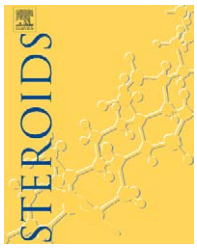
STEROIDS 71 (2006) 618–631

available at www.sciencedirect.com

journal homepage: www.elsevier.com/locate/steroids



Estrogen conjugation and antibody binding interactions in surface plasmon resonance biosensing

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ARTICLE INFO

Article history:
Received 16 November 2005
Received in revised form 23 March 2006
Accepted 23 March 2006
Published on line 15 May 2006

Keywords:
17β-Estradiol
Surface plasmon resonance
Anti-estradiol mAb
Immunoassay

ABSTRACT

Thioether-linked 3-mercaptopropionic acid derivatives of 17β-estradiol and estrone were formed at the A-ring 4-position of the steroids by substitution of their 4-bromo analogues. The carboxylic acid terminal was used to link to an oligoethylene glycol (OEG) chain of 15-atoms in length. The OEG derivative of 17β-estradiol was then in situ immobilized on a carboxymethylated dextran-coated gold sensor surface used to detect refractive index changes upon protein binding to the surface by surface plasmon propagation in a BIAcore surface plasmon resonance (SPR) instrument. Two other estradiol—OEG derivatives with Mannich reaction linkage at the 2-position and hemisuccinate linkage at the 3-position were also immobilized on the sensor surfaces for comparison. Binding performance between these immobilized different positional conjugates and monoclonal anti-estradiol antibody, raised from a 6-position conjugate, clearly demonstrated that both 2- and 4-conjugates, not conjugated through existing functional groups, gave strong antibody bindings, whereas the 3-conjugate through an existing functional group (3-OH) gave very little binding (2% compared to the 2-conjugate). Both 2- and 4-position conjugates were then applied in a highly sensitive estradiol SPR immunoassay with secondary antibody mediated signal enhancement that gave up to a 9.5-fold signal enhancement of primary antibody binding, and a detection limit of 25 pg/mL was achieved for a rapid and convenient flow-through immunoassay of estradiol.

1. Introduction

The production of coating steroid antigens for use in immunoassay is of great importance in the development of biosensors for steroid analysis. Biosensors are instruments that detect changes in chemical concentration by the use of a biochemical interaction, such as antibody/target binding, and convert the interaction into an electrical signal. One such transduction technique is surface plasmon resonance (SPR). SPR is an optical–electrical phenomenon whereby photons incident on a noble metal surface cause electrons in the metal to move as a plasmon and generate an electrical field. A change in the chemical environment of the noble metal sensing surface causes a shift in the angle or wavelength required to induce surface plasmon resonance, providing a means of detecting small mass changes on the sensing surface [1]. The presence of a small molecule, such as a steroid, can compete with labeled or surface bound steroid for binding to the antibody and thus can change the level of antibody/target binding detected. The use of biosensor technology has been greatly hindered by poor sensor surface stability resulting in a lack of repeatability in binding results and limiting the lifetime of the biosensor systems. Biosensors, especially those based on surface plasmon resonance also suffer from poor signal strength

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0039-128X/$ – see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.steroids.2006.03.004

so maximizing signal from specific binding is very important. There is a clear need to develop functionalized biosensor surfaces that are both stable and allow for maximum specific antibody binding through conjugation at points on the antigen that do not bear existing functional groups. By conjugating through existing functional groups, the antigenicity of the bound antigen is compromised, potentially reducing binding to antibodies that specifically recognize all functional groups on the analyte. Intuitively, greatest inhibition immunoassay sensitivity should be obtained by using conjugates that attach without modifying existing functional groups on the antigen both for production of the coating antigen and raising of the antibody, because with such conjugations the antibody should bind both the free analyte and the coating antigen strongly so that free analyte will significantly inhibit antibody binding to the sensor surface and at the same time there will be maximum surface binding signal in the immunobiosensor.

Most conjugation of the steroid hormones has involved attachment through an existing functional group, such as the formation of hemisuccinates of alcohols and carboxymethyloximes of carbonyl groups. This has certainly been the case for the estrogens 17β-estradiol and estrone [2,3]. Conjugations to estrogens have also been performed by introducing new functional groups such as amines and conjugating by such methods as diazo formation and glutaraldehyde linkage [4,5]. These methods however often involve many steps, with the diazo method often leading to self-conjugation of the antigen or intra-molecular coupling of the carrier protein, and the glutaraldehyde method produces unstable Schiff bases that need to be reduced, and can lead to self-conjugation. Mannich condensation is another popular method but, when done in its conventional one-step conjugation, a mixture of 2- and 4-position conjugated products results.

The use of thioethers to attach linkers or other substituents directly to the steroid without altering existing functional groups has been demonstrated for attachment at the 7-position of the estrogens [6] whilst carboxymethyloxime conjugation has been used for attachment at the 6-position [7,8]. Thioether attachment to the 4-position of the A-ring of the steroid has been achieved only through an epoxide-mediated route for estradiol [9,10] or by formation of the *o*-quinone of the 3-hydroxyl derivative, which leaves the final product still containing an unwanted 3-hydroxyl group [11–13]. Production of 4-position thioether bridged steroids with a non-aromatic A-ring has been achieved through reaction from the 6-bromo-derivative [14–16].

It has previously been shown for progesterone, that conjugation through the 4-position using a thioether linkage gives superior antibody binding over 7-position conjugation in flow-through biosensing formats [17]. It is therefore of interest to further extend the approach to produce the analogous conjugation for the estrogens through the 4-position on their aromatic A-rings. As for progesterone, the 4-position of the estrogens has the advantage that no epimeric mixtures are formed at point of attachment, and it can direct linkers away from functional groups. The position is also on the same side of the steroid as most of the conjugates commonly used to raise antibodies. Thioether linkages have also been shown in the past to have the necessary stability to function as coating antigens in SPR [18]. The carboxylic acid group then allows easy attachment of a range of linkers including oligoethylene glycol (OEG) chains. Such chains are water-soluble, have low immunogenicity [19,20] and their length can be easily incremented up or down as desired. Recent studies with progesterone immunoassay have shown that covalent immobilization of the amine terminal of OEG-derivatives of progesterone onto carboxymethylated dextran-coated gold biosensor surfaces is possible in situ in a controlled format, thus producing coating antigens that can be applied in surface plasmon resonance (SPR)-based assays using BIAcore instruments [18].

There is a dearth of reports from studies on the effects of changing conjugation position of steroids on the antibody binding and assay performance of small molecule conjugates either with carrier proteins or as coating antigens on solid surfaces. To rationally design immunobiosensors with maximum specific antibody-binding capacity, various linking positions and conjugation methods need to be examined. SPR biosensors provide a unique and convenient way of assessing antibody bindings in real-time for comparing different conjugations in a flow-through format. SPR has been applied to good effect to probe estrogen/receptor ligand interactions [21,22] and antibody binding of chemiluminescent estradiol conjugates [23]. It is also of great interest to develop new analytical techniques for sensitive detection of estrogens for reproductive and environmental monitoring. A consideration of the effects of the position of conjugation on antibody binding and assay performance in a flow-through SPR immunobiosensor is crucial to maximizing assay sensitivity.

In this study, we report the production of 17β-estradiol and estrone mercaptopropionate derivatives at the 4-position and the use of such derivatives to attach an OEG chain of 15-atoms in length. Use of the estradiol derivative as a surface coating antigen in SPR binding studies, in comparison to more conventional attachment methods, is discussed with respect to position of conjugation. Construction of a sensitive and convenient estradiol SPR immunoassay is also reported.

2. Experimental

2.1. Materials

All chemicals were reagent grade and used without further purification. All solvents were analytical grade for reactions and HPLC grade for chromatography. All dried solvents were dried over molecular sieves. 17β-Estradiol and *N*-hydroxysuccinimide (NHS) were purchased from ICN (Aurora, OH, USA), estrone from Acros Organics (Geel, Belgium), di-*tert*-butyl dicarbonate and 4,7,10-trioxa-1,13-tridecanediamine were purchased from Fluka Chemie (Buchs, Germany). All other chemicals were purchased from Sigma–Aldrich (Milwaukee, WI, USA). Silica column chromatography was carried out using silica gel, Merck 60 Å, grade 9385, 230–400 mesh. TLC was run on silica gel 60 F_{254} plates, aluminum-backed.

Monoclonal anti-17β-estradiol (E3550-29, US Biologicals, Swampscott, MA, USA) was raised to estradiol-6-17β-6-carboxymethyloxime-bovine serum albumin conjugate as

mouse anti-human. It had a calculated affinity constant of approximately 1×10^{10} L/M. The anti-mouse IgG (whole molecule) secondary antibody (M7023, Sigma, Milwaukee, WI, USA) was developed in rabbit as the IgG fraction of the antiserum. The SPR coupling kit was supplied by BIAcore (BR-1000-50, Uppsala, Sweden), consisting of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) and NHS solutions.

Melting point determinations were done using a Reichert Thermopan instrument and were uncorrected. ^{1}H, ^{13}C, and DEPT 135 NMR spectra were obtained on Bruker AC300 300 MHz and Bruker Avance 400 MHz spectrometers. Chemical shifts (ppm) were referenced to tetramethylsilane. Analytical reversed-phase HPLC was performed on a Shimadzu (Kyoto, Japan) Class VP instrument including an SPD-M10A VP UV–vis detector. Data was collected using Class VP software. Analyses were performed on a Sphereclone 3 μm ODS (2) column (150 mm × 4.6 mm internal diameter), at a flow rate of 1 mL/min at 35 °C.

Low-resolution mass spectra were obtained on a VG Platform II electrospray mass spectrometer (ESI-MS). High-resolution mass spectra were obtained on a VG-70SE mass spectrometer with MASPEC 2 data analysis software. BIAcore SPR analysis was done on a BIAcore 3000 instrument (BIAcore Inc., Piscataway, NJ, USA) using CM5 research grade chips and 10 mM (*N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]), 150 mM NaCl with 0.11% (w/v) ethylenediaminetetra-acetic acid and 0.05% (v/v) P-20 surfactant, pH 7.4 as running buffer. All BIAcore experiments were performed at 25 °C.

For statistical analysis, all error bars shown on graphs represent one standard deviation of the mean. All assay standard curves have been fitted to a four-parameter logistic plot using Sigma Plot 8.02 (SPSS, Chicago, IL, USA). All limits of detection (LOD) have been computed as the concentration corresponding to the blank less two standard deviations of the blank determination. The inhibitory concentration at 50% bound (IC_{50}) values have been computed as a parameter of the curve fitting. Standard deviations (S.D.) in the LOD and IC_{50} have been computed by inputting the standard deviation of a neighboring standard point into the rearranged four-parameter logistic equation, calculating from the S.D. in the response. Sensitivities represent the slope of the linear portion of the assay curve. The enhancement ratios are calculated by dividing the slope of the enhanced antibody-binding plot by the slope of the primary antibody plot. Errors quoted in the text are the standard error of the determination. All differences between association and dissociation constants and antibody binding values are statistically significant by *t*-test ($P < 0.02$).

2.2. 4-Bromo-17β-estradiol (1)

The method was adapted from previous reports [24,25]. 17β-Estradiol (800 mg, 2.94 mmol) was dissolved in dry EtOH (40 mL) and *N*-bromosuccinimide (522 mg, 2.94 mmol in 15 mL of dry EtOH) was added dropwise to the vigorously stirring solution. The solution was stirred for 24 h and a white solid formed within 1 h of reaction. The solid was collected by filtration and recrystalized from $CHCl_3$ to yield **1** as a white crystalline solid. Yield: 572 mg (55%). mp 203–205 °C (lit. [25] 207–208 °C). Analytical RP-HPLC [MeOH/H_2O (90:10, v/v), 283 nm]: R_t = 2.62 min 96%. IR: 1053, 1437, 2926, 3242, 3504 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 0.80 (3H, s, 18-CH_3), 1.98 (2H, dd, J = 2.7 Hz, J = 12 Hz, 12-H), 2.16 (2H, m, 11-H), 2.72 (1H, m, 9-H), 2.93 (1H, dd, J = 5.6, J = 11.9 Hz, 17-H), 3.75 (2H, q, J = 7.0 Hz, 6-H), 5.59 (1H, s, OH), 6.88 (1H, d, J = 8.5 Hz, 2-H), 7.21 (1H, d, J = 8.3 Hz, 1-H). ^{13}C NMR ($CDCl_3$): δ 11.1, 23.2, 26.7, 27.4, 27.6, 30.0, 31.3, 36.8, 38.2, 43.3, 44.3, 81.6, 113.1, 113.5, 125.2, 134.1, 137.2. ESI-MS *m*/*z* (MeOH, −45 V) 349.1, 351.1 $(M-H)^-$.

2.3. 4-Bromoestrone (2)

Estrone (400 mg, 1.48 mmol) was dissolved in dry EtOH (10 mL) and acetone (10 mL). *N*-Bromosuccinimide (263 mg, 1.48 mmol) was added to the vigorously stirring solution and the solution was stirred at room temperature for 24 h. The white solid formed was filtered off and washed with ethanol to yield **2**. The filtrate solvent was removed and the resultant solid recrystalised to increase yield. Yield: 221 mg (43%). mp 264–265 °C (lit. [26] 264–265 °C). Analytical RP-HPLC [MeOH/H_2O (90:10, v/v), 281 nm]: R_t = 2.55 min, 96%. IR: 1472, 1731, 3420 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 0.90 (3H, s, 18-CH_3), 5.37 (1H, s, OH), 6.86 (1H, d, J = 8.6 Hz, 2-H), 7.18 (1H, d, J = 8.6 Hz, 1-H). ^{13}C NMR ($CDCl_3$): δ 14.0, 21.8, 26.3, 26.9, 31.2, 31.7, 36.2, 37.8, 44.4, 49.1, 50.5, 113.2, 118.9, 125.3, 133.3, 137.0, 151.4, 222.6. ESI-MS *m*/*z* (MeOH, −40 V) 346.7 and 348.7 $(M-H)^-$. HRESI-MS: 348.0727 and 350.0708 (M^+) (calcd. for $C_{18}H_{21}O_2Br$, 348.0725 and 350.0705).

2.4. 3-(3,17β-Dihydroxyestra-1,3,5(10)-trien-4-yl) thiopropanoic acid (3)

Compound **1** (200 mg, 0.57 mmol) was dissolved in dry MeOH (20 mL). Methanolic potassium hydroxide (20 mL, 7.8 mg/mL) was added followed by 3-mercaptopropionic acid (550 μL). The solution was refluxed using oven-dried glassware and a drying tube for 24 h in the dark. The solvent was removed and the sample reconstituted in distilled water (50 mL). The aqueous phase was washed with ethyl acetate (2× 25 mL, 1× 50 mL). The ethyl acetate phase was then washed with distilled water and the product was reacted directly to the next stage. ^{1}H NMR ($CDCl_3$): δ 0.81 (3H, s, 18-CH_3), 1.38–2.3 (m, steroidal ring structure), 2.75 (3H, t, J = 4.6 Hz, 17-CH), 2.81 (2H, t, J = 4.5 Hz, S-CH_2), 6.89 (1H, d, J = 6.3 Hz, 2-H), 7.22 (1H, d, J = 6.7 Hz, 1-H) (spectrum was overlaid with 3-mercaptopropionic acid peaks). ^{13}C NMR ($CDCl_3$): δ 14.2, 21.2, 21.4, 22.8, 23.1, 24.0, 25.4, 26.8, 29.1, 29.8, 30.2, 31.0, 33.8, 34.2, 37.1, 50.9, 74.6, 90.5, 171.5, 194.0. ESI-MS (MeOH): 399.1 $[M+Na]^+$, 406.8 $(M+OMe)^-$.

2.5. 3-(3-Hydroxyestra-1,3,5(10)-trien-17-on-4-yl) thiopropanoic acid (4)

Compound **2** (150 mg, 0.43 mmol) was dissolved in dry MeOH (20 mL) and potassium hydroxide (15 mL, 23 mg/mL in dry MeOH) was added whilst stirring, followed by 3-mercaptopropionic acid (425 μL) and the solution heated under reflux for 24 h using oven-dried glassware and a drying tube. The sample was then cooled and solvent removed. The sample was reconstituted in distilled water (25 mL) and

washed with ethyl acetate (2× 12.5 mL, 1× 25 mL). The water was removed in vacuo and the sample separated by silica column chromatography using $CHCl_3$/MeOH (1:1) eluant to yield compound **4** as a white solid. Yield: 43 mg (27%). mp: 108–112 °C. IR: 1241, 1400, 1698, 1735, 3429 cm^{-1}. ^1H NMR ($CDCl_3 + d_3$-MeOH): δ 0.93 (3H, s, 18-CH_3), 2.54 (2H, t, J = 9.4 Hz, CH_2–COOH), 2.88 (2H, t, J = 8.8 Hz, CH_2-S), 6.81 (1H, d, J = 11 Hz, H-2), 7.22 (1H, d, J = 11 Hz, H-1). ^{13}C NMR ($CDCl_3 + d_3$-MeOH): δ 13.8, 21.6, 26.2, 26.7, 29.5, 31.0, 31.5, 35.9, 37.4, 38.5, 44.0, 48.0, 50.3, 113.0, 125.4, 133.6, 136.5, 150.6, 156.2, 175.4, 223.0. ESI-MS m/z (MeOH): 397.4 $(M + Na)^+$, 373.4 $(M - H)^-$.

2.6. 2,5-Dioxo-1-pyrrolidinyl 3-(3,17β-dihydroxyestra-1,3,5(10)-trien-4-yl)thiopropionate (5)

The entire semi-solid from the ethyl acetate phase of the compound **3** reaction was dissolved in dry *N,N′*-dimethylformamide (DMF) (1 mL), and dicyclohexylcarbodiimide (DCC) (176 mg in 1 mL of dry DMF) was added dropwise followed by NHS (98 mg in 1 mL of dry DMF). A white solid precipitated within 1 h and the reaction was left stirring in the dark overnight. The solvent was removed in vacuo and the sample separated by silica column chromatography using in succession, $CHCl_3$, $CHCl_3$/MeOH (15:1), $CHCl_3$/MeOH (10:1) and $CHCl_3$/MeOH (2:1) as eluant to yield compound **5** as a white solid. Yield: 141 mg (52%) from compound **1**. mp 194–196 °C. IR: 1242, 1576, 1626, 1736, 1783, 2851, 2929, 3327 cm^{-1}. ^1H NMR ($CDCl_3 + d_3$-MeOH): δ 0.79 (3H, s, 18-CH_3), 2.97 (4H, s, NHS), 3.05 (2H, t, J = 6.7 Hz, CH_2S, overlaid with 1H, s, H-9), 3.75 (1H, t, J = 10.9 Hz, H-17), 6.87 (1H, d, J = 11.3 Hz, H-2), 7.20 (1H, d, J = 11.4 Hz, H-1). ^{13}C NMR ($CDCl_3 + d_3$-MeOH): δ 11.1, 23.1, 24.7, 25.0, 25.5, 26.6, 27.4, 30.7, 31.2, 32.4, 32.9, 38.1, 43.2, 44.2, 52.9, 81.9, 112.8, 113.8, 125.6, 134.4, 136.5, 150.2, 162.6, 179.3. ESI-MS m/z (MeCN, 40 V): 515.5 $(M + MeCN + H)^+$.

2.7. 2,5-Dioxo-1-pyrrolidinyl-3-(3-hydroxyestra-1,3,5(10)-trien-3-ol-17-on-4-yl)thiopropionate (6)

Compound **4** (100 mg, 0.268 mmol) was dissolved in dry DMF (1 mL), and DCC (72 mg, 0.348 mmol, in 0.5 mL of dry DMF) was added to the rapidly stirring solution dropwise. This was followed by NHS (40 mg, 0.348 mmol, in 0.5 mL of dry DMF). The solution was left stirring at room temperature overnight in the dark. The solvent was removed in vacuo and the resulting semi-solid purified by silica column chromatography using $CHCl_3$/MeOH (15:1) eluant to yield compound **6** as a white solid. Yield: 40 mg (32%). mp 193–197 °C. IR: 1576, 1627, 1736, 1780, 2851, 2929, 3328 cm^{-1}. ^1H NMR ($CDCl_3 + d_3$-MeOH): δ 0.90 (3H, s, 18-CH_3), 2.89 (4H, s, NHS), 4.31 (1H, d, J = 9.4 Hz), 6.85 (1H, d, J = 11.4 Hz, H-2), 7.16 (1H, d, J = 11.4 Hz, H-1). ^{13}C NMR ($CDCl_3 + d_3$-MeOH): δ 13.9, 21.6, 24.8, 25.0, 25.5, 25.7, 26.2, 26.7, 31.1, 32.9, 34.0, 36.6, 37.6, 44.2, 48.0, 52.8, 113.0, 125.5, 133.6, 136.4, 150.6, 157.1, 162.7, 179.3, 220.9. ESI-MS m/z (MeOH 40 V): 471.6 $(M + H)^+$.

2.8. N-(t-Butoxycarbonyl)-4,7,10-trioxa-1,13-tridecanediamine (7)

Performed according to the method of [18] adapted from [27]. To return to free amine form, compound **7** was washed with aqueous sodium bicarbonate solution. Yield: 1.925 g (69%). Analytical RP-HPLC [MeOH/H_2O (60:40, v/v), 201 nm]: R_t = 1.34 min, 97%. IR: 1113, 1555, 1694, 2874, 2931 cm^{-1}. ^1H NMR ($CDCl_3$, compound **7** in R-NH_3^+ form): δ 1.43 (s, 9H, t-butoxy carbonyl (Boc) methyl), 1.77 (2H, quin, J = 6.3 Hz, CH_2–CH_2–NH_3^+), 1.94 (2H, quin, J = 5.8 Hz, CH_2–CH_2–NH–CO), 3.09 (2H, t, 6.1 Hz, CH_2–NH_3^+), 3.19 (2H, d of t, J_d = 5.9 Hz, CH_2–NH–CO), 3.53 (2H, t, J = 6.0 Hz, CH_2–O), 3.58 (2H, t, J = 3.3 Hz, CH_2–O), 3.62 (6H, m, CH_2–O), 3.66 (2H, t, J = 5.6 Hz CH_2–O). ^{13}C NMR ($CDCl_3$): δ 28.4, 29.7, 33.4, 38.4, 39.6, 69.4, 70.19, 70.22, 70.57, 70.60, 156.1. ESI-MS m/z (MeOH): 321.1 $(M + H)^+$.

2.9. N-(13-(t-Butoxycarbonylamino)-4,7,10-trioxatridecanyl)-3-(3,17β-dihydroxyestra-1,3,5(10)-trien-4-yl)thiopropanamide (8)

Compound **5** (50 mg, 0.075 mmol) was dissolved in dry DMF (1 mL). **7** (Na_2CO_3 washed, 21 mg, 0.064 mmol in 0.5 mL of $CHCl_3$) was added dropwise with vigorous stirring followed by triethylamine (100 μL, dried over molecular sieves). The solution was stirred in the dark at room temperature for 60 h. Solvent was then removed and the product separated by silica column chromatography using successively $CHCl_3$/MeOH (15:1), $CHCl_3$/MeOH (10:1), $CHCl_3$/MeOH (5:1), $CHCl_3$/MeOH (1:1) and then MeOH as eluant to yield compound **8** as a clear, colorless oil. Yield: 44 mg (86%). Analytical RP-HPLC [MeOH/H_2O (70:30, v/v), 207 nm]: R_t = 12.03 min, 95%. IR: 1542, 1655, 1695, 2919, 3410. ^1H NMR ($CDCl_3$): δ 0.77 (3H, s, 18-CH_3), 1.44 (9H, s, Boc CH_3), 2.56 (2H, t, J = 7.1 Hz, CH_2COO–), 2.99 (2H, t, J = 7.1 Hz Ar–S–CH_2), 3.60 (12H, m, OEG CH_2–O), 3.73 (1H, t, J = 8.5 Hz, H-17), 6.85 (1H, d, J = 8.5 Hz, H-2), 7.17 (1H, d, J = 8.5Hz, H-1). ^{13}C NMR ($CDCl_3$): δ 11.0, 23.2, 27.2, 28.1, 28.4, 28.5, 28.9, 29.2, 29.5, 29.7, 31.1, 34.2, 35.9, 36.5, 37.9, 38.1, 43.2, 44.2, 49.9, 69.5, 69.9, 70.1, 70.2, 70.5, 81.8, 82.3, 112.8, 113.7, 125.4, 134.2, 136.5, 150.3, 156.1, 170.9. ESI-MS m/z (H_2O, 60 V): 718.9 $(M + H_2O + Na)^+$.

2.10. N-(13-(t-Butoxycarbonylamino)-4,7,10-trioxatridecanyl)-3-(3-hydroxyestra-1,3,5(10)-trien-17-on-4-yl)thiopropanamide (9)

Compound **6** (37 mg, 0.079 mmol) was dissolved in dry DMF (1 mL). A solution of **7** (51 mg, 0.159 mmol, in 750 μL of dry $CHCl_3$) was added dropwise to the stirring steroid solution. Triethylamine (dry, 250 μL) was then added and the reaction stirred in the dark at room temperature for 60 h. After 48 h another 250 μL of dry $CHCl_3$ was added to aid solubility. The solvent was removed in vacuo and the sample separated by silica column chromatography using successively $CHCl_3$ and $CHCl_3$/MeOH (15:1) eluant to yield compound **9** as a waxy white solid. Yield: 27 mg (50%). Analytical RP-HPLC [MeOH/H_2O (65:35, v/v), 203 nm]: R_t = 12.76 min, 96%. IR: 1626, 1655, 1702, 1736, 2850, 2928, 3327 cm^{-1}. ^1H NMR ($CDCl_3$): δ 0.91 (3H, s, 18-CH_3), 1.44 (9H, s, Boc CH_3), 3.63 (13H, m, OEG), 4.62 (1H, d, J = 10.0 Hz), 6.83 (1H, d, J = 11.3 Hz, 2-H), 7.15 (1H, d, J = 11.7 Hz, 1-H). ^{13}C NMR ($CDCl_3$): δ 13.8, 21.6, 25.0, 25.6, 26.2, 28.4, 29.6, 31.1, 31.5, 33.8, 36.0, 37.6, 38.2, 39.3, 44.1, 48.9, 49.0, 50.3, 69.4, 69.9, 70.2, 70.5, 79.2, 113.0, 117.5, 125.3, 133.3, 136.6, 157.5, 161.9, 178.7, 221.8. ESI-MS m/z (MeOH, 40 V): 695.6 $(M + H_2O + H)^+$.

2.11. N-(13-Amino-4,7,10-trioxatridecanyl)-(3,17β-dihydroxyestra-1,3,5(10)-trien-4-yl)thiopropanamide (10)

Compound **8** (28 mg, 0.041 mmol) was dissolved in formic acid (4 mL) and stirred at room temperature for 4 h before removal of acids in vacuo. Yield: 24 mg (100%). Analytical RP-HPLC [MeOH/H_2O (60:40, v/v), 212 nm] R_t = 1.73 min, 98%. IR: 1653, 1718, 1734, 2847, 2929 cm^{-1}. ^1H NMR ($CDCl_3$): δ 0.85 (3H, s, 18-CH_3), 3.62 (13H, OEG CH_2-O), 4.80 (1H, t, J = 8.4 Hz), 6.86 (1H, d, J = 8.4 Hz, 2-H), 7.15 (1H, d, J = 8.4 Hz, 1-H). ^{13}C NMR ($CDCl_3$): δ 12.1, 23.3, 25.7, 26.4, 27.4, 27.6, 29.2, 31.1, 34.0, 35.9, 36.7, 36.9, 37.8, 43.0, 44.0, 49.7, 69.0, 69.7, 69.8, 69.9, 70.1, 70.4, 82.5, 113.0, 113.6, 125.4, 133.8, 136.5, 150.7, 161.2, 171.7. ESI-MS m/z (MeOH): 615.2 $(M + 2H_2O + H)^+$, 637.3 $(M + 2H_2O + Na)^+$.

2.12. 17β-Hydroxyestra-1,3,5(10)-trien-3-yl hemisuccinate (11)

Method adapted from [28]. 17β-Estradiol (100 mg, 0.367 mmol) was dissolved in pyridine (9 mL). Succinic anhydride (37 mg, 0.367 mmol in 1 mL of pyridine) was added dropwise to the rapidly stirring solution. The reaction was then stirred at 45 °C for 24 h. The pyridine was then removed in vacuo and the resultant sample purified by silica column chromatography using successively, $CHCl_3$, $CHCl_3$/MeOH (15:1), $CHCl_3$/MeOH (10:1) as eluant to yield compound **11** as a semi-solid. Yield: 33 mg (24%). IR: 1588, 1739, 3584 cm^{-1}. ^1H NMR ($CDCl_3 + d_3$-MeOH): δ 0.77 (3H, s, 18-CH_3), 2.74 (2H, t, J = 8.9 Hz, CH_2-ester), 2.84 (2H, t, J = 7.3 Hz, CH_2–COOH), 6.55 (1H, m, 4-H), 6.84 (1H, m, 1-H), 7.28 (1H, m, 2-H). ^{13}C NMR ($CDCl_3 + d_3$-MeOH): δ 11.1, 23.1, 26.2, 27.1, 27.3, 29.1, 29.5, 36.7, 38.6, 43.2, 44.2, 49.8, 50.1, 81.6, 118.5, 121.4, 126.4, 131.7, 138.0, 148.4, 171.6, 174.9. ESI-MS m/z (MeOH, 40 V): 395.3 $(M + Na)^+$.

2.13. N-(13-(t-Butoxycarbonylamino)-4,7,10-trioxatridecanyl)-3-(17β-hydroxyestra-1,3,5(10)-trien-3-yl)oxycarbonylpropanamide (12)

Compound **11** (72 mg, 0.194 mmol) was dissolved in DMF/$CHCl_3$ (1:1) (2.5 mL, dry). This solution was stirred whilst DCC (60 mg, 0.292 mmol, in 500 μL of dry DMF) was added dropwise followed by NHS (34 mg, 0.292 mmol, in 500 μL of dry DMF), also dropwise. The solution was stirred in the dark overnight. White solid was filtered from the solution and washed with $CHCl_3$ and DMF before removing all solvent in the filtrate under vacuum. The resultant white semi-solid was reconstituted in DMF/$CHCl_3$ (1:1) (2 mL, dry) and **7** was added (94 mg in 3.25 mL of dry DMF/chloroform (2:1)) dropwise to the stirring solution. Triethylamine (1 mL, dried over molecular sieves) was then added and the reaction stirred in the dark overnight. The solvent was then removed and the sample dried under vacuum. The sample was then separated by silica column chromatography using successively $CHCl_3$, $CHCl_3$/MeOH (15:1), and $CHCl_3$/MeOH (10:1) as eluant to yield compound **12** as a white semi-solid. Yield: 64 mg (49%). IR: 1627, 1654, 1702, 1736, 2850, 2926, 3326 cm^{-1}. ^1H NMR ($CDCl_3 + d_3$-MeOH): δ 0.77 (3H, s, 18-CH_3), 1.44 (9H, s, Boc methyls), 2.47 (2H, t, J = 9.3 Hz, hemisuccinate CH_2-amide), 2.65 (2H, t, J = 9.4 Hz, hemisuccinate CH_2-ester), 6.62 (1H, m, 2-H), 6.79 (1H, m, 4-H), 7.12 (1H, m, 1-H). ^{13}C NMR ($CDCl_3 + d_3$-MeOH): δ 11.1, 23.2, 25.7, 26.5, 27.4, 28.5, 28.9, 29.5, 29.6, 29.8, 30.1, 30.8, 33.9, 36.8, 37.5, 39.1, 44.1, 48.8, 48.9, 50.2, 69.6, 70.2, 70.6, 81.7, 83.3, 118.6, 121.5, 126.4, 131.8, 138.0, 148.5, 158.0, 172.1, 173.8. ESI-MS m/z (MeOH, 40 V): 697.5 $(M + Na)^+$. HRFAB-MS: m/z 675.4202 (MH^+) (calcd. for $C_{37}H_{58}O_9N_2$, 675.4202).

2.14. (13-Amino-4,7,10-trioxatridecanyl)-3-(17β-hydroxyestra-1,3,5(10)-trien-3-yl)oxycarbonylpropanamide (13)

Compound **12** (48 mg, 0.071 mmol) was dissolved in formic acid (4 mL) and stirred at room temperature for 2.5 h before adding chloroform (1 mL) to improve solubility and stirred for an additional 1.5 h. Solvent was removed in vacuo and the product column separated using MeOH/AcOH (10:1) eluant. AcOH was removed in vacuo. Yield: 36 mg (81%). Analytical RP-HPLC [MeOH, 203 nm]: R_t = 1.64 min, 95%. IR: 1414, 1561, 1638, 3434 cm^{-1}. ^1H NMR (D_2O): δ 1.14 (3H, s, 18-CH_3), 2.52 (2H, t, J = 6.0 Hz, CH_2-amide), 2.65 (2H, t, J = 7.2 Hz, CH_2-ester), 2.81 (2H, d, J = 12.0 Hz), 3.09 (2H, t, J = 7.4 Hz), 3.23 (2H, m), 3.55 (3H, m, OEG), 3.66 (11H, m, OEG CH_2–O), 6.68 (m, 2-H), 6.88 (m, 4-H), 7.23 (m, 1-H). ^{13}C NMR (d_3-MeOH): δ 21.1, 23.3, 29.8, 38.8, 70.1, 70.4, 83.4, 127.0, 174.8. ESI-MS m/z (MeOH, 40 V): 575.2 $(M + H)^+$.

2.15. 2-(N-(13-(t-Butoxycarbonylamino)-4,7,10-trioxa-1,13-tridecanyl)-(aminomethyl)-3,17β-dihydroxyestra-1,3,5(10)-triene) (14)

Estradiol (100 mg, 0.367 mmol) was dissolved in absolute EtOH (10 mL). Compound **7** (236 mg, 0.734 mmol in EtOH/water (4:1) (5 mL)) was added gradually to the solution whilst stirring. This was followed by 37% (v/v) formaldehyde solution (222 μL, 8.06 mmol) and the solution refluxed with stirring for 6 h. The solvent was removed under vacuum and the sample separated by silica column chromatography using $CHCl_3$, $CHCl_3$/MeOH (15:1), $CHCl_3$/MeOH (10:1) as eluant to yield compound **14** as a clear colorless oil. Yield: 30 mg (13%). IR: 1692, 3584 cm^{-1}. ^1H NMR ($CDCl_3 + d_3$-MeOH): δ 0.77 (3H, s, 18-CH_3), 1.43 (9H, s, Boc methyl), 2.80 (2H, m, CH_2–Ar), 3.60 (14H, m, OEG CH_2–O), 6.51 (1H, s, 4-H), 6.88 (1H, s, 1-H). ^{13}C NMR ($CDCl_3 + d_3$-MeOH): δ 11.1, 23.2, 26.5, 27.3, 28.2, 28.4, 28.5, 29.4, 29.4, 29.6, 30.1, 36.8, 38.2, 39.0, 43.3, 44.1, 48.3, 48.9, 49.5, 69.1, 69.3, 69.5, 70.2, 70.6, 72.1, 81.6, 82.4, 116.0, 117.3, 124.4, 133.0, 136.5, 151.8, 155.1. COSY ($CDCl_3 + d_3$-MeOH): no aromatic coupling cross-peaks. ESI-MS m/z (MeOH, 40 V): 605.5 $(M + H)^+$.

2.16. 2-(13-Amino-4,7,10-trioxa-1,13-tridecanyl)-(aminomethyl)-3,17β-dihydroxyestra-1,3,5(10)-triene) (15)

Compound **14** (21 mg, 0.034 mmol) was dissolved in formic acid (3 mL) and stirred for 4 h at room temperature before removal of acid in vacuo. The product was column purified using MeOH/AcOH (2:1) to elute the product. Yield: 15 mg (86%). Analytical RP-HPLC [MeOH, 203 nm]: R_t = 1.63 min, 97%. IR: 1134, 1421, 2981, 3438 cm^{-1}. ^1H NMR (d_3-MeOH) δ: 0.89 (3H, 18-CH_3), 2.83 (2H, m, 7-CH_2), 3.64 (m, OEG CH_2–O), 6.65 (4-H), 6.99 (1-H). ESI-MS m/z (MeOH): 505.3 $(M + H)^+$.

2.17. SPR chip surface immobilization

A CM5 sensor chip was docked in a BIAcore 3000 instrument and primed twice with running buffer. The surface was activated with BIAcore coupling solutions of EDC/NHS (1:1) (50 μL, 5 μL/min). A solution of the compound to be immobilized (**13**, **15** or **10**, 1 mg/mL in 1% (v/v) DMF in 10 mM phosphate buffered saline with 0.05% (w/v) Tween 20 (PBS/T) buffer, pH 9.7) was centrifuged to remove any insoluble material and then injected (1× 20 μL, 1× 80 μL, 1× 100 μL, 1× 200 μL, 5 μL/min). This was immediately followed by further injections of estrogen derivative at higher pH (1 mg/mL, 1% (v/v) DMF in PBS/T buffer, pH 11.7, 1× 200 μL, 2× 100 μL, 5 μL/min) and then deactivation with 1.0 M ethanolamine (50 μL, 5 μL/min). Compound **13** was immobilized in flow cell 2, compound **15** in flow cell 3 and compound **10** in flow cell 4. Flow cell 1 was activated as for the others and then immediately deactivated with ethanolamine (50 μL, 5 μL/min) without immobilization of estrogens to serve as a reference flow cell. The immobilization was repeated on another chip but at pH 4.2 to ensure alkaline degradation was not occurring to the surface coatings and antibody binding was checked as below.

2.18. SPR antibody binding studies

Plots of response versus primary antibody concentration with no secondary antibody enhancement were prepared using a BIAcore wizard program by injecting primary monoclonal antibody (60 μL, at 0, 0.25, 0.5, 1, 2, 5, 10, and 25 μg/mL, five replicates of each, 20 μL/min) and waiting 120 s before regeneration with two pulses of 50 mM NaOH, 10% (v/v) MeCN (20 μL each, 20 μL/min). These binding sensorgrams were then fitted using BIAevaluation 3.1 software and the affinity constants (K_A) and dissociation constants (K_D) for the interactions were calculated taking IgG mass at 150 kDa.

Plots of response versus secondary antibody concentration for secondary antibody signal enhancement were prepared by injecting monoclonal primary antibody (60 μL, 20 μL/min, 0.5 μg/mL) followed immediately by secondary antibody (60 μL; 0, 10, 20, 50, 100, 200, 300 μg/mL; 10 μL/min) and then a 120 s wait before regeneration as above.

Plots of response versus monoclonal antibody concentration with secondary antibody enhancement were prepared by injecting monoclonal primary antibody (60 μL; 0, 10, 25, 50, 100, 200, 350, 500 ng/mL; 20 μL/min) immediately followed by secondary antibody (60 μL, 200 μg/mL), a 120 s wait and then regeneration as above.

2.19. SPR 17β-estradiol assays

Assay curves for 17β-estradiol assay without secondary antibody enhancement were prepared by mixing monoclonal primary antibody (70 μL, 1 μg/mL) with 17β-estradiol solutions (70 μL; 0, 1, 5, 10, 50, 100, and 500 pg/mL, 1, 5, and 10 ng/mL; five replicates each) in a microwell plate, mixing using the automatic mixing function, incubating for 5 min at 25 °C and then injecting (60 μL, 20 μL/min), and then after a 120 s wait regenerating as above.

Assay curves for 17β-estradiol assay with secondary antibody enhancement were prepared by mixing monoclonal primary antibody (50 ng/mL, 70 μL) with 17β-estradiol solutions (70 μL; 0, 0.5, 1, 5, 10, 50, 100, and 500 pg/mL, 1 and 5 ng/mL, five replicates each) in a microwell plate, mixing, incubating and injecting as above. This injection was then immediately followed by injection of secondary antibody (60 μL, 200 μg/mL, 10 μL/min), a wait of 120 s and then regeneration as above.

3. Results and discussion

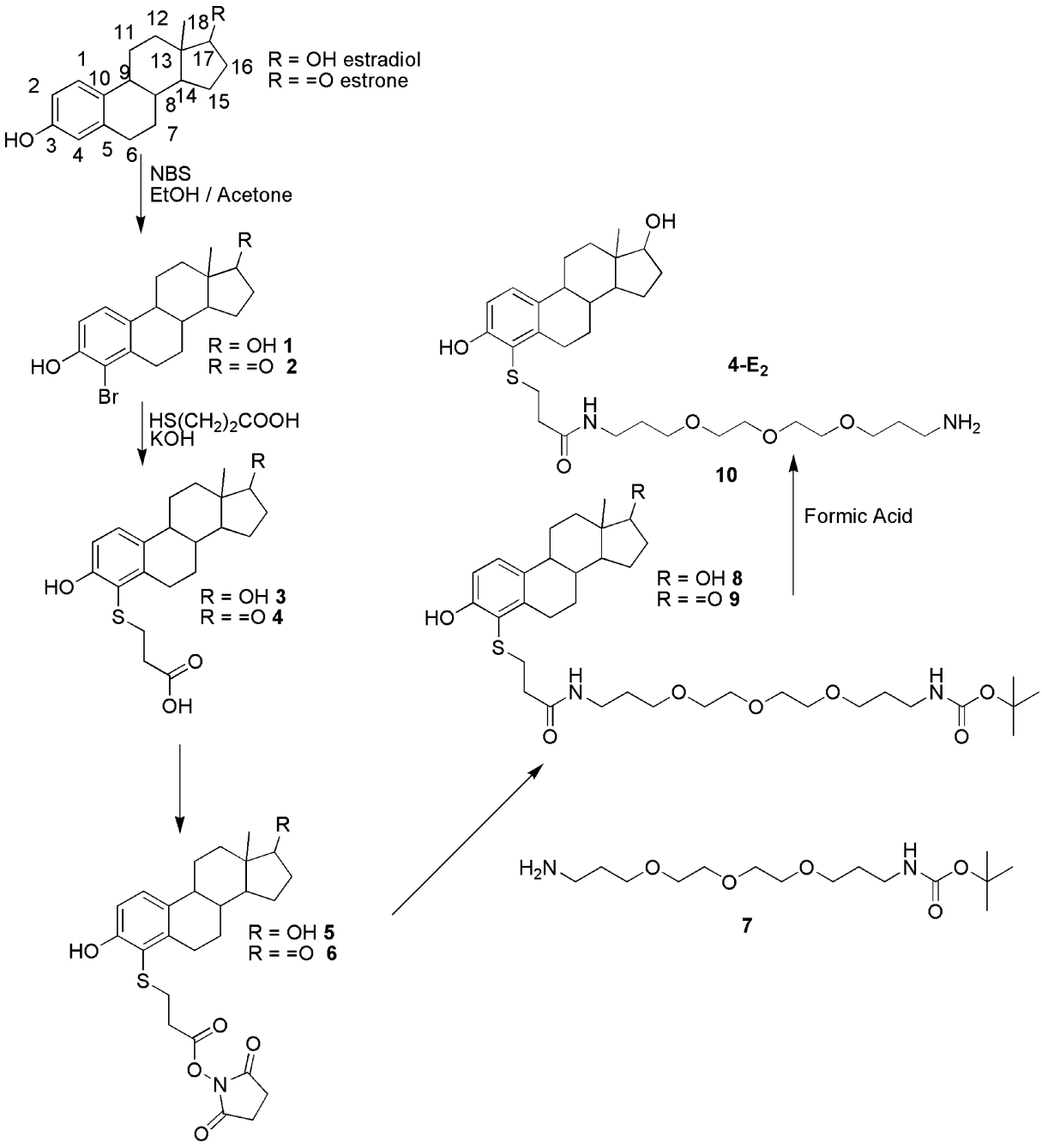
3.1. Synthesis of 4-position mercaptopropionate conjugates

To attach linkers by thioether bridging at the A-ring 4-position of non-aromatic steroids such as progesterone, it has been shown that formation of the 6-bromo-derivative and subsequent alkaline reflux with the thiol will produce the desired derivative. This method cannot be replicated when the A-ring of the steroid is aromatic, such as in the case of the estrogens. Whilst previous studies have used reaction via a 4,5-epoxide [9], a potentially simpler route is to form the 4-bromo-derivative and to follow this with an aromatic substitution of the bromo-derivative with the thiol, thus producing the linkage in two simple steps (Scheme 1).

4-Bromo-17β-estradiol was produced by adapting an existing generalized method whereby the steroid is simply mixed with *N*-bromosuccinimide in dry ethanol and the product precipitates [24,25] (Scheme 1). The existing methods [24,25] however provided no experimental details of the synthesis and so this paper is the first to provide clear details of a one-step synthesis of 4-bromoestradiol. Attempts have been made to synthesize 4-bromoestrone in one step but with limited success [25]. Instead, a two-step method whereby estrone is brominated with *N*-bromosuccinimide to form the 2,4-dibromoestrone followed by regioselective reductive dehalogenation using palladium on carbon has been previously adopted as the best method [29,30]. In this study it has been found that the two-step method resulted in a low yield of the product and so a method was developed whereby the 4-bromoestrone could be produced in one step by simply adjusting the solvent combination used in the reaction to precipitate the desired product (Scheme 1).

Production of derivatives with thioether linkages was then achieved by refluxing the corresponding bromo-derivatives in methanolic potassium hydroxide under dry conditions (Scheme 1). By simply extracting the product into ethyl acetate and washing with water it could be mostly purified, however in the case of the 17β-estradiol derivative there was still some residual 3-mercaptopropionic acid contamination. 4-Position attachment could be confirmed through the ^{1}H NMR signals, which clearly showed two doublets with chemical shifts consistent with those expected for 4-substituted estrogens.

This is a new example of replacement of an aryl bromo-derivative with an alkyl thiol group and speculatively may occur through a radical $S_{RN}1$ mechanism, as the 4-position is not activated toward nucleophilic aromatic substitution. This reaction attached a carboxylic acid group to the steroid A-ring without compromising existing functional groups and attached solely at the 4-position, therefore allowing



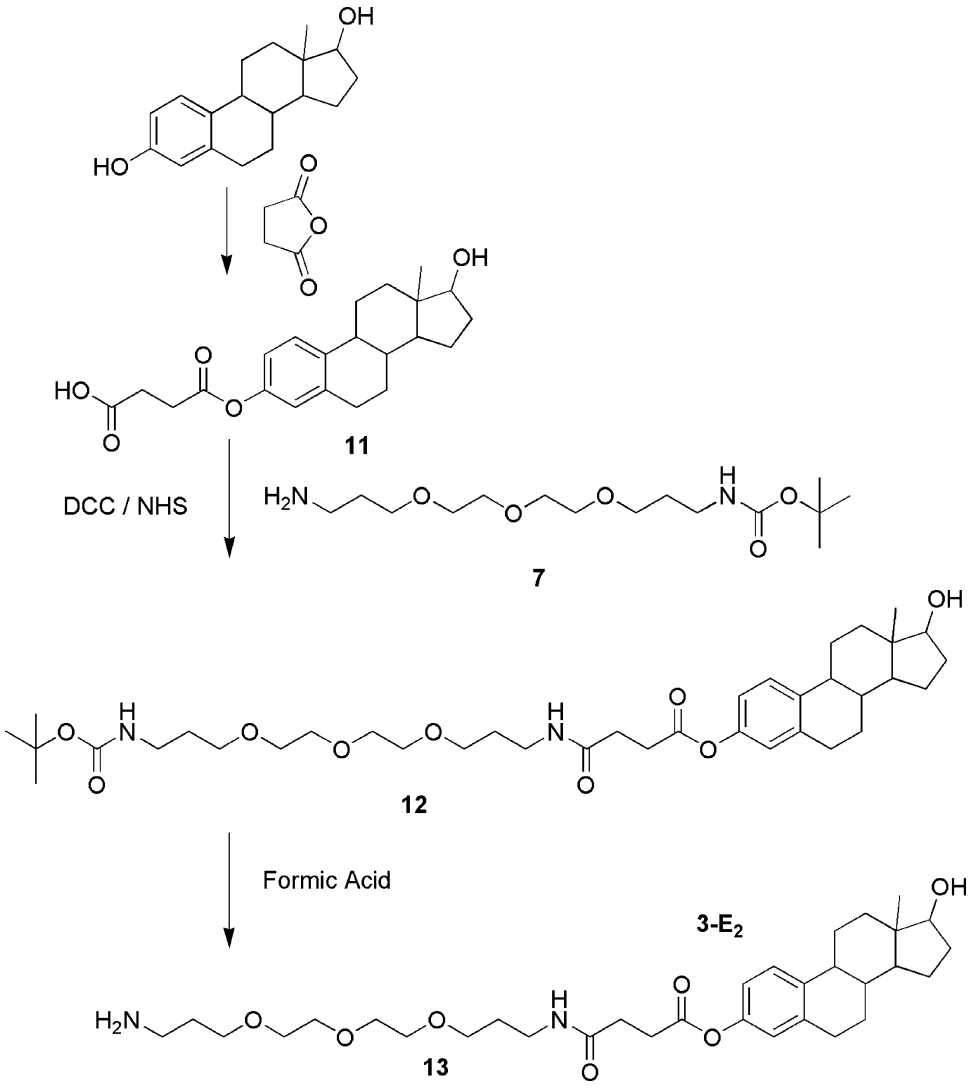
Scheme 1 – Synthesis of 4-position thiopropionate derivatives of 17β-estradiol and estrone and the attachment of oligoethylene glycol (OEG) chains.

attachment of a large range of different linkers tailored to individual requirements for various conjugations of the estrogens.

3.2. *Attachment of oligoethylene glycol linkers*

Oligoethylene glycol linkers are water-soluble, and as such, in aqueous media would be envisaged to improve the solubility of the estrogen derivatives for convenient in situ immobilization of these estrogens onto a solid surface such as a BIAcore biosensor chip in a flow-through format. Such water-soluble estrogen derivatives could also project the estrogen antigens into the aqueous phase effectively when tethered to a solid surface as a coating antigen. To attach such linkers to these derivatives, the carboxylic acid was first activated using NHS, with DCC used as a dehydrating agent (Scheme 1). This worked well for both steroids.

An OEG linker was attached to the estrogens by reaction with the corresponding NHS active ester (Scheme 1). The chain had an amine terminal at both ends and so one end was protected with a Boc protecting group to prevent dimerization. The reaction conditions were simple mixing and addition of



Scheme 2 – Synthesis of 3-hemisuccinate derivative of estradiol and attachment of an oligoethylene glycol (OEG) chain.

a mild base (triethylamine) and room temperature stirring in dry solvent.

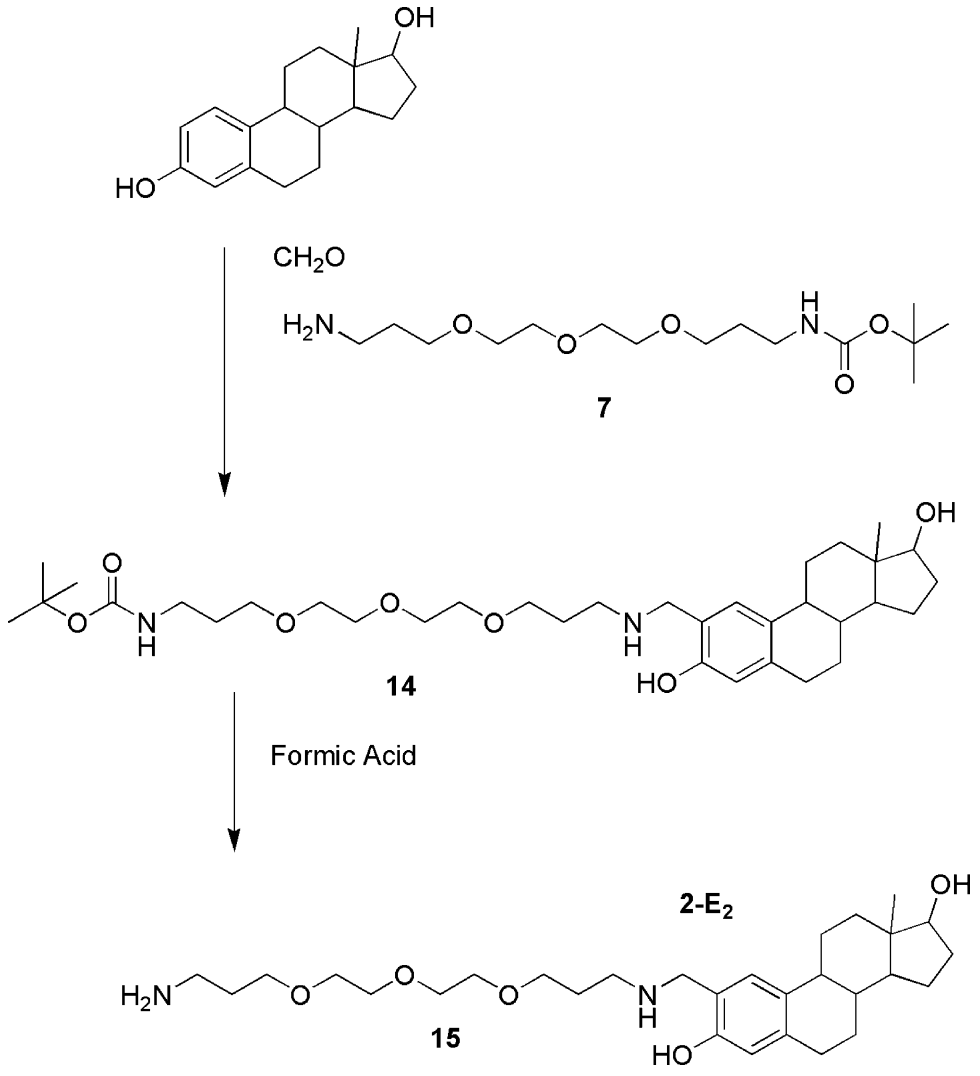
3.3. *Attachment at 3- and 2-positions*

To compare the newly synthesized 4-estrogen OEG derivative (4-E_2) as a coating antigen in a flow-through BIAcore SPR biosensor, it was necessary to synthesize reference compounds attached at other positions on the aromatic ring of the steroid. 3-Position conjugation through a hemisuccinate linkage via the 3-OH (3-E_2), and the 2-position via Mannich reaction (2-E_2) were selected. Estradiol-3-hemisuccinate was produced as reported previously [28], and a 15-atom OEG chain was then attached in the same way as for the 4-thioether derivatives (Scheme 2).

Mannich conjugations are normally done in one step with both the estrogen and the carrier protein present. This produces a mixture of 2- and 4-conjugated estrogen–protein conjugates. To compare the effect of position of attachment on the ability to bind primary antibody, it was necessary to isolate one of the positions by performing the Mannich reaction without the protein being present, but rather using mono-protected OEG with one free amine terminal exposed (Scheme 3). This reaction then enabled chromatographic purification of the main product. The yield of this reaction was quite low (13%); variable yields having been reported as a disadvantage with Mannich conjugations [31]. The product isolated was analyzed by ^{1}H NMR and 2-D COSY, both of which showed clearly that there was no coupling between the two proton singlet signals and the aromatic chemical shifts matched those expected for 2-position conjugation.

3.4. *Immobilization of the sensor surface*

To expose the amine terminal of the linker chain, all the estradiol-oligoethylene glycol derivatives were treated with formic acid to remove the Boc protecting group (Schemes 1–3). The estradiol derivatives could then be in situ immobilized on the sensor surface through their primary amine terminals. The carboxymethylated dextran surface of a BIAcore CM5 chip is functionalized with carboxylic acid groups that can be easily activated with a combination of NHS and EDC coupling



Scheme 3 – Synthesis of 2-E_2.

reagents. As for previous reports for progesterone [18], the surface was immobilized in situ by flowing high concentrations of the primary amines over the surface under basic conditions. To ensure surface saturation in each case, the concentrations were high (1 mg/mL) and a large volume of solution was flowed over the surfaces (800 μL per surface in total) giving a high excess of immobilizing agent. A very slow flow rate was used (5 μL/min) to maximize immobilization. Two alkaline pH were used in each case to help ensure that the maximum amount of steroid derivative was bound with the pH above the pK_a of the amine in each case. Each surface was immobilized under identical conditions with the first flow cell being simply activated and then deactivated with ethanolamine to act as the blank.

3.5. *Antibody binding studies using SPR*

It was necessary to use monoclonal antibody as the binding antibody because the use of commercial polyclonal antibody (E2885, Sigma, St. Louis, MO, USA) resulted in no detectable binding to any of the flow cells in this flow-through format. In the first instance, the binding of monoclonal antibody to the surfaces was examined (Fig. 1) and a plot of the binding response, in response units (RU), versus primary monoclonal antibody concentration was prepared (Fig. 2). This showed clearly very little antibody binding to the 3-E_2 conjugated surface and quite strong binding to the 4-E_2 conjugated surface but even stronger binding to the 2-E_2 conjugated surface. Antibody binding to the surface of the 4-E_2 conjugate was 84% that of the 2-E_2 conjugate at saturation (25 μg/mL primary antibody), whilst its binding to the 3-E_2 conjugated surface was only 2% that of the corresponding 2-E_2 (Fig. 2). This suggests that the formation of the conjugate through the 3-position of the steroid is greatly reducing the antibody binding capacity of the antigen, most likely through modification of the 3-hydroxy group of the steroid reducing its antigenicity when an antibody raised to the 6-position is employed. As 6-position conjugation involves attachment that does not compromise existing functional groups, the antibody would be expected to have a relatively high specificity to free estradiol.

Clearly, compromising one functional group of the estradiol has greatly reduced binding. This result has important

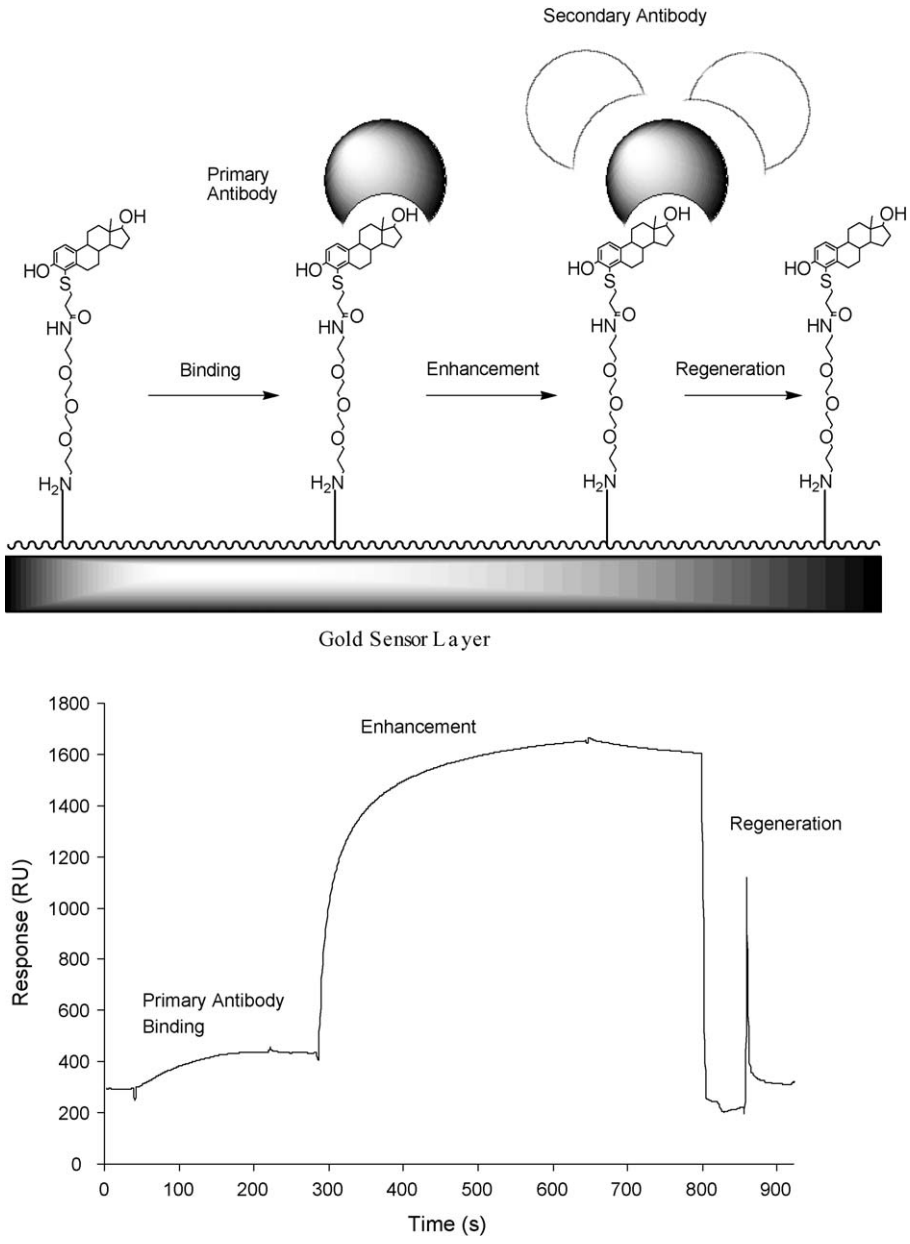


Fig. 1 – Schematic representation of primary antibody binding to 17β-estradiol-linker modified SPR sensor surface, subsequent binding of secondary antibody to enhance sensor signal and regeneration of the sensor surface. Signal responses are shown for each step as a plot of response vs. time, relating signal to binding event.

implications for biosensor design as the 3-position is widely used in enzyme and chemiluminescent label conjugations of estradiol and yet is of no use in flow-through biosensors using antibody raised to the 6-poistion conjugate. This result is different from that previously reported for enzyme-linked immunosorbent assay (ELISA) of progesterone, where significant antibody binding was obtained when the antibody was raised to the 6-position and the coating antigen was conjugated through the 3-position [32] indicating the importance of assay format in antibody binding performance. An antibody raised to the 3-position could be used to gain better biosensor signal but such an antibody would likely bind the 3-E_2 conjugated coating antigen more strongly than the free analyte and so immunoassay sensitivity would likely be reduced. Hemisuccinate linkages have been reported to be somewhat sensitive to alkaline degradation but this has not occurred in this case as immobilization at non-alkaline pH demonstrated comparable binding results for the hemisuccinate.

It is also interesting to note that the 2-E_2 system performs slightly better than that of the 4-E_2 (Table 1). As the antibody has been raised to the 6-position conjugate, it would be expected that any changes to the steroid structure on the opposite side of the antigen would greatly diminish the antibody binding. The 2-position however, appears to be near enough to the same side of the steroid as the conjugate used to raise the antibody for it to have no major effect on

Table 1 – Summary of 17β-estradiol immunoassay parameters of LOD, IC50, sensitivity, and signal enhancement with changes in primary antibody concentration and signal enhancement labeling[a]

Assay format	Conjugation position	mAb concentration (ng/mL)	LOD (pg/mL)	IC_{50} (pg/mL)	Sensitivity (RU mL/ng)	Enhancement ratio
mAb only	2	500	96 ± 32	1457 ± 9.6	22	n/a
mAb only	4	500	111 ± 33	1432 ± 14	16	n/a
mAb only	2	25	66 ± 27	414.9 ± 5.9	6.9	9.5
mAb only	4	25	95 ± 32	351 ± 14	4.1	8.9
Secondary antibody enhanced	2	25	25 ± 22	324 ± 21	65	9.5
Secondary antibody enhanced	4	25	61 ± 39	396 ± 28	41	8.9

[a] Errors quoted are standard errors.

the ability of the antibody to recognize the antigen; indeed the 2-E_2 gave the strongest antibody binding (Fig. 2). This result differs from that seen with fluoresecent conjugates of estradiol where a 2-position conjugation via a carboxypentyloxime linkage gave no binding to an antibody raised to the 6-carboxymethyloxime conjugate, whereas thioether conjugation through the 4-position did give binding in an ELISA format [9]. This difference is due either to the different format used (flow-through or ELISA) or the method of linkage at the 2-position. Speculatively, in a static ELISA format, 2-position of attachment close to the opposite side of the antigen from that used in raising the antibody may result in the linker and fluorescent label obstructing approach of the antibody to this side of the antigen whereas in flow-through formats the linker tether is suspended in the fluid stream away from the antigen allowing sterically favorable approach of the antibody. These results show how important the binding format and conjugation method is in determining levels of antibody binding.

The differences in antibody binding responses are also reflected in the calculated affinity and dissociation constants for the antibody binding to the sensor surfaces, Table 2. The affinity constants increase significantly when changing conjugation from 3-E_2 to 4-E_2 and then to 2-E_2, showing increasing antibody binding affinity. Correspondingly, the dissociation constants decrease as the affinity constants increase.

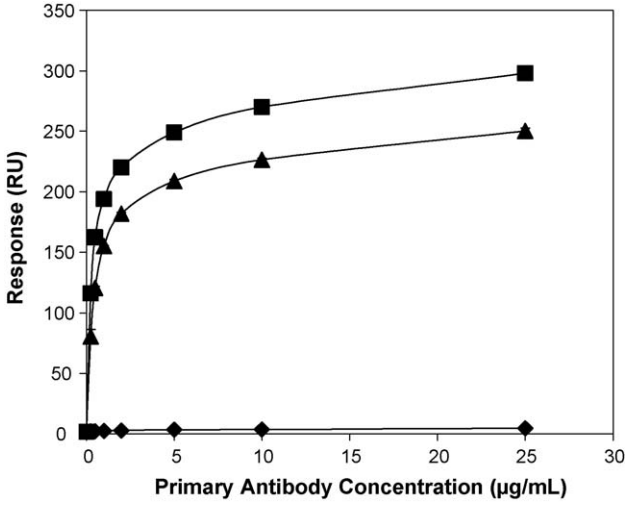


Fig. 2 – Plot of response (RU) vs. primary antibody concentration (μg/mL) for 3-E_2 (♦), 4-E_2 (▲) and 2-E_2 (■) conjugates (error bars too small to see).

Saturation of all binding sites occurred at close to 25 μg/mL of primary antibody under the flow-through conditions, with rapid decline in binding signal at concentrations less than 2 μg/mL. A primary antibody concentration of 0.5 μg/mL was adequate to achieve sufficient response for conducting an SPR immunoassay.

To produce sensitive immunoassays for small molecules where the competing antigen is immobilized on the surface, it is desirable to reduce the primary antibody concentration as much as possible so that smaller amounts of analyte can inhibit the antibody binding to the surface. The problem with this approach in SPR is that the lower the primary antibody concentration, the lower the signal and so the poorer the signal: noise ratio. One technique that has proved very successful with progesterone is to enhance the signal with secondary antibody that recognizes the bound primary antibody [18] (Fig. 1). Enhancements of signal up to eight-fold have been seen for progesterone using this method [18].

To determine the best secondary antibody concentration to use as a label for the binding, a plot of response versus secondary antibody concentration at fixed primary antibody was prepared (Fig. 3). This showed that primary antibody at 0.5 μg/mL had maximum binding with secondary antibody label at concentrations of about 200 μg/mL. Use of 300 μg/mL produced only approximately 4% additional binding but could potentially significantly increase non-specific binding. To determine just how much signal enhancement had been obtained and thus how low the primary antibody concentration could be reduced, a plot of response versus primary antibody concentration at fixed secondary antibody concentration was prepared (Fig. 4). By comparing the slopes of the plots in the linear region, this gave signal enhancements of

Table 2 – Affinity constants (K_A) and dissociation constants (K_D) for the binding of estradiol monoclonal antibody to SPR sensor surfaces with conjugation at the 2-, 3- and 4-positions[b]

Compound	Position of attachment	K_A (10^7 M^{-1})	K_D (nM)
15	2	148 ± 13	0.70 ± 0.06
13	3	2.02 ± 0.23	44.8 ± 7.7
10	4	95.6 ± 3.7	1.05 ± 0.04

[b] Errors quoted are standard errors, values are the average of five replicates.

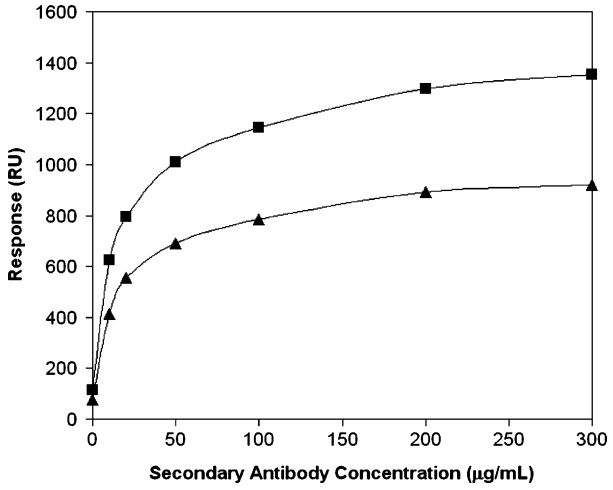


Fig. 3 – Plot of response (RU) vs. secondary antibody concentration (μg/mL) for 4-E_2 (▲) and 2-E_2 (■) linked conjugates (error bars too small to see).

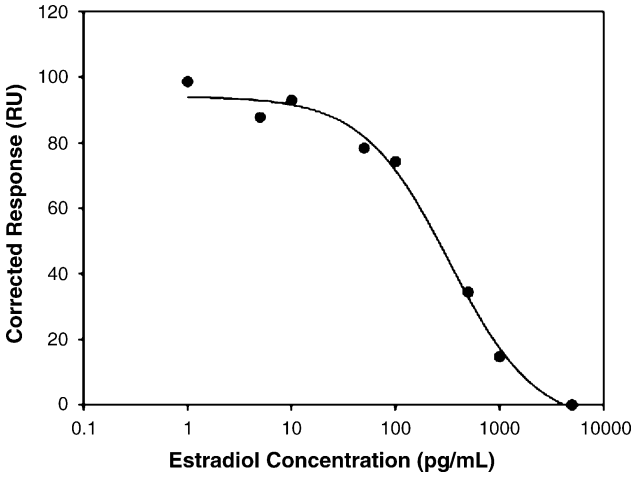


Fig. 5 – Estradiol immunoassay curve plot for secondary antibody enhancement and 2-E_2 conjugation showing the logistic-log fitted assay curve.

9.5-fold for the 2-E_2 surface and 8.9-fold for the 4-E_2 surface. These enhancements were slightly better than those seen previously with progesterone [18] and support the previous observation that secondary antibody can enhance primary antibody binding signal more than would be expected from simple one-to-one binding [18]. These plot also show that the primary antibody concentration can be reduced to 25 ng/mL and still maintain about 100 RU of specific binding after secondary antibody enhancement (Fig. 4).

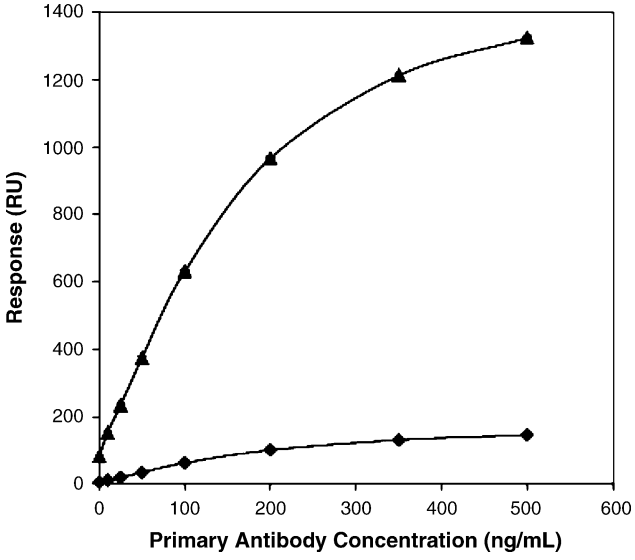


Fig. 4 – Plot of response (RU) vs. primary antibody concentration (ng/mL) using 2-E_2 conjugate and showing mAb binding only (♦) and secondary antibody-enhanced binding (▲). The linear portions of the curves are from 0 to 100 ng/mL and the line equations are $y = 0.574x + 3.98$ ($R^2 = 1.00$) for mAb only and $y = 5.43x + 92.7$ ($R^2 = 0.99$) for secondary antibody enhancement (error bars too small to see.

3.6. *Sensitive SPR assay of 17β-estradiol*

Having optimized the antibody concentrations that can be used, assays could be performed for 17β-estradiol. The first assay was constructed without secondary antibody signal enhancement and so used primary antibody at 0.5 μg/mL. The assay gave a limit of detection (LOD) of 96 pg/mL using the 2-E_2 surface (Table 1). It was not possible to conduct assays using the 3-E_2 surface because the binding signals were too small.

The assay was then repeated but adding the secondary antibody signal enhancement (Fig. 1) and using a final primary antibody concentration of 25 ng/mL (Fig. 5). This assay gave a LOD of 25 pg/mL with the 2-E_2 surface (Table 1). The LOD for the unenhanced primary antibody at the same concentration was 66 pg/mL (Table 1). The IC_{50} for the assay had dropped by approximately 75% upon secondary antibody enhancement whilst the sensitivity had increased three-fold (Table 1). These assays have the lowest LOD of reported SPR assays of estradiol and show how LOD for this steroid can be obtained that are comparable to certain ELISA tests [33,34] but much more rapid and simple. The only previous study of estradiol SPR immunoassay demonstrated eight-fold higher LOD, used manual mixing and injecting after prolonged incubation at 4 °C and used a much less stable protein conjugate format with conjugation through an existing functional group [35].

The in situ covalently immobilized sensor surface, like the previously reported progesterone surface [18], has proved to be quite stable over a large number of cycles (in excess of 200 cycles), with no noticeable decline in antibody binding capacity, a major factor in a practical biosensor.

Production of stable biosensor surfaces that project small molecule antigens with conjugation at positions not bearing functional groups is essential to retaining high specific binding signal in flow-through biosensors. A novel route has been demonstrated for the attachment of carboxylic acid groups to the 4-position of 17β-estradiol and estrone

via thioether bridging (Scheme 1) from the corresponding bromo-derivatives. OEG chains have been attached to provide a hydrophilic linker with low immunogenicity that allows good projection of the antigen in aqueous media (Scheme 1). Simple removal of a Boc protecting group enabled immobilization of the estrogen derivatives to the surface of a BIAcore chip so they can act as stable coating antigens in immunosensing of estrogens. A monoclonal antibody raised from a 6-position conjugate strongly bound both the 2- and 4-E_2 conjugates prepared without conjugation through existing functional groups, compared with very little binding to the 3-E_2 conjugate through an existing functional group (3-OH). Use of secondary antibody as a signal enhancement agent produced enhancements of 8.9–9.5-fold and a simple, sensitive, and highly automated assay constructed using this enhancement gave an LOD of 25 pg/mL (Table 1). These conjugation studies offer new insights into the effects of different conjugation methods on antibody binding in flow-through biosensor formats for small molecules and illustrate the importance of a systematic, rational design of biosensor surface chemistry to maximize immunoassay binding and sensitivity.

Acknowledgement

We thank the New Zealand Foundation for Research Science and Technology for funding this work and providing a Top Achiever doctoral scholarship for John Mitchell.

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