Three Minutes-Long Electrophoretically Assisted Zeptomolar Microfluidic Immunoassay with Magnetic-Beads Detection

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14. ABSTRACT
An ultrasensitive two-step immunoassay was developed for rapid assay of proteins and viruses. In the first step, electrophoresis was used to quickly bring soluble analytes from a flowing solution to a microarray of probe molecules immobilized on s semi-permeable membrane. in the second step, the captured analyte was detected by scanning the mcroarray with functionalized magnetic beads passed over the array surface by shear flow and pressed to the array surface by a magnetic field.

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Three Minutes-Long Electrophoretically Assisted Zeptomolar Microfluidic Immunoassay with Magnetic-Beads Detection

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Development of protein-detection techniques as sensitive as PCR-based assays presents a tremendous challenge in diagnostics. With benefits of amplification being unfeasible for proteins, limit of detection (LOD) may only be decreased by increasing detection sensitivity.[1] However, even an ultimate nanoscale biosensor capable of detecting a single analyte cannot have LOD much lower than femtamolar[2] because of the impractically long time needed for an analyte to encounter the biosensor at lower concentrations. To overcome this problem and further decrease LOD while accelerating the assay, a new approach was suggested recently in which passive diffusion of analytes is replaced by their active delivery to the sensor with an external force[3] (electric, magnetic or mechanical). LOD in such active assays may be extremely low because of analyte preconcentration and because of background reduction resulting from removal of weakly bound analytes with the force direction reversed.[3]

Though different elements of the active assay were reported previously,[3] this Communication describes for the first time an immunoassay method in which all the assay steps were performed actively in a single microfluidic device (schematically illustrated in Figure 1, panels A and B). The solution to be analyzed moves through a flow cell ($1.2 \times 8 \times 0.1$ mm^3) whose upper and lower walls are made of dialysis membranes. The bottom membrane has an antigen or antibody microarray on its upper surface. The membranes, penetrable to small ions, allow application of a normal electric field which draws charged analytes to the array surface, as illustrated in Figure 1A. While other similarly charged macro-ions are also brought to the microarray surface, they cannot establish strong bonds and are eventually removed by flow. The collected analytes are then detected with magnetic beads, as illustrated in Figure 1B. A suspension of magnetic beads, functionalized with probe molecules that specifically bind the analytes, flows through the cell while a magnet attracts the beads to the surface, causing them to slide over the array surface. When a bead meets a captured analyte, it becomes tethered temporarily or permanently.[4] By choosing the correct shear rate, beads can be removed from the areas between spots to reduce background as well as from spots of weakly binding cross-reactive antibodies, thus improving assay specificity.

A detailed description of the cell design, peripheral devices, preparation of beads and arrays is presented in Supporting Information. The method was tested using both antigen and antibody arrays. For the first test, an anti-streptavidin (SA)-IgG array was manufactured by electrospray deposition[5] on a dialysis membrane coated with a layer of oxidized dextran[6] and SA was electrophoretically

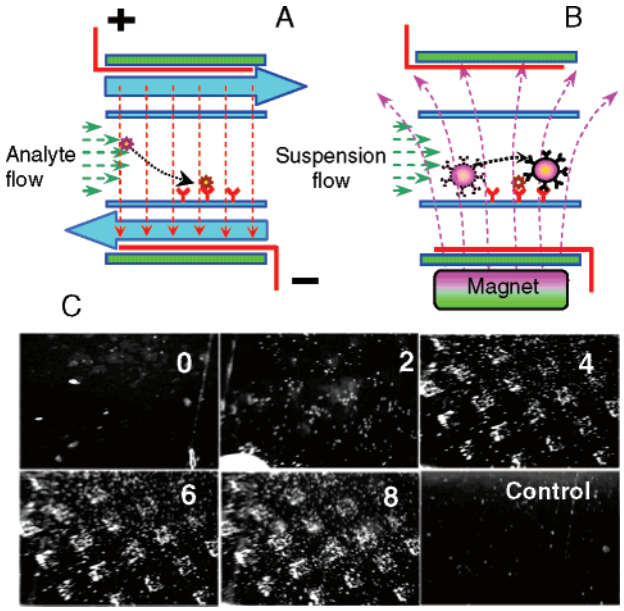

Figure 1. (A) Schematic of the electrophoretic capturing of charged analytes on an antibody array from flow. Green arrows denote flow of analyte, blue arrows denote flow of electrode buffer, red lines are platinum electrodes forming an electric field marked by red arrows. (B) Detection of captured analytes by scanning with functionalized magnetic beads. Suspension flow is marked by green arrows. (C) Dark-field images of an anti-SA-IgG array with SA molecules captured from 2×10^{-17} M solution of SA and detected by scanning with biotinylated magnetic beads. Distance between neighboring spots is 150 μm. Number in the upper right corner of each image in panel C indicates total time (in min) of capturing SA at a flow rate of 20 μL/min. Control image was obtained after capturing for 8 min under identical conditions from the same buffer solution without SA.

captured from a 2×10^{-17} M solution. Captured SA molecules were detected with biotinylated magnetic beads. The brightness of each spot was measured with a standard optical microscope and a CCD camera under dark-field illumination as the difference in gray level of the areas between the spots and that of spots bound with beads using SCION/NIH program, freely available on the Internet. The gray level of four to six spots was averaged on images taken at 8-bit resolution grayscale (255 levels) and subtracted from the average gray level of four to six areas between the spots. A pattern of bound beads was visually recognizable at spots brightness exceeding 3 units when more than five beads occupied each spot, 60×60 μm^2.

As illustrated in Figures 1C and 2A, a recognizable signal was obtained after 2−4 min of collection when 400−800 SA molecules had passed through the cell. Adding 1% serum ($\sim 5 \times 10^{10}$ molar excess of proteins) reduced the signal by only 10−15%, while adding 10% serum resulted in 50% inhibition of the signal. We

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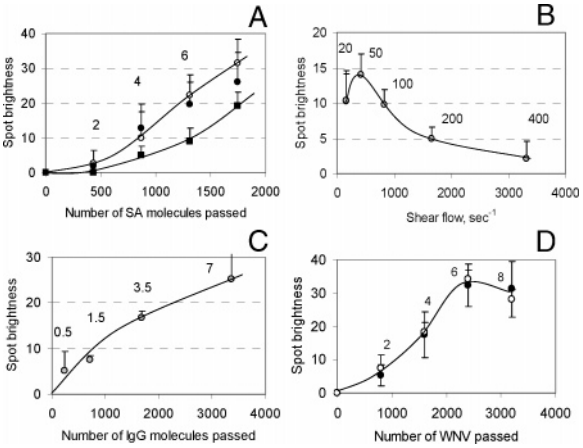

Figure 2. Dependence of spot brightness on the number of analyte molecules passed through the flow cell and on shear flow. (A) Streptavidin molecules captured on anti-SA-IgG array from buffer solution (empty circles), from the same buffer containing 1% dialyzed chicken serum (filled circles) and 10% chicken serum (filled squares) at a flow rate of 20 μL/min. (B) Dependence of the signal on the shear flow upon electrophoretic capturing of SA for 4 min. Numbers over points denote flow rate in μL/min. (C) Anti-Ova-IgG (commercial rabbit anti-Ova-IgG from Chemicon Intl, Temecula, CA, diluted $10^{12}-$fold) captured on an ovalbumin array at a flow rate of 60 μL/min. (D) Assay of viruses in serum from West Nile virus-infected chickens. The serum was dialyzed against water, diluted 1:200 with buffer, and WNV antigens were captured on an array of anti-WNV-IgG (solid circles). The same procedure was followed for a sample purified by exclusion chromatography (empty circles). The flow rate of the sample solution was 20 μL/min. The numbers above the experimental points in panels A, C, and D denote the time of capture in min. Other capturing parameters were a voltage of 110−150 V and current of 2−4 mA. Analytes were diluted with a buffer solution containing 20 mM imidazole, 10 mM glycine, 1% PVA, 1% PVP, and 0.1% Tween-20, pH = 8.5. The flow rate of the magnetic-bead suspension upon scanning was 5−10 μL/min. Flow rate of the external electrode buffer was 20 mL/min.

demonstrated that the efficiency of analyte capturing drops with increase in shear flow (Figure 2B). Similar sensitivity was obtained upon capturing antibody molecules (anti-ovalbumin-IgG) on an array of antigens (ovalbumin), as illustrated in Figure 2C. Negative controls in all these experiments were made using the same buffer solutions with or without serum but in the absence of analyte. No pattern of bound magnetic beads was observed (see Figure 1C as example).

In a third assay, sera from West Nile virus (WNV)-infected chickens were tested for the presence of serologically active WNV particles. A sample with a viral titer of 10^5 pfu/mL was inactivated with a 1−2 MRad radiation dose. Monoclonal anti-WNV protein E antibodies (Chemicon Intl., Temecula, CA) were arrayed and covalently linked to a layer of oxidized dextran on a dialysis membrane. Electrophoretically captured viral particles were detected by magnetic beads coated with the same antibody biotinylated as described[7] and attached to commercial SA-coated magnetic beads. As seen in Figure 2D, a reliable signal was observed after approximately 2 min when only ~700 viral particles passed through the cell. We concluded that numerous proteins present in the serum did not interfere notably with the assay because a twofold decrease in the total serum protein concentration after Sephacryl S-400 centrifugal exclusion chromatography did not change the signal intensity, as shown in Figure 2D. Chicken sera from control chickens not infected with WNV showed no bead binding after similar capture and scanning procedures.

In conclusion, replacing diffusion with active delivery of analytes and employing bead detection sensitive to a single analyte dramatically accelerated the immunoassay process and provided an extremely low LOD. Theoretically, all charged analytes may be collected on the array and every captured analyte could be discovered with scanning beads. In practice, detection was limited by the presence of defects on the array surface and by analyte losses due to adsorption to the walls and due to removal of analyte by beads during scanning (see example of such removal in section 8 in the Supporting Information). We believe that further refinement of the procedure and registration of every bead arrest by recording a movie will allow us to decrease the LOD still further, to a level now available only in the PCR-based techniques. The technology can be used in numerous applications where speed and/or extreme sensitivity are required such as in "warning"-type pathogen detectors, rapid immunoassay devices in emergency or surgery cabinets, or in an assay of biomarkers present at very low concentrations in biological fluids.

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Supporting Information Available: Detailed description of the flow cell design, peripheral devices, and general performance. This material is available free of charge via the Internet at http://pubs.acs.org.

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(4) Flow rate of suspension of magnetic beads $Q = 5-10$ μL/min corresponded to a shear rate, $S = 40-80$ sec^{-1}. At this shear-rate, a force applied to a single 20 nm long tether holding a magnetic bead, 1 μm in diameter, on the array surface is estimated as $T = 1.6-3.2$ pN in the Supporting Information.

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