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Antibody-functionalized SERS tags with improved sensitivity†

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Protein detection at the femtomolar level can be achieved by using metallic nanoparticle assemblies that function as surface enhanced Raman spectroscopy reporters and that contain suitable surface-bound recognition elements. Proper control of the interaction between nanoparticles within the assemblies is critical for achieving this performance.

Innovative biosensory approaches based on the properties of colloidal nanoparticle (NP) assemblies have emerged with the potential to reach single-molecule detection thresholds.^{1,2} Methodologies based on surface enhanced Raman spectroscopy (SERS) benefit from the electromagnetic field enhancement due to the collective excitation of electrons in metallic nanostructures.^{3,4} This enhancement can occur within inter-nanoparticle gaps,^{4b,5} and several strategies exist for achieving the requisite assemblies.^{6–8} Another useful distinguishing feature of SERS-based detection strategies arises from the narrow spectral bandwidths.^{8c,9}

We recently reported multi-NP structures termed antitags that incorporate SERS reporters within suitable interstitial sites and antigen-specific recognition elements for protein detection in heterogeneous assays.¹⁰ Antitags, as shown in Fig. 1a, consist of silver NPs (typically ~35 nm) held together by a dithiolated linker and an antibody-functionalized polyethyleneglycol (PEG) coating. This thin layer is bifunctional by design, and contains thiol groups for binding to the NP surface and carboxylic functionalities for coupling with suitable antibody probes.

Antitags have enabled sensitive SERS analogs of the enzyme-linked immunosorbent assay (ELISA). Picomolar detection limits have been reported.¹⁰ The overall action of the bioassay is depicted in Fig. 1b and is based on the binding specificity of antibody/antigen pairs and the signal amplification properties of SERS. The procedure is described below in more detail. Fig. 1a shows an idealized NP dimer, but it should be noted that higher order aggregates can also function as long as they

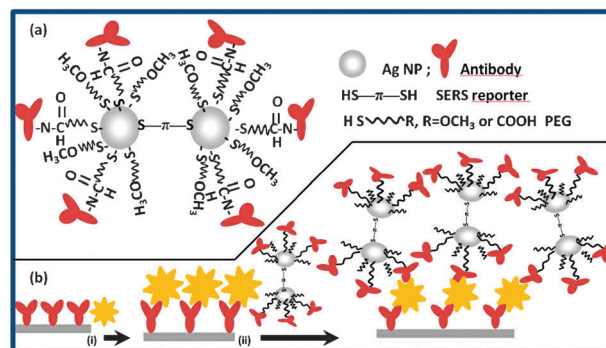


Fig. 1 (a) Schematic illustration of the antibody functionalized antitags. (b) SERS-based ELISA-analog detection. Human α -thrombin (Thr) antigen (yellow) is exposed to an epoxy functionalized glass substrate modified with a covalently immobilized layer of human α -thrombin mouse monoclonal capturing antibodies (pink). The surface-bound Thr subsequently couple with antitags via specific protein/antibody interactions. Drawing is not to scale.

do not non-specifically bind to the assay substrate. It is also worth pointing out that the ability to utilize different SERS labels paves the way for multiplexed detection on a single sensing surface, a feature currently not possible with the colorimetric or fluorometric ELISA counterparts.¹⁰ In this contribution, we show important improvements in antitag sensitivity by properly managing surface properties so that aggregation is minimized.

Antitags have been previously prepared by the sequence of steps highlighted in blue in Fig. 2.¹⁰ Biphenyl-4,4'-dithiol (DBDT) was first used to link the NPs together, step (i), and to serve as the SERS reporter. After centrifugation and isolation, step (ii), NP dimers and higher aggregates were stabilized by the addition of thiolated carboxylic-polyethylene glycol (HS-PEG-COOH), step (iii). This step minimizes protein-induced NP aggregation in subsequent treatments¹¹ and incorporates carboxylic functionalities that serve to anchor antibodies via carbodiimide-mediated amidation; see step (iv).¹² An additional methoxy-terminated PEG layer, step (v), is included to further stabilize toward against ligand exchange and aggregation.^{4b,13} However, examination of different batches revealed that the conditions used for centrifugation could influence the final distribution of NP aggregates, i.e. the ratio of monomer, dimer, trimer, and higher order species.

To address the batch-to-batch variability described above, a modified SERS antitag synthetic procedure was examined. The new process is highlighted as the sequence of steps

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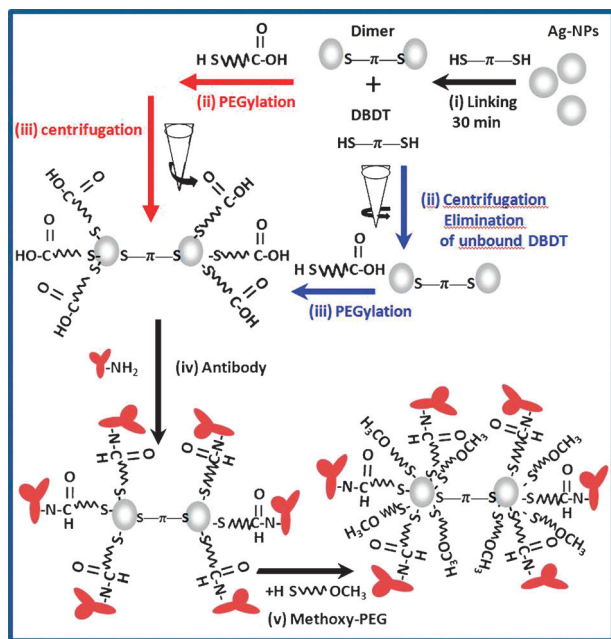


Fig. 2 Schematic of the preparation of SERS active antigens *via* new (red) and previous⁶ (blue) procedures.

highlighted red in Fig. 2. Essentially, HS-PEG-COOH is added to the reaction mixture prior to centrifugation with the idea that surface passivation *via* incorporation of polar functionalities and steric bulk would prevent undesired aggregation.¹⁴ Subsequent procedures after the colored sequences illustrated in Fig. 2 were kept similar.

Fig. 3 shows transmission electron microscopy (TEM) images collected from the NP distributions obtained *via* the two procedures illustrated in Fig. 2. These micrographs show that the preventive coating HS-PEG-COOH prior to centrifugation, *i.e.* the red sequence of steps in Fig. 2, yields a

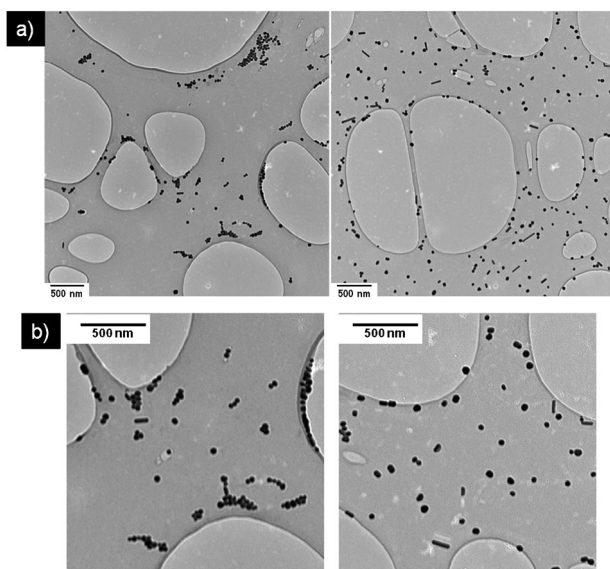


Fig. 3 (a) TEM micrographs of NPs obtained *via* centrifugation/passivation [left hand side, blue sequence in Fig. 2] and passivation/centrifugation [right hand side, red sequence in Fig. 2]. (b) Close up images.

distribution of aggregates that is biased toward a smaller number of incorporated NPs. The corresponding histograms of these distributions are plotted in Fig. S1 (ESI†).

The sandwich assay shown in Fig. 1b was used to detect the presence of human α -thrombin (Thr) antigen and thus demonstrate the sensing function of the new antigens. In this approach, the protein is first captured by human α -thrombin mouse monoclonal antibodies immobilized on an epoxy-functionalized glass substrate. Antigens with the reporting antibody specifically bind to the surface-bound Thr antigens. After washing away unbound antigens, the Raman signals can be correlated to surface bound Thr. Concentrations of 10^2 , 10^3 , 10^4 , 10^5 , 10^6 fM of Thr were examined using the diagnostic fingerprint of the DBDT-Raman reporter (1589 cm^{-1}), as obtained with a 633 nm laser source and 1 s exposure time. These test solutions were prepared by serial dilution in PBS buffer of a 1 nM thrombin stock solution. Fig. 4 displays representative spectra for the thrombin detection platform using the improved antigens.

Inspection of Fig. 4 reveals that the intensity of the peak increases with increasing Thr concentration. The signal at 0 fM is attributed to non-specific binding. Fig. 5 provides a more quantitative measure by plotting the ratio of the 1589 cm^{-1} peak intensity in the presence and absence of Thr as a function of concentrations. Each data point represents the average of four measurements from $15\text{ }\mu\text{m} \times 15\text{ }\mu\text{m}$ surfaces on the same substrate. A control sample was prepared under the same assay conditions with the absence of Thr (red curve in Fig. 4). Differentiation between control samples and thrombin coated surfaces exhibits a detection range from picomolar to femtomolar levels. The error bars represent the standard deviation as obtained from three different substrates. This dose-response curve was fitted linearly with a resulting R square value of 0.9962. The 100 fM limit of detection (LOD) is based on a 3:1 threshold ratio with respect to the control measurement.

The results shown in Fig. 5 demonstrate that using antigens prepared *via* the new procedure offers more than a 100- and 1000-fold improvement in the detection limit over that achievable with commercially available ELISA kits ($\sim 10\text{ pM}$)¹⁵ and the

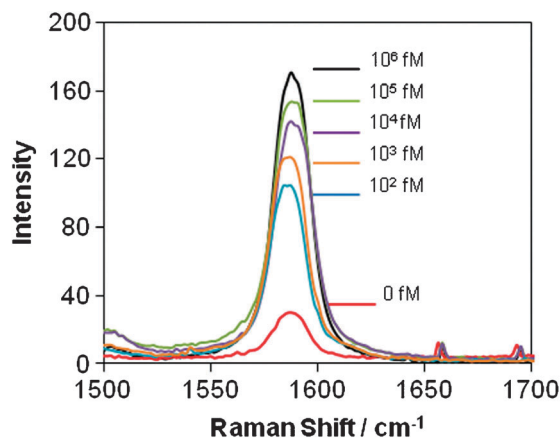


Fig. 4 Representative results from the SERS-based immunoassay detection of thrombin collected at different protein concentrations: 10^2 fM, 10^3 fM, 10^4 fM, 10^5 fM and 10^6 fM. The control sample (0 fM) is obtained in the absence of thrombin.

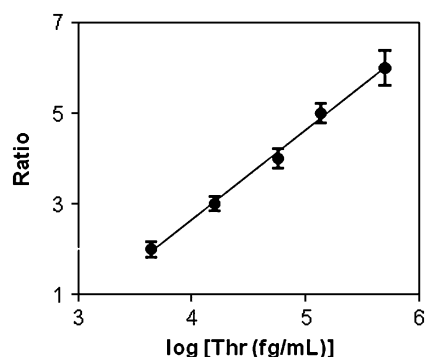


Fig. 5 Dose-response curve from the antibody-antigen binding affinity using a range of thrombin concentrations (100 fM–1 nM). The best-fit line is as follows: $y = 1.9688x - 5.2298$ ($R^2 = 0.9962$). The error bars represent the standard deviation. Ratio is the coefficient between the 1589 cm^{-1} peak intensity in the presence and absence of the protein.

performance of antitags prepared *via* the previous synthetic route (100 pM), respectively.¹⁰ In addition, this assay provides higher sensitivity than the previous literature report of 0.5 pM.^{2d,16}

In summary, it is possible to improve the sensitivity of antitag-mediated protein SERS detection by taking advantage of a synthetic procedure designed to mitigate the negative impact of nanoparticle aggregation. The key step involves the simple surface passivation using PEG immobilization prior to the purification steps. The overall detection platform is competitive with commercially available immunoassays.^{15–17} Furthermore, the sensitivity, the robustness and the high reproducibility of the prepared SERS bioassay provide a suitable platform for developing multiplexed sensitive detection of proteins and other target analytes.

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