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Wavelength-Ratiometric Plasmon Light Scattering-Based Immunoassays

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Abstract The application of a wavelength-ratiometric plasmon light scattering technique to immunoassays is demonstrated. A model immunoassay for anti-immunoglobulin G (IgG), constructed in gold colloid-modified high-throughput screening wells, was monitored by the changes in the intensity of scattered light (with transmitted light) from gold colloids as a result of antibody–antibody interactions. The quantitative determination of anti-IgG was undertaken by measuring the ratio of intensity of scattered light at both 590 and 500 nm. A white light-emitting diode (LED) and a fiber optic coupled fluorometer was used as an excitation source and the detection system, respectively. The visual confirmation of the quantitative nature of the measurement technique was done by digital photography. A lower detection limit of 0.05 μg/mL for anti-IgG was determined. The wavelength-ratiometric plasmon light scattering technique offers several advantages: (1) light at >500 nm can be used for reduced biological autofluorescence; (2) due to the ratiometric nature of these measurements, the fluctuations in the excitation or ambient light do not perturb the measured signal; and (3) with the addition of automated detection systems, multiple samples in a high-throughput format can potentially be assessed quickly and more efficiently.

Keywords Immunoassays · Light scattering · Surface plasmons · Gold colloids · Surface plasmon resonance

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Introduction

Immunoassays are widely used for the detection of antigen or antibodies present in biologically relevant samples [1]. The quantitative detection of antigen or antibodies are typically carried out by labeling either the antigen or the antibody with an enzyme [1], radioisotopes [2], magnetic particles [3, 4] or fluorophores [5, 6] and converting the readout signal to the concentration of the *unknown* biomolecules. Although well established, these methods either require expensive detection equipment or the expertise to both experimentally run and interpret the data. In this regard, the search for simpler highly sensitive techniques, which require little or no user expertise and yet yield simple visual readouts, is still ongoing.

In the last few decades, gold colloids have increasingly attracted the attention of scientists due to their unique optical properties [7, 8]. Gold colloids are known to both strongly absorb and scatter incident light depending on their size, shape, and proximity to other plasmon resonant nanostructures and as a function of the surrounding dielectric medium [7, 8]. To date, most applications of gold colloids have been concerned with the monitoring of the changes in their strong surface plasmon resonance (SPR) peak [9]. For biosensing purposes, when aggregated due to specific biorecognition events, the SPR peak of the gold colloids shifts to longer wavelengths along with a broadening in their absorption spectrum [10]. Drawing from their strong interactions with light, gold colloids have also been used as contrast agents for biomedical imaging using microscopy techniques [11, 12]. It was reported that light scattered from a single gold colloid can be equal to that of ≈10 [5] fluorescing fluorophores [7, 8].

More recently, several biosensing applications of gold colloids based on their ability to scatter light have also been

reported [13–15]. In these applications, the changes in the scattering of light by gold colloids decorated with oligonucleotides [15], antibodies [13], or polymeric molecules [16] were used to monitor the desired specific biorecognition events. In a series of recent reports, our laboratory has described several new approaches to aggregation-based biosensing using gold colloids and plasmon light scattering: (1) angular-ratiometric [17], (2) angular-dependent polarization [18], and (3) wavelength-ratiometric plasmon light scattering [16]. The first two approaches utilize the angular-dependent nature of light scattering governed by the Rayleigh theory [19] where light incident to *unaggregated* gold colloids is scattered in a $\cos^2\theta$ fashion. When aggregated due biorecognition events, gold colloids no longer scatter light in a $\cos^2\theta$ fashion but in an increased forward direction, a pattern underpinned by the Mie theory [17]. It is also known that the coupling of surface plasmon modes between two adjacent colloids occur within a distance of 2.5 times the diameter of the colloids [20]. The concentration of the desired biomolecules can be subsequently determined by simply measuring the ratio of intensity of scattered light at two arbitrary angles [17].

In the wavelength-ratiometric approach [16], the concentration of biomolecules of interest can be determined by measuring the ratio of scattered light from the aggregated gold colloids at two wavelengths (preferably wavelengths where the intensity is at a maximum and a minimum for a wide dynamic range). This technique offer several advantages over absorption-based sensing: (1) light at >500 nm can be used for suppressed biological background signal; (2) wavelength-ratiometric scattering measurements are not perturbed by fluctuations in the excitation or ambient light and can be also undertaken in conjunction with absorption measurements; and (3) the dynamic range of the scattered light can be increased by using larger size colloids, given that the colloids do not aggregate in a nonspecific fashion. Building on the experience gained from these studies, the proof-of-principle demonstration of a wavelength-ratiometric plasmon light scattering-based immunoassay, constructed in high-throughput screening (HTS) wells, is presented here. In this regard, commercially available HTS wells are modified with gold colloids, and subsequently, a model immunoassay is constructed with the addition of immunoglobulin G (IgG) and gold colloid-coated anti-IgG. The ratio of intensity of scattered light (with transmitted light) measured at 590 and 550 nm was used to determine the concentration of anti-IgG. A white light-emitting diode (LED) and a fiber optic coupled fluorometer was used as an excitation source and the detection system, respectively. In addition, the quantitative nature of the measurement technique was visually confirmed by photographs taken by a digital camera. A lower detection limit (LDL) of 0.05 μg/mL for anti-IgG was determined.

Materials and methods

Materials

Gold colloids 20 and 40 nm in diameter size and goat antirabbit gold colloid conjugate with 20 and 40 nm were purchased from Ted Pella, CA, USA. Rabbit IgG, goat antirabbit IgG, bovine serum albumin (BSA), and SigmaScreenTM poly-D-lysine-coated HTS plates (clear bottom, 96 wells) were purchased from Sigma-Aldrich. All chemicals were used as received. White LED was purchased from Radioshack.

Methods

Coating of HTS wells with gold colloid films

The coating of the HTS plates with gold colloids (20 and 40 nm) was achieved by incubating 0.5 mL of gold colloid solution inside the HTS wells overnight. The HTS wells were coated with gold colloids due to the binding of gold to the amine groups of the surface poly-lysine. The gold colloid-coated HTS wells were rinsed with deionized water at least three times prior to the scattering experiments to remove unbound material.

Model immunoassay

A model IgG–anti-IgG immunoassay was performed in a sandwich format as shown in Fig. 1. In this regard, a solution of IgG (0.1 mg/mL) was incubated in gold colloid-coated HTS wells at room temperature for 2 h. The unbound material was removed by rinsing the wells with a buffer solution at least three times. This resulted in noncovalent attachment of the IgG molecules onto the gold colloids. The IgG-deposited HTS wells were blocked with a solution of BSA (1% *w*/*v*) in order to minimize the nonspecific interaction of the anti-IgG/gold conjugates with the surface of the HTS wells. Then, 0.5 mL of a series of diluted solutions of anti-IgG/gold colloid conjugates (ranging from 1/1 to 1/1,000) were incubated in IgG–gold colloid-coated HTS wells at room temperature for 2 h. The unbound material was removed by rinsing the wells with a buffer solution multiple times. A 100-μL volume of phosphate buffer solution (pH7) was added to each well prior to the scattering measurements. Scattering light (white LED; Fig. 1, bottom) from gold colloids before and after the addition of anti-IgG were collected with a fiber optic system attached to a fluorescence spectrometer (HD2000) from Ocean Optics. The white light source (LED) and fiber optic was placed on the bottom and on the top of the HTS wells, as shown in Fig. 1, in a 180° geometry. Digital photographs of HTS

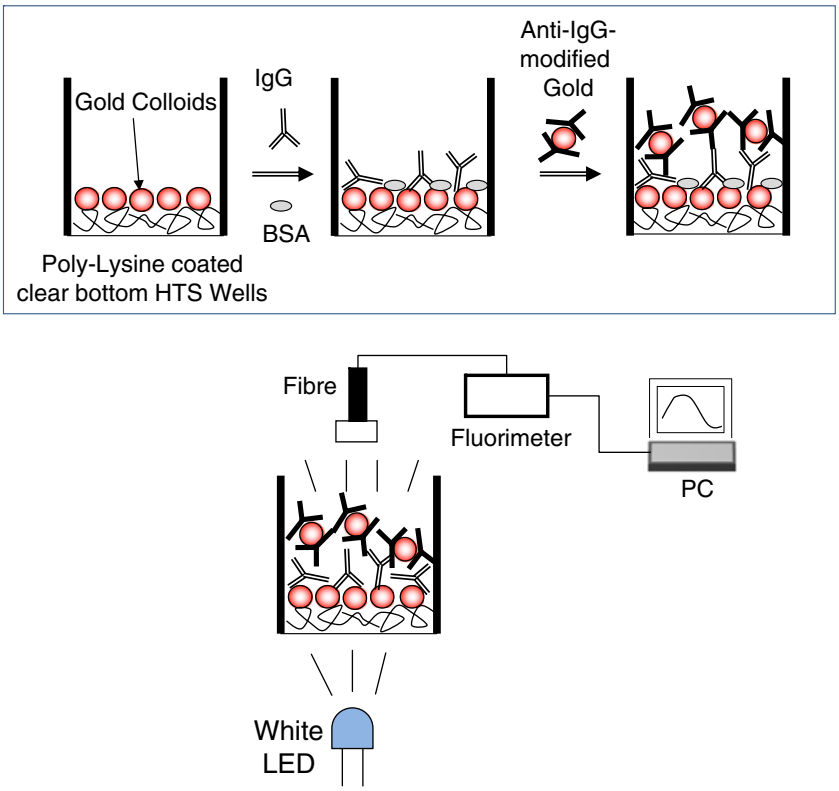

Fig. 1 Sample geometry in clear plastic-bottomed HTS wells for model wavelength-ratiometric plasmon light scattering-based immunoassays

wells after the assay is completed were taken with a Canon Powershot S50 Digital Camera.

Control experiments pertaining to the model immunoassay were performed by using *unconjugated* gold colloids instead of the anti-IgG/gold colloid conjugate.

Results and discussion

Since the new detection method described herein for immunoassays is based on the changes in the intensity of the scattered light by the gold colloids, it is pertinent to measure the wavelength-dependent scattered light from gold colloid-coated clear-bottom HTS wells before the immunoassay is constructed. These intensity values are used as the baseline value from which a deviation, due to close proximity of gold colloids as a result of biomolecular recognition events, is recorded. Figure 2 shows the scattering (and transmission) spectra of gold colloid-coated HTS wells and the spectrum of unpolarized white light (LED, measured from blank HTS wells). The presence of 20-nm gold colloids in the HTS wells increases the scattered light intensity at all measured wavelengths where a maximum intensity at 550 nm is observed. The coating of HTS wells with 40-nm gold colloids again results in increased scattered light (forward scattering at angles opposite to incident light) at all measured wavelengths where a slight red-shift in the maximum scattered light intensity to 590 nm is observed, as expected [17, 21]. It is important to note that, since the change in the intensity of scattered light from gold colloid-deposited HTS wells due to close proximity of gold colloids subsequently added in the last step of the immunoassay is recorded to determine the antibody concentration, the subtraction or normalization of the scattering spectrum of LED without gold nanoparticles is not required. It is also important to note the deposition of gold colloids onto commercially available poly-lysine-coated HTS wells is very reproducible: the variation in intensity measured at 550 nm from five different samples was ≈5% for all sizes of gold colloids.

To demonstrate the applicability of wavelength-ratiometric plasmon light scattering measurements to immunoassays, gold colloid-coated HTS wells were further coated with IgG and the model immunoassay is completed by incubating a solution of anti-IgG-modified gold colloids (20 and 40 nm, in separate experiments). Figure 3 shows the light scattering (and transmission) spectra collected from the model immunoassay using anti-IgG-modified gold colloids. The addition of anti-IgG-modified gold colloids onto IgG-modified HTS wells results in the following changes in the scattered light intensity: (1) a decrease at 550 nm and (2) an increase at 590 nm, as clearly evident from Fig. 3, insets. The changes in the scattered light intensity were more pronounced for the immunoassays

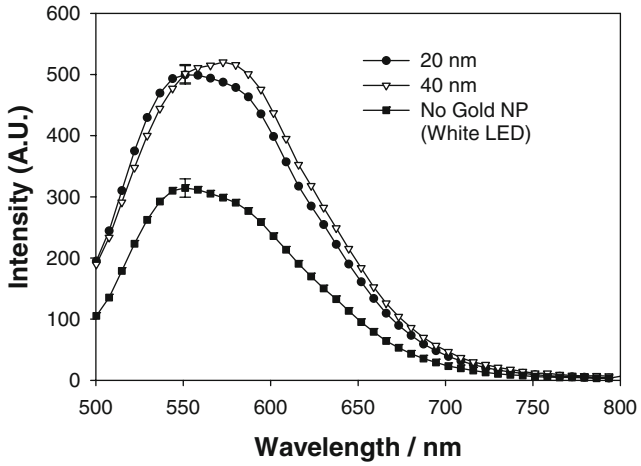

Fig. 2 Scattering spectra (also includes transmitted light) of gold colloid-coated HTS wells. The spectrum of white light (not normalized) used is also given for comparison. The variation in intensity measured at 550 nm from five different samples

carried out with 40-nm gold colloids. This is not a surprising observation, as the efficiency of scattering of light by gold colloids is known to increase as the size of the gold colloids is increased [7, 8]. It is important to note that, although one would expect even more pronounced changes in the scattered light intensity using larger gold colloids (>60 nm) and thus an increase in the sensitivity of the light scattering-based immunoassays, they were not used, however, in this study due to the self-aggregation of the larger gold colloids after their modification with an anti-IgG.

It is important to note that IgG and anti-IgG were attached to gold colloids by physical adsorption. Due to the random nature of the adsorption and the orientation of IgG and anti-IgG molecules (4×5×12 nm) on gold colloids, one can expect the distance between the gold colloids to vary between 4 and 24 nm. Since the reading of scattered light was taken from the entire individual HTS wells (5 mm in diameter; Fig. 1), the light scattering spectra (Fig. 3) reflects the average of scattered light from all possible IgG/gold and anti-IgG/gold conjugates. It is also important to note that the coating of the commercially available poly-lysine-coated HTS wells with gold colloids is reproducible and little to no variation in the extent of coating from well to well is expected.

The concomitant changes in scattered light intensity, measured at 550 and 590 nm as described above, afford the opportunity to eliminate the dependence of measured scattered light for the immunoassay on the intensity and its drift/fluctuations of the excitation light source. This is accomplished simply by taking the ratio of these intensities (I_{590}/I_{550}), which is *independent* of the intensity of excitation light source. Figure 4 shows the wavelength-ratiometric scattered light measured for immunoassays using anti-IgG-coated 20- and 40-nm gold colloids and a typical visual concentration-dependent response captured by a digital camera. Since gold colloids are employed as reporter molecules, the sensitivity of the immunoassays is influenced by the nonspecific adsorption of gold colloids. To determine the extent of nonspecific adsorption, a control experiment is performed with the largest concentration of unconjugated gold colloids (corresponding to ≥10 µg/mL in the actual immunoassay). The scattered light intensity measured from the control experiment is used to determine the LDL of the immunoassays. Figure 4, top, shows a wider dynamic range of I_{590}/I_{550} for the immunoassays using 40-nm gold colloids over the three-log concentration range with a LDL of 0.1 µg/mL anti-IgG. Despite a narrower I_{590}/I_{550} range, the LDL for the immunoassays using 20-nm gold colloids is ≈0.05 µg/mL, based on the signal-to-noise ratio (>1.5) using the experimental setup

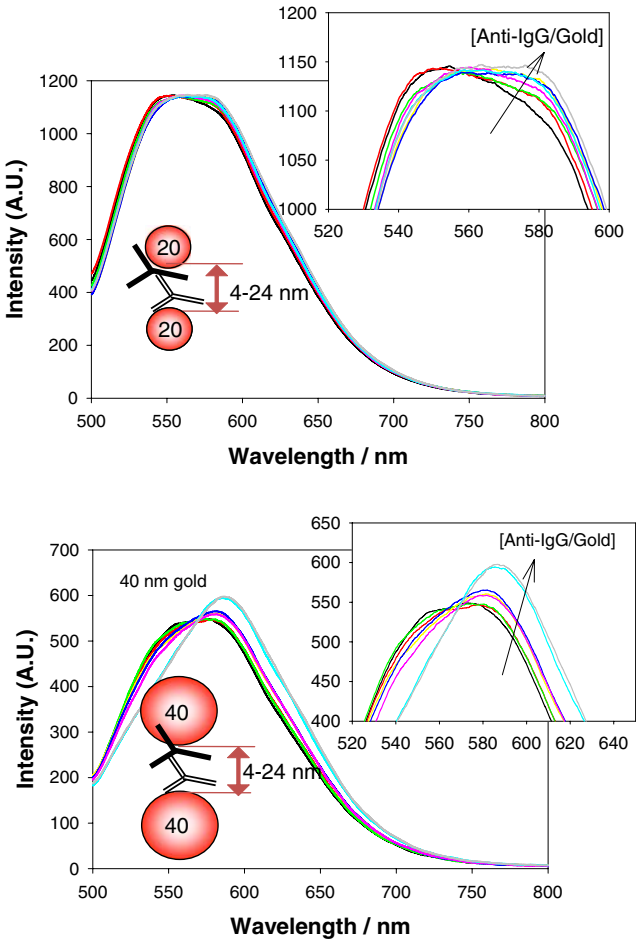

Fig. 3 Scattering spectra (also includes transmitted light) of gold colloid-coated HTS wells before and after the immunoassay is completed. *Top* anti-IgG-modified 20 nm gold (0.05, 0.01, 0.5, 5, 10, and 15 µg/mL in the direction of the *arrow*) *Bottom* anti-IgG-modified 40 nm gold (0.01, 0.05, 0.1, 1, 5, and 10 µg/mL in the direction of the *arrow*). The *arrow* indicates the increase in concentration of anti-IgG-modified gold colloids used as the detector antibody

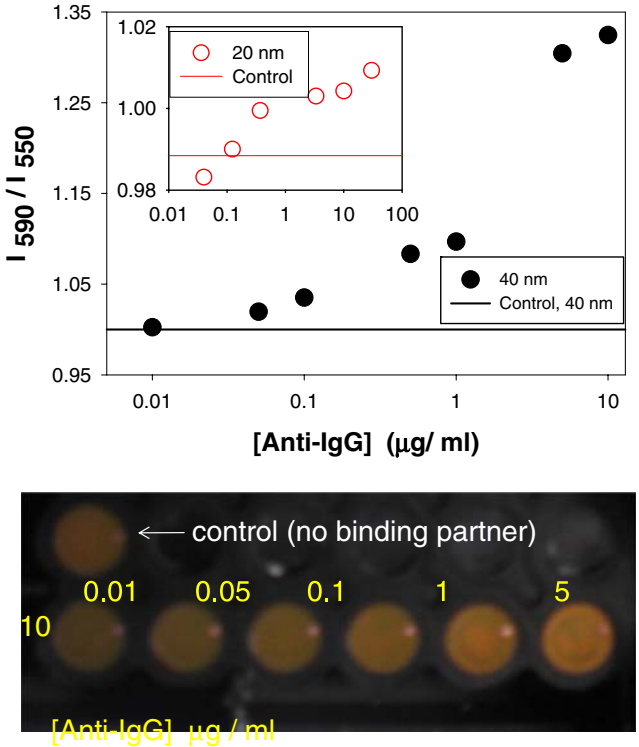

Fig. 4 *Top* Semilogarithmic plot of ratiometric scattered light (intensity at 590 nm divided by intensity at 550 nm) versus the concentration of anti-IgG-modified gold colloids. *Bottom* Digital photographs of a typical 40-nm gold colloid-coated HTS wells for a control experiment (no anti-IgG) and the actual assay

described here. The LDL could be improved by employing more sensitive commercially available detectors. This is due to fact that 20-nm gold colloids absorb light more efficiently than they in fact scatter light. On the other hand, 40-nm gold colloids can scatter light more efficiently and one can capture a concentration-dependent visual response using a digital camera without employing any emission filters as shown in Fig. 4, bottom.

It is important to note the advantages that the detection method have based on plasmon light scattering compared to existing detection strategies:

1. The excitation source is an inexpensive white light source (LED), although lasers and other light sources can also be employed.
2. The output signal is the ratio of intensity of scattered light measured at two unique wavelengths but at 180° to the excitation source, which alleviates the need to scan a range of wavelengths. Faster readout times subsequently affords for multiple samples in a high-throughput format to be assessed quickly and more efficiently. This is a notable step forward in the simplification and cost of the biosensing instrumentation. Moreover, the ratiometric output signal is *independent* of the intensity or drift/fluctuations of the excitation light, which allows the employment of this technique with potentially any excitation light source.
3. A quantitative readout from the immunoassay can be also undertaken visually without an additional excitation source, simply by comparing the brightness of the color developed as a result of the immunoassay against a precalibrated colored strip corresponding to a predetermined range of concentration of an unknown sample.
4. Fluorescence spectroscopy is the most commonly used detection technique in immunoassays today, which requires the use of fluorophores and moderately expensive instruments. However, fluorophores are prone to photodestruction over time by intense excitation intensities, unlike metal colloids which are not prone to photodestruction under the same conditions.
5. Most fluorescence-based systems require that the excitation light not be scattered into the detector. Most commonly, a 90° excitation/emission geometry is employed [22]. For our immunoassays, scattered light is detected in a parallel format (180°), Fig. 1, simplifying the instrumentation.
6. Unlike typical fluorescence-based immunoassays, the assay described here is potentially washless, since the scattering from the binding partners is red-shifted compared to the free unbound material. This is particularly important for the realization of point-of-care devices or field-deployable devices where access to laboratory instrumentation and procedures is limited.
7. In a recent paper [23], our laboratory has shown the application of low-power microwaves to colloidal nanoparticle-based assays, rapidly reducing the assay run time. The amalgamation of that approach [23] with the wavelength-ratiometric approach here would lead to a rapid yet simple sensing platform. Further studies are underway in this regard.
8. While we have demonstrated our sensing approach with a model immunoassay, IgG and anti-IgG, respectively, the technology could readily be applied to sense other proteins, RNA, and DNA targets.
9. The sensitivity of the immunoassay described here can be increased by employing covalent linkage procedure for the attachment of antibodies to gold colloids.

Conclusions

A wavelength-ratiometric plasmon light scattering-based technique for immunoassays, which can be carried out in a single assay or high-throughput format, has been developed. The technique is based on the changes in the intensity of scattered light by gold colloids brought into close proximity as a result of bioaffinity reactions. A *model*

immunoassay for anti-IgG was constructed in HTS wells to demonstrate the utility of the wavelength-ratiometric plasmon light scattering-based technique. In this regard, the HTS wells were sequentially coated with gold colloids (20 or 40 nm diameter) and IgG. The immunoassay was completed after the incubation of a solution of anti-IgG-modified gold colloids of the same size in the HTS wells. The bioaffinity reactions between IgG and anti-IgG brought the gold colloids within close proximity (4–24 nm), which resulted in a decrease and an increase in scattered light measured at 550 and 590 nm, respectively. The ratio of these intensity values followed a concentration-dependent trend where a wider dynamic range was observed with 40-nm gold colloids. A LDL of 0.05 μg/mL for anti-IgG was recorded. Wavelength-ratiometric plasmon light scattering measurements offer several advantages in terms of instrumentation simplicity and can be an alternative to the existing technologies commonly used in immunoassays today.

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