Multimeric Rhodamine Dye-Induced Aggregation of Silver Nanoparticles for Surface-Enhanced Raman Scattering

Matthew D. Bartolowits,‡,#,∥ Meiguo Xin,‡,⊥ Dino P. Petrov,† Thomas J. Tague, Jr.,§ and Vincent Jo Davisson*,†

†Department of Medicinal Chemistry and Molecular Pharmacology, Purdue University, West Lafayette, Indiana 47907, United States
‡Amplifi Sciences, LLC, 1281 Win Hentschel Blvd., West Lafayette, Indiana 47906, United States
§Bruker Optics, Inc., 19 Fortune Drive, Billerica, Massachusetts 01821, United States

ABSTRACT: Isotopic variants of Rhodamine 6G (R6G) have previously been used as a method of multiplexed detection for Surface Enhanced Raman Spectroscopy (SERS), including protein detection and quantification. Challenges exist, however, with producing long-term stable SERS signals with exposure to silver or gold metal surfaces without the use of additional protective coatings of nanomaterials. Here, novel rhodamine “dimers” and “trimers” have been created that demonstrate a higher avidity for metal nanoparticles and induce aggregation to create plasmonic “hotspots” as indicated by enhanced Raman scattering in situ. These aggregates can be formed in a colloid, on surfaces, or membrane substrates such as poly(vinylidene fluoride) for applications in biosciences. The integrity of the materials and Raman signals are maintained for months of time on different substrates. These dye materials should provide avenues for simplified in situ generation of sensors for Raman-based assays especially in settings requiring highly robust performance.

INTRODUCTION

Rhodamine 6G (R6G) has been widely utilized as a fluorescent probe and laser dye due to its outstanding optical properties (i.e., large molar extinction coefficient and fluorescence quantum yield). In previous work, isotopic variants of R6G derivatives have been synthesized and applied for quantitative Surface Enhanced Raman Spectroscopy (SERS) and SERS-based protein detection. The distinct Raman signals of D0-R6G and D4-R6G (Figure 1A) at 610 and 600 cm⁻¹, respectively, provide the basis for the accurate quantification of protein concentration, and this isotopologue strategy of substituting hydrogens for deuterium atoms on R6G has been employed for single molecule detection. Standardization methods using isotope dilution for enhanced Raman have been demonstrated for samples of biological fluids.

The application of SERS in quantitative bioassays motivates approaches to improve reproducibility and the robustness of molecular assemblies. The development of new bioassays suitable for clinical or point-of-care applications are especially timely. An established approach has been the use of core–shell nanomaterials, as individual reporters often termed nanotags or nanopores. Complementary approaches to arrive at substrates with stable arrays of SERS active gold or silver nanostructures continue to emerge. Several examples include metal functionalized “nanopillars” anchored on silicon wafers, paper dipsticks, and cotton swabs integrated with...
branched nanoantennas, printed gold nanoparticles on plastic substrates, cross-linked Au/Ag nanoparticles in mesoporous aerogels, gold-plated nanoporous membranes, and gold nanoparticles stabilized in filter paper. All of these approaches add complexity to either preparation of substrates or nanomaterials, substrates, or both. The robustness and costs of these methods for translation to commercial products remain to be realized.

An alternative approach is the in situ generation of nanoparticle assemblies capable of enhancing specific Raman signals at the point of analyte detection. A few recent examples have enabled biochemical measurement in complex matrices. However, the broad extensibility of these reported methods is not known at this time. Although dyes like R6G produce reliable Raman signals in silver colloid, the signal instability limits practical applications due to chemical instability of the dye–metal aggregates. Silver and gold nanoparticle aggregation can be induced through the addition of heat or salts to colloidal suspensions, but certain assay formats on different matrices may not be able to take advantage of this effect. To advance the in situ assembly of silver nanoparticle hotspots, the chemical features of dye molecules that promote interactions with silver and gold were employed to increase avidity for the metal surface by linking the dye units.

# RESULTS AND DISCUSSION

The basic strategy involves the covalent linkage of rhodamine molecules based upon previous work for substitution of the 2-carboxylic acid functional group. The symmetrical dimer molecules were synthesized through the linker N,N-dimethyl-1,6-hexanediame (Schemes S1 and S2). The coupling reaction was achieved with high yield (80–90%) using O-(1H-6-chlorobenzotriazole-1-yi)-1,1,3,3-tetramethyluronium hexafluorophosphate (HCTU) to form tertiary amides. Similarly, a rhodamine “trimer” was created using a trivalent, symmetric linker (Figure 1C and Scheme S3). Optical absorptivity of these compounds correlated with the number of dye fragments with the D0 dimer and trimer having molar extinction coefficients at 532 nm of 296 000 and 376 000 M⁻¹ cm⁻¹, respectively, which are both higher than that of R6G (116 000 M⁻¹ cm⁻¹).

The major Raman features of all the rhodamine variants are expected to be similar. However, the chemical properties of the dimer and trimer compounds should be distinct with respect to their interactions with metal nanoparticles. Isotopomeric variants of the dimer molecule were also prepared using the relevant isotopic substitution in R6G free acid based upon previous methods (Scheme S2). Four isotopically labeled variants of the dyme dimers were synthesized—D0, D4, D8, and D12—that are analogous to the substitution patterns of the R6G isotopomers in Figure 1A. Enhanced Raman spectra were obtained using two different substrates: gold-coated glass and PVDF.

The Raman transitions of each of the dimers were compared to R6G and the trimer. Raman shifts at 532, 633, and 785 nm (Figures S1–S3, respectively) indicate similar spectral features for the nondeuterium labeled R6G, D0 dimer and trimer. As expected from the previous work using R6G isotopologues the D4, D8, and D12 dimers showed distinct shifts in the 600 and/or 1350 nm range. Principal component analyses of the spectra also statistically validate these observations (Figures S4). The resulting spectra were of comparable intensity and shifts on the glass slides and PVDF membranes (Figure S5).

The exposure of the silver nanoparticle (SNPs) to the dimer or trimer resulted in visible aggregation at dye concentrations greater than 2.5 μM or 625 nM, respectively (Figure S6). R6G did not cause observable aggregation even at greater than 10-fold higher concentrations. The same effect was observed when using 20 or 100 nm silver nanoparticles or 50 nm gold nanoparticles (data not shown). Additionally, visible aggregation (noted by dark coloration and opaqueness) was observed even when dimer or trimer dyes were dried on either glass slides or PVDF before exposure to SNPs on the surface. These characteristics were not observed with R6G under any of the conditions tested. Furthermore, when the D0 dimer and R6G were added to SNPs at the same concentration (10 μM), the enhanced Raman for the dimer showed more than 10-fold greater signal-to-noise ratio relative to R6G (Figure 2A). When the concentration of R6G is taken to much higher concentrations (>100 μM), there begin to some visible signs of aggregation, but not with the sharp transition observed with the dimer and no increases in Raman signals. Additionally, extending the period of exposure of the dyes to the nanoparticle to greater than 1 h had no noticeable effect on the resulting levels of aggregation and Raman signal.

A competition experiment was executed by mixing R6G and D8 dimer to assess if there are observed differences in the association with hotspots as detected by enhanced Raman scattering. Aqueous solutions of D8 dimer and R6G were mixed in various proportions from 1:20 to 5:1, respectively, before exposure to constant amounts of SNPs (Figure 2B). At equivalent molar concentrations, the dimer signal (603 cm⁻¹) was 10-fold greater in intensity than that of R6G (615 cm⁻¹), based on signal-to-noise ratios of 238.9 and 24.6, respectively. The 603 and 615 cm⁻¹ peaks had the closest to equal signals when R6G was 10-fold higher in concentration than the D8 dimer. These observations are consistent with the distinct signal intensity SER(R)S for the D0 dimer.

Since aggregation of SNPs has been associated with longer-term stability, a comparison of 10 μM samples of R6G and D0 dimer with colloidal SNPs were spotted on PVDF membrane and a gold-coated glass slide. The enhanced Raman signals were measured at 532 nm using identical conditions for both samples on each matrix at 4 h and 90 d.
after depositing on the surfaces, with the dried PVDF membrane stored at room temperature, without specific protection from light. After the period of storage, the Raman spectra of the dimer had not significantly changed on either PVDF or glass (Figure 3A,B), while the signal of R6G had nearly completely been lost (Figure 3C). As confirmation that this observation was not dependent on visible aggregation (see Figure S6), a 313 nM colloidal sample of D0 dimer—well below the level of visible aggregation—was spotted on PVDF. In accordance with the previous observation of D0 dimer, after 90 d at room temperature, the Raman signal was not diminished (Figure S7).

In accordance with previous studies that have utilized isotopically labeled variants of fluorescent dyes, deuterium-labeled dimers should have distinct spectral shifts. To demonstrate these features, the D0 and D8 dimers were combined in increasing ratiometric mixtures in water before deposition onto gold-coated glass (Figure 4). The results show that the two isotopic variants of the dye dimer have unique spectral characteristics that can be exploited, as has been done in previous studies with Raman-active dyes, to enable relative quantification of one dye compared to the other.

The unique properties of the dyes described here could find application in bioassays. To enable general protein labeling by these compounds, variants of the D0 and D8 dimers were synthesized with a trivalent, symmetrical linker containing an NHS ester functional group (Figure 5A and Scheme S4).9

Generic goat antimouse IgG was labeled with the modified dimers. The extent of labeling was determined by the ratio of absorbances at 280 and 532 nm. A feasibility test for the detection of the IgG involved deposition onto low-fluorescence PVDF membrane, which is a generally useful protein binding substrate. The entire membrane was then exposed to PROTOGOLD gold staining solution (Ted Pella, Inc.), which generates Ag0 in situ. The deposited dye-labeled IgG could then be observed using chemical imaging with a Raman microscope down to sub-picogram levels (Figure 5B). Taken together with the fact that mixtures of isotopically labeled dyes can be used for purposes of quantification, the dye dimer-labeled IgG offers utility for common workflows involving antibody-based detection of bioanalytes on membranes. The assays could be performed with basic metal “staining” reagents, circumventing the need to formulate more sophisticated materials, such as coated nanoparticles.

To further demonstrate the feasibility of such an assay, solutions of human-derived serum albumin ranging from 2 nM to 2 pM were created in varying concentrations with phosphate-buffered saline (PBS) (pH 7.4). Strips (0.5 × 3 cm) were cut from low-fluorescence PVDF membrane and submerged into the solutions containing different concentrations of human serum albumin. The protein was probed by exposing the strips to mouse anti-human serum albumin monoclonal IgG, followed by exposure to secondary antibody composed of D0 dimer-labeled goat antimouse IgG. The PVDF strips were then treated with PROTOGOLD gold stain and L1 silver stain, before analysis by Raman spectroscopy at 532 nm. From the results, albumin present at a concentration of 2 pM in PBS could be detected with a

Figure 3. Stability of Raman signal over time. (A) Spectra of the D0 dimer were obtained on PVDF (A; green line) or a gold-covered glass slide (B; black line) at 532 or 785 nm, respectively. The two samples were assessed after 90 d at ambient temperature without protection from light using identical measurement conditions (green dotted line in A; red dotted line in B). Samples were prepared by mixing 10 μM D0 dimer with SNPs in water. Removal of the signal backgrounds (right panels in A and B) reveals that there was not substantial change in peak shape or intensities of the Raman signals even after 90 d of exposure. (C) When compared to the monomer, the D0 dimer (left panel) maintained a stable signal over time, while the R6G signal (right panel) is nearly completely absent.

Figure 4. Feasibility of using dimers for quantification. The D0 and D8 dimers were combined in ratiometric mixtures in water, were exposed to SNPs, and were then deposited on gold-coated glass. Consistent with prior studies involving isotopically labeled variant dyes, deuterium-labeled dimers have distinct Raman shifts, compared to the nondeuterated variant.

Figure 5. Dye-labeled IgG can be detected at low levels using basic staining procedures. (A) Variants of the D0 and D8 dimers were synthesized containing a trivalent linker containing an NHS ester functional group. This enables covalent attachment to protein lysine groups. (*) The locations of deuterium substitution for the D8 dimer. (B) When generic IgG antibody is labeled with the dye dimer(s), and spotted on PVDF, it can be easily detected with Raman to sub-picogram levels using basic Au/Ag staining conditions (see Experimental Section for details).
signal-to-noise ratio (S/N) of 4.4, based on the peak at 615 cm\(^{-1}\); however, these data are not shown, because the large amount of background signal (at that low level of detection) would make it impractical to try to integrate any peaks from the curve. The overall signal intensity and S/N increased in accordance with increased concentrations of serum albumin, with respective S/N ratios of 10.1, 23.4, and 31.4 (Figure 6).

![Figure 6](image-url) Dye-labeled IgG can be used as a probe to detect dilute protein in solution. Strips of PVDF were submerged in solutions of varying concentrations of human serum albumin in PBS buffer. Albumin was then probed for using mouse antihuman albumin, and analyzed by Raman spectroscopy at 532 nm. The resulting spectra were baseline corrected and vector normalized to equivalent scales using OPUS 7.5 software.

While this demonstration is not a complete or optimized analytical method, it does establish the feasibility of a simple “dipstick”-based assay for specific proteins adsorbed onto surfaces. Also note that, at points during the course of experimentation, nanoparticles were exposed to solutions of dyes in either ultrapure water or PBS at pH 7.4, and no effect on the resulting Raman signals’ intensities or stabilities (following exposure to dyes) was observed (data not shown). More acidic or basic solutions have not been tested, but along with more extensive mechanism studies of the aggregates will be an interesting subject for future investigations.

The combination of high Raman scattering dyes with isotopic labeling provides avenues for accurate and quantitative SERS. By leveraging increases in dye avidity for gold and silver nanoparticles exhibited by dimer and trimer rhodamine dyes, the direct applications in surface-enhanced Raman-based assays are enabled. The greater simplicity of the materials and processes and long-term stability of dye–metal aggregates offer a robust approach for \textit{in situ} generation of chemical sensors suitable for field-use and/or point-of-care on low-cost substrates. By synthetic modification, these dyes can be covalently incorporated by bioconjugation or other reactive surfaces typically used for bioanalytical methods such as enzyme-linked immunosorbent assay (ELISA), Western blotting, and microarrays. Along with prior efforts,\(^{29}\) this study establishes isotopically labeled dye dimer bioconjugation reagents for quantitative protein detection using \textit{in situ} generation of SERS active sensors. The extension of these dimeric dyes through chemical modification are anticipated to improve on the utility of SERS for applications such as robust, quantitative immunological assays.\(^{28}\)

### EXPERIMENTAL SECTION

**Synthesis of Dye Dimers and Trimer.** Synthetic details of the isotopically labeled dyes can be found in Supporting Information.

**Sampling and Analysis of Raman Spectra.** Raman spectra were obtained using a SENTERRA I Raman microscope (Bruker Optics, Inc.), with a 20× objective, 532 nm laser source, 5 mW laser power, 0.5 s integration time, and three coadditions. Spectral data were processed and analyzed using OPUS 7.5 software (Bruker Optics, Inc.). Graphs depicting Raman spectra were generated using OriginPro 2015 software.

**General Procedure for Depositing of Dye-Coated Nanoparticles on Glass or PVDF Substrates.** Five microliters (1 mg/mL) of 50 nm diameter silver citrate-capped nanoparticles (nanoComposix, Inc.) were pelleted by centrifugation and washed with ultrapure water three times. The pelleted SNP’s were dispersed into 20 μL of each of the respective dyes at equal concentrations (10 μM) in ultrapure water for 30 s. One microliter of the colloids was spotted onto a gold-coated microscope slide and allowed to air-dry. PVDF membranes were used as a second test matrix using the same sampling procedure.

**Labeling of IgG with Raman-Active Dyes.** One hundred microliters of goat antimouse IgG (2.12 mg/mL; Abcam, Plc.) was transferred to an Amicon Ultra 0.5 mL spin tube (MilliporeSigma), followed by 300 μL of 50 mM NaHCO\(_3\) (pH 9.0). The tube was centrifuged at 13 000g for 10 min, and 400 μL of additional 50 mM NaHCO\(_3\) (pH 9.0) was added. The tube was centrifuged and 50 μL aliquots of the supernatant were pipetted into two separate 500 μL Eppendorf tubes. Ten equivalents of either D0 or D8 NHS ester dimer (2 μM in 50 mM NaHCO\(_3\), (pH 9.0)) were added, and the solution was incubated at room temperature for 1 h, after mixing. The solutions were then transferred into two Amicon Ultra 0.5 mL spin tubes, followed by the addition of 300 μL of PBS at pH 7.4 with 0.1% Tween-20 (PBST), and centrifuged at 13 000g for 7 min. Four hundred microliters of additional PBST buffer was added, and the tubes were again centrifuged for 7 min; this step was repeated twice more, until the washing process was colorless. The solutions were then transferred to 500 μL Eppendorf tubes and diluted to a final volume of 100 μL using PBST. Concentrations of IgG were assessed via BCA.

**Generating Raman Spectra of Dye-Labeled IgG.** Raman spectra of dye dimer-labeled IgG were obtained using low-fluorescence, 0.45 μm pore size PVDF membrane (Bio-Rad Laboratories, Inc.). Solutions of dye-labeled IgGs were first prepared in ultrapure water; 2 μL of the samples were deposited on PVDF presoaked with methanol, and the membrane was allowed to air-dry for 20 min in the dark. At that time, the membrane was exposed to PROTOGOLD gold stain solution (Ted Pella, Inc.) for 3.5 h at room temperature in the dark. The membrane was then washed with ultrapure water and exposed to a mixture of equal volumes of LI silver solutions A and B (Nanoprobes, Inc.) for 15 min in the dark. At that time, the membrane was washed with ultrapure water and allowed to air-dry overnight at room temperature in the dark, prior to Raman analysis.

**Detection of Serum Albumin Using Dye-Labeled IgG.** Solutions of human serum albumin were prepared in PBS buffer at concentrations from 2 nM to 2 pM. Separately, 0.5×3 cm strips were cut from bulk low-fluorescence PVDF...
membrane. The strips were submergered in the different albumin solutions and then agitated at room temperature for 30 min; they were then removed from the albumin solutions, and excess liquid was blotted away. Following subsequent washing of the PVDF membrane with PBST buffer, and incubation of the strips in blocking buffer (3% BSA in PBST) for 1 h at room temperature, the strips were exposed to monoclonal mouse antihuman serum albumin IgG (2.0 mg/mL; Abcam, Plc.) in blocking buffer, washed with PBST, and then exposed to D0 dimer-labeled goat antimouse IgG in blocking buffer. The strips were washed with PBST and ultrapure water and then were allowed to dry at room temperature in the dark. The strips were then exposed to PROTOGOLD gold staining solution for 2.5 h at room temperature in the dark, followed by exposure to LI silver stain solution at room temperature for 10 min in the dark, washed with ultrapure water, and dried.

**ASSOCIATED CONTENT**

* Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.8b02970.

Experimental details and spectroscopic data (PDF)

Primary Raman spectral files (ZIP)

**AUTHOR INFORMATION**

*Corresponding Author*

E-mail: davisson@purdue.edu.

**ORCID**

Vincent Jo Davison: 0000-0003-1182-0007

**Present Addresses**

1amilh BioPharm Group, Cambridge, MA, United States. E-mail: mdbartol@gmail.com. (M.D.B.)

2Department of Food Science and Engineering, Foshan University, 528000, Guangdong, China. E-mail: meiguo@fusu.edu.cn. (M.X.)

E-mail: dicrmanp@gmail.com. (D.P.)

E-mail: Tom.Tague@bruker.com. (T.J.T.)

**Author Contributions**

These authors contributed equally.

**Notes**

The authors declare the following competing financial interest(s): V.J.D. and M.D.B. are shareholders in Amplified Sciences, LLC.

**ACKNOWLEDGMENTS**

This work was supported by grants from Millipore, Inc (V.J.D.), the Trask Fund of the Purdue Research Foundation (V.J.D.), and Leidos (V.J.D.). The authors gratefully acknowledge support for shared resources from the Purdue University Center for Cancer Research, NIH Grant No. P30 CA023168. We also wish to thank P. Bhandari for performing the principal component analysis work.

**REFERENCES**

1. Mandalá, M.; Serck-Hansen, G.; Martino, G.; Helle, K. B. The Fluorescent Cationic Dye Rhodamine 6G as a Probe for Membrane Potential in Bovine Endothelial Cells. *Anal. Biochem.* 1999, 274 (1), 1–6.

2. Matsumoto, Y.; Sasaoka, N.; Tsuchida, T.; Fujitaka, T.; Nagao, S.; Ohmoto, T. Fluorescent Dye Rhodamine 6G as a Molecular Probe to Study Drug Resistance of C6 Rat Glioma Cells. *J. Neuro-Oncol.* 1992, 13 (3), 217–222.

3. Martínez Martínez, V.; López Arbeloa, F.; Bañuelos Prieto, J.; López Arbeloa, I. Characterization of Rhodamine 6G Aggregates Intercalated in Solid Thin Films of Laponite Clay. 2 Fluorescence Spectroscopy. *J. Phys. Chem. B* 2005, 109 (15), 7443–7450.

4. Sasai, R.; Iyi, N.; Fujita, T.; Arbeloa, F. L.; Martínez; Takagi, K.; Iroh, H. Luminescence Properties of Rhodamine 6G Intercalated in Surfactant/Clay Hybrid Thin Solid Films. *Langmuir* 2004, 20 (11), 4715–4719.

5. Zhang, D.; Xie, Y.; Deb, S. K.; Davisson, V. J.; Ben-Amotz, D. Isotope Edited Internal Standard Method for Quantitative Surface-Enhanced Raman Spectroscopy. *Anal. Chem.* 2005, 77 (11), 3563–3569.

6. Blackie, E.; Ru, E. C. L.; Meyer, M.; Timmer, M.; Burkett, B.; Northcote, P.; Etchegoin, P. G. Bi-Analyte SERS with Isotopically Edited Dyes. *Phys. Chem. Chem. Phys.* 2008, 10 (28), 4147–4153.

7. Perera, P. N.; Deb, S. K.; Jo Davisson, V. J.; Ben-Amotz, D. Multiplexed Concentration Quantification Using Isotopic Surface-Enhanced Resonance Raman Scattering. *J. Raman Spectrosc.* 2009, 41 (7), 752–757.

8. Knudsen, G. M.; Davis, B. M.; Deb, S. K.; Loethen, Y.; Gudihal, R.; Perera, P.; Ben-Amotz, D.; Davisson, V. J. Quantification of Isotope Encoded Proteins in 2-D Gels Using Surface Enhanced Raman Resonance. *Bioconjugate Chem.* 2008, 19 (11), 2212–2220.

9. Deb, S. K.; Davis, B.; Knudsen, G. M.; Gudihal, R.; Ben-Amotz, D.; Davisson, V. J. Detection and Relative Quantification of Proteins by Surface Enhanced Raman Using Isotopic Labels. *J. Am. Chem. Soc.* 2008, 130 (30), 9624–9625.

10. Zrimsek, A. B.; Wong, N. L.; Van Duyne, R. P. Single Molecule Surface-Enhanced Raman Spectroscopy: A Critical Analysis of the Bialyrate versus Isotopologue Proof. *J. Phys. Chem. C* 2016, 120 (9), 5133–5142.

11. Zakel, S.; Rienitz, O.; Güttler, B.; Stosch, R. Double Isotope Dilution Surface-Enhanced Raman Scattering as a Reference Procedure for the Quantification of Biomarkers in Human Serum. *Analyst* 2011, 136 (19), 3956–3961.

12. Zakel, S.; Wundrack, S.; O’Connor, G.; Güttler, B.; Stosch, R. Validation of Isotope Dilution Surface-Enhanced Raman Scattering (IDERS) as a Higher Order Reference Method for Clinical Measurands Employing International Comparison Schemes. *J. Raman Spectrosc.* 2013, 44 (9), 1246–1252.

13. Li, J.-F.; Zhang, Y.-J.; Ding, S.-Y.; Panneerselvam, R.; Tian, Z.-Q. Core−Shell Nanoparticle-Enhanced Raman Spectroscopy. *Chem. Rev.* 2017, 117 (7), 5002–5069.

14. Yang, J.; Palla, M.; Bosco, F. G.; Rinzевичius, T.; Alstrom, T. S.; Schmidt, M. S.; Boisen, A.; Ju, J.; Lin, Q. Surface-Enhanced Raman Spectroscopy Based Quantitative Bioassay on Aptamer-Functionalized Nanopillars Using Large-Area Raman Mapping. *ACS Nano* 2013, 7 (6), S350–S359.

15. Webb, J. A.; Aufrecht, J.; Hungerford, C.; Bardhan, R. Ultrasensitive Analyte Detection with Plasmonic Paper Dipsticks and Swabs Integrated with Branched Nanoantennas. *J. Mater. Chem. C* 2014, 2 (48), 10446–10454.

16. Wu, W.; Liu, L.; Dai, Z.; Liu, J.; Yang, S.; Zhou, L.; Xiao, X.; Jiang, C.; Roy, V. A. L. Low-Cost, Disposable Flexible and Highly Reproducible Screen Printed SERS Substrates for the Detection of Various Chemicals. *Sci. Rep.* 2015, 5, 10208.

17. Gao, X.; Esteves, R. J. A.; Nahar, L.; Nowaczyk, J.; Arachchige, I. U. Direct Cross-Linking of Au/Ag Alloy Nanoparticles into Monolithic Aerogels for Application in Surface-Enhanced Raman Scattering. *ACS Appl. Mater. Interfaces* 2016, 8 (20), 13076–13085.

18. Penn, M. A.; Drake, D. M.; Driskell, J. D. Accelerated Surface-Enhanced Raman Spectroscopy (SERS)-Based Immunassy on a Gold-Plated Membrane. *Anal. Chem.* 2013, 85 (18), 8609–8617.

19. Ross, M. B.; Ashley, M. J.; Schmucker, A. L.; Singamaneni, S.; Naik, R. R.; Schatz, G. C.; Mirkin, C. A. Structure–Function Relationships for Surface-Enhanced Raman Spectroscopy-Active Plasmonic Paper. *J. Phys. Chem. C* 2016, 120, 20789.
(20) Li, M.; Li, J.; Di, H.; Liu, H.; Liu, D. Live-Cell Pyrophosphate Imaging by in Situ Hot-Spot Generation. *Anal. Chem.* 2017, 89 (6), 3532−3537.

(21) Yin, Y.; Li, Q.; Ma, S.; Liu, H.; Dong, B.; Yang, J.; Liu, D. Prussian Blue as a Highly Sensitive and Background-Free Resonant Raman Reporter. *Anal. Chem.* 2017, 89 (3), 1551−1557.

(22) Xuan, Z.; Li, M.; Rong, P.; Wang, W.; Li, Y.; Liu, D. Plasmonic ELISA Based on the Controlled Growth of Silver Nanoparticles. *Nanoscale* 2016, 8 (39), 17271−17277.

(23) Meyer, M.; Le Ru, E. C.; Etchegoin, P. G. Self-Limiting Aggregation Leads to Long-Lived Metastable Clusters in Colloidal Solutions. *J. Phys. Chem. B* 2006, 110 (12), 6040−6047.

(24) Dong, X.; Gu, H.; Kang, J.; Wu, J.; Yuan, X. Study on Surface-Enhanced Raman Scattering of Phenylalanine Using Different Aggregating Agents for Borohydride-Reduced Silver Colloid. 2009 2nd International Conference on Biomedical Engineering and Informatics 2009, 1−4.

(25) Su, X.; Zhang, J.; Sun, L.; Koo, T.-W.; Chan, S.; Sundararajan, N.; Yamakawa, M.; Berlin, A. A. Composite Organic−Inorganic Nanoparticles (COINs) with Chemically Encoded Optical Signatures. *Nano Lett.* 2005, 5 (1), 49−54.

(26) Brady, C. I.; Mack, N. H.; Brown, L. O.; Doorn, S. K. Self-Assembly Approach to Multiplexed Surface-Enhanced Raman Spectral-Encoder Beads. *Anal. Chem.* 2009, 81 (17), 7181−7188.

(27) Deb, S. K.; Davis, B.; Ben-Amotz, D.; Davisson, V. J. Accurate Concentration Measurements Using Surface-Enhanced Raman and Deuterium Exchanged Dye Pairs. *Appl. Spectrosc.* 2008, 62 (9), 1001−1007.

(28) Wang, Z.; Zong, S.; Wu, L.; Zhu, D.; Cui, Y. SERS-Activated Platforms for Immunoassay: Probes, Encoding Methods, and Applications. *Chem. Rev.* 2017, 117 (12), 7910−7963.