Force Discrimination for Enhanced Biosensor Specificity of Micro-Retroreflector Assays

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ABSTRACT

Immunoassays are commonly used in diagnostic assays, pathogen detection and in the detection of contamination of food supplies. The label is what is actually detected, and label detection sensitivity often limits assay sensitivity. This work introduces magnetic particles as light blocking labels in optical assays based on microfabricated retroreflectors. The detection surface is composed of arrays of micron-scale microfabricated retroreflector tetrads. Retroreflectors return light directly to its source and are readily detectable with inexpensive optics. The assay can easily detect the presence of a single particle bound to the surface. Results also show that one or two *Rickettsia conorii* bacteria can hold down a 1.0 µm magnetic particle coated with rabbit polyclonal anti-rickettsia antibodies, supporting the possibility of detecting a single target pathogen with this assay. The magnetic properties of the particles are used to discriminate against non-specific interactions, increasing the specificity of the assay.

Keywords: retroreflectors, bioassay, biosensor, immunoassay, diagnostics

1 INTRODUCTION

Many bioanalytic techniques employ a label, typically a dye, enzyme or fluor, to signal the presence of analyte. While these labels are common and well-developed there are difficulties associated with their use. Fluorescent labels emit isotropically, which results in most of the signal never being detected. They are also affected by photobleaching and degradation of signal over time. Enzymatic systems require extra time for the attached enzyme to convert substrate into a detectable signal. Most of these systems are limited by the sensitivity of the large, expensive equipment that is typically required for detection of the label.

Recently, there has been increasing interest in the use of small particles for detection assays. Particles offer an attractive alternative to traditional labels, though non-specific interactions present between the particles themselves and the particles and the surface can hinder assay specificity. The interactions can be due to van der Waals attractive forces between the objects, or the electrostatic and the steric stabilization forces between the proteins, but often both are involved [1]. Force discrimination can be utilized to remove the non-specifically bound beads. Micrometer-scale magnetic beads have been used in a fluidic force discrimination assay to detect femtomolar concentrations of DNA and proteins [2].

This work introduces microfabricated retroreflectors as a diagnostic platform for an immunoassay with magnetic particles used as the labels. Retroreflectors return light back toward the source, resulting in a high level of detectability and minimal loss of signal. The assay is designed so that in the absence of analyte the retroreflector is bright. A dimming-based assay requires careful sample preparation, which is greatly facilitated by the use of magnetic binding particles in a pre-washing step.

The detection surface is composed of arrays of microfabricated retroreflector tetrads. A detector tetrad is composed of four retroreflectors (Figure 1); one assay retroreflector with antibodies is surrounded by three reference reflectors without antibodies that are always bright. Magnetic particles are used in sample preparation to capture the target and then exposed to the detector. When the target is present the magnetic particles will bind to the region in front of the assay reflector and scatter some of the light, reducing the amount returned to CCD camera. The light intensity reflected from the assay reflector is compared to that of the three reference reflectors to detect the presence of bound magnetic beads without needing to optically calibrate the device, as shown in Figure 1. Both magnetic and fluidic force discrimination are used to remove the non-specifically bound particles from the surface.

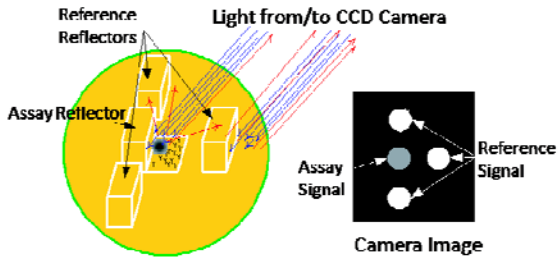

Figure 1: Diagnostic platform based on retroreflectors modulated by magnetic particles.

2 MATERIALS AND METHODS

All chemicals were used as received from the manufacturer. *Rickettsia conorii* bacteria killed by heating and polyclonal rabbit anti-Rickettsia antibodies were provided by Drs. Gary Wen and Juan Olano of the University of Texas Medical Branch (UTMB) at Galveston, TX. Polyclonal rabbit anti-*E. coli* antibodies were purchased from Fitzgerald Industries International. Dithiobis(succinimidyl propionate) (DSP) was from Pierce Biotechnology. Lysozyme, cytochrome c, bovine serum albumin, bovine serum, and phosphate buffered saline (PBS) were from Sigma-Aldrich. Tris-HCl and methylsulfoxide (DMSO) were from EM. Sodium Chloride was is from Mallinkrodt. HyHEL-5 monoclonal murine antibodies to hen egg lysozyme were prepared by the National Cell Culture Center. 2.8 µm Tosylactivated Dynabeads and 1.0 µm Carboxylic Dynabeads were purchased from Invitrogen and conjugated with antibodies using the manufacturer's recommended protocols. Silicone isolators, custom-made 2 mm wells and standard 9 mm wells, were from Grace BioLabs. Magnetic force discrimination was performed with Hall-probe calibrated neodymium permanent magnets purchased from K and J Magnetics. SEM images were taken using a Leo 1525 FE-SEM with Gemini column. The camera used for reflectivity measurements is a Sony CCD with 12x magnification.

2.1 Retroreflector Fabrication

Retroreflectors are fabricated by depositing a 5 µm layer of polyimide on a silicon wafer followed by spin-casting a thin 200 nm layer of PMMA [poly(methyl methacrylate)] on top. Electron beam lithography is then used to write the retroreflector pattern onto the PMMA layer. A thin layer of nickel is evaporated to protect the polyimide layer after the PMMA is dissolved away. Further etching transfers the nickel pattern onto the polyimide layer, leaving the retroreflector structure with very straight relatively smooth walls. Next, 10 nm of gold is evaporated to coat the entire structure.

2.2 Surface Preparation

Gold surfaces were cleaned with ethanol and rinsed with de-ionized water. Samples were then dried before surface activation using 4 mg/mL DSP in DMSO. Incubation with DSP was done for 30 min and samples were then rinsed twice with DMSO and water.

Capture antibodies are then spotted on the gold surface at 1.5 mg/mL and incubated for 2 hrs according to the protocol from Pierce. Samples were washed twice with PBS containing 0.1% Tween 20. The surface was then treated with 4% BSA in TBS to quench any remaining NHS groups. Similar activation procedures were followed to functionalize the surface with hen egg lysozyme (HEL) and cytochrome c.

2.3 Retroreflectors

Once the gold retroreflectors are functionalized with the capture antibody, they are dried and exposed to a helium ion beam so that only the region in front of the assay reflector is active; all references are deactivated. This is achieved by using the geometry of the tetrad since the ion beam only damages the antibodies in its line-of-sight. The retroreflector is exposed to the ion beam at an angle at which the assay region is protected by the reference reflectors. After the surface is selectively deactivated, it is incubated for at least 16 hrs in 4% BSA in TBS to passivate the surface. Before use, the retroreflectors are washed twice with PBS containing 0.1% Tween 20.

2.4 Magnetic Force Discrimination

For the HyHEL-5 lysozyme assay, gold surfaces were coated with hen egg lysozyme, cytochrome c or blocked with TBS containing 4%BSA using the same method as described above for the immobilization of antibodies. The 2.8 µm magnetic particles were functionalized with HyHEL-5 anti-lysozyme monoclonal antibodies. Functionalized gold surfaces were placed in a well with 150 µL of the HyHEL-5 particles and gently shaken at room temperature for 30 min. A horizontal magnetic force of 200 pN was applied to the samples for 2 min. The images taken by the CCD camera were done in solution.

For the *Rickettsia conorii* assay, gold surfaces were coated with rabbit polyclonal anti-Rickettsia antibodies or blocked with 4% BSA in TBS overnight. Both 2.8 µm and 1.0 µm particles were coated with rabbit polyclonal anti-*Rickettsia* antibodies or blocked. The particles were added to a solution of bovine serum containing heat sterilized *Rickettsia conorii* at 10^6 cells/mL, mixed end-over-end for 30 min and rinsed once with DI water. The particles and captured *R. conorii* were then added to the functionalized gold surface in a well and gently shaken at room temperature for 30 min. The samples were exposed to a horizontal magnetic force of 300 pN for 2 min and washed twice with water.

2.5 Fluid Channel

For initial fluid flow experiments a linear flow apparatus was used. Glass microscope slides were used to construct a 7.5 cm L x 0.6 cm W x 0.3 cm H fluid channel connected to a 15 mL syringe using 0.1 cm ID rubber tubing. The flow rate, due to gravity, was 25 mL/ min with a Reynolds Number of 160 corresponding to laminar flow. The samples were secured in the channel by double-sided silicon tape.

2.6 Combined Force Discrimination

Initial experiments integrating the magnetic and fluidic components of force discrimination have been conducted. Gold surfaces were coated with rabbit polyclonal anti-*E. coli* antibodies or blocked with 4% BSA overnight and the 2.8 µm particles were functionalized with rabbit polyclonal anti-*E. coli* antibodies as described above. The particles were added to a solution of bovine serum containing *E. coli*, mixed end-over-end for 30 min and rinsed once with DI water. The particles and captured *E. coli* were then added to the gold surfaces in the wells and gently shaken at room temperature for 30 min. The liquid was slowly removed from the wells so the particles remained on the surfaces. The sample was then placed in the fluid channel for 2.5 min with a flow rate of 25 mL/min of DI water (Re=160) and a 700 pN vertical magnetic force was applied.

2.7 Analysis

Samples were rinsed three times with DI water and allowed to air-dry overnight before viewing in the SEM. Light dimming measurements were done using a 50% mirror and CCD camera with intensity recorded by an attached computer and LabView analysis software.

3 RESULTS

Retroreflector signal attenuation experiments were done using the HyHEL-5/lysozyme system with an array of nine detector tetrads. HyHEL-5 anti-lysozyme antibody-functionalized 2.8 µm magnetic particles were exposed to the array which was coated with lysozyme. The signal attenuation in retroreflector brightness caused by the presence of a signal magnetic particle was measured and is reported in Figure 2. The presence of a single 2.8 µm particle on a retroreflector reduces the signal significantly. The intensity of a retroreflector with a particle is 33% less than the intensity of a retroreflector without a particle, indicating that single bead detection is possible. The repeatability of the measurement was determined by remounting the same sample in the reader 10 times and measuring the intensity of the same retroreflector. The error of measurement seen is 2% of the mean. Assuming the intensity distributions are Gaussian and normal, the probability of a false reading due to non-uniformity in retroreflector brightness is, at worst, about one in ten thousand.

Experiments to determine the number of *Rickettsia conorii* bacteria required to hold down a 1 µm magnetic particle were performed using polyclonal antibodies to *Rickettsia* immobilized on gold surfaces and the magnetic particles. *R. conorii* bacteria were incubated on the gold surfaces at 10^8 cells/mL before being exposed to 1 µm

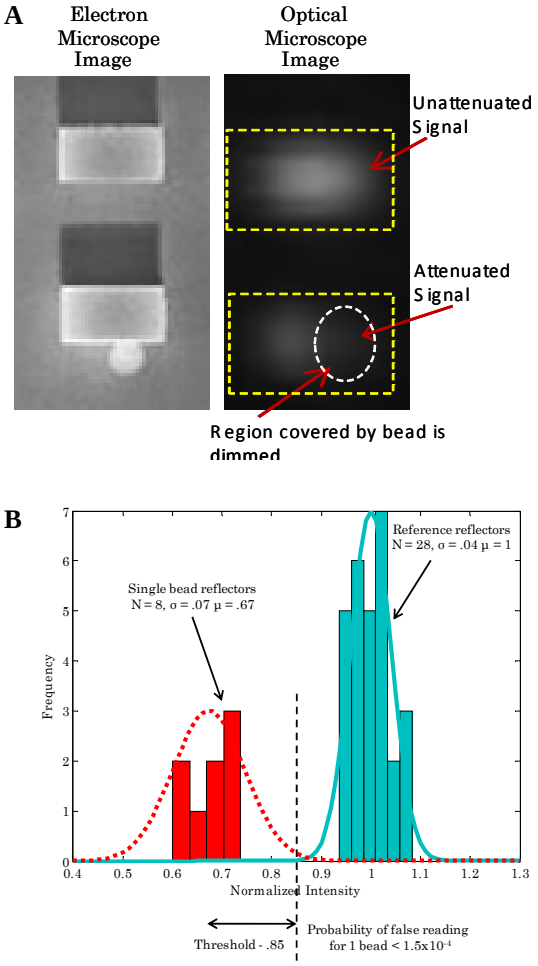

Figure 2: Dimming of signal seen by the presence of a 2.8 µm magnetic particle
(A) Images of "reference" reflector, top, and "assay" reflector (with particle), bottom. (B) Histogram of data collected which has been normalized to the mean value of the reference reflectors. The standard deviation of the assay and reference reflectors was 0.03 and 0.07, respectively. At a threshold of 0.85 is, then the probability of the brightness of a bead-bearing retroreflector being above the threshold is less than one in 10,000

magnetic particles. The entire surface was then coated with 5 nm of gold to show the bacterial cells holding down magnetic particles. This is shown in Figure 3.

Initial tests with magnetic particles show that the application of a magnetic field can be used to remove non-specific particles not bound by immobilized analyte. Results from the HyHEL-5/lysozyme assay show that a horizontal magnetic force of 200 pN from a Hall probe-calibrated permanent magnet is sufficient in discriminating against non-specific interactions between 2.8 µm HyHEL-5 coated magnetic particles and the protein-coated gold surface. Figure 4 shows the results of the magnetic stringency tests.

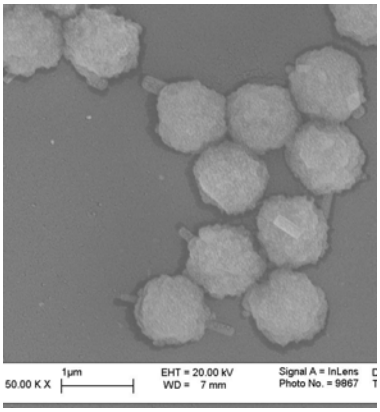

Figure 3: 1 µm magnetic particles held down by *Rickettsia conorii*

Similar results were obtained with the *R. conorii* system where the bacteria were captured from bovine serum by 2.8 µm anti-*Rickettsia* magnetic particles and then exposed to plain gold surfaces. The concentration of *R. conorii* in bovine serum was around 10^6 cells/mL and two different concentrations of magnetic particles were used, $1x10^6$ particles/mL and $3x10^6$ particles/mL. The higher magnetic particle to *R. conorii* ratio resulted in fewer particles bound to the surface. A horizontal magnetic force of 300 pN from a Hall probe-calibrated permanent magnet was applied and samples were washed twice with DI water. This treatment sufficiently discriminated against non-specific interactions between 2.8 µm anti-Rickettsia coated magnetic particles and the protein-coated gold surface.

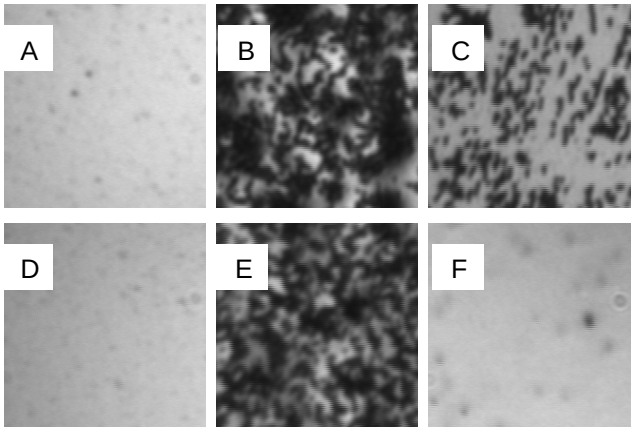

Figure 4: Magnetic pulloff stringency removes non-specifically bound particles for specificity enhancement: (A) Gold surface functionalized with HEL; (B) 2.8 µm anti-HEL magnetic beads on the surface in (A); (C) Horizontal magnetic force of about 200 pN leaves specifically-bound beads. (D) Gold surface functionalized with irrelevant cationic protein cytochrome C; (E) 2.8 µm anti-HEL magnetic beads non-specifically bound on the surface in (D); (F) Surface (D) after being exposed to a horizontal magnetic force of about 200 pN. Remaining specific beads in (C) ~ 450-500; unspecific beads in (F) ~ 10-15.

Preliminary experiments combining magnetic and fluidic force discrimination yielded similar results for the *E. coli* system on flat gold surfaces. The non-specially bound particles were removed from the flat surfaces. The retroreflector structures limited the shear force that could be applied to the particles on the surface in between the structures. Microfluidic components will be incorporated to increase the fluid force applied to the particles.

4 DISCUSSION

Retroreflectors offer an attractive platform for diagnostic applications. With their ability to reflect light from a wide degree of angles directly back to the source, they are highly visible and easily detectable with an inexpensive, simple optical setup. The required standard CCD camera and computer for analysis are easily available and can be moved as needed.

The development of a robust assay chemistry was achieved and applied to several model systems. The presence of a single magnetic particle on an assay retroreflector can easily be detected with the probability of a false reading being only one in ten thousand. Incorporating magnetic and fluidic force discrimination components will increase the specificity of the assay and its potential usefulness in clinical settings.

The retroreflectors can be patterned and mass-produced as assay chips for use in clinical applications. The chips will be prepared with antibodies to the causative agents of multiple illnesses. Such a platform diagnostic will be useful when a patient is suffering from non-specific symptoms such as fever and nausea. A sample from the patient can be analyzed using the chip and the cause of illness determined in a matter of hours. This will aid in providing the correct treatment in a timely manner. Most times, the causative agent will be common and minor, such as the flu. Occasionally the disease may be something more serious like bacterial meningitis where chances of recovery increase with prompt diagnosis and treatment. In rare cases, the cause of illness may be a bioterrorism agent or an emerging disease with serious implications for personal and public health, where rapid response is invaluable.

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