A Study on Progesterone Sensitive Determination 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: Progesterone, Monoclonal Rat and Mouse Antibody, Surface Plasmon Resonance

INTRODUCTION

An oscillation in charge density that occurs at the interface of two mediums with dielectric constants of opposing signs is known as the surface plasmon resonance (SPR) phenomenon. The electromagnetic wave, whose field vectors peak at the interface and evanescently fade into both mediums, is linked to the charge density waves.

The analyte may be detected by measuring the change in refractive index caused by the surface plasmon, which is excited at the interface between the metal film (gold-coated sensor chip) and a dielectric medium. When polarized light shined on a mirror with a diffraction grating on the surface, Wood1 saw a pattern of "anomalous" dark and bright bands in the reflected light. This was the first observation of the phenomenon. Otto, then Kretschmann, and Raether did not fully explain the phenomena until 1968. The user-friendly SPR technology has been used for a number of real-world

applications, such as chemical and biosensing, particularly real-time monitoring of biomolecular interactions, since it was first used as a biosensing approach by Lindelberg in 1983. Antigen-antibody interactions were the main focus of the early SPR sensing applications. Since then, SPR has been used to study a wide range of biomolecular interactions, including as antibody-antigen interactions, DNA hybridization, antibody conjugate immunoreactivity10, and quantitative immunological tests.

P4 is another name for the steroid hormone progesterone, 4-pregene-3,20-dione. Research has shown that progesterone regulates other hormones, which in turn helps to manage the ovulatory cycle's sequence. Progesterone measurement is thus a prime choice for an indicator of ovarian function. The current laboratory techniques for detecting progesterone are based on immunochemical techniques, which are very sensitive and specific because they concentrate on the interaction between the analyte and analyte-specific antibody. Labeled analyte or antibody was necessary for methods like radio immunoassays (RIAs) and enzyme immunoassays (EIAs). EIA test kits are commercially available and have been developed for on-site progesterone analysis. The number of tests offered per kit and the comparatively expensive cost for daily use are the main drawbacks of the onsite EIA test kit.

Steroids like P4 are too tiny to attach to the antibody and then immediately absorb onto a solid phase, like the surface of an SPR sensor. For surface immobilization, the tiny steroid is often conjugated to a protein. According to one research, the sensitivity of antibody binding is influenced by the linker length when P4 is conjugated to ovalbumin (OVA) with linkers of varying lengths, P4-4TP-OVA and P4-4TPH-OVA (Figure 1); the longer the linker, the higher the sensitivity of antibody binding.

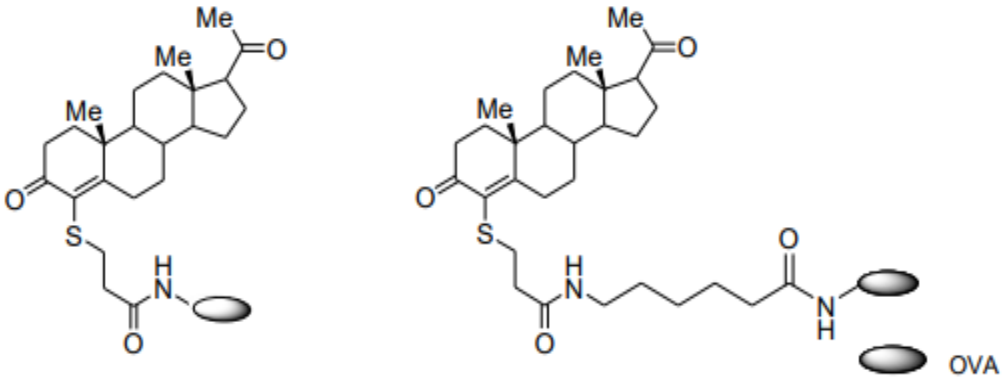


Figure 1. Structure of P4-4TP-OVA and P4-4TPH-OVA

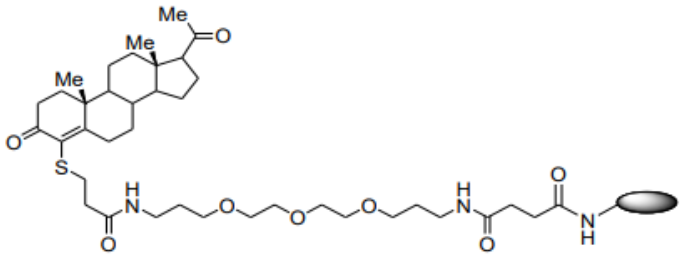


Figure 2. Structure of P4-OEG-OVA

Using a surface plasmon resonance immunoassay, we offer a quick and quantitative technique for determining P4 in solution using a recently synthesized P4-OEG-OVA (Figure 2) conjugate as a binding partner. An Anti-P4/P4 mixed solution was applied to the P4-OEG-OVA sensor surface in a competitive experiment.

EXPERIMENTAL

Instruments and chemicals

GE Healthcare Uppsalsa, Biacore X100 system was used to track the binding performance of monoclonal P4 antibodies to P4-conjugates in real time. 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 other materials were acquired from GE Healthcare (Uppsala) and used for SPR analysis. Rat monoclonal anti-progesterone antibody (P1922), mouse anti-progesterone monoclonal antibody (SE7720-1704) was acquired from Abd Serotech (Oxford, UK), progesterone (P8793-5G), ovalbumin (A-5503), and anti-mouse IgG (whole mouse) antibody generated in a rabbit (M6024) were all supplied by Sigma-Aldrich. Unless otherwise specified, all reactions were carried out in an argon environment.

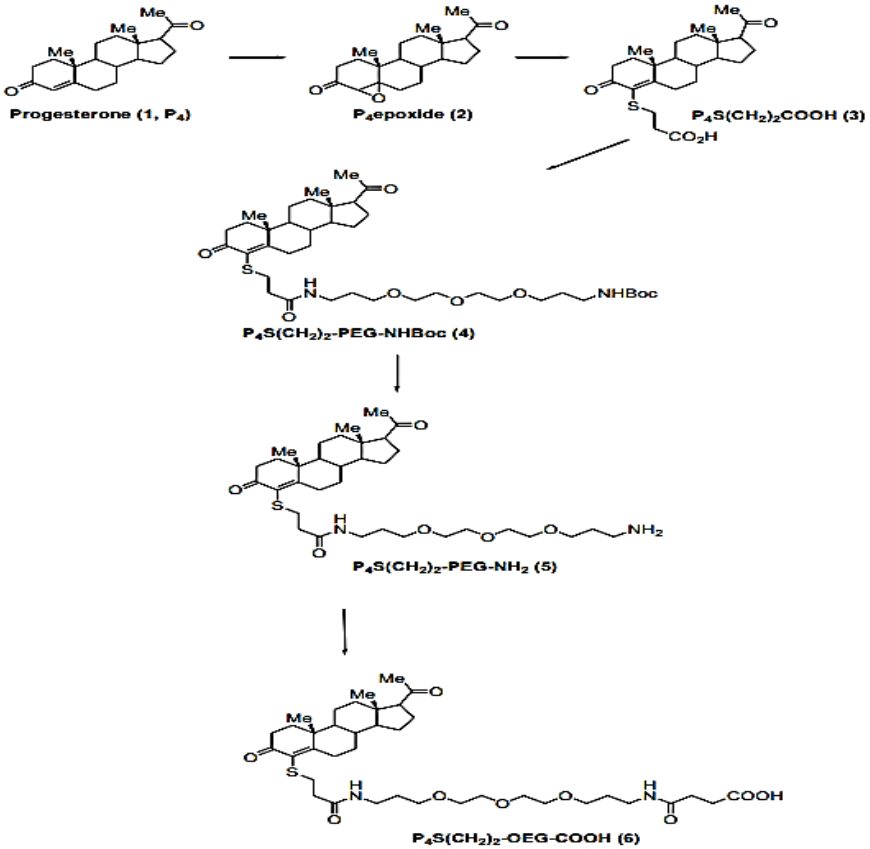
With the following exclusions, reagents were utilized directly and acquired from commercial providers. After distillation, N,N-Dimethylformamide was kept in argon-filled storage over four Ao molecular sieves. After being dried and distilled from CaH2, triethylamine was kept on top of KOH pellets. Using the specified solvents and Charlau 60 silica gel (230–400 mesh), flash chromatography was carried out. Precoated silica plates (Merck Kieselgel 60 F254) were used for thin layer chromatography (TLC), and chemicals were detected by UV fluorescence or staining with 10% concentrated sulfuric acid in methanol and heating. The Bruker 400/500 spectrometer was used to produce the 1H and 13C NMR spectra. Parts per million (ppm) are used to express chemical shifts for spectra in CDCl3. A VG 7070 mass spectrometer running at nominal accelerating voltage 70eV was used to capture high resolution mass spectra.

P4-OEG-OVA conjugate synthesis

Scheme 1 below lists the procedures needed to synthesize the P4-OEG derivative (6). The beginning substance was progesterone that was sold commercially.

Following the methods described in the literature, P4 (1) was first changed into the P4-epoxide (2) and subsequently into P4S(CH2)2COOH (3). Compound 3 was changed to 4, then to 5, and ultimately to 6. Scheme 1 now provides the details of these last three phases.

NMR and mass spectrometry have been used to identify and characterize compounds 2, 3, 4, 5, and 6.



Scheme 1 Synthesis of P4S(CH2)2OEG-COOH.

P4S(CH2)2-PEG-NHBoc (4) Synthesis

Drop by drop, P4S(CH2)2COOH (3,209 mg, 0.5 mmol, 1.0 eq in 2 mL of DMF) was treated with 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,Ndimethylformamide (DMF, 1.0 mL) at room temperature in a nitrogen atmosphere.

After 12 hours of stirring without light, the mixture's white precipitate was filtered and allowed to dry. The active ester in DMF (3) was mixed with an aqueous solution of polyethylene glycol (PEG) and amine derivative (240 mg, 0.75 mmol, 1.5 eq), then triethylamine (250 µL), and the mixture was swirled for 12 hours without light. After adding 50 milliliters of water, the mixture was extracted using dichloromethane (DCM, 20 milliliters x 3), rinsed with cold saturated NaHCO3 (20 milliliters x 2), water (20 milliliters x 2), and dried with magnesium sulfate. To get product (4) as yellow oil (yield 234 mg, 63%), the solvent was removed and the residue was refined using column chromatography eluting with DCM/MeOH 15/1.

1.62 (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), 3.45-3.62 (m, 12H), 3.65 (dt, J = 3.2, 15.0 Hz, 1H), 5.02 (s, 1H), 6.83 (s, 1H) 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. 13C NMR (100 MHz, CDCl3).

MH+, detected 721.4456 in HRMS (EI). 721.4462 is needed for C39H64N2O8S.

P4S(CH2)2-PEG-NH2 Synthesis (5)

For three hours at room temperature, the P4S(CH2)2-PEG-NHBoc (Boc-protected P4 derivative 4, 102 mg, 0.14 mmol) solution was agitated with two milliliters of formic acid. Compound 5 was obtained as yellow-orange oil when the solvent was extracted at lower pressure and dried. Without further purification, Compound 5 was used for the next step.

P4S(CH2)2-OEG-COOH (6) Synthesis

The solution of P4S(CH2)2-PEG-NH2 (5, 87 mg, 0.14 mmol, 1 eq) in 5 mL of toluene/methanol (4/1) was mixed with succinic anhydride (17 mg, 0.16 mmol, 1.16 eq), and the mixture was refluxed under nitrogen for one hour. Product 6 produced as yellow-orange oil after the solvent was eliminated and the resultant oil was refined using silica gel chromatography eluting with DCM/MeOH/HCOOH (10/1/0.1).

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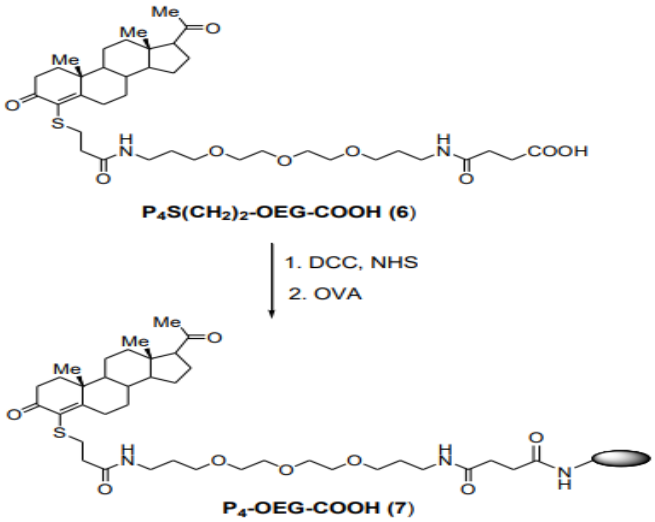
1H NMR (500 MHz, MeOH, d4): 0.69 (s, 3H), 0.94-1.14 (m, 4H), 1.21-1.37 (m, 2H), 1.29 (s, 3H), 1.39-1.41 (m, 2H), 1.57-2.01 (m, 11H), 2.05-2.12 (m, 2H), 2.15 (s, 3H), 2.25-2.39 (m, 5H), 2.87 (t, J = 6.7 Hz, 2H), 3.15 (t, J = 6.2 Hz, 2H), 3.20 (t, J = 6.2 Hz, 2H), 3.32 - 3.34 (m, 1H), 3.43-3.77 (m, 14H), 5.70 (s, 1H), 7.95 (s, 1H) ppm.

13.5, 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 are the values of 13C NMR (100 MHz, MeOH, d4).

HRMS (EI): MH+, 721.3870, but 721.4011 is needed for C38H60N2O9S.

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



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

A combination of DCC (1 M, 30 µL), NHS (1 M, 30 µL) in DMF (60 µL) was mixed with the P4 derivative (compound 6, 20 mg) and left to stir for two hours at room temperature. Before being dialyzed with Milli-Q H2O for two days (3 changes per day) and PBS buffer for one day (pH 7.4, 3 changes per day) at 4 °C, the mixture was added to OVA (20 mg, 0.5 µmol in cold phosphate buffered saline (PBS) and stirred overnight at 4 °C. The purified conjugate (7, 3.5 mL) was then collected after the solution was purified at room temperature using PBS buffer as the eluent on a PD10 desalting column.

P4 ligand immobilization on the CM5 surface

The P4 ligands were immobilized using the commercially available Biacore CM5 device. Mounting the sensor chip inside the SPR device (Biacore X100) activated both flow cells one (FC1) and two (FC2). At a flow rate of 5 µL/min, 70 µL of a 1:1 (v/v) combination of NHS (0.1 M) and EDC (0.4 M) were introduced into each flow cell.

Following surface activation, each P4 ligand was administered. At a flow rate of 5 µL/min, the P4 ligand solution was administered until the immobilization level reached around 6000 response units (RU). The sensor surface was deactivated by injecting a 90 µL, 1 M solution of ethanolamine hydrochloride, and then it was conditioned by injecting buffer. At 4 °C, the sensor chip was kept in storage.

RESULTS AND DISCUSSION

P4-OEG-OVA Synthesis (7)

Because of its tiny size, the P4 molecule was covalently bonded to a polyethylene oxide spacer in order to lessen steric effects on the sensor surface. The epoxidation procedure 20 was followed by linker conjugation in a novel way to synthesize the P4 derivatives. According to the literature, extending the spacer between the steroid and the chemically related protein, such ovalbumin (OVA), increased surface sensitivity. 16 OVA was selected because to its ability to connect the P4 ligand via its lysine residues, which also enabled effective conjugate immobilization on the SPR sensor surface.

The amine on the P4 linker to the carboxylic acid on the OVA is the typical method for linker OVA immobilization. The method we used in our work, however, was to attach the carboxylic acid functional group to the P4 linker and then conjugate it to the OVA amine residues. The technique produced a respectable yield and permitted control and stable conjugation.

Performance of P4-OEG-OVA binding on CM5 sensor chip

Following immobilization, P4-OEG-OVA's binding performance was evaluated on a CM5 sensor chip that is sold commercially. The impact of the extended linker between the P4 molecule and OVA on the binding performance was examined by testing the surface and comparing it to other sensor chips with distinct P4 derivatives (P4-4TP-OVA and P4-4TPH-OVA).

Rat Anti-P4

Based on the parameters of an earlier assay that utilized the OVA-P4 conjugate to a CM5 chip, a competitive P4 assay was created utilizing rat anti-progesterone (Anti-P4). 16 The P4-OEG-OVA binding sensitivity was compared with the P4-4TP-OVA and P4-4TPH-OVA sensor surfaces.

The P4 derivatives' binding performances made it abundantly evident that the longer the linker

between the P4 molecule and OVA, the higher the binding sensitivity. The reaction of P4-4TP-OVA was 87.6 RU with 20 µg/mL of the rat antibody solution, whereas the response of P4-4TPH-OVA was 145.3 RU, indicating a 65% increase in surface response. With 20 µg/mL of antibody, P4-OEG-OVA showed a response of 684.5 RU, indicating that a lengthy linker between the P4 molecule and the protein greatly improved binding effectiveness (Figure). The impact of the linker's length on antibody performance is evident, and prior research in the literature has not noted the extent of the improvement.

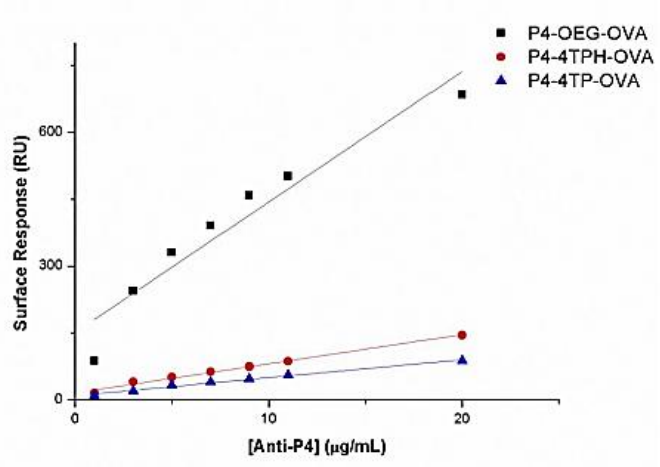


Figure 3. Rat Anti-P4 binding curves of P4-4TP-OVA, P4-4TPH-OVA, P4-OEGOVA conjugates. Binding performance of P4 derivatives increased as the linker length increased.

Anti-P4 Mouse

By changing the assay's constituent parts, such the antibodies, the sensitivity of the test may be improved. To optimize the assay performance, mouse anti-P4 was evaluated and contrasted with rat anti-P4. In comparison to the rat Anti-P4, the mouse Anti-P4 surface reaction showed a comparable pattern. When compared to the P4-4TPH-OVA and P4-OEG-OVA surfaces, the P4-4TP-OVA surface produced the least amount of surface response. Linker length continued to have a substantial impact on surface reaction (Figure 4).

The findings showed that mouse Anti-P4 more actively increased the surface sensitivity for all tested P4 ligands on a CM5 sensor chip when compared to rat Anti-P4. Therefore, mouse Anti-P4 was used to establish the inhibitory experiment.

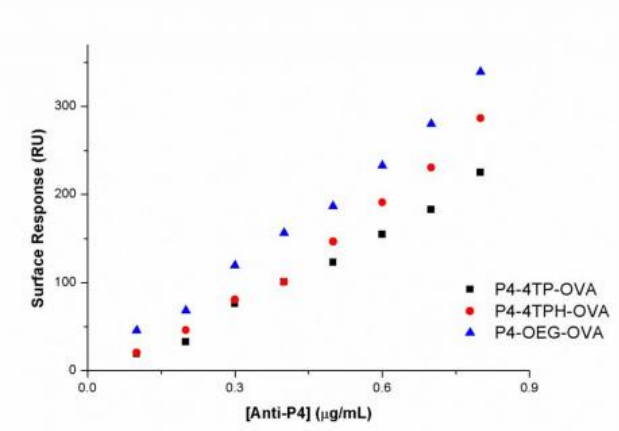


Figure 4. Mouse Anti-P4 binding curves of P4-4TP-OVA, P4-4TPH-OVA, P4-OEGOVA conjugates.

Assay for inhibition created using mouse anti-P4

Mouse AntiP4 was used to create the inhibition assays 21, 22 for each P4 sensor surface. The P4 test was developed using the observed binding curve for each P4 derivative. For every P4 derivative, the P4 standard curve was created using the inhibition assay technique. The findings showed that the half maximum effective concentration (EC50) of the P4-OEG-OVA sensor surface is 0.7561 ng/mL. To maximize the binding stability after each inhibition test, the sensor surface has to be completely regenerated.

The lengthy hydrophilic linker is likely to fold once a high concentration of P4 solution was injected, and it is also likely to stretch out to the solution throughout the experiment. As a consequence, after 20 buffer injections, the sensor surface could not be renewed.

The dose response curve (sigmoidal fitting) was fitted after the surface responses were plotted against the standard concentrations. To analyze dose response relationships and competitive binding tests (the competition of ligand for receptor binding), sigmoidal fitting is often used. The standard curve was used to calculate the half maximal effective concentration (EC50), the lowest concentration that can be differentiated from the background noise (EC20), and the maximum concentration that can be separated from the background noise (EC80).

The sensitivity and indication of an assay's most accurate working region were tracked using the EC50 of the standard curve.

Mouse Anti-P4 was utilized to create the P4 standard curves for all three linkers (Figure), and it was

evident that P4-OEG-OVA provided a larger surface response range (from 0 to around 20 RU) when the identical P4 standard solutions were used.

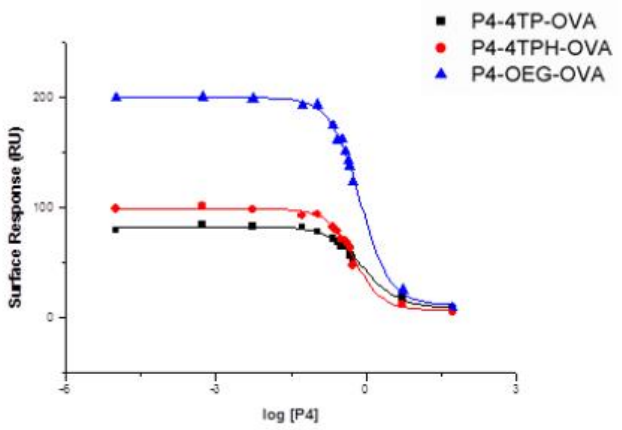


Figure 5. Mouse Anti-P4 standard curves of P4-4TP-OVA, P4-4TPH-OVA, and P4- OEG-OVA conjugates.

For the development of the test, a lower EC50 value is preferred. Therefore, in theory, P4-4TPH-OVA would be a superior surface than P4-OEG-OVA. Nevertheless, the P4- 4TPH-OVA was not appropriate for comparison with the other two linkers due to its very low EC20 value (0.73 ng/mL) (Table 1). In contrast to the P4-4TP-OVA surface, the P4-OEG-OVA surface exhibits a lower EC50 value while having a lower EC20 value. For sample analysis testing, the P4-OEG-OVA linker surface was thus preferred over the two short linker surfaces.

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

Sensor Surface	EC80 (ng/mL)	EC50 (ng/mL)	EC20 (ng/mL)
P4-4TP-OVA	0.27	0.8	2.38
P4-4TPH-OVA	0.38	0.53	0.73
P4-OEG-OVA	0.29	0.76	1.94

Conclusion

The findings of this investigation demonstrated that a crucial component of an SPR-based sensor is the linker length between the P4 molecule and OVA. The P4-linker-OVA system made it abundantly evident that the surface response rose in proportion to the linker size between the P4 molecule and protein (OVA). The P4-4TP-OVA and P4-4TPH-OVA sensor surfaces exhibited comparable behavior to the earlier study16; at 20 μg/mL, the surface response rose by 65% as the linker length increased. Compared to the two shorter linkers, the newly synthesized P4-OEG-OVA ligand shown a sharp improvement in sensitivity, which is ideal for assay development. Additionally, Mouse Anti-P4 improved surface response performance across all sensor surfaces. Since mouse anti-P4 demonstrated greater stability and sensitivity than rat anti-P4, it is essential to optimize the antibody binding condition in order to get a more economical analytical technique. The P4-OEG-OVA sensor surface is appropriate for the detection of low concentrations (ng/mL) of P4, with a working range of 0.29 ng/mL to 1.94 ng/mL, according to the inhibition tests. It has been shown that this immunoassay, which uses an SPR biosensor, is very sensitive for the quantitative measurement of progesterone.

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