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Surface plasmon resonance imaging based multiplex biosensor: Integration of biomolecular screening, detection and kinetics estimation

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ABSTRACT

We present a multiplex biosensing method to simultaneously screen targets of interest in a multiple target analyte sample and to extract the binding affinities of all interactant pairs from a single sensor surface using a commercial surface plasmon resonance imaging system. For demonstration, we have prepared our sensor disk with five different ligands varying from low molecular weight antibiotics to high molecular weight human IgG, all immobilized in a microarray format. The multi-target analyte sample was prepared by mixing five antibodies where each one is highly specific for one of the immobilized ligands in a range of concentrations for kinetics estimations. The key advantage of the newly developed approach is that many different types of assays can be performed simultaneously, however, care should be taken to understand the non-specific interactions between different analytes in the sample mixture and unintended ligands, and surface regeneration behavior of different ligand types. Other advantages include reductions in experimental and analysis time, reduced costs, and flexibility since the same microarray can be used for assays with a single target analyte specific for the single ligand.

1. Introduction

Biomolecular screening is normally performed using flow cytometry [1], enzyme linked immunosorbant assay (ELISA) [2], quantum dots [3], mass spectrometry [4] and optical biosensors such as wavelength interrogated optical biosensors [5] and surface plasmon resonance imaging (iSPR) [6–17]. iSPR [18] systems that are currently commercially available, such as, IBIS [19], GWC [20], Biacore flexchip [21], Genoptics [51] and Agilent [22] appear to be useful for making such multiplexed assays with the help of microarrays [23]. This interesting combination is reported for various screening applications in biomarker discovery [19] and fragment based drug discovery [24] as well as in disease diagnostics where small sample sizes are required [25]. The main advantage in combining iSPR and microarrays is that each spot in the array is an individual sensing area, which can be specific for a variety of different analytes [26]. Other advantages include that is a label-free technique where real-time binding kinetics information, such as the association rate (k_a) and dissociation rate constants (k_d) [27], can be directly extracted from the measured sensorgrams for each ligand spot simultaneously.

A number of application areas have been reported for equilibrium kinetics extraction for various application areas such as DNA–RNA hybridization [20], DNA–DNA interaction [28], peptide–protein interactions [29], epitope mapping [30], protein–carbohydrate interactions [31], polymer–enzyme studies [32], polymer–protein interactions [33], biomarker discovery [34] and chemical patterns through microfluidic channels for protein immobilization [35]. All of these articles deal with the injection of various concentrations of a single target analyte in serial dilutions with regeneration steps.

In order to increase the throughput and reduce assay times, we propose a new approach by injecting multiple target analytes in various concentrations with regeneration steps. In this way, the kinetics and affinity can be extracted not only for single interactant pairs, but also for all the interactant pairs that are present in the analytes in parallel. This method offers several advantages compared to conventional systems. The key advantage of the newly developed approach is that many different types of assays can be performed simultaneously using single sensor surface [41], however, care should be taken to understand the non-specific interactions between different analytes in the sample mixture and unintended ligands, and surface regeneration behavior of different ligand types. Other advantages include reduced sample and reagent volumes, reduced time for experimental procedures and real-time binding information from the interactant pairs.

Multiple target analytes have been previously reported for biodetection purposes [36,37]. We have recently reported on the advantages of an integrated microarray-iSPR system for kinetic

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analysis [38]. Combining these two techniques leads to a new integrated measurement approach where microarrays with multiple ligand types, for biomolecule screening and kinetics extraction, are subjected to multi-target analyte samples, where each target molecule in the sample is specific for one of the ligands in the microarray sensing surface.

For biological screening applications, it is not necessary to inject various analyte concentrations, however, the extension of experiments with injection of various concentrations of analytes leads to more reliable kinetics and affinity parameter estimation of the biomolecular interactant pairs.

A demonstration of our approach has been conducted and we have prepared sensor disks with five different ligands varying from low molecular weight antibiotics to high molecular weight human IgG to show the effectiveness of the approach even with a large molecular weight variation of the immobilized ligands. The multiple target analytes were prepared by mixing five antibodies in a single sample, where each one is highly specific for one of the immobilized ligands. Concentration ranges of single target analytes and also multiple target analytes were prepared for the typical kinetics experiments. The binding kinetics and affinity of interactant pairs obtained from the single target analytes acts as a reference value for the assay.

2. Materials and methods

Experiments have been performed to screen multiple biomolecular interactions and to extract binding affinities from multiple biomolecular interactant pairs from an analyte solution containing a mixture of different targets using a single sensor surface. Various ligand types with replicates were immobilized in a microarray format. The experiments were conducted by injecting various mixtures of monoclonal antibodies and quantitatively comparing the extracted kinetics parameters k_a, k_d, R_m and $K_D = k_d/k_a$ [27] with results extracted from single target antibody samples. All experiments were conducted with serial analyte injections of varying concentrations separated by surface regeneration steps.

2.1. Multi-ligand immobilization

Prior to ligand spotting, the functionalized hydrogel sensor disk (HC-80m, XanTec, Germany) was activated with 750 μL of 400 mM 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) (Sigma, The Netherlands) and 750 μL of 100 mM N-hydroxysuccinimide (NHS) (Sigma, The Netherlands) for 20 min followed by rinsing with 0.25% (v/v) acetic acid. The activated disk was dried for 30 min under continuous dry nitrogen flow. Five ligand spots of 0.7 mg/ml β2-microglobulin (β2M) in sodium acetate buffer (pH 5.4), four spots of 0.5 mg/ml human IgG in MES buffer (pH 5.4), six spots of 10 mg/ml neomycin in MES buffer (pH 4.5) and five spots of 2.5 mg/ml gentamycin in MES buffer (pH 4.5) and four spots of 1 mg/ml human IgG Fab fragments (Jacksons Immuno Research, USA) in MES buffer (pH 5.4) were printed (TopSpot, Biofluidics, Germany) on the sensor surface [39]. Even though, we need approximately 4 μL of each sample for a single spot, the final spot is made of 1 nL of the respective sample. All the chemicals mentioned above except the human IgG Fab fragments were purchased from Sigma, The Netherlands. The respective spot arrangements are shown in Fig. 1a. The fabricated microarray was incubated in a humidity chamber for one hour. Following protein immobilization, the sensor surface was blocked with 1 M ethanolamine (Sigma, The Netherlands). The sensor containing the protein microarray was mounted in the IBIS-iSPR [12,14,38] (IBIS Technologies BV, Enschede, The Netherlands) with a drop of refractive index matching oil (n_{oil} = 1.518, Cargille Lab, USA). The system was equilibrated using 1 mL binding buffer in a flow-cell at a flow-speed of 2 μL/s at 25 °C. A representative real-time iSPR image of the fabricated microarray spots is shown in Fig. 1b. In order to reduce the sample volume to 125 μL, a back-and-forth mixing technique was used. After defining the ROIs of 30 × 30 pixels each, corresponding to 225 μm × 225 μm, the SPR-dip was measured. A baseline measurement was made by injecting the binding buffer for 120 s. In addition to 24 ligand spots, 8 separate ROIs are placed on blank spaces for referencing.

Table 1
Analyte concentrations used in multi-ligand/multi-analyte kinetics experiment.

Sample	Antibodies	Concentrations (nM)
1	mAb β2-microglobulin	22, 44, 88, 176 and 352
2	mAb neomycin	7, 14, 28, 56 and 112
3	mAb gentamycin	5, 10, 20, 40 and 80
4	Fab antihuman IgG	33, 65, 130, 260 and 520
5	Goat antihuman IgG (H + L)	166, 332, 664, 1328 and 2656

2.2. Multi-analyte kinetic screening

The various monoclonal antibodies (mAb) used in these experiments were mAb for β2-microglobulin (Abcam, UK), mAb for neomycin and mAb for gentamycin (Meridian Life Sciences, USA), and Fab specific antihuman IgG (Jacksons Immuno Research, USA). Polyclonal goat antihuman IgG (H + L) (Zymed, USA) was used to study the difference between the interaction of polyclonal antibody and Fab of antihuman IgG to the Fab of human IgG and human IgG, respectively. Multiple target analytes as well as single target analyte were prepared in HBS-EP buffer (GE Healthcare/Biacore, Sweden). The different analyte concentrations used in these experiments are shown in Table 1. The first sets of experiments were performed with varying concentrations of single target analytes for the kinetics parameter estimation (Fig. 1c) in a serial order. The approach described in this article uses multiple target analytes (each target molecule in the mixture is specific to single immobilized ligands) from which responses of all the interactant pairs are measured simultaneously (Fig. 1d). Multiple target analytes used in these experiments also have the same final concentrations as single target analytes and were prepared accordingly. Mixture 1 is a combination of mAb for neomycin, mAb for β2M, and Fab of antihuman IgG, mixture 2 is a combination of mAb for neomycin, mAb for β2M and mAb for gentamycin, and mixture 3 is a combination of all the 4 antibodies. In each case, regeneration was done with 10 mM glycine–HCl (pH 1.6) between each analyte injection. In this case, the association and dissociation profiles were measured for 1200 and 600 s, respectively.

2.3. Binding kinetics model

The 1:1 interaction model represented by $A_i + B_j \underset{k_d}{\overset{k_a}{\rightleftarrows}} A_iB_j$, where i is the number of analytes and j is the number of ligands and k_a and k_d are the association and dissociation rates, respectively, has been used for all parameter extractions. The affinity constant is defined as $K_D = k_d/k_a$ [27]. The observed SPR response R_t is proportional to the formation of '*AB*' complexes at the surface with respect to the ligand density. Accordingly, the maximum signal R_m represents the maximum ligand capacity that can bind with analytes without any dissociation of the *AB* complex and is proportional to the active ligand density at the surface.

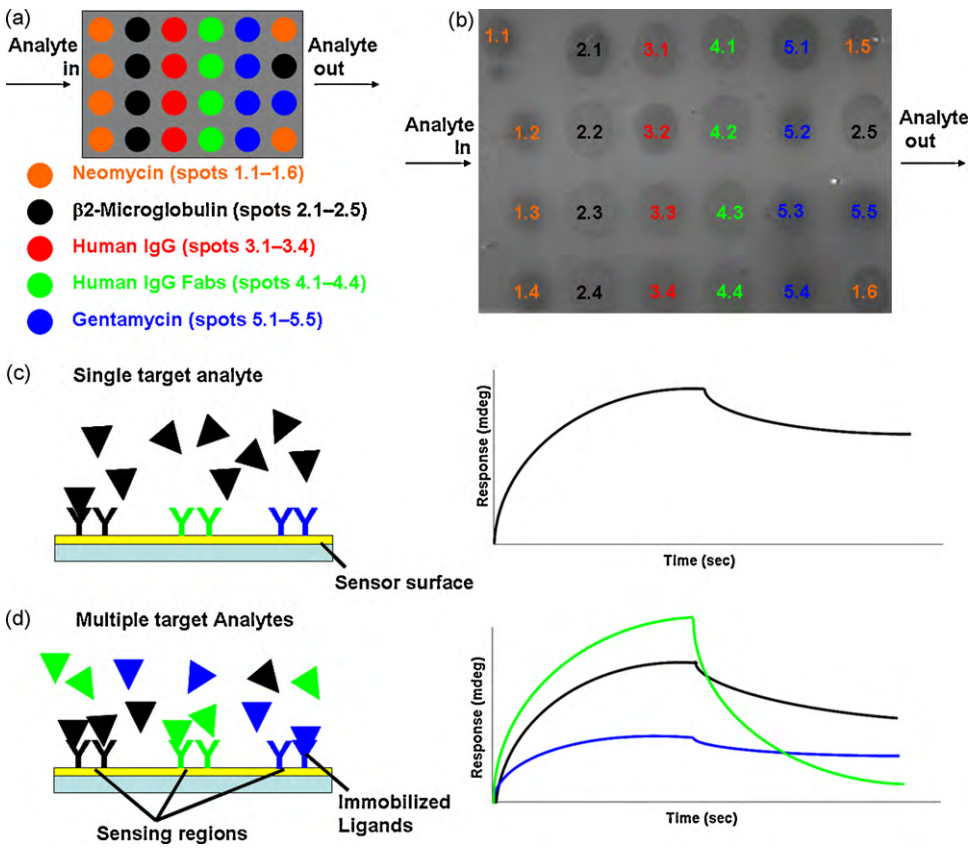

Fig. 1. (a) Schematic illustration of the immobilized spot locations. (b) Real-time iSPR image of the array of 24 immobilized spots. The numbers represent the spot numbers with respect to the molecules used: (1) color indicates the respective color of the molecules indicated in (a). (c) Schematic illustration of the classical experiment where single target analytes are injected over the array and its measured response for the specific spot. (d) Schematic illustration of newly developed approach while injecting a multiple target analytes (each specific for different ligands immobilized) and its measured responses for all the different spots. (For interpretation of the references to color in this artwork, the reader is referred to the web version of the article.)

2.4. Data analysis

Data analysis was performed with the SPRint software (IBIS Technologies BV, Enschede, The Netherlands) and kinetics parameter extraction was performed using Scrubber 2 (Biologic software, Australia) [27]. All model functions are plotted in orange color in the respective sensorgrams. Microsoft Excel was used to calculate the average and standard deviations.

3. Results and discussion

The experiments were conducted with multiple target analytes as well as single target analytes. All experiments described in this article were conducted using a single sensor disk. The details of the interaction scheme for the various analytes and ligands are shown in Table 2. The miniaturization of assays offers several advantages compared to conventional systems including reduced sample and reagent volumes, reduced time for experimental procedures and real-time binding information from the interactant pairs. Some disadvantages include cross-reactivity of different targets to unintended ligands on the surface, and regeneration non-uniformity between different ligand types, as well as, possible aggregation of biomolecules in solution when different targets with large differences in molecular weights are used [37,40]. However, the latter disadvantage is minimal as most of the antibodies belong to the family of Immunoglobulin G with a molecular weight of ~150 KDa.

All experiments were done using conventional biomolecular interaction kinetics where a series of serially diluted analyte samples are injected over the immobilized ligands on the sensor surface. In this article, we not only describe the biomolecule screening approach but also the measurement of kinetics and affinity of five different interactant pairs in parallel for each injection of multiple target analytes, which can reduce the total experimental time. In conventional SPR systems, one ligand is immobilized prior to binding measurements. However in our case, the immobilization procedure is done offline, which requires some time for the biomolecules from the 1 nL solution to adsorb to the sensing surface. This method requires longer time than the conventional immobilization method. At the same time, we can use multiple ligands (approximately 400 spots) and all immobilized in parallel that can lead to a high throughput assay. There is no need for extra time for multi-ligand immobilization in our case. The results from the various combinations of multiple target analytes and single target analytes have been compared.

Each of the ligands used in this experiment is spotted multiple times in order to check the reproducibility of the measurement, but another approach could be to use a serial dilution of the ligands spotted to the sensor chip. The spotting is performed outside the iSPR system. Hence, with the present spotting method for ligand immobilization, exact quantification of the spots is not possible. However, quantification is done indirectly with the extracted R_m values when the data is fitted to the model function. If the response deviations are high between spots of the same component, there is evidence that the immobilization is not uniform.

Although the 1:1 interaction model has been used to extract kinetics parameters from all interactant pairs presented in this article for the sake of simplicity. Some poor fits have been shown due to the fact that the biomolecule is bivalent or multivalent that can lead to various effects including avidity [45], heterogeneity of ana-

Table 2
Reaction scheme lists the analytes that are reactive to the specific ligand types in the array.

S.No.	Analytes	Immobilized ligands				
		Neomycin	β2M	Human IgG	Fab human IgG	Gentamycin
1	mAb neomycin	+	–	–	–	–
2	mAb gentamycin	–	–	–	–	+
3	mAb β2M	–	+	–	–	–
4	Fab antihuman IgG	–	–	+	+	–
5	Goat antihuman IgG (H + L)	–	–	+	+	–
6	Mixture 1	+	+	+	+	–
7	Mixture 2	+	+	–	–	+
8	Mixture 3	+	+	+	+	+

Table 3
Interactant pairs that follow 1:1 interaction model functions.

S.No.	Analytes	Immobilized ligands				
		Neomycin	β2M	Human IgG	Fab human IgG	Gentamycin
1	mAb neomycin [42]	Yes	–	–	–	–
2	mAb gentamycin [42]	–	–	–	–	No
3	mAb β2-microglobulin [43]	–	Yes	–	–	–
4	Fab antihuman IgG [44]	–	–	No	Yes	–
5	Goat antihuman IgG (H + L) [44]	–	–	No	No	–
6	Mixture 1	Yes	Yes	No	Yes	–
7	Mixture 2	Yes	Yes	–	–	No
8	Mixture 3	Yes	Yes	No	Yes	No

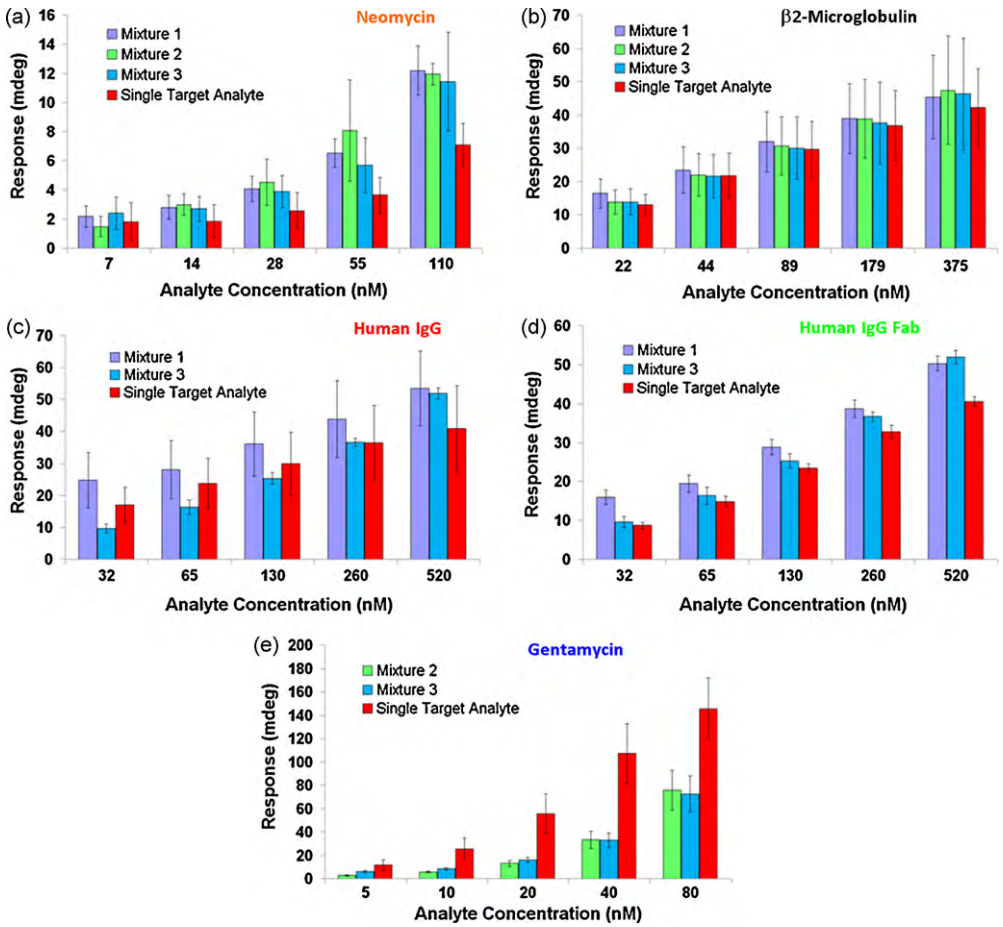

Fig. 2. Response analysis of all the ligand–ligate pairs for varying analyte concentrations (Table 1). Mixture 1 is the mixture of mAb for neomycin, mAb for β2-microglobulin and Fab specific antihuman IgG. Mixture 2 is the mixture of mAb for neomycin, mAb for β2-microglobulin and mAb for gentamycin. The mixture is the combination of all the five antibodies. (a) Neomycin–mAb for neomycin; (b) β2-microglobulin–antiβ2-microglobulin; (c) human IgG–Fab of antihuman IgG; (d) Fab of human IgG–Fab of antihuman IgG; (e) gentamycin–mAb for gentamycin. The number of analytes injected is 5. The number of sample points considered for standard deviation estimation is 4.

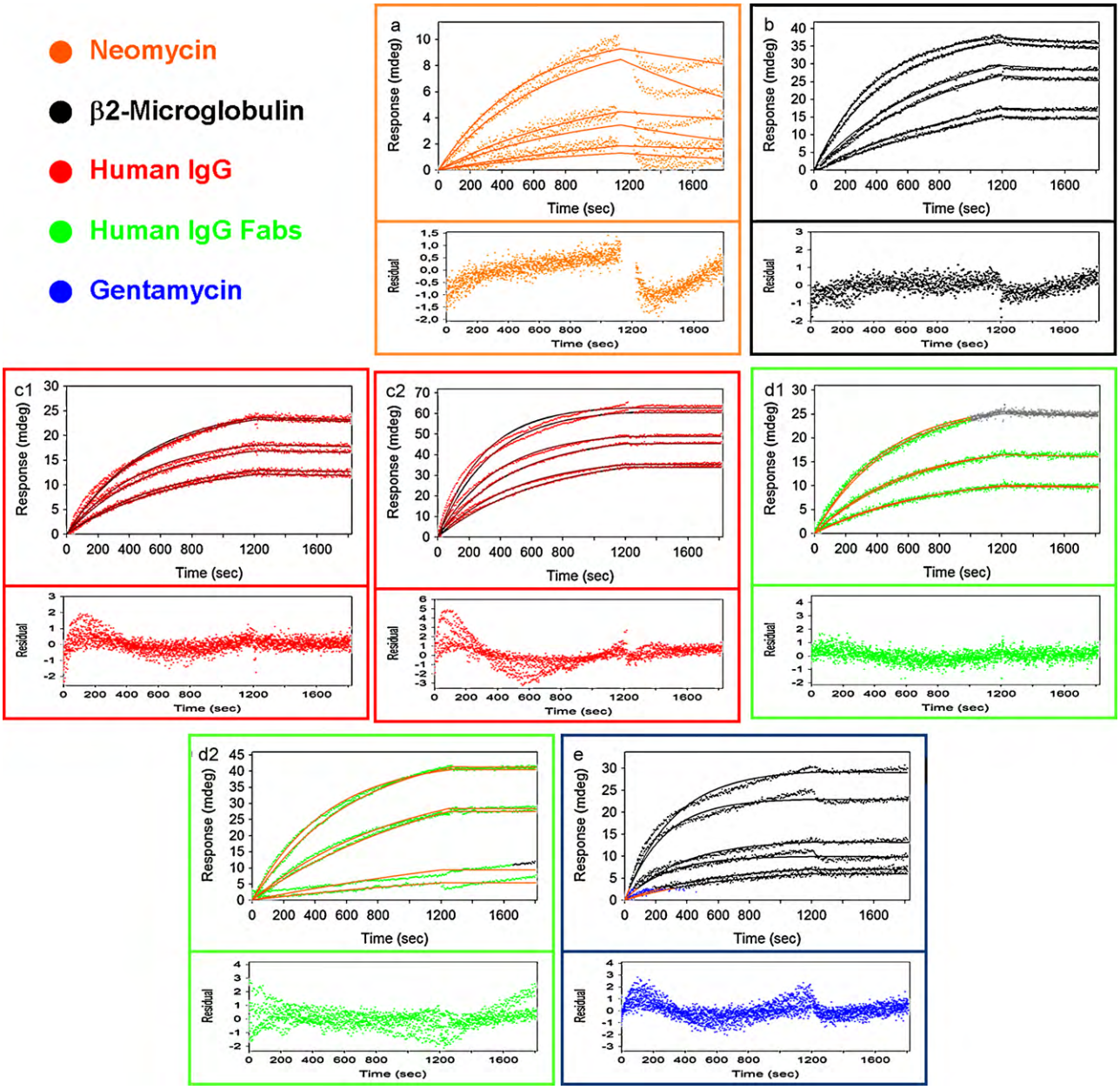

Fig. 3. iSPR sensorgrams recorded for various analyte concentrations (Table 1) with duplicate injections. (a) Neomycin–mAb for neomycin; (b) β2-microglobulin–antiβ2-microglobulin; (c1) human IgG–antihuman IgG Fabs; (c2) human IgG–antihuman IgG; (d1) Fab of human IgG–Fab of antihuman IgG; (d2) human IgG Fab–Fab of antihuman IgG; (e) gentamycin–mAb for gentamycin. Orange curves show the 1:1 model fit functions. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

lytes [46], other ligand heterogeneity factors affecting the binding process includes, conformational change [46], and mass transport effects [47]. However, to demonstrate the concept of integrated screening, detection and kinetics estimation possibilities, we have considered the 1:1 interaction model in this article. Since the model used is not suitable for some interactant pairs (e.g. neomycin, gentamycin), the extracted kinetic data for such model systems are not reliable. Table 3 describes the model fit scheme for the various interactant pairs used in this article. The deviation from this model is clearly observable in some cases and explained in the respective section of this paper.

From Fig. 1b, spots 2.1 (β2M) and 5.1 (gentamycin) show a ligand immobilization problem, where the intensity of the SPR image is different compared to the quadruplet spots of the same ligand. The heterogeneity of ligand spots is typically caused by a leakage in the microchannel in the TopSpot print head. This type of problem was also observed in the SPR-dip measurement plot (not shown) for β2M and gentamycin. The initial SPR-dips for the neomycin showed small variations, which can have direct influence on the responses measured during the interaction processes (results not shown).

The measured responses are plotted as bar plots (Fig. 2) for all individual ligand types and the standard deviation was calculated from the various ligand spots with the same concentration. These are, neomycin (Fig. 2a), β2-microglobulin (Fig. 2b), human IgG (Fig. 2c), Fab of human IgG (Fig. 2d) and gentamycin (Fig. 2e). The responses shown here are extracted from the various SPR sensorgrams at the transition from the association and dissociation phases (t = 1200 s). When multiple target analytes were used, the response was enhanced as previously reported [37,40]. There is no

direct co-relation between varying composition of antibodies in the mixture and the variation in enhanced response levels. However, there is not much deviation between the responses observed for the single target analyte and multiple target analytes used in the case of β2-microglobulin. Higher analyte concentrations, show larger deviations. But these deviations are lower compared to the other components used. Since the experiment was done with serial injections, the reduced signal could also be due to the regeneration steps. In reality, the numbers of regeneration steps are minimal because of the multiple target analytes used. High molecular weight components cause steric hindrances when they started to accumulate on the sensor surface, which might create pockets for other molecules to sit resulting in non-specific binding. This could be the reason for the slightly enhanced response measured in the case of multiple target analytes compared to single target analyte measurements. It does not appear to have a significant impact as there is little variation in the extracted kinetics and affinity of the interactant pairs. However, in the case of gentamycin, single target response is higher compared to the multiple target analyte samples. The exact reason for this specific difference is not known.

Sensorgrams obtained for the large analyte concentrations have been neglected in this paper as they typically deviate from the 1:1 interaction model irrespective of the valency of the biomolecules. Large analyte concentrations lead to saturation effects that can result in slightly different degrees of heterogeneity on the surface [48]. In addition, if the surface is loaded with large amounts of biomolecules then mass transport limitations can also introduce deviations from the 1:1 model behavior [49].

Conventional kinetics experiments were performed with the fabricated microarray with varying concentrations of single target analytes. The sensorgrams and model fits are shown in Fig. 3 with duplicate injections of the same analyte to check the reproducibility of the fabricated spots. Neomycin results (Fig. 3a) indicate antibody re-binding where response increases in the dissociation phase are evident. The re-binding effects are reproducible independent of the analyte concentration for neomycin. However, the model fit is in good agreement with the measured data in the association phase. Another reason for the increased signal in the dissociation phase may be due to the back-and-forth dissociation phase buffer flow. The back-and-forth operation is commonly in IBIS-iSPR system to reduce the amount of samples and buffer solutions used and this could be easily changed to flow-through approach if necessary. When kinetics information is of absolute importance, the flow-through approach is recommended. With a flow-through approach, we have not observed such re-binding behavior for neomycin [52]. In the cases where the measured interaction correlates well to the 1:1 model, such as β2-microglobulin (Fig. 3b) and human IgG Fabs (Fig. 3c1 and d1 and d2), the deviation between duplicate injections had a negligible effect. When both the ligand and analytes are Fab fragments, the sensorgrams are very reproducible (Fig. 3d1) and the model fit is in good agreement with the measured responses. Therefore, it is reasonable to consider Fab fragments rather than

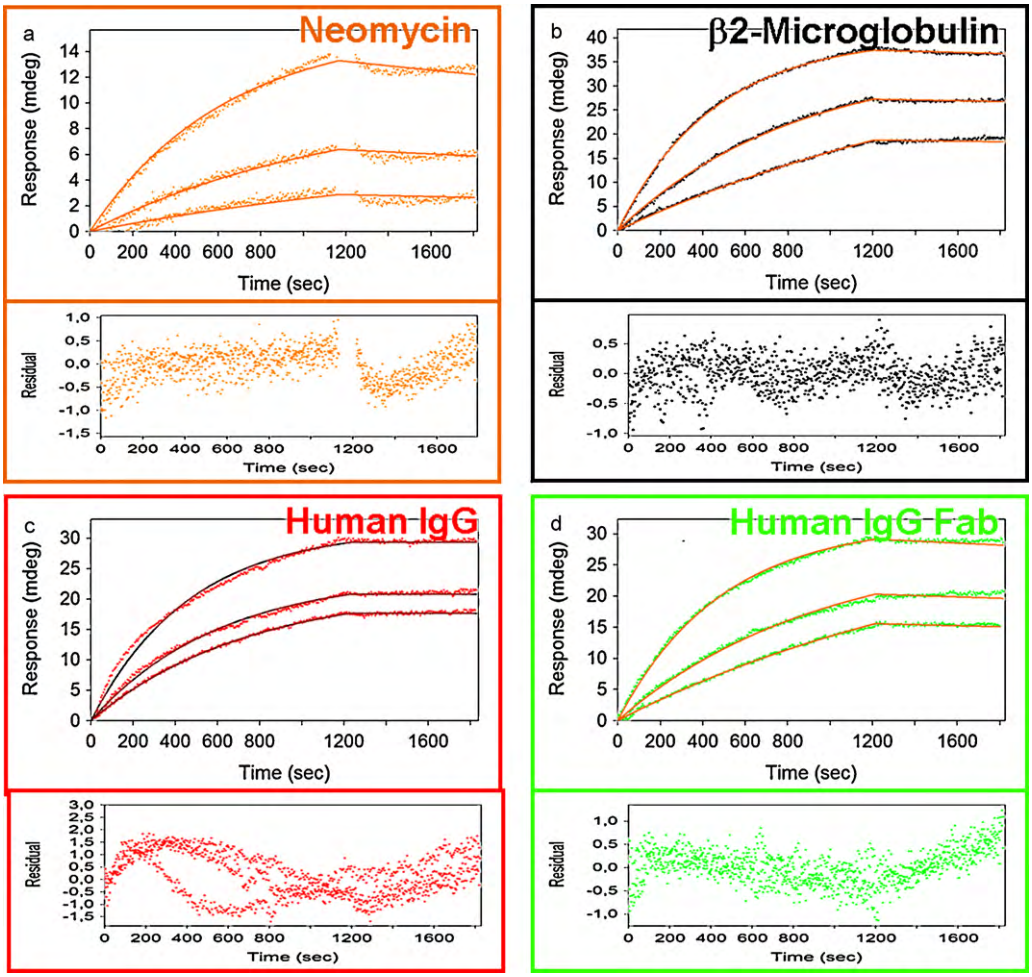

Fig. 4. Sensorgram recorded for the injection of sample mixture 1 (mAb for neomycin, mAb for β2M and Fab of antihuman IgG) with varying sample concentrations same as listed in Table 1. Orange curves show the 1:1 model function. (a) Neomycin; (b) β2-microglobulin; (c) human IgG; (d) human IgG Fab. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Table 4
Extracted kinetics parameters with various mixtures of analytes and pure antibody samples. The number of analytes considered for kinetics estimation is 3. The number of sample points considered for standard deviation estimation is 4.

Ligand–ligate pairs	k_a ($\times 10^4$ M^{-1} s^{-1})	k_d (s^{-1})	R_m (m°)	K_D (nM)
1. Mixture 1 results				
Neomycin	–	–	–	–
Microglobulin	2.8 ± 0.3	$(6.7 \pm 12.1) \times 10^{-3}$	34.2 ± 10.5	1.0 ± 0.2
HIgG	2.1 ± 0.3	$(5.7 \pm 2.3) \times 10^{-5}$	38.1 ± 10.1	2.8 ± 1.5
HIgG Fab	1.6 ± 0.2	$(7.1 \pm 1.5) \times 10^{-5}$	32.1 ± 1.1	4.3 ± 1.0
2. Mixture 2 results				
Neomycin	–	–	–	–
Microglobulin	2.3 ± 0.2	$(4.4 \pm 8.0) \times 10^{-3}$	34.5 ± 10.7	1.1 ± 0.6
Gentamycin	–	–	–	–
3. Mixture 3 results				
Neomycin	–	–	–	–
Microglobulin	2.3 ± 0.3	$(1.1 \pm 0.1) \times 10^{-4}$	36.7 ± 11.5	4.7 ± 0.7
HIgG	2.3 ± 0.2	$(3.2 \pm 0.3) \times 10^{-5}$	35.8 ± 9.1	1.4 ± 0.2
HIgG Fab	1.5 ± 0.3	$(7.1 \pm 1.6) \times 10^{-5}$	40.6 ± 6.6	4.5 ± 0.7
Gentamycin	–	–	–	–
4. Single target analyte results				
Neomycin	–	–	–	–
Microglobulin	2.5 ± 8.8	$(1.2 \pm 3.3) \times 10^{-4}$	36.3 ± 9.6	5.0 ± 2.8
HIgG/AHIgG Fab	2.3 ± 0.3	$(2.9 \pm 0.2) \times 10^{-5}$	29.1 ± 10.7	1.3 ± 0.2
HIgG/AHIgG	0.6 ± 0.1	$(5.8 \pm 1.8) \times 10^{-6}$	66.5 ± 15.9	0.9 ± 0.3
HIgG Fab/AHIgG Fab	0.6 ± 0.03	$(3.0 \pm 0.1) \times 10^{-5}$	29.4 ± 2.8	5.2 ± 0.2
HIgG Fab/AHIgG	0.2 ± 0.03	$(4.5 \pm 0.1) \times 10^{-3}$	73.7 ± 8.4	1.7 ± 0.2
Gentamycin	–	–	–	–

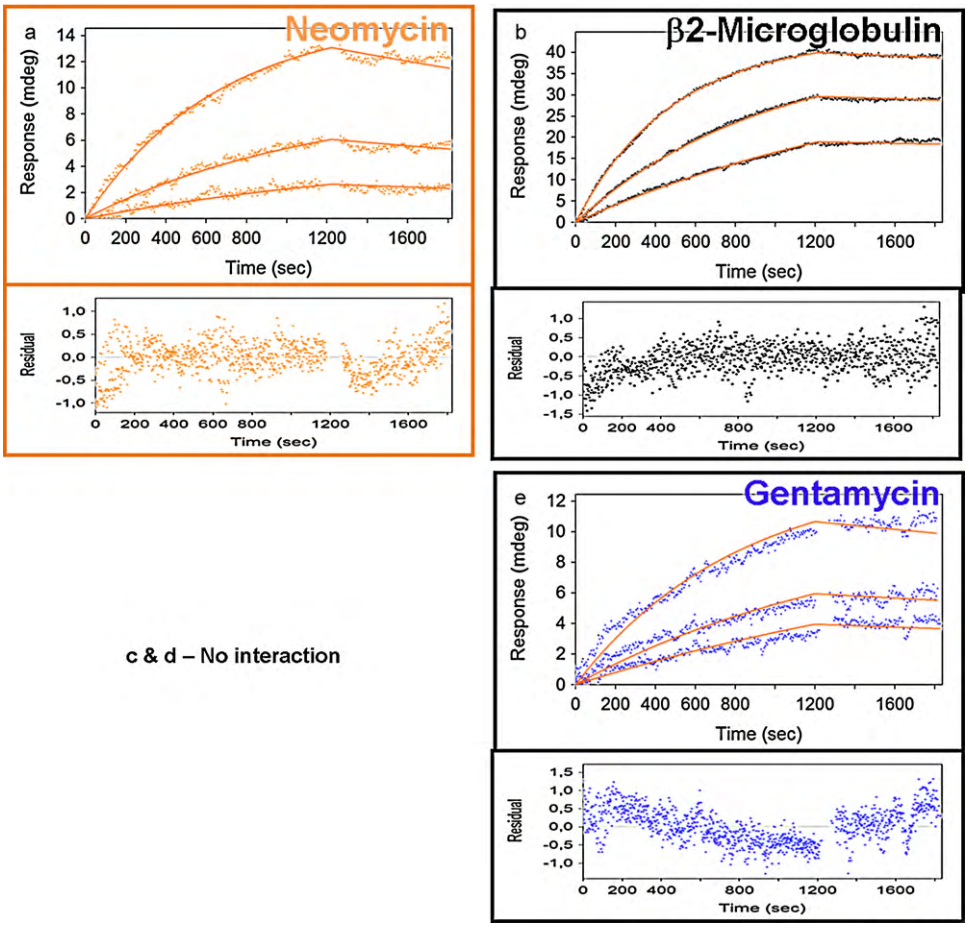

Fig. 5. Sensorgram recorded for the injection of sample mixture 2 (mAb for neomycin, mAb for β2M and mAb for gentamycin) with varying sample concentrations same as listed in Table 1. Orange curves show the 1:1 model function. (a) Neomycin; (b) β2-microglobulin; (c and d) human IgG and human IgG Fabs – no interactions; (e) gentamycin. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

the entire molecule when designing an assay [44]. Knowing that human IgG–antihuman IgG interactions (Fig. 3c2) do not follow a 1:1 interaction [44], we have clearly observed poor fits and the sensorgrams of duplicate injections were not reproducible. Gentamycin (Fig. 3e) sensorgrams show a biphasic behavior and hence the fitting to 1:1 model function does not work either, as shown in the sensorgram (Fig. 3e). This small difference in the responses of the duplicate injections does not affect the extracted kinetics and affinity constants. The missing points in the sensorgrams (e.g. Fig. 3a) were removed due to spikes that were observed during the buffer change for dissociation phase measurements.

Another type of screening involves the same analyte that could be reactive to many immobilized ligands [50], for example in drug discovery [51]. To demonstrate this, we have considered human IgG and fragments of human IgG both immobilized on the sensor surface and the experiments were conducted with Fab specific anti-

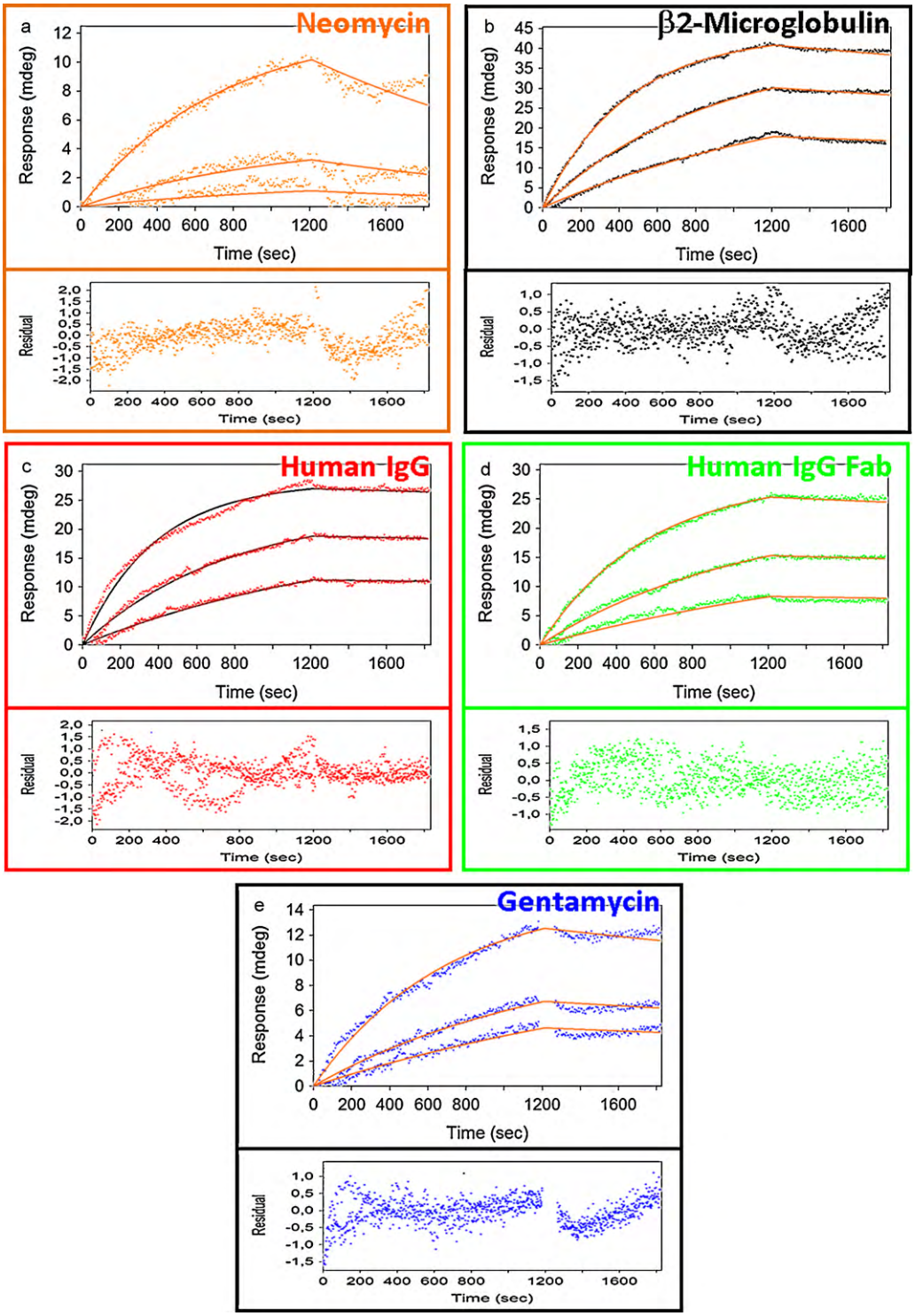

Fig. 6. Sensorgram recorded for the injection of sample mixture 3 (mAb for neomycin, mAb for β2M, mAb for gentamycin and Fab of antihuman IgG) with varying sample concentrations same as listed in Table 1. (a) Neomycin; (b) β2-microglobulin; (c) human IgG; (d) human IgG Fab; (e) gentamycin. Orange curves show the 1:1 model function. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

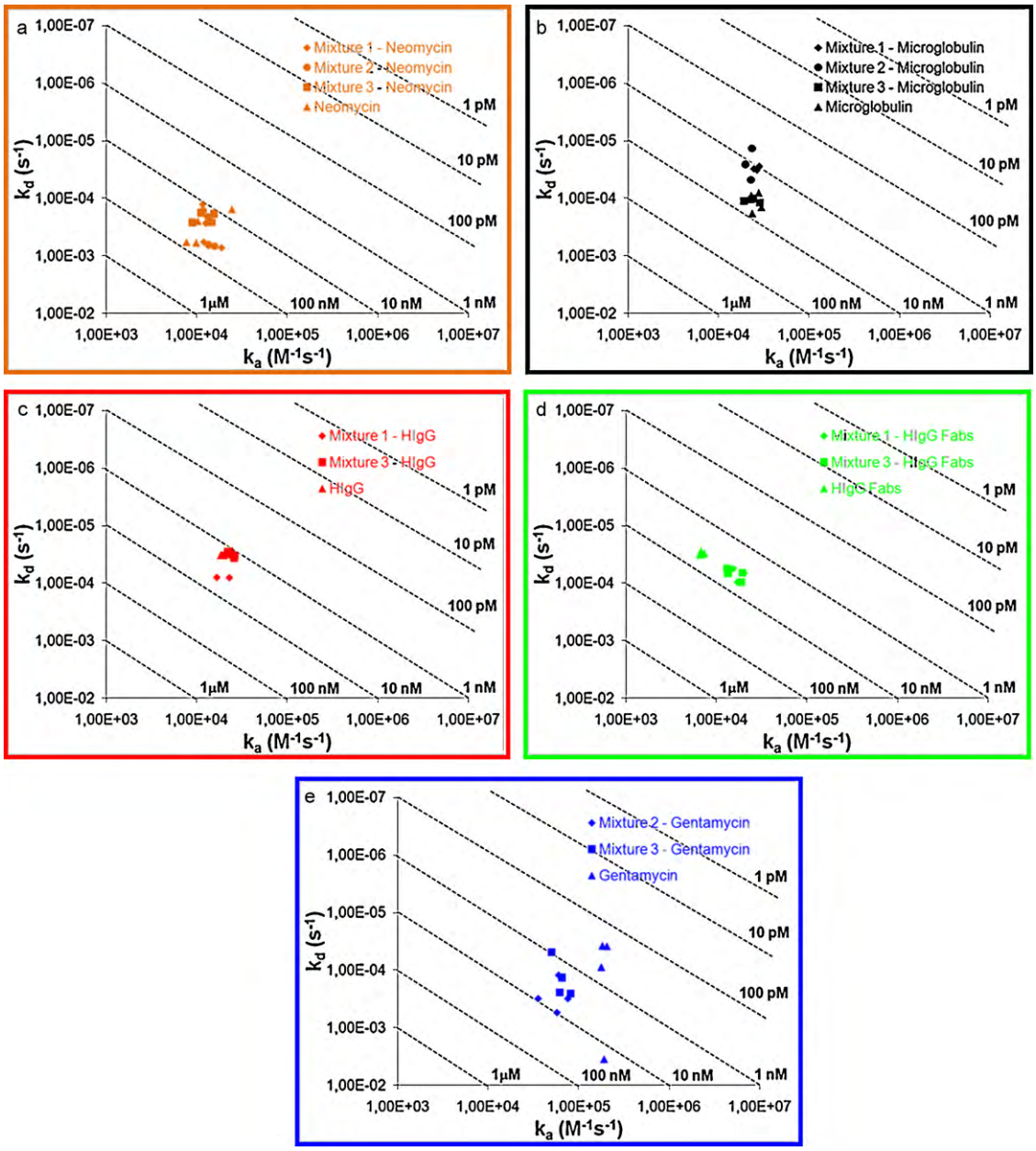

Fig. 7. Kinetic distribution plot for various ligand–ligate pairs. The result shown here is plotted from the parameter extracted from various ligand spots and also various sample mixtures used as well as with the pure ligate component. (a) Neomycin–mAb for neomycin; (b) β2M–antiβ2M; (c) human IgG–antihuman IgG Fabs; (d) human IgG Fabs–antihuman IgG Fabs; (e) gentamycin–mAb for gentamycin.

human IgG and antihuman IgG. The kinetics and affinity extracted clearly show deviations in the measurements. Fab of human IgG reacts with Fab specific antihuman IgG as well as when one of the components (either ligand or analyte) is Fab (Fig. 3c1 and d2); thus negligible deviations in the model fit. The small deviation in the model fits observed for the high analyte concentration sensorgrams (Fig. 3c1) is most likely caused by high analyte concentrations. The extraction of accurate kinetics and affinity parameter values from a polyclonal antibody with 1:1 interaction model is not possible, further experiments were performed only with monoclonal antibodies.

Mixtures of mAb for neomycin, mAb for β2M and Fab of antihuman IgG is represented as mixture 1. This mixture is reactive with 4 ligand types and are not reactive to gentamycin spots. Fig. 4a shows the sensorgrams and fit obtained for neomycin spots when the spots are exposed to mixture 1 samples. This shows clearly the same behavior observed for the single analyte specific for neomycin spots. The fits to the 1:1 model in all cases are in good agreement with the resultant measured data. The slight deviation in the fits at higher concentrations of mixture 2 specific for human IgG spots (Fig. 4c) is due to the multivalency of the human IgG which has 2 (H + L) sites for Fab specific antihuman IgG. As in the single analyte responses described above, β2M (Fig. 4b) and human IgG Fabs (Fig. 4d) show good agreement with measured data.

The sample mixture 2 consists of mAb for neomycin, mAb for β2M and mAb for gentamycin. Since mixture 2 is without antihuman IgG, we don't expect any reaction with immobilized human IgG or its Fab fragments. For neomycin spots (Fig. 5a) and β2M spots (Fig. 5b), the trend with respect to the measured responses and model fit function is similar to the other sample mixtures or single target analytes. However, gentamycin responses (Fig. 5e) show comparatively lower measured intensity and hence the 1:1 model

fit is in fair agreement with the measured data. Additionally, biphasic behavior was not observed with the concentrations used for kinetics estimation.

The mixture of all five antibodies is represented as sample mixture 3. The resultant sensorgrams and fits are shown in Fig. 6. The results are quite comparable with those of the other sample mixtures (Fig. 6a–d), except gentamycin (Fig. 6e). Gentamycin spot responses are quite similar to that of the sensorgrams obtained with the other sample mixtures, however, deviates from the single target analyte specific for gentamycin. The reason for this variation is not exactly known and it could be due to the multivalent behavior of the antibody or the gentamycin molecules.

All the extracted kinetics parameters for all the sample mixtures as well as single target analytes are listed in Table 4. Since the re-binding effect was not considered in this parameter estimation, the extracted parameters show large variations in the case of neomycin (extracted parameters are not reported due to bad fit). Since we have not considered the biphasic behavior of the gentamycin interactions, we have observed large deviations in the kinetics and affinity parameters extracted using the 1:1 interaction model. In other cases, such as, human IgG, human IgG Fabs and β2M, the extracted kinetics and affinity parameters are in very good agreement. Fab of human IgG interactions with polyclonal antihuman IgG shows higher affinity because of the multivalent behavior of the molecules and hence the dissociation becomes slower when compared to the Fab specific antihuman IgG. When designing the kinetics experiments, it is desirable to choose long dissociation times such that accurate dissociation rate constants are extracted. When the immobilized ligand is multivalent, as in human IgG, it does not show a large variation in the affinity constant extracted using the 1:1 interaction model when it interacts with antihuman IgG or Fab specific antihuman IgG. At present, the reason for this not exactly known.

One of the major disadvantages of a multiplexed assay is that an initial calibration is required to quantify all the interactant pairs involved in the experiments. However, this could indirectly be used as an initial screening to identify cross contamination. Optimizing regeneration conditions that should be same for all the interactant pairs is problematic in some cases and should be considered carefully.

The distribution of the extracted affinity was low between various ligand spots fabricated when the interactant pairs follows the 1:1 interaction model. The affinity distributions are presented by the plot of k_d versus k_a (Fig. 7). The deviations in the affinity constants for neomycin (Fig. 7a), β2M (Fig. 7b), human IgG (Fig. 7c), human IgG Fab fragments (Fig. 7d) and gentamycin (Fig. 7e) are between 10 and 100 nM, less than 1 and 10 nM, 1 and 10 nM, 4 and 6 nM and 0.1 and 15 nM, respectively. The large deviation in the neomycin and gentamycin is due to a poor fit to the 1:1 model. The distribution of affinity constants in quadruplet spots might also be due to our spotting technique, which requires the diffusion of molecule from a 1 nL volume to the surface over a fixed time. Other properly controlled microfluidic immobilization might avoid this problem.

An important point to consider while designing such an assay is that there may be cross interaction of the analyte sample to other immobilized ligands, which can lead to inaccurate kinetics estimation. There might also be heterogeneity in the analyte sample that could lead to experimental error due to the varying molecular weights of the multiple components used in the mixture of analytes. Vigorous mixing of bio-samples typically leads to the agglomeration that can lead to steric hindrance on the sensor surface, which could block the immediate available site for the other molecules to reach. This is due to the improper orientation of immobilized ligands which cannot be controlled precisely with the existing spotting techniques.

Our approach is limited not only for the analysis that we have demonstrated but also for the assays where the antibodies have to immobilized on the sensor surface for certain reasons. The important point of consideration at this case is the orientation of the antibodies on the surface which is extremely important for reliable biomolecular interaction measurements. However, there would not be any difference in the extracted affinity constants.

4. Conclusion

The design of an integrated multi-ligand/multi-analyte detection assay with the estimation of kinetics and affinity parameters was successfully demonstrated using five different interactant pairs on a single sensor surface. The kinetics and affinity parameters were extracted for all the interactant pairs, by injecting the mixture of various antibodies and are in very good agreement with results from conventional measurements using a single analyte. This also gives information about specificity of the multiple targets used. The experimental time was reduced for such experiments when compared to typical kinetics experiments and this approach could be extended further to more interactant pairs as long as the molecules do not cross-react, which can lead to non-specific binding and inaccurate kinetics estimation. This new approach facilitates the simultaneous screening and kinetics estimation of various multi-parameter samples, e.g. drug targets for drug discovery, bio-warfare agents, food screening, biomarker discovery, and antibody production.

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Richard B.M. Schasfoort (1959) graduated as a biotechnology major from the University of Groningen in 1984. He defended his Ph.D. thesis regarding the development of a biosensor at the University of Twente in 1989. He joined The Netherlands Organization for Applied Scientific Research (TNO-Zeist) as project manager biosensors. In 1994 he founded IBIS Technologies BV where he was responsible for the development and sales of SPR-based biosensors. In 1998 he became senior scientist at MESA+ Research Institute, Faculty of Electrical Engineering, University of Twente. After receiving the NWO (Netherlands Organisation for Scientific Research) grant "vernieuwingsimpuls" in 2002, he established the Biochip group at the Faculty of Science and Technology, MESA+, University of Twente. The Biochip Group operated as an independent research unit running 8 projects with 12 members by December 2006. After reorganizing the Biochip group he joined IBIS Technologies partly as chief sales officer in June 2008. Recently he shifted from the BIOS group (MESA+ Institute) of Prof. Albert van den Berg to the Medical Cell BioPhysics Group (MCBP) (MIRA Research Institute) of Prof. Leon Terstappen for 0.2 fte. Dr. Schasfoort is editor of the Handbook of Surface Plasmon Resonance and published ~60 peer reviewed papers.