Micro-Retroreflectors in Immunoassays and Biosensors

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ABSTRACT

Many current bioanalytical and diagnostic methods rely on labeling the species of interest and the use of specialized detection equipment to monitor the label. Incorporation of retroreflectors into immunoassays and biosensors offers a novel and economical solution to problems associated with these methods and provides a detection assay useful in clinical and defense applications. Spotting of various detection antibodies on various test surfaces was done to test the viability of a "syndrome chip" for use in clinical diagnostics. Magnetic particles were used to incorporate magnetic stringency into the assay, which when combined with microfluidics will increase the specificity of the system. Such a chip would be activated with capture antibodies for several common disease vectors as well as some that are less common and may have bioterrorism applications.

Keywords: retroreflectors, bioassay, biosensor, immunoassay, diagnostics

1 INTRODUCTION

Many current bioanalytic techniques rely upon the detection of a label to confirm the presence of analyte. Of the labels available, fluorescent, radioactive, and enzymatic tags are among the most prevalent. While these tags are prevalent and well-developed there are difficulties associated with their use. Fluorescent and radioactive tags emit isotropically, which results in most of the signal never being detected. Fluorescent labeling also suffers from photobleaching and degradation of signal over time. Enzymatic systems require extra time for the attached enzyme to convert substrate into a detectable signal. All of these systems use large, expensive detection equipment that is typically anchored to one location.

Over the last few years, there has been increasing interest in the use of small particles for detection assays. Labeled gold nanoparticles immobilized on surfaces through molecular interactions can be detected by wavelength-dependent absorption. If necessary, silver enhancement of the gold particles can be done to increase detectability [1]. Magnetic particles have been used in similar applications, where the density of bound particles is directly related to the captured analyte. Detection in such a system is either done by optical counting or through the use of a magnetic sensor [2].

Micro-fabricated retroreflectors propose an attractive solution to the difficulties with current techniques referenced earlier. Retroreflectors are designed to return light back toward the source, resulting in a high level of detectability and minimal loss of signal. This work proposes the incorporation of gold and magnetic particles into a retroreflector based detection system. The system is designed so that in the absence of analyte the retroreflector is bright. When analyte is present, particles decorated with antibodies assemble in front of the reflector. Immobilized particles scatter or absorb the incoming light and dim the retroreflector in comparison with surrounding references reflectors. The degree of reduction is proportional to the number of captured particles and therefore the amount of analyte. Light is provided using a standard broad spectrum bulb and detection is done with a CCD camera connected to a computer for image capture and analysis. Because they are anufactured through lithographic techniques already widely used in the semiconductor industry, large-scale production of retroreflectors can be implemented with ease.

2 MATERIALS AND METHODS

All chemicals used in this work are used as is from the manufacturer. Gold pads and retro-reflectors are produced by Tim Sherlock. Norwalk virus-like particles (VLP) and antibodies were acquired from the lab of Dr. Robert Atmar at the Baylor College of Medicine in Houston, TX. *Rickettsia conorii* bacteria killed by heating and antibodies were provided by Drs. Gary Wen and Juan Olano of the University of Texas Medical Branch (UTMB) on Galveston, TX. Dithiobis(succinimidyl propionate) (DSP) and the NAb Protein A/G spin kit were from Pierce Biotechnology. 2-[methoxypoly(ethyleneoxy)propyl] trimethoxysilane (PEG-silane) was purchased from Gelest in Morrisville, PA. Lysozyme, bovine serum albumin, and phosphate buffered saline (PBS) were from Sigma-Aldrich. Tris-HCl and methylsulfoxide (DMSO) are from EM. Sodium Chloride was is from Mallinkrodt. D1.3 and HyHEL-5 monoclonal murine antibodies to hen egg lysozyme were obtained from National Cell Culture Center. Dynal brand MyOne 1 μm carboxylated and Dynabeads 2.8 μm tosylactivated magnetic beads were purchased from Invitrogen and conjugated with antibodies using attached protocols. Goat anti-mouse IgG labeled 40 nm

nanoparticles, unconjugated 40 nm gold nanoparticles, and Nanoparts antibody-gold conjugation kit were purchased from Ted Pella, Inc. Silicone isolators, custom-made 2 mm wells and standard 9 mm wells, were from Grace BioLabs. 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. Particle counting and density determinations were done using the ImageJ image analysis program from NIH.

2.1 Retreflector Fabrication

Retroreflectors are fabricated by coating a silicon wafer with 2.5 microns of polyimide and 200nm of resist. A lithography step is used to generate the retroreflector pattern as openings in the resist and a 50nm thick nickel coating is deposited using thermal evaporation. After a lift-off step, which leaves behind only the nickel that coated the base of the resist openings, the patterns are transferred into the polyimide in an O_2/CF_4 reactive ion etch, leaving the retroreflecting structure with very straight relatively smooth walls. Next, gold is evaporated to coat the entire structure.

2.2 Surface Preparation

Gold surfaces were cleaned for one minute in 0.01 M HCl and rinsed with de-ionized water and ethanol. In experiments where there is silicon oxide surrounding the gold, passivation was performed using 3 mM PEG-silane in toluene for 18 hours [3]. 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. Gold pads were then treated with 4% BSA in TBS to quench any remaining NHS groups.

Similar activation procedures were followed for gold pads and retroreflectors.

2.3 Retroreflectors

Once the capture antibody was deposited, retroreflectors were washed twice with PBS containing 0.1% Tween 20 and rinsed three times with DI water and dried with helium. A second layer of gold was then angle-evaporated so that the area directly in front of the assay reflector is left with active antibodies, while the rest of the surface is covered with a fresh layer of gold. The active assay region is protected by a reference reflector that blocks the second gold layer in the line-of-sight deposition.

Retroreflectors were then incubated for at least 16 hrs in 4% BSA in TBS to quench any remaining NHS groups and passivate the fresh gold surface. Before use, the retroreflectors are washed twice with PBS containing 0.1% Tween 20.

2.4 Analyte Assays

Direct detection assays were done by immobilizing HyHEL-5 antibody to lysozyme directly on the gold surface. Presence of the HyHEL-5 was confirmed by 40 nm gold nanoparticles coupled with goat anti-mouse IgG antibodies. In competitive binding assays, soluble HyHEL-5 was added as competitor to bind with the nanoparticle bound antibody.

Standard assays were done by incubating analyte with the capture antibody coated surface at 0.01 mg/mL for 1 hr. Samples were then washed with PBS containing 0.1% Tween 20. Incubation of the sample with antibody-coated detection particles is done to confirm presence of the analyte. Incubation is done with light mixing for 20 minutes with the 1 and 2.8 μm magnetic particles and for at least 3 hrs with the 40 nm gold nanoparticles. Unbound particles are removed using two rinses with PBS containing 0.1% Tween 20 and magnetic pull-off.

2.5 Magnetic pull-off

Magnetic stringency tests were done by immobilizing analyte on gold surfaces using the DSP chemistry described in Section 2.2. 1 μm or 2.8 μm magnetic beads coupled with antibody were incubated with the sample for 30 min. A magnetic field was applied to remove unbound particles, with surface imaging being done with a CCD camera. SEM images were also used to determine particle coverage.

2.6 Analysis

Samples were rinsed three times with DI water and allowed to air-dry overnight before viewing in the SEM. Particle counting was done with ImageJ software and used to determine density and surface coverage.

Light dimming measurements were done using a 50% mirror and CCD camera with intensity recorded by an attached computer and LabView.

3 RESULTS

Initial tests done with immobilized HyHEL-5 for direct detection and competitive binding assays were done on flat gold surfaces with no mixing. Incubation was done overnight to allow for the particles to diffuse to the surface. The experiments revealed a linear relationship between the coverage of gold nanoparticles on the surface and the reduction in light intensity returned from the gold surface. The results are summarized in Figure 1 below.

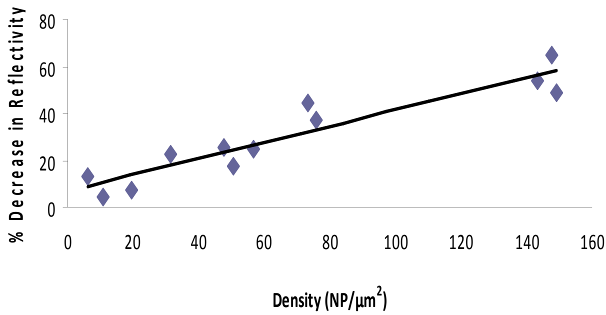

Figure 1: Decrease in reflectivity vs. nanoparticle density

System specificity was confirmed by using two gold pads, one spotted with polyclonal rabbit anti-Norwalk IgG antibodies and the other with hen egg white lysozyme. The pads were incubated with sample solution containing 0.01 mg/mL Norwalk VLPs for one hour and 0.01 mg/mL HyHEL-5. Samples were then incubated with 40 nm gold nanoparticles conjugated with goat anti-mouse IgG antibodies to detect HyHEL-5 and 1 µm magnetic particles conjugated with polyclonal antibodies to Norwalk virus for VLP detection. This resulted in selective accumulation of the detection particles on the pads with their associated antigens.

Similar experiments were done with the heat-killed *Rickettsia conorii* bacteria. Polyclonal antibodies to Rickettsia were immobilized on gold pads and incubated with Rickettsia at 10^8 cells/mL before being exposed to 1 µm magnetic particles with Rickettsia antibodies. 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 2 below.

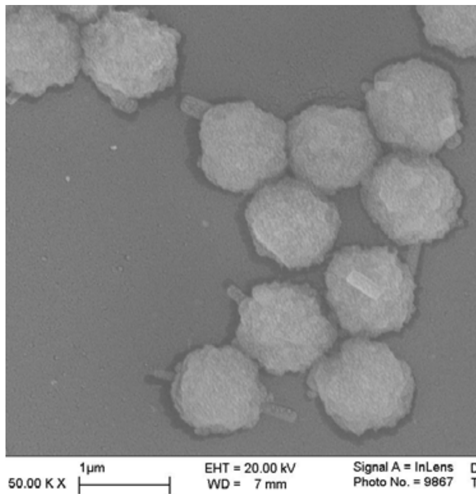

Figure 2: 1 µm magnetic particles held down by Rickettsia bacteria

Initial tests with magnetic particles show that the application of a magnetic field can be used to remove particles not bound by immobilized analyte. Preliminary tests were done using lysozyme coated gold samples and 2.8 µm beads conjugated with HyHEL-5. A magnetic force of 200 pN was applied to the samples to remove the particles. Figure 3 shows gold surfaces after the magnetic stringency test. The left-hand sample was the negative control coated with cytochrome C, while the sample on the right was treated with lysozyme.

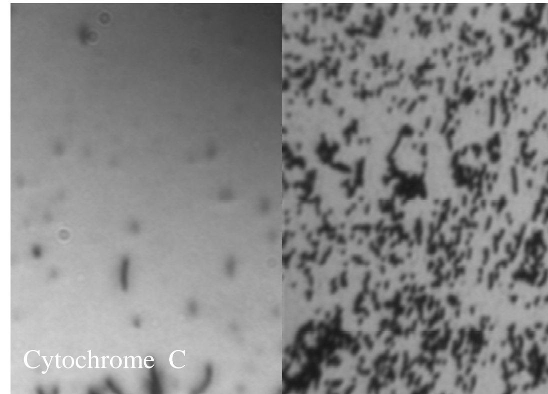

Figure 3: Results of magnetic stringency tests of 2.8 µm beads conjugated with HyHEL-5 antibody.

Retroreflectors were printed in groups of nine tetrads, seen in Figure 4. Each tetrad is arranged is arranged so that the front retroreflector acts as a shield during the deposition of the second gold layer and protects the assay region directly in front of the center feature. The second layer of gold blocks the immobilized antibodies over the rest of the surface. Direct detection assays for HyHEL-5 were used with retroreflectors to show the selective assembly of gold nanoparticles in the assay region of the retroreflectors. Figure 5 shows the shadow cast by the second gold deposition and the accumulation of particles within the shadowed region. Fabrication of the retroreflectors is highly reproducible with standard deviations of signal returned from the features under 4%.

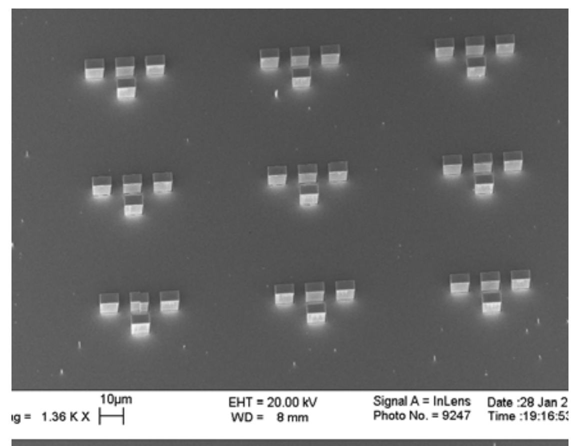

Figure 4: Micro-fabricated retroreflectors printed in tetrads. The front feature protects the assay region and serves as a reference with the two outer reflectors.

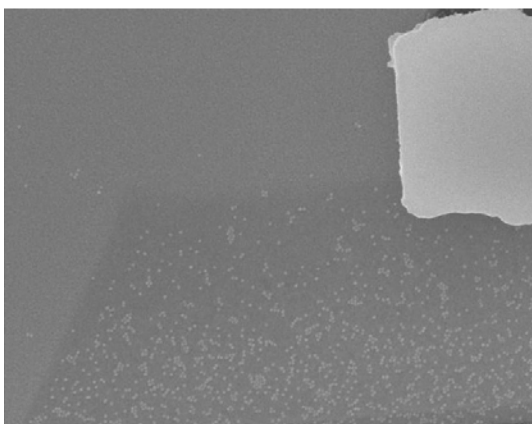

Figure 5: Accumulation of gold nanoparticles inside shadowed region of retroreflector

4 DISCUSSION

Retroreflectors offer an attractive platform for diagnostic applications. Their property of returning light back to the source minimizes lost signal and means that sophisticated equipment is not needed for detection and analysis. The required materials of a 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 accumulation of particles was shown to produce a measurable reduction in assay signal that will scale with the amount of immobilized analyte. Incorporation of fluidic or magnetic discrimination will increase the specificity of the assay and it's 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 many illnesses. Such a "syndrome" chip will be useful in doctor's offices when a patient is suffering vague 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 easily treatable, such as the flu. Occasionally the disease may be something more serious like bacterial meningitis where chances of recovery increase with prompt treatment. In rare cases, the cause of illness may be a bioterrorism agent with serious implications for personal and public health.

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