



Quantitative label-free screening for antibodies using scattering biophotonic microarray imaging

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ABSTRACT

A biophotonic array based on gold nanoparticles functionalized with antigen proteins has been used to determine the concentrations of the respective antibodies in solution. Four proteins—fibrinogen, bovine serum albumin, transferrin, and C-reactive protein—were used to construct a test array with the assay repeated a number of times. The antibody–antigen association and dissociation rate constants were determined for the antibody assays from a series of calibration experiments. The label-free determination of the unknown antibody concentrations was performed using two related kinetic analyses. From these results, the current array assay sensitivity is 250 ng ml⁻¹ with an accuracy of 15% using an 8-min kinetic measurement and a 16-spot averaged assay.

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Microarrays for high-throughput screening technology are being developed for a number of applications in chemical biology [1–4], most of which are based on fluorescence detection schemes. Alternative label-free screening platforms are also being investigated for a number of applications based on acoustic biosensors, calorimetric biosensors, and (most prominently) surface plasmon resonance (SPR)¹ biosensors [5–9]. Application of plasmon biosensor platforms has been found in the fields of gene expression [10], immunosensors for monitoring the number of colony-forming units of *Escherichia coli* in water samples [11], and protein function screening [4], to note just a few examples. There are two distinct advantages of the label-free screening technologies: (i) no chemistry is required to label the biomolecules and (ii) the concentration of target species in solution can be determined by monitoring the kinetics of a specific binding event. Knowledge of the kinetic parameters of the specific interaction is required to extract the absolute concentration of the protein or target species. Quantification of cytokines and chemokines has been reported by fluorescence-based methods [3], although these methods are vulnerable to nonspecific fluorescence quenching that may depend on the concentration of species in the target medium. This is particularly difficult for complex media such as whole blood serum samples.

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¹ Abbreviations used: SPR, surface plasmon resonance; UV, ultraviolet; TRA, human transferrin; BSA, bovine serum albumin; FBR, human fibrinogen; CRP, human C-reactive protein; CTAB, cetyltrimethylammonium bromide; aCRP, goat polyclonal antibody to CRP; IgG, immunoglobulin G; aFBR, goat polyclonal antibody to FBR; aBSA, sheep polyclonal antibody to BSA; aTRA, sheep polyclonal antibody to TRA; DTSP, dithiobis-succinimidyl propionate; PBS, phosphate-buffered saline; RI, refractive index; RU, response units; PSA, prostate-specific antigen; aPSA, antibody to PSA; IgE, immunoglobulin E.

Label-free multiplex array sensing [4,6] enables the collection of high-quality data-rich information on a profile of species, such as in the blood, in contrast to the often small numbers of molecules used for diagnosis. Screening provides a data-driven discovery process that is more than a biomarker fishing exercise simply looking for marker proteins of a potential disease. Monitoring the changes in the blood proteome, for example, enables the rapid establishment of a multicomponent profile from which a systemic view of a diseased or healthy state can be achieved. For this to be successful, the multiplexed array platform must be cost-effective with the array of assays on each chip viewed as a consumable.

Label-free biosensing using particle-based arrays has been reported by a number of groups [12,13], and recently by our group [14,15], using simple ultraviolet (UV)–visible spectroscopic methods [12,13,16] and single nanoparticle scattering [17]. We recently reported an apparent significant advantage for nanoparticle-based sensors over continuous gold surface plasmon sensors. For a continuous gold surface, the plasmon field penetrates some 200 nm into the medium above the surface. The particle-localized surface plasmon field, however, does not penetrate more than approximately 20 nm into the medium, sampling a small volume of space dominated by the antibody–antigen bilayer. Therefore, the fraction of volume of the plasmon field occupied by the assay for the particle is much larger than the assay occupancy of the plasmon field on a continuous surface. Hence, a larger refractive index change or refractive index contrast [14] allows the particle plasmon to be more sensitive to changes in the local environment than to the nonspecific changes in the medium.

The success of the plasmon-based sensors in determining the concentration of the target species in solution requires a quanti-

tative kinetic analysis of the specific interactions at the gold surface [18]. The biospecificity of the sensor response is usually associated with an antibody–antigen interaction, and the successful determination of the target molecule concentration requires the kinetic analysis of the response to be sensitive and accurate. Therefore, a kinetic assay requires the variation of the pseudo-first-order rate constant with concentration to be measured, forming a calibration curve from which the concentration may be determined. Accurate measurement of the association and dissociation rate constants for the interaction is, therefore, a critical measurement, and this has been performed for a few antibody–antigen pairs as we reported recently [14], but the number of available data sets in the literature is much smaller than would be expected. In the current article, we report the results of a blind trial for the determination of the concentration of antibodies in solution using the kinetic parameters derived for the specific interactions among four different proteins and their respective antibodies. These proteins were printed in a 64-spot array, and the kinetic assays were performed to determine the unknown solution concentration. Two kinetic analysis techniques were used, and the relative merits of both methods are discussed. From these results, we conclude the current sensitivity and accuracy of the array reader platform.

Materials and methods

The current sensitivity and accuracy analysis was performed on a biophotonic array platform, which uses gold nanoparticles that are printed into the array format using an inkjet printer initially as seed particles. A particle growth or development step then occurs to produce larger particles within each of the array spots. The arrays are then returned to the array printer and functionalized with the target protein/antigen for the proposed antibody assay. Interrogation of the array is performed with the array reader, allowing near-field illumination of the sample, and the light scattered by the particles is then collected by a video camera at a 15-Hz frame rate. The antibody mixture is passed over the microarray within a flow cell that has been optimized for geometry, volume, and flow rate to ensure concentration-limited kinetics. The variation of the scattered light intensity due to proteins binding to the sensor surface with time produces the kinetic trace, and this is analyzed to determine the concentration of the antibody in the solution. The experimental procedure has been described in detail elsewhere [14,15].

Chemicals

Human transferrin (TRA, 4 mg/ml) was obtained from Invitrogen. Bovine serum albumin (BSA, 98%), human fibrinogen (FBR, 60% with 40% buffer salts; the protein content is >80% clottable FBR), human C-reactive protein (CRP, >90%, 2.3 mg/ml), gold(III) chloride trihydrate ($\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$, 99.9%), cetyltrimethylammonium bromide (CTAB, 95%), and sodium citrate ($\text{C}_6\text{H}_5\text{Na}_3\text{O}_7 \cdot 2\text{H}_2\text{O}$, 99.9%) were obtained from Sigma–Aldrich. Goat polyclonal antibodies to CRP (aCRP, 10 mg/ml, immunoglobulin G [IgG] fraction) and to FBR (aFBR, 10 mg/ml, IgG fraction) were obtained from Abcam. Sheep polyclonal antibodies to BSA (aBSA, 23 mg/ml, IgG fraction) and to TRA (aTRA, 34.3 mg/ml, IgG fraction) were supplied by AbD Serotec UK. Dithiobis-succinimidyl propionate (DTSP, 97%) was obtained from Fluka. If not mentioned otherwise, the protein and antibody solutions were prepared and used in standard phosphate-buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4 , and 1.5 mM KH_2PO_4 , pH 7.3) containing 0.005% (v/v) Tween 20 surfactant supplied by Sigma–Aldrich. Ascorbic acid ($\text{L}+\text{C}_6\text{H}_8\text{O}_6$, 99%) was supplied by Acros.

Biophotonic microarrays

Preparation of scattering photonic sensor arrays based on the surface synthesis of gold nanoparticles has been described elsewhere [14,15]. The current work used glass substrates (microscope slides, $25 \times 25 \times 1$ mm) with 8×8 rectangular arrays of gold nanoparticle-coated round spots of approximately 200 μm diameter and with horizontal and vertical pitches of 300 and 380 μm , respectively (Fig. 1A). The array spots are initially printed with 4-nm seed gold particles and substrates were removed from the inkjet printer (Arrayjet). The seed nanoparticles were then grown within the spot using a growth mixture of ascorbic acid, auric chloride, and CTAB. The particle growth was influenced by the presence of the surfactant and produced truncated polyhedron-shaped particles that were 130 ± 6 nm in size. The biofunctionalized sensors were prepared by printing the target proteins onto the array spots according to the array key shown in Fig. 1B.

Two-step coupling chemistry was used to tether the target proteins to the gold surface covalently. The gold surface was first activated by incubation in 1 mg/ml water-free DTSP solution in a 1:1 mixture of isopropyl alcohol and dimethyl sulfoxide. Then concentrated protein solutions (1 mg/ml BSA, 0.6 mg/ml FBR, 1 mg/ml CRP, and 4 mg/ml TRA, all PBS buffered) were printed over the gold-covered areas. DTSP contains a succinimide ester group that reacts with primary amines on the surface of the proteins, forming a peptide bond. Each array spot received approximately 0.3 nl of the corresponding protein solution, and after allowing 2 h for protein immobilization, the sensor slides were washed in PBS and stored at 5 °C until use. In total, eight sensor slides were prepared: two were discarded due to poor printout quality, three were used in calibration experiments, and the other three were used in the series of blind tests.

Array reader

Kinetic characterization of antibody–antigen binding was performed with a simple array reader instrument that follows the intensity variations of the scattered light from dark-field illuminated sensor photonic array in real time. The basic design and detailed description of the reader have been presented elsewhere [14], and the only difference relevant to the current study was the replacement of a telescope expanded diode laser light source with a fiber-coupled laser (diode continuous wave, 635 nm, Oz Optics) collimated with a single lens to form a beam of approximately 5 mm diameter, which is sufficient to illuminate the whole 8×8 spot sensor array (Fig. 1). A schematic of the array reader, including the injection systems, is shown in Fig. 2. The absolute sensor re-

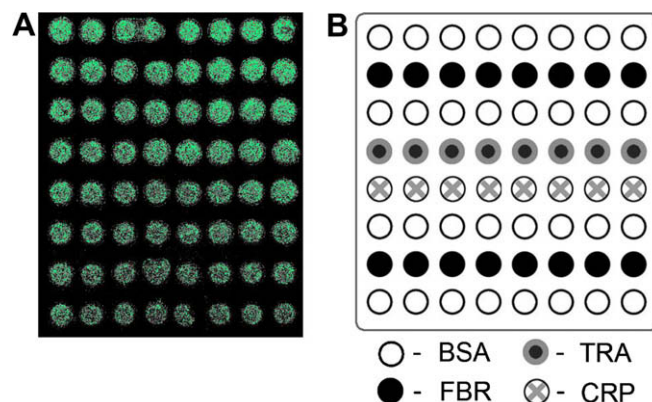


Fig. 1. Sensor array. (A) Slide illuminated in the array reader. (B) Protein functionalization legend.

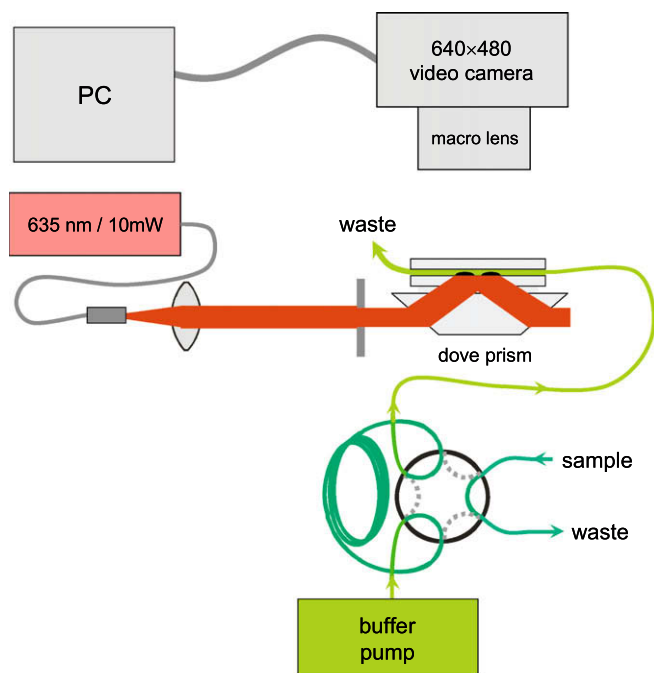


Fig. 2. Schematic of the array reader instrument.

sponse was calibrated after the protein binding by measuring the scattering brightness change for each spot as the bulk refractive index (RI) is changed from water to 1% by weight of isopropyl alcohol in water solution ($\Delta RI \sim 8.5 \times 10^{-4}$ RIU [19]). All data presented in the subsequent figures are in response units (RU), with 1 RU conventionally equivalent to a 10^{-6} RI change in the bulk analyte.

Results

The sensitivity of the antigen functionalized surface of each of the array spots needs to be calibrated; hence, series of responses from known antibody analyte concentrations were recorded. The

antibody–antigen binding was studied as follows: (i) the installed microarray was stabilized in a flow of PBS for 10 to 20 min with a short (~ 30 -s) initial injection of 10 mM phosphoric acid solution; (ii) four antibody-containing analyte solutions were injected sequentially in random order with approximately 8 min of exposure time; and (iii) the sensor surface was regenerated with a 1-min injection of 10 mM phosphoric acid solution; steps (ii) and (iii) were repeated for each of the target concentrations. Six kinetic traces were obtained for each of four antibodies in a concentration range from 12.5 to 100 nM in PBS. A typical example of the recorded data is shown in Fig. 3, where each instrument response trace corresponds to an average signal from a subset of the array spots functionalized with the same proteins.

The microarray contains a number of repeats of each antibody assay: aCRP is measured 8 times, aFBR—16 times, aBSA—32 times, and aTRA is measured 8 times, as shown in the key for the array in Fig. 1B. The response of each related assay group may be averaged

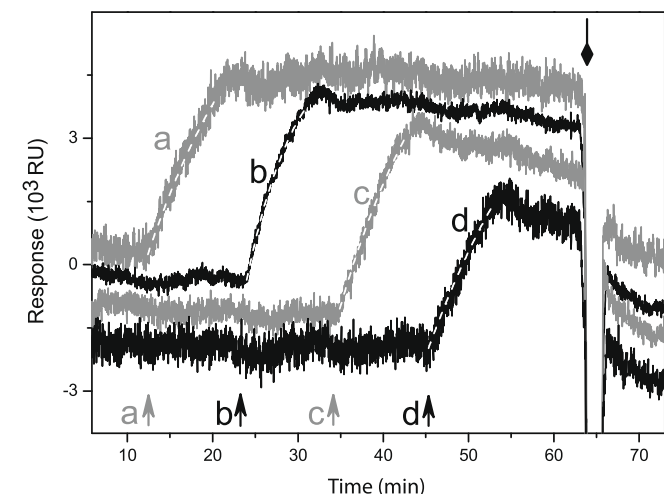


Fig. 3. Transient kinetic responses for the spot-averaged response of all similar assays performed on the array. Each of the assay spots is functionalized with TRA (a), BSA (b), FBR (c), and CRP (d) proteins. The traces are separated vertically for presentation. At the times marked with arrows, 25 nM of each antibody solution was added in turn: (a) aTRA; (b) aBSA; (c) aFBR; (d) aCRP. The diamond indicates a 1-min regeneration wash with 10 mM phosphoric acid. The dashed lines indicate the fit to Eq. (2).

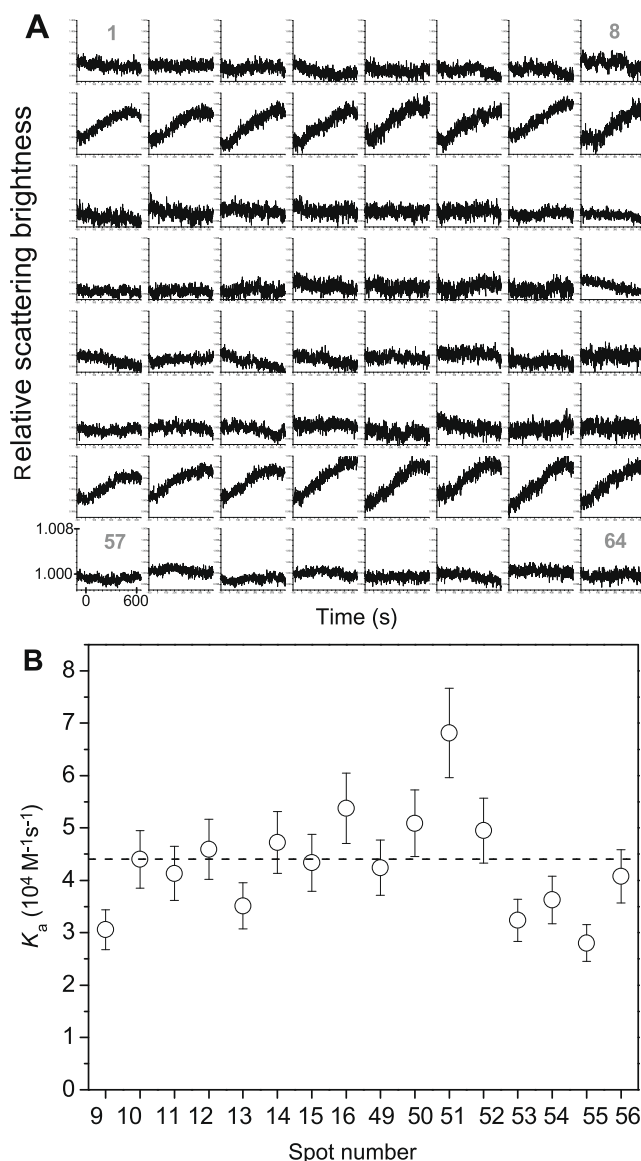


Fig. 4. Kinetic response of the array to 25 nM aFBR. (A) Transients observed at each spot of the 64-spot array. Only the spots functionalized with FBR (Fig. 1) show binding. (B) Adsorption rate constants (k_a) derived from each of the 16 FBR-functionalized assay spots. The dashed line indicates a 16-spot averaged value, and the distribution has a relative standard deviation of 23%.

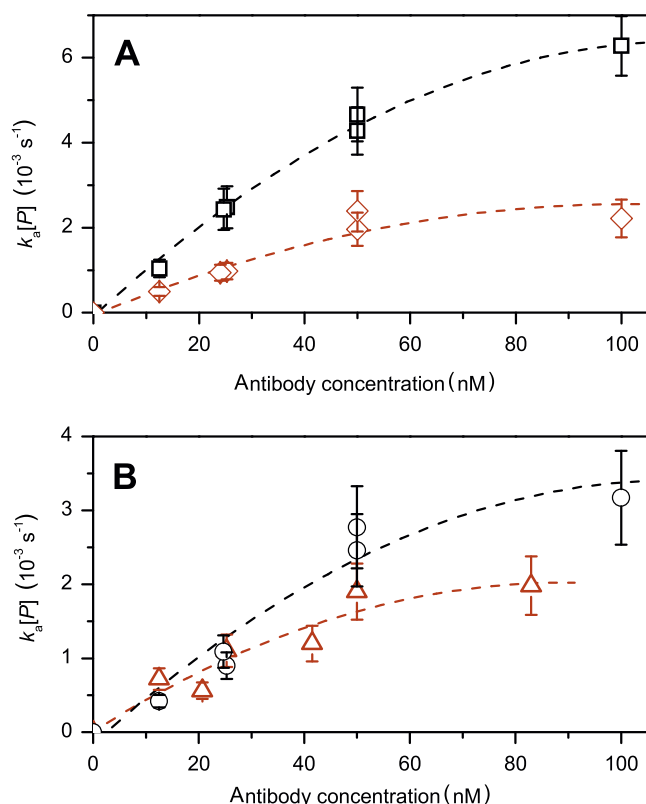


Fig. 5. First-order antibody-antigen association rate constants derived from kinetic experimental data: (A) $\square$, aBSA-BSA; $\diamond$, aTRA-TRA; (B) $\circ$, aCRP-CRP; Δ , aFBR-FBR. The dashed lines are spline fits to indicate the trend.

together to produce the traces seen in Fig. 3, or they may be analyzed individually as presented in Fig. 4A for the aFBR binding kinetics. It can also be seen that no significant nonspecific binding was observed on any of the assays for the other target antibodies. The association rate constants derived individually from the 16

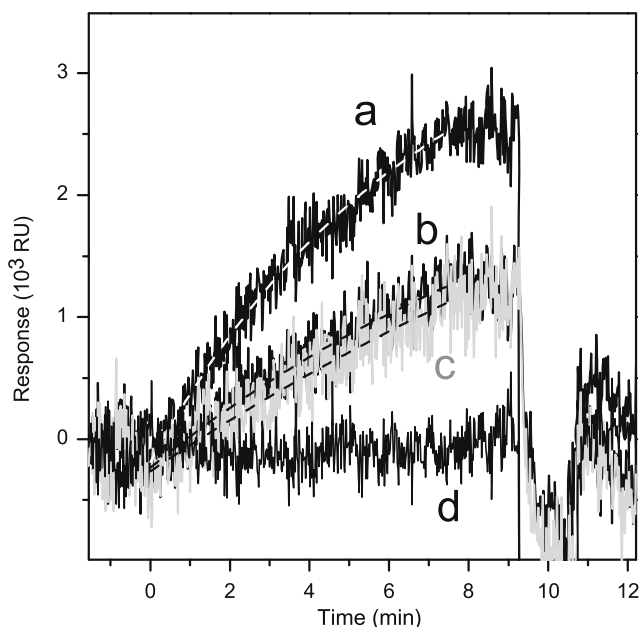


Fig. 6. Kinetic response of the array assay for test experiment 1: (a) BSA; (b) TRA; (c) FBR; (d) CRP. The dashed lines indicate the fits to Eq. (2).

aFBR assays are presented in Fig. 4B. The calibration experiments were performed for four different concentrations from which the association rate was derived in each case; these data are shown in Fig. 5 to produce a calibration curve for each assay. The methods of analyzing the data are discussed below.

The blind tests were performed with three sample mixtures containing varying concentrations of each of the antibodies; these concentrations were not revealed until the quantitative analysis of the data was performed. The measurements were carried out using the same sequence as presented above with the test solution mixtures injected in step (ii). Fig. 6 shows the kinetic traces from blind test solution 1, with the traces presented as averages over all of the spots on the array for each assay.

Discussion

The principal objective of this study was the validation of the current array reader design and biophotonic arrays for quantitative assays of antibodies in solution for high-throughput screening applications. Quantitative determination of the specific antibody concentrations is based on the initial calibration to determine the sensitivity and accuracy of each antibody assay; from this, the kinetic parameters can then be derived. Once the array assays have been calibrated, the concentration of an analyte in solution in a blind trial can then be determined absolutely.

Antibody-antigen binding kinetics

The kinetics of antibody-antigen binding can be described in terms of a simple adsorption model with the corresponding differential equation:

$$\frac{d\theta}{dt} = k_a[P](1 - \theta) - k_d\theta, \quad (1)$$

where θ is the surface coverage, a fraction of the occupied binding sites; $[P]$ is the concentration of antibody in solution; and k_a and k_d are the adsorption and desorption rate constants, respectively. The adsorption transients are shown in Fig. 3 for the early adsorption phase, which is then followed with a switch to PBS running buffer at the end of the adsorption phase to observe desorption of the antibody from the surface. The desorption phase may be described as a single exponential decay, with the rate depending on the surface coverage and the desorption rate constant: $\theta = \theta_0 \exp(-k_d t)$; the derived k_d rate constants are listed in Table 1. The integrated form of Eq. (1) with respect to the association phase is the following:

$$\theta = \frac{\vartheta}{\vartheta_m} = \frac{k_a[P]}{k_a[P] + k_d} (1 - \exp(-(k_a[P] + k_d)t)), \quad (2)$$

where ϑ refers to the experimental observable, namely change in scattering brightness, and ϑ_m corresponds to the maximum brightness change so that $\theta = 1$, or complete coverage of the available active sites on the surface is achieved. The analysis does not require an absolute determination of the surface coverage or the number of binding sites available on each protein.

The model in the differential or integrated form in Eqs. (1) and (2) describes a simple 1:1 interaction for the antibody and the antigen surface, neglecting interactions between antibodies on the surface and allowing the rate of adsorption to depend only on the number of available binding sites. A study of the adsorption phase over the concentration range of interest in the calibration experiments should produce a straight-line plot for $k_a[P]$ versus $[P]$ for each array spot, from which a global fit to the data from all spots will allow k_a to be determined.

Table 1

Association and dissociation rate constants derived from kinetic experimental data.

Antibody–antigen	This work		Reference values	
	k_a ($10^4 \text{ M}^{-1} \text{ s}^{-1}$)	k_d (10^{-4} s^{-1})	k_a ($10^4 \text{ M}^{-1} \text{ s}^{-1}$)	k_d (10^{-4} s^{-1})
aBSA–BSA	9.0 ± 0.8	4.0 ± 0.8	13 ± 2^a 14.6^b	4 ± 2^a 3.52^b
aFBR–FBR	3.4 ± 1.0	2.5 ± 0.5		
aTRA–TRA	4.4 ± 0.6	2.3 ± 0.5		
aCRP–CRP	5.3 ± 0.8	1.5 ± 0.8		

Note. Values are means $\pm$ 2SD.^a Ref. [14].^b Ref. [23].

The spot-to-spot variation in the performance of the array assays is an important characteristic in determining the accuracy and sensitivity of the array assays. The response of the array to aFBR is shown in Fig. 4, and there is a clear positive specific binding response to 25 nM aFBR on the array spots functionalized with FBR (rows 2 and 8) and no nonspecific binding on any of the other spots. The derived k_a constants for the 16 assay spots are presented in Fig. 4B, which shows some statistical variation with a standard deviation of 23% in the determination of k_a . The spot-to-spot variation observed for the nanofabricated array spots may be compared with the continuous gold surface array platform on the Biacore Flexchip instrument of 9% [20]. The cause of the variation may arise from a number of factors: (i) variability in the number of nanoparticles on the surface of each spot and, hence, the number of scatter centers; (ii) considerable variability within the spot of the shape and size of the nanoparticles and, hence, the variability in the efficiency and sensitivity of the scatter particles; (iii) variability across the area of the laser beam in the intensity of the laser light; and (iv) variability in the protein printing. The intensity variation across the spots is random, however, and averaging the kinetic traces together over a number of repeats of the assay has the expected square root improvement in the signal-to-noise ratio (Fig. 3), with the signal in the BSA channel (32-spot average) being approximate two times less noisy than that in the TRA and CRP channels (8-spot average). The noise source most likely to dominate the observed kinetic data comes from the variation in the laser intensity across the array area.

The analysis of the data with Eqs. (1) and (2) suggests that a plot of the pseudo-first-order rate constants versus concentration should be a straight line, and this would form a calibration curve. However, the observed plot of $k_a[P]$ versus $[P]$ (Fig. 5) is not linear but rather monotonically increasing and may be used as an empir-

ical calibration of the sensor response. The simple model does not consider any of the more complex surface interactions, including mass transport at high analyte concentrations and the effects of steric hindrance on the binding of the antibody to the sensor surface. For lower concentrations of antibody (<50 nM), the response of the assays is linear and the simple kinetic analysis is sufficient. The resulting association rate constants (k_a) derived from the concentration range from 0 to 50 nM are listed in Table 1 and are average values for all of the spots functionalized with the appropriate protein. Comparisons are also made with our previous work and the literature values where possible. The relative errors in the association rate constants correlate with the number of assays performed on the array with the exception of the aFBR–FBR case. Fibrinogen in the blood naturally polymerizes, and this may have enhanced the sensing properties of the tethered protein in the formation of the assay surface. The observed rate constant uncertainties are typical for antibody–antigen measurement [14], as shown in a recent benchmark study that explored the variability in biosensor measurements; the reported antibody–antigen rate constant associated errors were in the range of 20% to 100% depending on the instrument and the laboratory within this global benchmark study [21].

Low analyte concentrations allow the approximation $\exp(-\alpha t) \sim (1 - \alpha t)$, valid for small α , to be used, and Eq. (2) reduces to the following expression:

$$\vartheta \approx \vartheta_m k_a [P] t, \quad (3)$$

implying that parameters ϑ_m and k_a are strongly correlated and, therefore, are poorly determined separately by fitting the data. Allowing these parameters to be coupled, the calibration can be expressed in the coupled $\vartheta_m k_a$ parameter, analogous to an initial rate analysis. The dependence of the array sensitivity on ϑ_m variation

Table 2

Quantitative analysis of the blind test data.

	Test sample concentration (nM)	k-Derived concentration (nM)	k-Derived error (%)	$\vartheta \times$ k-Derived concentration (nM)	$\vartheta \times$ k-Derived error (%)
Test sample 1					
aBSA	17.5	22.1	26	15.3	–13
aFBR	15.0	11.1	–26	12.9	–14
aCRP	0	<2.9	–	0 ^a	–
aTRA	17.5	9.7	–45	15.1	–14
Test sample 2					
aBSA	0	<1.2	–	0 ^a	–
aFBR	5.0	<1.7	–	5.4	8
aCRP	17.5	10.3	–41	10.5	–40
aTRA	17.5	12.1	–31	16.9	–4
Test sample 3					
aBSA	15.0	21.4	43	15.7	5
aFBR	0	<1.7	–	0 ^a	–
aCRP	0	<2.9	–	0.3	–
aTRA	35.0	26.1	–25	35.1	1

^a Fixed at zero level (assumed baseline).

may be eliminated by careful and reproducible fabrication of the assay spots.

A comparison between the responses of the assays performed on continuous gold surface SPR platforms, such as Biacore and SensiQ, shows that the response of the nanoparticle-localized surface plasmons to the binding assay is greater than 5000 RU (Fig. 3), whereas typical SPR instrument values are less than 1000 RU [21,22]. This is expected given that the smaller penetration depth of the nanoparticle plasmon field compared with that of a continuous gold surface results in better RI contrast. The biological assay occupies approximately 50% of the plasmon field for the nanoparticle compared with approximately 5% of the plasmon field for the continuous gold surface. This results in a sensitivity that is two to five times larger for the current antibody assays [7,14].

Quantitative antibody screening

The analysis of the blind sample test solutions must be performed independently to extract the unknown antibody concentrations. The analysis must be quantitative and robust, ideally requiring that the correlation coefficients between parameters of the kinetic fit should be negligible. The generic approach is to fit the data from the blind tests to the integrated kinetic dependence (Eq. 2). Knowledge of the association rate constants for each of the assays presented in Table 1 allows deriving the concentration of the antibody. Using this procedure, the derived antibody concentrations are presented in Table 2 as k -derived values. These concentrations may be compared with the known concentrations in each of the three test solutions and show an average error in excess of 35%, reflecting the high correlation between k_a and ϑ_m discussed above.

The second analysis method employs initial rate parameter $\vartheta_m k_a$ for the interpretation of the calibration curves and subsequent determination of the unknown concentrations. The obtained concentrations are listed in Table 2 as $\vartheta \times k$ -derived values. There is a significantly better determination of the unknown concentrations, with approximately 15% discrepancy between the known test sample composition and $\vartheta \times k$ -derived values. The 15% deviation is very similar to that reported with the multiple-instrument investigation of the association constant for PSA–aPSA (prostate-specific antigen–antibody to PSA) binding studies [22], potentially a fundamental limit to the accuracy of antibody–antigen binding analyses pointing toward an intrinsic variability in the antibody itself and the binding specificity [21].

The kinetic analysis for each of the assays indicates detection limit, sensitivity, and accuracy values that are different for each of the antibodies owing to the different association constants. Analysis of the minimum detectable concentration for each species based on the signal-to-noise ratio of a single spot assay for a kinetic trace monitored over 8 min with an observed 15% uncertainty suggests the following sensitivities: aBSA = 6.8 ± 1.0 nM (1020 ± 150 ng ml^{−1}), aTRA = 7.4 ± 1.1 nM (1110 ± 170 ng ml^{−1}), aCRP = 8.2 ± 1.3 nM (1230 ± 190 ng ml^{−1}), and aFBR = 6.8 ± 1.0 nM (1020 ± 150 ng ml^{−1}). The minimum detection limit and accuracy values can be improved by multiple repetitions of the assay on the array so that a 16-spot assay will reduce the noise by a factor of 4, suggesting a sensitivity of 2 nM (250 ng ml^{−1}).

Conclusion

The four-protein functionalized biophotonic microarray using nanoparticle light scattering centers was successful in determining the absolute concentration of antibodies in solution as a label-free screening technology. The current detection limit is typically 250 ng ml^{−1} potentially in whole serum with an accuracy of 15% for a 16-spot assay with an 8-min kinetic response measurement.

At these sensitivity levels, the antibody assays could be used for detecting antibodies during the onset of infection where concentrations may reach the $\mu\text{g ml}^{-1}$ level or in determination of allergy where an allergic response is considered as significant with immunoglobulin E (IgE) levels above 170 ng ml^{−1}.

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