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Autoassembly Protein Arrays for Analyzing Antibody Cross-Reactivity

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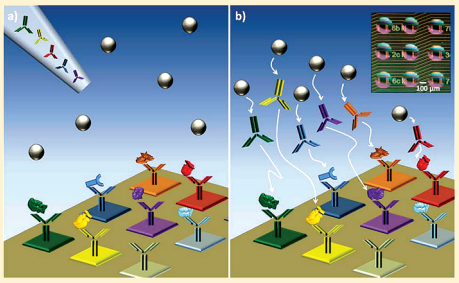
Supporting Information

ABSTRACT: We report an autoassembly protein array capable of rapidly screening for aberrant antibody−antigen binding events. Our technique combines magnetic nanoparticle technology with proximity-based, magnetically responsive nanosensors for rapid (under 15 min) and high-density screening of antibody cross-reactivity at sensitivities down to 50 fM in a homogeneous assay. This method will enable the identification of the precise cause of aberrant or cross-reactive binding events in an easy-to-use, rapid, and high-throughput manner.

KEYWORDS: Biosensors, nanobiotechnology, antibody cross-reactivity, giant magnetoresistance, magnetic nanoparticles



In recent years, researchers have recognized the tremendous potential of protein microarrays. Over a decade ago, the DNA microarray revolutionized the way in which biologists analyze gene expression.[1−3] DNA arrays have been successfully implemented in a variety of applications ranging from genome-wide screens for chromosomal abnormalities to the identification of coregulated gene networks during embryonic development. However, while gene function studies that utilize mRNA expression levels are somewhat informative, they often do not correspond well with the abundance of protein levels in the cell.[4] Accordingly, many researchers have adopted protein microarrays to directly investigate protein expression patterns and protein function.[5,6] Protein microarrays make it possible to study the expression of the entire proteome (or a subset of the proteome) in a multiplex format. Although this advance addresses a crucial limitation, protein microarrays have enjoyed limited success thus far compared to DNA microarrays due to the highly complex nature of the antibody−antigen interaction.[4] Here we introduce a novel nanosensor-based technique that can simplify and enhance the reliability of protein array-based analysis, allowing the field to unlock the true potential of protein microarrays.

The complexity of protein interaction presents a number of significant challenges. Unlike the predictable sequence-specific hybridization chemistry of nucleic acids, proteins exhibit incredible diversity in their functional groups, affinities, and secondary and tertiary structure. In addition, after translation, proteins typically undergo multimerization and post-translational modification, such as acetylation, glycosylation, and phosphorylation, making the protein structure even more diverse. As a result, protein replication or amplification is not possible with current tools, limiting the sensitivity of protein microarrays. Furthermore, antibodies only bind to a small portion of the target protein, known as the epitope. Given the incredibly complex structure of each protein, antibodies in a high density protein array often bind aberrantly to epitopes with identical or similar structure in off-target proteins,[7,8] resulting in nonspecific cross-reactive signals. (In this paper, we refer to cross-reactions and aberrant binding events synonymously, since both are undesired or unexpected binding events beyond the specific binding between a pair of targeted protein and antibody.) This cross-reactivity problem is exacerbated when researchers employ polyclonal antibodies, which are mixtures of antibodies that bind to multiple different epitopes on a particular protein. Not surprisingly, the literature is filled with examples of such cross-reactive monoclonal and polyclonal antibodies that have necessitated reassessment of data or even retraction of experimental findings.[9] The constant push to increase the density of protein arrays will only further compound this problem. However, no universally accepted method for assessing antibody cross-reactivity exists.[9]

To address these concerns, we have designed a simple and sensitive nanosensor-based immunoassay capable of rapidly characterizing antibody cross-reactivity. This assay employs high density arrays of giant magnetoresistive (GMR) nanosensors[10,11] and magnetic nanotags, as diagrammed in Figure 1. Furthermore, we have designed this assay as a one-step, wash-free process employing the site-specific autoassembly characteristics of macromolecular complexes.

Our innovation relies on magetoresistance, a property of GMR sensors rooted in quantum mechanics, by which a change in the external magnetic field induces a spin-dependent change in the electrical resistance of the device. In particular, spin valve type GMR nanosensors have high linearity and low noise, making them ideal for quantitatively detecting magnetic nanotags.[12,13] Prior work has demonstrated that by implementing a traditional sandwich assay using magnetic nanotags, GMR spin valve biosensors are capable of multiplexed protein detection at femtomolar to attomolar sensitivities.[14,15]

The traditional sandwich assay utilized in our prior work has been completely redesigned here. In this report, we demonstrate

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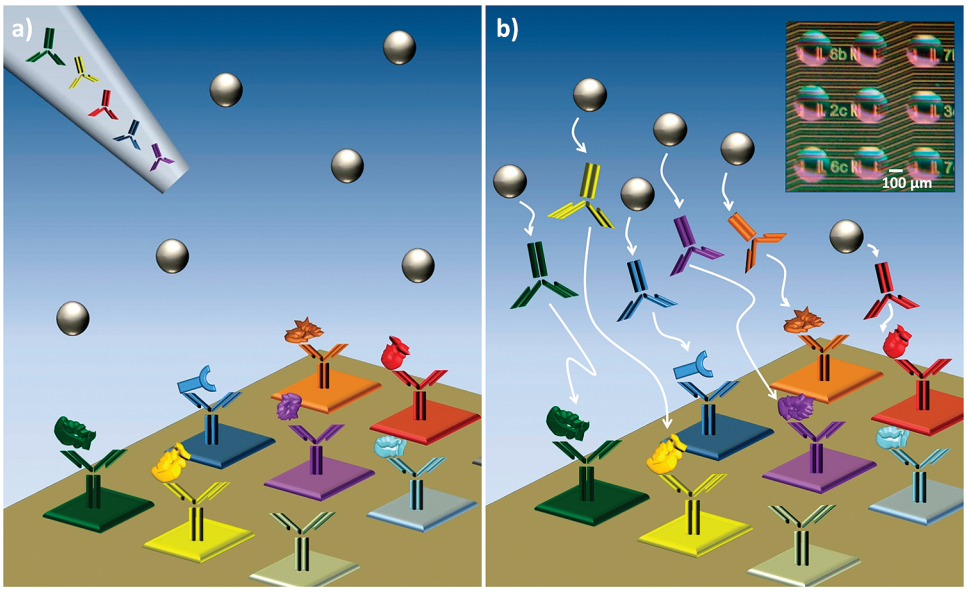

Figure 1. Schematic representation of the autoassembly immunoassay where each square represents a 100 μm × 100 μm GMR nanosensor and each color represents a unique target antibody and antigen. (a) After immobilizing unique capture antibodies over a unique, individually addressable sensor and incubating with the protein of interest, the magnetic nanotags are added in solution above the sensor. Since there is no chemistry to link the magnetic nanotags to the captured antigen, no signal is detected by the underlying sensor. (b) As each of the detection antibodies are sequentially introduced, they are capable of linking the streptavidin labeled magnetic nanotags to the captured analytes. In the presence of captured analyte, the magnetic nanotags will congregate over the corresponding GMR sensors in high enough concentration to be detected. Insert: optical microscopy of a section of the array of nanosensors. Each square in the array is one sensor and each circle is a nanoliter droplet of capture antibody uniquely functionalized over the sensor surface.

a novel autoassembly immunoassay by leveraging the proximity-based detection capabilities and unique magnetic properties of our GMR-based biosensor system (elaborated in Supporting Information, Figure S1). As described below, we allow the antibody and magnetic nanoparticle to bind on the surface of the sensor all in the same solution, removing the wash steps required for traditional sandwich assays (Figure 1). Thus, by taking advantage of what we term autoassembly, this assay requires only minimal human intervention and requires no laboratory training to perform. The assay works as follows. Arrays of GMR sensors are prefunctionalized with a panel of capture antibodies targeting the proteins of interest. Upon sample incubation in the reaction well, the biomolecules of interest are selectively captured by antibodies that have been immobilized directly over GMR sensors (alternatively, the target proteins can be directly immobilized on the sensor surface, though this may lead to undesirable conformational changes in protein structure). To illustrate, we employ piezoelectric robotic spotter technology (Scienion sciFlexarrayer, BioDot) to spot 350 picoliter droplets of capture antibody onto individually addressable GMR nanosensors in the array (Figure 1b insert). The user then adds a solution of magnetic nanotags labeled with streptavidin to the reaction well. At this point, no reaction takes place because complementary reaction chemistry between the captured antigen and magnetic nanotag is not yet present in the well (Figure 1a). Detection antibodies labeled with biotin are sequentially introduced. Upon introduction, each detection antibody diffuses throughout the reaction well and acts to link the magnetic nanotags to surface-immobilized analyte, creating a signal in the underlying GMR sensor (Figure 1b). In this way, the cross-reactivity of each successive antibody in a panel can be assessed with only one additional wash-free step. No washing steps are required between sequential additions of detection antibody or after adding magnetic nanotag solution. The assay is run in an open-well format, removing the need for complex microfluidic plumbing or external pneumatic pressure controllers.

In a demonstration experiment, we monitored GMR signal in real-time to show that addition of sample and magnetic nanotags without detection antibody produces no significant rise in the GMR signal. However, once the detection antibody is introduced, the magnetic nanotags congregate over the sensor surface in high enough density to be detected by the underlying GMR sensor (Figure 2a). As noted above, each sensor in the array is individually addressable and monitored in real-time. Therefore, upon sequential addition of different detection antibodies at unique time points, it is possible to isolate the signal and potential for cross-reaction from each antibody addition—an important advance that is unique to our new method.

Currently, fluorescent or colorimetric detection is implemented when performing protein microarrays. These technologies typically have a detection limit of ~1 pM, 2 orders of linear dynamic range, and assay times of approximately half a day.[9] While alternative methods such as mass-spectrometric antigen identification and two-dimensoinal polyacrylamide gel electrophoresis have been demonstrated, their protocols are technically challenging, expensive, and even more time-consuming. In addition, although the Western blot has been used in the past to examine cross-reactivity, this technique has the potential to misrepresent antibody specificity due to the highly denaturing conditions of the assay.[9] Furthermore, while cross-reaction may be apparent, Westerns cannot identify the source of the aberrant binding event, providing minimal information for future experiments. In short, all of these existing solutions possess severe limitations that have prevented protein microarrays from realizing their potential.

In contrast, the autoassembly immunoassay presented here is simple to implement because it lacks tedious washing steps,

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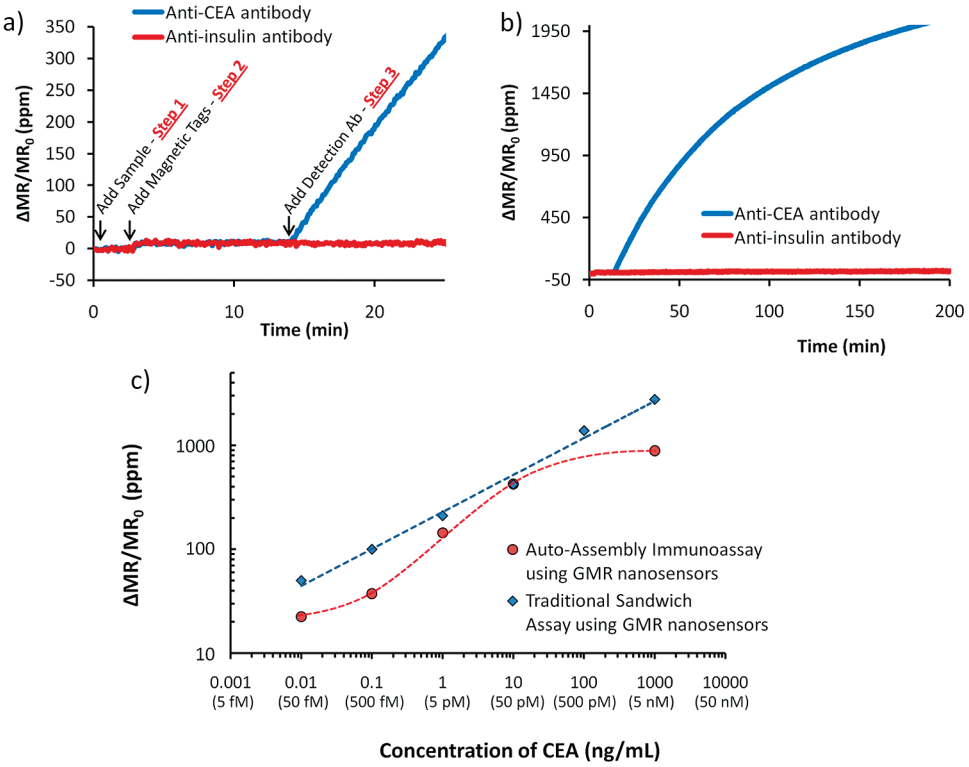

Figure 2. (a) Real-time monitoring of sensors during autoassembly immunoassay. Addition of the sample and magnetic nanotags contribute negligible signal (upon addition of the magnetic nanotags, there is a very small signal rise due to detection of magnetic nanotags in solution above the sensor). However, once the detection antibody is introduced, the magnetic nanotags are clearly measurable on sensors functionalized with the appropriate capture antibody and antigen. The negative control sensors, coated with a noncomplementary antibody, anti-insulin antibody, remain flat, indicating negligible nonspecific binding. The *y*-axis units are the change in magnetoresistance normalized to the initial magnetoresistance presented in parts per million (ppm). (b) Full scale graph of anti-CEA detection antibody binding selectively to captured CEA protein when performing the autoassembly immunoassay. (c) Superimposed calibration curves comparing the autoassembly assay to the traditional sandwich assay using GMR nanosensors in both assays. Although the linear dynamic range is not quite as good as the traditional immunoassay using GMR biosensors, the autoassembly assay is still quite sensitive with a lower limit of around 50 fM.

utilizes simple salt buffers that do not denature the antibodies or proteins, and achieves at least an order of magnitude higher sensitivity in less than 15 min. Indeed, by employing magnetic sensing rather than optical detection, we can achieve detection down to 50 fM in a 25 μL sample (Figure 2b). For the sake of completeness, we note that the linear dynamic range and lower limit of detection using the autoassembly immunoassay on our GMR sensor array are not as broad as in the standard sandwich immunoassay on our GMR sensor array.[15]

To illustrate the application to the identification of aberrant antibody binding events, we performed a trial screen for antibody cross-reactivity. To begin, we examined the cross-reactivity of an antiepidermal growth factor receptor (EGFR) antibody to a panel of 20 unique target proteins. We spotted antibodies to each of the 20 target proteins on the sensor, followed by addition of the target proteins themselves, which were thereby immobilized to the surfaces over their respective sensors. For the experiment, we added magnetic nanotags, followed by the addition of biotinylated anti-EGFR detection antibody. While the majority of proteins revealed no detectable cross-reaction, the assay was able to highlight two sensors in addition to EGFR that were bound by the anti-EGFR antibody; the epithelial cell adhesion molecule (EpCAM) and human trophoblast cell-surface antigen (TROP2, also termed GA733-1, M1S1, or EGP-1) (Figure 3a,b). Control experiments in which Trop2 and EpCAM were absent, and in which EGFR protein was absent, each revealed no cross-reactivity, confirming that the cross-reaction was due to detection antibody−antigen interaction and not due to detection antibody binding to capture antibody (Supporting Information, Figures S2 and S3). Similarly, when anti-Trop2 detection antibody was introduced instead of anti-EGFR antibody, we observed cross-reactive binding between anti-Trop2 antibody over the sensor functionalized with anti-EGFR antibody (Supporting Information, Figure S4).

From a survey of the literature, it is not surprising that these two additional proteins exhibited cross-reaction, as both EpCAM and Trop2 contain EGF-like domains.[16] Therefore, EGFR protein, which is capable of binding EGF-like domains, most likely bound to the EGF-like domains on captured Trop2 and EpCAM protein. Consequently, a more complex sandwich structure was first formed over the anti-Trop2 antibody-coated sensor on which Trop2 protein was captured. Subsequently, the EGF-like domain on Trop2 was bound by EGFR, and finally, EGFR was bound by the anti-EGFR detection antibody (Figure 3a). While the aberrant binding events we present here can be explained by the structure of the reacting proteins, the majority of cross-reactive binding events are not so easily predicted. In many situations, antibodies may recognize a nonlinear conformational protein epitope that cannot be predicted via sequence homology.[17−20]

When performing a traditional protein microarray, detection antibodies are added simultaneously (in a cocktail) instead of sequentially (i.e., only one detection antibody at a time). However,

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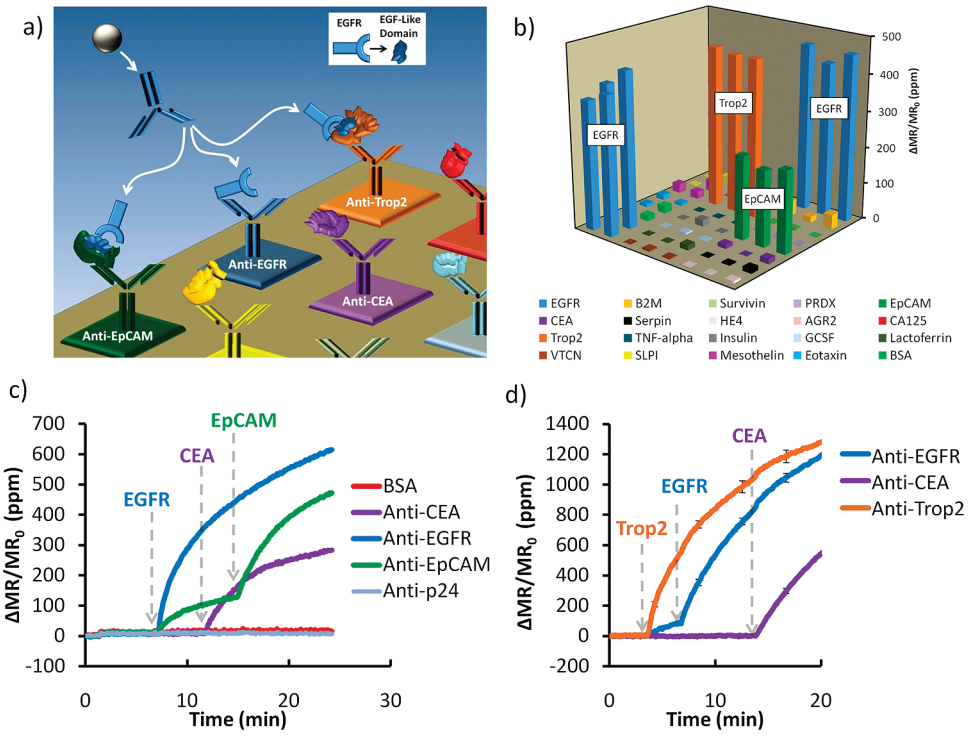

Figure 3. (a) Schematic representation of anti-EGFR antibody binding over sensors immobilized with EpCAM protein, EGFR protein, and Trop2 protein to illustrate one possible mechanism of antibody-cross reactivity. (b) Twenty unique capture antibodies were selectively immobilized on unique sensors in replicas of 3−7. All 20 proteins were incubated at high concentration (approximately 10 ng/mL). After washing, magnetic nanotags were added followed by anti-EGFR antibody. The autoassembly assay was run for 5 min resulting in signal over the EGFR sensors, Trop2 sensors and EpCAM sensors. (c) Autoassembly immunoassay where anti-EGFR detection antibody was introduced first and exhibited cross-reactivity with anti-EpCAM immobilized sensor. Upon addition of anti-CEA detection antibody and anti-EpCAM detection antibody, no additional cross reactivity was observed. (d) Autoassembly immunoassay where anti-Trop2 detection antibody was introduced first and exhibited cross-reactivity with anti-EGFR immobilized sensor. Addition of anti-CEA detection antibody at the end, however, exhibited no cross reactivity.

if the prior experiment had been performed like a traditional protein microarray sandwich assay, using a cocktail of detection antibodies to all 20 proteins, the origin of the signals from the sensors immobilized with Trop2 capture antibody or the sensors immobilized with EpCAM capture antibody would certainly be ambiguous and potentially misleading. Distinguishing the amount of signal generated over the Trop2 sensor by anti-Trop2 detection antibody binding to Trop2 protein from the amount of signal generated by anti-EGFR detection antibody binding to EGFR captured on the EGF-like domain of Trop2 protein would be impossible. Indeed, every antibody that is currently in use in a protein microarray with unrecognized cross-reactivity would present the same concern.

Using our autoassembly immunoassay, we can address this issue by simply introducing detection antibody sequentially in time to isolate each detection antibody that is aberrantly binding. Doing sequential addition with traditional protein microarrays would be prohibitively time-consuming, since these tools require lengthy incubation times and several washing steps for each antibody. However, using the autoassembly immunoassay presented here, we can simply and rapidly screen through a panel of potentially cross-reactive antibodies.

By sequentially adding each antibody of interest to the GMR protein array at unique time points, it is possible to rapidly identify the source of aberrant binding events in real-time. To illustrate, in Figure 3c we first added anti-EGFR antibody to the reaction well. Not only did the anti-EGFR antibody bind to the EGFR protein, but it also bound over the anti-Trop2 protein, as described above. Upon addition of the anti-CEA detection antibody, however, no such cross-reaction was observed, and only the sensor with anti-CEA capture antibody exhibited a binding curve. Signal over the noncomplementary antibody and BSA control sensors remained flat, indicating no settling or nonspecific binding of the magnetic nanotags (Supporting Information, Figure S5). Similarly, when anti-Trop2 detection antibody was added, it not only bound to the sensor immobilized with anti-Trop2 capture antibody, but also bound to the sensor immobilized with EGFR protein (Figure 3d). Anti-CEA antibody, however, again exhibited no cross-reaction (refer to Supporting Information, Figure S6 for demonstration of control experiments that reveal no cross-reactivity). This is the first demonstration of a technology capable of rapidly isolating individual cross-reactive binding events in a multiplex manner. The technology is potentially high-throughput because the number of nanosensors on a single chip is highly scalable.

In the literature, both monoclonal and polyclonal antibodies have been shown to cross-react with epitopes on nontarget antigens. In fact, physicians have utilized cross-reactive antibodies for the diagnosis of medical disease. The Venereal Disease Research Laboratory (VDRL) test, for example, is a very common blood test for syphilis that detects nonspecific antibody in human serum that reacts with beef cardiolipin.[21] Such a large incidence of antibody cross-reactivity is clearly worrisome, as the aberrant binding events that have been reported most likely just scratch the surface of the problem. Even more problematic is the use of antibodies with unknown cross-reactivity properties. With the

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drive to increase protein microarray density, identifying such aberrant binding events is of paramount importance, and implementing easy-to-use methods to do so must become standard practice. However, no standardized methods currently exist. Our unique approach has the ability address this problem and dramatically improve the fidelity of the protein microarray in a simple, rapid and potentially high-throughput fashion. This technology can be instrumental in helping the protein microarray deliver on its vast promise.

■ ASSOCIATED CONTENT

Supporting Information. Experimental materials and methods and supplementary figures and text. This material is available free of charge via the Internet at http://pubs.acs.org.

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