Biosensors and Bioelectronics 24 (2009) 2177–2183

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

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

Ultrasensitive detection of testosterone using conjugate linker technology in a nanoparticle-enhanced surface plasmon resonance biosensor

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ARTICLE INFO

Article history:
Received 8 September 2008
Received in revised form 7 November 2008
Accepted 20 November 2008
Available online 30 November 2008

Keywords:
Testosterone
Surface plasmon resonance
Linker
Conjugate
Saliva

ABSTRACT

A rationally designed oligoethylene glycol linker conjugate to testosterone was synthesised and covalently immobilized on a surface plasmon resonance (SPR) biosensor surface. The sensing surface was stable for more than 330 binding and regeneration cycles allowing a high degree of re-use. This surface was then used in the development of an ultrasensitive immunobiosensor system for testosterone in buffer utilizing both secondary antibody and gold nanoparticle signal enhancement. The mechanism for the increased sensitivity results from increased binding mass and a gold plasmon coupling effect. The addition of a secondary antibody with an attached gold nanoparticle increased the signal sensitivity of the assay 12.5-fold compared with primary antibody alone. In the enhanced format the assay had limits of detection (LOD) of 3.7 pg ml^{-1} with standard in running buffer, and 15.4 pg ml^{-1} in a stripped human saliva matrix. This immunobiosensor system has sufficient sensitivity to measure testosterone across the broad physiologically relevant range in male saliva (29–290 pg ml^{-1}) in under 13 min allowing monitoring of testosterone in near real-time.

1. Introduction

Testosterone is an anabolic androgenic steroid that acts as the principal male sex hormone. The measurement of testosterone levels is of increasing interest for sports endocrinology (Crewther et al., 2006; Loebel and Kraemer, 1998) and the study of medical conditions such as testosterone deficiency syndrome (Tostain and Blanc, 2008). Measurement in saliva offers a viable alternative to invasive blood sampling as the hormone concentrations are well correlated with serum and plasma free concentrations (Arregger et al., 2007; Ohzeki et al., 1991; Magrini et al., 1986). Salivary testosterone is mostly in the free form rather than bound to sex hormone binding globulin (SHBG) (Khan-Dawood et al., 1984) and the free form of steroids is arguably the most relevant fraction to determine bioavailability and potential physiological function (Vining et al., 1983). Furthermore, the relative ease and non-invasive nature of saliva sampling promotes subject compliance and allows large numbers of samples to be collected if required (Read et al., 1990; Magrini et al., 1986), without specialist sampling support.

A number of radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA) techniques exist for testosterone measurement in a variety of media including saliva (Wheeler, 2006; Granger et al., 2004) as well as alternative plate-based immunoassays such as time-resolved fluorescence immunoassay (Tschöp et al., 1998). These techniques are typically laboratory-based, labour-intensive, have high costs per sample, have long wait times for results (typically hours to days) and for the case of RIA use hazardous radioisotopes.

Biosensors could offer a means for rapid and low cost measurement of testosterone with results available at the point of sample collection. Surface plasmon resonance (SPR) is a flow-through biosensor technology that measures very small changes in refractive index on a noble metal surface when mass binds to that surface and is used as a transduction approach in many immunobiosensor assays (Homola, 2008). One of the most popular commercial SPR systems available is the BIAcore™ which can utilize carboxymethylated dextran polymer surfaces for the conjugation of proteins or other amine-bearing molecules (Fivash et al., 1998). Concentrations of the target antigen can be determined by binding of a primary antibody to the immobilized antigen in a competitive assay format with the option of further signal enhancement by use of a secondary antibody (Mitchell et al., 2006). Until recently SPR lacked the necessary sensitivity to detect small molecules at the concentrations in which they are found in many biological fluids (often below 1 ng ml^{-1}). In addition to their use for large molecule SPR immunoassays (Lyon et al., 1998), nanoparticle labels have now been utilized for small molecule assays to maximise sensitivity and reduce limits of detection (LOD) (Mitchell et al., 2005). In such assays it is critical to use rationally designed linker conjugates of the target antigen for immobilization to the sensor surface

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doi:10.1016/j.bios.2008.11.018

to allow maximum antibody recognition through relief of steric hindrance and conjugation at a site distant from key functional groups. Antigen conjugates incorporating attachment of a 15-atom linker to the 4-position of the steroid have been developed for the hormones progesterone (Mitchell et al., 2005; Wu et al., 2002), estradiol, estrone (Mitchell et al., 2006) and catecholamines such as dopamine (Mitchell et al., 2007). Similar linker technology mediated through thiol attachment has also been studied for cortisol (Sharpe et al., 2008).

Testosterone has a physiological range in human adult male saliva of 29–290 pg ml^{-1} (Ellison et al., 2002) and so a highly sensitive SPR system would be required to measure it. Previous reports studying testosterone immobilized on an SPR biosensor use unstable streptavidin immobilization with biotin coupling at the 3- and 19-positions of the steroid through existing functional groups and only study binding kinetics, not an immunoassay (Kaiser et al., 2000). Binding between the related androgen, dihydrotestosterone, and SHBG has also been studied by SPR (Luppa et al., 2006; Metzger et al., 2003). There is a dearth of studies that use SPR biosensors for measurement in complex biological media. Most small molecule work to date has been done with milk (Gillis et al., 2002; Gustavsson et al., 2004), with studies on interleukin-8 (Yang et al., 2005) and phenytoin (Fu et al., 2007) in human saliva.

We report the synthesis of a novel linker conjugate of testosterone and apply it as a coating antigen in the sensitive detection of testosterone using an SPR-based biosensor. The assay is developed in buffer where different enhancement techniques using a secondary antibody conjugated to either horse radish peroxidase (HRP) or a gold nanoparticle are compared. These techniques would be suitable for extracted hormone samples in buffer, allowing hormone measurement in a wide range of matrices. In addition, the gold nanoparticle signal enhancement method is extended to a human saliva matrix.

2. Experimental

2.1. Materials and statistics

Testosterone was from Steraloids (A6950-000), activated charcoal was from Merck (1.02186.1000) and PEG-400 from Prolabo (26 602-290). All other chemical reagents were from Sigma–Aldrich. Monoclonal primary antibody (mAb) to testosterone was from US Biologicals (raised in mouse, T2950-22) and anti-mouse immunoglobulin G (IgG)–HRP secondary antibody (A9044) and anti-mouse IgG (M7023) were from Sigma. BIAcore™ SPR experiments were performed on a BIAcore 2000™ instrument with CM5 dextran chips supplied by BIAcore™. Running buffer was HEPES (0.01 M) buffered saline (0.15 M) with EDTA (3 mM) and Tween-20 (0.005%, v/v) (HBS-EP). Assay curve data were fitted to four parameter logistic curves using SigmaPlot™ software and this was also used to translate response variability into concentration errors. LOD were calculated as the concentration corresponding to the blank value less two standard deviations of the blank (Chard, 1995). IC_{50} was the 50% bound figure from the curve fitting. Sensitivity was calculated as the slope of the linear portion of the assay curve and is independent of any non-specific binding (nsb) by reference flow cell subtraction. Standard errors are quoted. Outlier treatment was as for Mitchell et al. (2005). All synthesis methods and characterisation data for synthesised compounds are in Supplementary Material.

2.2. Immobilization of testosterone derivative

A CM5 dextran chip was docked into a BIAcore 2000™ instrument and flow cell two of four was immobilized with steroid as follows. *N*-Ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide (EDC) (0.4 M) was mixed 1:1 with NHS (0.1 M) and injected (2× 50 μL at 5 μL/min) to activate the surface. This was immediately followed by compound **6** (4× 100 μL at 5 μL/min, 1 mg ml^{-1} in 1% (v/v) dimethylformamide (DMF) in 0.01 M phosphate buffered saline pH 9). The surface was then deactivated with ethanolamine (1 M, pH 8.5). Flow cell 1 was activated and then deactivated as a reference flow channel.

2.3. Secondary antibody-HRP signal enhancement

To optimize loading of secondary antibody-HRP conjugate, mAb (200 ng ml^{-1}, 60 μL in running buffer) was injected immediately followed by IgG–HRP (60 μL, 10 μL/min) at dilutions of 0, 0.001, 0.002, 0.004, 0.01, 0.013, 0.02 and 0.04 in running buffer. Five replicates were run of each concentration. Regeneration was by injection of 20% (v/v) acetonitrile in 50 mM NaOH (20 μL). To determine the relationship between signal and primary antibody concentration, mAb (60 μL) was injected at concentrations of 0, 5, 10, 25, 50, 100, 150, 200 ng ml^{-1} immediately followed by IgG–HRP (0.02 dilution factor, 60 μL, 10 μL/min). Five replicates of each concentration were run with regeneration as above. Immunoassay of testosterone was run by mixing mAb (50 ng ml^{-1}) 1:1 with testosterone standard in running buffer (0, 1, 10, 25, 50, 100, 250, 1000, 5000, 10,000 pg ml^{-1}) in a vial using automated BIAcore™ functions and incubating the mixture at room temperature for 5 min before injecting 60 μL. This was immediately followed by injection of IgG–HRP (0.02, 60 μL, 10 μL/min). There were five replicates of each point, with regeneration conducted as described above.

2.4. Nanogold signal enhancement in buffer

Gold colloid (25 nm diameter, 0.01% (w/v) $HAuCl_4$) was prepared by the citrate reduction method (Frens, 1973) and secondary antibody was conjugated according to an established method (Mitchell et al., 2005) to give a final secondary antibody concentration of 300 μg ml^{-1}. Secondary antibody–gold conjugate loading was optimized by injecting mAb (50 ng ml^{-1}, 60 μL) immediately followed by IgG–gold 25 nm (60 μL, 10 μL/min) at dilutions of 0.5, 0.25, 0.125 in 10% (v/v) PEG-400 in deionized water, regeneration as above. The relationship between signal and mAb concentration was determined as for the IgG–HRP enhancement but using IgG–gold conjugate at 0.125 dilution in place of IgG–HRP and using mAb concentrations of 0, 1, 2.5, 5, 10, 25, 50 ng ml^{-1}. An immunoassay of testosterone was constructed as above but with IgG–gold 25 nm (0.125 dilution) in place of IgG–HRP, primary mAb at 9 ng/ml injected concentration and using testosterone standards of 0, 1, 5, 10, 25, 50, 100, 250, 1000, 5000 pg ml^{-1}.

2.5. Assay in human saliva

Saliva was collected from a male subject and endogenous steroids were stripped by addition of activated charcoal (Carter, 1978) (10 mg ml^{-1}), shaking at room temperature for 18 h at 560 rpm and then centrifuging (twice at 4620 × *g*, 20 min) to remove charcoal. An assay for detection of testosterone in stripped human saliva was performed as for the IgG–gold enhanced buffer assay but with testosterone standards prepared in stripped human saliva (four replicates) and with IgG–gold at 0.0625 dilution in 7.5% (w/v) PEG-4000 in deionized water. A more rapid salivary testosterone assay was then developed using the same format as above but with no pre-incubation of standard and antibody, all BIAcore™ injection formats changed to "quickinject" and IgG–gold injected at 20 μL/min reducing assay cycle time to less than 13 min and primary mAb injected concentration of 4.5 ng/ml. Saliva collection was approved by the New Zealand Northern Y Regional Ethics Committee (NTY/07/02/017).

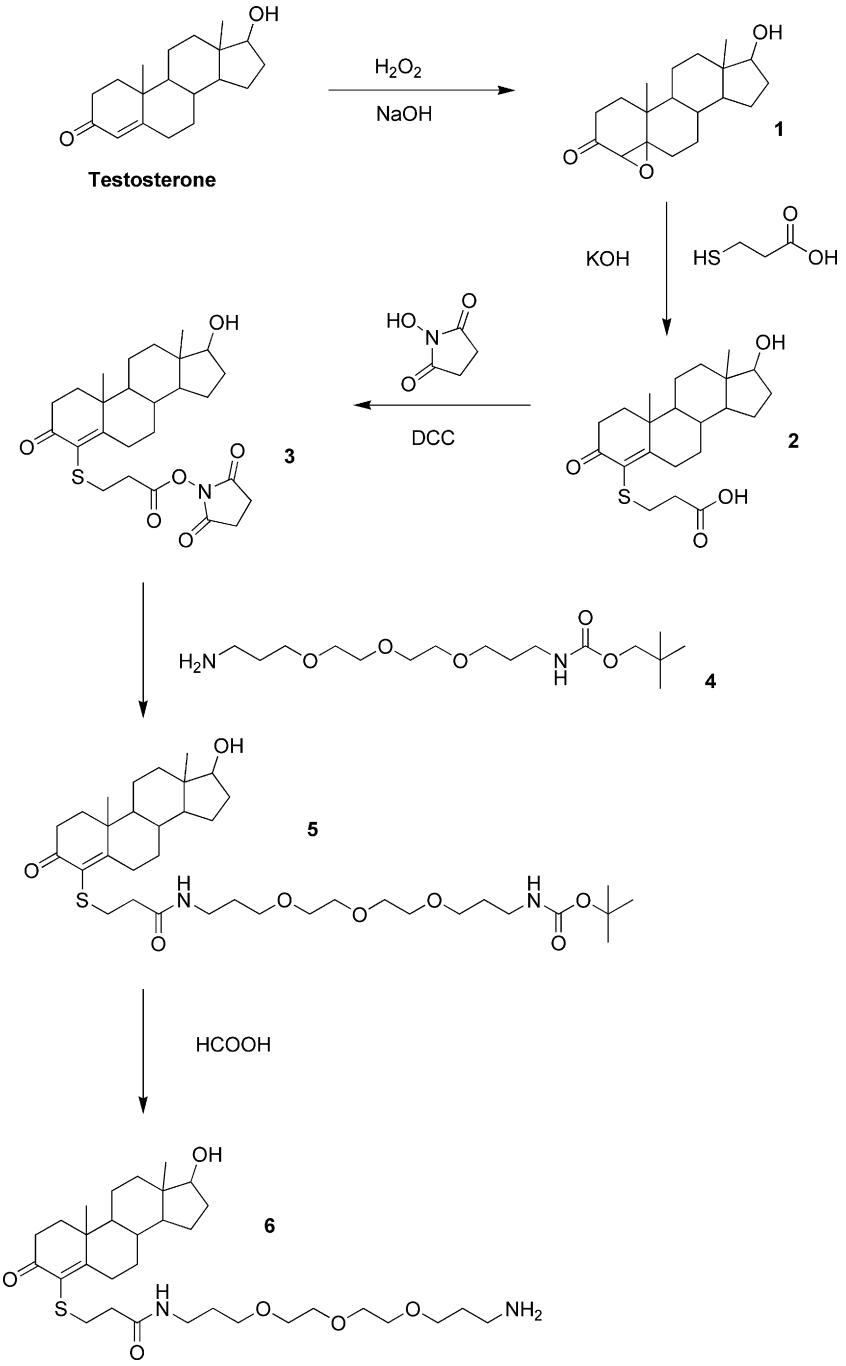
3. Results and discussion

3.1. Synthesis of testosterone linker conjugate

Previous reports have indicated that conjugation through the 4-position of steroid hormones for immobilization in flow-through SPR biosensors is preferred for optimal antibody recognition and signal response (Wu et al., 2002). The synthesis of a testosterone linker conjugate through the 4-position is summarised in Scheme 1. For testosterone, it is possible to form an epoxide across the 4, 5-double bond of the A-ring and then attach a thioether moiety under alkaline conditions (Camerino et al., 1956; Hosoda et al., 1979). From this derivative it was then possible to activate the carboxylic acid terminus with NHS to make it more reactive to primary amines (Hosoda et al., 1979) and then attach an oligoethylene glycol linker with amine termini, one of which is protected with a Boc protecting group. The derivative is stable in this form for an extended period and can be easily de-protected to form a primary amine for immobilization.

3.2. Immobilization of testosterone linker conjugate

The simple *in situ* immobilization technique resulted in a high yield of immobilized steroid and a resultant high binding capacity. The use of covalent attachment meant that the surface was very stable, lasting for more than 330 binding and regeneration cycles despite the use of harsh regeneration conditions (NaOH and acetonitrile). The stability limit of this surface has not yet been reached



Scheme 1. Synthesis scheme for production of testosterone–oligoethylene glycol linker conjugate.

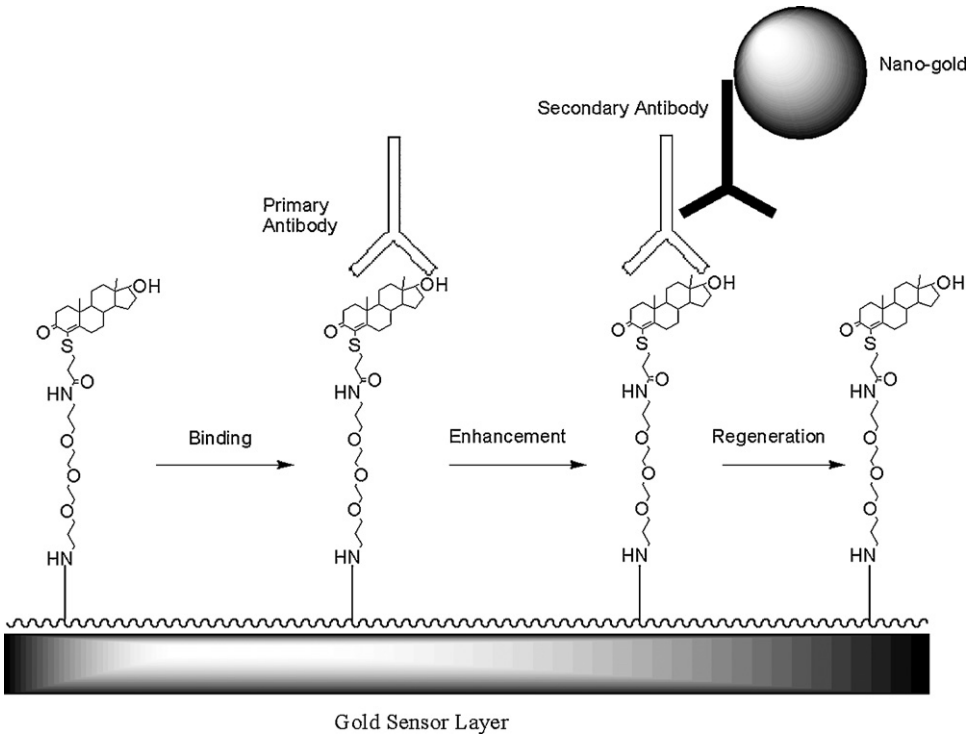


Fig. 1. Schematic of the SPR immunosensor assay binding process with nanogold labelling.

and similarly constructed surfaces have lasted more than 1100 cycles (Mitchell et al., 2005). In comparison, immobilizing proteins can lead to denaturation upon successive exposure to regeneration solutions thus resulting in a much shorter surface lifetime. The testosterone-linker surface is illustrated in Fig. 1. The oligoethylene glycol linker has low immunogenecity (Kurusu et al., 2003; Otsuka et al., 2003) which minimises non-specific binding to the linker unit whilst providing a hydrophilic chain to project the antigen into the polar running phase and can be used successfully in immunoassay of a range of small molecules (Mitchell et al., 2005; Mizuta et al., 2008).

Competitive immunoassay of small molecule targets using SPR offers a potentially rapid method of measuring concentration of the analyte without the need to immobilize antibody on the sensing surface, Fig. 1. This means that a highly stable surface with covalently attached coating antigen can be used that does not suffer from the unstable coatings of antibody or protein conjugate immobilized systems and the primary antibody can act as the signal generator.

3.3. Enhancement using IgG–HRP conjugate

When high sensitivity is required, one can bind a secondary antibody to add mass to the surface and thus increase signal, Fig. 1. Previous reports utilizing small molecule immobilization have shown that secondary antibodies can enhance signal well beyond the enhancement expected from a simple 1:1 binding event (Mitchell et al., 2005, 2006). Once the testosterone biosensor surface was constructed, a number of secondary antibodies were tested for their specific and non-specific binding in buffer and an IgG–HRP conjugate was chosen for its low non-specific binding properties (20 RU at 0.02 dilution) combined with excellent signal enhancement comparable to that of a standard secondary IgG (7.3-fold). To determine the loading of IgG–HRP required for maximum signal enhancement, a plot of response vs. IgG–HRP concentration was prepared (Fig. S-1 supplementary material). Binding response had largely reached saturation at a 0.02 dilution and so this was adopted as the best compromise between assay signal and per cycle material cost. It was necessary to maximise secondary antibody signal enhancement so that the loading of primary antibody used was as low as possible to ensure the lowest possible LOD.

To determine the degree of signal enhancement obtained, a plot of the relationship between signal strength and primary antibody concentration was prepared for both the total signal response and the signal arising from the primary antibody alone, Fig. 2A. The signal enhancement as determined from the ratio of the slopes of the linear plots was 7.3-fold, comparable to other secondary antibody systems (Mitchell et al., 2005). The non-specific binding arising from the secondary antibody is the intercept of the total response line and so is not part of the signal enhancement determination.

A competitive assay format was developed for measuring testosterone in buffer using IgG–HRP signal enhancement and yielded a highly sensitive assay standard curve with a LOD of 35 pg ml^{-1}, assay 1B, Table 2. The variability in the assay signal responses was so low that the un-enhanced primary antibody bindings could generate a reliable buffer assay with an even lower LOD of 21 pg ml^{-1}, assay 1A, Table 2. Whilst primary antibody binding-based assays are useful in buffer they can be unusable when the primary antibody is mixed with a biological sample such as saliva where there is some variable salivary non-specific binding. In these cases one can generate an assay curve from the secondary labelling response of IgG–HRP or IgG–gold conjugates which excludes the non-specific binding component of the saliva; hence it is always valuable to study the enhanced assays. Another enhancement technique, using IgG–nanogold conjugate, was then investigated to further improve the signal strength of the assay whilst retaining low LOD.

3.4. Enhancement using IgG–nanogold conjugate

Gold nanoparticles can add substantial additional bound mass to the sensing surface but also undergo SPR themselves and so can have a cooperative effect in enhancing the SPR signal (Lyon

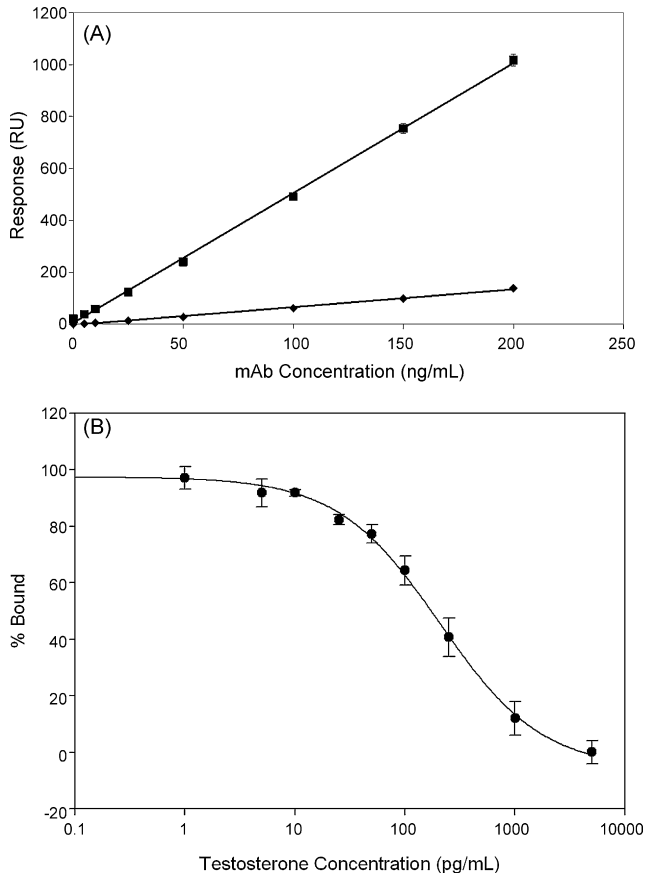


Fig. 2. (A) Plot of signal response (RU) vs. mAb concentration for mAb signal alone (♦) (linear fit: $y = 5.0017x + 5.512$, $R^2 = 0.9991$) and with IgG–HRP signal enhancement (■) (linear fit: $y = 0.6864x - 2.8776$, $R^2 = 0.996$). (B) Assay standard curve for mAb only response in buffer.

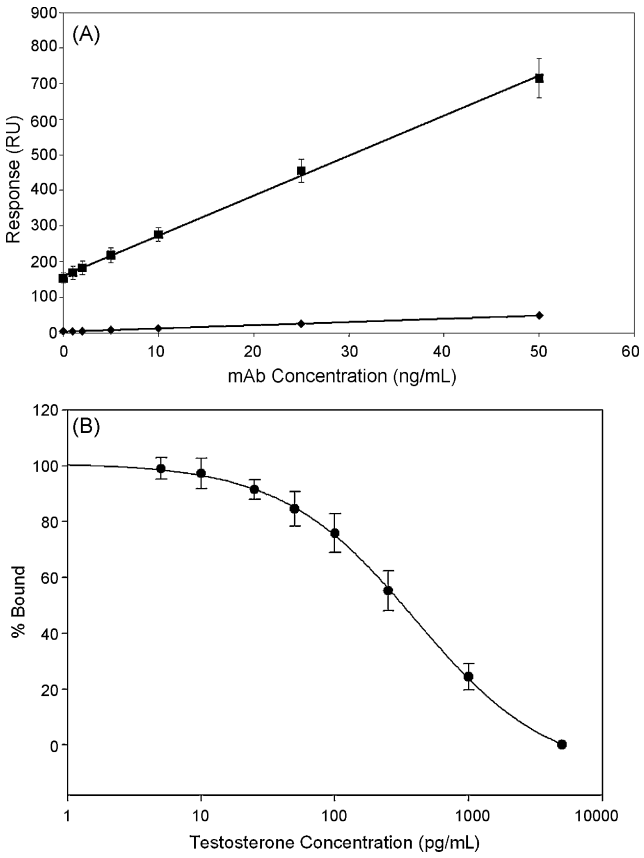


Fig. 3. (A) Plot of signal response (RU) vs. mAb concentration for mAb signal alone (♦) (linear fit: $y = 11.246x + 160.57$, $R^2 = 0.9988$) and with secondary antibody–nanogold signal enhancement (■) (linear fit: $y = 0.9005x + 3.4431$, $R^2 = 0.9992$). (B) Assay standard curve for IgG–nanogold enhanced assay in stripped saliva, longer format.

et al., 1998). They have been used for signal enhancement in SPR sandwich assays for protein analytes (Lyon et al., 1998) but only recently for SPR immunoassay of small molecule antigens (Mitchell et al., 2005). Previously, a 25 nm gold sol has been found to be optimal for use with a secondary antibody in an SPR immunosensor with an underlying dextran surface (Mitchell et al., 2005). Such an IgG–nanogold conjugate was produced by charge immobilization on a citrate reduced colloid. When the conjugate was tested in the SPR system it was found that use of a high loading of polyethylene glycol (PEG) in the diluent of the gold conjugate improved the stability of the conjugate by reducing settling of the colloid.

To determine the effect of dilution of the gold conjugate on levels of both specific and non-specific binding in the immunosensor, several dilutions were used to enhance primary antibody response and their non-specific binding tested. The data showed no significant decline in signal enhancement for dilutions from 0.5 to 0.125 but did show a significant decline in non-specific binding (38% reduction), Table 1. This implies that at these loadings there has been saturation binding of the primary antibody. This experiment indicated signal enhancement factors of about 13-fold which is consistent with previous reports with different antigen (Mitchell et al., 2005) for a dextran, 25 nm nanogold system, Table 1. To optimize the primary antibody concentration and determine the relationship between signal and primary antibody loading, a plot was constructed across various primary antibody concentrations for both primary antibody only response and nanogold enhanced response, Fig. 3A. Based on the ratio of the slopes of enhanced and un-enhanced linear plots, the enhancement across the concentration range was 12.5-fold, indicating consistent enhancement regardless of primary antibody concentration.

With the additional signal enhancement from the nanogold conjugate it was possible to reduce primary antibody to such an extent that a low LOD could be obtained at 23 pg ml^{-1}, assay 2B, Table 2. This assay, unlike the IgG–HRP enhanced assay, demonstrated a linear response range from 25 to 250 pg ml^{-1} encompassing the normal physiological range in saliva of healthy human males (Ellison et al., 2002). The assay sensitivity of 122 RU ml/ng ensured that sufficient signal could be obtained for good resolution of concentrations in the range of interest. This assay is therefore valuable for the determination of testosterone in saliva samples that have been extracted and reconstituted in buffer; and if the samples are concentrated as part of the extraction process it may also be extended to measurement in female saliva samples where testosterone levels are even lower.

Surprisingly, the un-enhanced primary antibody only response gave sufficiently precise results to be used in formation of an assay curve despite the very low signal, Fig. 2B. This assay proved to have a lower LOD of 3.7 pg ml^{-1}, probably due to a reduction in signal variability associated with the absence of a second binding event, assay 2A Table 2. This LOD is comparable to that previously reported for an SPR immunoassay of progesterone using a SAM layer approach (Yuan et al., 2007). However, the current testosterone assay has the advantage of not using thiol dative bonds of limited stability in immobilization or needing any form of signal enhancement, thus increasing the durability of the assay system whilst reducing cost and assay cycle time for buffer-based assay. The CVs for the signal were 3.9% indicating high precision despite the lower signal response sensitivity, assay 2A Table 2. Signal enhancement is

Table 1
The effects of nanogold dilution factor on binding signals and sensor signal enhancement factor for the SPR immunosensor.

Nanogold dilution factor	Specific binding (RU)	Non-specific binding (RU)	Enhancement factor
0.5	651.5 ± 0.1	239.7 ± 0.1	13.1 ± 0.03
0.25	705.2 ± 0.1	189.8 ± 0.1	12.9 ± 0.02
0.125	664.9 ± 0.1	149.2 ± 0.1	13.2 ± 0.03

Table 2
Assay parameters for the assay formats developed for testosterone using the SPR immunosensor.

Assay format	Primary antibody concentration (ng/ml)	Incubation time (min)	Limit of detection (pg/ml)	IC_{50} (pg/ml)	Signal sensitivity (RU ml/ng)
1A. Buffer, primary Ab	25	5	21 ± 6.6	385 ± 12	21.5 ± 0.8
1B. Buffer, IgG–HRP	25	5	35 ± 11	396 ± 22	180 ± 48
2A. Buffer, primary Ab	9	5	3.7 ± 0.9	206 ± 26	11.6 ± 0.9
2B. Buffer, gold	9	5	23 ± 1.5	288 ± 48	122 ± 3
3A. Saliva, primary Ab	9	5	36 ± 13	299 ± 79	8.1 ± 0.6
3B. Saliva, gold	9	5	66 ± 15	397 ± 60	128 ± 9
4. Saliva, gold	4.5	0	15.4 ± 7.3	408 ± 12	45 ± 4.3

still required though, as whilst this assay performs well in buffer, the presence of saliva is likely to compromise un-enhanced assay performance. The lower IC_{50} of both un-enhanced and enhanced assays (assays 2A, 2B Table 2) reflects a significant shift of the assay curve to lower concentrations. With the construction of an assay system capable of detecting testosterone at physiologically relevant concentrations in buffer it was then advantageous to convert this system to measure in human saliva.

3.5. SPR biosensing in human saliva

The human salivary matrix is a very complex and viscous fluid that is difficult to use in biosensor settings. The components of saliva can potentially interfere with antibody/antigen binding events and result in fouling of fluidic systems and non-specific binding to sensor surfaces (Yang et al., 2005). These problems are further compounded in the case of steroid hormone measurements as only very low concentrations of steroids are present in the saliva, especially in the case of testosterone, reflecting only a small percentage (about 2%) of the blood levels (Rilling et al., 1996).

The nanogold enhanced testosterone assay was adapted for use in saliva by running standards in stripped saliva. This enhancement was needed as the use of stripped saliva meant primary antibody only responses had too low a signal to noise due to interferences from the matrix. By charcoal stripping the saliva, the steroid component is removed along with a portion of the large molecule content which reduces the non-specific binding but still provides an approximation to un-treated saliva which is arguably a better standard diluent than synthetic saliva since synthetic saliva does not approach the chemical complexity present in real saliva. To further minimise any settling of the conjugated gold colloid with time, the gold diluent treatment used a higher mass PEG, which can also reduce non-specific binding (Mitchell et al., 2005) and the gold was diluted further to obtain reduction in non-specific binding.

Reliable assay curves were obtained for both primary antibody only and nanogold enhanced bindings despite interferences from the stripped saliva, Fig. 3B. The presence of stripped saliva caused the assay curves to shift to higher concentrations and thus result in poorer LOD than for the buffer, assays 3A, 3B Table 2. Speculatively, this may be the result of salivary components obstructing binding between the free testosterone and the antibody thus resulting in higher testosterone concentrations being needed for inhibition of binding to the surface. The presence of salivary matrix greatly compromised the performance of the un-enhanced assay resulting in an LOD of 36 pg ml^{-1} (assay 3A, Table 2), about 10-times higher than for buffer and not quite adequate for salivary testosterone detection. LOD for the gold enhanced assay was also increased but to a much lesser extent (less than 3-fold to 66 pg/mL, assay 3B, Table 2) and signal sensitivity was retained.

To measure testosterone in human saliva at physiologically significant concentrations, the LOD must be reduced further and this was achieved by halving the primary antibody concentration. This assay data is shown in Table 2, assay 4. In this assay the addition of albumin to the primary antibody diluent further reduced non-specific binding from the stripped saliva standards down to near zero. As the primary antibody concentration was now so low, it was not possible to obtain an assay curve from its non-enhanced response as the variable salivary non-specific binding was now too high relative to the primary antibody signal thus requiring the continuation of nanogold signal enhancement. When nanogold enhancement was employed the LOD was found to have improved to 15.4 pg ml^{-1} which is comparable to the enhanced buffer assay. These results indicate how an enhanced SPR immunoassay can produce required assay curve results in the presence of a complex biological matrix where un-enhanced assays cannot. This highly sensitive assay now opens the way to potential measurement of testosterone in male saliva at its physiological concentrations without extraction and represents the first testosterone assay system using an SPR biosensor. Furthermore, the assay cycle time in this format has now been reduced to less than 13 min per sample. In future, real saliva samples could be measured by the immunobiosensor and the results compared to existing lab-based techniques such as radioimmunoassay.

4. Conclusion

Ultrasensitive measurement of the important steroid hormone testosterone has been achieved through use of SPR biosensor technology. A specialized linker conjugate of testosterone has been synthesised for covalent attachment to a dextran SPR surface as the coating antigen. This surface has demonstrated stability for in excess of 330 binding cycles enabling a high throughput of samples over a single surface. The relationships between signal response and the use of enhancement labels incorporating secondary antibody and/or nanogold colloid were examined and binding parameters optimized. Assay formats were employed utilizing both forms of enhancement for measurement of testosterone in buffer but it was also demonstrated that such assays could be performed in buffer without enhancement due to high precision at low binding signals. However, this performance could not be extended to stripped saliva matrix. The assays were then adapted to measurement in stripped human saliva and showed that with the use of nanogold

enhancement it was possible to achieve an immunosensor assay using SPR with the sensitivity required for measurement at physiologically relevant concentrations, with assay cycle times of under 13 min. This rapid assay system opens the way to potential detection of salivary testosterone in near real-time for physiological and clinical investigation.

Acknowledgements

This work was funded by the New Zealand Foundation for Research Science and Technology (C06X0206 and C06X0410). We thank Dr. Yinqiu Wu for helpful discussions.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2008.11.018.

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