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Detection of Human Proteins Using Arrayed Imaging Reflectometry

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Abstract

Assays built upon protein arrays are critical tools in determining the basic nature of biology, and have considerable promise in diagnosing human disease. These protein arrays aid in the elucidation of mapping pathway interactions, disease biomarker discovery, and regulatory processes. The solutions used in these experiments, including cellular lysate and serum, are inherently complex mixtures and are high in total protein content. Therefore, array based assays must be robust and maintain a high level of selectivity and sensitivity. We report herein that Arrayed Imaging Reflectometry (AIR), a label-free biosensing platform we have previously disclosed, is highly suitable for the detection of human proteins in complex solutions. In particular, we demonstrate array-based detection of cytokines in buffered solutions, and in undiluted human serum.

Keywords

proteomics; cytokines; biosensor; arrayed imaging reflectometry

1. Introduction

A challenge for any protein biosensor platform is to remain quantitative over the extent of a target's physiological relevance. The balance between dynamic range (quantitation of signal over orders of magnitude in target concentration) and sensitivity (both in terms of limit of detection, as well as signal disparity distinguishing comparable concentrations) is often difficult to maintain, as many proteins exist in low copy number (Anderson and Anderson, 2002), or the concentration span between basal and upregulated levels is large (Laack et al., 2002; Le et al., 2005). This difficulty is enhanced when attempting to selectively detect the target molecule in a complex solution (Yocum et al., 2005; Gong et al., 2006). A salient example of this challenge is the detection and quantitation of serum biomarkers of various ailments: for example, cytokines and chemokines (markers of infection and/or inflammation) can have concentration ranges between single pg/mL to hundreds of ng/mL, depending on their role in a specific response pathway (Fenton et al., 1997; Barrios-Rodiles et al., 1999; Ichiyama et al., 2004). In the case of detection from human serum, the technique must also be able to discern

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the presence of a protein that is in essence a few parts-per-billion “sample impurity” in over 60 mg/mL total protein (Altıntaş and Denizli, 2006).

Enzyme-linked immunosorbant assays (ELISAs) are the archetypal biomolecular interaction detection technique, and have been used previously to detect proteins in human serum (Knipping et al., 1995; Boakle et al., 2006). However, since ELISAs are performed *de facto* through sample dilution and generate signal via conjugation of a fluorophore or enzyme turnover, the technique’s performance in truly complex solutions is difficult to discern. Alternative strategies are required to circumvent the cost disadvantages (both in assay time and in the compulsory secondary reagents) and the potentially detrimental effects arising from reporter conjugation to a protein in the detection scaffold. An example of such a biomolecular sensing platform is surface plasmon resonance (SPR; Homola et al., 1999). SPR has, in various configurations, been used as a tool to study protein biomarkers in complex solutions (Cui et al., 2003; Battaglia et al., 2005). Only recently has a group reported the detection of nanogram-per-milliliter quantities of human proteins from undiluted serum (Masson et al., 2007). Although this work utilized a human protein spiked into pure *bovine* serum, this is clearly a significant step forward. Nevertheless, further research is required to investigate the potential of monitoring multiple targets concurrently, as well as broadening this avenue of research into a method that operates in a fully human system.

We have previously reported the initial demonstration and characterization of an optical biosensing technique called Arrayed Imaging Reflectometry (AIR; Lu, et al. 2004; Mace, et al. 2006). The basis of AIR is the creation and perturbation of an antireflective coating on a silicon substrate. This antireflective layer is simply a thin film of thermally grown silicon dioxide manufactured to a specific thickness dictated by the wavelength of light chosen to illuminate the substrate’s surface. If the light is s-polarized and incident onto the silicon dioxide/silicon substrate, at a precise angle determined by their refractive indices, total destructive interference of reflected light will occur. Fixing the wavelength, polarization, and incident angle then allows for monitoring sub-Ångstrom changes in the thickness of the antireflective coating. Modifying the silicon dioxide surface with probe molecules (for example, antibodies) covalently attached to a chemical scaffold allows for the observation of probe/target complex formation by quantifying the reflectance changes concomitant with the thickness increase due to the interaction. An important feature of the AIR method is that this is accomplished in an arrayed format, allowing numerous probe spots to be simultaneously monitored.

Herein we provide the first demonstration of the use of AIR chips bearing immobilized antibodies to human proteins. The antibody arrays are employed in order to detect various cytokines, proteins involved in the body’s inflammatory response (Hanada and Yoshimura, 2002) and reaction to Gram-negative bacterial infection (Netea et al., 2003), among other biological pathways. We examine the selectivity of our antibody arrays, and by using a single antibody system, anti-IFN γ and IFN γ , we explored the limit of detection for an antibody-based AIR sensor. Finally, the current capability of the technique to detect native proteins from undiluted human serum is investigated and discussed.

2. Materials and Methods

2.1 Chip Functionalization

Silicon wafers with approximately 1400 Å of thermal silicon dioxide (Rochester Institute of Technology) were diced into 2 cm $\times$ 1 cm chips. Individual chips had their oxide thicknesses finely tuned to 1365 Å using successive rounds of dilute hydrofluoric acid washes as monitored by spectroscopic ellipsometry (J.A. Woollam M2000 spectroscopic ellipsometer; Lincoln, NE, USA). Etched chips were then washed in a 1:1 solution of hydrochloric acid:methanol for 30

minutes. After thorough washing in glass distilled deionized water (ddH₂O) and drying under a stream of nitrogen, the chips were submerged in a solution of γ -aminopropyl triethoxysilane (SigmaAldrich, APTES; 0.25% v/v APTES in 4.75% ddH₂O and 95% acetone) for 15 minutes. After successive washes in ddH₂O, the chips were dried under a stream of nitrogen and cured in an oven at 100°C for 15 minutes. Upon cooling to room temperature, a solution of glutaraldehyde (Alfa Aesar; 1.25% v/v glutaraldehyde in 100 mM Na₂HPO₄, 150 mM NaCl, pH 7.3) was added to the chips for 60 minutes. The chips were then washed with ddH₂O, acetone, and ddH₂O, and dried under a stream of nitrogen.

2.2 Array Fabrication

All monoclonal antibodies and cytokines for this study were purchased from R & D Systems, Inc. (Minneapolis, MN, USA), with the exception of anti-human IgG, which was purchased from Sigma Aldrich. Antibodies were diluted in MPBS (10 mM Na₂HPO₄, 10 mM NaH₂PO₄, 150 mM NaCl, pH 7.2) to a stock concentration suitable for arraying (500 μ L for each cytokine antibody and 50 μ g/mL for anti-human IgG). All cytokine proteins were stored at high concentrations (>10 μ g/mL) in a 1% bovine serum albumin (BSA, 20% ultrapure solution from Invitrogen) solution in MPBS for stability. Upon utilization, cytokines were sequentially diluted tenfold in an MPBS-BET buffer (MPBS buffer supplemented with 1% BSA, 3 mM EDTA, and 0.005% Tween-20); MPBS-BET buffer was also applied to a separate chip for all negative control experiments. Human serum was purchased from Equitech-Bio, Inc. (Kerrville, TX, USA) and was used undiluted.

Arrays were created manually by pipetting 1.5 μ L of antibody solutions in a defined pattern of spots. Once the full array was prepared, the chips were incubated at 4°C for six hours. Excess liquid was then removed from the array, and the chips were washed with ddH₂O at 4°C. The wet chips were then immediately immersed into a blocking solution containing 200 μ g/mL BSA in HBS (20 mM HEPES, 150 mM NaCl, pH 7.2). After blocking for two hours, the chips were rinsed with ddH₂O, blotted at an edge to remove excess liquid, and had target solutions directly applied. Target solutions were allowed to incubate for 60 minutes. The chips were then washed with ddH₂O and dried under a stream of nitrogen in order to be imaged.

2.3 Image Acquisition and Data Analysis

Chips were mounted onto a bench-top reflectometer as described previously (Mace et al., 2006) and images were acquired at an appropriate exposure time (30 ms for selectivity and sensitivity arrays, and 5 ms for arrays exposed to serum). Images were then loaded into ImageJ (Abramoff et al., 2004) in order to analyze the reflectance from each spot in the array. Briefly, each spot was manually selected and a histogram intensity profile was computed from the defined region of interest. The intensity data were imported into Origin (v7.0; OriginLab Corporation, Northampton, MA, USA), and the profiles were truncated to remove the high intensity aberrations resulting from the outer “coffee stain” ring and the centroid. The resultant profiles were then fit to a Gaussian distribution and the mean was utilized as the characteristic parameter for the spot. In order to determine the signal change for each spot in the array, the intensity value for the experimental spot (arrays introduced to target solutions) were normalized to the intensity value for the identical spot on the control array (arrays receiving MPBS-BET only). The reflectometer detector is an 8-bit CCD camera, and, therefore, the quantification of reflectance changes will be limited to a maximum of 255 arbitrary units.

3. Results and Discussion

ELISA assays typically utilize secondary antibodies in order to detect binding of antigens to their primary, capture antibodies. Label-free detection platforms, on the other hand, operate as direct assays and the monoclonal antibodies of which they make use inherently confer a certain

level of selectivity. However, when creating an analytical array for use with complex samples, lack of cross reactivity should not be taken for granted. To this end, we subjected a multiplexed array, consisting of antibodies to five individual cytokines (interleukin-5 [IL-5], interleukin-8 [IL-8], interleukin-12 [IL-12], interferon- γ [IFN γ], and tumor necrosis factor- α [TNF α]), to various solutions of target cytokines. The results of these experiments are depicted in Figure 1. In the first experiment, a solution containing 1 $\mu\text{g/mL}$ of TNF α and 1 $\mu\text{g/mL}$ of IFN γ was introduced to the array. As expected, large signal increases were observed for both anti-TNF α and anti-IFN γ probe spots. For the remaining probes, effectively serving as controls, only nominal deviations from background reflectances were measured. The fact that any change at all in “control” spots is seen is likely more indicative of the difficulty in unequivocally normalizing the signal from multiple control antibodies than any actual change (material addition or subtraction) in the probe spot itself. The second experiment was identical to the first, except that it also included 500 ng/mL of IL-5 in the target mixture. TNF α and IFN γ responses returned to similar levels as in the first experiment, while we also see a marked increase in reflectance from the anti-IL-5 probe spot.

Reflectance changes are a function of several variables: the affinity constant for the antibody/antigen complex (information not generally available from commercial suppliers), the multimeric state of the target protein, and the size of the target protein (for the protein targets discussed here, these are: TNF α , 51 kDa, homotrimer; IFN γ , 34 kDa, homodimer; IL-5, 34 kDa, homodimer). Another factor is that these solutions are not pure, *per se*; all target solutions contain 1% BSA (i.e. 10 mg/mL) as a carrier in order to stabilize the target molecules of interest. Therefore, assuming that all target molecules remain free in solution, even at such a high concentration (1 $\mu\text{g/mL}$), the proteins of interest are 10,000-fold impurities relative to the BSA carrier.

Our previous report on the sensitivity of AIR (Mace et al., 2006) employed a relatively well-understood model protein/protein system from pathogenic *E. coli* (Luo et al., 2000). However, the affinity of that interaction was weak (Ross et al., 2007) compared to what one would expect for an antibody/antigen interaction. If one were to modestly assume that the increase in affinity for an antibody-antigen interaction relative to our previously examined protein-protein recognition system provides at least an order of magnitude increase in sensitivity, in terms of detected target concentration, then observations of target at a concentration in the tens of pg/mL could be expected as a detection limit. In order to test this assertion, serial dilutions of IFN γ into MPBS-BET were prepared in order to examine concentrations spanning 250 ng/mL to 25 pg/mL. The reflectance changes were calculated for all four spots in the array, corresponding to both the experimental anti-IFN γ and control anti-human IgG probes. The normalized response profile was well correlated with concentration in this range, albeit subject to significant variability at very low (sub ng/mL) concentrations (Figure 2a): the 25 pg/mL IFN γ response cannot be distinguished, within error, from the MPBS-BET control, and reflectance changes for 250 pg/mL and 2.5 ng/mL also overlap within error. However, it is important to note that we observe clear detection of 250 pg/mL of target IFN γ (40 p.p.b. impurity with respect to BSA).

Analysis of AIR images suggests that errors observed at very low target concentrations are due in large part to irregularities in spot morphology. For example, Figure 2b shows an image acquired at 30 ms of the detection of 250 ng/mL IFN γ . The outer “coffee stain” ring can be explained either by drying (Deegan et al., 1997) or transport effects (Deng et al., 2006) at the liquid/surface/air interface. Similarly, the centroid within the probe spot is potentially caused by these same phenomena upon the creation of a similar liquid/air/substrate interface when the probe solution is initially introduced to the chip surface. Finally, we observe a “comet tail” emanating from probe spots on many of our chips. This arises from excess probe molecules reacting with aldehyde groups during our rinse procedure. As the size and shape of this “tail”

is difficult to control, this adds additional binding area (not included in our image analysis) to the sensor surface. We anticipate that most, if not all, of these issues can be addressed by optimization of spotting solutions (for example, through the use of various additives) and wash conditions. Efforts along these lines are currently underway.

With a successful demonstration of the selective detection of cytokines in a solution containing a high total protein concentration in hand, the next test of the method was to determine in a qualitative sense whether protein detection in a truly “complex” biological solution is possible. To that end, we performed a set of assays using undiluted human serum. AIR chips bearing antibodies to TNF α , IFN γ , and human IgG were monitored for changes in signal with respect to the background control image, as well as the *chip*'s background as a metric for non-specific binding (Figure 3). As expected, application of undiluted human serum caused significant non-specific binding over the whole of the chip surface, necessitating a decrease in the exposure time to 5 ms in order to avoid saturating the CCD. Non-specific binding did not obscure observation of target binding, however, as there was a sizeable reflectance increase (clearly differentiated from nonspecific binding) from the anti-human IgG probes, consistent with the known high IgG concentration in human serum (Pieper et al., 2003). Interestingly, we also observed a large increase in intensity for TNF α relative to background. Dot-blot experiments, a variant of a Western Blot where native proteins are directly immobilized into a paper membrane support, confirmed the presence of TNF α in the serum sample, as well as suggesting the presence of a smaller amount of IFN γ (not detectable by AIR). While considerable research remains to verify selectivity and determine limits of detection, this initial, qualitative test effectively demonstrates the feasibility of the AIR platform in serum protein analysis.

4. Conclusions

Through the use of immobilized antibody arrays, we have accomplished the first detection of human proteins using arrayed imaging reflectometry. Using a multiplexed array of five cytokine antibodies, we demonstrated selective detection of IL-5, IFN γ , and TNF α . Building on the results of these experiments, we investigated the sensitivity and dynamic range of a selected antibody/antigen interaction. We were able to definitively detect concentrations of IFN γ to at least 250 pg/mL, and over four logs of concentration. We anticipate that detection at 25 pg/mL is approachable with further optimizations of our assay conditions, and quantification over this concentration range would allow for ELISA-level sensitivity in a simple label-free platform. Finally, we examined the presence of basal levels of TNF α and IFN γ in undiluted human serum. Despite observing a large reflectivity increase across the entire sensor surface due to nonspecific binding, we were readily able to detect substantial binding to immobilized antibodies to TNF α and IgG.

In sum, these results show for the first time that AIR can be used to detect human proteins, and may be useful for the multiplexed detection of proteins in serum. Conversion of this initial qualitative success into a broadly applicable quantitative assay will require improvements in spot morphology and surface blocking. Additionally, expansion of the arrays to allow the use of several antibodies targeting multiple epitopes on the same antigen will increase confidence levels in the detection. Efforts along those lines are in progress.

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Figure 1.

Selectivity results for an array of five cytokine antibodies. In experiment A (light gray), the array was exposed to 1 $\mu\text{g/mL}$ of TNF α and 1 $\mu\text{g/mL}$ of IFN γ . In experiment B (dark gray), the array was exposed to the same TNF α /IFN γ mixture as in experiment A, but with an additional supplement of 500 ng/mL IL-5.

Figure 2.

Detection of IFN γ . (a) Reflectance change profile (N=4) for target solutions of IFN γ in the range of 250 ng/mL to 25 pg/mL. (b) Image of the array used for detecting 250 ng/mL IFN γ . The top row corresponds to four anti-IFN γ spots. The bottom row, not readily visible in this image, lies directly beneath the top row and corresponds to four anti-human IgG control spots.

Figure 3. Detection of human proteins from undiluted serum. (a) Background, control image showing the array of anti-IFN γ (top left, mid bottom), anti-TNF α (bottom left, mid top), and anti-human IgG (right column). (b) Chip exposed to 100% serum. (c) Quantification of signal changes, corrected for background reflectance increases, for all probe spots (N=2) on chips exposed to undiluted serum. The corrected IgG response represents saturation of the detector.