Embedding Raman Tags between Au Nanostar@Nanoshell for Multiplex Immunosensing

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Raman spectroscopy, which has incomparable advantages in multiplex molecular identification due to its intrinsic narrow fingerprint spectrum,[1] suffers from low scattering cross-section. The discovery of surface-enhanced Raman scattering (SERS) phenomenon overcome this limitation,[2] and opens the doors for its application in detecting trace analytes.[3] The major contribution of this enhancement effect comes from the enhanced local electromagnetic (EM) field, due to the excitation of surface plasmon resonances in noble metal nanocrystals, which then radiate a dipolar field leading to redistribution of the local field and a greatly enhanced EM field at specific positions (termed “hot spots”) around the nanocrystal. To maximize SERS enhancement factor, “hot spots” capable of achieving single molecule sensitivity have been generated by forming either random aggregates or molecular linked nanostructures, creating local field strength over one thousand times of the incident EM field.[4] However, this approach tends to lack reproducibility and reliability for practical applications. Recently, creating internal “hot spots” have gathered significant interests, where reporter molecules are embedded in between noble metal layers.[5] The strong coupling between the adjacent gold core and gold shell leads to the incident electromagnetic field being squeezed into the gap,[6] creating enhanced local electromagnetic field and thus SERS signals. These bright SERS-active core@tag@shell nanostructures can be ideal for quantitative SERS analysis, as the reporter molecules are sandwiched between the core and shell, making them stable even when outer surfaces are modified under harsh conditions.

Imunoassay is a commonly deployed method for disease diagnosis, drug discovery, environmental monitoring, and food safety,[7] which requires fast, cost-effective, and reliable results for practical applications. The bright and stable SERS signals from the core@tag@shell nanostructures make them attractive for indirect quantification of bioanalytes in immunoassays, as SERS-based immunoassay techniques possess unique advantages such as high sensitivity, anti-photobleaching, and multiplex detection capability.[8] For reporter molecules exposed on the surface of noble metal nanoparticles as in common SERS tags, the Raman signals could be altered due to changes in the microenvironment or competitive absorption of species used for target-specific antibody modifications.[9] Using hidden reporter molecules embedded at the internal “hot spots” will undoubtedly 1) increase the detection sensitivity by using a brighter SERS tag, and 2) improve SERS tag stability and detection reproducibility.

Herein, we present the synthesis of nanostructures where Raman reporter molecules were embedded in between gold nanostars (AuNSs) and gold nanoshell, and their application in magnetic field assisted multiplex immunosensing for the first time. Compared to gold nanoparticles (AuNPs), AuNSs provide higher SERS enhancement factor due to lighting rod effect, as their sharp tips concentrate EM field significantly.[10] The overall SERS signal from AuNS@tag@shell was more than an order of magnitude stronger than that of similarly prepared AuNP@tag@shell nanostructures. These bright SERS tags were then evaluated in an immunoassay, where antibody labeled immunomagnetic beads (IMBs) were used as capture probes. Results show that the SERS-based immunoassay using AuNS@tag@shell is highly specific with sensitivity down to 0.1 pg mL\(^{-1}\), a four orders of magnitude improvement over similarly designed immunoassay but using AuNP@tag@shell instead. Finally, different reporter molecules with various end functional groups can also be successfully embedded inside the gold nanoshell, and a proof of concept duplex detection has been demonstrated, showing excellent selectivity.

As illustrated in Figure 1a, Au overcoated Au nanostar nanostructures with embedded Raman tag molecules were prepared by using 1,4-benzenedithiol (BDT) labeled Au nanostars as the cores, followed by an Au overlay growth using ascorbic acid (AA) as reducing agent and cetyltrimethyl ammonium chloride (CTAC) as capping agent. Transmission electron microscopy (TEM) and UV–vis absorption spectroscopy were used to characterize the morphological and optical changes associated with this coating process. As seen in Figure 1b,c, TEM images of AuNSs before and after being overcoated showed the growth initiated from AuNS core and expands outward, which effectively decreased the aspect ratio of the branches protruding from the gold core. As a result, the gold nanostar surface plasmon resonance slightly red-shifted first from ≈815 nm after being modified with BDT, and then blue-shifted down to ≈650 nm after the
gold nanoshell coating (Figure 1d). The colloidal stability of the as-made AuNS@BDT@Au was checked by monitoring their solution absorbance over time. As shown in Figure S1 in the Supporting Information, only slight peak broadening was observed for solution kept on shelf for a week, indicating little nanoparticle aggregation. In comparison, when NaCl was added into the solution to intentionally induce aggregation, significant peak broadening and damping were seen. In a similar fashion, AuNP@BDT@Au were also prepared by using AuNP instead of AuNS. UV–vis absorption spectrum and TEM image of these overcoated AuNPs can be found in Figure S2 in the Supporting Information.

The Au overlayer coating can greatly enhance Raman scattering signals from BDT molecules absorbed on AuNS, as shown in Figure 1e, which is expected due to the locally generated “hot spots” at noble metal nanostructure junctions. Assuming the same number of molecules in both structures, the extra enhancement factor is calculated to be ≈20. This is the lower bound of the enhancement factor, as some of the BDT molecules may detach from AuNS surface during the subsequent gold nanoshell growth process. Similarly, SERS intensity was also greatly enhanced for AuNP@BDT@Au over that of AuNP@BDT (Figure S3, Supporting Information). Due to significantly larger electromagnetic field enhancement in AuNS especially at the sharp tips, AuNS@BDT exhibit much higher initial SERS signals than AuNP@BDT, and the overall Raman scattering signal from AuNS@BDT@Au was 2–3 times stronger compared to AuNP@BDT@Au even with the Au nanostructure concentration at an order of magnitude lower, indicating each AuNS@BDT@Au was ≈20–30 times brighter than AuNP@BDT@Au on average. To further prove that the enhanced Raman signal is not simply due to the increased nanoparticle sizes (as larger particles scatter light more strongly), another type of Raman tag molecules 3-fluorothiophenol (3-FTP, vide infra) were adsorbed onto the outer surfaces of AuNS@BDT@Au nanostructures, exhibiting only about 5% of Raman scattering signals of that of BDT molecules (Figure S4, Supporting Information). Again, this is consistent with the estimated 20 times extra enhancement factor brought by the nanoshell overcoating.

With the bright SERS tags made available, Figure 2a depicts the operating principle of the SERS-based immunoassay for
biodetection in an antibody/antigen/antibody sandwich-type configuration. Firstly, the capture antibody (anti-mouse IgG) modified N-Hydroxysulfosuccinimide sodium salt (NHS)-activated IMBs were mixed with a phosphate buffer saline (PBS) solution containing the target antigen (mouse IgG), forming IMBs-anti-IgG/IgG complex. Meanwhile, AuNS@BDT@Au were conjugated with the detection antibody (AuNS@BDT@Au-anti-IgG). Secondly, AuNS@BDT@Au-anti-IgG was bound to IMBs-anti-IgG/IgG though specific antibody-antigen interactions, leading to the formation of the IMBs-anti-IgG/IgG-anti-IgG-AuNS@BDT@Au sandwich immunocomplex. Lastly, the immunocomplexes were characterized by SERS (Figure 2b–d) and TEM (Figure 2e).

The sensitivity and specificity of SERS-based immunosensing were analyzed by performing quantitative immunoassay, with the concentration of mouse IgG antigens varied over a wide range from $10^{-6}$ to $10^{-13}$ g mL$^{-1}$, and a blank control carried out without adding any antibodies. Figure 2b displays the SERS spectra collected at different antigen concentrations together with the blank control. Using the characteristic Raman peak of BDT at 730 cm$^{-1}$ for quantification, a clear signal appeared for antigen concentration as low as 0.1 pg mL$^{-1}$, which became more intense with increasing mouse IgG concentration. In contrast, when using the less bright AuNP@BDT@Au nanostructure as SERS tags, the same immunoassay was only effective down to 1.0 ng mL$^{-1}$ level (Figure 2c), demonstrating about four orders of magnitude higher sensitivity of AuNS@BDT@Au SERS tags over AuNP@BDT@Au nanostructures.

The working calibration curve can be obtained by plotting the measured SERS signal intensity as a function of the known added analyte concentration (Figure 2d). The resulting semilog plot is fitted by a 4-parameter sigmoidal curve, as typically observed in immunoassays such as ELISA. Based on 3σ criterion, the limit of detection (LOD) is at least 0.1 pg mL$^{-1}$ for the AuNS@BDT@Au based immunoassay, and 1 ng mL$^{-1}$ for the AuNP@BDT@Au based immunoassay. The significantly improved detection sensitivity is due to the SERS signal’s nonlinear dependence on analyte concentrations. For example, the average intensity ratio of AuNS@BDT@Au based immunoassay at 730 cm$^{-1}$ over that of AuNP@BDT@Au was about 20, similar to their solution properties. However, the weaker signal of AuNP@BDT@Au made detection below ng mL$^{-1}$ range difficult, while AuNS@BDT@Au based immunoassay showed clearly detectable signal even at 0.1 pg mL$^{-1}$ concentration. This result highlights the importance of preparing bright SERS tags for sensing applications.

The simultaneous detection of multiple analytes in one sample is a critical demand for the development of more effective and facile biological sensing. An important advantage of SERS based immunoassay is their ability to simultaneously detect multiple targets using different SERS-tags as reporting agents, since characteristic Raman scattering peaks are very narrow. For this matter, we have fabricated various SERS-labeled nanoparticles by embedding different Raman active molecules inside the gold shell. Besides BDT, other thiolated molecules with various end functional groups such as 3-FTP, mercaptobenzoic acid (MBA), and 4-nitrobenzenethiol (4-NBT) can be all successfully embedded in between AuNS and gold nanoshell. Figure 3a shows the SERS spectra of the different SERS-labeled nanoparticles with distinct fingerprint peaks, making them useful as unique Raman codes for multiplex detection.

As a proof of concept, simultaneous detection of dual antigen targets was tested using BDT and 3-FTP tagged nanostructures, with their characteristic Raman peaks centered at 730 and 870 cm$^{-1}$ used for the quantitative evaluation of duplex immunoassay. Goat anti-mouse IgG and goat anti-rabbit IgG polyclonal antibodies were conjugated with the AuNS@BDT@Au nanoparticles and AuNS@3-FTP@Au nanoparticles, respectively. In a separate reaction vessel, goat anti-mouse IgG and goat anti-rabbit IgG antibodies were simultaneously conjugated to magnetic beads as capture substrates for immunocomplex formation. Then, mouse IgG and rabbit IgG mixed in the concentration ratios of 1:0, 100:1, 1:1, 1:100, and 0:1 (Figure 3b) were exposed to the mixture of the two Raman tags and capture substrates. Finally, SERS spectrum was taken to perform duplex detection. As shown in Figure 3c, with the mouse IgG concentration decreased, the SERS intensity of BDT at 730 cm$^{-1}$ decreased as well. In the meantime, the rabbit IgG concentration in the mixture was increasing, and the SERS intensity of 3-FTP at 850 cm$^{-1}$ concomitantly increased. More importantly, in the absence of rabbit IgG (Figure 3c trace A) or mouse IgG (Figure 3c trace E), there were no strong signal of 3-FTP or BDT observed in the Raman spectrum even with the non-matched antigens at μg mL$^{-1}$, showing no serious cross-reaction occurred. Therefore, highly specific simultaneous detection of two analytes has been demonstrated using our designed SERS tags with good sensitivity. However, for the duplex detection, the detection sensitivity was lower than the above demonstrated pg mL$^{-1}$ level, possibly due to immunoassay interference, which would require further experimental optimization such as the use of blocking agents as well as alternate assays.[11] Similar level of interference has been spotted in the work by Choo and co-workers, where cross-reactivity produced signals comparable to that from detection antibody at 0.1–1 ng mL$^{-1}$ range.[8c]

Compared to other Au nanostructure based SERS immunoassays, our AuNS@tag@shell based detection scheme has unique advantages. For example, Choo and co-workers developed a fast on-chip immunosensing assay using hollow Au nanoshapers,[12] but due to low signal intensity, the LOD for rabbit IgG is 1–10 ng mL$^{-1}$. Wang and co-workers synthesized Au mesowafers with rough surfaces, and used Raman tag molecules as linker for bioconjugation, demonstrating Ig mg mL$^{-1}$ detection sensitivity.[7d] As the tag molecules were directly involved in bioconjugation, the required specific functional groups will limit their selections. By embedding reporter molecules at the internal “hot spots” can not only increase the detection sensitivity by creating a brighter SERS tag with multiplexing capability, but also improve SERS tag stability and reproducibility.

In conclusion, by embedding Raman reporter molecules between AuNS core and a gold shell, we have developed SERS tags with greatly enhanced signals. The Au overcoating enhanced SERS intensities by 20 times compared to that without. These SERS tags containing AuNS core showed at least an order of magnitude stronger scattering signal than those with AuNP as the core. Moreover, different reporter
molecules with various end functional groups other than thiols can be embedded inside the gold shell, making them useful for multiplex detection, as each molecule has unique and narrow fingerprint spectrum. Using these bright SERS tags together with magnetic field assisted enrichment, we have designed highly specific and sensitive immunoassay, with protein biomarker detectable at concentration as low as 0.1 pg mL$^{-1}$, about four orders of magnitude more sensitive than AuNP based immunoassays. As the detected Raman signal depends nonlinearly on analyte concentrations, it is highly important to create bright SERS tags for immunosensing applications. Finally, multiplex detection was successfully realized by simultaneous spectral identification of dual analytes in solution. The demonstrated sensitivity, selectivity,
and multiplexing capabilities of our developed SERS tags make them highly promising for biosensing applications.

**Experimental Section**

**Materials:** Mouse IgG antigen, rabbit IgG antigen, goat anti-mouse IgG, and goat anti-rabbit IgG polyclonal antibody were purchased from Beijing Biosynthesis Biotechnology Co., Ltd.; the IMBs BeaverBeads MagNHS was obtained from Beaver Biosciences Inc.; BDT, MBA were purchased from Sigma, and 4-NBT was purchased from Aladdin. De-ionized water was used throughout the experiment, and the Raman spectra were collected with 3 s integration time under a 10× objective. Three spectra from different sites were collected from each sample and averaged to represent the SERS results.

**Synthesis of Gold Nanocrystals:** Spherical AuNPs were prepared by citrate reduction of HAuCl₄.[33] In a typical process, 1.0 mL of 10 mg mL⁻¹ sodium citrate was added to 50 mL of boiling solution containing HAuCl₄ (0.24 × 10⁻³ M). The solution was then kept boiling for 15 min. Afterward, the solution was allowed to cool to room temperature and stored at 4 °C before use.

AuNSs were synthesized following a seed-mediated growth method that was modified from previously reported works.[14] Briefly, the 12 nm seed solution was prepared according to the standard sodium citrate reduction method. Nanostars were grown by sequentially adding 40 µL of 1 M HCl, 400 µL of the gold seed solution, 400 µL of 2% SDS, 400 µL of 3 × 10⁻³ M AgNO₃, and 200 µL of 0.1 M AA into a solution containing 40 mL of 0.25 × 10⁻³ M HAuCl₄·4H₂O under vigorous stirring. After 30 s the solution color turned rapidly from light red to greenish-black. The obtained AuNSs were then centrifuged at 3200 g for 20 min and redispersed in 40 mL of water.

**Embedding Molecules inside Au:** AuNP@BDT@Au nanoparticles were synthesized by following the procedure reported by Singamaneni with some modifications.[33] 10 µL of 1 × 10⁻³ M BDT was added to 4 mL of AuNPs (1 × 10⁻⁹ M) under sonication for 1 h. Then, BDT modified nanoparticles (AuNP@BDT) were centrifuged twice at 10000 rpm for 10 min and resuspended with 2.0 mL of 0.2 M CTAC. The gold shell was grown by adding 200 µL of 10 × 10⁻³ M HAuCl₄·4H₂O, 400 µL of 0.2 M AA to the above suspension of AuNP@BDT nanoparticles, and the mixture was allowed to react for 20 min. The obtained nanoparticles were centrifuged and redispersed into 4 mL of water.

AuNS@BDT@Au or AuNS@3-FTP@Au nanoparticles were prepared in the same way as described above. 10 µL of 1 × 10⁻³ M probe molecule (BDT or 3-FTP) was added to 4 mL of AuNSs (0.1 × 10⁻⁹ M) under sonication and was centrifuged after 1 h. Then, 2.0 mL of 0.2 M CTAC, 100 µL of 10 × 10⁻³ M HAuCl₄·4H₂O, 200 µL of 0.2 M AA was added to the reporter-labeled nanostar and then stirred for 20 min. Afterward, the nanoparticles were centrifuged twice at 2700 g for 10 min and redispersed into 4 mL of water.

**Antibody Conjugation:** First, the surface of Au nanostructures was modified with carboxyl groups by adding 20 µL of 1 × 10⁻³ M dihydroxylic acid to 1 mL of AuNS@BDT@Au solution for 1 h, followed by removal of unabsorbed reagents through centrifugation. Next, the carboxyl groups on the surface were activated by adding 100 µL of 10 mg mL⁻¹ EDC and NHS solution for 30 min, and then separated by centrifugation. Subsequently, 100 µL of 1.0 mg mL⁻¹ anti-IgG polyclonal antibody was added to the activated nanostructures for 1 h at 35 °C, and reacted overnight at

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**Figure 3.** a) The SERS signature spectra of the other three AuNS@tag@Au nanoconstructs: from top to bottom are 3-FTP, MBA, and 4-NBT, respectively; b) different concentration used for the pair of mouse IgG and rabbit IgG in a duplex immunoassay; c) SERS spectra of the mixture solution for two targets (labeled with BDT and 3-FTP respectively) with different concentrations as shown in (b).
4°C. Finally, 100 μL of 2% BSA was added to the above antibody-bound nanostructures solution to make sure that no active sites on nanostructures were left. The mixture was incubated for 1 h at 35°C, centrifuged and resuspended in 1.0 mL of PBS buffer (pH 7.4).

**Immunomagnetic Beads for Bioseparation and Immunoassay:** Firstly, 4 μL of 10 mg mL\(^{-1}\) NHS-activated magnetic beads were washed with 100 μL of 1 × 10^{-3} M ice-cold HCl by magnetic separation process. Subsequently 100 μL of 1.0 mg mL\(^{-1}\) polyclonal antibody (goat anti-mouse or goat anti-rabbit IgG) was added to the beads and incubated in the shaker for 1 h at 35°C, and then incubated overnight at 4°C. After that, non-specific sites on IMBs were blocked by 100 μL of 2% BSA at 35°C for 1 h. Finally, IMBs were washed with PBS (containing 0.05% Tween-20) for three times and resuspended in 100 μL of PBS (pH 7.4) for further use.

**Sandwich Immunoassay:** The mouse IgG antigen solutions with different concentrations were obtained by a serial dilution with PBS (pH 7.4). 10 μL IMBs (≈40 μg) were added into each mouse IgG antigen solution and incubated at 35°C for 1 h under shaking, which were then collected by magnetic separation and washed with PBS buffer solution containing 0.05% Tween-20 three times to remove unbound antigens. These antigen-conjugated IMBs were further incubated with 100 μL of AuNS@BDT@Au nanoparticles for 1 h at 35°C. The resultant sandwich immunocomplexes were separated with a magnetic bar and washed with PBS buffer solution containing 0.05% Tween-20 and deionized water for several times to remove nonspecific binding events. Then, the resultant mixture was pipetted onto a silicon wafer for SERS detection.

**Multianalyte Immunoassay:** The mixture of goat anti-mouse IgG and goat anti-rabbit IgG antibodies were conjugated to IMBs (IMBs-mix) as capture substrate. In a separate solution, goat anti-mouse IgG antibodies conjugated AuNS@BDT@Au nanoparticles (AuNS@BDT@Au-mouse) and goat anti-rabbit IgG antibodies-conjugated AuNS@3-FTP@Au nanoparticles (AuNS@3-FTP@Au-rabbit) were chosen as SERS probes. Then, the duplex immunomassays were conducted as follows: 10 μL IMBs-mix, 50 μL AuNS@BDT@Au-mouse and 50 μL AuNS@3-FTP@Au-rabbit were added to 400 μL mixed antigens of mouse IgG and rabbit IgG. After being incubated for 2 h at 35°C, the immunocomplexes were separated with a magnetic bar, and the resultant was washed with PBS buffer solution containing 0.05% Tween-20 and deionized water for several times. Finally, the obtained immunocomplexes were transferred to a silicon wafer for SERS detection.

### Supporting Information

**Supporting Information is available from the Wiley Online Library or from the author.**

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