

A Whole Blood Immunoassay Using Gold Nanoshells

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A rapid immunoassay capable of detecting analyte within complex biological media without any sample preparation is described. This was accomplished using gold nanoshells, layered dielectric-metal nanoparticles whose optical resonance is a function of the relative size of its constituent layers. Aggregation of antibody/nanoshell conjugates with extinction spectra in the near-infrared was monitored spectroscopically in the presence of analyte. Successful detection of immunoglobulins was achieved in saline, serum, and whole blood. This system constitutes a simple immunoassay capable of detecting sub-nanogram-per-milliliter quantities of various analytes in different media within 10–30 min.

The availability of a rapid, in situ whole blood assay with the capacity to detect a variety of selected analytes would greatly benefit point-of-care or public health applications, where there is a strong demand for rapid, high-throughput screening of blood-borne species. Immunoassays, with their high affinity and specificity, show the most promise for implementing such a system. Conventional approaches, such as the enzyme linked immunosorbent assay (ELISAs) are not a feasible approach for such a system since they require time-consuming sample purification/separation, incubation, and rinsing steps prior to analysis. Homogeneous immunoassays are, however, free of any separation steps and may provide a more amenable strategy in the development of a near-instantaneous, in situ whole blood assay. Surface plasmon resonance (SPR),¹ microelectromechanical cantilevers,² piezoelectric devices,^{3,4} and microparticle agglutination assays⁵ are a few of the promising approaches for homogeneous assays in development; however, none of these systems have to date produced a viable whole blood analytical tool. In this paper, we demonstrate a nanoshell-based, rapid near-infrared (near-IR) homogeneous im-

muoassay capable of detecting low concentrations of analytes in whole blood within minutes without any sample preparation.

Nanoshells are a layered, spherical nanoparticle consisting of a dielectric core (silica) surrounded by a thin metal shell (gold or silver), possessing a tunable plasmon resonant response that gives rise to intense optical absorption and scattering.⁶ In this core-shell topology, the plasmon resonance frequency of the nanoparticle can be varied systematically by modifying the relative dimensions of the core and the shell layers.⁷ Nanoshells can be designed and fabricated with optical resonances from the visible to the midinfrared regions of the spectrum,⁸ easily spanning the near-infrared region of the spectrum (700–1300 nm), where optical transmission through tissue is optimal.⁹ To date, nanoshells have demonstrated their usefulness in applications ranging from photooxidation inhibition in photoluminescent polymer films,¹⁰ to Raman sensors that can be optimized to specific pump laser wavelengths,¹¹ to optically triggered drug delivery¹² and optomechanical materials.^{13,14} Since gold nanoshells offer the added features of high biocompatibility and facile bioconjugation to antibodies via long-standing protocols adapted from gold colloid bioconjugate chemistry, their near-infrared optical properties make them an ideal, if not unique, vehicle for a whole blood immunoassay.

The underlying mechanism behind the nanoshell-based aggregation immunoassay is similar to that of conventional latex agglutination (LA) and sol particle immunoassays, which have existed for decades.^{15,16} When antibody-conjugated particles are presented with a multivalent analyte, multiple particles will bind

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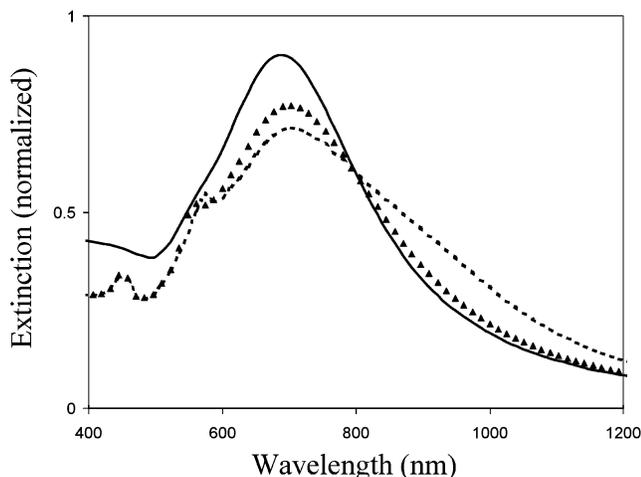


Figure 1. Calculated extinction spectrum of a single nanoshell with a 96-nm-diameter core and a 22-nm-thick shell (solid line) and a dimer system composed of two such nanoshells whose surfaces are separated by 10 (---) and 40 nm (\blacktriangle).

to the analyte, forming particle dimers and higher order aggregates of particles of various morphologies in the sampling medium. Unfortunately, LA and sol particle assays are difficult to perform within whole blood due to blood's high turbidity and strong visible extinction, contaminating any detectable signal from LA or sol particle agglutination. Furthermore, the colloidal stability of latex particles is precarious and subject to numerous forces, including pH, salinity, and the molecular species used for sensitization, often requiring the use of surfactants or steric exclusion species to improve stability.^{17,18} Regardless of recent developments, the biologically complex high saline whole blood environment has stifled the development of a LA system in whole blood, limiting its use to dilute, buffered serum samples. Nanoshells, in contrast, are quite stable with respect to aggregation and flocculation within whole blood, and their antigen-induced aggregation can be detected via spectral red-shifting in the near-infrared, where whole blood is more transmissive, permitting detection of nanoshell aggregation in whole blood.

The plasmon resonant spectral response of nanoparticle aggregates differs significantly from that of isolated, dispersed plasmon resonant nanoparticles.¹⁹ Nanoshell dimers produce a second, significantly red-shifted plasmon resonance that is excited when the polarization of incident light is along the axis of the pair of nanoparticles. This red-shifted "dimer" resonance occurs over a range of internanoparticle spacings on the order of the dimension of the antibody/antigen/antibody linker between nanoshells. The red-shifted aggregate plasmon is simultaneously accompanied by a decrease in the amplitude of the single nanoshell plasmon resonance in the overall spectral response.¹⁹ Figure 1 shows a theoretical single nanoshell plasmon resonance spectrum, calculated using Mie scattering theory, shown in comparison to its angle-averaged dimer resonance counterpart, calculated using the finite difference time domain method, for two different nanoshell separations. By comparing these two spectra,

we see that the additional red-shifted feature of the aggregate plasmon is also accompanied by a decrease in the plasmon response at the single nanoshell plasmon maximum. These spectra indicate that an optically straightforward method for detecting nanoparticle aggregate formation is to monitor the decrease in extinction at the single nanoshell plasmon resonance frequency as aggregation proceeds. This aggregate-induced red shift in nanoparticle resonance has been well documented for other metal colloids.^{16,20,21}

In the experiments reported here, the presence of analyte was detected optically in a solution of nanoshell-conjugated antibodies exposed to prepared samples of known analyte concentration by monitoring the decrease in the single nanoshell plasmon resonance extinction. Using different antibody/analyte pairs, successful detection of immunoglobulins was achieved in saline, serum, and 20% whole blood with detection limits on the order of 100 pg/mL within 10–30 min.

EXPERIMENTAL SECTION

Gold/Silica Nanoshell Fabrication. Metal nanoshells were fabricated as previously described.⁷ Briefly, silica nanoparticles were grown by the Stöber method,²² in which tetraethyl orthosilicate (Aldrich) was reduced in NH_4OH in ethanol. Particles were sized by scanning electron microscopy (SEM) (Phillips XL30); polydispersity of <10% was considered acceptable. The particle surface was then terminated with amine groups by reaction with (aminopropyl)triethoxysilane (APTES, Aldrich) in ethanol.

Very small gold colloid (1–3 nm) was grown using the method of Duff et al.²³ This colloid was aged for 2 weeks at 4 °C and then concentrated using a rotary evaporator. Aminated silica particles were then added to the gold colloid suspension. Gold colloid adsorbs to the amine groups on the silica surface, resulting in a silica nanoparticle decorated with gold colloid at a surface area coverage of typically 25–30%. Gold/silica nanoshells were then grown by reacting HAuCl_4 with the silica/colloid particles in the presence of formaldehyde. This process reduces additional gold onto the adsorbed colloid, which acts as nucleation sites, causing the surface colloid to grow and coalesce with neighboring colloid, forming a complete metal shell. Nanoshell formation was assessed using a UV–vis spectrophotometer (Hitachi U-2001) and SEM.

For this application, gold nanoshells resonant at 720 nm were fabricated. SEM confirmed the fabrication of a 96-nm-diameter core with a 22-nm gold shell, creating a 720-nm resonant gold-silica nanoshell. UV–vis spectroscopy confirmed the placement of the extinction peak centered at 720 nm and is in agreement with Mie scattering theory predictions for particles with these dimensions and composition.⁶

Synthesis of Thiolated Polyethylene Glycol (SH-PEG). SH-PEG was synthesized by reacting PEG-amine (MW 5000, Shearwater Polymers, 2M2V0H01) with 2-aminothiolsane (Sigma, I-6256) for 1 h. The product was then dialyzed (MWCO 500, Spectrum Labs) against DI H_2O for at least 1 h to remove excess reagent. The SH-PEG yield was determined colorimetrically at 412 nm after reacting with Ellman's reagent (5,5'-dithio-bis(2-nitrobenzoic acid), Sigma). Product was stored at –20 °C.

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Preparation of OPSS-PEG-Antibodies. Antibodies were tethered to nanoshell surfaces using PEG linkers. Orthopyridyl disulfide-PEG-*n*-hydroxysuccinimide (MW 2000, OPSS-PEG-NHS, Shearwater Polymers, 2Z3K0D12) was reacted with each antibody at 1:1 molar ratio in 100 mM sodium bicarbonate, pH 8.5, overnight on ice.

Determination of Immobilized Antibody Activity. An enzyme immunoassay was performed on the antibody/nanoshell conjugates to assess the density of active antibodies on nanoshell surfaces after conjugation. OPSS-PEG-anti-rabbit IgG (AR, Sigma, R5506) was prepared as described above and assembled onto nanoshells by mixing OPSS-PEG-AR (5 $\mu\text{g}/\text{mL}$) with a nanoshell suspension (1.98×10^9 particles/mL) in 1.8 mM potassium carbonate for at least 1 h. Remaining nonspecific adsorption sites on the nanoshell were blocked with 1.04 μM PEG-SH for an additional hour. For a negative control, antibody-free nanoshells were incubated with PEG-SH blocking agent at the same concentration. Conjugate solutions were centrifuged at 650 g for 4 min and resuspended in 100 mM phosphate buffered saline (PBS) to remove excess antibody and PEG-SH blocking agent. Conjugate solutions were then incubated with 10 $\mu\text{g}/\text{mL}$ horseradish peroxidase-labeled rabbit IgG (HRP-rb-IgG, Sigma, A6917) for 1 h at RT. Samples were centrifuged and resuspended 2 times in PBS to remove excess HRP-rb-IgG. Tetramethylbenzidine dihydrochloride (TMB) peroxidase substrate (Sigma, T3405) was suspended in 0.05 M phosphate citrate buffer and 0.006% hydrogen peroxide. The capture of HRP-rb-IgG onto nanoshells was quantified by mixing 1 part TMB solution with 9 parts nanoshell conjugate solution for 10 min. TMB development was stopped with 2.0 N H_2SO_4 , and absorbance was read at 450 nm. Using Mie scattering theory, UV-vis analysis of sonicated, dispersed nanoshell conjugate solutions revealed the total nanoshell surface area in solution, permitting calculations of antibody concentration per unit nanoshell surface area.

Detection of Rabbit IgG. An immunoassay for rabbit IgG was performed in saline, bovine serum, and whole human blood. OPSS-PEG-anti-rabbit IgG (AR, Sigma, R5506) was prepared as described above and assembled onto nanoshells by mixing OPSS-PEG-AR (10 $\mu\text{g}/\text{mL}$) with a nanoshell suspension (4.87×10^9 particles/mL) in 1.8 mM potassium carbonate for at least 1 h. Remaining nonspecific adsorption sites on the nanoshell were blocked with 1.04 μM PEG-SH for an additional hour.

Rabbit IgG (Sigma, N4142) was dissolved in 100 mM phosphate buffered saline (PBS) at 2.2, 1.1, 0.22, 0.11, 0.022, and 0.0 $\mu\text{g}/\text{mL}$. Whole human blood was collected with porcine heparin (10 units/mL) anticoagulant and stored at 4 $^\circ\text{C}$ until needed. Prior to analysis, whole blood was diluted to 33% (v/v) in lysis buffer (10 mM HEPES, 50 mM β -glycerophosphate, 70 mM NaCl, 2 mM EDTA, 1% Triton X100, and 1 \times Sigma protease inhibitor cocktail).

The nanoshell suspension was then diluted to 1.83×10^9 particles/mL using either 100 mM PBS, fetal bovine serum (FBS, Biowhittaker), or 33% whole blood. A 480- μL portion of these suspensions was used for each sample. Before analyte addition, nanoshell extinction at 720 nm (A^{720} , 0.45 cm path) was measured for $t = 0$ in saline, serum, and blood. A 20 μL portion of rabbit IgG was then added to each nanoshell aliquot. Samples were mixed, and extinction at 720 nm was measured between 1 and 30 min.

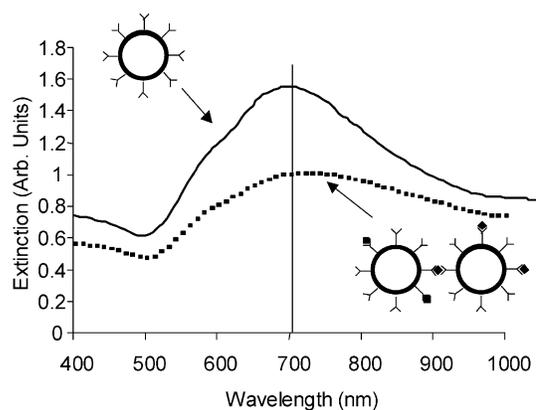


Figure 2. UV-vis spectrum of disperse nanoshells fabricated with a 96-nm-diameter core and 22-nm-thick gold shell (—); spectrum of nanoshells/antibody conjugates following addition of analyte (- - -). Extinction reduction upon aggregation in the presence of analyte was monitored at 720 nm, as indicated.

RESULTS AND DISCUSSION

The presence of active immobilized antibody on nanoshell surfaces was confirmed via enzyme immunoassay. PEG-SH is a linear chain molecule capable of assembling into a dense monolayer on the gold nanoshell surface. Although this molecule does an excellent job of sterically stabilizing the nanoshells in a saline environment, previous work by Bain and Whitesides has shown that thiol-mediated assembly of heterogeneous solutions of different thiol species is a thermodynamic process largely determined by the free energy of assembly onto the 2D surface.²⁴ These findings suggest the potential for displacement of SH-PEG-antibodies in favor of the SH-PEG blocker; therefore, desensitizing the nanoshell conjugates and compromising the assay performance. Despite these findings, we found that a sufficient amount of anti-rb-IgG remained on nanoshell surfaces and proceeded to capture HRP-rb-IgG analyte at a concentration of 2.45 pmol/cm² with a standard deviation of 0.31 pmol/cm². These values are in good agreement with those reported for protein coverage on other gold substrates.^{25,26} Antibody-free PEG-nanoshells, in contrast, displayed minimal nonspecific adsorption of analyte, with a coverage of 0.193 ± 0.036 pmol/cm², which is 12.7-fold less than anti-rb-IgG nanoshells. In light of these findings, we conclude that if PEG/antibody displacement is occurring within our system, it is minimal within the regime of concentrations and incubation times used in this study.

The concept of an optically detected assay based on nanoshell aggregation in the presence of analyte is depicted in Figures 1 and 2. A stable solution of disperse antibody-conjugated nanoshells possesses a strong extinction peak at 720 nm. Upon the addition of analyte, the 720-nm peak begins to diminish and broaden as a result of spectral red-shifting from redistributed particle-particle aggregate resonances. These figures are in good agreement with results reported by Leuvering and colleagues for their gold sol particle immunoassay performed at 530 nm.¹⁶ By measuring the reduced extinction at the particles' original 720 nm resonance, we were able to quantitatively identify IgG analyte in saline, serum, and whole blood.

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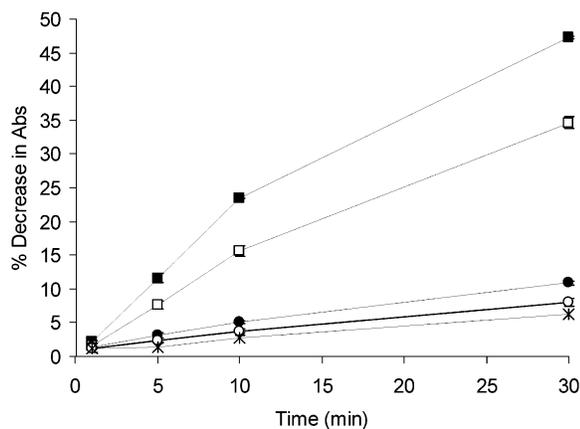


Figure 3. Time series plot of nanoshell aggregation in the presence of 88 (■), 44 (□), 8.8 (●), 4.4 (○), and 0.0 (×) ng/mL IgG. Absorbance is expressed in percentage decrease from $t = 0$. All error bars depict standard deviations.

AR-PEG-OPSS–nanoshell probes quantitatively detected a range of final analyte concentrations (88–0.88 ng/mL) in a log-linear fashion within 10 min. Plotted as the percent decrease in absorbance from $t = 0$, Figure 3 shows a time series of the assay performance for five analyte concentrations in 100 mM PBS. Despite the high saline content, analyte-free samples show virtually no aggregation after 30 min. In contrast, all other analyte concentrations induced aggregation assessed by the drop in extinction at 720 nm. Higher analyte levels (88, 44 ng/mL) demonstrate rapid aggregation kinetics at early times. The corresponding change in absorbance becomes moderate at later times as the percent change of the large aggregates becomes smaller. The fast rates of aggregation of this assay allow one to rapidly determine analyte levels. All five IgG concentrations were statistically discernible after 10 min. Compared to quantitative LA methods that require sophisticated instrumentation, such as dynamic light scattering, nephelometry, and photon correlation spectroscopy, the nanoshell immunoassay is a very simple method that requires only a basic spectrophotometer.

These initial studies focused on analyte detection in aqueous/saline solutions, which bear little resemblance to the protein-rich, optically turbid medium of blood and other biological fluids. As the studies above have shown, high saline environments do not hinder the assay's performance, because PEG-derivatized nanoshells are very stable in suspension. However, the protein-rich environment of serum and blood introduces a multitude of proteins potentially capable of initiating nonspecific aggregation and causing background noise. Furthermore, the optically turbid medium of blood may attenuate the optical signal due to scattering. To address these issues, analyte detection was performed in saline, serum, and whole blood. As shown in Figure 4, readings from rabbit IgG in saline, FBS, and 20% whole blood (final concentration) show similar trends for all concentrations. Detection of IgG within the protein-rich serum environment demonstrates good analyte specificity. Like their saline counterpart, FBS samples were capable of detecting 0.88 ng/mL IgG after only 10 min. Rabbit IgG was also detected successfully in 20% whole blood but with a limit of detection of 4.4 ng/mL. Despite the elevated detection limit in blood, all three assays displayed a log-linear performance over the range of 88–4.4 ng/mL after only 10 min. Compared to

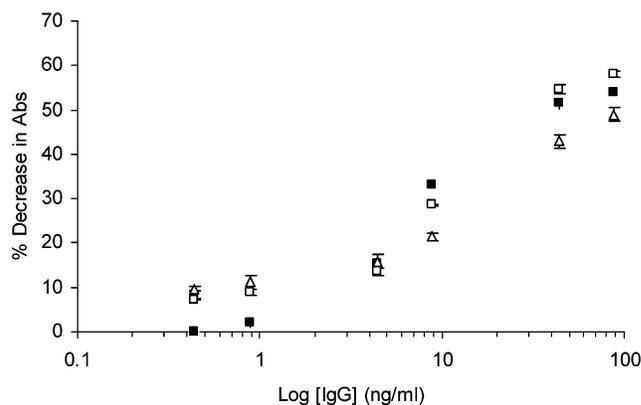


Figure 4. Analyte detected in saline (□), serum (■), and 20% whole blood (Δ) at 30 min.

saline performance, sensitivity was reduced 17 and 24% in serum and whole blood, respectively, as determined by the reduction in slope over the 88–4.4 ng/mL range. This reduced sensitivity may be attributed to a variety of factors, such as nonspecific binding with serum/blood proteins or nanoshell/antibody degradation via blood-borne proteolytic enzymes. Viscosity is another likely contributor. Blood plasma/serum is about 1.8 times as viscous as water, whereas whole blood can be up to 8 times as viscous. Since a nanoparticle's Brownian motion is inversely proportional to the medium's viscosity, fluids with greater viscosity decrease particle velocity and, therefore, reduce the rate of nanoparticle–nanoparticle interactions, which are critical to inducing aggregation. Despite the reduced performance in serum and whole blood, all three assays performed quite well, resulting in a simple, quantitative homogeneous assay capable of detecting over the range of 0.8–88 ng/mL analyte in saline, serum, and 20% whole blood within 10–30 min. To our knowledge, this is the first *in situ* whole blood immunoassay to report sensitivities on this order in under 30 min.

To demonstrate both the inter- and intra-assay reproducibility of this system, fresh reagents were prepared, and the assay above was repeated in saline at week 0 and week 1. Samples were stored at 4 °C between experiments. After 1 week in storage, the nanoshell suspension resuspended easily upon vortexing; however, some extinction signal was lost (~12%) over this period, resulting in an 11% reduction in the slope over the 4.4–88 ng/mL range. This may be due to nonspecific aggregation upon settling or antibody destabilization, since no preservatives, such as thimerosal, sodium azide, or glycerol, were used during storage. Despite a loss in slope, the assay still performed well after 1 week, retaining the same detection limits and quantitatively detecting IgG over the linear region between 88 and 4.4 ng/mL (Figure 5).

The findings above demonstrate the intra-assay reproducibility of the antibody/nanoshell conjugates over extended periods of time. In addition, we can examine interassay reproducibility by comparing the performance of two separate experiments, such as those of Figures 4 and 5. Signal intensities were ~19% less in the studies of Figure 5 with an 11% decrease in sensitivity over the 88–4.4 ng/mL region, but such deviations are expected. Differences in pH, nanoshell surface chemistry, antibody activity, temperature, and numerous other factors may contribute to differences in assay performance from batch to batch. This is not an absolute method of measurement. Like any other immunoas-

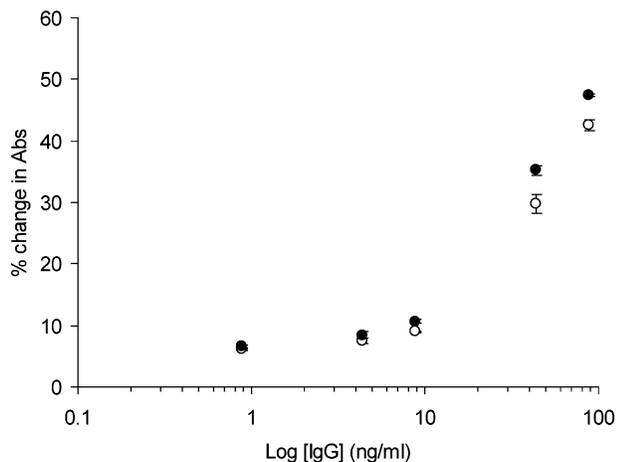


Figure 5. Detection of IgG analyte at day 0 (●) and day 7 (○) in saline.

say, standards would need to be run in conjunction with chemometric analysis to compensate for these deviations in order to make this assay a useful quantitative tool. These are necessary measures required for every immunoassay and do not compromise the simplicity of its design.

CONCLUSION

This new immunoassay shows promise in meeting the strong demand for a simple, rapid, in situ whole blood immunoassay. The simple thiol-mediated assembly of OPSS-PEG-antibodies onto nanoshells could be extended to any number of antibody/analyte

immunoassay systems, creating a robust immunoassay capable of detecting a variety of clinically relevant blood-borne analytes. Furthermore, the simplicity of this assay (mix, wait, and measure extinction) requires less technical proficiency than conventional ELISAs and LA assays and reduces the risk of experimentation error. Assay performance was comparable to conventional immunoassays, and analyte detection proceeded in a quantitative fashion over the range of 88–0.8 ng/mL within 10–30 min. In addition to low variances within each experiment, these assays also displayed good reproducibility after storage for 1 week (longer storage time have not been investigated). This demonstrates that, in the absence of analyte, these colloidal suspensions not only maintain stability in solution, but also retain their activity for analyte in the high saline and protein environments of serum and whole blood. Only after analyte addition does rapid aggregation kinetics take hold. Hence, we have demonstrated a simple rapid immunoassay capable of detecting picogram-per-milliliter quantities of various analytes in different media, using spectrophotometry—a simple, low-cost method of detection.

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