Rapid ultrasensitive measurement of salivary cortisol using nano-linker chemistry coupled with surface plasmon resonance detection†

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Cortisol detection in saliva is of great interest for the diagnosis of various disease states and the monitoring of stress in humans. Currently, measurements are performed predominantly by radioimmunoassay (RIA) which is expensive, labour intensive, uses hazardous radioisotopes and involves extensive delays in obtaining results. A rationally designed cortisol-linker conjugate allowing high assay sensitivity was employed as a coating antigen in a microfluidic surface plasmon resonance (SPR) biosensor immunoassay for the ultrasensitive and rapid detection of salivary cortisol. Detection of cortisol is by competitive immunoassay using a secondary antibody for signal enhancement. The method requires no chemical extraction or complex sample pre-treatment despite high saliva viscosity. The cortisol assay was optimized for maximum sensitivity in buffer before being adapted for the salivary matrix, where it showed a limit of detection of 49 pg/mL. The results showed good correlation to RIA ($r = 0.94$). The biosensor assays showed an inter-assay coefficient of variation (CV) of 13.5% and recoveries close to 100%. The covalently immobilized sensor surface provided stable responses for more than 140 binding and regeneration cycles, enabling re-use. Cortisol in saliva was detected across the physiologically relevant range using the SPR immunobiosensor by employing a rationally designed assay format including signal enhancement for maximum sensitivity. The system can handle saliva matrix effects by use of chemical treatment during the assay to reduce non-specific binding to sensor surfaces. This sensor system provides an automated, high sensitivity analytical tool capable of yielding results in approximately 15 min. This biosensor could potentially be used for active stress-monitoring and in the diagnosis of disease.

Introduction

Cortisol is a key steroid hormone involved in the restoration of homeostasis after stress. Its level in human blood serum is a well-known indicator of stress[1–3] and a number of immunoassay systems are available for its measurement.[4–6] Cortisol levels in serum also change substantially with Addison's disease and Cushing's syndrome.[7,8] More recent research has indicated that salivary cortisol concentrations are well correlated with serum free cortisol,[9,10] as the non-polar steroid can effectively pass through the lipid membranes of the salivary glands. From a physiological perspective, the fact that salivary cortisol represents free or bioavailable cortisol can be argued to be an advantage when assessing hypothalamic pituitary adrenal axis function compared with measurement of total plasma levels.[11] Another key advantage of saliva over plasma is that measurement is non-invasive. This reduces patient stress and compliance issues particularly where subjects require regular monitoring with repeated sampling.[12,13] Furthermore, the taking of saliva samples can be done by the patient in their own home rather than requiring trained personnel. Measurement in saliva offers a convenient non-invasive method of profiling cortisol concentrations, and a number of radioimmunoassay (RIA) and ELISA kits have been developed for the salivary matrix.[14–16] These techniques, however, suffer from significant limitations such as long wait times for results (usually one to two days), high labour requirements, high costs per sample analyzed, problems of reliability in detecting steroids in the complex salivary matrix, little or no automation, and the handling of hazardous radioisotopes for RIA.

There are a number of applications that would benefit from more rapid monitoring of salivary cortisol fluctuations in humans. These include the identification of stress in workers and athletes, so that timely interventions can be made to minimize stress responses, and the diagnosis and management of diseases such as Addison's disease and Cushing's syndrome.[17,18] Microfluidic biosensor technology offers a potential means of automating immunoassays and producing concentration measurements so that individual results can be available in minutes rather than days.

Surface plasmon resonance (SPR) is one promising biosensor transduction technology that uses very small changes in the refractive index of a chemically modified noble metal surface to detect binding of mass on the surface.[19,20] It is possible to immobilize small molecules onto the sensing surface and then bind primary monoclonal antibodies in a competitive assay format, with the additional option of

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secondary antibody signal enhancement to increase bound mass and sensitivity.[21–23] The biosensor operates in a flow-through mode where solutions are pumped over the sensing surface, and the refractive index changes upon binding and therefore assay concentration data are obtained in real-time. One of the most popular commercial SPR instruments available is the BIAcore™ system, which utilizes a carboxymethylated dextran polymer on the gold sensor surface to present carboxylic acid groups for immobilization of proteins and/or antigens. Recent advances in small molecule sensing on SPR platforms have enabled highly sensitive detection of some important steroid biomolecules through incorporation of convenient labeling nanochemistry and rational design of highly stable sensing surfaces.[21–23] Previous studies have established that linking a steroid antigen to a sensing surface in a flow-through biosensor *via* the 4-position of the steroid, with use of a linker of about 15 atoms in length, confers a significant increase in binding strength through the relief of steric hindrance.[21–23] This approach can be utilized for cortisol to produce an amine-terminating linker conjugate that can be covalently immobilized on a polymer surface, as opposed to less stable thiol immobilization.[24]

Despite SPR being widely used, there is a paucity of studies on its application to biological samples. The most common medium investigated is bovine milk for detecting compounds such as reproductive hormones and β-lactams,[25,26] but these have relied on extensive dilution of samples in applications requiring only high limits of detection. For human saliva, only interleukin-8 [27] and phenytoin[28] immunoassay with SPR has been reported and these techniques require laborious pre-concentration or pre-conditioning and filtration of samples. Furthermore, there have been no reports on using SPR for detection of salivary cortisol. The measurement of cortisol in human saliva offers the significant challenges of a very low concentration range (typically 0.1–10 ng/mL) and a complex biological matrix containing high levels of mucins and protein, with potential problems of high non-specific binding or the need for laborious solvent extraction.

We report the development of a rapid, ultrasensitive, microfluidic SPR immunoassay system for cortisol detection. The cortisol analyte is derivatised and covalently conjugated to a carboxymethylated dextran biosensing surface for maximum antibody binding and immobilization stability utilizing specialist conjugation chemistry. In the immunoassay, cortisol-containing sample (saliva or buffer) is mixed with primary antibody and injected into the flow-through biosensor where it competes with surface immobilized cortisol for binding to the antibody. Surface-bound primary antibody is then bound by a secondary antibody to greatly enhance the biosensor signal by increasing bound mass on the surface, which can then be regenerated using high pH and chaotropic reagent ready for a new assay cycle (Fig. 1). In this format, the biosensor signal is inversely related to the cortisol concentration.

The assay performance was evaluated in buffer and then adapted for use in human saliva employing a special chemical agent to minimize non-specific binding in the microfluidic channel whilst retaining specific antibody binding. The assay detection limits were assessed in saliva and the assay was then correlated against radioimmunoassay using a set of saliva samples from male volunteers.

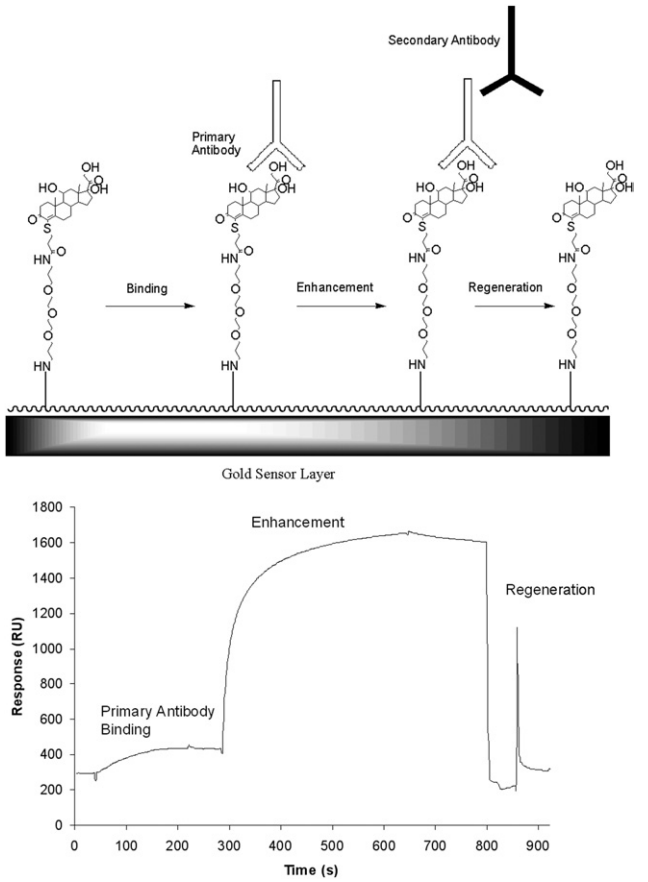

Fig. 1 Binding schematic of the SPR biosensor cortisol assay with a sensorgram plot of response (response units (RU)) *vs.* time (s) showing the responses obtained on primary antibody binding, secondary antibody signal enhancement and surface regeneration.

Materials and methods

Materials

Cortisol was obtained from Steraloids (Q3880-000) and sodium dodecyl sulfate (SDS) from J.T. Baker (4095-02). Activated charcoal was from Merck (1.02186.1000). All other chemical reagents were from Sigma Aldrich. Monoclonal primary antibody (mAb) to cortisol was from US Biologicals (raised in mouse, C7904-11B) and anti-mouse IgG-horse radish peroxidase (HRP) secondary antibody (A9044) and anti-mouse IgG (M7023) were from Sigma. BIAcore™ SPR experiments were performed on BIAcore 2000™ and BIAcore 3000™ instruments with CM5 dextran chips supplied by BIAcore™. Running buffer for all experiments was HEPES (0.01 M) buffered saline (0.15 M) with EDTA (3 mM) and Tween-20 (0.005% v/v) (HBS-EP). The radioimmunoassay kit was from Diagnostics Systems Laboratories USA (DSL-2000) and was adapted for use in saliva based on previous reports.[12,13]

Synthesis of cortisol derivative

Please see the ESI† for synthetic methods and characterization data for the cortisol derivative and all intermediates.

Immobilization of cortisol derivative

A CM5 dextran chip was docked into a BIAcore 2000™ or 3000™ instrument and flow cell 2 or 4 was immobilized with steroid as follows. *N*-Ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide (EDC) (0.4 M) was mixed 1 : 1 with *N*-hydroxysuccinimide (NHS) (0.1 M) and injected (2 × 50 μL at 5 μL/min) to activate the surface. This was immediately followed by compound **6** (see ESI†) (4 × 100 μL at 5 μL/min, 1 mg/mL 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.

Cortisol immunoassay in buffer

All flow rates for antibody binding were 20 μL/min unless otherwise stated. All assay curves were fitted to a four-parameter logistic curve fit using SigmaPlot™ software. Limits of detection (LOD) were computed as the concentration corresponding to the blank value less two standard deviations of the blank ($n = 5$). Sensitivity was calculated as the slope of the linear portion of the assay curve. A secondary antibody dilution plot was prepared by injection of monoclonal antibody (mAb) (11 ng/mL, 60 μL) followed by anti-mouse IgG (0, 5, 10, 25, 50, 75, 150, 300 μg/mL, 60 μL) with five replicates for each point. The surface was regenerated with 200 mM NaOH 20% v/v MeCN (20 μL). A mAb dilution plot was prepared by injection of varying concentrations of mAb (0, 5, 10, 25, 50, 100, 130, 160 ng/mL, 60 μL) followed by anti-mouse IgG (300 μg/mL). There were five replicates of each point with regeneration as above. A buffer assay standard curve for cortisol was constructed by mixing mAb (50 ng/mL) 1 : 1 with cortisol in running buffer (0, 1, 5, 10, 25, 50, 100, 250, 1000, 5000, pg/mL) in a 96-well microtitre plate. Following 5 min of incubation at room temperature, 60 μL of standard was injected, and this was immediately followed by injection of anti-mouse IgG (300 μg/mL, 60 μL). The surface was regenerated as before. There were five replicates of each point.

Collection and treatment of human saliva

Participants gave informed consent to the donation of saliva. The project was approved by the New Zealand Northern Y Regional Ethics Committee (NTY/07/02/017). All donors were healthy males. The donors were instructed to chew a small tab of sugar-free gum and discard the first 30 s of saliva generated. They then, whilst continuing to chew, produced 8–10 mL of saliva, which was collected directly into a polypropylene tube. The saliva was then immediately frozen in a −20 °C freezer and then transferred to a −80 °C freezer for longer storage. When required for analysis, the samples were thawed at room temperature and centrifuged (14 000 × *g*, 5 min) and the supernatant was transferred to the BIAcore™ sampling rack for analysis. The freeze–thaw process precipitated salivary mucins and other proteins. Human saliva to be used as diluent for the standards was stripped with activated charcoal[29] using the supernatant after centrifugation (10 mg/mL loading, shaken at 560 rpm overnight) and was then centrifuged again (4620 × *g*, 15 min) to remove the charcoal.

Cortisol immunoassay in human saliva

Monoclonal antibody (mAb) was injected (11 ng/mL, 60 μL) and various anti-mouse secondary antibodies were then bound and assessed for non-specific binding and signal enhancement with an IgG-HRP conjugate being selected at 1 : 50 dilution (1 : 25 dilution can also be used for higher signal enhancement). A mAb dilution plot was prepared by injecting varying concentrations of mAb (0, 5, 10, 25, 50, 100, 130, 160 ng/mL) (60 μL in running buffer) followed immediately by IgG-HRP (1 : 50, 60 μL, 10 μL/min). The surface was regenerated using 200 mM NaOH, 20% v/v MeCN (20 μL). Each mAb concentration was run in quadruplicate. A saliva assay curve for cortisol was constructed by mixing mAb (50 ng/mL) 1 : 1 with cortisol standard in running buffer (0, 5, 10, 25, 50, 100, 250, 1000, 5000, 10 000 pg/mL) in a 96-well microtitre plate and then injecting immediately (60 μL). This was immediately followed by injection of IgG-HRP (1 : 25, 60 μL). The surface was regenerated with 50 mM NaOH 20% v/v MeCN. There were five replicates of each point.

Correlation to radioimmunoassay

Saliva samples ($n = 40$) collected from 40 different healthy male donors at various times throughout the working day (0900 h–1700 h) were run in the assay. These were each run in duplicate along with cortisol standards in charcoal-stripped saliva (0, 25, 50, 100, 250, 500, 1000, 2500, 10 000, 25 000 pg/mL) using the same saliva method as above but with mAb at 75 ng/mL in HBS-EP with 0.01% w/v SDS. Intra-assay CV was calculated as the mean of the CV of 40 human saliva samples from 40 different male subjects assayed in duplicate. Inter-assay CV was calculated as the mean of the CV of four human saliva samples from four different male subjects assayed in duplicate over two days. Analytical recoveries were determined at spiked concentrations of 0.1, 1 and 10 ng/mL cortisol. Using the secondary antibody response only, the data were fitted as above and the formula used to calculate concentrations. The correlation, regression coefficient and confidence intervals for the relationship between the RIA and SPR methods were calculated using Spearman's rank correlation coefficient due to the data not being normally distributed.

Results

Synthesis of cortisol derivative

Synthetic details are provided in the ESI,† including some adapted methods.[30–33] A carboxylic acid group was attached to cortisol for linker attachment at the 4-position by formation of an epoxide across the 4,5-double bond by a modification of existing methods.[28–30] This was then reacted with 3-mercaptopropionic acid to yield a carboxylic acid function in good yield and high purity with modifications from a previous method.[31] This acid was then activated with NHS to produce a compound highly reactive with primary amines and this was isolated. Reaction was then possible with a non-immunogenic oligoethylene glycol linker with one of its primary amine termini protected by a *t*-butoxycarbonyl protecting group. This cortisol linker conjugate could then be treated with formic acid as required to cleave the protecting group and leave a reactive

primary amine for conjugation to the carboxylic acid-bearing dextran-sensing surface of an SPR biosensor. All reactions occurred in acceptable yields.

Immobilization of cortisol derivative

By using EDC/NHS activation of the dextran carboxylic acid modified surface, it was possible to immobilize the steroid *in situ* conveniently by simply flowing it over the surface at alkaline pH. Sufficient quantities of the steroid were used to ensure saturation of the surface binding sites. The conjugated surface was then fully stable, giving reliable responses for more than 140 binding and regeneration cycles, thus showing a clear ability to process large numbers of samples. The stability limit of this surface has not been reached and other surfaces similarly constructed have lasted for more than 1100 cycles.[23]

Cortisol immunoassay in buffer

An assay for cortisol in buffer was constructed using the covalently immobilized SPR sensor surface and a schematic of a typical assay cycle is given in Fig. 1. To determine the optimal concentration of secondary antibody needed for high signal enhancement and to determine the relationship between response and secondary antibody concentration, a secondary antibody dilution plot was prepared for the sensing surface (Fig. 2). Under these conditions, the primary antibody is bound to the surface at a constant concentration and the secondary antibody is bound at varying concentrations. Optimal response was given at 300 μg/mL of secondary antibody and this was applied in optimizing the primary antibody loading. The primary antibody dilution plot is given in Fig. 3, top. The secondary antibody signal enhancement as determined by the ratio of the slopes of the enhanced and un-enhanced lines of the plot is 10.3-fold, slightly better than that seen in previous reports.[21–23] When translated to an assay standard curve (Fig. 3, bottom), this buffer format gave a limit of detection of 13 pg/mL and a sensitivity of 72 RU.mL/ng (Table 1).

Cortisol immunoassay in human saliva

To determine the effect on assay sensitivity and performance of running the assay standard curve in saliva, the medium was changed to human saliva, stripped with activated charcoal to remove endogenous cortisol, and the assay conditions altered to accommodate it; this included using anti-mouse IgG-HRP conjugate to reduce secondary antibody non-specific binding. The primary antibody concentration plot was determined (Fig. 4, top), and the enhancement decreased to 7.3-fold. Testing was performed to establish whether saliva needed to be diluted before use in the assay to preserve the specific binding signal. Results showed that dilution of stripped saliva had no effect on this signal and that saliva could be used without prior dilution, with the viscosity of the liquids being acceptable for the instrument to function. A higher sample throughput was achieved by removing the pre-incubation for the sample and antibody, and by reducing the time allowed for secondary antibody binding. The concentration of primary antibody was also increased so that the curve would better cover the physiological range (assay dynamic range

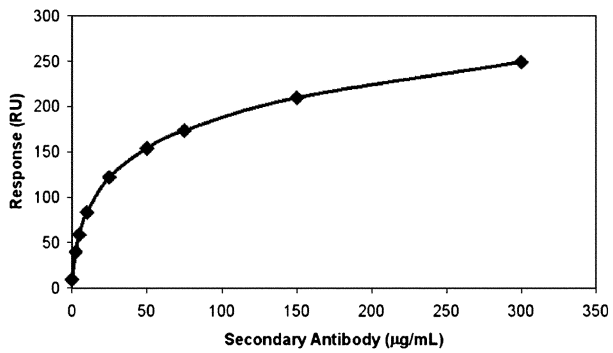

Fig. 2 Plot of total response *vs.* secondary antibody concentration for the cortisol SPR biosensor in buffer.

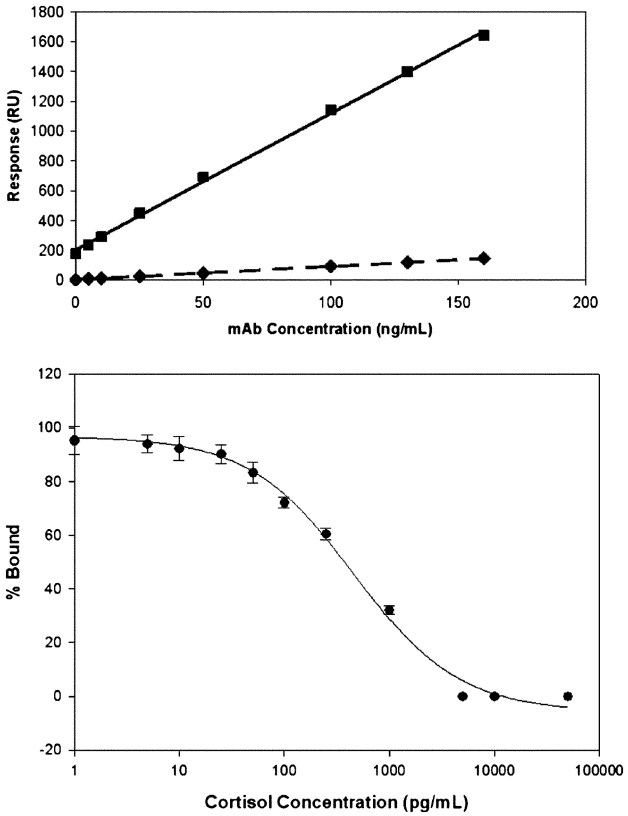

Fig. 3 Top: plot of response *vs.* primary antibody concentration for primary antibody-only (---◆---) and secondary antibody-enhanced response (—■—) for the cortisol biosensor in buffer. The linear fit equations are: $y = 0.892x + 3.80$ ($R^2 = 1.000$) for primary antibody and $y = 9.16x + 204$ ($R^2 = 0.999$) for secondary antibody. Bottom: cortisol assay standard curve in buffer.

Table 1 Assay standard curve parameters for cortisol SPR immunoassay in buffer (HBS-EP) and human saliva (charcoal-stripped)

Medium	Limit of detection (pg/mL)	IC_{50} (pg/mL)	Sensitivity (RU.mL/ng)
Buffer	13	376	72
Human saliva	49	297	162

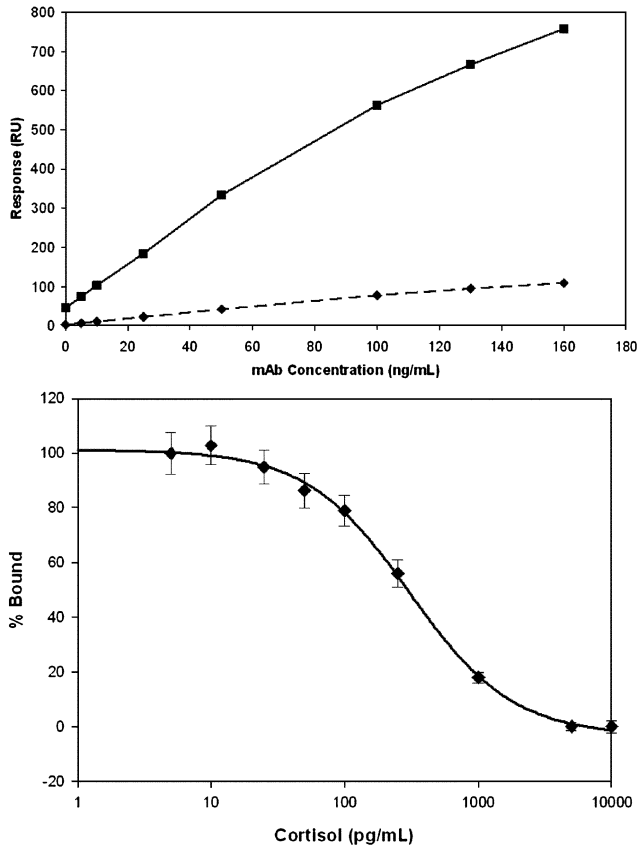

Fig. 4 Top: primary antibody dilution plot for the cortisol biosensor showing primary antibody-only response (---◆---) and the IgG-HRP secondary antibody-enhanced response (—■—). The equations for fitted linear regression lines up to 50 ng/mL were: $y = 0.789x + 2.34$ ($R^2 = 1.000$). Above 50 ng/mL the curves flatten, indicating saturation binding to the surface. Bottom: cortisol assay standard curve in human saliva.

of 91–934 pg/mL, higher concentration samples can be diluted as required). This assay format when used with saliva gave a limit of detection of 49 pg/mL and a sensitivity of 162 RU.mL/ng (Fig. 4, Table 1).

Correlation to radioimmunoassay

To cope with variations in the levels of mucins and proteins between samples causing variations in non-specific binding levels, SDS was introduced to the antibody diluent and the assay applied to the measurement of human samples from a number of subjects. The data were plotted against measurements made on the same samples by radioimmunoassay and the correlation is plotted in Fig. 5 ($n = 40$). The scatter gives a strong correlation of $r = 0.94$ (95% CI; 0.88, 0.97). As well as establishing a correlation with an existing technique, these data were gathered to ensure that the assay could cope with samples from a significant number of people whose salivary matrices may differ. The cortisol concentration intra-assay CV was 15.3% and the signal response intra-assay CV was 8.5%. The cortisol concentration inter-assay CV was 13.5%. Analytical recovery was 96.8% at 0.1 ng/mL, 102.8% at 1 ng/mL and 102.4% at 10 ng/mL cortisol concentration.

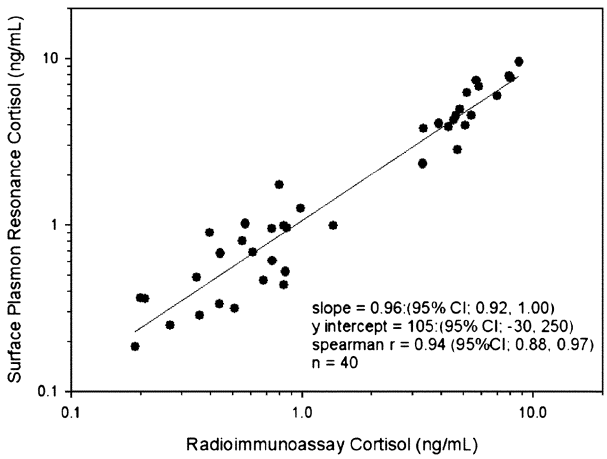

Fig. 5 Correlation between SPR salivary cortisol determination and RIA cortisol determination for samples from 40 healthy men taken at random times throughout the day, using regression analysis, as described in the Materials and methods section.

Discussion

The first step in developing the SPR biosensor platform for cortisol in human saliva was the synthesis of a cortisol derivative to conjugate to the sensing surface. Previous studies have shown that conjugation *via* the 4-position of a steroid with a linker length of about 15 atoms gives the best presentation of the antigen for antibody binding.[21–23] The use of an oligoethylene glycol linker ensures low antibody binding to the linker itself[34,35] and the covalent attachment to the sensor surface ensures a very stable sensing surface that can withstand the harsh regeneration conditions needed to remove the antibodies. The use of epoxide chemistry allows conjugations to the 4-position of steroids bearing a 4,5-double bond. High chemical stability ensures good sensor baseline stability and highly repeatable antibody bindings, critical for a reliable and reusable sensor. The immobilization process is very simple and can be run automatically on the BIAcore™ software. This is the first report of this type of linker conjugate for cortisol using a terminal amine for immobilization.

To determine whether the sensor would be sensitive enough to detect human salivary cortisol in its physiological range of 0.1–10 ng/mL, it was necessary first to determine sensor performance in buffer before introducing the challenges of a salivary matrix. This involved the optimization of the secondary antibody loading, by determining how total response varied with the concentration of the antibody. A secondary antibody concentration of 300 μg/mL was used to avoid the problems of higher non-specific binding whilst retaining maximum signal enhancement (Fig. 2). By altering the primary mAb concentration and examining how both total enhanced and primary antibody-only responses varied, the level of enhancement from the labeling antibody could be determined. For a reliable SPR assay, a signal strength of 100 RU is usually sufficient on a BIAcore™ system and so the primary antibody concentration that could give this response was chosen. It is desirable to reduce the primary antibody concentration as much as possible without compromising signal strength too much, as lower concentrations yield lower limits of detection in competitive immunoassays such as this.

When these optimized concentrations were applied in the assay, it yielded a limit of detection well below that required for cortisol detection in saliva (13 pg/mL compared with the 100 pg/mL required). This buffer-based assay format is also valuable in the detection of cortisol in extracted samples that have been reconstituted in buffer.

Human saliva is a very complex matrix consisting of a high level of mucins and proteins, all of which can contribute both to the viscosity of the fluid and to the level of non-specific binding that can occur on sensor or immunoassay surfaces, which may distort results. Biosensors that have measured from biological matrices such as milk in the past have tended to rely on dilution or extensive pre-treatment or filtration to reduce these effects. It was therefore of interest to see whether dilution would have any effect on the level of both non-specific binding and specific binding from antibodies, and whether the instrument would require prior dilution before it could handle these samples. The results from this study indicated that dilution in buffer up to 1 : 8 for saliva : buffer had no effect on either total or non-specific binding for this sensor, and so dilution was not necessary for the effective operation of the BIAcore™. However, it was found that there were variations in the levels of non-specific binding between individuals that necessitated the use of SDS to minimize this binding and any effects it might have on the assay responses. SDS is a well-known cleaning agent for the removal of proteins from sensor surfaces and tubing and is commonly used in regeneration solutions. When used at 0.01% w/v in the antibody diluent, we have found that SDS is very effective in removing salivary non-specific binding for this biosensor but has no effect on primary antibody binding. This treatment is necessary to ensure the reliability of the SPR method and removes the need for solvent extraction of the samples or similar complex pre-treatments.

Even with saliva present in the assay matrix, this did not increase the limit of detection above the lower physiological limit, with a final LOD of 49 pg/mL. In fact, the primary antibody concentration was then deliberately increased to adjust the assay curve range to fit the physiological concentration range better. For application with saliva, the labeling secondary antibody was changed to an IgG-HRP conjugate, as this demonstrated lower non-specific binding whilst still delivering good enhancement of signal.

To determine how well the assay could measure cortisol in human saliva samples, it was applied to measurements across 40 individuals and the results compared with those from radioimmunoassay (Fig. 5). This indicated a good correlation between the two methods, with a slope close to 1, and confirmed the efficacy of the technique relative to an established radioimmunoassay technique. It also indicates that the SPR technology can cope with variations in the salivary matrix between individuals and give reasonable results as determined by radioimmunoassay. The parameters of variability were all acceptable and indicated reasonable reliability. The assay only requires 50 μL of saliva per sample replicate, so subjects would only need to donate a small volume of saliva each time, making multiple analyses of salivary cortisol from one subject easy. Binding and regeneration can be completed in 10 min for each sample, allowing a fast throughput of samples with data available as soon as the cycle is complete rather than having to wait for an entire plate of samples to be finished. This enables rapid hormone measurement. The cost per sample is also very low after instrument purchase, as no radiolabels are used, the primary antibody concentrations are very low and the system is automated.

Conclusion

A rationally designed cortisol derivative has been synthesized and immobilized to form a highly stable SPR biosensing surface in microfluidic channels of a BIAcore™ instrument. This surface has been used to produce a very sensitive immunoassay of cortisol in buffer that has then been adapted for use in human saliva, with special measures to reduce non-specific binding without the need for complex sample pre-treatment. These highly sensitive measurements have been possible in a microfluidic system despite the high viscosity and great complexity of the human saliva matrix and can be made without the need to pre-concentrate samples, thus representing a further advance for lab on a chip systems measuring in complex biological media. The assay measurements compare well with traditional radioimmunoassay and do not use hazardous materials. This biosensor system provides an automated, rapid analytical technology for monitoring of cortisol in human saliva and can be applied to a range of diagnostic and stress-monitoring applications.

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