SERS Immunoassay based on the Capture and Concentration of Antigen-Assembled Gold Nanoparticles

Arielle Lopez,^a Francis Lovato,^a Soon Hwan Oh,^b Yen H. Lai,^a Seth Filbrun,^a Elizabeth A. Driskell,^b and Jeremy D. Driskell^{a}*

^a Department of Chemistry, Illinois State University, Normal, IL 61790

^b Department of Pathobiology, College of Veterinary Medicine, University of Illinois at Urbana-Champaign, Urbana, IL 61802

*corresponding author: jdriske@ilstu.edu; (p) 309-438-2663; (f) 309-438-5538

ABSTRACT

A simple, rapid, and sensitive immunoassay has been developed based on antigen-mediated aggregation of gold nanoparticles (AuNP) and surface-enhanced Raman spectroscopy (SERS). Central to this platform is the extrinsic Raman label (ERL), which consists of a gold nanoparticle modified with a mixed monolayer of a Raman active molecule and an antibody. ERLs are mixed with sample, and antigen induces the aggregation of the ERLs. A membrane filter is then used to isolate and concentrate the ERL aggregates for SERS analysis. Preliminary work to establish proof-of-principle of the platform technology utilized mouse IgG as a model antigen. The effects of membrane pore diameter and AuNP size on the analytical performance of the assay were systematically investigated, and it was determined that a pore diameter of 200 nm and AuNP diameter of 80 nm provide maximum sensitivity while minimizing signal from blank samples. Optimization of the assay provided a detection limit of 1.9 ng/mL, 20-fold better than the detection limit achieved by an ELISA employing the same antibody-antigen system. Furthermore, this assay required only 60 minutes compared to 24 hours for the ELISA. To validate this assay, mouse serum was directly analyzed to accurately quantify IgG. Collectively, these results demonstrate the potential advantages of this technology over current diagnostic tests for protein biomarkers with respect to time, simplicity, and detection limits. Thus, this approach provides a framework for prospective development of new and more powerful tools that can be designed for point-of-care diagnostic or point-of-need detection.

KEYWORDS: Immunoassay; Surface-enhanced Raman Spectroscopy; Dynamic Light Scattering; Gold Nanoparticles

1. Introduction

Sensitive point of care/need (POC) platforms are the next critical step in the advancement of medical diagnostics, environmental analysis, food safety, and defense [1]. Rapid results and convenience of these platforms will facilitate more effective disease management or enable rapid response to an immediate bio-warfare threat. Lateral flow immunoassays (LFAs) have found the most commercial success and are the most widely used format for POC diagnostics because they provide rapid results and convenience at a low cost [2]. However, LFAs also have a number of weaknesses and opportunities for improvement in POC diagnostics exist [2].

Standard LFAs are typically based on a colorimetric readout and suffer from two major limitations: poor detection limits and lack of multiplexed detection. It is well-established that LFAs do not afford the same detection limits as laboratory-based assays [3, 4]; thus, these POC platforms often suffer from a high rate of false negatives. This is exemplified by the CDC guidelines for primary care providers which recommend secondary testing of any sample that produces a negative result by a LFA. Given the impact of poor detection limits and potentially severe consequences, numerous works toward improving detection limits have been reported, including sample concentration via isotachopheresis [4] and more sensitive readout strategies based on enzymes [5], chemiluminescence [6] and fluorescence [7-9]. However, each of these improvements to achieve better detection limits sacrifices the low cost, “one-step” procedure, and/or visual readout of traditional LFAs.

In general, LFAs are not amenable to multiplexed detection and separate LFA devices are needed to test for each antigen/target of interest. Thus, the development of a multiplexed LFA would potential reduce sample consumption and further reduce costs. One approach that has been developed for multiplexed detection in a LFA is based on quantum-dot barcodes [10].

While effective for multiplexed detection, portable instrumentation is not readily available for POC testing.

Recently developed SERS-based immunoassays provide ultralow levels of detection and multiplexed detection [11-20], thereby overcoming the limitations of LFAs. Moreover, numerous affordable handheld Raman spectrometers are available to support the development of SERS-based POC diagnostics. However, the format of SERS-based assays are often time-consuming and labor-intensive; thus, not suitable for POC diagnostics [21]. To leverage the benefits of SERS-based detection, POC formats are needed. Toward this end, efforts include the development of SERS-LFA [22] and a one-step homogeneous assay [16]. Most recently, our group has developed a rapid SERS immunoassay in which antibody antigen binding is accelerated via active transport through an antibody-modified gold filter [17]. However, in that work, the multi-step procedure is not ideal for POC diagnostics and the binding efficiency was determined to be flow rate dependent.

Herein we developed a rapid, simple, and sensitive SERS assay in which antigen mediates the aggregation of modified gold nanoparticles (AuNP) and the SERS-active AuNP aggregates are captured and concentrated on a nanoporous membrane. The assay platform was developed for mouse IgG as a model antigen and utilized commercial off the shelf components. The analytical figures of merit of the optimized SERS assay, including sensitivity, detection limit, and specificity, were directly compared to an established ELISA, and the platform was successfully applied to the analysis of IgG in whole mouse serum. The commonplace components as well as the modular design of the assay enable a straightforward approach to POC testing of a variety of analytes.

2. Experimental

2.1. Reagents and materials

Gold nanoparticles (AuNP; 60 nm, 2.6×10^{10} particles/mL and 80 nm, 1.1×10^{10} particles/mL) were purchased from Ted Pella, Inc (Redding, CA). Sodium borate buffer (50 mM, pH 8.96), phosphate-buffered saline (PBS; 10 mM, pH 7.4), goat anti-mouse IgG polyclonal antibody, mouse IgG (1.0 mg/mL), rabbit IgG (11.3 mg/mL), and human IgG (2.5 mg/mL) were obtained from Thermo Scientific (Rockford, IL). Bovine serum albumin (BSA) and 4-nitrobenzenethiol (NBT) were purchased from Sigma-Aldrich (St. Louis, MO). Mouse serum was acquired from Santa Cruz Biotechnology. Polycarbonate track etched (PCTE) membrane filters with a nominal pore diameter of 0.2 μm were purchased from Millipore. All chemicals were analytical grade. All aqueous solutions were prepared with NANOpure deionized water from a Barnstead water purification system (18 M Ω).

2.2. Preparation of Extrinsic Raman Labels (ERLs)

Extrinsic Raman labels (ERLs) were prepared by co-adsorbing a Raman reporter molecule and an antibody onto gold nanoparticles according to a previously reported procedure, with slight modifications [20]. In a microcentrifuge tube, 1000 μL of AuNP was combined with 40 μL 50 mM borate buffer, 20 μL 1 mM NBT, and 10 μL goat anti-mouse IgG polyclonal antibody. The mixture was allowed to incubate overnight on the benchtop at room temperature. The ERL suspension was centrifuged at 5000g for five minutes. The supernatant was decanted and the AuNP pellet was resuspended in 1000 μL of 2 mM borate buffer with 1% BSA (pH 8.6). This centrifugation/resuspension process was repeated twice more, with the final suspension in 2 mM borate buffer containing 1% BSA and 1% NaCl at pH 8.6.

2.3. Antigen-mediated assembly and capture of ERLs

Mouse, rabbit, and human IgG standard solutions were prepared through serial dilutions using PBS. Antigen (10 μ L) was introduced to a suspension of ERLs (90 μ L) and allowed to incubate at room temperature for one hour. The membrane filters were treated with 200 μ L of 1% BSA for 20 min to block non-specific binding. A custom built blotting apparatus consisted of two small Teflon sheets (13 $\times$ 39 $\times$ 3 mm, each), a solid lower sheet to serve as the base and an upper sheet with a 4.5 mm hole. Four sheets of blot paper were layered on the Teflon base and the membrane was placed on top, BSA treated side up. A rubber O-ring (4-mm inner diameter) was placed between the membrane and top Teflon sheet, lining up with the hole as the top sheet was pressed down with gentle pressure so as not to crease the filter.

Using a micropipette, 80 μ L of the ERL-sample mixture were blotted onto the filter membrane, then washed with 120 μ L of 2 mM borate buffer with 1% NaCl unless otherwise stated via micropipette. ERL solution was allowed to completely flow through the filters by capillary action before introducing the buffer wash. Filters were allowed to dry before SERS analysis.

2.4. ELISA

Dilutions of mouse IgG were added 100 μ L per well in a 96 well Immulon 2HB microtiter plates and incubated 18 hours at 4 $^{\circ}$ C. Wells were washed and then blocked with Super Block. Goat anti-mouse IgG antibody was applied to pre-adsorbed plates at a dilution of 1:100. After washing, HRP labelled rabbit anti-goat IgG (Thermo-Fisher) was applied as the secondary antibody (1:12000) to all wells. After the final rinsing step, 1-step Turbo TMB ELISA (Thermo-Fisher) was added for the substrate and the reaction was stopped with 1 M H₂SO₄. Absorbance

was measured at 450 nm on a 96-well format plate reader (SpectraMax Gemini reader and SpectraMax software). Absorbance readings against mouse IgG concentration were plotted.

2.5. Instrumental characterization and assay readout

Dynamic light scattering (DLS) was used to measure the hydrodynamic diameter of aggregated ERLs upon the addition of antigen. Measurements were taken with a BI-90 Plus (Brookhaven Instrument Corporation, New York) equipped with a 658-nm laser and an avalanche photodiode detector (Perkin) with the detection angle of backscattered light set to 90°. The mean hydrodynamic diameter for each sample was calculated from three, 30-s runs using MAS OPTION particle sizing software to perform cumulants analysis.

SERS measurements were performed using an Enwave Optronics, Inc. ProRaman-L-785B Analyzer, using a diode laser at the fixed wavelength 785 nm. The laser was focused to a 100- μ m spot with a 10 $\times$ objective and the power was adjusted to 30 mW at the sample surface. The instrument was equipped with a high-sensitivity CCD thermoelectrically cooled to -60 °C. Membrane filters were placed onto a rotating sample stage and the rotor was tuned to a speed of approximately 2.5 rotations per second during 10-s spectral acquisitions. Five spectra were collected for each sample and the spectra were averaged. Sample rotation/rastering during spectral acquisition increases sampling area and effectively averages signal from heterogeneous locations. This sampling method has been established as an effective means of improving signal reproducibility, particularly non-uniform samples such as randomly spaced ERL aggregates on a membrane [23].

3. Results and discussion

3.1. Assay design

The design of the SERS-based aggregation immunoassay is presented in Fig. 1. Central to this platform is the ERL reagent, which upon mixing with the sample, undergoes antigen-mediated aggregation. After incubation of the ERL reagent and sample, the mixture was drawn through a PCTE membrane via capillary action. The pores of the membrane filter were selected such that ERL aggregates formed in the presence of antigen were too large to pass through the membrane and therefore, were captured on the membrane surface. However, excess single ERLs, or in the absence of antigen, all ERLs, freely passed through the filter pores.

To establish proof-of-principle, a model antibody-antigen system was employed. ERLs were synthesized with a goat anti-mouse IgG polyclonal antibody and mouse IgG served as the targeted antigen. The following sections detail the optimization of this platform, which includes characterizing antigen-mediated assembly of ERLs and elucidating the effects of membrane pore size, ERL size, and sample volume on assay performance. Using optimized parameters we established the analytical figures of merit and applied the assay to the analysis of a serum sample.

3.2. Characterization of antigen-mediated assembly of ERLs

As a first step in establishing this SERS-based immunoassay platform, antigen-mediated assembly of ERLs was characterized. Toward this end, 90 μL of ERLs consisting of 60 nm AuNP modified with a co-immobilized layer of NBT and goat anti-mouse IgG antibody were mixed with 10 μL of 5 $\mu\text{g/mL}$ mouse IgG. Dynamic light scattering was utilized to monitor the formation of ERL aggregates as a function of time (Fig. 2). The initial mean hydrodynamic diameter of the ERL suspension measured 82 nm, consistent with the expected diameter resulting from a 60 nm AuNP core and an adlayer of IgG antibody [24]. Moreover, absorbance and fluorescence based protein analysis of ERLs further confirms the adsorption of 362 ± 64

antibodies per AuNP, which is consistent with a theoretical close-packed adlayer of antibody (Supplementary Material; Fig. S1). As shown in Fig. 2, the mean hydrodynamic diameter increased after introduction of the target mouse IgG antigen, reaching a maximum size increase of ~100 nm after 90 minutes of incubation. These aggregation kinetics are consistent with other groups reporting that 80% of aggregation is completed in ~60 minutes [25]. Importantly, the antibody-antigen binding time in this homogeneous format significantly outperforms that reported for heterogeneous binding and is approaching practical timescales for point-of-care diagnostic assays [26, 27]. To demonstrate that the increase in ERL size is due to specific antigen interactions and not due to salt-induced aggregation [28], ERLs were also mixed with PBS. Fig. 2 shows that no significant change in ERL size is measured via DLS over 2 hours in a high ionic strength environment and confirms the stability of the ERLs.

ERLs were mixed with standard solutions of antigen ranging from 0.5-50,000 ng/mL to evaluate concentration dependent aggregation. Fig. 3 presents the mean hydrodynamic diameter as a function of antigen concentration with the corresponding size-distribution histograms provided as supplementary material (Fig. S2). The shape of the concentration dependent curve is similar to results previously reported for DLS-based aggregation assays [29, 30]. At concentrations below 50 ng/mL, a change in mean diameter is not detected. At this low concentration range, ERL aggregates may be present; however, the mean diameter is dominated by the excess ERLs in suspension. As the antigen concentration increases, an increase in mean diameter is detected as more aggregates are formed and less free ERLs are present. At an optimal antigen concentration, e.g., 5000 ng/mL, the ratio of ERLs and antigen are similar to result in the formation of large multi-ERL aggregates and the maximum measured mean diameter. This is referred to as the hook point. At concentrations greater than that of the hook point, e.g., >5000

ng/mL, the antigen saturates the binding sites of the ERLs to yield fewer aggregates and the measured mean diameter decreases from that of the hook point.

It may be speculated that plasmonic coupling between the AuNP cores of the antigen-assembled ERLs could facilitate direct detection via SERS. To test this premise, SERS spectra were collected from the antigen-mediated ERL aggregated suspensions (Supplementary Material; Fig. S3). As is evident, no SERS bands are observed. It is well-established that plasmonic coupling between adjacent nanoparticles only supports SERS when the interparticle spacing is on the order of a few nanometers or less [31, 32]. This result supports a recent finding by our group that a hydrated antibody layer surrounding a AuNP is sufficiently large to inhibit plasmonic coupling between aggregated ERLs and prevent the SERS phenomenon [33].

3.3. Filtration and SERS detection of assembled ERLs

In an effort to concentrate aggregated ERLs and reduce interparticle spacing via dehydration, the ERL-sample mixtures were passed through a polycarbonate track etched membrane (PCTE) with 200 nm diameter pores via capillary action. The membranes were allowed to dry and then analyzed via SERS to acquire spectra from ERL aggregates. SERS spectra, characteristic of the ERL (4-NBT), were readily detected providing evidence of effective capture, concentration, and dehydration of the ERL aggregates (Supplementary Material; Fig. S4). The strongest SERS band centered at 1338 cm^{-1} was due to the symmetric nitro stretch and its intensity was used to quantify the capture of ERL aggregates. The SERS intensity as a function of antigen concentration is plotted in Fig. 4. The SERS calibration curve is similar in shape to the DLS calibration curve. This is expected since only aggregates are captured on the membrane while excess single ERLs are anticipated to pass through the pores. However, there are also inconsistencies with the DLS curve. For example, no SERS signal is detected at or

below an antigen concentration of 50 ng/mL. In this low concentration region of the curve the ERLs are in much greater excess than antigen and the antigen binding sites are saturated with bound ERLs. To a first approximation, a simple geometric analysis suggests that sterics prevents many large ERLs from binding to one antigen given the relatively small size of the antigen, e.g., mouse IgG, compared to the ERL. (Supplementary Material; Fig. S5). It is likely that many small aggregates such as dimers are present at low antigen concentrations; however, the membrane pore is too large to effectively capture the dimers of ERLs fabricated with 60 nm AuNP cores.

3.4. Optimization of ERL size

In an effort to more effectively capture small aggregates or dimers that form when low antigen concentrations mediate the assembly of ERLs, the effect of increasing the AuNP size was investigated. ERLs were prepared with 60 nm and 80 nm AuNP cores. These ERLs were then mixed with low concentrations of mouse IgG, e.g., 0, 0.5, 5, and 50 ng/mL, incubated for 30 min, and passed through a 200 nm pore size PCTE membrane. SERS spectra were collected from the surface of these membranes and the intensity of the 1338 cm^{-1} band is plotted in Fig. 5. Signal was detected only for the 50 ng/mL sample when 60 nm ERLs were employed; however, a concentration dependent signal was detected for all tested concentrations when 80 nm ERLs were used. These data suggest that small aggregates did form at these low antigen concentrations; however, the collective aggregate is sufficiently small to be captured by the 200 nm pore membrane when using 60 nm AuNP to prepare the ERLs. In contrast, the 80 nm AuNP is sufficiently large that small aggregates of these ERLs are effectively captured by the 200 nm pore membrane.

It should be noted that decreasing the pore size is an alternative means to improving the capture efficiency of small ERL aggregates. PCTE membranes with a pore diameter of 100 nm

are also commercially available and were evaluated. However, significant background signal was observed even in the absence of antigen, suggesting that single, non-aggregated ERLs are too large to pass through the 100 nm pore (data not shown). Previous work has demonstrated that 60 and 80 nm AuNP yield the largest SERS enhancement [17, 34]; thus, no effort was made to reduce the AuNP size to be compatible with the 100 nm pore membranes.

3.5. Effect of sample volume

In an effort to increase the analytical signal, the effect of sample volume passed through the membrane was investigated. ERLs (90 μ L) were mixed with 500 ng/mL mouse IgG (10 μ L) as a positive control or PBS (10 μ L) as a negative control, and 20, 40, or 80 μ L volumes of these sample/ERL mixtures were passed through separate membranes. The SERS intensity as a function of sample volume is plotted for both the positive and negative samples in Fig. 6. The signal for the positive control sample increased with a corresponding increase in sample volume. No signal was detected for 20 μ L of the negative controls sample, while the 40 and 80 μ L negative control samples each produced a signal just above the baseline noise. It is anticipated that larger volumes would result in greater analytical signal to further improve the analytical sensitivity. However, 80 μ L was used in subsequent assays as it is a good balance of analytical signal and reasonably small sample volumes.

3.6. Analytical performance- detection limit, reproducibility, specificity

The analytical performance of the SERS assay, including reproducibility, detection limit, and specificity were evaluated using optimized conditions, e.g., AuNP size, membrane pore diameter, and sample volume. Four assays for mouse IgG were performed on two different days using four independently prepared ERLs and four independently prepared sets of mouse IgG

standard solutions. The average signals and associated standard deviations are plotted as a function of antigen concentration in Fig. 7. The inter-assay reproducibility is acceptable as is evident in the calibration curve error bars. The percent relative standard deviation was dependent on the antigen concentration and ranged from 9% to 49%. The largest %RSD was obtained for the highest antigen concentration and likely results from the formation of many large aggregates of varying sizes that are unevenly distributed on the membrane.

The detection limit was calculated from the mean calibration data of the four independent assays and was defined as the antigen concentration that produces a signal equal to the blank signal plus three times the standard deviation of the blank signal. The optimized assay provided a detection limit of 1.9 ng/mL (~13 pM) for mouse IgG. It is important to note that the antibody binding affinity has a significant effect on immunoassay detection limit; thus, it is not appropriate to compare this detection limit to the detection limit of other assay platforms evaluated using different antibodies. Thus, to accurately assess the detection limit of this platform, an ELISA was performed using the same antibody-antigen system. The calibration curve for the ELISA analysis of mouse IgG is included as Supplementary Material (Fig. S6) and ELISA provided a detection limit of 35 ng/mL. This direct comparison of the immunoassays establishes that the SERS platform is capable of detecting low levels of antigen and provides two orders of magnitude improvement in detection limit compared to a well-established platform.

The selectivity of the SERS assay was evaluated against off-target human and rabbit IgG. ERLs, constructed with anti-mouse IgG antibodies, were mixed with mouse IgG (500 ng/mL), rabbit IgG (50 μ g/mL), human IgG (50 μ g/mL), or PBS; allowed to incubate 60 min; and passed through membrane filters. Each sample was run in duplicate and SERS spectra were collected from the filters (Fig. 8). The signal for the rabbit IgG (154 ± 7 cts/s) and human IgG (265 ± 47

cts/s) off-targeted controls were just above the noise in the spectral baseline even though the IgG levels were 100 times greater than that of the mouse IgG which provided a signal of 8605 ± 954 cts/s. These data clearly demonstrate high specificity of the assay for the target antigen.

3.7. Application to the analysis of mouse serum

The performance of the developed SERS immunoassay was validated by determining the concentration of IgG in whole mouse serum. IgG levels in mouse serum is in the range of 5–13 mg/mL, which is outside of the dynamic range of this SERS assay. Therefore, the mouse serum was diluted 1:25,000 in PBS to reduce the IgG level to an appropriate concentration, and the diluted serum was analyzed using the developed SERS assay (Fig. S7). The serum IgG concentration was determined to be 10 ± 3 mg/mL, based on a best fit of the calibration data in Figure 7 to a one-site ligand binding curve. This result falls within the accepted range and is similar to the value previously measured by our group [17]. Importantly, these results demonstrate that a more complex background does not diminish the performance of the SERS assay by increasing nonspecific binding or interfering with antibody-antigen binding.

4. Conclusions

Herein we demonstrate a simple, rapid, and sensitive strategy for protein POC detection. Using mouse IgG as a model antigen, we have developed an optimized a SERS-based immunoassay that affords the speed of a homogeneous assay and the sensitivity of a heterogeneous assay. Direct comparison to an ELISA establishes that this novel approach significantly reduces assay time (24 h versus 60 min) and lowers the detection limit (35 ng/mL versus 1.9 ng/mL), without sacrificing specificity. Furthermore, this assay has been validated by accurately quantifying IgG levels in serum without requiring sample pretreatment other than dilution. This platform could easily be implemented for the detection of a variety of antigens

with the appropriate selection of antibody to construct the ERLs. In addition to the ease of use, speed, sensitivity, and flexibility, SERS-based detection affords the potential for multiplexed detection [20, 35, 36]. This novel platform provides a new opportunity to extend the application of immunoassays from the research laboratory to a number of on-site analyses, including POC diagnostics and biodefense.

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Figure Captions

Figure 1. Illustration of the assay including (1) sample introduction and antigen-specific aggregation of the ERLs and (2) passage of the ERL/sample mixture through a porous membrane to capture and concentrate the aggregate's for subsequent SERS analysis.

Figure 2. Time dependent aggregation of ERLs upon the addition of mouse IgG (positive control; black circles) or PBS (negative control; red squares) measured as an increase in hydrodynamic diameter via DLS.

Figure 3. Aggregation of ERLs as a function of mouse IgG concentration measured as an increase in hydrodynamic diameter via DLS. The size increase is relative to the size of the ERLs for the negative control sample. Each data point is the average of three measurements and the error bars represent the standard deviation.

Figure 4. Calibration curve for the SERS assay employing 60 nm ERLs. The SERS intensity of the symmetric NO₂ stretch at 1338 cm⁻¹ is plotted as a function of mouse IgG concentration. Each data point is the average of five measurements and the error bars represent the standard deviation.

Figure 5. Effect of ERL size. The SERS intensities of the symmetric NO₂ stretch at 1338 cm⁻¹ are compared for assays employing ERLs prepared with 60 nm and 80 nm AuNP. Error bars represent the standard deviation of three independent assays.

Figure 6. Effect of assay volume. The SERS intensities of the symmetric NO₂ stretch at 1338 cm⁻¹ are compared for assays in which 20, 40, or 80 μL of the ERL/sample mixture were passed through the membrane filter. Error bars represent the standard deviation of three independent assays.

Figure 7. Calibration curve for the SERS assay employing 80 nm ERLs. The SERS intensity of the symmetric NO₂ stretch at 1338 cm⁻¹ is plotted as a function of mouse IgG concentration. Each data point is the average of four independent assays with five measurements recorded for each independent assay. The error bars represent the standard deviation in signal among the independent assays.

Figure 8. Evaluation of assay specificity. ERLs prepared with anti-mouse IgG were used to analyze samples containing mouse IgG, rabbit IgG, human IgG, and PBS.

figure 1

1. Antigen-mediated assembly of ERLs

2. Filtration of ERL aggregates

figure 2

figure 3

figure 4

figure 5

figure 6

figure 7

figure 8

Graphical abstract

Negative

Positive