Video Article

Fabricating a UV-Vis and Raman Spectroscopy Immunoassay Platform

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Abstract

Immunoassays are used to detect proteins based on the presence of associated antibodies. Because of their extensive use in research and clinical settings, a large infrastructure of immunoassay instruments and materials can be found. For example, 96- and 384-well polystyrene plates are available commercially and have a standard design to accommodate ultraviolet-visible (UV-Vis) spectroscopy machines from various manufacturers. In addition, a wide variety of immunoglobulins, detection tags, and blocking agents for customized immunoassay designs such as enzyme-linked immunosorbent assays (ELISA) are available.

Despite the existing infrastructure, standard ELISA kits do not meet all research needs, requiring individualized immunoassay development, which can be expensive and time-consuming. For example, ELISA kits have low multiplexing (detection of more than one analyte at a time) capabilities as they usually depend on fluorescence or colorimetric methods for detection. Colorimetric and fluorescent-based analyses have limited multiplexing capabilities due to broad spectral peaks. In contrast, Raman spectroscopy-based methods have a much greater capability for multiplexing due to narrow emission peaks. Another advantage of Raman spectroscopy is that Raman reporters experience significantly less photobleaching than fluorescent tags[1]. Despite the advantages that Raman reporters have over fluorescent and colorimetric tags, protocols to fabricate Raman-based immunoassays are limited. The purpose of this paper is to provide a protocol to prepare functionalized probes to use in conjunction with polystyrene plates for direct detection of analytes by UV-Vis analysis and Raman spectroscopy. This protocol will allow researchers to take a do-it-yourself approach for future multi-analyte detection while capitalizing on pre-established infrastructure.

Video Link

The video component of this article can be found at https://www.jove.com/video/54795/

Introduction

Typical sandwich immunoassays indirectly detect the presence of an antigen using two antibodies. The capture antibody is bound to a solid surface and forms an antibody-antigen complex when in proximity to an appropriate antigen. A detection antibody is then introduced and binds to the antigen. After washing, the antibody/antigen/antibody complex remains and is detected by the labeled detection antibody as demonstrated in **Figure 1A**. Typical detection is done by a fluorescent or colorimetric detector, limiting multiplexing to 10 analytes due to broad spectral peaks[2,3]. In contrast, Raman-based systems have much narrower emission peaks resulting in enhanced multiplexing capabilities with sources claiming simultaneous detection of up to 100 analytes[2,3].

Many literature sources are available which cover important aspects related to immunoassays[4-6] such as step-by-step details to create personalized ELISA kits. Unfortunately, these protocols are for fluorescent or colorimetric detection, limiting multiplexing capability of customized immunoassays. To address this need, we present a detailed procedure to fabricate the UV-Vis/Raman immunoassay published previously[7] for a direct immunoassay as illustrated in **Figure 1B**.

This protocol includes the fabrication of functionalized gold nanoparticle-based probes, illustrated in **Figure 2**. The procedure to make the Raman/UV-Vis probes begins by binding Raman reporters to the surface of gold nanoparticles (AuNPs). The AuNPs are then functionalized with antibodies that are associated with polyethylene glycol (PEG). Remaining binding sites on the AuNPs are blocked by binding methoxy polyethylene glycol thiol (mPEG-SH) to AuNPs to prevent subsequent non-specific binding during analysis. The prepared AuNP probes are tested by binding to antigens fixed to the wells of a polystyrene plate as illustrated in **Figure 1B**. Upon washing the plate, the AuNP probes are detected using UV-Vis spectroscopy while the associated Raman reporters are detected with Raman spectroscopy. Combining UV-Vis and Raman spectral data provides two methods of analyses, enhancing the capabilities of this immunoassay.

Protocol

1. Preparation of Buffers

1. **Phosphate Buffered Saline (PBS)**
   1. Dilute 50 ml of 10x PBS with 450 ml HPLC grade water to make a 1x PBS concentration. Sterile filter the solution with a 0.22 µm filter.
   2. Store solution at room temperature.

2. **Preparation of Tris Buffered Saline + Tween 20 (TBST)**
   1. Dilute 50 ml of 10x Tris Buffered Saline (TBS) with 450 ml HPLC grade water to make a 1x concentration. Add 250 µl of Tween-20 for a 0.05% (v/v) of Tween-20. Sterile filter the solution with a 0.22 µm filter.
   2. Store at room temperature.

3. **Preparation of Human Serum Albumin (HSA) Blocking Solution**
   1. Weigh 0.45 g of HSA into 15 ml of sterile filtered 1x PBS to make a 3% w/v HSA solution. Vortex solution until HSA is fully dissolved.
   2. Store HSA solution at 4 °C.
      NOTE: Bovine Serum Albumin (BSA) can also be used as a blocking solution.

4. **Preparation of PEGylated antibody (PEG-Ab) solution**
   NOTE: The antibody solution must be free from carrier or stabilizing proteins such as BSA, which would interfere with conjugation reactions by competing for the n-hydroxysulfosuccinimide (NHS) binding sites. If the antibody comes in a Tris or glycine buffer solution, it must undergo a buffer exchange to prevent amines or ammonium salts from interfering with the NHS conjugation reaction. If the antibody is in a lyophilized form, it can be resuspended according to the manufacturer's recommendation at a concentration of 1-10 mg/ml.
   1. For antibodies in a Tris or glycine buffer, perform a buffer exchange to 100 mM sodium bicarbonate using a desalting column. Use the 100 mM buffer to raise the pH to approximately 8.5 to speed up the conjugation reaction.
   2. Hydrate ortho-pyridyl disulfide-PEG-NHS (OPSS-PEG-NHS) with 100 mM sodium bicarbonate to a volume of 1 ml at a concentration of 1 mg/ml or greater.
      NOTE: OPSS-PEG-NHS should be made fresh and used within approximately 20 min. The NHS group on the OPSS-PEG-NHS has a half-life of approximately 20 min in an aqueous solution at pH 8.5.
   3. Add OPSS-PEG-NHS to the antibody solution at a 2:1 ratio (PEG: Antibody) conjugation ratio to be used for the test samples. In a separate microcentrifuge tube, add OPSS-PEG-NHS to the antigen solution at a 2:1 conjugation ratio to be used for the control.
      NOTE: The 2:1 ratio is assuming a 50% conjugation efficiency. The objective is to label each antibody with one PEG chain. In this step, over-labeling is better than under-labeling. Use the following equation to determine the appropriate volumes of OPSS-PEG-NHS and antibody solution:
      $$2 = \frac{V_{PEG}}{V_{Ab}} \frac{C_{PEG}}{C_{Ab}}$$
      where *V* is volume, *C* is concentration expressed in molecules or antibodies per ml. Subscripts *PEG* and *Ab* are OPSS-PEG-NHS and antibody, respectively. The final volume should be approximately 250 µl.
   4. Incubate PEG-Ab solution at 4 °C for 8 hr or overnight. Store solution in working aliquots of approximately 25 µl at -20 °C to limit the freeze thaw cycles and make sure to use low binding tubes.

2. Prepare UV-Vis/Raman Probes

1. **Prepare bare AuNP solution**
   1. Prepare a 2 ml solution of AuNPs with a concentration of approximately 1 x 10^{11} particles per ml.
      1. If the AuNPs need to be concentrated, fill low binding centrifuge tubes with 2,000 µl of stock AuNP and centrifuge at 5,000 x g for 20 min or until the supernatant is clear. Remove the supernatant by pipetting, being careful not to disturb the AuNP pellet.
      2. Combine the remaining AuNP solutions into one tube and estimate the concentration by obtaining a UV-Vis measurement and comparing values to known concentrations as this is a linear relationship.

2. **Determine the appropriate Raman reporter labeling ratio**
   1. Prepare a working solution of the Raman reporter dissolved in methanol. This concentration will be dependent on the reporter used. In this work, prepare 3,3′-diethylthiatricarbocyanine iodide (DTTC) at a working solution of 200 µM.
   2. Assuming a final volume of 100 µl for each well, add enough of the working reporter solution to each well of the first row of a 96-well plate such that the Raman reporter will range in concentrations from 0.2 µM to 10 µM. Add enough HPLC grade water to each well such that the volume is 80 µl. Add 20 µl of AuNP to each well making a final volume of 100 µl for each well. An example is provided in **Table 1**.
   3. Measure the UV-Vis spectra from 400 to 700 nm using a plate-reading UV-Vis spectrophotometer. The appropriate concentration is the highest concentration with defined peaks for the UV-Vis spectra. Repeat step 2.2.2 at increasing concentrations until the highest concentration ratio of Raman reporters to AuNPs is found.
      NOTE: The dye and the AuNP shape, size, and manufacturer influence the appropriate concentration. Therefore, the steps listed must be evaluated and altered depending on the components used. This protocol involved the use of a positively charged dye. As such, binding between the AuNP and reporter was improved by using negatively charged AuNPs. This was done by using citrate capped AuNPs. See the Discussion section for further details.

3. **Binding Raman reporter and PEG-Ab to AuNP**
    1. Prepare two 1.5 ml batches of AuNP and Raman reporter at the previously determined concentration, allowing the Raman reporter to bind to the AuNPs for 30 min at room temperature.
    2. Add the PEGylated antibody (PEG-Ab) to one batch of the AuNP and Raman reporter solution to create a 200:1 ratio of antibodies to particles. This solution will be for the test samples. In a separate microcentrifuge tube, add the PEGylated antigen to the other batch of the AuNP and Raman reporter solution at a 200:1 ratio of antibody to particles to be used as the control. Incubate the solutions for 30 min at room temperature.
    NOTE: The ratio of antibodies to particles will be specific to the AuNPs and dye used and should be optimized for each individual case. The objective here is to have the highest ratio of antibodies for the AuNP probes to bind to while preventing aggregation of the particles. Use the following equation to determine the appropriate volumes to add together:
    $$200 = \frac{V_{Ab}}{V_{AuNP}} \frac{C_{Ab}}{C_{AuNP}}$$
    where *V* is volume, *C* is concentration expressed in particles or antibodies per ml. The final volume should be approximately 1.5 ml.

4. **Block remaining sites on the AuNP surface with mPEG-SH.**
    1. Prepare mPEG-SH by dissolving solid methoxy polyethylene glycol thiol to a 200 µM concentration using water. Vortex the solution until mPEG-SH is completely dissolved.
    2. Add mPEG-SH at a 40,000:1 ratio to the AuNP-PEG-Ab solution made in step 2.3. Incubate the solution at room temperature for 10 min to ensure the remaining sites on the gold nanoparticle are blocked. Use the following equation to determine the appropriate volumes to add together:
    $$40{,}000 = \frac{V_{SH-PEG}}{V_{AuNP-PEG-Ab}} \frac{C_{SH-PEg}}{C_{AuNP-PEG-Ab}}$$
    where *V* is volume, *C* is concentration expressed in particles or antibodies per ml. The final volume should be approximately 1.5 ml.

5. **Recover functionalized Raman probes.**
    1. Centrifuge particles at 5,000 x g for 20 min in low bind centrifuge tubes or until the supernatant is clear. Remove the supernatant by pipetting being careful not to disturb the AuNPs.
    2. Resuspend the particles with 1 ml of 1x PBS solution that was made previously. Estimate the AuNP concentration by taking a UV-Vis measurement of a small volume of solution (3 µl) and compare the results to measurements from a known AuNP concentration. Adjust the volume such that the final solution is at least 1 x 10^{11} particles per ml.
    3. Store solutions at 4 °C until it is used for functionalizing of the immunoassay plate. Use the solutions within one week.

	Volumes to add of each component (ml)		
DTTC final concentration (mM)	DTTC working solution (200 mM)	AuNP	Water
0.2	0.1	20	79.9
0.6	0.3	20	79.7
1	0.5	20	79.5
2	1.0	20	79
5	2.5	20	77.5
7	3.5	20	76.5
10	5.0	20	75

Table 1. DTTC dilution example. Various dilutions of DTTC and the associated volumes of stock DTTC, gold nanoparticle solution, and water.

3. Immunoassay Plate Preparation

1. **Bind desired antigen to the immunoassay plate.**
    1. Prepare enough diluted antigen (50 µg/ml) to fill the polystyrene wells. Vortex the solution, and immediately add the solution to the plate wells. Allow the antigen to bind to the plates for 1 hr at room temperature.

2. **Wash off unbound antigens.**
    1. Remove the excess antigen solution by dumping solution into a disposal container and hitting the plate against a paper-towel-covered tabletop.
    2. Add TBST to the wells to wash the surface then remove the wash in the same manner as stated previously. Repeat this step two more times.

3. **Block remaining binding sites on the plate to prevent non-specific binding.**
    1. Add 70 µl of HSA blocking solution to each well of the plate and incubate at room temperature for 30 min.
    2. Remove and rinse the plate using the same procedure as specified in step 3.2. Cover the plate and store dry at 4 °C until ready for further use.

4. **Functionalize immunoassay plate.**
    1. Add 70 µl of the probe nanoparticles prepared in Section 2 to the first column of a 96-well plate and dilute subsequent columns using a 1:2 serial dilution. Allow the plate to incubate for at least 1 hr. An example of how to prepare the immunoassay plate is given in **Figure 3**.

2. Wash the plate with TBST five times as detailed in steps 3.2, making sure to dispose of the AuNPs appropriately. After the final wash, add 70 µl of 1x PBS to each well and cover with a plate seal.
   NOTE: The control samples should be clear. If non-specific binding has occurred, the control samples will have a similar color as the test samples.

5. **Test assay sensitivity by UV-Vis and Raman spectroscopy.**
   1. For each well, measure the UV-Vis spectra ranging from 400 to 700 nm using a plate-reading UV-Vis spectrophotometer.
   2. Using an inverted Raman microscope, focus the objective onto the surface of the well that has the AuNP probes. Obtain a Raman spectra of the well. Collect a spectrum ranging from 1,800 cm^{-1} to 400 cm^{-1}. Repeat this step for all wells.
   3. Using an appropriate spectral software, perform an 11th order polynomial baseline correction for the Raman spectra and a 3rd order polynomial for the UV-Vis spectra.
   4. Using an appropriate spectral software, normalize the Raman and UV-Vis spectra. Set the maximum value to 1 and scale all other values accordingly. To normalize the Raman spectra, select a unique polystyrene peak and set it equal to 1 and scale all other values accordingly.
   5. Using an appropriate spectral software, perform peak integration for each spectrum. For Raman spectra, the peak representing the Raman reporter must be in a region absent of polystyrene peaks. To perform peak integration, specify the integral boundaries for the desired peak and record the desired peak area for all samples including the controls.
   6. Plot the average peak area of interest as a function of the log of the AuNP concentration with error bars for each point indicating its associated standard deviation. Fit these calibration points to a 4-parameter logistic curve.
   7. Determine the mean value of the blank by averaging the area of the peak of interest for a blank sample. Determine the standard deviation of these areas; this is the standard deviation of the blank.
   8. For the same peak analyzed in the previous step, find the standard deviation of that peak area for the lowest concentration.
   9. Calculate the limit of the blank and lower limit of detection as specified in the Representative results section. Use these values with the 4PL calibration curves to determine the LLOD in terms of AuNP concentration.

Representative Results

In this study, 60 nm gold particles were used for UV-Vis spectroscopy. UV-Vis absorption spectra from 400 to 700 nm were collected and the peak areas for each AuNP concentration were determined using an open source spectral analysis software[8]. Prior to peak integration, the collected spectra underwent baseline correction using a three-point polynomial fit. Peak areas were used to generate a logarithmic calibration curve as demonstrated in **Figure 4**. It should be noted that **Figures 4** and **5** incorporated logarithmic calibration curves. The use of non-linear calibration curves can significantly expand the dynamic range of an assay and has become an accepted practice for various immunoassays that require low range detection capabilities[9,10].

To quantitatively assess the sensitivity of the assay, the limit of the blank (*LOB*) and the lower limit of detection (*LLOD*) was calculated as follows

$$LOB = \bar{x}_{BLANK} + 1.645\sigma_{BLANK}$$

$$LLOD = LOB + 1.645\sigma_{Low}$$

where standard deviation of the blank and of the lowest sample concentration is σ_{BLANK} and σ_{Low}, respectively, while $\bar{x}_{BLANK}$ is the mean value of the blank[11,12]. Using these definitions, as well as the generated 4-parameter logistic calibration curve, the LLOD for UV-Vis was 3.5 pM of gold nanoparticles.

Using a Raman spectroscopy setup detailed previously[7], a 785 nm inverted Raman microscope was used to collect spectra from the DTTC Raman reporter associated with the functionalized immunoassay plate. Operating parameters included 7 mW laser power and a 10 sec acquisition time. Spectra underwent baseline correction (11th order polynomial) and peak integration. **Figure 5** shows the 4-parameter logistic calibration curve generated for the DTTC peak areas for the 493 cm^{-1} and 508 cm^{-1} DTTC peaks. As the exact concentration of Raman reporter bound to the AuNP surface was unknown, the calibration curve was based on AuNP concentration. Using the equations described above, the LLOD was determined to be 1.7 pM of AuNP.

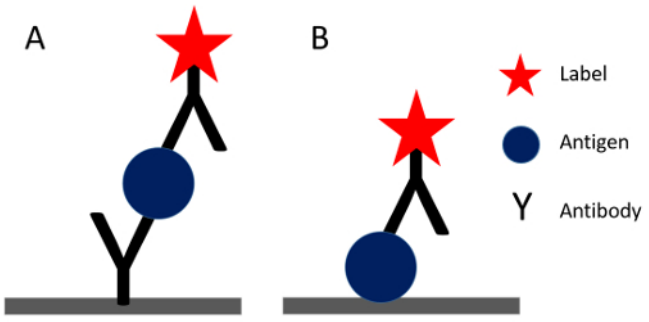

Figure 1. Illustration of direct and indirect immunoassay analysis. Illustration of indirect (**A**) and direct (**B**) detection schemes for immunoassays. Please click here to view a larger version of this figure.

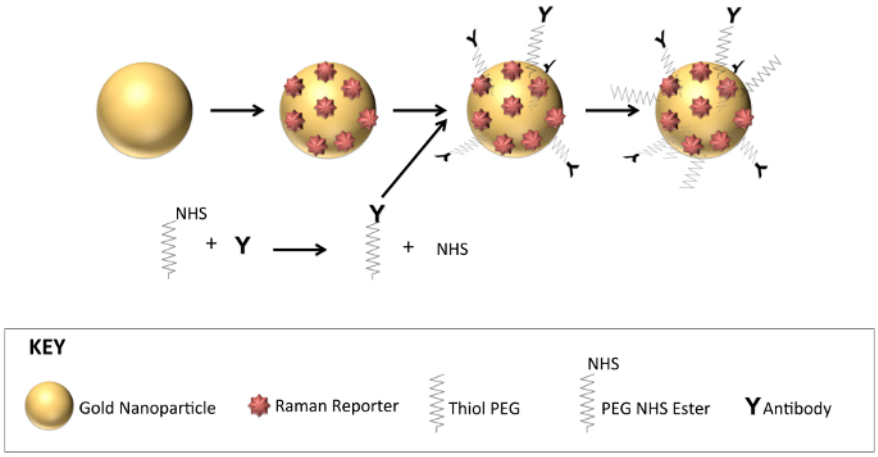

Figure 2. Nanoparticle probe fabrication illustration. Process of functionalizing Raman/UV-Vis probes for immunoassays. Please click here to view a larger version of this figure. Please click here to view a **much** larger version of this figure.

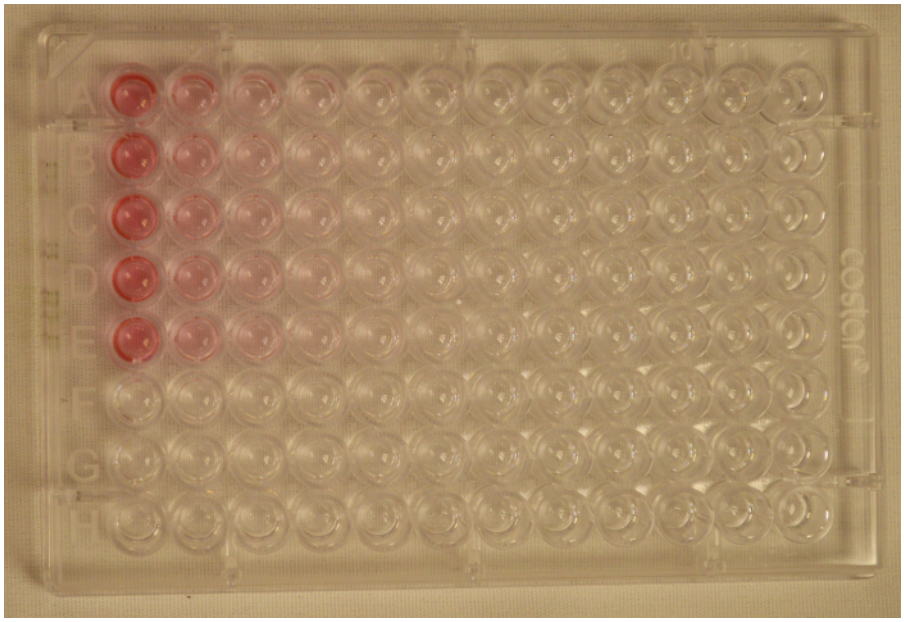

Figure 3. Prepared immunoassay plate. Image of a prepared immunoassay plate. Rows A through E are tests samples while rows F through H are control samples. Column 1 contains the undiluted nanoparticles and every subsequent column has half the concentration of AuNP probes. Please click here to view a larger version of this figure.

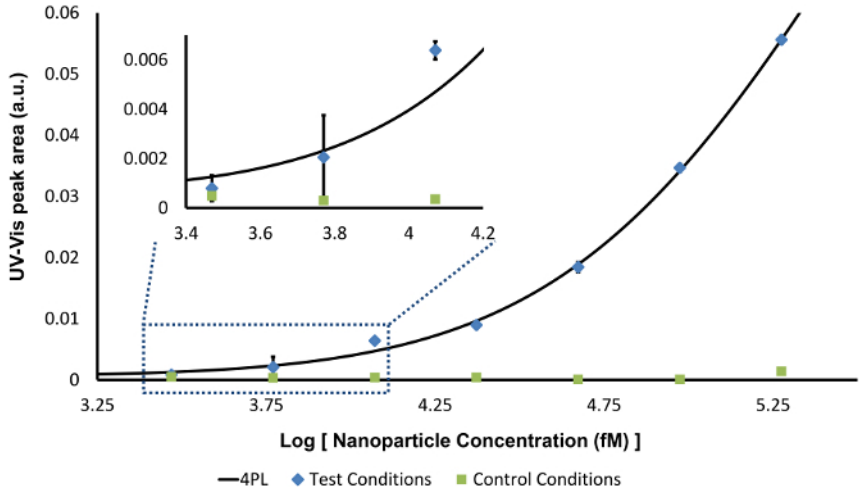

Figure 4. UV-Vis calibration curve for immunoassay using nanoparticle probes. Logarithmic calibration curve for the UV-Vis peak areas to nanoparticle concentration. The fitted line is a 4-parameter logistic (4PL) curve. Error bars indicate the peak area standard deviation. Please click here to view a larger version of this figure. Please click here to view a **much** larger version of this figure.

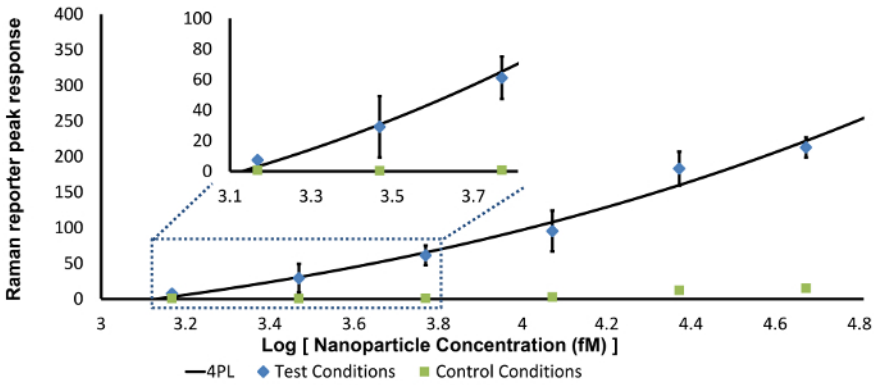

Figure 5. Raman calibration curve for immunoassay using nanoparticle probes. Calibration curve correlating Raman reporter peak area to gold nanoparticle concentration. The fitted line is a 4-parameter logistic (4PL) curve. Error bars indicate the peak area standard deviation. Please click here to view a larger version of this figure. Please click here to view a **much** larger version of this figure.

Discussion

In the detailed protocol, there are several critical points to address. One issue is the choice of Raman reporter and gold nanoparticle. Although the protocol was written to be adapted for individual use, the Raman reporter DTTC was used as an example. DTTC is a positively charged reporter and binds to negatively charged surfaces such as citrate capped AuNPs. This protocol can be adapted for negatively charged reporters by using gold nanoparticles with a positive surface charge. For example, polyethyleneimine (PEI) capped AuNPs provide a positive surface charge and better binding with negatively-charged reporters.

Maintaining the balance between proteins and nanoparticles is a critical step of this protocol. This balance is achieved by adding PEGylated antibodies at an optimized antibody to gold nanoparticle ratio of 200:1. If the PEGylated antibodies are added to the gold nanoparticle solution at a significantly higher ratio than this, ion-induced particle aggregation may occur. Alternatively, at too small of a ratio, protein aggregation and insolubility would occur. This ratio must be determined in each individual case.

Another critical protocol step is the conjugation of OPSS-PEG-NHS to the antibody. This step is preceded by suspending OPSS-PEG-NHS in sodium bicarbonate where the NHS group binds to the antibody. This conjugation step competes with the unfavorable hydrolysis reaction as detailed previously[7]. The hydrolysis reaction is more likely to happen over time, and as such, the OPSS-PEG-NHS to antibody binding should be performed immediately.

The end product of the protocol is an immunoassay which can be tested for sensitivity by construction of a calibration curve using UV-Vis and Raman spectroscopy. The results indicate that the immunoassay is comparable to other bioassays[13-17] which have detection limits of 1 pM. Not only is the Raman immunoassay sensitivity competitive with other bioassays, it has the potential for improved sensitivity by means of surface-enhanced Raman spectroscopy (SERS). SERS incorporates the use of gold nanoparticles or a roughened gold surface to enhance the Raman emission. As this protocol already includes the use of gold-nanoparticles probes, it is well suited for development into a SERS immunoassay. In the future, this technique could be used for development of a light scattering immunoassay that could be used to detect many protein analytes

simultaneously. As biomarker profiling becomes increasingly important for the diagnosis and treatment of a wide variety of diseases, this technique may have profound clinical applications.

Common problems associated with this protocol include aggregation of gold nanoparticles, insufficient binding of blocking proteins, binding of non-specific proteins, and a weak Raman signal. These problems are listed in **Table 2** along with the possible cause of the problem and action steps to address each problem. Other problems can arise due to limitations of the protocol. First, this protocol is limited to AuNP concentrations up to 5 x 10^{12} particle/ml as higher concentrations tend to cause aggregation. The technique relies on stable, unaggregated nanoparticles for estimation of the nanoparticle concentrations. If there is aggregation, the estimation of nanoparticle concentration will be biased. The protocol is also limited to Raman reporters with peaks strong enough to overcome the polystyrene background and are unique from polystyrene. Lastly, the use of traditional 96-well plates limit the protocol to use of an inverted Raman microscope due to the height of the plates. Otherwise, a low magnification must be used for the Raman microscope objective to accommodate the 96-well plate height.

Symptom	Probable Cause	Corrective Action
Gold nanoparticles aggregate after centrifugation and resuspension.	The Raman reporter concentration is too high.	Test a range of Raman reporter concentrations as specified in section 2.2 or this protocol.
	The antibody to AuNP ratio is too high.	Reduce the number of PEGylated antibodies bound to the nanoparticle surface to increase particle stability.
	The mPEG-SH blocking agent isn't binding to the AuNP surface.	Prepare mPEG-SH solution fresh prior to nanoparticle blocking.
Non-specific binding to the immunoassay plate surface	The immunoassay plate is insufficiently blocked.	Prepare blocking solution fresh prior to plate functionalization.
	The molecular weight of mPEG-SH is not large enough.	Ensure that the mPEG-SH molecule used for blocking has a molecular weight of 5,000 kDa or greater.
Weak or absent Raman signal	The Raman reporter is not binding to the particle surface.	Ensure the Raman reporter is allowed to bind for at least 30 minutes prior to addition of the PEGylated antibody.
	The molecular weight of mPEG-SH is not large enough.	Ensure that the nanoparticle capping agent has the appropriate charge for ionic Raman reporter binding.

Table 2. Troubleshooting for common problems. List of common problems encountered during the protocol with the associated causes and corrective action items.

In this manuscript, we have presented a protocol for custom fabrication of a nanoparticle-probe based immunoassay for analysis using UV-Vis/Raman spectroscopy. The protocol includes functionalization of gold nanoparticles with Raman reporters and immunoglobulins for direct detection of antigens bound to a polystyrene plate. The protocol can be adapted to suit a particular Raman reporter and associated excitation wavelength. Gold nanoparticle shape and size can also be altered. However, solution ratios for appropriate binding will vary according to the Raman reporter used as well as the nanoparticle size, shape, and manufacturer. The protocol has been written to cue researchers of when solution ratios must be determined for each unique arrangement and thereby allow for custom fabrication according to research needs. Unlike the typical fluorescent/colorimetric immunoassay protocols, this protocol holds the potential for greater multiplexing capabilities while capitalizing on a pre-existing infrastructure.

Disclosures

The authors declare that they have no competing financial interests.

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