http://dx.doi.org/10.5012/bkcs.2012.33.11.3681

Avidin Induced Silver Aggregation for SERS-based Bioassay

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Received June 26, 2012, Accepted August 14, 2012

We developed a simple and effective method for the SERS-based detection of protein-small molecule complexes and label-free proteins using avidin-induced silver aggregation. Upon excitation with light of the appropriate wavelength (633 and 532 nm), the aggregated silver nanoparticles generate a strong electric field that couples with the resonance of the molecules (atto610 and cytochrome *c*), increasing the characteristic signals of these molecules and resulting in sensitive detection. The detection limit of biotin with the proposed method is as low as 48 ng/mL. The most important aspect of this method is the induction of silver aggregation by a protein (avidin), which makes the silver more biocompatible. This technique is very useful for the detection of protein-small molecule complexes.

Key Words : Avidin, SERS, Bioassay, Quantitative analysis

Introduction

Surface-enhanced Raman scattering (SERS)-based biomolecular detection and applications have been widely developed in workflow research.[1-5] SERS-based methods offer promising opportunities in biology, medicine, and pharmacy, and allow studies of the relationships between the structure and function of proteins.[6-9] The SERS technique generally benefits from the great increase of the intrinsically weak Raman signals caused by the presence of nano-sized metallic structures, for example, when the target molecule is attached to colloidal silver or gold clusters. Low detection limits, allowing even single-molecule assays; narrow spectral bandwidths; the ability to quench fluorescence; and optical labels make SERS a good choice for DNA or RNA analysis, genetics and proteomics studies, medical diagnostics, and the detection of chemical warfare agents.[10-13] SERS-based protein detection and analysis of protein-protein and protein-molecule interactions are the most rapidly developing methods that are widely used in biochemistry.[14-17]

There are two types of protein research methods based on SERS, label-based and label-free methods. Label-based detection is an indirect detection strategy that employs a Raman-active molecule with high Raman scattering cross-sections, which can provide great SERS enhancement. Many research groups have recently proposed the use of SERS-active probes and Raman dye-labeled gold nanoparticles to detect proteins.[4-6,18-21] Some groups have used fluorescein-labeled antibodies to recognize antigens on metal substrates probed by SERS.[18,19] A reagent-based protein assay based on SERS was also employed for ultrasensitive protein detection.[20,21] Label-free detection methods can directly detect target protein signal, resulting in more reliable and convenient detection for any analyte. New methods for protein detection based on SERS have been developed. For example, a method that combines electrophoresis, Western blotting, colloidal silver staining and SERS techniques for label-free protein detection has been developed, and another new method combines enzyme-linked immunoadsorbent assay technology and SERS for immunoassays.[4]

Metal colloids (*e.g.*, gold, silver and metal composites) are commonly used SERS-active substrates for proteins. These materials exhibit excellent plasmon resonances, resulting in the strong scattering and absorption of light, as well as enhanced optical fields near the metal surface. The aggregation of metal colloids induced by the target molecule itself or by the addition of chemicals is essential to achieve high SERS enhancement. There are many types of aggregation agents used for the preparation of SERS-active substrates, such as NaCl, H_2SO_4, $NaSO_4$ and $MgSO_4$. However, most chemicals are not compatible to biomolecules.[22,23]

In this study, we developed an effective protocol in which avidin induces the aggregation of silver colloids, which exhibit great SERS activity. The aggregation of silver nanoparticles was induced by a strong electrostatic interaction with avidin, and biotin conjugated with atto610 was subsequently attached to the silver nanoparticles that were aggregated by the biotin-avidin interaction. This aggregation resulted in excellent SERS enhancement of the labeled atto610 molecule, allowing the detection of the protein-ligand complex (avidin-biotin). Furthermore, this method allows the direct and rapid detection of label-free proteins (cytochrome *c*).

Experimental Section

Materials. Avidin, atto610-biotin, cytochrome *c* (cyt *c*), bovine serum albumin (BSA), silver nitrate and sodium citrate were all purchased from Sigma-Aldrich Co., Ltd. All reagents and materials were used without further purifi-

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cation. Ultrapure water (18.0 MΩ cm^{-1}) was used throughout the present study.

Buffers and Colloidal Silver. The phosphate-buffered saline (PBS; 0.01 M, pH 7.0) used in this study contained 0.8% NaCl, 0.02% KH_2PO_4, 0.02% KCl and 0.12% $Na_2HPO_4 \cdot 12H_2O$. A blocking buffer was prepared by dissolving BSA in phosphate-buffered saline (PBS, containing 1% BSA). Colloidal silver was prepared by the aqueous reduction of silver nitrate (10^{-3} M, 200 mL) with trisodium citrate (1%, 4 mL). The plasmon maximum of the silver colloid was located at 415 nm.[24]

Freshly prepared avidin (3 μL, 25 μg/mL in PBS buffer) was placed in a cuvette that contained 200 μL of silver colloids. Two minutes later, a phosphate buffer containing 1% BSA was used to block the nonspecific adsorption of avidin to the silver colloid. After 15 minutes, different volumes of atto610-biotin (200, 100, 50, 10, 5, 1 and 0.5 μL and blank sample) were put into the cuvettes, which were then incubated at room temperature for 15 minutes. In label-free protein detection, freshly prepared avidin (3 μL, 25 μg/mL in PBS buffer) was placed in a cuvette that contained 200 μL of silver colloids. Two minutes later, cyt *c* with different concentrations (200, 50, 20 and 5 μg/mL) were put into the cuvettes.

Characterization of Substrates. The UV-vis spectra of silver colloid and aggregated silver colloid were obtained with a Scinco UV-spectrophotometer and SERS substrates were characterized by field emission scanning electron microscope. Transmission electron microscopy (TEM) image of silver colloid and aggregated silver colloid were obtained using a JEOL JEM-2010 Luminography (Fuji FDL-5000) Ultramicrotome (CRX).

SERS Measurements. SERS spectra were recorded using a Jobin Yvon/HORIBA LabRam ARAMIS Raman spectrometer. The radiations from an air cooled frequency doubled Nd:Yag laser (532 nm) and HeNe laser (633 nm) were used as an excitation sources. The typical exposure time for each SERS measurement in this study was 30 s and 10 s with one accumulation.

Results and Discussion

We prepared silver colloids following the method of Lee and Merisel,[24] and the maximum plasmon absorption of the silver colloids was located at 415 nm (Figure 1). Figure 2(a) shows the TEM image of the silver colloids. Figure 3 is statistics the size of silver nanoparticles by using the TEM image from three independent preparations. As shown in Figure 3, the size of most silver nanoparticles was 60-70 nm. A previous study showed that aggregated silver nanoparticles exhibit high SERS enhancement.[17] In this study, we first aimed to induce the aggregation of silver nanoparticles using a biocompatible reagent (avidin) to provide the highest plasmon coupling and the maximum SERS enhancement of the target signal. Under neutral pH conditions, silver colloids are negatively charged, whereas avidin (isoelectric point = 10.5) is rich in positively charged amino groups. Thus, it is possible to obtain aggregated silver nanoparticles using avidin as an aggregating reagent. Scheme 1 shows the formation of aggregated silver nanoparticles. After adding avidin to the silver colloid, the color of the silver colloid changed from yellow-green to red-brown. TEM and UV-vis spectroscopy were employed to monitor the variation of the silver nanoparticles. A TEM image of the aggregated silver nanoparticles is shown in Figure 2(b). In contrast to the image of

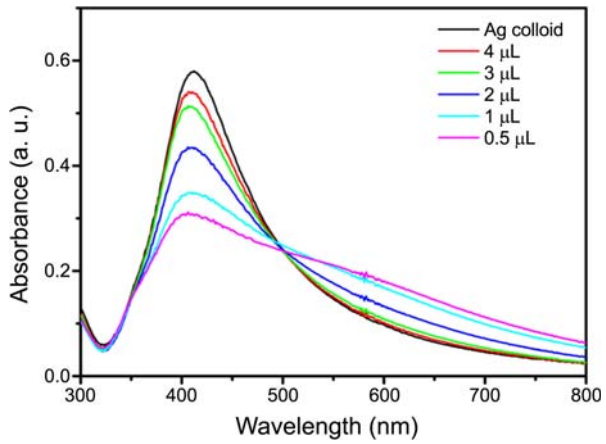


Figure 1. UV-vis spectra of silver colloid with addition of 4, 3, 2, 1 and 0.5 μL avidin (0.25 mg/mL).

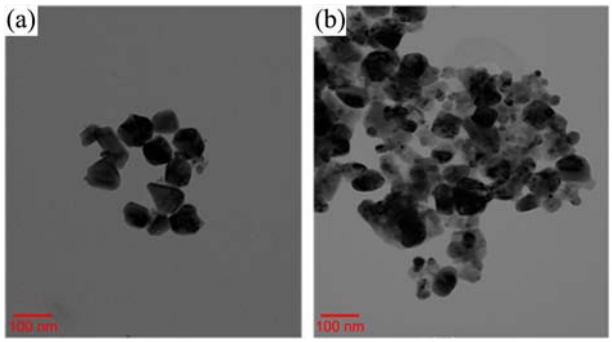


Figure 2. (a) TEM images of silver colloid itself and (b) after addition of avidin (0.25 mg/mL, 3 μL).

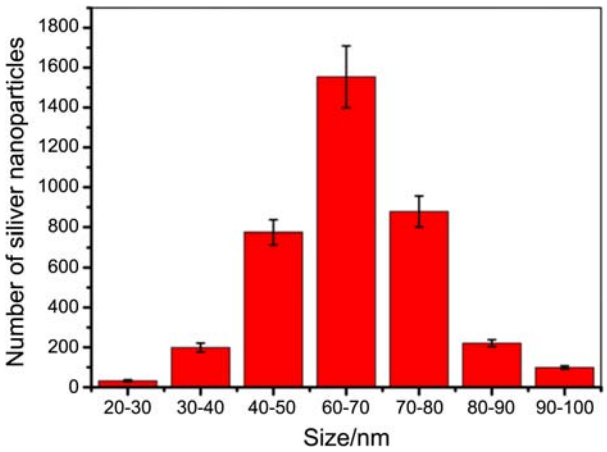
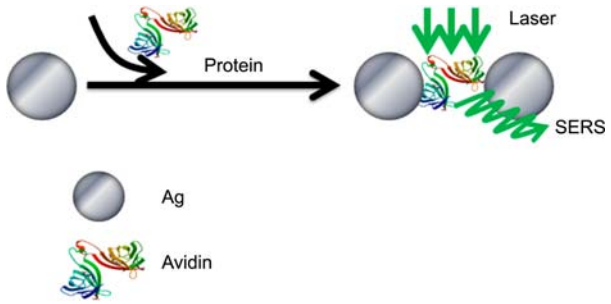


Figure 3. Statistics the size of silver nanoparticles by using the TEM image from three independent preparations.



Scheme 1. Schematic diagram representing the formation of silver aggregates.

the original sample, aggregated silver nanoparticles can be observed after the addition of avidin. Figure 1 shows the UV-vis spectra of the silver nanoparticles after adding different volumes (from 4 to 0.5 μL) of avidin. As the amount of added avidin increases, the intensity of the original absorption peak decreases and shows a red shift, which indicates that the size of the aggregates increased. This result is in good agreement with the TEM image shown in Figure 2(b). The avidin-aggregated silver nanoparticles are more biocompatible than particle produced with other aggregation methods.[22,23]

Based on its ability to tightly bind to avidin, biotin is often used as a protein target. Biotin is used in commercially available biochemical assays and has wide applications in biological laboratories. In addition, the avidin-biotin complex can be used as a model complex system for protein-drug interaction studies. To obtain the highest SERS enhancement of silver aggregates induced by avidin (25 μg/mL), a volume-dependent SERS experiment was conducted in which different volumes of avidin were used. Figure 4 shows the SERS spectra of atto610 in the presence of different volumes of avidin. When increasing the volume of avidin, the SERS intensities also increased, indicating that the plasmon coupling and the degree of silver aggregation affect the SERS intensity. The data revealed that 3 μL is the best candidate for silver aggregation in this proposed SERS-based study. The following SERS experiments employed 3 μL of avidin solution as an aggregation reagent. It is very likely that the bands at 1637 and 1551 cm^{-1} in the SERS spectra of atto610 are due to the aromatic C=C stretching vibrations, whereas the band at 1172 cm^{-1} can be attributed to the C-H in-plane vibration.[25,26]

The ability to perform quantitative analysis is an important feature of any bioanalytical technique. In this study, the avidin-biotin system was employed to investigate the quantitative aspects of this approach. In a series of measurements, the concentrations of both avidin and the aggregated silver nanoparticles were kept constant while the concentration of biotin was varied from 4.9×10^{-3} mg/mL to 2.4×10^{-5} mg/mL. Figure 5 shows the SERS spectra of the atto610-biotin/avidin complex for different the concentrations of biotin. Biotin solutions with different concentrations were examined by the method depicted in Scheme 1, and their SERS spectra are shown in Figure 5(a). The higher the concentration of biotin was, the more intense the SERS signals were. Therefore, one can easily determine the concentration of biotin indirectly from the intensity of the characteristic SERS band of atto610. The detection limit of the present method was found to be as low as 4.8×10^{-5} mg/mL. The

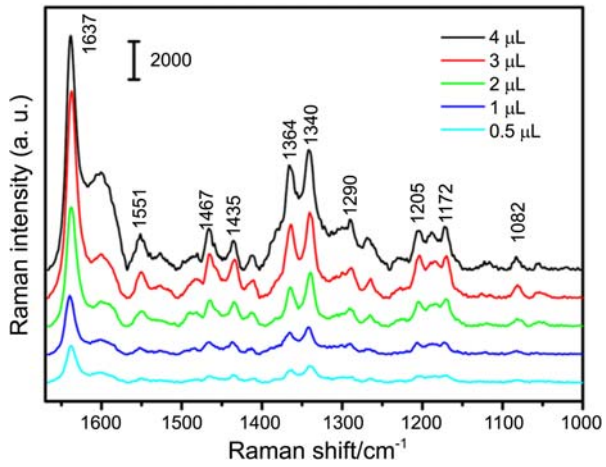


Figure 4. SERS spectra of atto610 with different volume of avidin (from 4 to 0.5 μL).

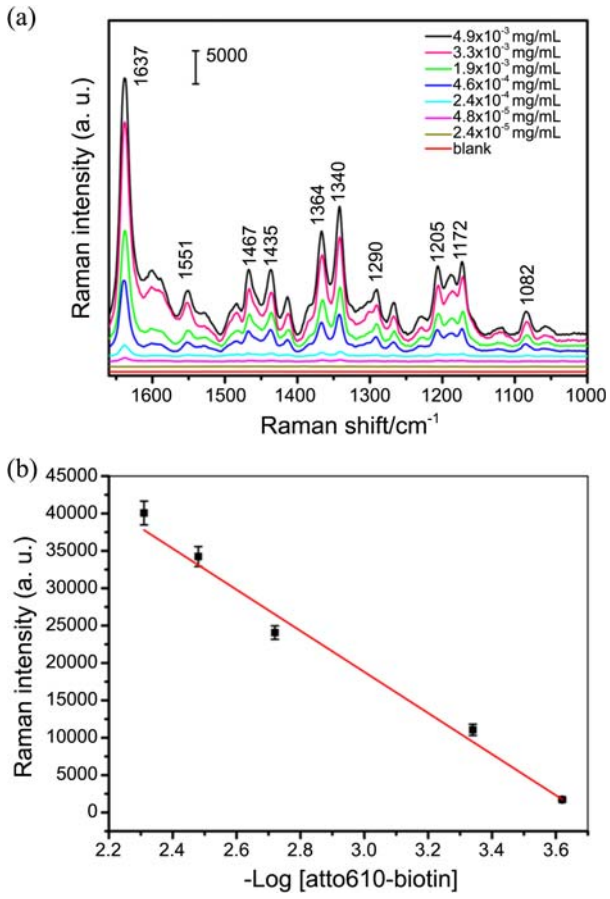


Figure 5. (a) Concentration-dependent SERS spectra of atto610 with biotin concentration range from 4.9×10^{-3} to 2.4×10^{-5} mg/mL and blank sample. (b) Concentration-dependent SERS intensities of the band at 1637 cm^{-1} of atto610. Error bars indicate the standard deviations from four independent measurements.

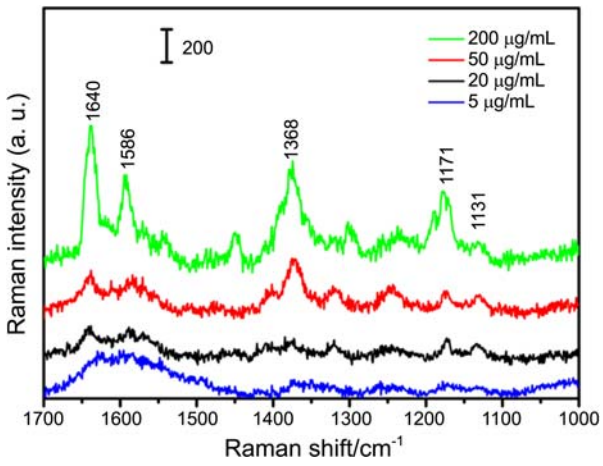


Figure 6. Concentration-dependent SERS spectra of cyt *c* (from 200 to 5 μg/mL).

intensity of the band at 1637 cm^{-1} versus the concentration of biotin is plotted in Figure 5(b). Interestingly, the proposed SERS method has a linear response over the concentration range of the proposed protein-small molecule assay. This linearity implies that quantitative analysis is feasible in this concentration range (4.9 μg/mL to 240 ng/mL). The standard curve of the proposed method yielded the equation $Y = 1031304–27499X$, $R^2 = 0.989$, where Y represents the SERS intensity of the band at 1637 cm^{-1} for atto610-biotin and X is the concentration of atto610-biotin. Error bars indicate the standard deviations from four independent measurements. The greatest advantage of this proposed method is the rapid and effective detection of protein-small molecule interactions. The strong SERS activity of this proposed method indicates that it has great potential for the detection of protein-ligand interactions.

To investigate SERS ability of this proposed substrate in biomolecular assay, we used the substrate to detect label-free protein. The label-free detection of proteins can provide direct spectroscopic data about the target molecules. Accordingly, a great deal of effort is currently being invested in label-free biosensors and detection. We carried out SERS experiments to explore the potential of the proposed avidin-aggregate silver substrate to detect cyt *c* at low concentrations. We found that cyt *c* with the heme cofactor, which has a high scattering section, was a good candidate for SERS-based research and detection. Figure 6 shows the SERS spectra of different concentrations of cyt *c* adsorbed on the avidin-aggregated silver nanoparticles. The observed Raman bands are in good agreement with those previously reported, and the bands at 1640, 1586, 1368, 1171 and 1131 cm^{-1} are all assigned to the heme group, which is a cofactor of cyt *c*.[27-31] The presence of "hot spots" from the aggregated silver nanoparticles would provide a huge SERS enhancement for label-free protein detection.

Conclusion

We proposed a simple, fast, and effective biomolecular assay method. Avidin-induced silver aggregation is a convenient method for the preparation of substrates with high SERS activity. The greatest advantage of the proposed avidin-aggregated silver nanoparticles is the better biocompatibility relative to chemically aggregated nanoparticles. Furthermore, the proposed silver aggregates can be used for label-free protein assays, as demonstrated by the high SERS enhancement for a cofactor protein (cyt *c*). This proposed silver aggregation method provides a starting point for the development of avidin-biotin-based SERS assays.

Acknowledgments. This study was supported by the National Research Foundation of Korea (NRF) grants funded by the Korea government (MEST) (No. 2011-C00052 and No. 2009-0087013), the Korean Federation of Science and Technology Societies Grant funded by Korea Government (MEST, Basic Research Promotion Fund), and the BK 21 program from the Ministry of Education, Science and Technology of Korea. The authors thank the Central Laboratory of Kangwon National University for the measurements of Raman spectra.

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