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Allergen immobilisation and signal amplification by quantum dots for use in a biosensor assay of IgE in serum

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ABSTRACT

The production of biosensors for point of care diagnostics usually requires the immobilisation and storage of protein (for example, antigen or antibody) on a sensor surface, in a manner that retains a high degree of activity and low levels of non-specific binding. These characteristics have been assessed for polymer immobilised antigens (allergens) using an IgG binding assay and demonstrated further by assay with serum containing reactive IgEs.

The activity of allergens immobilised on sensor chips using copoly(DMA–NAS–MAPS) and a spotting technique, as well as the specificity of their binding interactions with cognate immunoglobulins was assessed using Dual Polarisation Interferometry (DPI). The data obtained indicate that the allergens studied remain stable over long periods of time (at least 114 days). This performance compared favourably with other immobilisation methods. Allergen coated chips were tested in an anti-casein IgE assay using human serum from allergic and non-allergic donors. Detection of both total Ig and specific IgE was demonstrated using a secondary anti-IgE antibody. Furthermore, optical signal enhancement with streptavidin conjugated quantum dots was shown to yield responses for samples below 0.84 ng/mL (0.35 KU/L) of IgE, which overlap with the industrial quasi-standard ImmunoCAP® and is the clinically relevant threshold used to classify serum samples from allergic individuals.

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1. Introduction

The study of molecular recognition events, such as antigen–antibody interactions, in both research and clinical settings can benefit from the development of peptide and protein microarrays that have the potential to allow such analyses to proceed in a time efficient and high throughput fashion with low reagent consumption. One of the key issues for the successful translation of protein microarray technology into diagnostics is the ability to control the quantity and the integrity of surface immobilised proteins. A suitable immobilisation strategy has to achieve the maximum binding capacity, keep the capture reagents in a functional state (even for long term storage) and prevent non-specific interactions in order to provide the high signal to noise ratio that is a prerequisite for high sensitivity (Hartmann et al., 2009).

Determining the optimal surface chemistry for each application is critical and influences the reliability of any protein microarray experiment (Rusmini et al., 2007). Previously, a functional polymer named copoly(DMA–NAS–MAPS), obtained by radical copolymerisation of *N*,*N* dimethylacrylamide, *N*-acryloyloxysuccinimide, and 3-(trimethoxysilyl)propyl methacrylate has been used to immobilise allergens on coated glass and silicon slides (Cretich et al., 2010). This allowed the efficient measurement of their interactions with allergen-specific Immunoglobulin E (IgEs) that are the universally accepted biomarkers for allergy testing in blood. The coating is prepared by a “dip and rinse” approach, where a silicon oxide substrate is immersed in an aqueous solution of the copolymer (10 mg/mL) at ambient temperature followed by washing with water. This procedure produces a thin film with an average thickness of a few nanometres (Cretich et al., 2009a) by a combination of physisorption and chemisorption. The coating is stable under aqueous conditions, where it swells up to 15 nm (Yalcin et al., 2009), and provides a generic covalent immobilisation strategy as the active esters present are able to react with surface exposed amine groups of proteins.

In many applications, such as point-of-care allergy tests, it is necessary to immobilise proteins onto a sensor surface and to store them for an extended period of time.

Covalent immobilisation is the preferred method for attaching proteins to sensor surface as this approach leads to formation of a strong and stable linkage. The most widely used method to

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functionalize Si/SiO_2 sensors involves monodimensional silane coatings. However, the direct attachment of a protein to a surface without a spacer can cause steric constraint of the protein's reactivity or interaction capability compared to the protein in solution (Kusnezow and Hoheisel, 2003). Moreover, multiple direct contacts with the surface can induce denaturation. By introducing a spacer between the protein and the reactive group on the surface, these effects can be minimised. An effective way to space a protein from the surface is by the introduction of a polymeric coating (Milum and Sadana, 1997) which, besides reducing steric hindrance, increases the density of protein per unit area by providing a three dimensional binding scaffold.

The suitability of copoly(DMA–NAS–MAPS) for such a task, together with its stability and capacity to reduce the level of non-specific binding is studied here using Dual Polarisation Interferometry (DPI).

DPI is a surface-based optical technique that can measure both the amount of material deposited upon a SiO_xN_y chip surface and the average structural properties (thickness and density) of the layer formed (Swann et al., 2004). It is commonly used to study interactions between proteins and can give quantitative data including the binding stoichiometry and surface coverage. DPI is an evanescent waveguide technique in which light travels down adjacent sensing and reference waveguides and diffracts at the output to give a Young's interference pattern in the far field. The evanescent field is highly sensitive to changes in refractive index in the vicinity of the sensing surface, therefore, addition of layers to the sensing region of the chip will result in changes in the position (phase) of the fringes generated. The DPI supports both transverse magnetic (TM) and transverse electric (TE) polarisation modes, which generate different evanescent fields at the sensing surface. This means that the phase measurements can be used to provide sensitive thickness and density values of the layer, and allow for the further calculation of mass deposited. DPI has been used to study both interactions and resulting conformational changes of a range of biomaterials including proteins, lipids and DNA (Sanghera et al., 2009; Wang et al., 2010; Coan et al., 2012).

Here, a DPI assay was used to measure the interaction of copoly (DMA–NAS–MAPS) immobilised allergens with commercially available antibodies, which act as a mimic of the allergen-reactive IgEs present in human sera. These measurements allow analysis of the specificity of allergen–Immunoglobulin interactions and the effect of storage, over a range of time periods under various conditions, on the activity of the immobilised allergen. Furthermore, changes in the resistance of the polymer coated surface to fouling under various conditions over the range of storage periods is assessed.

The assay is then tested under quasi-clinical conditions with casein as the immobilised allergen against human serum samples containing controlled levels of IgE. The contribution from specific and non-specific binding is assessed from total serum protein containing IgG and IgE, using the primary binding response followed by a secondary antibody in a "label free" format. The secondary antibody response is then further tested using streptavidin conjugated quantum dots (QDs), which act as signal enhancement and improve the sensitivity in the low IgE concentration regime.

2. Experimental section

2.1. Materials

All chemicals and proteins were purchased from Sigma-Aldrich (Poole, UK) unless stated otherwise. DPI sensor chips (unmodified SiO_xN_y glass, amine functionalised or PEG-Biotin, FB80 *Ana*Chip plus) were obtained from Biolin Scientific SA (Gothenburg, Sweden). Quantum dots (Qdot 655) streptavidin conjugate was purchased from Invitrogen (Paisley, UK), BS^3 (Bis[sulfosuccinimidyl] suberate) was from ThermoScientific (Rockford, IL).

2.2. Sensor chip polymeric coating

Unmodified SiO_xN_y *Ana*chips (Biolin Scientific SA, Gothenburg Sweden) compatible with DPI experiments were used in this study. The planar *Ana*chips were coated with copoly(DMA–NAS–MAPS) by immersing the slides into a 1% (*w*/*v*) polymer solution in ammonium sulphate followed by rinsing with water and drying under vacuum at 80 °C.

2.3. Chip bio-functionalization

Allergen proteins, purchased from Sigma-Aldrich were spotted at 1 mg/mL in PBS (pH 7.4) using a FlexArrayer S5 piezo-electric spotter by Scienion, (Berlin, Germany). Two thousand protein spots (400 pL each) were deposited onto only one of the two active sensing channels present on the coated *Ana*chip and incubated overnight. The remaining active succinimide ester groups were then blocked by incubation of the spotted chips in 50 mM ethanolamine, 0.1 M Tris (HCl), pH 9, briefly rinsed with water and dried under a stream of Nitrogen. The functionalised chips were stored dry for various time periods either at room temperature or 4 °C before DPI data was acquired.

The same procedure was performed on amine functionalised chips using physisorption as the immobilisation mechanism, or on amine chips functionalised with BS^3 before blocking the unreactive sites with ethanolamine.

2.4. Casein specific IgE serum preparation

The concentration of IgE was determined in KU/L using ELISA, however for consistency between the different assays in this paper the ng/ml value is given as the concentration with KU/L values in brackets. Three sera to be used as negative samples were purchased from SeraCare Life Science and one serum reported as containing more than 240 ng/mL (100 KU/L) casein IgE, evaluated by Phadia ImmunoCAP technology, was purchased from Laboratoire Cerba. Specific IgE REAST kit, calibrators and biotinylated Casein (F78) allergen were purchased from Dr Fooke Laboratorien. Assay procedure as described by the kit provider was strictly followed, a cubic spline curve fitting was used to process data and estimate casein specific IgE content.

"Negative" sera were analysed without any dilution, sera with highly concentrated IgE were diluted 1:25 and 1:50 in negative serum.

A serum sample at high IgE level (120 ng/mL) (50 KU/L) was prepared by diluting the positive serum in a mix (1:1:1) of the three negative sera. Other levels (60 ng/mL, 24 ng/mL, and 2.4 ng/mL) (25 KU/L, 10 KU/L, and 1 KU/L) were prepared by serial dilution of the 120 ng/mL (50KU/L) sample in the negative serum mix. IgE concentrations of prepared samples were assessed by ELISA using the same method.

2.5. Dual Polarisation Interferometry (DPI)

All DPI measurements were acquired using an *Ana*light 4D (Farfield Group, Manchester, UK) running *Ana*light DAQ software. The experiments were performed at 20 °C and the running buffer used, unless stated otherwise, was 50 mM Tris (HCl) pH 7.6, 150 mM NaCl, 0.02% (*v*/*v*) Tween 20. After insertion of the functionalised chip containing immobilised allergen into the fluidic compartment of the *Ana*light 4D the proteins were hydrated by continuous flow of running buffer at 50 μL/min over

the surface for ~30 mins. Injection of 2 × 200 μl of 10% (*v*/*v*) glycerol (at 50 μL/min) ensured removal of any trapped air within the fluidic channels. The activity of the immobilised allergen was probed by injection of 200 μL of various concentrations of a reactive antibody in the presence of 10 mg/mL bovine serum albumin (at 5 μL/min). At the completion of the experiment 3 × 200 μL of 100 mM glycine (pH 2) was injected (at 50 μL/min) to remove the bound immunoglobulin molecules and a standard calibration procedure was performed using solutions of 80% (*w*/*v*) ethanol and MQ H_2O. The data were analysed using *Ana*light Explorer software and calculations of specific IgG mass bound were obtained after subtracting the phase change observed on the control channel from that of the allergen channel, and by including a step function (TM + 5.95 rads, TE + 4.8 rads) to mimic the properties of the protein layer that was immobilised *ex situ*.

To estimate the variability of the measurements and apply errors, β-lactoglobulin was immobilised on three chips (as described above) simultaneously and under identical conditions. DPI measurements of the interaction between these three chips and two injections of 4800 ng/mL anti-β-lactoglobulin (Bethyl Laboratories, Montgomery, TX, USA), 10 mg/mL BSA were then performed using PBS (pH 7.4), 0.02% (*v*/*v*) Tween 20 as the running buffer. The values obtained for specific mass binding were used to obtain a % CV error for these measurements of 22.2%.

Serum assays were performed in PBS running buffer with 0.02% (*v*/*v*) Tween 20. After hydration and injections (× 2) of 10% (*v*/*v*) glycerol, 200 μL serum was added to the allergen spotted chip at 5 μL/min. Following a 30 min rinse in buffer at 50 μL/min, 200 μL of 100 μg/mL biotinylated secondary antibody (Anti-IgE) was added at 10 μl/min followed by 200 μL of 5 nM Streptavidin coated Quantum Dots (SA-QDs). The chips were then stripped and calibrated at the end of the experiment as above.

Characterisation of the Streptavidin coated Quantum Dots and comparison with Streptavidin was performed by binding them to a biotin functionalised chip to form a complete monolayer. This was performed after chip calibration in PBS, with three sequential injections of 5 nM SA-QDs (180 μL at 30 μL/min) or a single injection of 25 μg/mL (420 nM) streptavidin so that saturation was achieved. The layer densities and mass per unit areas were calculated from the thickness and RI according to the de Feijter equation (Coan et al., 2012), using a dn/dc value of 0.182 mL/g (Vöros, 2004). For the SA-QD this corresponds to apparent density and mass values, as the high RI QD core is likely to affect the actual dn/dc value of the particle. For the purpose of the analysis undertaken here this is sufficient. The estimated molecular weight of a particle on the surface can be determined from the thickness and mass per unit area, assuming a spherical shape and hexagonal close packing.

3. Results and discussion

3.1. Measurement of specific versus non-specific binding on copoly (DMA–NAS–MAPS) coating

Each sensor chip was functionalised *ex situ* with copoly(DMA–NAS–MAPS) as described in the experimental section, then spotted with allergen on only one of the two working channels before blocking. Two common milk allergens were tested: β-lactoglobulin and α-lactalbumin. Reaction of the surface bound allergen with cognate antibody (rabbit polyclonal anti β-lactoglobulin and α-lactalbumin) was studied by DPI *in situ* (Fig. 1). The antibody was injected across both channels in running buffer at a range of concentrations (0–4800 ng/mL) in the presence of 10 mg/mL bovine serum albumin (BSA). The data obtained for both the β-lactoglobulin (Fig. 1-a) and α-lactalbumin (Fig. 1-b) functionalised

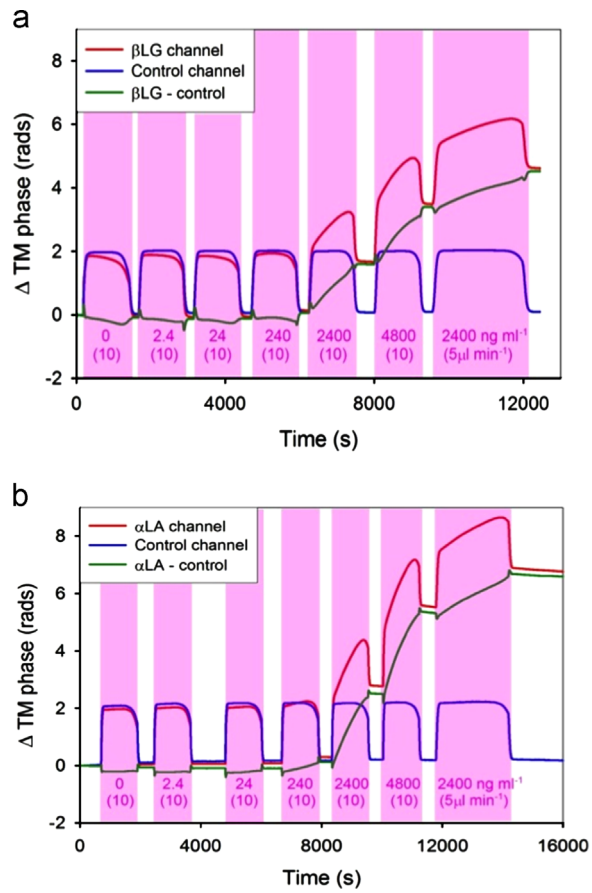

Fig. 1. Dual polarisation interferometry (DPI) data showing the response upon injection of antibodies. (a) Anti-β-lactoglobulin and (b) anti-α-lactalbumin were injected over both channels of chips functionalized by complementary allergens (a) β-lactoglobulin (β-LG) and (b) α-lactalbumin (α-LA) immobilised *via* copoly(DMA–NAS–MAPS) on one channel only (red line). The control channel contains only blocked copoly(DMA–NAS–MAPS) without allergen (blue line). Green lines depict specific ΔTM response of antibody after subtraction of the control. The concentration of antibody injected and the flow rate used is reported in pink. The antibody injections each contain 10 mg/mL bovine serum albumin (BSA) and little non-specific binding is observed under these conditions. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

chips show a specific binding response on the channel containing the allergen only, as demonstrated by the long lived increase in TM phase change on one channel only. The DPI experiment allows calculation of the mass of specifically bound antibody (after subtraction of the non-specifically bound mass) at the end of the measurement and indicates that, in these examples, 1.9 ng/mm^2 anti-α-lactalbumin and 1.1 ng/mm^2 anti-β-lactoglobulin antibodies specifically bind the immobilised allergens (Table 1).

Prior to the antibody injections, the baseline recorded during the buffer rinse, showed good stability, with no loss of material observed on either antigen or polymer control channel. This indicates that both the polymer layer as well as the antigen are stably immobilised on the sensor surface.

During the period of each BSA/Antibody injection there is a transient increase in TM phase of ~2 rads on both channels (pink and blue line in Fig. 1), which is due to the bulk RI effect of the high (BSA) protein concentration (this is in the expected range for the bulk RI change due to 10 mg/mL protein, corresponding to a RI change of 1.8×10^{-3} RIU as determined from the dn/dc value for protein). In each instance it is clear that, whilst a considerable amount of antibody binds to the allergen (pink line in Fig. 1), very little protein is observed to bind non-specifically to the polymer

Table 1
Details of the stability of dry-stored allergens immobilised on DPI chips as probed by their interaction with antibodies. The residual activity of the protein can be assessed from the mass of specifically bound IgG measured and the non-fouling properties of copoly(DMA–MAPS–NAS) can be demonstrated from the % non-specific binding (NSB) measured. r.t. is room temperature.

Immobilised allergen	Injectant	Chip storage days	Chip storage temp. (°C)	Specific IgG mass bound (ng mm^{-2})	NSB (%)
β-LG	Anti β-LG + 10 mg/mL BSA	0	–	1.1 ± 0.1	4.7
β-LG	Anti β-LG + 10 mg/mL BSA	42	r.t.	0.8 ± 0.1	7.8
β-LG	Anti β-LG + 10 mg/mL BSA	42	4	1.3 ± 0.2	2.0
β-LG	Anti β-LG + 10 mg/mL BSA	114	r.t.	0.8 ± 0.1	3.4
β-LG	Anti β-LG + 10 mg/mL BSA	114	4	1.2 ± 0.1	2.0
α-LA	Anti α-LA + 10 mg/mL BSA	73	4	1.9 ± 0.2	2.4

coated surface (Fig. 1, blue line), as evidenced by the return of the phase change to pre-injection levels. This is particularly striking as the surface is challenged with not only antibody of various concentrations but also with a high concentration of BSA, the human homologue of which is the most highly concentrated protein in blood. An estimate of the non-specific binding (NSB) observed on the surface can be made by comparing the overall TM phase change observed on the active allergen channel with that observed on the ethanolamine blocked polymer channel. The NSB is given as the amount of antibody and BSA bound on the control (polymer only) channel expressed as a percentage of that bound on the allergen channel, which provides an estimate of % NSB. Indeed, for the binding experiments described in Fig. 1, the NSB is 4.7% for β-lactoglobulin/BSA and 2.4% for α-lactalbumin/BSA (Table 1).

The experiments described above clearly demonstrate the specific binding of antibodies to the surface immobilised proteins and, importantly, quantify the level of non-specific binding when the chip is coated by copoly(DMA–NAS–MAPS). As a further control for the specificity of the observed binding, another experimental format was used, where α-lactalbumin was immobilised on only one channel, with the other channel contained blocked copoly(DMA–NAS–MAPS). Initial injections of 4800 ng/mL anti-β-lactoglobulin (mixed with 10 mg/mL BSA in PBS (pH 7.4), 0.02% (*v*/*v*) Tween 20) yield no long-lived response, indicating that protein–protein binding does not occur unless cognate binding partners are injected. Indeed, a final injection of 4800 ng/mL anti-α-lactalbumin displays a response characteristic of a specific interaction, indicating that the immobilised α-lactalbumin remains active and binds only to its complementary antibody. Data are shown in the Supplementary Information (Fig. 1-S).

3.2. Immobilised proteins stability over time

The fact that the DPI assay can measure specific binding of proteins to *ex-situ* prepared chips enables its use to estimate the stability of proteins immobilised using copoly(DMA–NAS–MAPS) on sensor surfaces. A series of chips were treated, as before, with polymer and spotted with β-lactoglobulin on only one channel before being blocked. These functionalised chips were stored dry at either room temperature or 4 °C over extended time intervals before an antibody binding assay was performed. The data acquired after 0, 42 and 114 days (Fig. 2-S in the Supplementary Information) indicate that the immobilised protein is still capable of binding antibody after dry storage over this time period as demonstrated by the long-lived TM phase change. The calculated mass values (Fig. 2-S, Table 1) account for the amount of specific protein binding, as they are determined by subtracting the amount of binding to the channel containing only blocked polymer from the amount bound to the active allergen channel. At each time-point studied, the mass of IgG bound is higher for the chips stored at 4 °C than those stored at room temperature and shows no loss of activity compared to the freshly prepared sensor, indicating that the proteins immobilised on chips stored at 4 °C retain activity better than those stored at room temperature.

For evaluation of the immobilisation technique in comparison with other alternatives, the experiments were repeated with chips where the allergen was immobilised by either physisorption to an amine functionalised sensor chip or coupled to the chip using the bifunctional amine–amine cross-linker BS^3. The data for the assay performed immediately after functionalization and after storage for 42 days are shown in Fig. 3-S in the Supplementary Information. The sensor response is clearly larger on both these chip surfaces, probably reflecting a higher degree of binding of the allergen to the surface. However the level of NSB is also significantly higher. The unfunctionalised amine chip (without BS^3) is the worst in this respect, with NSB comprising up to ~50% of the signal. This is presumably due to the highly charged nature of the underlying chip surface. The allergen spotted onto the BS^3 modified amine chip showed the highest response (~13 rad TM phase change) but still an appreciable level of NSB (> 10%). In terms of the achievable limit of detection of such an assay NSB is likely to be the limiting factor, rather than the instrument sensitivity, which is over three orders of magnitude smaller (in the < 1 mradian range).

From the immobilisation conditions studied, the polymer method clearly provides a constant, if slightly lower level of active allergen (presumably due to a lower immobilisation yield) compared to that of the other surfaces, but with significantly better signal to noise ratio due to the low level of NSB. The level of specific antibody binding observed is none the less still appreciable, with 1.9 ng/mm^2 of antibody measured for anti α-lactalbumin corresponding to 30% of an antibody monolayer; an amount which may even be desirable in terms of the linearity of binding response and subsequent detection with a secondary antibody.

3.3. IgE serum assays

In order to evaluate the performance of the sensor in a serum assay for allergen specific IgE detection, experiments were performed with sera containing controlled quantities of IgE, made by combining human serum samples from a highly casein allergic individual with serum from a non-allergic individual as described in the experimental section.

No casein specific IgEs were detected using the ELISA method in any of the 3 "negative" sera tested individually or as a mix at equal ratio to the detection limit of 0.84 ng/mL (0.35 KU/L). This is the threshold used by the ImmunoCAP® to classify serum samples from allergic individuals according to clinical relevance (Fall et al., 2003). The pool of these sera can be used as diluent to prepare the serum samples with controlled IgE levels.

Casein specific IgE concentration in serum from an allergic individual was determined in duplicate, the average concentration was 1.25×10^3 ng/mL (524 KU/L) with a standard deviation of 364.8 ng/mL (152 KU/L).

Considering this concentration, a sample predicted to have 120 ng/mL (50 KU/L) casein specific IgE concentration was prepared by diluting this serum 10.5 fold in the pool of negative sera. Other samples were prepared by serial dilution in the pool of negative sera, with predicted IgE concentrations of 60 ng/mL, 24 ng/mL and 2.4 ng/mL (25 KU/L, 10 KU/L and 1 KU/L).

The IgE concentrations of the prepared samples were determined by ELISA assays, where the actual values measured were 76.56 ± 16.32 ng/mL, 46.08 ± 3.12, 23.28 ± 2.88 and 1.92 ± 0.24 (31.9 ± 6.8 KU/L, 19.2 ± 1.3, 9.7 ± 1.2 and 0.8 ± 0.1). Full results of the assays are shown in Table 1S in the Supplementary Information.

Fig. 2 shows an experiment where a series of serum samples containing increasing concentrations of casein specific IgE (from 0 to 76.56 ng/mL (31.9 KU/L)) were injected over a copoly(DMA–NAS–MAPS) chip with one channel spotted with casein and the other channel unspotted and blocked. The negative control serum sample shows very similar binding to both channels. This can be attributed to a degree of NSB to the underlying polymer structure. After this injection, at sequentially higher casein IgE concentrations, little extra binding is observed on the control channel but increasing levels of specific binding are observed on the casein spotted channel. The total specifically bound response (casein channel − control channel, Fig. 2, green line) after addition of the final 76.56 ng/mL (31.9 KU) IgE sample was >10 rad. This value corresponds to a high level of binding to the surface and is close to that expected for a monolayer of immunoglobulins. This amount is due, not only to the IgE in the sample, but also to casein-specific Immunoglobulin G (IgG) present in the serum samples as human serum samples contain both allergen specific IgEs and IgGs. In a typical assay, when allergens are immobilised on a surface, both IgGs and IgEs compete for the binding with the same allergen. Whilst the IgGs are present in larger quantities, only the IgEs are relevant for allergy diagnostics. The use of a secondary (detection) antibody specific either for human IgGs or human IgEs is needed to distinguish between the two antibody isotypes and for precise quantification of each of them.

Addition of the Anti-IgE secondary IgG to this surface resulted in a comparatively small TM phase response of 0.8 rad. Attributing this signal to the secondary binding to the IgE component of the total specifically bound immunoglobulins at the surface (14 rad), and accounting for the molecular weights of the species (IgE = 180 kDa and IgG = 150 kDa), we can determine the magnitude of the phase response of IgE on the surface (IgE = $0.8 \times 180/150 = 0.96$ rad). Thus, the proportion of IgG in the signal (93% by mass, or 94% by moles) can also be calculated.

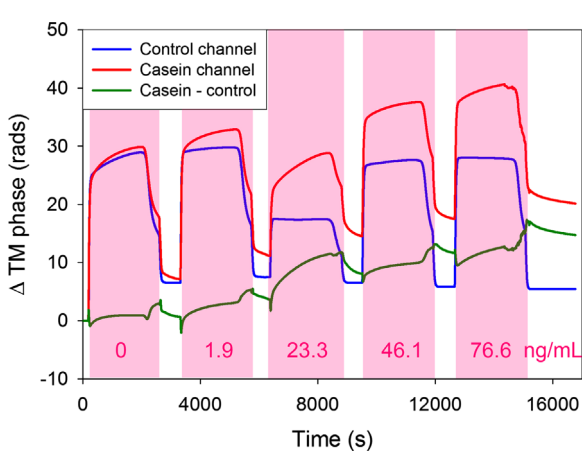

Fig. 2. IgE human serum assay. Injections: negative control serum and IgE containing sera. The total specifically bound amount (casein channel − control channel) sample is reported in green. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

A relatively smaller contribution from the IgE in the sample (with respect to IgG) is in accordance with the typical blood serum content of the two antibody isotypes, however the observed surface composition, 7%, is nonetheless an order of magnitude greater than the expected solution value, ~0.75%, (IgE levels in a normal individual are ~75 ng/mL, compared to 10 mg/mL for IgG (Cretich et al., 2009b)). Relating the expected proportions on the surface to the proportion of IgE to IgG in the sample requires consideration of both the relative concentrations as well as the corresponding kinetic rate constants and affinities.

Surface plasmon resonance measurements of polyclonal IgE and IgG species revealed that their affinities diverge widely, being in the range of 10^{-10} and 10^{-11} M for IgE, but only 10^{-6}–10^{-8} M for human IgG (Jin et al., 2008; El-Khouly et al., 2007). Moreover, murine monoclonal IgG1 antibodies against the allergens showed affinities of 10^{-7}–10^{-8} M. The most significant contribution to the increased affinity of IgE being predominantly due to an enhanced association rate constant (K_a).

Given these typical values, even if IgE is present at two orders of magnitude lower concentration than IgG in the sample, the higher affinity (by approximately three orders of magnitude) can account for the one order of magnitude enhanced composition of IgE over IgG observed on the surface compared to that expected to be present in solution.

3.4. Signal amplification by quantum dots

Concentration dependent DPI serum assays were performed on copoly(DMA–NAS–MAPS) coated and casein spotted chips. The injection sequence used was: Serum (with controlled IgE concentration), secondary antibody (anti-IgE, biotinylated), Streptavidin conjugated quantum dot (SA-QD). Each assay was performed on a fresh sensor channel and the response upon injection of secondary antibody (anti-IgE, biotinylated) followed by that of SA-QD can be seen for an example dataset in Fig. 3-a. A range of experiments were performed where different samples were injected prior to the addition of secondary antibody (Fig. 3-b), these included a control with no serum, 0 ng/mL casein specific IgE control serum and IgE controlled serum of nominal concentration between 1.92 and 76.56 ng/mL (0.8 and 31.9 KU/L), as measured by ELISA. The signal upon injection of secondary antibody with the zero IgE concentration sample subtracted is also shown (Fig. 3-c) and indicates a concentration-dependent response. The amplification effects of the SA-QD are clearly observable when the plots are overlaid and the binding data for the zero casein IgE serum sample subtracted (Fig. 4-S). A clear IgE concentration dependence for both the secondary antibody binding and the SA-QD binding can be demonstrated by the DPI data (Figs. 3-b, -c and 4). In Fig. 3-b it can be observed that for the zero IgE sample (black line) and serum sample containing 1.92 ng/mL (0.8 KU/L) IgE (red line) a small phase loss is observed after injection of the secondary IgG, this is not observed for the control experiment (green line) where the serum injection is omitted. This indicates that injection of the secondary IgG promotes a small net loss of adsorbed serum protein from the surface of the chip. The reason for this is unclear, but may reflect displacement of non-specifically bound material prompted by the very slightly different composition of the IgG buffer. Consequently this effect reduces the low concentration sensitivity of the net secondary IgG binding response. This is an unavoidable consequence of such a label-free methodology which probes total surface composition. At higher concentrations the response is approximately linear with IgE concentration.

The assay response can be amplified by the addition of a further component, namely the Streptavidin coated Quantum Dots

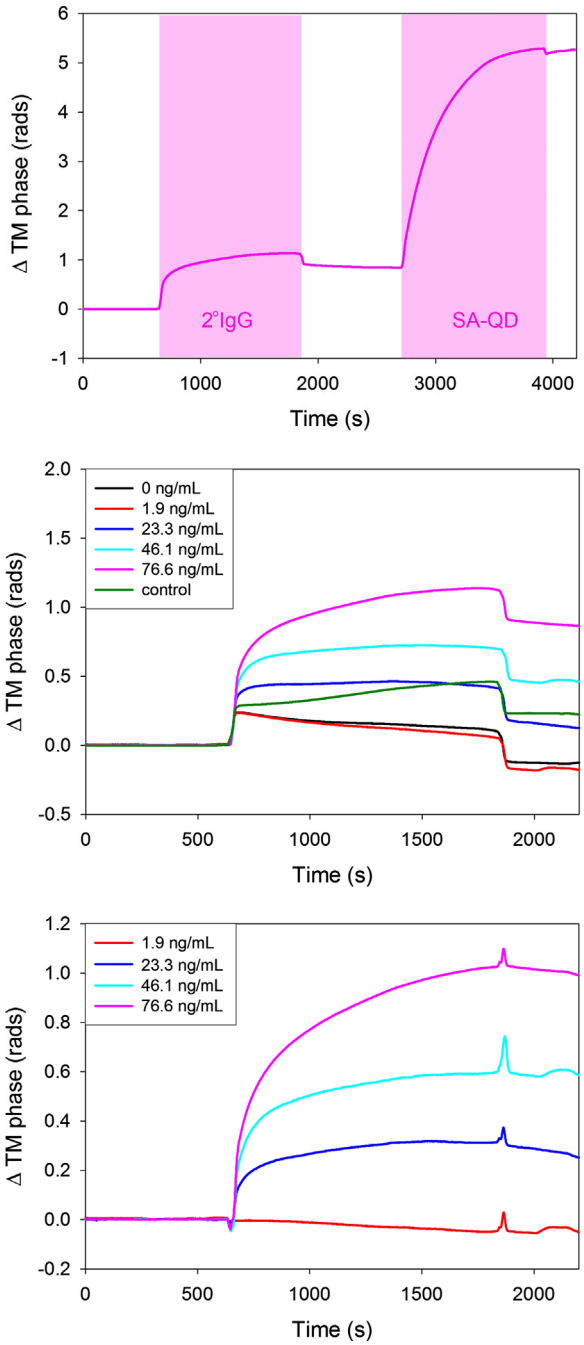

Fig. 3. (a) Example dataset showing the IgE detection steps following the injection of serum sample containing 76.56 ng/mL (31.9 KU/L) IgE (not shown) – the addition of 100 mg/mL biotinylated secondary antibody (anti-IgE) and then 5 nM SA-Q are marked on the trace. (b) Response on injection of secondary detection antibody (anti-IgE) for all of the serum samples. The 0 ng/mL sample is negative control serum and the "control" sample is data from an experiment where the serum injection was omitted from the sequence. (c) Normalised secondary antibody response, after subtraction of response from the 0 ng/mL negative control serum sample. The anti-IgE biotinylated secondary antibody is used to distinguish between allergen specific IgEs and IgGs and only detect specific IgEs. Allergen specific IgGs contained in the serum are not relevant for allergy diagnostics. (For interpretation of the references to colour in this figure, the reader is referred to the web version of this article.)

(SA-QDs), which bind to the biotinylated secondary IgG (Figs. 3 and 4). This amplification step can be undertaken to reduce the detection limit observed above for the "label free" method, which in this case is not governed by the sensitivity of the instrument, as much as by the surface/protein assembly and serum

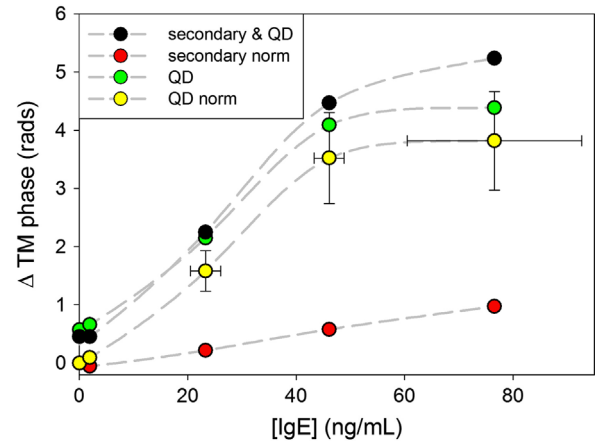

Fig. 4. IgE human serum assay: final responses as a function of IgE concentration when using quantum dots to amplify the signal of secondary antibody. Secondary & QD is the total response due to 2°-IgG and QD binding, secondary norm is the 2°-IgG response after subtraction of the 0 KU/L negative control, QD is the response from just the QD binding and QD norm is after subtraction of the 0 ng/mL negative control response. Error bars on [IgE] represent the error determined on ELISA calibration of the standard serum samples ($n=4$), error bars on the TM phase response represent 22% CV determined by reproducibility assays of the protein spotted polymer surface ($n=3$).

containing samples being measured. In the case of an IgE assay this is a requisite step due to the low level of IgE that is required to be determined due to clinical relevance (0.84 ng/mL or 0.35 KU/L). Whilst other materials can be used for this amplification step (a range of streptavidin coated nanoparticles are available) the Quantum dots represent a convenient multifunctional label. The RI sensitive properties are being used here rather than the more commonly utilised fluorescent properties, but the SA-QDs are also strongly optically absorbing, so can be used to provide a colorimetric assay. Thus, this methodology could be directly transferred and compared across multiple detection platforms and measurement techniques.

The SA-QDs injections show positive responses for all IgE sample concentrations. The no-serum control shows no SA-QD binding, whilst the no added IgE "zero" concentration serum sample shows a small binding response. Fig. 4 reports results of the IgE human serum assay as a function of IgE concentration when using QDs for signal amplification. The plotted errors in the IgE concentration are determined from the ELISA concentration determination experiments and those in the TM phase response are set at $\pm 22\%$ as determined for the specific antibody binding reproducibility assays (see Methods). The IgE sample assays show IgE concentration dependent responses. The 1.92 ng/mL (0.8 KU/L) assay shows a small but significantly larger response than the "zero" serum, and the higher concentrations show a 6–7 fold enhancement over the secondary IgG response. At the highest IgE concentration (76.56 ng/mL) (31.9 KU/L) the SA-QDs response shows a reduced enhancement, suggestive of saturation.

In order to gain a greater understanding of how the SA-QDs contribute to the DPI signal they were characterised (in a separate set of experiments) by binding them as a monolayer onto a biotinylated sensor chip, shown in Fig. S5, together with a Streptavidin monolayer binding by way of comparison. As well as the phase change, a clear drop in the fringe contrast is also observed for the SA-QD binding, which is not seen for the SA monolayer. This is due to absorption of light in the sensing waveguide by the quantum dots and represents another potential way of quantifying their presence, namely by their optical extinction coefficient. It was anticipated that the SA-QDs would have a large contribution from the high RI of the QD core (>2) (Nor and Mohd, 2012) as well as from the polymer and multiple streptavidin molecules attached to it, that would contribute

Table 2
Dimensional data for monolayers of SA-QD and SA on a biotinylated sensor chip.

	TM (radian)	Refractive index	Thickness (nm)	Mass (ng/mm^2)	Density (g/cm^3)
SA-QD	21.682	1.3817	24.0	6.19	0.258
SA	8.641	1.4001	6.4	2.30	0.359

to an enhanced DPI response. The properties of both a monolayer of SA and of SA-QDs are given in Table 2. The measured characteristics of the SA monolayer are in agreement with previously determined values and correspond to the expected monolayer dimensions (Swann et al., 2004). The SA layer density reflects the fact that the monolayer is not close packed and that approximately a quarter of the layer volume is made up of protein. The measured thickness of the SA-QDs layer (24 nm) is just above the approximate range quoted by the product sheet for the product series of QDs: the particles are sized 15–20 nm in total, with 5–10 streptavidin molecules attached to the QD core. The low layer RI measured here suggests that despite the high RI of the QD core, the polymer coated shell and loose arrangement of SA molecules around the periphery dominate the refractive index contribution from the particle. Taking the SA and SA-QD as spherical particles and from the layer thickness and mass per unit areas calculated, we can derive an apparent molecular weight for the SA-QD nanoparticle. Using this method the molecular weight of the SA is calculated as 49 KDa, and the SA-QDs as 1.8 MDa. Allowing for a similar non-optimal packing of the SA-QDs as the SA, which has a supplier quoted molecular weight of 60 KDa, gives us 2.2 MDa for the SA-QD. This represents a 15 fold increase over the molecular weight of an IgG molecule (150 KDa). In this case, the observed 6–7 fold signal enhancement suggests that approximately half the secondary IgG on the surface is able to bind a SA-QD nanoparticle.

The 5 rad response observed for the SA-QDs binding in the 76.56 ng/mL (31.9 KU/L) IgE assay also suggests the surface is at ~25% its SA-QDs maximum capacity. This may be sufficient to account for the slightly sub-linear response at 76.56 ng/mL (31.9 KU/L) due to some IgE molecules being too close to one other for each one to bind a separate SA-QDs nanoparticle. Linearity would give an upper detection limit of ~288 ng/mL (120 KU/L), but the effect of occlusion of IgE means that significantly higher concentrations would probably be required to completely pack the surface, giving an extended if non-linear dynamic range.

At the low concentration end, the limit of detection with the SA-QDs is reduced to the single KU/L level. The noise of the measurement technique (< 1 mradian) and the sensitivity of the SA-QD/IgE response of ~12 ng/mL/radian (5 KU/L/radian) would suggest the potential for the measurement to discriminate samples at the 0.0024 ng/mL (0.001 KU/L) level. However, as the zero serum sample represents an IgE concentration of below 0.84 ng/mL (0.35 KU/L), a lower or more precise limit value cannot be determined from these experiments.

4. Conclusions

The long term stability and non-fouling properties of the copoly(DMA–NAS–MAPS) coating was evaluated using the measurement of TM phase change determined by Dual Polarisation Interferometry. The data for all experiments indicate that very little non-specific binding, of either IgG or BSA present in the injected solution, is observed (Table 1). Moreover, across all time points and in both conditions tested the overall phase response observed at the end of each experiment on the control channel as a percentage of that measured on the allergen channel does not show an increase over an extended storage period, indicating that copoly(DMA–NAS–MAPS) is highly stable under these conditions. The activity of the spotted allergen also remained constant over the time period studied. In addition, the performance of the polymer coated surface was shown to be enhanced over that of alternative approaches.

The functionality of the coated chips in a serum IgE assay was tested and showed specific responses down to low levels of IgE. Detection of allergen specific IgE is a challenging task requiring sensitivity down to the ng/mL level. The measurements obtained with the industrial quasi-standard ImmunoCAP®, are expressed in units (KU/L) or in specific IgE classes (0 – 6). Any analytical technique for the examination of specific IgE levels should be able to distinguish the classes 0 and 1. Class 0 comprises IgE concentrations of up to 0.84 ng/mL, which is equivalent to 0.35 KU/L (Fall et al., 2003). With signal enhancement the assay performed by DPI provided responses to below 0.84 ng/mL (0.35KU/L) of IgE which overlaps with ImmunoCAP®. The relevance of this result is striking when considering some of the issues that make IgE assays in serum a demanding analytical task. Such challenges include the fact that most of the IgE-binding epitopes are conformational, meaning that their native structure must be retained during the assay to be properly recognised. Another hurdle to obtaining sensitive and specific responses in such assays is the fact that in serum, IgE binding can be hindered by the large concentration of competing allergen specific IgG.

To summarise, the results of the DPI investigations indicate that blocked copoly(DMA–NAS–MAPS) is a suitable non-fouling coating and is also robust to allergen storage over the time periods and conditions studied here. Dry-form reagent storage is particularly useful to simplify assay automation, improving assay repeatability, to reduce training requirements and to facilitate integration into lab-on-a-chip devices.

Acknowledgements

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2013.08.019.

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Supplementary information

Allergen immobilization and signal amplification by quantum dots for use in a biosensor assay of IgE in serum.

Geoffrey W. Platt, Francesco Damin, Marcus J. Swann, Isabelle Metton, Gilbert Skorski Marina Cretich and Marcella Chiari

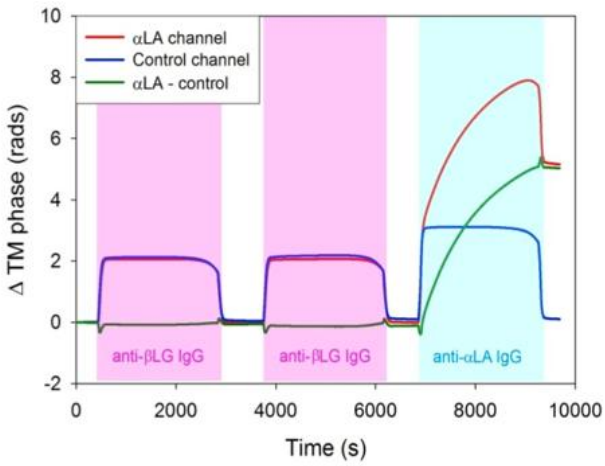

Figure 1-S: Injection of antibodies (plus 10 mg/mL BSA) across a DPI chip containing α-lactalbumin (αLA) immobilised with copoly(DMA-MAPS-NAS) on one channel only. Addition of anti-β-lactoglobulin (highlighted in pink) elicits no long term binding, whereas addition of anti-α-lactalbumin (highlighted in light-blue) results in a stable long-term interaction between the antibody and the cognate immobilised allergen, demonstrating the specificity of the assay. Green line highlight the specific αLA signal subtracted by the control.

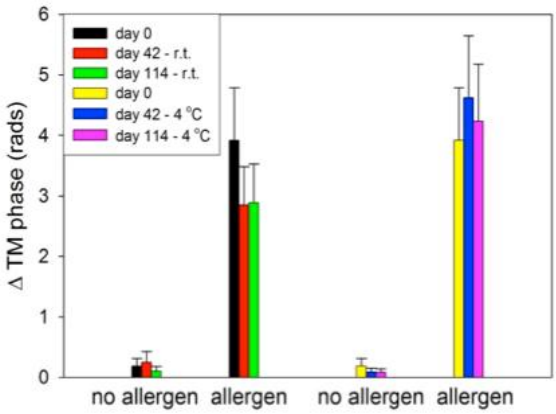

Figure 2-S: Total TM phase change observed by DPI upon completion of the IgG titration across chips containing allergen immobilised (using copoly(DMA-NAS-MAPS)) on one channel only. The chips were stored dry over a range of intervals either at room temperature (r.t.) or at 4 °C before analysis. A comparison of the response observed on the channel containing allergen with the control channel (no allergen) allows an estimate for the non-specific binding of protein to the surface polymer upon storage under each condition.

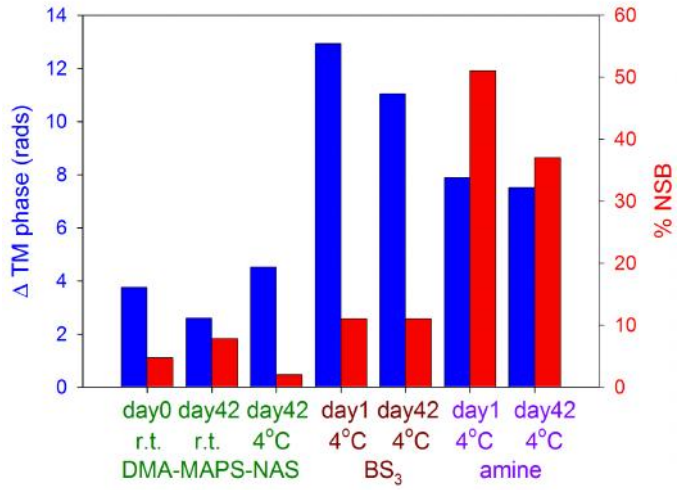

Figure 3-S: Activity and % NSB as a function of storage conditons and surface functionalisation. The columns on the left are data from copoly(DMA-NAS-MAPS) coated chip recorded on various days (at RT or 4 °C) and the columns on the right are those from the chips where protein β-LG was spotted on BS^3 or amine.

Table 1-S: IgE content in serum samples prepared at 4 casein specific IgE concentrations as determined by ELISA.

IgE content		**Sample 1**	**Sample2**	**Sample 3**	**Sample 4**
Predicted [IgE] KU/L		50	25	10	1
Assay 1	Replicate 1	27.8	18.9	11.2	0.8
	Replicate 2	25.0	17.7	10.1	0.8
Assay 2	Replicate 1	35.2	19.9	9.1	0.7
	Replicate 2	39.8	20.5	8.6	0.7
Average [IgE] KU/L		**31.9**	**19.2**	**9.7**	**0.8**
Standard deviation		6.8	1.2	1.2	0.1
CV %		21%	6%	12%	10%

Formattato: Posizione: Orizzontale: 2,12 cm, Rispetto a: Pg, Verticale: 0,9 cm, Rispetto a: Paragrafo, Orizzontale: 0,25 cm, Testo intorno

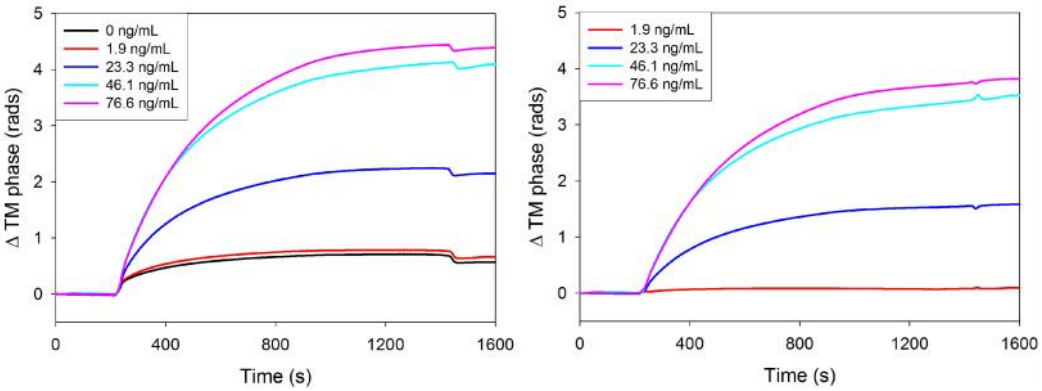

Figure 4-S: Left panel (SA-QDs response): IgE human serum assay. Injections: negative control serum and IgE containing sera. Right panel (Normalized SA-QDs response) signals with zero IgE control subtracted.

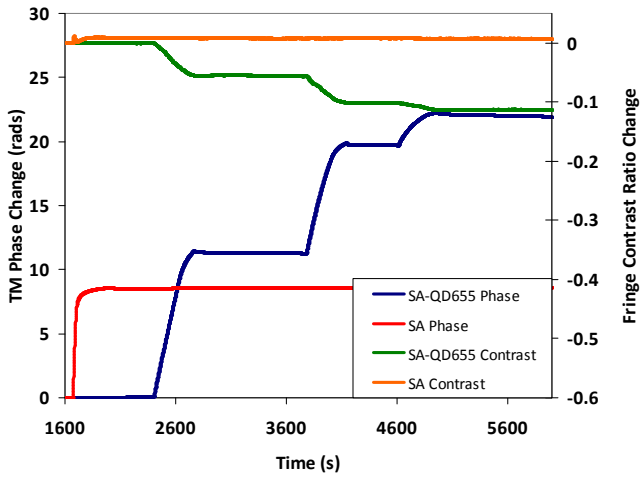

Figure 5-S: Monolayer binding response of SA-QD binding to a biotinylated chip, 3 sample injections at 5 nM (TM Phase change - blue and fringe contrast - green) and the comparative binding response for a monolayer of Streptavidin, one injection at 420 nM (TM Phase change – red and fringe contrast orange).