Multiplexed Protein Detection Using Antibody-conjugated Microbead Arrays Assembled on a Microfabricated Electrophoretic Device

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ABSTRACT

We present a method for conducting sensitive, multiplexed immunoassays on antibody-conjugated microbeads that have been assembled into an array in a microfabricated electrophoretic device. This device improves upon our previous work through the use of more robust dielectric and counter electrode materials. The array of micron to submicron wells is fabricated in a silicon dioxide film on a gold-coated wafer and the counter electrodes consist of a series of gold lines fabricated on a glass coverslip. These substrates are assembled together with pre-cut double-coated adhesive tape to form microfluidic channels. Antibody-conjugated microbeads are then introduced into the fluidic channels and assembled into high-density arrays using a series of electric pulses. Our method and device can be used to produce dense antibody arrays with extremely small footprints in less than 30 seconds.

Keywords: immunoassay, microbeads, assembly, electric field, array

1 INTRODUCTION

The analysis of proteins in a high-throughput manner is of paramount importance in research and clinical diagnostics, proteomic studies, biomarker analysis, and drug discovery [1, 2, 3, 4]. Established methods such as enzyme-linked immunosorbent assays (ELISA), western blots, and gel electrophoresis are often time-consuming and require large sample and reagent volumes. Furthermore, they are only capable of interrogating a small number of proteins at a time [5, 6]. These issues can be addressed by the use of protein and antibody microarrays. Microarrays are typically fabricated by individually spotting each unique protein or antibody onto the substrate. This process is inefficient and results in spot sizes on the order of tens of micrometers [7]. A more scalable approach is through the application of bead based assays. The use of micron and sub-micron microbeads allows for much smaller sample sizes, greater multiplexing, and incorporation into microfluidic devices.

There are many established methods for the assembly of antibody-conjugated microbeads in microfluidic platforms, including evaporation, gravitational settling, fluidic trapping, electrostatic attraction, and micromanipulation [8, 9, 10, 11, 12]. These methods often result in poor filling efficiency, less order, and a high defect rate. In addition, some of these methods only manipulate a small number of microbeads at a time or rely on approaches that may not be suitable for preservation of biomolecule function [13]. Methods for producing high-density arrays of microbeads with near perfect order are much more advantageous over other assembly methods that lack structure. Arrays offer the ability to spatially separate unique antibody-conjugated microbead species from one another in a very small footprint, resulting in reduced sample and reagent volumes. The microbead arrays can also be integrated into microfluidic devices to enable higher multiplexing, more sensitive imaging, and high-throughput data collection.

We present a method for the rapid assembly of antibody-conjugated microbead arrays in a microfabricated electrophoretic device. Following assembly, we demonstrate the ability to perform sensitive immunoassays within the device. The device consists of microfabricated arrays of wells on a gold primary electrode in which the microbeads are actively assembled by electrophoresis. Compared to our previously reported methods of microbead assembly, the device now incorporates substantial improvements in materials and fabrication allowing for a much more robust device [14]. Previously, the microfabricated wells were fabricated from a negative tone photoresist (SU-8). These wells were prone to defects caused by limitations of photolithographic patterning. We now fabricate the microwell structures in silicon dioxide (SiO_2), which allows for higher resolution lithography and is more robust with regard to cleaning processes and electrophoretic conditions. In addition, the counter-electrode is now composed of a series of gold lines on a glass coverslip. In previous work, the counter electrode consisted of an indium-tin oxide (ITO) film on a glass substrate, which tends to degrade when exposed to electrophoretic conditions and leads to reduced light transmission. This significantly affects the ability to perform sensitive imaging and detection. By using a series of gold lines, we can still maintain consistent assembly across the entire chip while providing unobstructed viewing windows between the lines for high-sensitivity imaging.

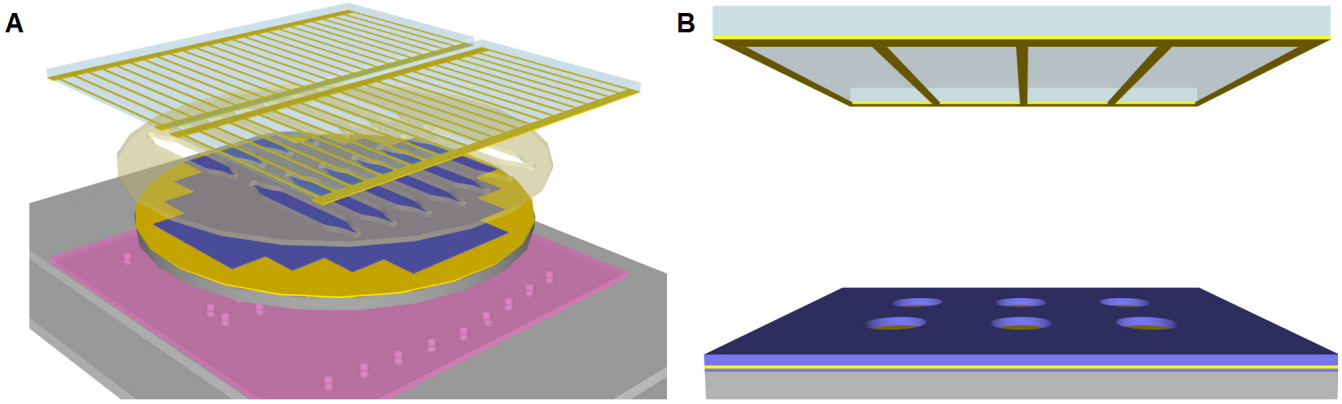

Figure 1. A microfabricated electrophoretic device. (A) An exploded view of the microfabricated electrophoretic device. (B) A cross-sectional view of a microfluidic cell formed between the gold-coated silicon wafer with the array of wells and the glass coverslip with the counter electrode lines. These drawings are not to scale.

2 EXPERIMENTAL METHODS

2.1 Fabrication of microwell arrays in SiO_2

Arrays of microwells were fabricated in a silicon dioxide (SiO_2) film on gold-coated wafers using a metal etch mask and inductively coupled plasma reactive ion etching (ICP/RIE). Silicon wafers were first cleaned and coated with SiO_2 via plasma-enhanced chemical vapor deposition (PECVD). A metal stack consisting of titanium, gold and titanium were then sequentially deposited via sputter coating. An additional layer of SiO_2 was then deposited via PECVD and a photolithographic lift-off process was used to generate an array of wells in a nickel film on this oxide layer. CHF_3-based ICP/RIE was then used to etch wells in the upper oxide and titanium films. The remaining nickel was removed via wet etching with nitric acid.

2.2 Fabrication of counter electrode lines

Long, narrow metal lines were fabricated on glass coverslips using a photolithographic lift-off process. Briefly, a series of parallel lines were first patterned in a thin layer of photoresist on the glass substrate. Next, titanium and gold films were sequentially deposited via sputter coating. The photoresist and undesired metal were then removed with heated solvents and ultrasonic agitation. The remaining gold lines were used as counter electrodes in the electrophoretic device. These line were typically 10-30 µm wide with a pitch of 100-300 µm spacing to allow for imaging of the array in the assembled device.

2.3 Device assembly

The assembly of the microfabricated electrophoretic device has been described elsewhere [14]. Briefly, holes are drilled in the silicon wafer containing the array of wells on a gold film, which is then mounted onto an aluminum plate using double-coated adhesive tape. Another layer of double-coated tape containing cutouts that define the flow cells is then attached to the top side of the wafer. Glass coverslips containing the counter electrode lines are then attached to the wafer, thus forming the tops of the flow cells. Electrical connections are made to the counter electrode lines and the gold film on the wafer using copper tape. An exploded view of the device is shown in Figure 1A. A cross-sectional view of one of the flow cells formed between the wafer and the coverslip is shown in Figure 1B.

2.4 Preparation of antibody-conjugated microbeads

Biotinylated antibodies (rabbit anti-chicken/turkey IgG (H+L) and goat anti-rat IgG (H+L), Invitrogen Corp.) were conjugated to either 0.4 µm or 1.0 µm streptavidin-coated beads (Bangs Laboratories, Inc.) by adding drop-wise a suspension of microbeads to the biotinylated antibodies in phosphate buffered saline (PBS). After adding the entire bead suspension to the antibody solution, the mixture was shaken at room temperature for 1 hr. The microbeads were then washed with PBS and stored at 4 °C until needed.

2.5 Assembly of antibody-conjugated microbeads

The assembly of protein-conjugated microbeads has been described elsewhere [14]. In this work, the same process has been employed for the assembly of antibody-conjugated microbeads. Briefly, antibody-conjugated microbeads were washed with a low-conductance buffer (LCB) and then introduced into the flow cell. A function generator was then used to apply a 3.0 V DC potential across the electrodes in the flow cell. The waveform consisted of 1 Hz pulses at a 10% duty cycle. Approximately 15-30 pulses were applied to direct the assembly and capture of microbeads on the array.

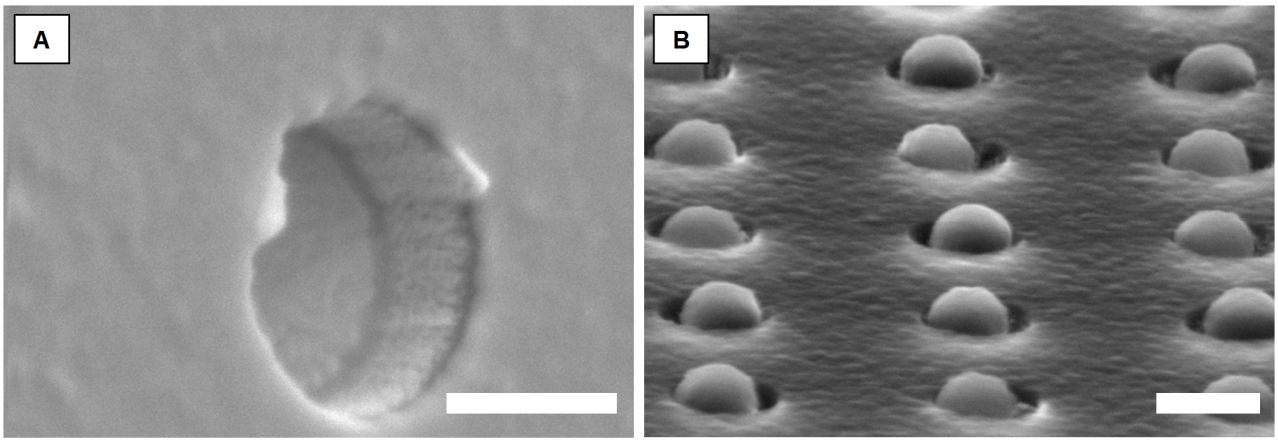

Figure 2. A microwell on a gold-coated wafer and an antibody-conjugated microbead array. (A) A single 0.6 μm well in SiO_2. The nickel etch mask is still intact. (B) 0.4 μm antibody-conjugated microbeads assembled into 0.5 μm wells. Scale bars are 0.5 μm in both images.

2.6 Microbead-based immunoassays

Equal amounts of two antibody-conjugated microbead populations (rabbit anti-chicken/turkey IgG microbeads and goat anti-rat IgG microbeads) were either combined at a final concentration of ~0.2% or diluted with plain, streptavidin-conjugated microbeads to keep the suspension at a concentration of ~0.2% solids but limit the proportion of antibody-conjugated microbeads to 2–20% of all the microbeads. Assembly was performed as described above and once assembled, the chamber was washed with PBS with 0.02% Tween-20 (PBS-2T). A blocking solution containing 6% bovine serum albumin (BSA), 10% bovine serum and 1% Tween-20 was then introduced into the chamber. After a 60 min incubation, the chamber was washed with PBS-2T. Fluorophore-labeled detection antibodies (Alexa Fluor 680 donkey anti-rabbit IgG (H+L) and Alexa Fluor 568 donkey anti-goat IgG (H+L), Invitrogen Corp.) were combined and diluted in PBS with 1% BSA, 10% bovine serum and 0.02% Tween-20, and then loaded into the chamber at concentrations ranging from 250 nM (40 μg/mL) to 100 pM (16 ng/mL). After a 60 min incubation, the chamber was washed with PBS-2T and the array was imaged on a fluorescence microscope.

2.7 Imaging

Imaging was performed on a fully automated epifluorescence microscopy system consisting of an Axio Observer.Z1 inverted microscope with a 40x/1.3 NA oil objective and a Definite Focus system (Carl Zeiss, Inc.). Excitation was achieved using a Lambda DG-5 light source (Sutter Instrument Co.) and images were acquired with an iXon+ 885 EMCCD (Andor Technology, PLC). The array scanning was performed using a BioPrecision 2 motorized stage (Ludl Electronic Products, Ltd.). All operations were controlled using custom software and the images were analyzed with ImageJ [15].

3 RESULTS AND DISCUSSION

3.1 Assembly of antibody-conjugated microbeads

We have demonstrated that an electrophoretic microfluidic device can be used to direct the assembly of antibody-conjugated microbead arrays in a rapid and efficient manner. As shown in Figure 2, the antibody-conjugated microbeads are captured within wells that have been etched in a silicon dioxide film on a gold-coated wafer. The assembly process takes less than 30 seconds and extremely high filling efficiencies can be achieved with both micron and sub-micron beads.

We have shown that by controlling the proportion of each microbead in suspension, we can control the proportion of each microbead type assembled in the array (Figure 3). This capability will support multiplexing of microbead-based immunoassays. It will also allow us to control the number of each type of microbead on the array, which is crucial to the sensitivity of the immunoassay.

3.2 Design of Counter-electrodes

The pitch of the counter electrode lines was chosen to maintain uniform microbead assembly as well as to enable unobstructed imaging between them when imaged with a 40x objective and a camera with a 8 mm × 8 mm EMCCD sensor. Lines too close to one another result in shadows in the field of view. These shadows reduce the fluorescence signal of the beads in those areas; thus hindering imaging and detection. Conversely, lines that are too far apart result in non-uniform assembly of the microbeads. This is due to a non-uniform electric field near the surface of the array. For future work, modeling of the electric field with respect to counter-electrode line width and pitch will enable further optimization.

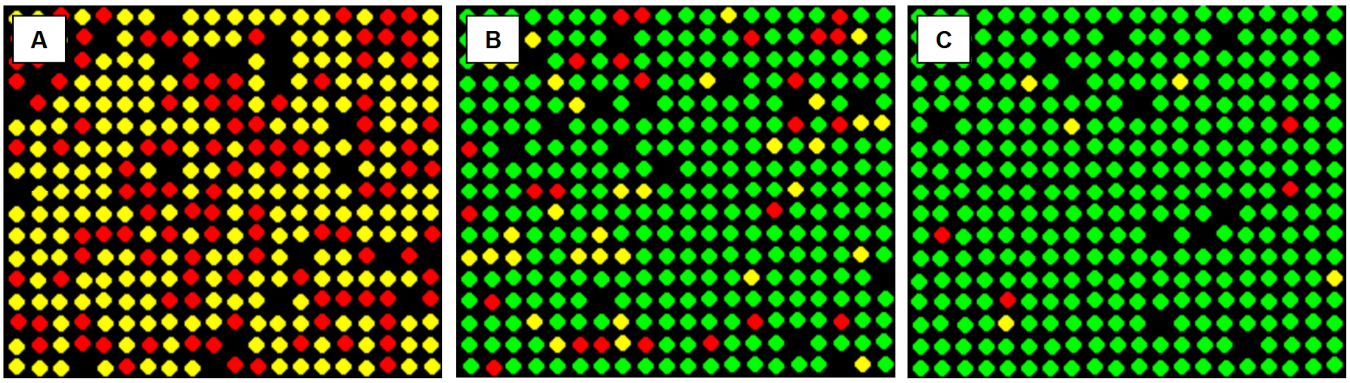

Figure 3. Fluorescence micrographs of small portions of high-density arrays of 1 µm streptavidin and antibody-conjugated microbeads assembled via electrophoretic deposition. (A), (B) and (C) Composite, false-colored fluorescence images after direct detection of two different antigens. The percent of microbeads conjugated with antibodies were 100% in (A), 20% in (B) and 2% in (C). Antigen concentrations were 250 nM in (A), 1 nM in (B) and 100 pM in (C). The pitch for the 1 µm bead arrays is 2.4 µm. The plain streptavidin-conjugated microbeads are visible as green false-colored microbeads. Red microbeads have been detected by Alexa 680-donkey-anti-rabbit and yellow microbeads by Alexa 568-donkey-anti-goat secondary antibodies. For clarity, the images from each fluorescent channel were first thresholded and then the microbeads were reconstructed and colored prior to creating the composite images.

3.3 Direct detection of antigens

Direct immunoassays were conducted to demonstrate that antibody-conjugated microbeads could be assembled on our platform and that the antibodies are able to withstand the electrophoretic conditions associated with our assembly process. The integrity of the antibodies was confirmed directly through the use of fluorophore-labeled detection antibodies. In these assays, the concentration of the detection antibodies was varied from 250 nM to 100 pM and the results were assessed qualitatively (Figure 3). Future work will utilize sandwich-type immunoassays for quantitative detection of protein antigens in solution and to determine the parameters to optimize conditions for maximum sensitivity.

4 CONCLUSIONS

We have demonstrated the ability to perform direct, multiplexed immunoassays on antibody-conjugated microbead arrays that have been rapidly assembled via an electric field. Antibody functionality was verified after microbead assembly, indicating this device is well suited for chip-based protein detection assays. We have also developed a more robust device using microfabricated wells in SiO_2 on a gold substrate and a counter-electrode consisting of a series of gold lines patterned on glass coverslips. With the addition of a microbead encoding scheme, this device will be capable of much higher multiplexing. Moreover, investigation into the ability to accelerate antigen transport using the electric field could enhance the sensitivity of this device and drastically decrease assay times.

REFERENCES

[1] R. Bellisario, R. J. Colinas and K. A. Pass, *Early Hum. Dev.*, **64**, 21-25 (2001).

[2] J. Burbaum and G. M. Tobal, *Curr. Opin. Chem. Biol.*, **6**, 427-433 (2002).

[3] V. Knezevic et al., *Proteomics*, **1**, 1271-1278 (2001).

[4] G. MacBeath and S. L. Schreiber, *Science*, **289**, 1760 - 1763 (2000).

[5] E. Engvall and P. Perlmann, *Immunochemistry*, **8**, 871-874 (1971).

[6] J. Renart, J. Reiser and G. R. Stark, *Proc. Natl. Acad. Sci. U. S. A.*, **76**, 3116-3120 (1979).

[7] J. W. Silzel, B. Cercek, C. Dodson, T. Tsay and R. J. Obremski, *Clin. Chem.*, **44**, 2036-2043 (1998).

[8] T. M. Blicharz et al., *Anal. Chem.*, **81**, 2106-2114 (2009).

[9] A. Goodey et al., *J. Am. Chem. Soc.*, **123**, 2559-2570 (2001).

[10] M. Herrmann, E. Roy, T. Veres and M. Tabrizian, *Lab Chip*, **7**, 1546-1552 (2007).

[11] X. Qiu et al., *Biomed. Microdev.*, **11**, 1175-1186 (2009).

[12] V. Sivagnanam, B. Song, C. Vandevyver and M. A. M. Gijs, *Anal. Chem.*, **81**, 6509-6515 (2009).

[13] A. Winkleman, B. D. Gates, L. S. McCarty and G. M. Whitesides, *Adv. Mater.*, **17**, 1507-1511 (2005).

[14] K. D. Barbee, A. P. Hsiao, M. J. Heller and X. Huang, *Lab Chip*, **9**, 3268-3274 (2009).

[15] M. D. Abramoff, P. J. Magelhaes and S. J. Ram, *Biophotonics Intl.*, **11**, 36-42 (2004).