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Whole-Blood Immunoassay Facilitated by Gold Nanoshell–Conjugate Antibodies

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Summary

In this chapter, we outline a simple procedure using gold nanoshells as a substrate for an immunoassay that is capable of detecting subnanogram levels of analyte within whole blood on the order of minutes. Unique to metallic nanoshells is their optical tunability over a large range of wavelengths. We describe the design of nanoshells that attenuate light strongly in a region of light where blood does not (i.e., the near infrared [IR]), permitting optical detection of nanoshells in whole blood. We also describe a procedure to monitor the analyte-induced aggregation of antibody-conjugated nanoshells in whole blood using near-IR light. The immunoassay is fast and specific, requires no separation/purification steps, is simple to perform (mix and sit), and uses common laboratory equipment for detection (spectrophotometer). Preparation of the antibody-nanoshell conjugates is described, along with the design and optimization of the whole-blood nanoshell-based immunoassay system.

Key Words

Nanoshells; immunoassay; whole blood; silica cores; gold colloid.

1. Introduction

We describe a procedure using metal nanoshell technology for a new immunoassay capable of detecting analyte in whole blood with minimal sample preparation that is completed on the order of minutes ***(1)***. To date, there are very few homogeneous immunoassays capable of performing in whole blood. Existing methods often employ the agglutination of sensitized erythrocytes, which, although effective, are limited in scope owing to the difficulty of sensitizing erythrocytes. The method described herein is simple; is capable of incorporating a variety of antibody/analyte pairs; and could be extended into areas

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such as public health or point-of-care applications where there is a strong demand for the rapid, high-throughput screening of blood-borne species.

Silica-gold nanoshells are a subclass of a larger family of metal nanoshells that consist of layered, spherical nanoparticles possessing a dielectric core (silica) surrounded by a thin metal shell (gold) ***(2)***. These nanoparticles possess a plasmon resonance that gives rise to intense optical absorption and scattering. The unique property of nanoshells is that their plasmon resonance—also their absorption resonance—is tuned by changing the relative dimensions of the core and shell, permitting peak optical extinctions spanning the visible to the mid-infrared (IR) region of the spectrum ***(3)***. The application outlined in this chapter hinges on the ability to fabricate nanoshells with intense attenuation in the near-IR (700–1300 nm), a region of light where blood provides strong optical transmission owing to its deficiency of near-IR chromophores. This situation permits the photometric detection of nanoshells in whole blood.

Development of the immunoassay begins with assembly of antibodies onto the nanoshell surfaces, forming antibody-nanoshell immunoconjugates. Then, in a manner similar to latex particle agglutination, nanoshell conjugates are placed in an analyte-containing solution, where multiple particles bind to the multivalent analyte, causing agglutination of the particles. However, unlike latex particles, the optical resonance of nanoshells is strongly modified by an additional optical absorption at longer wavelengths just as aggregation commences. This phenomenon leads to a reduction in the original near-IR extinction of the nanoshell solution (**Fig. 1**). We have reported that, over the appropriate concentration ranges, analyte induces aggregation of the particles in a concentration-dependent fashion, leading to a concentration dependent reduction in near-IR extinction, observable by photometry ***(1)***. This method provides an easy immunoassay; simply mix the nanoshell immunoconjugates with the blood sample, let them sit, and measure the extinction. A sensitive and quantitative assay is possible in approx 5 min.

2. Materials

1. Tetrakis(hydroxymethyl)phosphonium chloride (THPC) (Aldrich).
2. Chloroauric acid ($HAuCl_4$) (Aldrich).
3. Tetraethyl orthosilicate (TEOS) (Aldrich).
4. Polystyrene particles (1 μm) (2 wt%) (Aldrich).
5. Formaldehyde.
6. Antibody (analyte specific, free of stabilizing proteins, stored in amine-free buffer).
7. Analyte standards.
8. Whole-blood specimen (heparinized, 10 U/mL).
9. Whole-blood standard (heparinized, 10 U/mL, analyte free).
10. Silica-gold nanoshells (720-nm resonant, 156-nm total diameter).

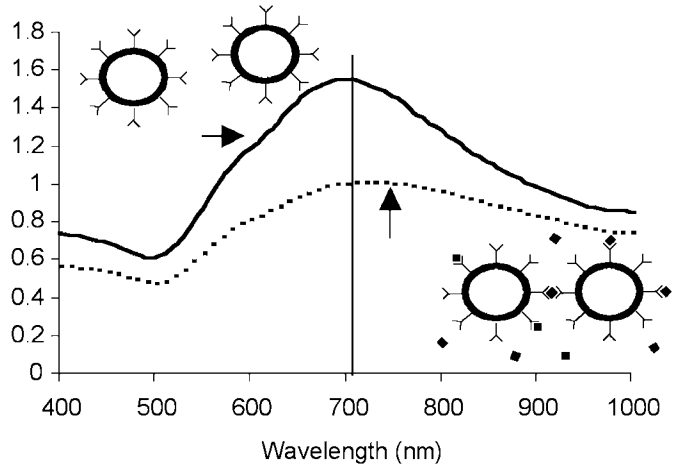

Fig. 1. Well-dispersed antibody-nanoshell conjugates in the absence of analyte possess a well-defined extinction peak in the near-IR. In the presence of the complementary analyte, multiple nanoshells bind to the analyte, causing agglutination and a corresponding reduction in the extinction peak.

11. Deionized (DI) water, 18.2 mΩ-cm purity.
12. 2-Iminothiolane (Traut's Reagent; Sigma, St. Louis, MO).
13. 5,5′-Dithio-*bis*(2-nitrobenzoic acid) (Ellman's Reagent; Sigma).
14. Polyethylene glycol (PEG)-amine (mol wt = 5000; Shearwater Polymers).
15. Dialysis membrane (500 mol wt cutoff; Spectrum).
16. Orthopyridyl disulfide-polyethylene glycol-*N*-hydroxysuccinimide (OPSS-PEG-NHS) (mol wt = 2000; Shearwater Polymers).
17. 100 m*M* Phosphate-buffered saline (PBS) (pH 7.4).
18. 1.8 m*M* Potassium carbonate buffer.
19. 10 m*M* Sodium bicarbonate buffer (pH 8.5).
20. Lysis buffer: 8 mg/mL HEPES, 26.7 mg/mL NaCl, 4.4 mg/mL EDTA, 72 mg/mL β-glycerophosphate, 6.7% Triton X.
21. X10 Protease inhibitor cocktail (Sigma).

3. Methods

3.1. Fabrication of Silica-Gold Nanoshells

The following protocol describes the fabrication of a 720-nm resonant silica-gold nanoshell with a 106-nm-diameter core and a 25-nm-thick gold shell using methods outlined by Oldenberg et al. ***(3)*** (*see* **Note 1**). The procedure begins with the growth of the silica cores. The silica particles are then functionalized with amine groups so that when the aminated particles are placed in a gold colloid solution, the colloid adsorbs to the aminated silica surface. The

gold colloid serves as nucleation sites for further electroless plating of gold. During this procedure, the gold colloid grows on the silica surface. Eventually, the gold particles begin to coalesce, forming a complete metal shell. Although this fabrication step is somewhat involved, a batch of nanoshells and its precursors are stable in solution for months, if not years, and therefore do not require recurrent synthesis. However, if desired, this step can be averted; the nanoparticles may be purchased from Nanospectra, L.L.C., Houston, TX.

3.1.1. Growth of Gold Colloid

Growth of gold colloid is performed using the method of Duff and Baiker ***(4)***. Because it must be done at least 3 wk in advance of nanoshell growth, it should be performed first.

1. In a clean 250-mL beaker, add 180 mL of DI water and 1.2 mL of 1 *M* NaOH.
2. In a separate container, make a stock solution of 400 µL of THPC in 33 mL of DI water.
3. Add 4 mL of this THPC solution to the NaOH. Stir the NaOH and THPC for 5 min.
4. Prepare a solution of 1% $HAuCl_4$. The solution must age at least 3 d.
5. To the NaOH/THPC solution, quickly add 6.75 mL of the $HAuCl_4$ under rapid vortexing. The color should instantly change to a light/midbrown. Age for 3 wk at 2–4°C. Before use the colloid needs to be concentrated 10- to 20-fold using a rotovap. This can be done either before or after aging.

3.1.2. Growth of Silica Cores

Growth of the 106-nm silica cores is done via the Stöber method ***(5)***, in which TEOS (Aldrich) is reduced in NH_4OH in ethanol. This procedure is moisture sensitive and, for best results, should be done under inert gas.

1. In a 50-mL beaker, mix 45 mL of ethanol with 2.8 mL of concentrated NH_4OH (14.8 *N*).
2. While vortexing, add 1.5 mL of TEOS. React for at least 8 h.
3. Size the particles via electron microscopy. For this application, nanoshells should possess a diameter of 106 ± 10 nm or better (*see* **Notes 2** and **3**).

3.1.3. Amination of Silica Cores

The silica cores must be functionalized with amine groups (aminated) to serve as adsorption sites for the gold colloid. Amine groups are incorporated onto the silica surface using aminopropyltriethoxy silane (APTES) as described next.

1. While vortexing the silica solution from **Subheading 3.1.2.**, add 126 µL of APTES. This is enough APTES to assemble 10 monolayers onto the particles.
2. Let react for at least 8 h.

3.1.4. Rinsing of Cores

1. Boil the aminated cores for 2 h, adding additional ethanol as necessary to maintain a 50-mL volume.
2. Centrifuge the cores at 2000*g* for 30 min. Discard the supernatant and resuspend the pellet in 50 mL of ethanol using probe sonication for at least 5 min.
3. Repeat **step 2** two more times but resuspend the aminated cores to a total volume of 10 mL during the last centrifugation (instead of 50 mL).

3.1.5. Coating of Cores With Gold Colloid

1. Determine the final concentration of aminated silica cores using the EM latex technique as follows. Mix silica particles with 1-µm polystyrene particles in DI water. Using a hemacytometer, count the polystyrene microparticle concentration in the mixture. Using scanning electron microscopy, measure the ratio of aminated cores to microparticles. Determine the aminated particle concentration by multiplying the microparticle concentration by (aminated particles/microparticles). Record the silica concentration.
2. In a 50-mL centrifuge tube, add 1×10^{12} aminated cores in ethanol.
3. Add excess gold colloid to the aminated particles. To determine the amount needed, calculate the amount of gold colloid needed to cover >150% of the silica particle surface area. For 3 nm of gold colloid, this would be $>7.5 \times 10^{15}$ particles. If the colloid were concentrated 10-fold in **Subheading 3.1.1.**, then the concentration would be about 7.5×10^{15}/mL. In this case, one would add at least 1.0 mL of gold colloid to the solution in **step 1**. Mix thoroughly. React for at least 8 h.
4. Remove excess gold colloid by first suspending the solution to 40 mL in DI water, then centrifuging at 2000*g* for 30 min. Discard the supernatant, and resuspend via probe ultrasound into 40 mL of DI water. Repeat two more times.

3.1.6. Growth of Nanoshells

1. At least 16 h prior to growth of nanoshells, prepare 200 mL of 1.8 m*M* K_2CO_3 supplemented with 3 mL of 1% $HAuCl_4$.
2. In ultraviolet-visible (UV-vis) cuvets, mix the $HAuCL_4$ from **step 1** and the silica-gold colloid suspension (**step 4** in **Subheading 3.1.5.**) at different volumetric ratios of $HAuCl_4$:silica-gold colloid (e.g., 20:1, 10:1, 5:1).
3. Add 20 µL of 30% formaldehyde to each solution. Mix thoroughly. Solutions should change color from a light red to a blue/green. The reaction should reach completion after 5–10 min.
4. Record the ratio of $HAuCl_4$:silica-gold colloid that grows a nanoshell with a peak extinction at 720 nm (*see* **Fig. 1**).
5. In a clean beaker, scale up the ratio from **step 2** to make 200 mL of nanoshells.
6. After growth, centrifuge the nanoshells once at 650*g* for 12 min. Discard the supernatant and resuspend in a solution of 1.8 m*M* K_2CO_3 at a concentration that provides an equivalent absorbance of 12.0 at 720 n*M* with a 1.0-cm path length (A^{720}) (*see* **Note 4**).

3.2. Binding of Antibodies to PEG Tethers

Antibodies are tethered to nanoshell surfaces using PEG linkers (*see* **Note 5**). These linkers are a heterobifunctional PEG compound. On one end of the PEG is an NHS that is used to couple the PEG to the antibody's amine residues, forming an amide linkage. On the other end is an OPSS whose sulfur groups bind strongly to the gold surface of the nanoshell. The use of PEG linkers serves two purposes: (1) it provides an easy method of self-assembly of antibodies onto the gold surface, for the gold-sulfide bond is much stronger than the electrostatic forces that drive protein-gold chemisorption; and (2) it helps lift the antibody off of the surface, permitting greater conformational freedom of the molecule and therefore improving antibody binding. All preparations and procedures in this section should be performed on ice.

1. Suspend antibody in 10 m*M* sodium bicarbonate (pH 8.5) at a concentration between 1 and 10 mg/mL. Antibody solution should be free of stabilizing proteins (i.e., no bovine serum albumin or other serum proteins). Additional protein will compete for binding sites onto the gold nanoshell surface and reduce the surface density of antibody on the antibody-nanoshell conjugates.
2. Prepare OPSS-PEG-NHS at a 10-fold excess concentration of the antibody in 10 m*M* sodium bicarbonate (average mol wt of IgG is ~150,000). Beware that NHS groups have a very short half-life (~20 min) in an aqueous environment and, thus, should be reacted with the antibody as quickly as possible on suspending in buffer.
3. Add 1 part OPSS-PEG-NHS to 9 parts antibody. Vortex, and let sit for >2 h.
4. Store OPSS-PEG-antibody in frozen working aliquots.

3.3. Synthesis of PEG-Thiol

PEG-thiol (PEG-SH) is a linear chain molecule capable of assembling into a densely packed monolayer on the gold nanoshell surface via the thiol residue. By occupying any remnant sites on the nanoshell surface left unoccupied by OPSS-PEG-antibody, this molecule helps both to deter nonspecific protein adsorption onto the metal surface and to stabilize sterically the nanoshells in a complex saline environment such as whole blood. Although PEG-SH is commercially available, this compound can be made from a PEG-amine precursor using simple chemistry at a considerably lower cost (*see* **Note 6**).

1. Prepare a 1 m*M* PEG-amine solution in 100 m*M* PBS.
2. Prepare Traut's Reagent at 10 m*M* in DI.
3. Mix equal parts of PEG-amine and Traut's Reagent.
4. Vortex, and react for 1 h at room temperature.
5. Dialyze the product against DI water for at least 2 h using a dialysis membrane with a molecular weight cutoff between 500 and 1000. The dialysate should be changed at least three times. Store produce in frozen aliquots at less than 0°C.

3.4. Fabrication of Antibody-Nanoshell Conjugates

Fabrication of antibody-nanoshell conjugates involves two steps: (1) determining the optimal concentration of PEG-SH necessary to stabilize sterically nanoshells in a saline environment, and (2) assembly of OPSS-PEG-antibody onto the nanoshells at the optimal concentration.

3.4.1. Determining Optimal PEG-SH Concentration to Stabilize Nanoshells in Saline

1. Serially dilute the PEG-SH prepared in **Subheading 3.3.** over a range of 1:1–1:1000 in DI water.
2. In a standard 1-cm UV-vis cuvet, add 50 µL of each PEG-SH dilution to 450 µL of nanoshells from **Subheading 3.1.6.**
3. Make two additional control samples that contain 50 µL of DI water and 450 µL of nanoshells. Mix all samples thoroughly and let sit for 1 h.
4. Add 55 µL of 10% NaCl to each sample from **step 2**, and to one of the samples in **step 3**. To the other sample in **step 3**, add 55 µL of DI water. Let sit for 30 min. The NaCl will aggregrate nanoshells possessing insufficient quantities of the sterically stabilizing PEG-SH.
5. Add an additional 3.0 mL of DI water to each sample. Mix well and record the A^{720}.
6. Report the data as the percentage decrease in absorbance from the control in **step 4** possessing no NaCl and no PEG-SH. On examining the results, there should be a PEG-SH threshold concentration at which a reduction in PEG-SH results in a dramatic decrease in absorbance. This is the cutoff concentration below which the PEG-SH is too low to stabilize the nanoshells (*see* **Note 7**). Hence, the optimal concentration for PEG-SH stabilization is the concentration just above this threshold that stabilizes the nanoshells in NaCl. Record this value.

3.4.2. Assembly of OPSS-PEG-Antibody Onto Nanoshells

For a given concentration of analyte and nanoshells, there is an optimal concentration of antibody on the nanoshells that will promote aggregation in a concentration-dependent fashion. It is the purpose of this section to determine that optimal value.

1. Prepare different concentrations of PEG-antibody in DI water, ranging between 100 and 1 µg/mL (e.g., 100, 50, 10, 5, 1 µg/mL).
2. For each concentration of PEG-antibody, add 200 µL of antibody to 1.8 mL of nanoshell solution. Vortex. One hour is sufficient time for completion of antibody-nanoshell conjugation.
3. To each sample in **step 2**, add 222 µL of PEG-SH at the optimal concentration determined in **step 6** of **Subheading 3.4.1.**
4. Prepare the analyte standard solutions. These solutions should be prepared over a concentration range that spans the expected concentration range of the unknown

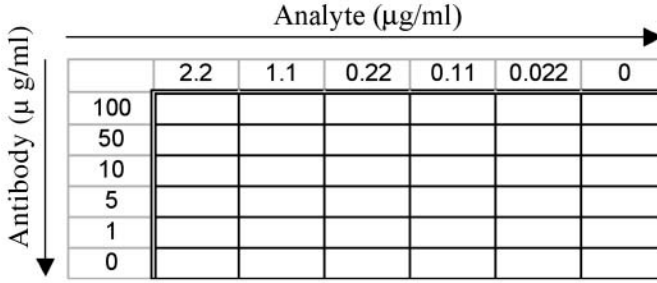

Fig. 2. Diagram of 2D matrix for determining optimal antibody and analyte concentration for maximal agglutination.

samples. For instructional purposes, suppose that the physiological range of interest for the analyte under investigation is 2.2–0.022 µg/mL. A reasonable range of standards would then be 2.2, 1.1, 0.22, 0.11, 0.022, and 0 µg/mL in 100 m*M* PBS. This will be the range used throughout the rest of the protocol.

5. Set up a two-dimensional (2D) matrix of cuvets, such that there is a a cuvet for each antibody-nanoshell and analyte pair (*see* **Fig. 2** and **Note 8**).
6. In each row, add 252 µL of nanoshells at the appropriate antibody concentration as outlined in **step 5**.
7. Add 420 µL PBS to each cuvet. This step places the antibody-nanoshell conjugates in a physiological buffer, a step that is necessary to promote optimal antibody activity. Doing so prior to assembly of OPSS-PEG-antibody and PEG-SH would cause premature aggregation of the particles.
8. Record the A^{720} for each cuvet. This is the absorbance at $t = 0$.
9. Add 28 µL of analyte of appropriate concentration to each sample in the matrix, paying careful attention to the time when the analyte was added to each sample.
10. Record the decrease in absorbance over time (e.g., 10, 30, 60 min).
11. By plotting the percentage decrease in absorbance from $t = 0$, there should be an optimal antibody concentration and time point that provides a quantitative, log-linear reduction in absorbance over the concentration range of interest. Record this concentration and the elapsed time.

3.5. Whole-Blood Immunoassay

After optimization of the assay in saline, the system is ready to be performed in whole blood. This assay is very similar in design to the saline assay performed in **Subheading 3.4.**, with a few minor adjustments of volume and time that are necessary because the whole-blood specimens already contain the analyte and are not spiked (*see* **Note 9**).

3.5.1. Preparation of Reagents and Samples

The optimal preparation of antibody-nanoshell conjugate was determined in **Subheading 3.4.2.** The same preparation is used in the whole-blood assay;

steps 1 and **2** from **Subheading 3.4.3.** therefore need to be repeated and scaled up. To determine the degree of scale-up, consider that assaying one blood sample will require about 3 mL of nanoshells (this includes all standards and controls). Suppose that only one blood sample requires analysis. This would require 3 mL of nanoshells/assay × 1 assay = 3 mL of antibody-nanoshells. The original protocol outlined in **steps 2** and **3** from **Subheading 3.4.2.** makes 2.222 mL of antibody-nanoshells. Therefore, a scale-up of twofold would prepare sufficient amounts of conjugate (4.444 mL).

1. In 100 m*M* PBS, serially dilute a standard solution of analyte, spanning the range of interest for the assay (e.g., 2.2, 1.1, 0.22, 0.11, 0.022, 0 μg/mL). This is the same preparation as that in **step 4** of **Subheading 3.4.2.**
2. Collect the blood. This procedure requires two blood specimens. In tube 1, collect the analyte-free sample of blood for the standards, and in tube 2 collect the patient's specimen containing analyte of unknown concentration. Both samples should be freshly drawn and heparinized (10 U/mL).
3. Prepare lysis buffer working solution by mixing 1.5 mL of lysis buffer with 1.0 mL of protease inhibitor cocktail.
4. Lyse both the analyte-free blood and the blood specimen by mixing 3.75 mL of the whole blood from **step 3** with 1.25 mL of lysis buffer working solution. Vortex until the blood is completely lysed, changing from a turbid solution to a clear red (about 1 min). After lysis, add an additional 6.25 mL of 100 m*M* PBS to each blood specimen.

3.5.2. Running of Standards

1. Zero the spectrometer using a solution of 280 μL of PBS and 420 μL of blood lysate from tube 1.
2. Set up 1 cuvet for each standard. To each of these, add 252 μL of nanoshells and 420 μL of the blood lysate from tube 1.
3. Add 28 μL of the corresponding standard to each cuvet. Mix thoroughly.
4. Measure the A^{720} precisely 20 s after mixing each sample. Then record the A^{720} at the optimal time of completion, as determined in **step 11** from **Subheading 3.4.2.**

3.5.3. Running of Samples

1. Zero the spectrometer using a solution of 280 μL of PBS and 420 μL of blood lysate from tube 2.
2. For each sample, add 420 μL of blood lysate from tube 2 to a cuvet containing 28 μL of PBS. This should be done in triplicate ($n = 3$).
3. Add 252 μL of nanoshells to each cuvet. Measure the A^{720} precisely 20 s after mixing each sample. Then record the A^{720} at the optimal time of completion, as determined in **step 11** of **Subheading 3.4.2.**

3.5.4. Analysis

1. Prior to setting up a standard curve from the data in **Subheading 3.5.2.**, divide all standard concentrations by 5 (i.e., 2.2, 1.1, . . . μg/mL now becomes 0.44,

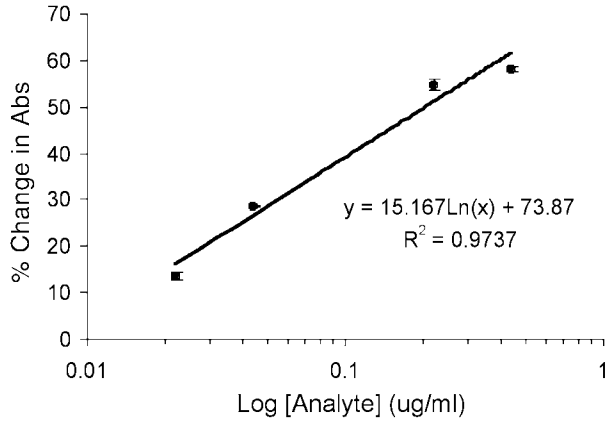

Fig. 3. Standard curve depicting log-linear behavior of antibody-nanoshell agglutination in presence of analyte.

0.22, . . .). Doing so accounts for the dilutions and volumes used in this study. The standard data now represents the original concentration of analyte in the 100% whole-blood specimen.

2. Fit a logarithmic curve to the data in **Subheading 3.5.2.** (*see* **Fig. 3**).
3. Using this trend, extrapolate the original sample concentrations in **Subheading 3.5.3.**

4. Notes

1. Although these particles are resonant at 720 n*M* and perform well in whole blood, nanoshells with resonances anywhere between 700 and 900 n*M* should also perform well in whole blood.
2. This protocol outlines one specific recipe for a 106-n*M* core; however, a core diameter ranging anywhere between 95 and 110 n*M* would suffice. What is more important is that the particles be monodisperse; that is, their standard deviation should be <10% of the particle diameter.
3. Results from this procedure are highly sensitive to reagent volumes. Increasing amounts of either NH_4OH or TEOS typically produce larger particles. In addition, improved results can be achived if the TEOS is distilled prior to reacting.
4. Most UV-vis spectrometers are incapable of detecting an absorbance beyond 2.0. To make this measurement, a fraction of the solution is removed and diluted 10-fold prior to measurement. The stock solution concentration is then adjusted until the 10-fold dilution reads 1.2.
5. When choosing the appropriate antibody for this assay, one needs to be mindful of whether the antibody is polyclonal or monoclonal. Although monoclonal antibodies (MAbs) are typically more specific, they only bind to one epitope. Hence, two separate MAbs will have to be used, with each binding to a separate epitope on the analyte. Using a polyclonal antibody, however, requires only one preparation.

6. A number of different molecular weight PEG compounds have been examined to assess nanoshell stability in a saline environment. Studies to date have shown that PEG with a mol wt of <5000 fails to stabilize the nanoparticles. Although larger PEG compounds (10,000 and 20,000) also stabilize the nanoshells, they are not recommended for this application. Larger PEG chains will sterically deter antibody-antigen binding on the nanoshell surface.
7. The use of excessive quantities can also promote nanoshell aggregation, a phenomenon that may be owing to steric exclusion forces.
8. This experiment is designed such that it can be performed in a standard 1-cm cuvet, which, although available in most laboratories, requires large volumes of sample and nanoshells. It should, however, be possible to perform this assay on a much smaller scale (on a 96-well tray perhaps), permitting rapid, high-throughput screening of multiple samples.
9. **Suheading 3.5.1., step 1** can be performed well in advance of the whole-blood immunoassay. Preliminary studies show that antibody-nanoshell conjugates can be stored for weeks with minimal loss of activity (in the absence of preservatives). The purchase of, or advance preparation of, the conjugates makes the remaining whole-blood assay (**Subheading 3.5.**) a simple technique capable of completion within 1 h.

Acknowledgments

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