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Increasing the Sensitivity of Lateral Flow Diagnostic Assays with Ultra-bright Nanoparticle Reporters

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Lateral flow assays are one class of diagnostic assay that detects or quantifies biomolecules in complex samples, including blood, urine, saliva or other fluids [Wong 2009]. These rapid tests are self-contained, portable diagnostic devices that are easy to use, fast, and inexpensive. Lateral flow test devices can be stored at ambient temperature, have a long shelf life, and provide diagnostic results without sample processing or additional equipment, making this format ideal for point-of-care and field-based diagnostics. Hundreds of millions of lateral flow rapid tests (also known as immunochromatographic assays) are used each year for a wide variety of applications including the ubiquitous pregnancy test (**Figure 1**). The sensitivity of these assays depends in part on the components of the test, methods of treating the components and sample, and the properties of the nanoscale reporter particles that generate a signal during use. The reporter particles are labelled with a molecule – often an antibody or nucleic acid – that will recognize an analyte in the sample and bind to a specific physical location on the strip. To maximize the sensitivity of the assay the reporter should be as "bright" as possible to generate the largest signal intensity per binding event. The reporter also needs to be very small, typically on the nanoscale, so that it can effectively bind to molecular targets.

Figure 1: Lateral flow rapid test for determining pregnancy.

Lateral flow assays consist of a series of inexpensive, paper-like components that are assembled onto a backing card for handling (**Figure 2**). Components of lateral flow strips typically include:

- Sample pad: Provides sample absorption and controls distribution and flow of sample onto the conjugate pad
- Conjugate pad: A medium for dispensing and drying nanoparticle-antibody conjugates ensuring controlled release of conjugate onto the nitrocellulose membrane upon hydradation
- Nitrocellulose membrane: Provides the ideal solid phase for immobilizing Test line and Control line reagents
- Wicking pad: Provides uniform capillary flow through the membrane, absorbs applied sample, and prevents backflow

Each component overlaps by at least 1 mm which allows for the sample to flow unimpeded from the sample pad to the wicking pad.

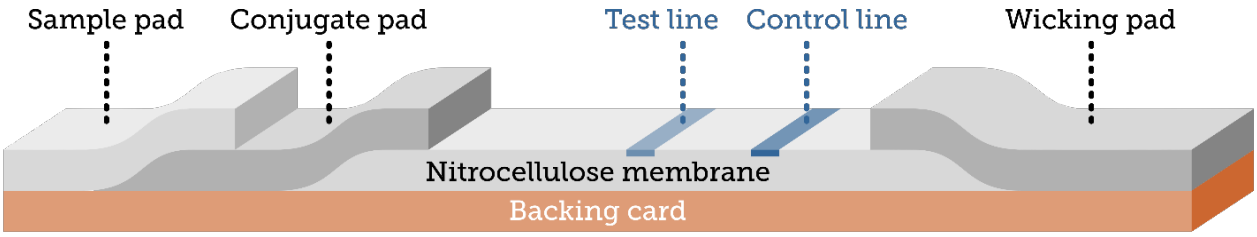

Figure 2: Lateral flow rapid test for determining pregnancy.

To use a lateral flow rapid test, a liquid sample such as blood, serum, plasma, urine, saliva, or solubilized solids is added directly to the sample pad and is drawn through the lateral flow device by capillary action. The sample pad can be treated with reagents that adjust the pH of the sample and can contain filters to remove unwanted particulates such as red blood cells. The sample then flows to the conjugate pad which contains strongly colored or fluorescent nanoparticles with antibodies conjugated to their surface. When the sample reaches the conjugate pad, the nanoparticles are rehydrated and mix with the sample. This mixture then flows through the nitrocellulose membrane across one or more test lines and a control line. The test and control lines consist of immobilized proteins that can interact with the sample and generate a signal corresponding the amount of analyte or conjugate present. The remaining fluid is absorbed by a wick pad that is designed to modulate capillary rise and prevent backflow. Once all the sample has finished running, results can be read via visual interpretation or with a reader for the presence of test line(s) and control line.

Lateral Flow Assay Formats

The two most common lateral flow assay formats are called "sandwich" and "competitive" (**Figure 3**). The sandwich assay format is typically used for detecting larger analytes that have at least two epitopes (binding sites). Usually, an antibody to one epitope is conjugated to the nanoparticle (detection antibody), and an antibody to another epitope is used for the assay's test line (capture antibody). If there is analyte in the sample, it will bind to both the detection and capture antibodies, creating a sandwich between the nanoparticle conjugate and the test line to produce a positive signal. If no analyte is

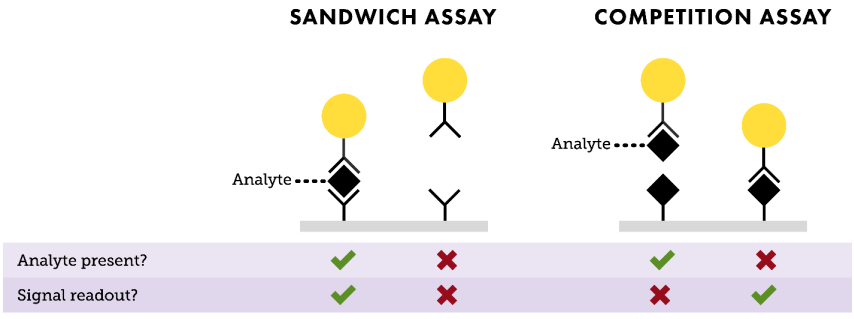

Figure 3: Depiction of "Sandwich" and "Competition" assay formats. In a sandwich assay, a positive signal indicates the presence of analyte and the test line intensity is proportional to the amount of analyte available in the sample. For a competitive assay, a strong signal on the test line means little or no analyte is present. This signal intensity is inversely proportional to the analyte concentration.

present, the nanoparticle conjugate will not bind to the capture antibody and no signal is observed on test line. Sandwich assays result in a signal intensity at the test line that is directly proportional to the amount of analyte present in the sample.

The competitive format is used for detecting analytes when antibody pairs are unavailable or if the analyte is too small for multiple antibody binding events, typical for steroids and drugs, for example. In this format, the test line typically contains the analyte molecule, usually a protein-analyte complex. If the target analyte is present, the analyte will bind to the conjugate and prevent it from binding to the analyte at the test line. If the analyte is not present, the conjugates will bind to the analyte at the test line, yielding a signal. In the competitive format, the signal intensity is inversely proportional to the amount of analyte present in the sample.

In both assay formats, an anti-species antibody used as the control line binds the conjugated antibody regardless of test line result. Presence of a control line indicates proper flow and demonstrates functionality of the assay.

Nanoparticles as Reporters in Lateral Flow

Lateral flow tests generate an optical signal that arises from strongly colored or fluorescent particles bound to test lines on a white nitrocellulose strip. This signal can be read by eye (qualitative or semi-quantitative) or by an optical reader (quantitative). To maximize the sensitivity of the test, each binding event between an analyte and reporter particle should produce the strongest signal possible. While larger particles will typically provide a stronger signal per binding event and be easier to visualize, particles that are too large or too dense will not easily flow through the nitrocellulose membrane and will have limited opportunity to bind to the test line. Thus, particles with sizes between 20 nm and 500 nm in diameter are typically selected for use in lateral flow assays. Fluorescent particles can also be employed, and the same general rules apply: the more potent the fluorescence per binding event, the stronger the signal.

One of the most common types of particles used in diagnostic assays are gold nanoparticles, often referred to as gold colloid [Wilson 2008] Gold nanoparticles have unusual optical properties that make them exceptionally strong absorbers of light. 40 nm diameter gold nanospheres have a peak absorbance at ~520 nm, resulting in a ruby red colored test line. The gold surface has a natural affinity for antibodies and other proteins, allowing for the fabrication of nanoparticle-antibody conjugates by simply mixing gold nanoparticles with an affinity ligand. Other sizes and shapes of nanoparticles have also been used as lateral flow probes. Gold nanoshells with a 150 nm diameter provide a higher contrast per binding event and typically provide a 5–20 fold increase in sensitivity when compared to 40 nm gold nanospheres. Gold nanoshells are prepared by the electroless deposition of metallic gold on the surface of a silica particle that is studded with small gold nanoparticles [Oldenburg 1998]. The small gold seeds grow larger until they coalesce into a complete shell (**Figure 4**).

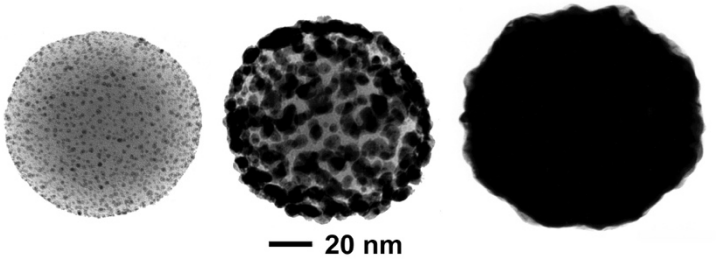

Figure 4: Transmission electron microscopy images of different stages of nanoshell growth. Initially, thousands of ~2 nm gold nanoparticle seeds are bound to positively charged silica nanospheres (left). Gold ions in solution are electrolessly deposited onto the bound gold seed growing the seeds larger (middle) until they coalesce into a complete shell (right).

By controlling the silica core size and the thickness of the gold shell during gold nanoshell synthesis, the peak wavelength of the nanoshells can be tuned to change the particle's color. To maximize sensivity in lateral flow assays, blue-grey 150 nm gold nanoshells with a 120 nm diameter silica core and a ~15 nm gold shell have been developed. The silica core has a much lower mass than gold, improving the settling time and flow characteristics in the nitrocellulose membrane compared with solid gold particles. **Figure 5** shows a comparison between the optical extinction (scattering + absorption) per particle of 40 nm gold nanospheres and 150 nm nanoshells. Because of their stronger extinction per particle, each nanoshell binding event has a much higher contrast against the nitrocellulose substrate resulting in increased assay sensitivity.

An image of test and control lines using gold nanoshell reporters with a corresponding scanning electron microscope image is shown in **Figure 6**. Individual nanoshells bound to antibodies immobilized on the nitrocellulose membrane can be identified as light-colored spheres on the darker nitrocellulose fibers. Even at the relatively sparse coverage density on the membrane, the control line appears very dark demonstrating that each nanoshell binding event generates a high contrast signal against the white nitrocellulose background.

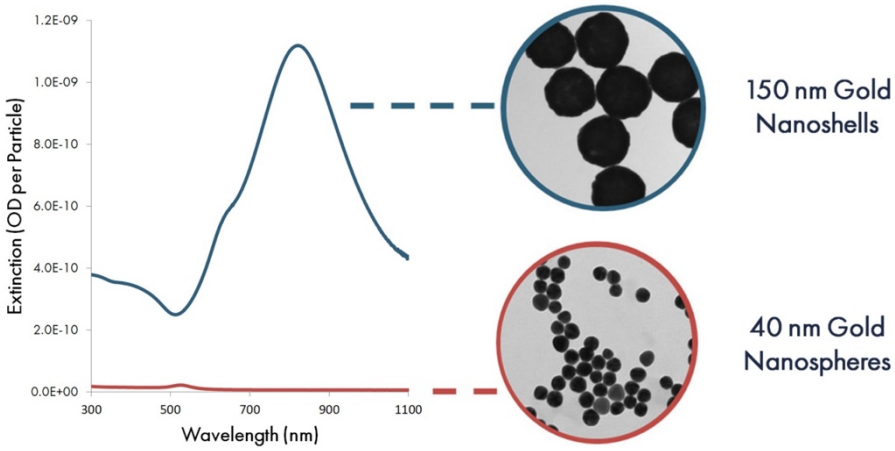

Figure 5: Per particle optical extinction (optical density per particle) as a function of wavelength for 40 nm gold nanospheres and 150 gold nanoshells.

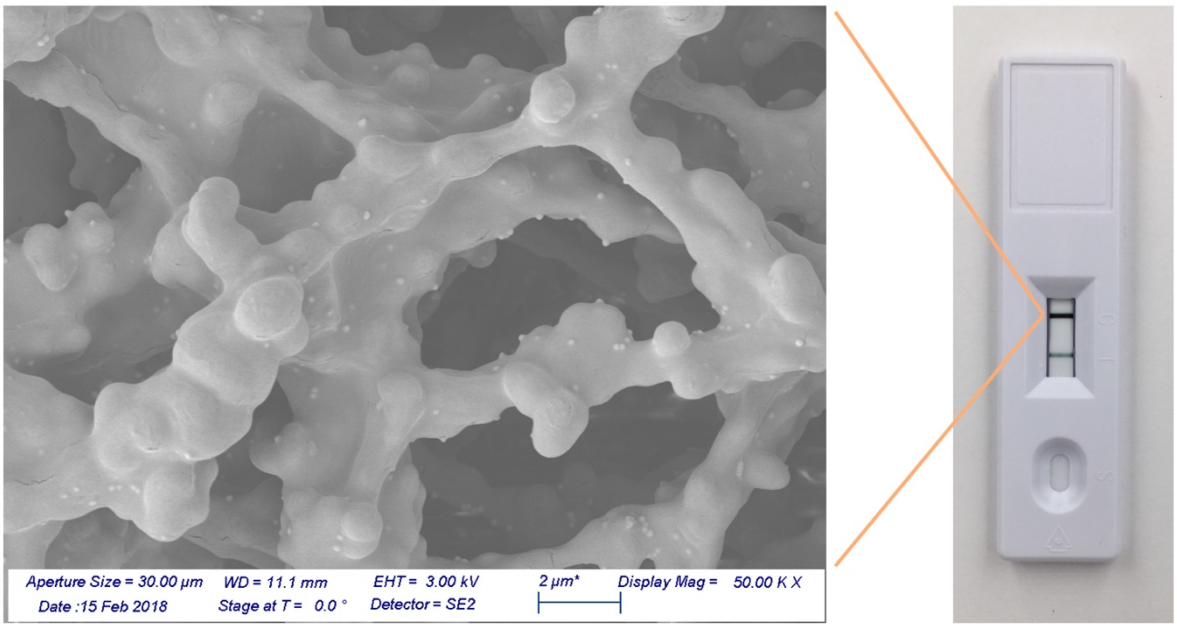

Figure 6: Gold nanoshells bound to a nitrocellulose membrane.

Ultimately, the sensitivity of the assay is determined by the number of reporters that must bind to see a visible test line. To measure how many particles are required to obtain a visual signal, streptavidin-coated 40 nm gold nanospheres and streptavidin-coated 150 nm gold nanoshells were captured on a biontinylated test line and the signal analyzed with an optical reader (**Figure 7**). With 40 nm gold nanospheres there is a visible test line when 5 million particles are added to the assay. With gold nanoshells only 500,000 particles are required to see a line, an order of magnitude decrease that yields increased assay sensitivity.

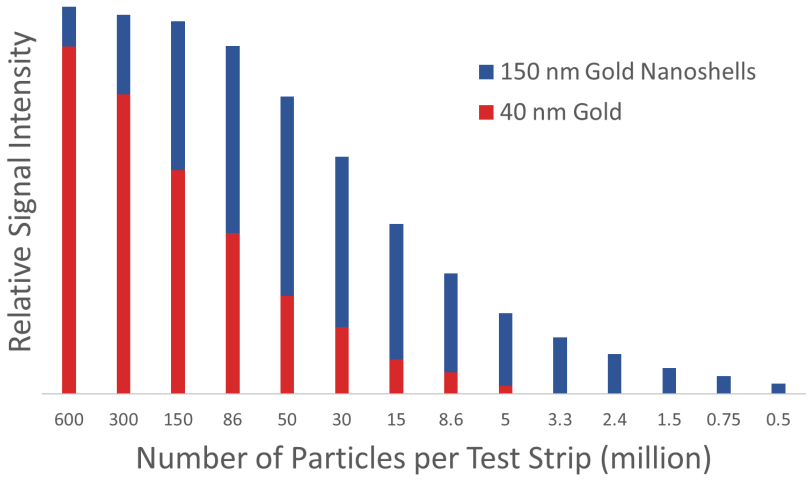

Figure 7: Lateral flow assay results where streptavidin functionalized 150 nm gold nanoshells and 40 nm gold nanoparticles are run on a nitrocellulose strip and captured on a biotinylated BSA test line. The visual detection limit of gold nanospheres is 5,000,000 while nanoshells can be detected at 500,000 binding events.

To see how the absorbance per particle affects assay sensitivity, 40 nm diameter gold nanoparticles and 150 nm diameter gold nanoshells were used to detect Troponin I, a cardiac marker, in a lateral flow sandwich assay (**Figure 8**). The limit of detection with the 40 nm gold nanoparticles

was 0.5 ng/mL while the Troponin I detection with 150 nm gold nanoshells was an order of magnitude better at 0.05 ng/mL.

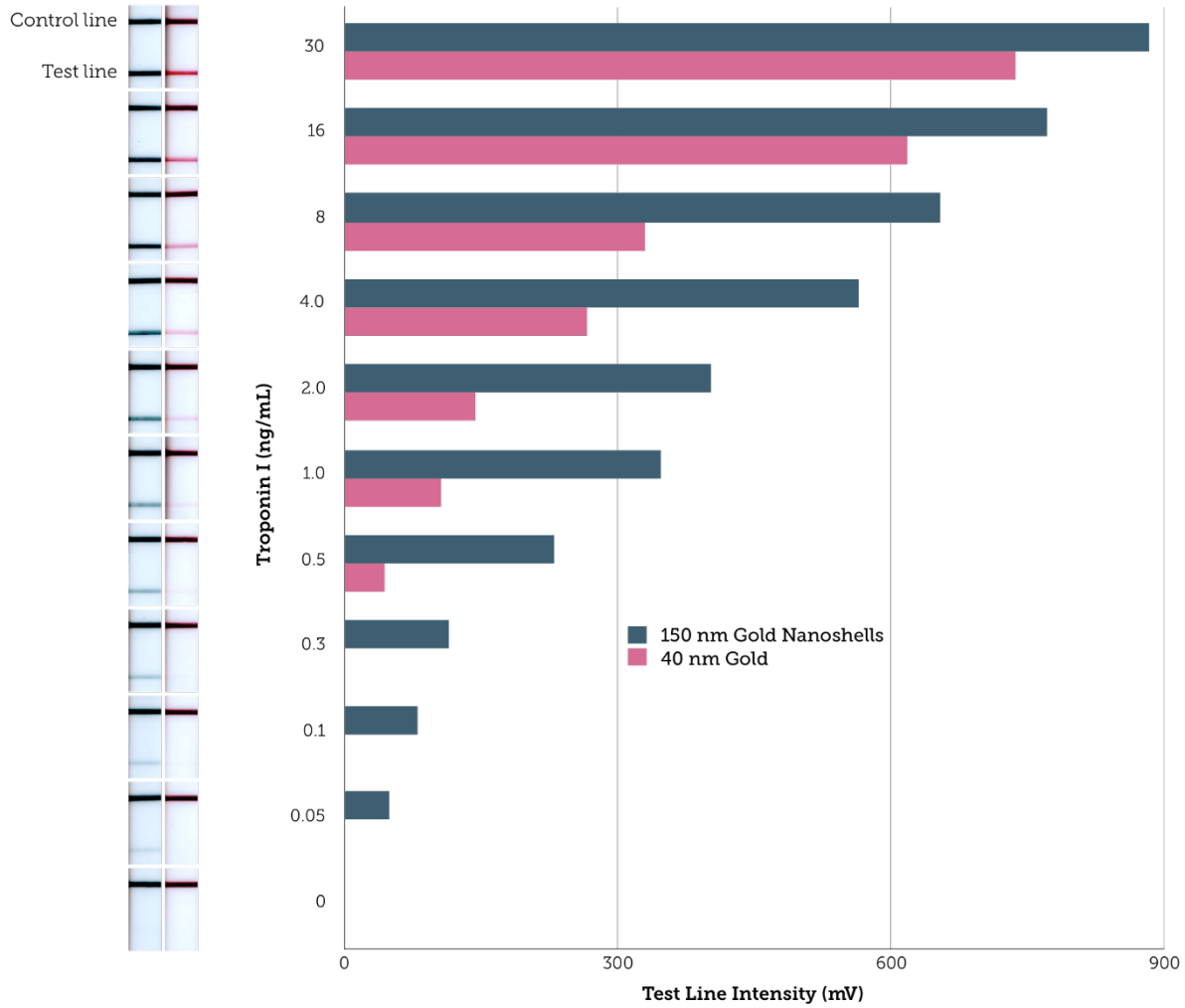

Figure 8: Lateral flow assay results where streptavidin functionalized 150 nm gold nanoshells and 40 nm gold nanoparticles are run on a nitrocellulose strip and captured on a biotinylated BSA test line. The visual detection limit of gold nanospheres is 5,000,000 while nanoshells can be detected at 500,000 binding events.

Covalent Linkage Chemistry for High Stability Reporters

Another important consideration when developing high-sensitivity lateral flow assays is how the antibodies or other proteins are bound to the nanoparticle surface. Robust and effective binding is critical for maximizing the sensitivity and selectivity of the assay. The process of binding antibodies to the surface of a reporter

particle is referred to as conjugation and the resulting antibody-coated particle is referred to as a conjugate [Hermanson 2013]. Passive adsorption (physisorption) is the traditional method for attaching proteins to lateral flow nanoparticle probes and is widely used. By taking advantage of various forces between molecules and surfaces at a specific pH (e.g. van der Waals and ionic forces) antibodies will spontaneously bind to a bare gold nanoparticle surface to form a conjugate. The antibody is typically added in excess to ensure there is complete coverage of the surface of the nanoparticle. Any free antibody remaining in solution is removed via centrifugation or filtration after the conjugation is complete.

An alternative method of forming conjugates is to covalently bind the antibody to the nanoparticle surface. Covalent binding provides several advantages over passive adsorption. The covalent conjugation chemistry is typically more reproducible than physisorption and, for some antibodies, covalent chemistry is necessary to prepare stable conjugates. Covalent binding chemistry also allows for more control over the amount of antibody on the surface of the nanoparticles and the antibody orientation. One common method of fabricating covalent conjugates utilizes carboxyl (carboxylic acid) functionalized gold surfaces. The carboxylic acid on the nanoparticle can be linked to primary amines in the lysine residues of the antibody or protein using EDC and sulfo-NHS reagents to form amide bonds between these functional groups. A typical IgG antibody will have 80–100 lysine residues, of which 30–40 will be accessible for EDC/NHS binding. Advantages of covalent binding include:

- Less antibody is needed to maximize sensitivity, reducing the overall cost of an assay
- Greater stability can be obtained with covalent conjugates, solving challenges associated with difficult sample matrices and high salt/detergent buffering environments
- Conjugates are easily prepared without requiring extensive salt or pH optimizations, saving time when performing antibody screening experiments
- The antibody-to-particle ratio can be precisely controlled, which is important for adjusting the dynamic range in competitive assays and optimizing sensitivity with antibodies with varying binding kinetics

Interpreting Results from Lateral Flow Tests

The results from a lateral flow test can be either qualitative (if an analyte is or isn't present, within the limits of detection), semi-quantitative (analyte present at low, medium, or high levels) or quantitative (determination of a precise amount of analyte). The pregnancy test is an example of a qualitative "yes"/"no" assay, where a positive test line signal correlates to elevated levels of the hCG hormone in urine, indicating that the user is pregnant. For quantitative diagnostics, the test line intensities are compared to a calibration standard and converted to an analyte concentration value. Quantitative assays can be used to measure the concentration of a specific analyte or biomarker instead of simply indicating the presence or absence. For example, someone who has high stress may want to accurately measure the concentration of their cortisol

levels over time to determine if stress mitigation interventions are working. To accurately measure the test line intensity, the result must be analyzed by a strip reader. The recent commercialization of small form factor, inexpensive, mobile-centric readers is transforming the lateral flow assay industry. Simple methods of quantifying the output of lateral flow assays without the use of stand-alone bench top readers opens up a tremendous opportunity for home use of point-of-care diagnostics. **Figure 9** shows a variety of disposable and benchtop reader formats. For benchtop units, a lateral flow strip is inserted into a cartridge, the signal intensity on the completed test is analyzed with a photodiode, and the results are sent back to a mobile phone to display and interpret the result. The calibration curve for any given assay can be built into the software such that test line signal intensity can be automatically converted to an analyte concentration and presented to the user. Disposable readers have all of the necessary hardware to perform the analysis on the device itself, further simplifying the user experience.

Figure 9: Various Lumos Diagnostic reader formats for lateral flow diagnostics.

Conclusions

Ultra-bright reporter particles that are based on the unique optical properties of gold nanoshells significantly increase the sensitivity of lateral flow immunoassays. When combined with recent developments in portable reader technology, a new class of point-of-care diagnostics are coming to market that have the sensitivity, specificity, and reproducibility of laboratory-based tests in a much less expensive and more convenient test format. In addition to lateral flow, gold nanoshells have application in a wide variety of diagnostic and detection applications including surface enhanced Raman spectroscopy, flow cytometry, and molecular imaging. With simple to use covalent coupling chemistry and easy to follow protocols, gold nanoparticle conjugates are an important tool in many biotechnology applications.

References

1. Hermanson, G.T. "Bioconjugate Techniques" Academic Press, 2013.
2. Oldenburg, S. J., R. D. Averitt, S. L. Westcott, and N. J. Halas. "Nanoengineering of optical resonances." Chemical Physics Letters 288, no. 2 (1998): 243-247.
3. Wilson, R. "The use of gold nanoparticles in diagnostics and detection", Chemical Society Reviews, 2008, 37, 2028-2045 10.1039/B712179M
4. Wong RC and Tse HY, "Lateral Flow Immunoassay", Springer, New York 2009