Biosensor Based Protein Profiling on Reverse Phase Serum Microarray

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Abstract

The reverse phase serum microarray format enables multi-parallel and simultaneous analysis of literally thousands of samples, a feature which is of utmost importance for protein profiling of clinical samples. We have here screened 2400 serum samples for their potential IgA deficiency by using a fluorescence based reverse phase serum microarray platform and a biosensor based label-free microarray platform for verification and also compared our microarray-results to clinical routine ELISA. We have been able to identify possible IgA-deficiencies and to show the suitability of our microarray-platforms for large-scale screening of clinical serum samples.

The two microarray methods show reproducibility and correlation towards each other and low variation between replicates within each platform. Both of the microarray platforms show less agreement towards ELISA. The fluorescence based microarray method has been shown to be applicable for large-scale screening of clinically important serum samples for detection of possibly IgA-deficient patients. Furthermore, it was found that the microarray based biosensor method could be used for determining the relative differences in concentration of IgA between the samples.

Keywords: Reverse phase serum microarray; Protein profiling and screening; SPR; Biosensor; Label-free

Introduction

The reverse phase microarray format enables multi-parallel and simultaneous analysis of literally thousands of samples, a feature which is of utmost importance for protein profiling of clinical samples and which is also often a limiting factor. Other microarray based technology platforms might have the capacity to profile large numbers of targets and analytes, but is usually restricted in the multiplexing dimension of samples. In the reverse phase microarray platform the samples are immobilised on the substrate in an array configuration and the affinity reagents are subsequently applied as detection reagents. This enables large numbers of samples being profiled for one or a few targets simultaneously under the same experimental conditions while using low sample volumes. On the other hand, in the forward phase array where the affinity reagents, antibodies or other binding molecules, are immobilised on the functionalised surface, the samples containing the antigens are then applied to the array. This allows for the simultaneous analysis of a few samples towards a high number of targets.

The majority of the described applications of reverse phase microarrays have so far mainly involved various forms of either cell lysates [1,2] or tissue lysates [3,4]. We have previously described the application of serum microarrays. This have been in the context of analysis of IgA levels in 2000 patients [5], screening for IgA deficiency of samples. In the reverse phase microarray platform the samples are immobilised on the substrate in an array configuration and the affinity reagents are subsequently applied as detection reagents. This enables large numbers of samples being profiled for one or a few targets simultaneously under the same experimental conditions while using low sample volumes. On the other hand, in the forward phase array where the affinity reagents, antibodies or other binding molecules, are immobilised on the functionalised surface, the samples containing the antigens are then applied to the array. This allows for the simultaneous analysis of a few samples towards a high number of targets.

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There is an inherent sensitivity issue with reverse phase serum microarrays. This limitation arises due to the large dynamic range of proteins present in blood where many proteins are present in low pg/ml and thereby IM [8]. Proteins that are present in such low concentrations in whole blood will be represented by only a few molecules if the total sample volume is in the sub-nano litre scale spotted in the array which greatly impedes sensitive measurements. Efforts are needed and ongoing to develop methodologies and techniques for increased detectability utilizing various types of signal amplification as for example reviewed by Nong et al. [9] for DNA-based technologies. It is although still in its current direct setup a platform suitable for detecting medium to highly abundant proteins while low abundant proteins will be challenging to detect reliably. Within the IgA profiling the limits of detection has been found to be in the high ng/ml to the low µg/ml range [5].

When conducting large-scale antibody based protein profiling experiments and screening of samples, confirmation and verifications of early indications are of fundamental importance. Initial technical verifications usually involve repeated measurements and various replicates and often before looking into different and most often also larger sample sets, there is also a value to confirm screening results with alternative methods and technologies. One such alternative is to use a biosensor based label-free platform avoiding the need for a secondary reagent since no labelled detection molecule is utilized. Measuring a property of a binding event itself also allows for real-time measurement and extraction of reaction kinetics of the event. Label-free methods can be based on, the polarization state of reflected light [10,11], interference fringes [12-14], or a number of other properties [15-20]. Surface plasmon resonance (SPR) sensors are based on the changes in refractive index that are produced by the interaction between molecules immobilised on a detection surface and molecules in a liquid sample flowing over the detection surface [21-25].

The majority of currently available biosensor instruments does not allow for multi-parallel analysis in an array-based format, but there are some platforms that can provide the necessary sample throughput [26].

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This makes them suitable as alternative platforms for confirmation of results obtained from large-scale screenings on reverse phase fluorescent microarrays.

We have previously developed a reverse phase microarray platform for large-scale simultaneous probing of serum samples [5]. We have now applied this platform to 2423 serum samples from children in order investigate the feasibility of using this platform in large-scale screening of children for detection of IgA-deficiency in combination with a biosensor based microarray platform for validation. A subset of those samples with indications of being IgA deficient were transferred to a SPR-based platform in order to confirm the results, we have also compared the two microarray platforms to the commonly used ELISA.

Materials and Methods

Experimental setup

2423 serum samples from children were printed on glass slides and analysed for their IgA content. 182 of those samples were reprinted on glass slides and reanalysed as well as printed on a sensor chip for SPR-analysis. This selected set consisted of 28 samples with a concentration of IgA of 0.3 mg/ml or less, 100 samples that showed large discrepancies between ELISA and fluorescence microarrays, and a 54 sample set that were randomly chosen from the sample pool. A comparison between replicates and methods were performed as well as a comparison with ELISA-values for those samples.

Fluorescence based microarray

The serum samples were diluted 1:10 PBS with 0.5% Tween20, loaded onto 384 well plates in volumes of 30 µl/well (Genetix) and printed in duplicate (in 14 identical blocks) on Corning Epoxide slides (Corning) using a non-contact printing robot (Nano-plottet 2.0, Gesim). The slides were incubated in a humidity chamber (75%) for 16 hours at 20°C and blocked with Super Block solution (Pierce) using an air-brush pistol. Polyclonal rabbit anti-human IgA antibodies (DAKO) were added at a concentration of 46 ng/ml. Alexa Fluor 555 goat-anti-rabbit IgG (Molecular Probes) was used as a secondary antibody at a concentration of 33 ng/ml. The slides were scanned in a G2S65BA array scanner (Agilent) with the photomultiplier tube set to 100% for both channels and the scan resolution set to 10 µm. The resulting images were analysed with GenePix-Pro 5.1 (Molecular Devices) using non-circular feature alignment.

Biosensor based label-free microarray

A total of 200 samples consisting of 182 serum samples with known IgA levels and 18 control samples where diluted 1:10 in 0.5% Tween20 in 1x PBS and printed in duplicates with a non-contact microarray printer (NanoPlottet2, GeSiM).

Blocking of the detection surface was conducted in the FlexChip instrument (GE Healthcare,Biacore Systems) by filling the flowcell with 0.1% Tween 20 in 1xPBS with 10% Bovine Serum Albumin (BSA Cohn fraction V, protease free, Saveen Werner) five times for five minutes each. A baseline was established by flowing the running buffer (0.1% Tween 20 in 1xPBS) through the flow cell for ten minutes before the first antibody injection.

As a negative control anti-rabbit IgG antibody (Jackson ImmunoResearch) was used, anti-human HSA antibody (Jackson ImmunoResearch) was used as a positive control, anti-human IgG antibody (DakoCytomation) was used for verification of normal IgG levels in the samples and a blank sample consisting of 0.1% Tween 20 in 1xPBS was used to verify that no unspecific binding occurred due to the dilution buffer.

The antibodies where diluted in running buffer to final concentrations of 50 µg/ml in separate sample tubes and sequentially recirculated through the flowcell for five minutes each with a five minute disassociation phase in between every injection. The order of injection was; anti-rabbit IgG, anti-human IgA, anti-human C3, anti-human IgG, anti-human HSA, 0.1% Tween 20 in PBS (Figure3).

The resulting binding curves yields full kinetic data for the binding by extracting two report points, binding early and binding late, from the association phase and two report points, stability early and stability late from the dissociation phase. In this work only the late stability values were used in the comparison between platforms. This was repeated on two detection chips to yield a total of four binding curves for each serum sample.

ELISA

Total serum IgA levels were determined by sandwich ELISA using polyclonal rabbit anti-human IgA antibodies (DAKO) and alkaline phosphatase-conjugated rabbit anti-human serum IgA antibodies (Jackson ImmunoResearch), added at a concentration of 1.2 µg/ml and 0.6 µg/ml respectively. Polystyrene plates (Corning) were coated over night at room temperature with 100 µl per well of the primary antibody diluted in carbonate bicarbonate buffer (0.05M). The plates were washed four times with phosphate-buffered saline (PBS) with 0.5% Tween 20 between the incubations. The samples were three-fold serially diluted in PBS with 0.5% Tween 20. All samples were titrated against a six-fold serially diluted standard, ranging from 3.1 ng/ml to 100 ng/ml. The samples, the standard dilutions and a blank (PBS with 0.5% Tween 20) were added in duplicate (100 µl/well) and incubated over night at room temperature. The alkaline phosphatase-conjugated antibodies were added (100 µl/well) and incubated for 2 hours in room temperature. p-Nitrophenyl phosphate (Sigma-Aldrich) was added and the absorbance was read on a Vmax microplate reader ( Molecular Devices). A mean concentration was obtained for each sample using DeltaSoft JV 1.8 (Biometallics). In addition, total serum IgG levels were determined in the individuals with IgA deficiency using sandwich ELISA. Polyclonal rabbit anti-human IgG antibodies (DAKO) and alkaline phosphatase-conjugated polyclonal rabbit anti-human IgG antibodies (DAKO) were added at a concentration of 0.6 µg/ml and 1.1 µg/ml respectively. The same protocol as for determination of serum IgA was followed.

Data analysis and normalisation

For both array platforms a simple global median normalisation was performed in order to correct for experimental artifacts. This was done by multiplying each data point with the ratio between the median of the individual slide and the median of all slides. The median intensity of each spot was averaged based on replicates and the correlation between the microarrays and ELISA was calculated. A scaling factor was calculated between the data from the arrays and the data from ELISA as the ratio between the median of the array data and the median of the ELISA data and applied to the array data for scaling to mg/ml for comparison with ELISA values. The coefficient of variation and Pearson correlation were calculated between replicate ELISA experiments, replicate microarray printings and between replicate SPR-sensor chips. All statistical analysis and normalisation were done using R, a language for data analysis and graphics (www.r-project.com).
Results and Discussion

We have in a high-throughput fashion screened 2400 serum samples from children to identify possible IgA-deficiencies with the goal of utilizing a fluorescence-based microarray platform as a tool for large-scale screening of clinically relevant samples and to use a biosensor-based label-free reverse phase microarray platform for validation. We have confirmed the results on a subset of 182 samples consisting of samples that were identified as IgA-deficient on ELISA and samples that showed large discrepancy between the two platforms. This was done using a microarray-based biosensor with label-free surface plasmon resonance detection and the results from both microarray-based platforms were compared with results from ELISA in order to compare the results obtained from the microarray platforms to a clinically common platform and to investigate the suitability of the SPR-based platform for detecting IgA-deficiencies (Figure 1).

Fluorescence microarrays

Printing the serum samples yielded spots with uniformly homogenous morphology. The correlation between replicate slides and within slides were both r=0.98 and between printings r=0.90. This high correlation between technical replicates show that the protocol used enables consistent generation of high quality sample spots which is necessary in order to ensure that sufficient precision in determining deficient samples is achieved and to minimise the risk of producing false negatives (Figures 2A–2C). Separation between deficient samples and non-deficient samples were achieved to a satisfactory degree making it possible to identify possible deficiency samples that can be further analysed in order to validate their lack of IgA (Figure 4D).

This means that it is possible to use this protocol for high-throughput screening of serum samples for determining which samples show low IgA-levels that could be a result of IgA-deficiency in the patient. This would best be achieved by first performing a large initial screening of samples in order to investigate the relative amount of IgA and then reanalyse the lowest range to confirm possible deficiencies. The initial large-scale screening can be performed upon thousands of samples simultaneously and thus minimise the amount of time and money needed to perform each sample analysis. This kind of screening is best performed in a microarray format with fluorescence labelling due to the possibility to easily print and analyse samples in the tens of thousands. The subsequent reanalysis of the lowest ranged samples is necessary in order to ensure that sufficient precision in determining deficient samples is achieved and to minimise the risk of producing false negatives (Figures 3A–3C). Separation between deficient samples and non-deficient samples were achieved to a satisfactory degree making it possible to identify possible deficiency samples that can be further analysed in order to validate their lack of IgA (Figure 4D).

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SPR-microarrays

We printed 182 serum samples with known IgA levels and 18 control samples in duplicates forming a total of 400 spots resulting in 400 simultaneous binding curves per injection (Figure 3). Using duplicate chips and six injections we obtained a total of 4800 binding curves. The duplicate positive binding curves showed a correlation between chip replicates of r=0.98 as well as sufficient separation between deficient and non-deficient samples confirming the results from the fluorescence based analysis. Interrogating the immobilised samples with an anti-IgG antibody showed that there were no correlation between the IgA-levels and the general IgG-levels ensuring that the results are specific to the IgA-levels (Figure 3C). We have chosen to use the stability latepoint of the dissociation part of the binding curve for measuring the IgA-levels but the results were similar for the binding early, binding late and stability early measurements meaning that either of these points on the binding curve could be used for determining the relative amount of IgA.

Comparison with ELISA

When comparing the two microarray-based platforms to ELISA the results from the microarray-based platforms proved to be more consistent with each other than with the results from ELISA (Figures 4A–4C). ELISA show lower correlation between replicates (r=0.57 vs. r=0.90 for the fluorescence-based platform and r=0.98 for the SPR-
based platform) which implicates overall better robustness for the microarray platforms. This is of high importance in a clinical setting since it would mean that fewer technical replicates are needed to minimise the risk of false positives or false negatives.

This inconsistency between the two microarray methods and ELISA becomes more apparent at increasing IgA concentration which might mean that ELISA lacks accuracy at high concentrations of the target molecule. Although when using ELISA to identify samples with IgA deficiency, defined as serum IgA levels below 0.07 mg/ml, and plotting the two groups for all three platforms they all show good separation between the deficient samples and the normal samples. The lower correlation between replicates as well as the low correlation between the two microarray platforms and ELISA ($r = 0.66$ for ELISA vs. fluorescence based arrays and $r = 0.76$ for ELISA vs. SPR-based arrays) might be an effect of the low throughput of ELISA that requires the samples to be analysed in small batches over a long period of time while the high-throughput array-platforms allow analysis of large sample cohorts under the same experimental conditions. It could otherwise have been expected that ELISA and the fluorescence-based microarray platform would have behaved more similar to each other than to the SPR-platform due to the similar setup using primary and secondary labelled binders, while the SPR-platform only need the primary binder and therefore should be better suited for analysing complex samples.

Conclusions

We have in this work analysed 2400 serum samples in a large-scale screening on a microarray platform based on fluorescent labelling and confirmed the results on a microarray platform based on surface plasmon resonance. Sufficient separation between deficient and non-deficient samples was achieved for identification of deficient samples even though no depletion of the samples was performed before hand. No correlation between IgA-levels and general IgG-levels could be found meaning that identified IgA-deficiency samples are not suffering from general IgG-deficiency.

For benchmarking purposes we have compared the two microarray platforms to ELISA. We found that the fluorescently labelled and the SPR-based microarray platforms show higher correlation between replicates than ELISA and confirm each other with better correlation towards each other than towards ELISA. ELISA show increasing disagreement with the microarray-based methods at higher concentrations of the target and show a low reproducibility of results which imply a lower accuracy for ELISA when analysing for IgA in serum.

This means that utilising a microarray-based platform with fluorescence-based detection appears highly suitable for screening large cohorts of samples to determine their relative concentration of IgA. The set of samples that show the lowest relative concentration can then be reanalysed on a SPR-based method to screen that smaller subset of samples in an effective way of validating possible IgA-deficiencies.
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