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Sensitive Determination of Progesterone using Surface Plasmon Resonance Sensing

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Abstract

For the quantitative analysis of progesterone (P4) in liquid media, we developed a simple and highly sensitive immunoassay using a surface plasmon resonance (SPR) biosensor. P4 derivatives with varied linker size were conjugated to ovalbumin (OVA) to form protein conjugates. The conjugates were immobilised on a carboxymethylate dextran coated sensor chip (CM5) via amine coupling and followed by the development of inhibition immunoassays. The sensitivity of each P4-linker-OVA conjugate was investigated using both monoclonal rat antibody (rat Anti-P4) and mouse antibody (mouse Anti-P4). The newly synthesised P4-OEG-OVA ligand showed a dramatic enhancement in response compared to P4-4TP-OVA and P4-4TPH-OVA with rat Anti-P4. Mouse Anti-P4 also enhanced the surface response for all ligands with lower Anti-P4 concentration required. The inhibition assay of P4-OEG-OVA with mouse P4-Anti showed the working range for progesterone to be 0.29 ng/mL - 1.94 ng/mL. In addition, the high stability of the P4-OEG-OVA surface gave consistent antibody binding capability after more than 1000 binding/ regeneration cycles.

Keywords: Surface Plasmon Resonance; progesterone; monoclonal rat and mouse antibody

INTRODUCTION

The surface plasmon resonance (SPR) effect is a charge density oscillation that exists at the interface of two media with dielectric constants of opposite signs. The charge density waves are associated with the electromagnetic wave, the field vectors of

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which reach their maxima at the interface and decay evanescently into both media. The surface plasmon is excited at the interface between the metal film (gold coated sensor chip) and a dielectric medium, and causes a measurable change in refractive index, which can be used for sensing the analyte. The phenomenon was first observed in 1902 by Wood,[1] who observed a pattern of "anomalous" dark and light bands in reflected light when polarized light shone on a mirror with a diffraction grating on the surface. The phenomenon was not completely explained till 1968 by Otto[2] then Kretschmann and Raether.[3] SPR was first applied as a biosensing technique by Liedberg in 1983[4] and since then the user friendly SPR technology has been applied to several practical applications including chemical and biosensing especially real time analysis of biomolecular interactions.[5] The earlier applications of SPR sensing were focused on antigen-antibody interactions.[6] Since then many examples of biomolecular interactions have been studied using SPR including antibody-antigen interactions,[7,8] DNA hybridization,[9] immunoreactivity of antibody conjugates[10] and quantitative immune assays.[11-13]

Progesterone, 4-pregene-3,20-dione also known as P4 is a steroid hormone. Studies have shown that progesterone plays a role in regulating the sequence of the ovulatory cycle by controlling other hormones. Therefore measurement of progesterone is an ideal candidate as a marker for monitoring ovary function. Current methods for progesterone detection in a laboratory are based on immunochemical methods, which focus on the reaction between analyte and analyte specific antibody, and hence are highly specific and sensitive. Techniques such as radio immunoassays (RIAs)[14], and enzyme immunoassays (EIAs) required labelled analyte or antibody. EIA test kits[15] have been developed for onsite progesterone analysis and are also commercially available. The major disadvantage of the onsite EIA test kit is the number of tests available per kit and the costs are relatively high for daily application.

Steroids such as P4 are too small to directly absorb onto a solid phase such as a SPR sensor surface while retaining binding to the antibody. It is common to conjugate the small steroid to a protein for surface immobilisation. One study showed that when P4 is conjugated to the ovalbumin (OVA) with different length linkers P4-4TP-OVA and P4-4TPH-OVA (Figure 1), the sensitivity of the antibody binding is affected by the linker length, as the linker length increased the sensitivity of antibody binding is increased.[16]

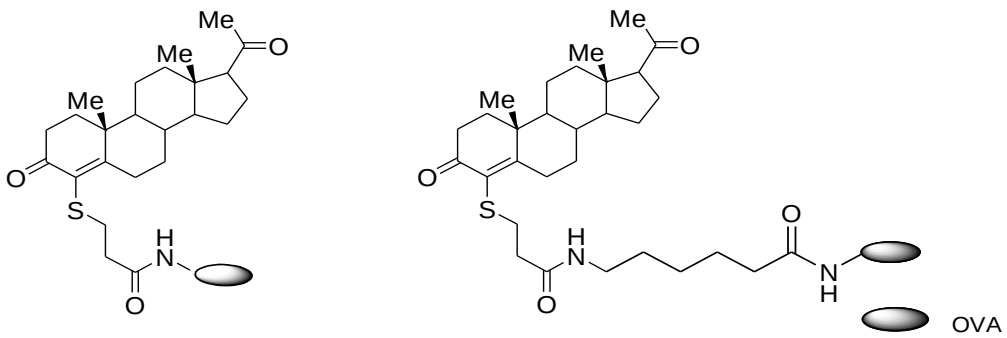


Figure 1. Structure of P4-4TP-OVA and P4-4TPH-OVA

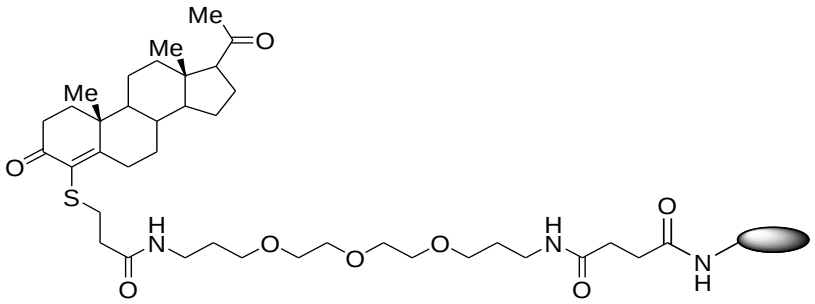


Figure 2. Structure of P4-OEG-OVA

In this present study, we report a rapid and quantitative method for P4 determination in solution with a newly synthesised P4-OEG-OVA (Figure 2) conjugate as binding partner using a surface plasmon resonance immunoassay. A competition assay was carried out using an Anti-P4/P4 mixed solution over the P4-OEG-OVA sensor surface.

EXPERIMENTAL

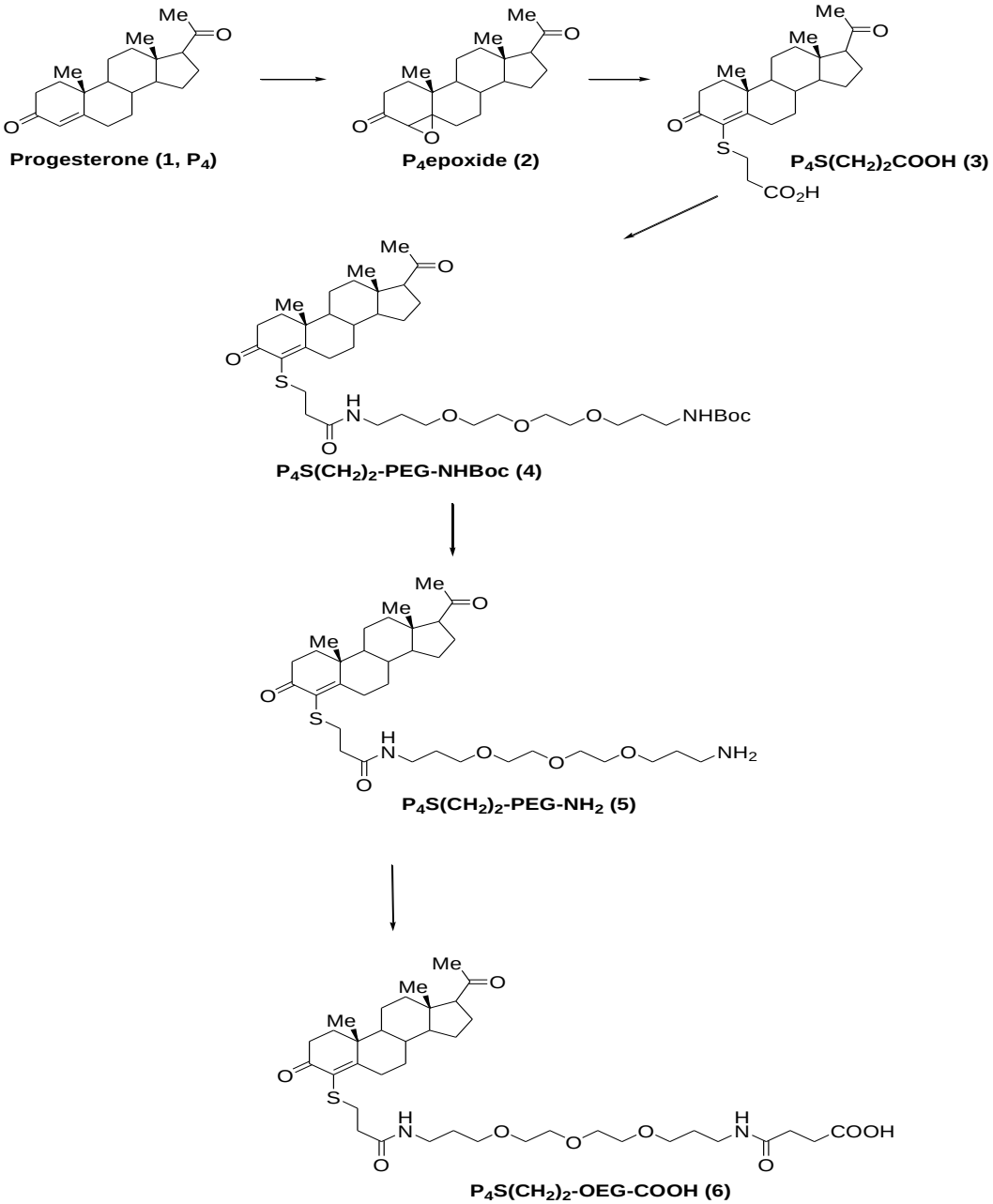
Chemical and instruments

A Biacore X100 system (GE Healthcare Uppsalsa, Sweden) was used to monitor the real-time binding performance of monoclonal P4 antibodies to P4-conjugates. The reagents used for SPR analysis included the CM5 sensor chip, HBS-EP$^+$ running buffer (0.01 M HEPES pH=7.4, 0.15 M NaCl, 3mM EDTA and 0.005% surfactant P20) and these were purchased from GE Healthcare (Uppsala, Sweden). Progesterone (P8793-5G), ovalbumin (A-5503), anti-mouse IgG (whole mouse) antibody produced in a rabbit (M6024), rat monoclonal anti-progesterone antibody (P1922) were from Sigma-Aldrich, and mouse anti-progesterone monoclonal antibody (SE7720-1704) was obtained from Abd Serotech (Oxford, UK).

All reactions were conducted under an argon atmosphere unless otherwise noted. Reagents were obtained from commercial suppliers and used directly with the following exceptions. N,N-Dimethylformamide was distilled and stored over 4 A^o molecular sieves under argon. Triethylamine was dried and distilled from CaH_2 and stored over KOH pellets. Flash chromatography was performed using Scharlau 60 silica gel (230-400 mesh) with the indicated solvents. Thin layer chromatography (TLC) was carried out on precoated silica plates (Merck Kieselgel 60 F_{254}) and compounds were visualized by UV fluorescence or by staining with 10% concentrated sulphuric acid in methanol and heating. ^1H and ^{13}C NMR spectra were obtained using Bruker 400/500 spectrometer. Chemical shifts for spectra in $CDCl_3$ are given in parts per million (ppm). High resolution mass spectra were recorded using a VG 7070 mass spectrometer operating at nominal accelerating voltage 70eV.

Synthesis of P4-OEG-OVA conjugate

The steps involved in the synthesis of the P4-OEG derivative (**6**) are given in Scheme 1 below. Commercially available progesterone was used as the starting material. Firstly, P4 (**1**) was converted to the P4-epoxide (**2**) and then to P4S$(CH_2)_2$COOH (**3**) following literature procedures[17-19]. Compound **3** was converted to **4** and then to **5** and finally to **6** and these latter three steps are now given and are outlined in Scheme 1. Compounds **2**, **3**, **4**, **5** and **6** have been identified and characterised by NMR and mass spectrometry.



Scheme 1 Synthesis of P4S$(CH_2)_2$OEG-COOH.

Synthesis of P4S$(CH_2)_2$-PEG-NHBoc (4)

A solution of 1,3-dicyclohexylcarbodiimide (DCC, 134 mg, 0.65 mmol, 1.3 eq), and N-hydroxysuccinimide (NHS, 74 mg, 0.065 mmol, 1.3 eq) in N,N-dimethylformamide (DMF, 1.0 mL) were added drop wise to P4S$(CH_2)_2$COOH (**3**, 209 mg, 0.5 mmol, 1.0 eq in 2 mL of DMF) at room temperature under an atmosphere of nitrogen.

The mixture was stirred for 12 hr in the absence of light and the resulting white precipitate was filtered and dried. An aqueous solution of the polyethylene glycol (PEG) and amine derivative (240 mg, 0.75 mmol, 1.5 eq) was added to the active ester in DMF (**3**) followed by triethylamine (250 μL) and stirred under the absence of light for 12 hr. 50 mL of water was added to the mixture, and extracted with dichloromethane (DCM, 20 mL x 3) then washed with cold saturated $NaHCO_3$ (20 mL x 2), water (20 mL x 2) and dried with $MgSO_4$. The solvent was evaporated and the residue was purified by column chromatography eluting with DCM/MeOH 15/1 to afford the product **(4)** as yellow oil (yield 234 mg, 63%).

^{1}H NMR (500 MHz, $CDCl_3$): □□0.62 (s, 3H), 0.93-1.43 (m, 4H), 1.18 (s, 3H), 1.19-1.34 (m, 5H), 1.38 (s, 9H), 1.50-1.79 (m, 4H), 1.84-1.91 (m, 1H), 1.94-2.04 (m, 2H), 2.07 (s, 3H), 2.09-2.18 (m, 2H), 2.31 (t, *J* = 6.9 Hz, 2H), 2.43-2.50 (m, 3H), 2.80 (t, *J* = 6.9 Hz, 2H), 3.15- 3.18 (m, 2H), 3.33 (q, *J* = 6.4 Hz, 2H), 3.45-3.62 (m, 12H), 3.65 (dt, *J* = 3.2, 15.0 Hz, 1H), 5.02 (s, 1H), 6.83 (s, 1H) ppm.

^{13}C NMR (100 MHz, $CDCl_3$): □□13.4, 18.1, 21.1, 22.9, 24.3, 25.0, 25.6, 28.5, 29.1, 30.0, 30.6, 30.7, 31,5, 32.1, 33.9, 34.3, 34.6, 35.3, 36.7, 37.6, 38.6, 41.0, 41.4, 43.9, 54.1, 55.9, 63.9, 69.5, 69.7, 70.2, 70.5, 128.7, 156.1, 171.1, 176.0, 195.4, 209.3 ppm.

HRMS (EI): MH^+, found 721.4456. $C_{39}H_{64}N_2O_8S$ requires 721.4462.

Synthesis of P4S$(CH_2)_2$-PEG-NH_2 (5)

The solution of P4S$(CH_2)_2$-PEG-NHBoc (Boc-protected P4 derivative **4**, 102 mg, 0.14 mmol) was stirred with formic acid (2 mL) for 3 hr at room temperature. The solvent was removed under reduced pressure and dried over to give compound **5** as yellow-orange oil. Compound **5** was used for the next step without further purification.

Synthesis of P4S$(CH_2)_2$-OEG-COOH (6)

Succinic anhydride (17 mg, 0.16 mmol, 1.16 eq) was added to the solution of P4S$(CH_2)_2$-PEG-NH_2 (**5**, 87 mg, 0.14 mmol, 1 eq) in 5 mL of toluene/methanol (4/1) and the mixture refluxed under nitrogen for 1 hr. The solvent was removed and the resulting oil was purified by silica gel chromatography eluting with DCM/MeOH/HCOOH (10/1/0.1) and product **6** formed as yellow-orange oil.

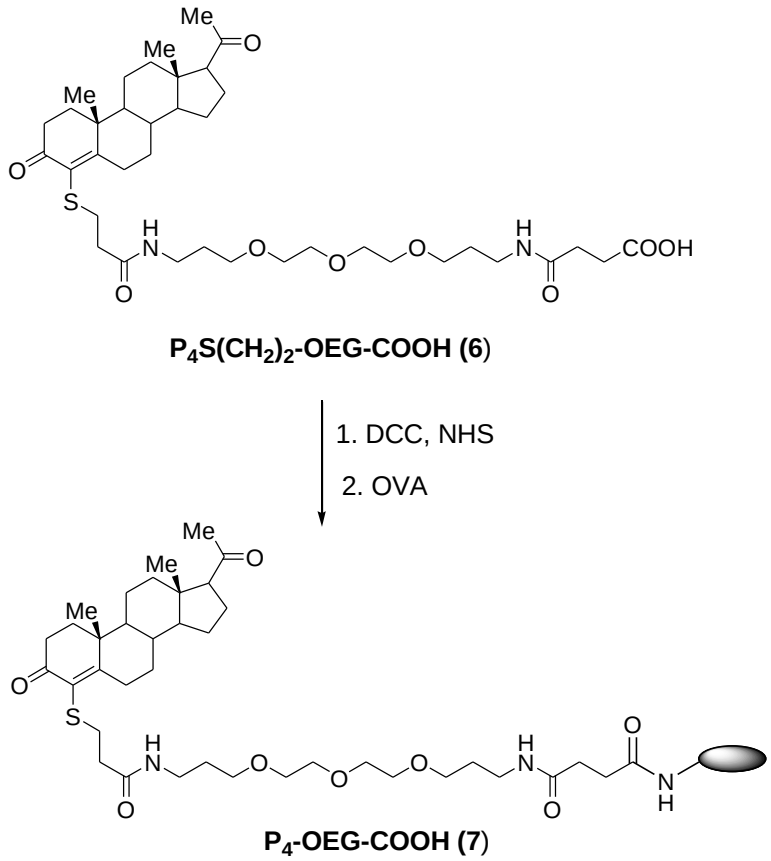
^{1}H NMR (500 MHz, MeOH, d_4): □□0.69 (s, 3H), 0.94-1.14 (m, 4H), 1.21-1.37 (m, 2H), 1.29 (s, 3H), 1.39-1.41 (m, 2H), 1.57-2.01 (m, 11H), 2.05-2.12 (m, 2H), 2.15 (s, 3H), 2.25-2.39 (m, 2H), 2.43-2.76 (m, 5H), 2.87 (t, *J* = 6.7 Hz, 2H), 3.15 (t, *J* = 6.2 Hz, 2H), 3.20 (t, *J* = 6.2 Hz, 2H), 3.32 - 3.34 (m, 1H), 3.43-3.77 (m, 14H), 5.70 (s,

1H), 7.95 (s, 1H) ppm.

^{13}C NMR (100 MHz, MeOH, d_4): □□12.5, 17.04, 20.9, 22.6, 24.0, 24.7, 25.4, 27.6, 28.4, 30.3, 30.5, 31,8, 33.4, 33.9, 34.3, 35.2, 38.4, 41.3, 43.8, 54.1, 55.8, 63.2, 68.2, 69.9, 70.2, 77.7, 77.9, 78.2, 127.4, 158.4, 174.1, 176.7, 176.9, 196.0, 210.8 ppm.

HRMS (EI): MH^+, found 721.3870. $C_{38}H_{60}N_2O_9S$ requires 721.4011.

Conjugation of the P4 derivative (6) to ovalbumin (OVA) to give P4-OEG-OVA (7)



Scheme 2. Synthesis of P4-OEG-OVA (7)

The P4 derivative (compound **6**, 20 mg) was added to a mixture of DCC (1 M, 30 μL), NHS (1 M, 30 μL) in DMF (60 μL) and stirred at room temperature for 2 h. The mixture was added to OVA (20 mg, 0.5 μmol in cold phosphate buffered saline (PBS), and stirred at 4℃ overnight, and was then dialysed with Milli-Q H_2O for 2 days (3 changes per day), then with PBS buffer for 1 day (pH 7.4, 3 changes per day) at 4 ℃. The solution was then purified on a PD10 desalting column at room temperature using PBS buffer as the eluent and the purified conjugate (**7**, 3.5 mL) was collected.

Immobilisation of P4 ligands onto the CM5 surface

The commercially available Biacore CM5 chip was used for the immobilisation of the P4 ligands. Both flow cell one (FC1) and flow cell two (FC2) were activated by mounting the sensor chip within the SPR instrument (Biacore X100). Each flow cell was exposed to 70 μL of a 1:1 (v/v) mixture of EDC (0.4 M) and NHS (0.1 M) at the flow rate of 5 μL/min.

Each P4 ligand was injected after the surface activation process. The P_4 ligand solution was injected at a flow rate of 5 μL/min till the immobilisation level was approximately 6000 response units (RU). A solution of ethanolamine hydrochloride solution (90 μL, 1 M) was injected to deactivate the sensor surface followed by buffer injections to condition the sensor surface. The sensor chip was stored at 4 ℃.

RESULTS AND DISCUSSION

Synthesis of P4-OEG-OVA (7)

The size of the P4 molecule is relatively small; hence it was covalently linked to a polyethylene oxide spacer to reduce steric effects on the sensor surface. The P4 derivatives were synthesised via a new route following the epoxidation method [20], and followed by linker conjugation. In the literature, the enhancement in surface sensitivity was achieved by the extension of the spacer between the steroid and the chemically linked protein such as ovalbumin (OVA).[16] OVA was chosen because the P4 ligand can be linked through the lysine residues on the OVA and it allowed efficient immobilisation of the conjugate on to the SPR sensor surface.

The common approach to linker OVA immobilisation is via the amine on the P4 linker to the carboxylic acid on the OVA. However, in this paper we took the approach of placing the carboxylic acid functional group on the P4 linker, and then conjugating it to the amine residues of OVA. The method allowed control and stable conjugation and resulted in a reasonable yield.

P4-OEG-OVA binding performance on CM5 sensor chip

The binding performance of P4-OEG-OVA was tested after the immobilisation onto a commercially available CM5 sensor chip. The surface was tested and compared to other sensor chips with different P4 derivatives (P4-4TP-OVA and P4-4TPH-OVA) to observe the effect of the elongated linker between the P4 molecule and OVA on the binding performance.

Rat Anti-P4

A competitive P4 assay was developed using rat Anti-progesterone (Anti-P4) based on the conditions used in a previous assay which employed the OVA-P4 conjugate to a CM5 chip.[16] P4-4TP- OVA and P4-4TPH-OVA sensor surfaces were also used and

compared with the P4-OEG-OVA binding sensitivity.

The binding performances for the P4 derivatives clearly indicated that the binding sensitivity was enhanced with increasing length of linker between the P4 molecule and OVA. With 20 µg/mL of the rat antibody solution, the response of P4-4TP-OVA was 87.6 RU whereas with P4-4TPH-OVA it was 145.3 RU, which was a 65% increase in surface response. P4-OEG-OVA had a response of 684.5 RU with 20 µg/mL of antibody which clearly demonstrated that with a long linker between the P4 molecule and the protein, the binding performance significantly increased (Figure). The effect of the length of the linker on antibody performance is clearly noticeable and the magnitude of the enhancement has not been observed in previous studies in the literature.

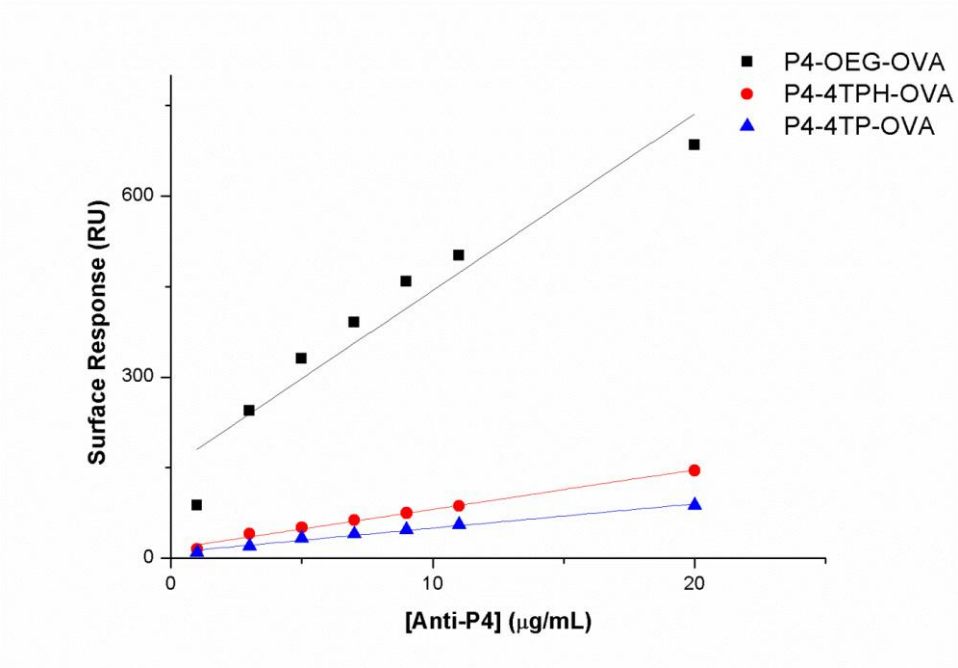


Figure 3. Rat Anti-P4 binding curves of P4-4TP-OVA, P4-4TPH-OVA, P4-OEG-OVA conjugates. Binding performance of P4 derivatives increased as the linker length increased.

Mouse Anti-P4

The sensitivity of an assay can be enhanced by varying the assay components such as the antibodies. Mouse Anti-P4 was tested and compared with Rat Anti-P4 in order to maximise the assay performance. The surface response from mouse Anti-P4 followed a similar trend compared with the rat Anti-P4. The P4-4TP-OVA surface generated the lowest surface response compared with the P4-4TPH-OVA and P4-OEG-OVA surfaces. The effect of linker length on surface response was still significant (Figure 4).

In comparison with rat Anti-P4, the results indicated that mouse Anti-P4 more actively enhanced the surface sensitivity for all tested P4 ligands on a CM5 sensor chip. Hence, mouse Anti-P4 was used for inhibition assay development.

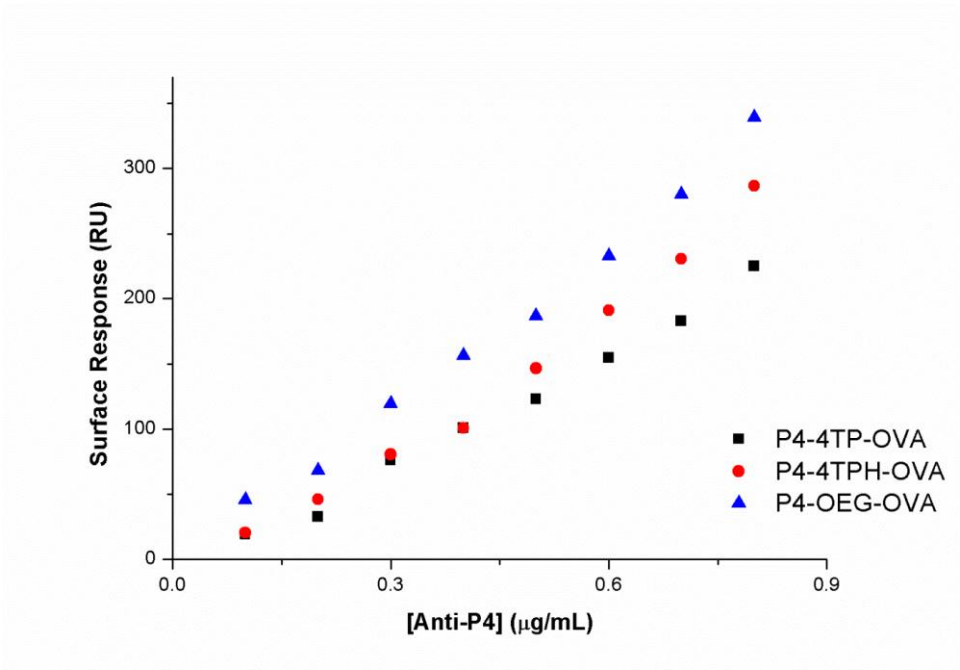


Figure 4. Mouse Anti-P4 binding curves of P4-4TP-OVA, P4-4TPH-OVA, P4-OEG-OVA conjugates.

Inhibition assay developed with mouse Anti-P4

The inhibition assay [21, 22] for each P4 sensor surface was developed using mouse Anti-P4. The observed binding curve for each P4 derivative was used to carry out P4 assay development. The P4 standard curve for each P4 derivative was established using the inhibition assay method. The result indicated that the P4-OEG-OVA sensor surface has a half maximal effective concentration (EC50) of 0.7561 ng/mL. After each inhibition assay, the sensor surface required complete surface regeneration to optimise the binding stability.

The long hydrophilic linker is prone to extend out to the solution as the assay is performed and is also suspected of being folded after a high concentration of P4 solution was injected. This resulted in the inability to regenerate the sensor surface after 20 injections of buffer.

The surface responses were plotted versus the standard concentrations and the dose response curve (sigmoidal fitting) was fitted. A sigmoidal fitting is commonly used to analyse dose response relationship, and competitive binding assays (the competition of ligand for receptor binding). The half maximal effective concentration (EC50), the lowest concentration that can be distinguished from the background noise (EC20) and the highest concentration that can be distinguished from the background noise (EC80)

were determined from the standard curve.

The EC50 of the standard curve was used to monitor the sensitivity and the indication of the most accurate working area of an assay.

The P4 standard curves for all three linkers were established with mouse Anti-P4 (Figure) and it was clear that P4-OEG-OVA gave a greater surface response range (from 0 to approximate 20 RU) when the same P4 standard solutions were used.

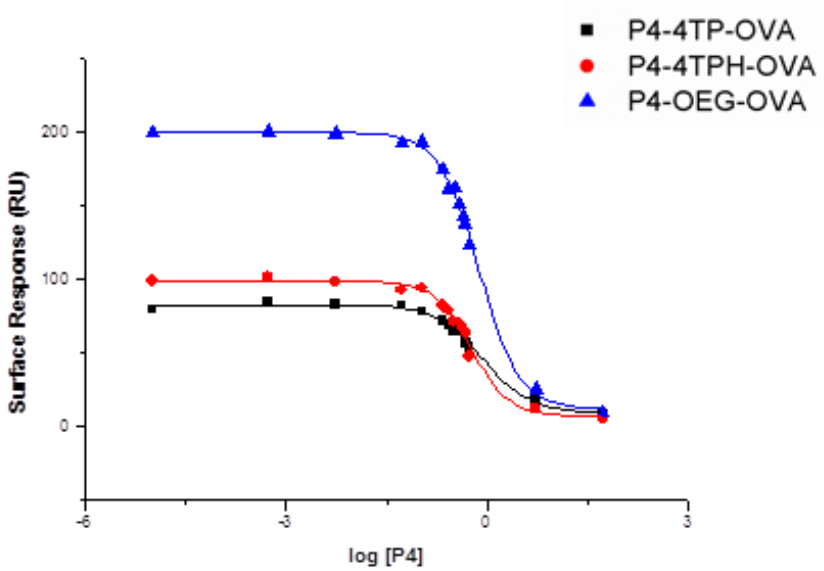


Figure 5. Mouse Anti-P4 standard curves of P4-4TP-OVA, P4-4TPH-OVA, and P4-OEG-OVA conjugates.

The lower EC50 value is desirable for the assay development. Hence, theoretically P4-4TPH-OVA would be a better surface than P4-OEG-OVA. However, the P4-4TPH-OVA also has a very low EC20 value (0.73 ng/mL), hence it was not suitable to compare with the other two linkers (

Table **1**). Although the P4-OEG-OVA surface has a lower EC20 value compared with the P4-4TP-OVA surface, it has a lower EC50 value. Hence the P4-OEG-OVA linker surface was more desirable compared to the two short linker surfaces and for sample analysis tests.

Table 1. Summary of EC20, EC50 and EC80 for each sensor surface

	P4-4TP-OVA	P4-4TPH-OVA	P4-OEG-OVA
EC80 (ng/mL)	0.27	0.38	0.29
EC50 (ng/mL)	0.80	0.53	0.76

EC20 (ng/mL)	2.38	0.73	1.94

CONCLUSION

In this study, the results showed that the linker length between the P4 molecule and OVA is an important feature for a SPR based sensor. The P4-linker-OVA system clearly demonstrated that as the linker size between the P4 molecule and protein (OVA) increased the surface response increased. P4-4TP-OVA and P4-4TPH-OVA sensor surfaces behaved similarly to the previous study[16], as the length of the linker increased the surface response increased by 65% at 20 µg/mL. The newly synthesised P4-OEG-OVA ligand showed dramatic increase in sensitivity than the two shorter linkers, which is desirable for assay development. Mouse Anti-P4 also enhanced the performance of the surface responses for all sensor surfaces. The mouse Anti-P4 showed stability and better sensitivity than rat Anti-P4, hence it is essential to optimise the antibody binding condition to achieve a more cost effective analysis method. The inhibition assays indicated the P4-OEG-OVA sensor surface is suitable for the detection of low concentrations (ng/mL) of P4,the working range being 0.29 ng/mL to 1.94 ng/mL. The development of this immunoassay using a SPR biosensor has been shown to be highly sensitive for the quantitative analysis of progesterone.

ACKNOWLEDGMENT

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REFERENCES

[1] Wood, R., 1902, "On a Remarkable Case of Uneven Distribution of Light in a Diffraction Grating Spectrum ," *Philosophical Magazine* , 4, pp. 396-402.

[2] Otto, A., 1968, "Excitation of Nonradiative Surface Plasma Waves in Silver by the Method of Frustrated Total Reflection," *Zeitschrift fuer Physik*, 216, pp. 398-410.

[3] Kretschmann, E., 1971, "Determination of the Optical Constants of Metals by Excitation of Surface Plasmons," *Zeitschrift fuer Physik* , 241, pp. 313-324.

[4] Liedberg, B., Nylander, C., and Lunstrom, I., 1983, "Surface Plasmon Resonance for Gas Detection and Biosensing," *Sensors and Actuators*, 4, pp. 299-304.

[5] Phizicky, E. M., and Fields, S., 1995, "Protein–Protein Interactions: Methods for Detection and Analysis," *Microbiological Review* , 59, pp. 94–123.

[6] Luo, J., Zhuo, J., Zou, W., and Shen, P., 2001, "Antibody-Antigen Interactions Measured by Surface Plasmon Resonance: Global Fitting of Numerical Integration Algorithms," *Journal of Biochemistry*, 130, pp. 553–559.

[7] Kaganer, E., Pogreb, R., Davidov, D., and Willner, I., 1999, "Surface Plasmon Resonance Characterization of Photoswitchable Antigen-Antibody

Interactions," *Langmuir* , 15, pp. 3920-3923.

[8] Lipschultz, C. A., Li, Y., and Smith-Gill, S., 2000, "Experimental Design for Analysis of Complex Kinetics using Surface Plasmon Resonance," *Methods, (Orlando, Florida)* 20, pp. 310-318.

[9] Thiel, A. J., Frutos, A. G., Jordan, C. E., Corn, R. M., and Smith, L. M., 1997, "In situ Surface Plasmon Resonance Imaging Detection of DNA Hybridization to Oligonucleotide Arrays on Gold Surfaces," *Analytical Chemistry*, 69, pp. 4948-4956.

[10] Adamczyk, M., Mattingly, P. G., Shreder, K., and Yu, S., 1999, "Surface Plasmon Resonance (SPR) as a Tool for Antibody Conjugate Analysis," *Bioconjugate Chemistry*, 10, pp. 1032-1037.

[11] Laricchia-Robbio, L., Balzan, S., Ghione, S., Montali, U., and Revoltella, R. P., 1998, "Detection of Digitalis Compounds using Surface Plasmon Resonance-Based Biosensor," *Biosensors & Bioelectronics*, 13, pp. 1055-1060.

[12] Mullett, W., Lai, E. P., and Yeung, J. M., 1998, "Immunoassay of Fumonisins by a Surface Plasmon Resonance Biosensor," *Analytical Biochemistry*, 258, pp. 161-167.

[13] Mullett, W. M., Lai, E. P. C., and Yeung, J. M., 2000, "Surface Plasmon Resonance-Based Immunoassays," *Methods (Orlando, Florida)* 22, pp. 77-91.

[14] Pulley, S. L., Wallace, L. D., Mellieon H. I. Jr., and Stevenson, J.S., 2013, "Ovarian Characteristics, Serum Concentrations of Progesterone and Estradiol, and Fertility in Lactating Dairy Cows in Response to Equine Chorionic Gonadotropin," *Theriogenology* , 79, pp. 127-134.

[15] Nebel, R. L., Altemose, D. L., Munkittrick, T. W., Sprecher, D. J., and McGilliard, M. L., 1989, "Comparisons of Eight Commercial On-Farm Milk Progesterone Tests," *Theriogenology* , 31, pp. 753-764.

[16] Wu, Y. Q., Mitchell, J., Cook, C., and Main, L., 2002, "Evaluation of Progesterone-Ovalbumin Conjugates with Different Length Linkers in Enzyme-Linked Immunosorbant Assay and Surface Plasmon Resonance-Based Immunoassay," *Steroids*, 67, pp. 565-572.

[17] Hosoda, H., Miyairi, S., and Nambara, T., 1980, "Studies on Steroids. Part CLV. Synthesis of Corticosteroid Haptens Possessing the Bridge at the C-4 Position," *Chemical & Pharmaceutical Bulletin*, 28, pp. 1294-1299.

[18] Yoshida, H., Nakai, S., Nimbari, F., Yoshimoto, K., and Nishimura, T., 1989, "Synthesis of 11 Alpha-Hydroxyprogesterone Haptens," *Steroids*, 53, pp. 727-738.

[19] Hosoda, H., Ushioda, K., Shioya, H., and Nambara, T., 1982, "Studies on Steroids. CLXXIV. Synthesis of Haptens for use in Immunoassays of Δ4-3-Keto Steroids," *Chemical & Pharmaceutical Bulletin*, 30, pp. 202-205.

[20] Abul-Hajj, Y. J., 1986, "Synthesis and Evaluation of 4-(Substituted thio)-4-Androstene-3,17-Dione Derivatives as Potential Aromatase Inhibitors," *Journal of Medicinal Chemistry*, 29, pp. 82-584.

[21] Haughey, S. A., Campbell, K., Yakes, B. J., Prezioso, S. M., DeGrasse, S. L., Kawatsu, K., and Elliot, C., 2011, "Comparison of Biosensor Platforms for Surface Plasmon Resonance Based Detection of Paralytic Shellfish Toxins," *Talanta*, 85, pp. 519-526.

[22] Fonfria, E. S., Vilarino, N., Campbell, K., Elliot, C., Haughey, S. A., Ben-Gigirey, B., Vieites, J. M., Kawatsu, K., and Botana, L. M., 2007, "Paralytic Shellfish Poisoning Detection by Surface Plasmon Resonance-Based Biosensors in Shellfish Matrices," *Analytical Chemistry*, 79, pp. 6303-6311.