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Research paper

Label-free monitoring of antibody–antigen interactions using optical microchip biosensors

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ABSTRACT

A rapid, label-free optical biosensor system for sensitive monitoring of bio-molecular interactions in real-time is presented. SpectroSens™ sensor chips are based on integrated planar Bragg gratings sensitive to localised changes in refractive index. Bio-molecule recognition is imparted by functionalisation of the sensing surface with antibodies against targets of interest. In this study, antibodies against selected proteins were oriented with recombinant Protein A/G, which was covalently immobilised to the sensor chip via amine coupling to a glutaraldehyde-activated silane layer. Immunoassays for the detection of rabbit IgG and ovalbumin proteins as model antibody–antigen interaction systems were performed. Binding of complementary antigens to respective antibody-functionalised sensors manifested as changes in wavelength of light reflected from the optical sensors. Quantitative binding kinetics with detection sensitivities in the mid ng/ml range were obtained for both antigens using this planar, two-dimensional surface coating. Data presented demonstrate the suitability of SpectroSens™ sensors as a valuable tool in life science research and development for monitoring bio-specific interactions, protein concentration determination and antibody selection; the optical integration and analytical characteristics of these sensors suggest that they may find numerous applications in bio-pharmaceutical development and clinical diagnostics.

1. Introduction

The detection and quantification of biological entities are central to many areas of life science research and development, and healthcare, ranging from the discovery of new drug targets and monitoring of bio-pharmaceutical manufacture to disease diagnosis. Real-time, label-free detection techniques are highly desirable for these purposes as they circumvent the need to modify target molecules and allow monitoring of reaction kinetics. Biosensor technology has received heightened interest over the last two decades, since the launch of the BIAcore instrument based on Surface Plasmon Resonance in late 1990 (Karlsson et al., 1991). Whilst now commonplace in the laboratory for label-free measurement of antibody–antigen interactions, limitations associated with versatility have prompted the development of alternative sensing platforms with improved characteristics. Recent technological innovations have produced numerous sensing devices capable of rapid, sensitive, label-free detection of bio-molecules; different approaches include electrochemical detection (Darain et al., 2003; Tang et al., 2005), surface-acoustic waves (Rupp et al., 2008), quartz crystal microbalances (Liu et al., 2003) and a multitude of optical transducers, comprising interferometric devices (Schmitt et al., 2007), ring resonators (Ramachandran et al., 2008), fibre-optic sensing (Tazawa et al., 2007) and optical waveguide sensors (Zourob et al., 2005), and this list is by no means exhaustive.

Recently, a novel optical microchip sensing platform, SpectroSens™, exploiting optical integrated circuits ubiquitous in the telecommunications industry, has been reported in its capacity for the detection of biological agents (Bhatta et al.,

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2010). SpectroSens™ sensor chips comprise high-precision planar Bragg gratings, which reflect light at precisely defined wavelengths that are defined by the average refractive index within the sensing region in accordance with Bragg's Law:

$$\lambda_{max} = 2\ \Lambda\ n_{eff}$$

Hence, changes in the immediate environment surrounding the grating result in observable shifts in reflected light from the sensor chip. Functionalisation of the sensing surface with antibodies selected against potential targets determines sensitivity to specific analytes. Complementary antigen-binding to target-selective antibodies results in localised changes in refractive index, which are measured by the associated changes in sensor peak reflection. The sensor response correlates to the increase in surface mass associated with the binding interaction between antigen and antibody and is therefore directly proportional to the analyte concentration in the sample medium under mass-transport limiting conditions. This manuscript describes the characterisation of SpectroSens™ optical sensor chips for the quantitative monitoring of antibody–antigen interactions using immunoglobulin G (IgG) and ovalbumin (OVA) as model antigens.

2. Materials and methods

2.1. Materials

All chemicals were of analytical grade unless otherwise stated. Albumin from bovine serum (BSA, electrophoresis grade), 3-aminopropyltriethoxysilane (APTES) (99%), Dulbecco's phosphate buffered saline (PBS), pH 7.4, glutaraldehyde solution, Grade II, 25% (v/v) and sodium cyanoborohydride were purchased from Sigma-Aldrich Company (Dorset, UK). Acetone (HPLC grade) and Decon 90 liquid detergent were purchased from Thermo Fisher Scientific Ltd. (Leicestershire, UK).

Affinity-purified goat polyclonal anti-rabbit IgG antibody (lyophilised powder), chromatographically-purified rabbit IgG (5 mg/ml in 10 mM PBS, 0.1% sodium azide) and mouse IgG (2.5 mg/ml in 10 mM PBS, 0.1% sodium azide) were purchased from Zymed Laboratories, Invitrogen Ltd. (Paisley, UK). Recombinant protein A/G from *Escherichia coli* (lyophilised powder) was obtained from Pierce, Thermo Fisher Scientific (Leicestershire, UK). Antigen-affinity-purified rabbit polyclonal anti-OVA antibody and ovalbumin protein (Grade V) were supplied by DSTL (Porton Down, UK).

2.2. Sensor chip fabrication

Spectrosens™ chips (20×10 mm) were fabricated using the direct UV writing method described previously (Emmerson et al., 2002). The overclad layer directly above the sensing region was then removed using a wet etch process and coated with a high index titanium oxide layer (Sparrow et al., 2009). Finally, chips were fibre-pigtailed to enable spectral measurements.

2.3. Sensor surface functionalisation

Functionalisation of sensing surfaces was carried out as described in Bhatta et al. (2010). Briefly, sensor chips were cleaned by immersion in Decon 90 for 5 min under agitation, after which they were rinsed with distilled water and then acetone, before drying under nitrogen. Chips were then incubated with 4% (v/v) APTES in acetone for ~30 min under agitation, before extensive rinsing of the chips with acetone and subsequent drying under nitrogen. The resultant silane layer was left to cure at 110°C for 1 h. Activation of the silanised surfaces was carried out by incubation with 2.5% (v/v) glutaraldehyde in PBS for 1 h, after which excess glutaraldehyde was removed by extensive rinsing of chip surfaces using PBS.

2.4. Antibody immobilisation

Activated chips were exposed to Protein A/G (2 mg/ml) in 40 mM sodium cyanoborohydride in PBS for approximately 40 min before rinsing in PBS. Test chips were exposed to either goat anti-rabbit IgG antibody (500 μg/ml) or rabbit anti-OVA antibody (500 μg/ml) in PBS for approximately 45 min and then washed with PBS. Control chips were exposed to mouse IgG antibody (500 μg/ml) for the same time period. All chips were then incubated with 1% (w/v) BSA in PBS for approximately 30 min.

2.5. Detection of rabbit IgG and ovalbumin protein antigens

Protein detection was evaluated using a SIS:Lab II instrument produced by Stratophase Ltd., UK. Sample delivery to the sensor chips was achieved by means of a fluidic module comprising syringe pumps upstream of PTFE flow cells (FlowCubes) of ~5 μl volumes (dimensions: 5 mm (l)×5 mm (w)×0.2 mm (h)). Antibody-functionalised sensor chips were mounted in the FlowCubes and equilibrated with phosphate-buffered saline (PBS, pH 7.4) until a stable baseline was achieved. Selective binding of corresponding antigen partners was assessed by introduction of rabbit IgG to anti-rabbit IgG-functionalised sensor chips and addition of ovalbumin protein to anti-OVA functionalised sensor chips in a flow-through format. Antigen binding to mouse IgG (control antibody)-functionalised sensor chips was monitored in parallel to assess the extent of non-specific binding. Sensor chips were exposed to varying concentrations of each antigen (ranging from 500 ng/ml to 20 μg/ml for rabbit IgG antigen and between 250 ng/ml and 20 μg/ml for ovalbumin protein) for 10 min before re-introduction of PBS to remove any loosely bound antigen. All experiments were carried out in triplicate using a flow rate of 50 μl min^{-1} with PBS running buffer at room temperature.

3. Results and discussion

The purpose of this work was to evaluate the use of Spectrosens™ optical microchip sensors for the real-time analysis of bio-molecular binding interactions. The optical chip design used in this study comprised two discrete refractive index sensing regions and a single temperature sensitive region (used previously in Bhatta et al., 2010). Detection sensitivity and specificity were ensured through the directed immobilisation of analyte-selective antibodies on the top-surface of the sensor chip.

3.1. Real-time monitoring of antibody immobilisation

Titania-coated SpectroSens™ surfaces were functionalised by exploiting the well-documented self-assembly of organosilanes on metal oxide surfaces (Liedberg and Cooper, 1998). Recombinant Protein A/G was attached to aminosilanised sensor surfaces via amine coupling using a glutaraldehyde cross-linker; this allowed orientation of capture antibodies via their Fc-binding regions. The structure of the bio-recognition layer is illustrated in Fig. 1.

Surface-immobilisation of the detection antibodies is critical to the performance of the biosensor and was assessed by monitoring the magnitude of increase in the sensor response associated with antibody binding in real-time (Fig. 2); continued investigation into alternative coupling chemistries to improve the activity of immobilised antibodies is ongoing.

To demonstrate the potential of the SpectroSens™ system, two model antibody–antigen systems were chosen for analysis; the binding interaction with rabbit IgG using anti-rabbit IgG-functionalised sensor chips and detection of ovalbumin protein using anti-OVA-functionalised sensor chips were performed. For both assays, antigen binding to mouse IgG-functionalised sensor chips was monitored in parallel as sensor controls.

Real-time sensorgrams demonstrating typical antibody immobilisation and subsequent antigen detection sequences for both IgG and ovalbumin immunoassays as monitored by SpectroSens™ are illustrated in Fig. 2.

Stage 1 demonstrates the covalent coupling of Protein A/G to activated aminosilane-functionalised SpectroSens™ chips; the initial increase in sensor reflected wavelength is due to a change in bulk refractive index associated with the relatively high concentration of Protein A/G used (2 mg/ml); the shift in the baseline of approximately 100 pm upon return to PBS indicates the true magnitude of Protein A/G binding. Stage 2 illustrates the binding of capture antibodies, which is associated with a wavelength shift of between 400 and 550 pm for all antibodies; anti-rabbit IgG (solid line) and mouse IgG (dashed line) in Fig. 2A and anti-OVA (solid line) and mouse IgG (dashed line) in Fig. 2B. Stage 3 represents a blocking stage and shows negligible signal changes indicating near complete saturation of the sensor surface during the previous antibody immobilisation stages. Stage 4 within the insets of each graph shows the real-time sensor responses associated with the binding of complementary antigens to the antibody-functionalised sensors; Fig. 2A, Stage 4 shows the specific increase in sensor peak wavelength of approximately 20 pm over ~15 min associated with the binding interaction between rabbit IgG (2 μg/ml) and immobilised anti-rabbit IgG, whilst negligible changes were observed on the control antibody-functionalised sensor. Similarly, Fig. 2B, Stage 4 shows the relatively large increase in wavelength shift (~240 pm over 15 min) associated with binding of a high concentration of ovalbumin protein (20 μg/ml) to surface-

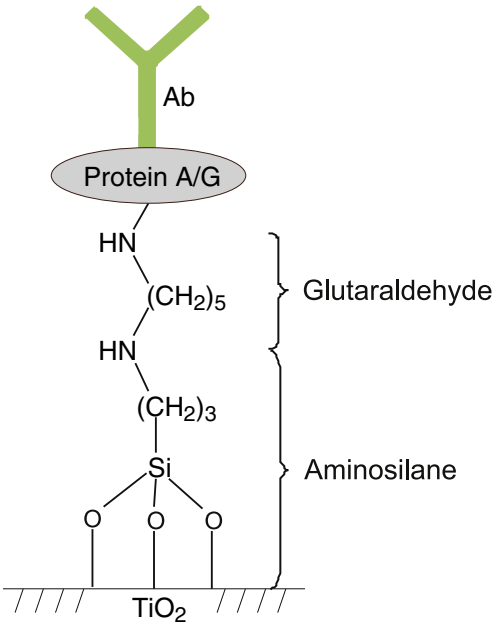

Fig. 1. Schematic illustration of antibody immobilisation. Analyte-specific antibodies were oriented via recombinant Protein A/G, which was covalently immobilised to the aminosilanised titanium oxide sensor surface via cross-linking with glutaraldehyde.

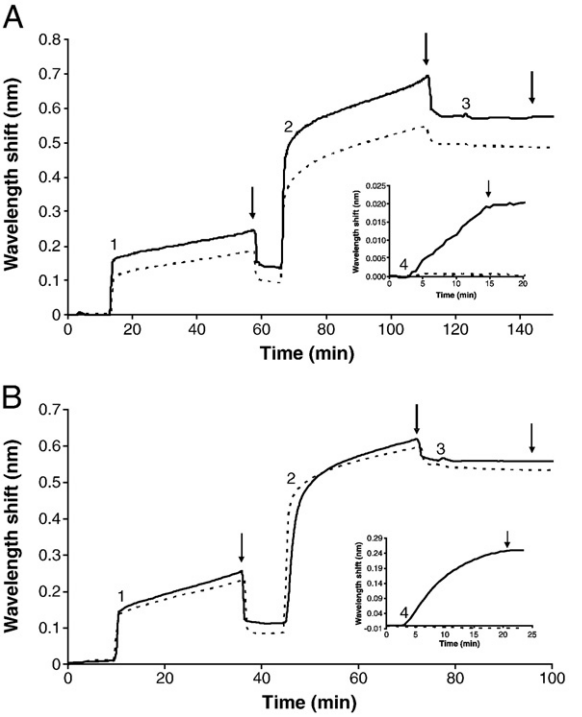

Fig. 2. Real-time SpectroSens™ sensorgrams illustrating antibody immobilisation and subsequent antigen binding (A) IgG binding assay, (B) Ovalbumin binding assay. Sensors were equilibrated in PBS running buffer at 50 μl/min. (A) 1. Introduction of protein A/G linker, 2. Addition of anti-rabbit IgG test antibody, (−), Mouse IgG control antibody (−−), (3) Addition of BSA blocking agent. Downward arrows represent introduction of PBS buffer. Inset displays magnified subsequent antigen binding profile (4) Addition of rabbit IgG (2 μg/ml). (B) 1. Introduction of Protein A/G linker, 2. Addition of Anti-OVA test antibody (−), Mouse IgG test antibody (−−), 3. Addition of BSA blocking agent. Downward arrows represent introduction of PBS buffer. Inset displays magnified subsequent antigen binding profile 4. Addition of OVA protein (20 μg/ml).

immobilised anti-OVA and no significant changes in response were observed on the sensor control.

3.2. IgG binding assay

In order to quantitate SpectroSens™ sensor responses, the binding interactions of increasing concentrations of rabbit IgG (between 500 ng/ml and 20 μg/ml) with surface-immobilised anti-rabbit IgG antibodies were monitored by recording the associated changes in sensor peak wavelength in real-time. The concentration dependency of the IgG binding kinetics is depicted in Fig. 3.

Evident from these data are the differences in the rate of increase in sensor wavelength associated varying IgG concentrations, enabling quantification of the SpectroSens™ response through determination of the initial binding rates from the linear regions of the binding curves. A calibration graph displaying the rate of change in sensor peak wavelength as a function of rabbit IgG concentration is shown in Fig. 3B; this shows a linear relationship within the concentration range analysed with a correlation coefficient of 0.987. The extrapolated limit of detection for the assay (based on the antigen concentration producing a sensor wavelength shift that is 3 times the standard deviation for repeated measurements ($n=3$) of blank samples) is approximately 250 ng/ml. These data demonstrate satisfactory detection sensitivity and dynamic range of the anti-IgG SpectroSens™ chips, suitable for quantification of many clinical and pharmaceutically relevant analytes.

3.3. Ovalbumin binding assay

Changes in SpectroSens™ sensor responses associated with the binding interactions between different concentrations of ovalbumin protein (ranging from 250 ng/ml to 10 μg/ml) in the liquid sample and surface-immobilised anti-OVA antibodies were monitored in real-time. Fig. 4 illustrates the observed changes in sensor reflected wavelength as a function of ovalbumin concentration.

Analogous with the IgG assay, the SpectroSens™ response to ovalbumin binding constituted increases in sensor reflected wavelength over time, the profiles of which correlated with antigen concentration. Fig. 4B represents these data in a calibration graph, which reveals a linear correlation between initial sensor response rate and ovalbumin concentration between the concentration range tested, with a correlation coefficient of 0.993. The extrapolated limit of detection for this

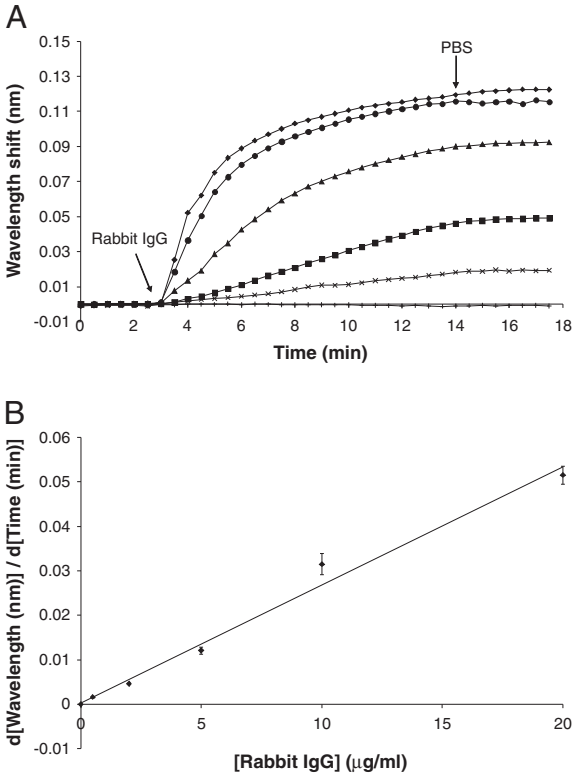

Fig. 3. Rabbit IgG antibody binding kinetics. (A) SpectroSens™ sensorgram demonstrating binding kinetics (averaged data ($n=3$)) associated with binding of varying concentrations of rabbit IgG to anti-rabbit IgG-functionalised SpectroSens™ chips (20 μg/ml (♦), 10 μg/ml (•), 5 μg/ml (▲), 2 μg/ml (■), 0.5 μg/ml (×) and 0 μg/ml (+)). (B) Calibration graph illustrating the rate of change in sensor peak wavelength as a function of rabbit IgG concentration. Error bars represent one standard deviation from the mean ($n=3$). Data points fitted to a linear regression $y=0.0027x+0.0003$, $R^2=0.987$.

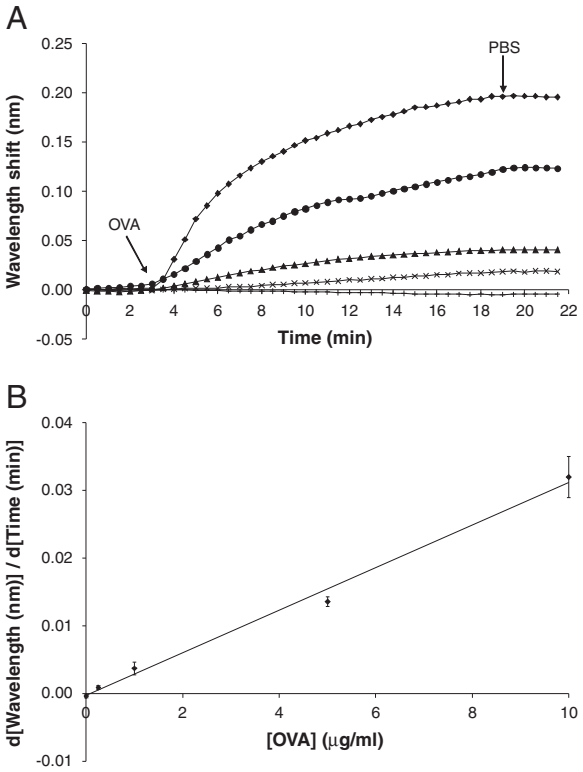

Fig. 4. Ovalbumin protein binding kinetics. (A) SpectroSens™ sensorgram demonstrating binding kinetics (averaged data ($n=3$)) associated with varying concentrations of ovalbumin (10 μg/ml (♦), 5 μg/ml (•), 1 μg/ml (▲), 0.25 μg/ml (×) and 0 μg/ml (+)). (B) Calibration graph illustrating the effect of ovalbumin concentration on the rate of change in sensor peak wavelength. Error bars represent one standard deviation from the mean ($n=3$). Data points fitted to a linear regression: $y=0.0031x-0.0002$, $R^2=0.993$.

assay was equal to the minimum concentration tested at 250 ng/ml, consistent with that obtained for the IgG assay.

Whilst reasonable detection sensitivities have been achieved for both model assays studied using SpectroSens™ chips, expansion of the measurable range of potential analytes could be realised by further improvements to the assay detection limits. Currently, minimum detectable analyte concentrations are predominantly defined by the limited availability of active detection antibodies as a consequence of using a monolayer-based sensor surface-functionalisation protocol. Current work is focused on the use of branched or dendritic molecular linkers (Tomalia et al., 1985) providing a high abundance of reactive functional groups, with a view to increasing antibody immobilisation density and improving antigen binding capacity. An alternative approach involves the development of a suitable 3-dimensional hydrogel matrix to increase the sensor surface area available for active antibody attachment (Löfas and Johnsson, 1990). Both of these approaches are envisaged to significantly enhance antibody performance and consequently increase sensor sensitivity; it is anticipated that improved sensor performance resulting from these surface-chemistry modifications will be the subject of future publications.

3.4. SpectroSens™ chip reproducibility

To investigate the inter-chip variability of the SpectroSens™ response, six independent sensor chips were functionalised with anti-rabbit IgG antibodies under the conditions described previously. The performance of these anti-rabbit IgG-functionalised sensors was then assessed to evaluate the consistency of IgG binding signals; kinetic data for two different IgG concentrations were obtained using three independent anti-rabbit IgG-functionalised sensor chips per concentration tested (Fig. 5). Whilst some variability in the IgG binding kinetics is observed, the maximum standard deviation ($n=3$) of sensor reflected wavelength noted for these chips is approximately 7 pm for the SpectroSens™ responses to each IgG concentration, which corresponds to <10% and <15% of the total mean sensor responses to 5 μg/ml and 2 μg/ml IgG respectively; reasonable deviations upon consideration of the multiple variables associated with the SpectroSens™ chip development process. Sensor reproducibility is cumulatively affected by batch-batch variations in the underlying sensor chip, both from the UV writing process and titanium dioxide coating procedure (influencing inherent sensitivity to refractive index), as well as inter-chip variability in the sensor surface modification procedures and consequently efficiency of antibody immobilisation; the latter is believed to be the most dominant source of variability in sensor response at present.

Ongoing work is heavily focused on the fabrication of multi-channel SpectroSens™ chips comprising multiple discrete sensing regions and concomitant development of integrated multi-channel fluidics to enable patterning of multiple antibodies on a single sensor chip providing a true multiplexing solution, which is envisaged to significantly improve sensor reproducibility. The multi-parameter detection functionality will eliminate any issues resulting from inter-chip variations associated with separate surface-functionalisation procedures and/or underlying chip fabrication by enabling referenced biological interaction measurements (binding to test antibody channels versus control antibody channels) on a single chip.

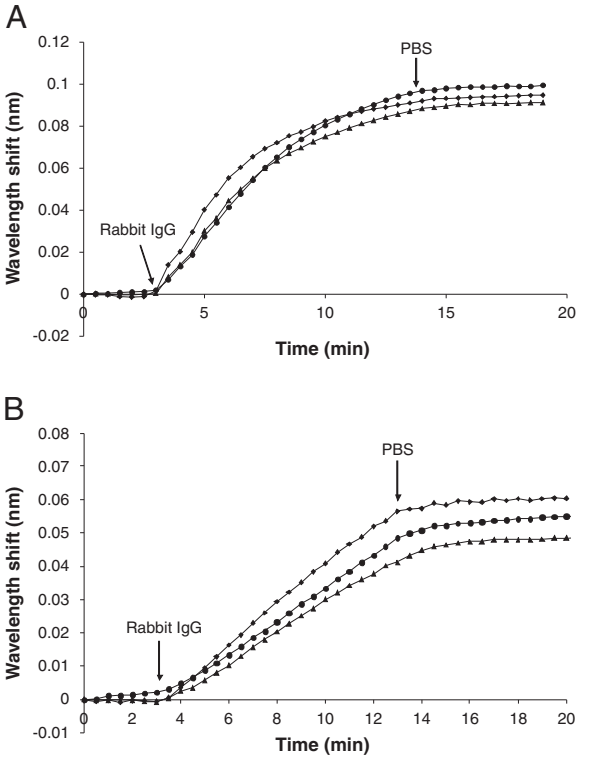

Fig. 5. Inter-chip reproducibility. Sensorgrams illustrating variability of the SpectroSens™ response to binding of rabbit IgG (A) 5 μg/ml and (B) 2 μg/ml using 3 independent anti-rabbit IgG-functionalised sensor chips for each concentration.

4. Conclusions

The use of SpectroSens™ optical sensor chips for label-free, real-time monitoring of antibody–antigen interactions has been demonstrated. Highly selective SpectroSens™ responses manifested as increases in sensor peak reflection wavelength associated with antigen binding to complementary antibody-functionalised sensors. The rate of change of sensor reflected wavelength is directly proportional to the concentration of target analyte in the test sample, enabling quantification of the sensor responses. Detection sensitivities in the mid ng/ml range were obtained for both IgG and ovalbumin antigens. Continued improvements to sensor surface chemistry to enhance antibody performance in combination with assay multiplexing on individual sensor chips is ongoing and is envisaged to significantly improve both the sensitivity and reproducibility of the sensor response. These data highlight the potential exploitation of SpectroSens™ optical microchips in a variety of applications including life science research and development, bio-therapeutic development, bio-marker discovery and clinical diagnostics.

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