Investigating a Functionalized Terthiophene Surface for the Detection of Progesterone Using Surface Plasmon Resonance

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***Abstract*—Herein we report a rapid and sensitive surface Plasmon resonance (SPR) biosensor to detect progesterone (P_4), a model analyte of small molecules. A terthiophene scaffold with an oligoethylene glycol (OEG) linker is used as an anchor for the P_4-ovalbumin (OVA) immobilization on gold to form a stable, regenerable SPR surface. Subsequently, inhibition SPR immunoassays based on this terthiophene/OVA-P_4 surface have demonstrated that P_4 limit of detection (LOD) in buffer solution was 0.13 ng ml^{-1}. Due to the rapidity of each measurement (less than 5 min per measurement), and the high sensitivity of the assay, we believe the sensing platform may hold great potential for P_4 detections.**

***Index Terms*—terthiophene, progesterone, surface plasmon resonance, biosensor**

I. Introduction

There is a current need for the reliable, rapid and sensitive detections of trace amounts of small molecules (<2kDa) in the area of food safety screening for drug residues, detection of biological toxins, environmental applications, and biomedical analyses. This is particularly true of the steroids, a major class of small molecule hormone such as progesterone. Progesterone (P_4) is a steroidal hormone which is directly involved in the reproductive pathways of many animals, including cows. Its presence in bovine milk is a well-studied indicator of the bovine estrous cycles [1]. The current method used to detect P_4 is an enzyme-linked immunosorbent assay (ELISA), and although ELISAs display high sensitivity, they are time-consuming, labor-intensive and can require relatively expensive reagents. Being able to rapidly and sensitively detect P_4 would be of great use to the agricultural community.

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A typical range of concentrations for P_4 testing is between 0 and 50ng/ml. This range is chosen because during the normal bovine estrous cycle P_4 values of above 4ng/ml indicate luteal activity [2]. Being able to identify concentrations above 4ng/ml P_4 from basal levels would therefore be an indication of where in the cycle the cow currently sits. In turn, predictions of this will allow more accurate timings, reducing the cost of incorrectly timed inseminations and promoting successful pregnancies.

The sensing platform for P_4 is often a competitive assay [3]-[6], with a surface bound progesterone sensor. A direct sensing method is not desirable for P_4, due to the small size of the hormone and hence the small sensor response it elicits. There are examples of alternative assays, but these often require labeling [7], [8]. Additionally using antibodies at the sensor surface can be detrimentally affected by the poor stability of antibodies [6].

Surface Plasmon Resonance (SPR) is a surface-sensitive optical technique whereby the Plasmon produces optical electric fields which are localized approximately 250nm out from the sensor surface. Its resonance condition is extremely sensitive to the Refractive Index (RU) of the analyte sample in contact with a gold surface. Therefore, SPR not only has potential to provide fast, sensitive, portable and cost-effective analytical methods, but also can serve as an excellent screening tool for development of other biosensors. Effective fabrication to functionalize the gold sensing surface for immobilizing biological materials such as antibodies is crucial in designing SPR biosensors. This is usually accomplished by covalent binding *via* self-assemble monolayers (SAMs) [5] or using gold-binding polypeptides (GBP) [9] and protein G. One excellent example is commercially carboxymethylated dextran-based BIAcore CM5 chips which have high binding capacity and high surface stability, but these chips require

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multiple steps for surface cleaning and functionalization, are difficult to prepare and also are expensive. Recently, a simple liquid phase deposition (LPD) method has been successfully fabricated to apply organic/inorganic hybrid thin films onto the SPR gold surface [10]. However, the binding capacity and surface stability of these films are lower than those of the CM5 chips. Finding a simple, cheap fabrication method with high binding capacity and surface stability on the SPR gold surface has been a great challenge for SPR biosensor development.

Here, we investigate a new sensing surface whereby a novel carboxylated terthiophene scaffold is used for the formation of a self-assembled monolayer anchor for rapid and highly sensitive SPR detection of P_4.

II. Experimental

A. Reagents and Instrumentation

4-Pregnen-3, 20-dione 3-O-Carboxymethyloxime (P_4-3-O-CMO) was purchased from Steraloids (Newport, U.S.A.). HBS buffer (10 mM HEPES, pH 7.4, 150mM NaCl, 3.4mM EDTA, and 0.05% surfactant), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), *N*-Hydroxysuccinimide (NHS) and SPR gold chips (BR-1004-05) were purchased from GE Healthcare Life Science (Auckland, New Zealand). *N, N'*-Dicyclohexyl-carbodiimide (DCC), phosphate buffered saline tablets (PBS, pH 7.4) and all other reagents were purchased from Aldrich, and used without purification. The terthiophene-OEG-CO_2H (Fig. 1b) was synthesized according to previously developed methods [11].

Surface Plasmon Resonance (SPR) measurements were carried out on a BIAcore Q (BIAcore AB, Inc., Piscataway, NJ, USA). Reactive ion etching (RIE) was performed on a March CS-1701 (Nordson MARCH, California, USA) with an oxygen plasma (set at 50W for 30s and 50% O_2).

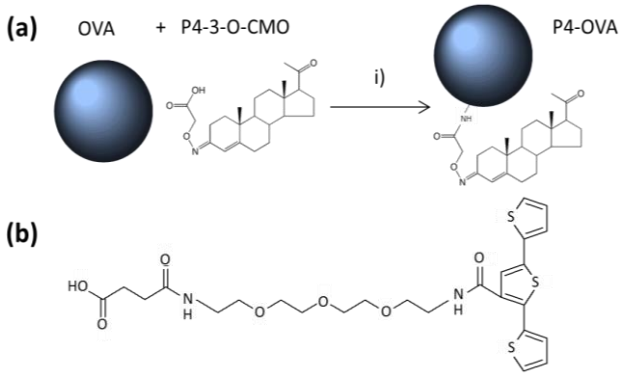

Figure 1. a) Synthesis of P_4-OVA conjugates, i) DCC/NHS/DMF, OVA in phosphate buffer at 4 ℃ overnight, b) Chemical structure of the terthiophene-OEG-CO_2H.

B. Preparation of the P_4-OVA Conjugates

The P_4-OVA conjugates were prepared based on a method previously reported, Fig. 1a [12]. Namely, to a solution of P_4-3-O-CMO in DMF, DCC and NHS are added, and the mixture left to react in a solution of OVA in phosphate buffer at 4 ℃. Dialysis of the product was then performed in de-ionized water followed by PBS with Tween 20. The dialyzed conjugate was then collected.

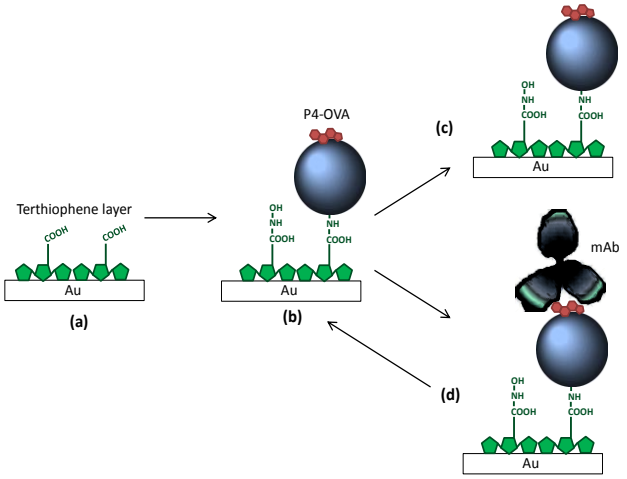

Figure 2. Sequential assay format: a) a terthiophene functionalized gold surface, b) a P_4-OVA immobilized sensor surface, c) when there is a certain amount of P_4 in the sample/standard all mAb is saturated with P_4, hence none can bind to surface P_4, d) when the mAb is not saturated with P_4, unsaturated mAb will bind to surface immobilized P_4. After regeneration the sensor surface is once again as in b).

C. Preparation of Terthiophene Functionalized SPR Chips

Following the RIE clean (2 min), SPR chips were immediately immersed in terthiophene-OEG-CO_2H solution (10mM, ethanol: water 1:3) for 15h (Fig. 2a), and washed with water and assembled into a sensor holder. Immobilization of P_4-OVA conjugate was performed by 150 µl injection of 390mM EDC/100mM NHS, followed by P_4-OVA conjugates 150 µl at 5 µl/min. Ethanolamine solution (1M, pH 8.5, 50 µl at 5 µl/min) was then used to cap any remaining activated sites, and final injections of regeneration solution, 10mM NaOH 10 µl at 20 µl/min, were performed to stabilize the sensor surface (Fig. 2b).

III. Results and Discussion

A. Preparation of Terthiophene Functionalized SPR Chip

A clean gold surface is required before the self-assembly of the terthiophene-OEG-CO_2H molecules. Previous methods used a very harsh piranha solution, or a mixture of sulfuric acid and hydrogen peroxide, for gold cleaning [5]. Here we used a simple RIE method to clean the SPR gold surface in a much quicker (2 min) and safer way. Following immobilization of P_4-OVA conjugates on the SPR surface, the multiple antibody binding/regeneration cycles from the present work have shown the terthiophene-OEG/OVA-P_4 surface is remarkably stable, demonstrated by its stable baseline. The terthiophene platform may provide enhanced stability over some anchors such as alkanethiol mSAM surfaces [5]. Although both alkanethiol and terthiophene surfaces can be prone to oxidation and hence removal [13], terthiophenes have multi-pronged binding, hence

may maintain contact with the gold surface for longer. Additionally the immobilized OVA conjugate has been previously shown to improve stability [5].

B. Antibody Binding Test

The sensor surface showed good responses to the mAb concentrations tested, with SPR response unit (RU) values of between 26±1 and 324±16 (Fig. 3). The concentration of mAb chosen for the P_4 assay was set at 2.5 µg/ml as this value gave a reasonable response (>100 RU), while not saturating the surface ensuring easy surface regeneration.

C. Progesterone Assay

The developed assay involved a P_4-immobilized surface onto which *anti*-progesterone antibody could bind in the absence of free P_4. The concentrations of P_4 investigated were between 0.01 and 100ng/ml (Fig. 4). Each concentration was repeated at least 3 times and showed good reproducibility. All sensor responses were as expected, indicating good surface regenerability – of importance as poor regeneration can result in false positives or baseline drift. The assay demonstrated good sensitivity over the range 0-50ng/ml.

The limit of detection (LOD) was determined as the concentration corresponding to the mean response for the negative P_4 standard minus three times the standard deviation [14], and is 0.13ng/ml (132pg/ml). This LOD is lower than many other reported levels, such as 3.56ng/ml by Gillis *et al.* using a CM5-chip with real milk samples and 95% maximum binding or effective dose (ED 95) of 0.4-0.6ng/ml after assay alteration [3], [4]; 372.7pg/ml and 4.9pg/ml (after enhancement) from previous research using a mixed self-assembled monolayer [5]; and 1ng/ml and 143pg/ml (with pre-labeling with gold-labels) using a CM5-chip [6]. Another comparison can be made by examining the antibody binding sensitivity based on the slope of the antibody binding curve. The sensor surface detailed in this research showed a much higher slope in the binding curve (Fig. 4) than our previous research with a Biacore™ CM5-chip [10].

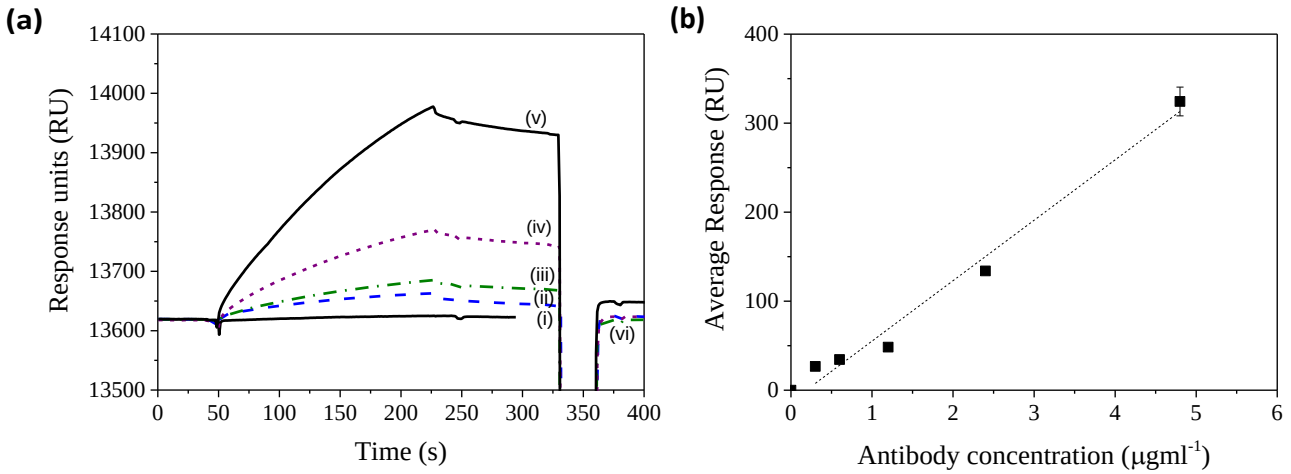

Figure 3. a) Overlay of SPR sensorgrams: i) HBS, ii) 0.3 µg/ml, iii) 1.2 µg/ml, iv) 2.4 µg/ml and v) 4.8 µg/ml mAb. vi) sensor regeneration; b) an antibody binding curve. Error bars represent standard deviation of the measurements (n=3).

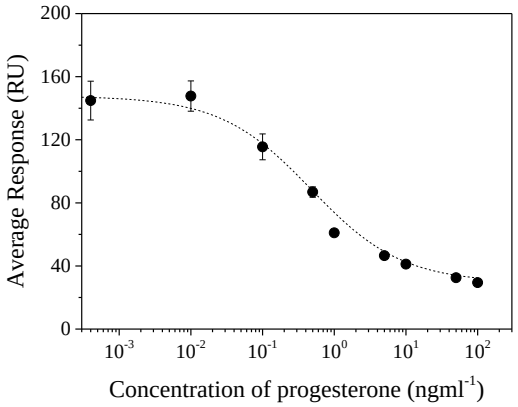

Figure 4. An inhibition assay curve of P_4 standards versus sensor response. Error bars indicate the standard deviations from triplicate measurements.

CM5-Chips have some advantages; but with the carboxylated-dextran coating being approximately 100nm, sensitivity may be hindered due to the increased distance to the sensing event. In comparison, with a terthiophene attachment there can be as little as a single monolayer which forms, shortening the distance between the gold chip and the sensing event.

Other detection methods, such as immunosensors can also be used to detect P_4, with varying LODs. Kreuzer *et al.* developed an immunosensor which was able to achieve a detection limit of 3±2ng/ml [7], while Arévalo *et al.* calculated an LOD of 0.2ng/ml, however this required labeled P_4 [8]. Arévalo *et al.* comment that the electrochemical immunosensor had higher sensitivity as compared to ELISA methods, however with similar LODs. Achieving an LOD similar to the current conventional method used (ELISA) with a much more rapid process shows the great promise of the present terthiophene-oligoethylene glycol CO_2H scaffold as a new fabricating material on gold sensor surfaces for development of SPR or other gold-surface based assays.

IV. Summary

This research demonstrates that terthiolphene molecular scaffolds have the great potential as new fabricating materials for SPR and other gold-based biosensor developments because of their simple fabrication approach (one step), amenability to manipulation for introducing various molecular linkers (OEG etc) and different functional groups (CO_2H etc). The developed SPR biosensor surface showed a stable baseline with good responses to monoclonal *anti*-progesterone antibody. An inhibition P_4 assay also showed excellent reproducibility, sensitivity and a low LOD. It is suggested the improved assay performance with this platform arise from the multiple benefits including: 1) Multiple attachments from terthiophene molecules on gold to form a stable SPR surface; 2) The OEG linker to reduce steric hindrance between the antibody-binding site and the gold surface; and 3) 3D-structures of the OVA-P_4 conjugate to favor antibody bindings and surface regenerations. This, coupled with the rapidity of the assay (each measurement is completed within 5min including regeneration) shows the promise of P_4 sensing with this platform and SPR.

Although the demonstrated sensitivity of the assay is high, there would be an advantage in improving this further. In real milk samples, although the typical concentration of P_4 would be easily detectable with the developed sensor platform, in order to minimize the matrix effects arising from milk significant dilution of the milk samples is often required [3], [4]. We suggest enhancement with nanoparticles may provide a valuable assay addition [5].

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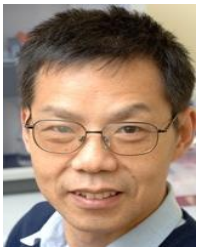

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