the BIAevaluation software, the parameters of binding kinetics and equilibrium binding constants were derived. The results are summarized in Table 1. The kinetic analysis of BPA binding to human nuclear receptors rarely has been reported in the literature. In 2007, Matsushima et al. [20] found that BPA binds strongly to ERRγ, with a dissociation constant (K_D) of 5.5×10^{-9} M using the saturation binding assay method with tritium-labeled BPA ([^{3}H]BPA). The K_D obtained in the present study is generally greater, however, in the range 1.5 to 8.2×10^{-8} M. This is probably because 1 of the hydroxyl groups of the BPA molecule is utilized for immobilization, thus weakening the interactions with the ERRγ receptor. The experimental results indicate that binding does occur; however, the binding property could be different from the system using native BPA to bind with estrogen receptor via 2 hydroxyl groups (the hydrogen bonding defines the affinity of ligand for receptor) [20]. In the present study, the immobilized BPA with 1 of the key hydroxyl groups replaced by the linking agent may possibly change the orientation of BPA within the receptor binding pocket. It is worth noting that this is the key difference of the present method from the radioactive and fluorescence polarization methods. The dissociation rate constant (k_d) was found to be 2.8×10^{-3} s^{-1} to 13.2×10^{-3} s^{-1}, and the association rate constant k_a was approximately 1.2 M^{-1} $s^{-1}$to 7.1×10^{5} M^{-1} s^{-1}. These kinetics results are comparable to data previously reported [21].

Binding relationship between ERRγ and BPA-O-C8-NN-C8-NNH$_2$ in a competitive binding assay

After optimizing the conditions for the interactions between ERRγ and BPA-O-C8-NN-C8-NNH$_2$ immobilized on the sensor chip surface, a competitive inhibition assay was performed to study the interaction behavior of ERRγ toward the immobilized BPA and the trace-free BPA in solution. Bisphenol A solutions with various concentrations in the range of 0.01 ng/mL to 1.0 ng/mL were incubated with 1 nM ERRγ. During the incubation, ERRγ proteins were supposed to bind with the BPA molecules present in the sample solution and reach an equilibrium state. When the solution was injected into the flow cell immobilized with BPA-O-C8-NN-C8-NNH$_2$ molecules, only the unbound ERRγ proteins could bind to the immobilized BPA molecules on the sensor chip surface, giving a response signal. The sample solutions also were injected into the reference flow cell for background correction. Figure 6A shows the sensorgrams of the reference flow cell after injection of ERRγ/BPA solutions. No specific binding occurred between the C6-NNH$_2$ surface and the ERRγ and BPA molecules. In the sensorgrams obtained from the flow cell with the BPA-O-C8-NN-C8-NNH$_2$–immobilized sensor chip, dose-dependent response curves were observed (Figure 6B and C). Compared with the results of ERRγ binding assays as shown in Figure 6B, however, the increase in the concentration of BPA in the sample solution decreased the response signals in general, and a clear trend in which the BPA concentration is inversely proportional to the response was observed. This is because a high concentration of free BPA molecules in competitive binding assays will occupy most of the ERRγ, and therefore, a lower amount of ERRγ would be able to interact with the immobilized BPA on the sensor chip surface. A small response signal was thus observed. The experimental results obtained are also in agreement with the proposed working principle of this system. However, this system only shows a limited BPA concentration range (0.01–1.0 ng/mL), giving a linear range of BPA concentrations versus response signals. When the concentration of BPA was higher than 1.0 ng/mL, the response signals reached a plateau (minimum point of response signal). This is because almost all ERRγ proteins in solution were occupied and unavailable for binding

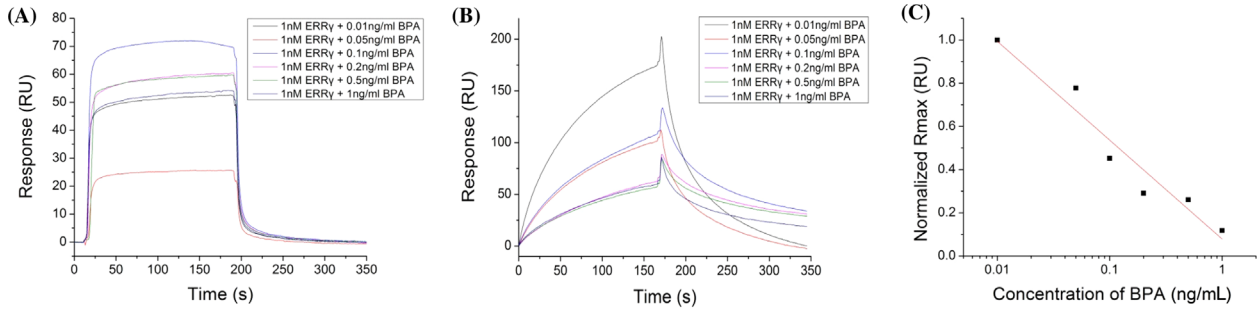

Figure 6. (**A**) Sensorgrams of different concentrations of bisphenol A (BPA) in the presence of 1 nM estrogen-related receptor γ (ERRγ) injected to the C6-NNH$_2$–coated reference sensor chip surface. (**B**) Sensorgrams of different concentrations of BPA in the presence of 1 nM ERRγ injected to the BPA-O-C8-NN-C8-NNH$_2$–coated sensor chip surface corrected by the reference flow cell. (**C**) Bisphenol A dose-dependent response curves from the maximum response unit (R_{max}) obtained after fitting using the BIAevaluation software and normalized with an R_{max} of 0.01 ng/mL BPA in the presence of 1.0 nM ERRγ injected to the flow cell ($r^2 = 0.96$). (All results are corrected with the reference channel.)

to the immobilized BPA molecules in the flow cell. Therefore, a further increase in the amount of BPA molecules could not decrease the response signals further. After the normalization with R_{max}, a linear relationship ($R^2 = 0.96$) was obtained, as shown in Figure 6C, by plotting the free BPA concentrations against the response signals. The experimental results of the competitive binding assay further support that ERRγ proteins significantly interacted with the tagged BPA molecules of the ligand BPA-O-C8-NN-C8-NNH_2.

CONCLUSIONS

A new BPA derivative was synthesized and successfully immobilized onto a CM5 sensor chip surface by the typical amine coupling method. The BPA-O-C8-NN-C8-NNH_2–immobilized sensor chip can be used effectively for investigating the binding with ERRγ at a very low concentration (0.1 nM) using surface plasmon resonance. The kinetic parameters of the dissociation constant were in the range 1.5×10^{-8} M to 8.2×10^{-8} M, and the association constant was in the range 1.3×10^{7} M^{-1} to 7.2×10^{7} M^{-1}. The dissociation rate constant was in the range 2.8×10^{-3} s^{-1} to 13.2×10^{-3} s^{-1}, and the association rate constant was approximately in the range 1.2×10^{5} M^{-1} s^{-1} to 7.1×10^{5} M^{-1} s^{-1}. Furthermore, a competition interaction study between the free and immobilized BPA was performed by an inhibition assay with 1 nM ERRγ; ERRγ proteins significantly interacted with the tagged BPA molecules through the BPA-O-C8-NN-C8-NNH_2 ligand. In the BPA concentration range of 0.01 ng/mL to 1.0 ng/mL, a linear relationship ($R^2 = 0.96$) was obtained between BPA concentrations and response signals. The CM5 sensor chip immobilized with BPA-O-C8-NN-C8-NNH_2 can be regenerated easily using a high-concentration running buffer for repeated use more than 150 times and is stable at room temperature for more than 2 mo.

SUPPLEMENTAL DATA

Schemes S1–S2.
Figures S1–S10. (573 KB PDF).

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Data Availability—Data, associated metadata, and calculation tools are available in the Supplemental Data or from the authors (wingleung@ied.edu.hk).

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