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Label-Free Detection of Native Proteins by Surface-Enhanced Raman Spectroscopy Using Iodide-Modified Nanoparticles

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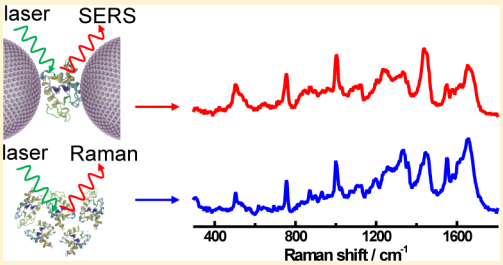
Supporting Information

ABSTRACT: Proteins perform vital functional and structural duties in living systems, and the in-depth investigation of protein in its native state is one of the most important challenges in the postgenomic era. Surface-enhanced Raman spectroscopy (SERS) can provide the intrinsic fingerprint information of samples with ultrahigh sensitivity but suffers from the reproducibility and reliability issues. In this paper, we proposed an iodide-modified Ag nanoparticles method (Ag IMNPs) for label-free detection of proteins. The silver nanoparticles provide the huge enhancement to boost the Raman signal of proteins, and the coated iodide layer offers a barrier to prevent the direct interaction between the proteins and the metal surface, helping to keep the native structures of proteins. With this method, highly reproducible and high-quality SERS signals of five typical proteins (lysozyme, avidin, bovine serum albumin, cytochrome *c*, and hemoglobin) have been obtained, and the SERS features of the proteins without chromophore were almost identical to the respective normal Raman spectra. This unique feature allows the qualitative identification of them by simply taking the intensity ratio of the Raman peaks of tryptophan to phenylalanine residues. We further demonstrated that the method can also be used for label-free multiplex analysis of protein mixture as well as to study the dynamic process of protein damage stimulated by hydrogen peroxide. This method proves to be very promising for further applications in proteomics and biomedical research.



Proteins perform vital functional and structural duties in living systems. The detection and characterization of proteins are extremely important for drug screening, early diagnosis, and clinic therapy, leading to the vast development of proteomics.[1−4] The formidable challenges in the emerging field of proteomics are to study large numbers of proteins whose abundance spans some 10 orders of magnitude[5] and identify their interactions and functions.[6] Most conventional approaches including mass spectroscopy (MS),[7,8] X-ray crystallography,[9,10] NMR,[11,12] and enzyme-linked immunosorbent assay (ELISA)[13] have made significant contributions to the progress of proteomics. But most of them are complex and expensive for routine use. Therefore, developing a high-efficiency, ultrasensitive, cost-effective, and simple detection technique for proteins is of great benefit to the advance of proteomics.

In 1980, Cotton et al. combined surface-enhanced Raman scattering (SERS) with resonance Raman scattering (RRS), also called as surface-enhanced resonance Raman scattering (SERRS), for detecting cytochrome *c* (cyt *c*) and myoglobin on a silver surfaces.[14] This work opened a new field for SERS in the biological application. Thereafter, SERS on flavoenzyme, or even the reaction center of *Rhodopseudomonas sphaeroides*, have been reported.[15] SERS is a noninvasive spectroscopic technique with single-molecule sensitivity,[16,17] benefited mainly from the enhanced electromagnetic field of nanostructure of Ag, Au, and Cu under the excitation of appropriate laser wavelengths. It inherits the capability of providing molecular fingerprint information of Raman spectroscopy, which endows it unique molecular selectivity and multiplex ability[18] in systems containing liquid. It has found increasing applications in biological studies, such as detection of DNA,[19] proteins,[20] carbohydrates,[21] and cells.[22] SERS detection of biological systems is often classified into label and label-free strategies. In the label method, the extrinsic Raman labels (usually dyes with strong resonance Raman signal) are usually covalently attached to metal nanoparticles, followed by modification with bimolecular ligands to form SERS probes. The SERS probes are then used to detect target analytes via biomolecule−ligand recognition.[20,23] The label method detects the signals of the probes rather than that of the biomolecules themselves. Therefore, it was also known as the indirect or extrinsic SERS method. It has found successful application in the trace detection[20] and SERS imaging of living cells.[24] However, there are still some challenges, including synthetic challenges and false positive issues. In contrast, the label-free method fully conveys the advantage of Raman spectroscopy, as it provides the inherent vibrational properties of the biomolecules to be detected.[6,25] The label-free method is also termed as the direct

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or intrinsic SERS method. The work related to the label-free SERS detection of proteins can be divided into two categories, with and without chromophores. Proteins with chromophores (e.g., cyt *c*, hemoglobin, and myoglobin) were well-characterized[26−28] with good reproducibility due to their strong SERRS effect. In these studies, the Raman signals were dominantly contributed by the chromophore center of the proteins and information could be obtained related to the conformation and orientation of the proteins,[29,30] charge-transfer processes,[26] and etc. For the majority of proteins without chromophores, their signals are much more difficult to be obtained. The obtained signals were primarily generated by amino acid residues and amide backbones,[31,32] which may be able to provide useful information of the constituent and structure of proteins.

In recent years, substantial attempts were made in the development of the method of SERS-based label-free identification of proteins. Zhao, Ozaki, and their co-workers conducted systematic work to promote the reliability and versatility of the method.[32−37] The three major concerns for SERS-based label-free identification of proteins are sensitivity, reproducibility, and native state. The sensitivity depends mainly on the design of SERS-active nanoparticles (NPs) or nanostructures. Silver NPs were widely used since they show the highest SERS activity. Several approaches have been developed to increase the number of "hot spots" and to increase the chance to trap the target protein molecules in the "hot spot" to improve the detection limit, such as addition of aggregation agents,[33] silver staining,[36,38] hybrid self-assembly of proteins and silver nanoparticles driven by heat,[34,35] convection,[39] or hydrophobicity.[40]

The second and the major challenge of the SERS-based label-free approach is the poor reproducibility, especially for proteins without chromophores. It has been found that spectra of the same proteins obtained by different groups showed different spectral features.[33,34,39] Some spectra showed an amide I mode,[41,42] whereas others did not.[43,44] The reproducibility is influenced by interfacial properties of NPs, structural flexibility of proteins,[25,45] different adsorption orientations of proteins,[30] and laser-induced reaction.[46] We and the others have found that the reproducibility can be improved distinctly after cleaning the surface of SERS substrates.[22,47,48] Han et al. found that the reproducibility can be effectively improved by conducting SERS measurement of target proteins in aqueous solutions to reduce laser-induced effect.[33]

The last but not the least concern is to retain the proteins in their native state during the detection process. Study of the structure of protein in its native state is vitally important for illuminating the protein folding mechanism and protein−ligand interactions and functions. However, it is well-known that the SER spectra of proteins are normally quite different from the corresponding normal Raman spectra. The following reasons may account for the difference: (1) In order to preserve the native structure of protein, it normally requires the protein in an aqueous environment. However, most SERS experiments were operated in a dry state. (2) The strong interactions between protein molecules and the surface of SERS substrates may alter the structure of proteins.[28] (3) The selective enhancement of certain vibrational modes as a result of the surface selection rule of SERS may also be a reason for the difference between the SERS and the corresponding normal Raman spectra.[30,49] Two recent reports are particularly interesting. They successfully addressed the above issues and obtained the SERRS of native proteins. Feng and Tachikawa obtained the SERRS signal of native met-myoglobin in a Raman flow system by reducing the interaction time,[28] and Sivanesan et al.[50] obtained the SERRS of native cyt *c* by using mecaptoundecanoic acid modified Ag NPs to decrease the chemical effects and interaction.[46] However, these important progresses are still limited to proteins with resonance Raman effect, and it is still a great challenge to detect the SERS of native proteins in the absence of resonance Raman effect.

Among all the above-mentioned challenges, reproducibility is the most critical point as it is extremely important for a wide application of SERS to biological systems. In our previous study,[22] we have proposed an iodide adsorption method to eliminate the surface impurities, which greatly improved the reproducibility of SERS signals of membranes of living cells on self-assembled SERS substrates. Unfortunately, we could not obtain any signal of protein without chromophores on the iodide-modified SERS substrates. It may be understood that the proteins are too large to squeeze into the "hot spots" in the gap of immobilized nanoparticles.

Herein, we proposed a facile method to enable reliable label-free SERS detection of the native structures of a wider range of proteins. The iodide-modified silver colloids were mixed with target proteins. The colloidal state of Ag NPs will help to keep the native structures of proteins and promote the photostability of samples. The iodide modification affords a one-atom-thick monolayer on the surface without producing interfering signal. It could not only clean the surface but also avoid the strong chemical interaction between the metal surface and the proteins and reduce the possibility of denaturation. We were able to obtain SER spectra of proteins with excellent reproducibility and sensitivity. The spectra exhibited almost identical features to that of the normal Raman spectra of protein solutions, implying the native structures were preserved. We applied this method to study five typical proteins, all of which displayed distinctive fingerprint features. The method also allowed simultaneously detection of multiple proteins and in situ monitoring the conformational changes of proteins. The result indicates the method is very promising for high-throughput proteomics and studying the dynamics of protein.

EXPERIMENTAL SECTION

Chemicals. Hen egg white lysozyme, avidin, and cyt *c* were purchased from Bio Basic Inc. Hemoglobin and bovine serum albumin (BSA) were obtained from Worthington and AMRESCO, respectively. High-purity water (Milli-Q, 18.2 MΩ cm) was used throughout the study.

Preparation of Iodide-Modified Ag NPs (Ag IMNP). Colloidal silver was prepared via reduction of $AgNO_3$ by sodium citrate using the modified method of Lee and Meisel.[51] In brief, 200 mL of 1 mM $AgNO_3$ was heated to boil under vigorous stirring, followed by the addition of 6 mL of 1% sodium citrate. The mixture was kept boiling and stirring for 1 h and turned yellow-green. The average diameter of the Ag NPs was 50 nm according to the scanning electron microscopy (SEM) characterization (Supporting Information Figure S1). To prepare Ag IMNPs, 4.5 mL of silver colloid was centrifuged (5000 rpm, 10 min) and the supernatant was removed. An amount of 50 μL of the concentrated colloid was mixed with 50 μL of 1 mM potassium iodide, which was incubated for 20 min at 25 °C to ensure a complete modification of the surface of Ag NPs with iodide. After that, the Ag IMNPs were mixed with 50

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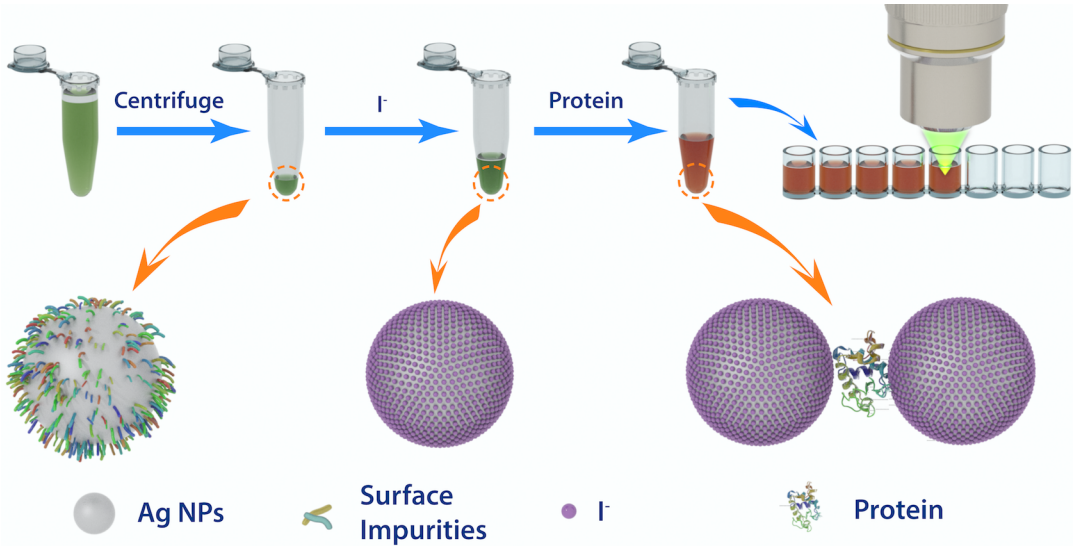
Scheme 1. Schematic Diagram of the Method Proposed for Protein Detection



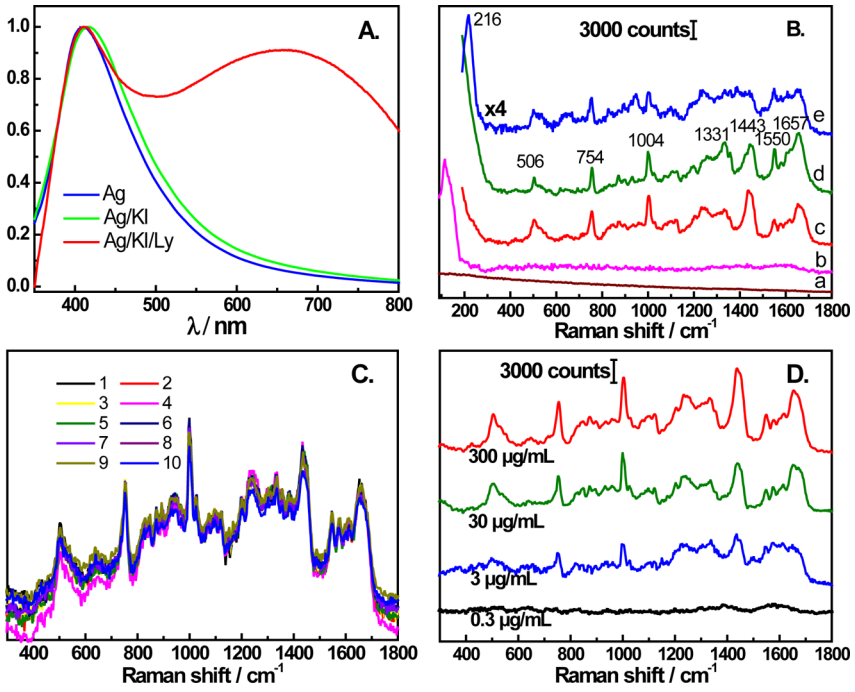


Figure 1. (A) UV−vis spectra of centrifuged silver colloid, Ag IMNPs, and Ag IMNPs mixed with lysozyme. (B) SER spectra of Ag NPs (a), Ag IMNPs (b), 300 μg/mL lysozyme with Ag IMNPs (c), 300 μg/mL lysozyme with Ag NPs (e), and normal Raman spectra of 100 mg/mL lysozyme solution (d). The laser power was 5 mW in curves a−c, 20 mW in curve d, and 2 mW in curve e. The acquisition time was 30 s. (C) Ten sequential SER spectra of 300 μg/mL lysozyme with Ag IMNPs. (D) SER spectra of lysozyme at different concentrations as indicated in the figure detected with Ag IMNPs.

μL of protein aqueous solution at room temperature and immediately added to 96-well plates for SERS measurements.

SERS Measurements. Normal Raman and SER spectra were acquired using a confocal Raman system (Xplora, Horiba) using a 532 nm laser as the excitation light. The laser power was around 5 and 20 mW for SERS and normal Raman measurements, respectively. The typical exposure time for protein measurements was 30 s unless otherwise stated. In the normal Raman and SERS experiments of the solution samples, the laser beam was directly focused into the solution in the 96-well plates. A 10× objective (NA = 0.25) was used. In the normal Raman of solid protein experiments, a 50× objective (NA = 0.50) was used. All the spectra were present only after baseline correction by a polynomial fitting method.

UV−Vis Absorption Measurements. All the absorption spectra were collected with a Shimadzu 2550 UV−vis spectrometer at room temperature. Here all the samples were diluted 100 times compared with those in SERS experiments to avoid the light absorption by the sample.

RESULTS AND DISCUSSION

The reason to modify the silver NPs with iodide is to adjust the interfacial properties of the NPs in hope of changing the way of the interaction between proteins and SERS-active NPs to

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preserve the native structures of proteins and improve the reliability of the SERS signal. Scheme 1 illustrates the procedure for the native protein detection and the models of the corresponding nanoparticles step by step. The UV−vis and SER spectra of the products in each step are presented in Figure 1, parts A and B. The silver colloid was first centrifuged to remove most of the surface capping agents, which resulted in 50 μL of concentrated colloid. The UV−vis absorption band was located at 410.5 nm, and no Raman signal can be detected in this case. Then, 50 μL of 1 mM KI was added into the colloid at 25 °C, thoroughly mixed, and the mixture was incubated for 20 min to obtain the Ag IMNPs with a full monolayer of adsorbed I^-. The UV−vis absorption peak of the mixture Ag IMNPs slightly red-shifted to 416.5 nm, indicating that no evident aggregation occurred. A strong Ag−I band at 108 cm^{-1} and clean background in the region of 200−1800 cm^{-1} were observed in the SER spectra of the Ag IMNPs, demonstrating that the surface of Ag NPs had been successfully modified with iodide and the signal of Ag IMNPs themselves will not interfere with those of analytes. At last, the prepared Ag IMNPs were mixed with 50 μL of 1 mg/mL lysozyme (a basic protein containing 129 amino acids) aqueous solution at room temperature, and the color of the colloids turned red immediately. Meanwhile, a new broad UV−vis absorption peak at 660 nm was observed, indicating that the protein has induced the aggregation of Ag IMNPs. A high-quality SER spectrum of lysozyme was obtained and is shown in Figure 1B.

From Figure 1B, it is obvious that the SER spectrum obtained from the 300 μg/mL lysozyme solution is almost identical to that of the normal Raman (NR) spectrum from the 100 mg/mL solution, implying that the native conformation of lysozyme can be maintained due to its weak interaction with Ag IMNPs. More importantly, the SER spectrum (Figure 1B, curve c) of lysozyme provides abundant peaks from proteins, such as bands assigned to the disulfide bond (506 cm^{-1}), aromatic residues of tryptophan (Trp) (754, 1004, 1550 cm^{-1}), phenylalanine (Phe) (1004 cm^{-1}), aliphatic residues CH_2 (1443 cm^{-1}), amide I (1657 cm^{-1}), and amide III (1233 cm^{-1}),[31,32] which are characteristic peaks and important signatures of the structure and conformation of proteins. We then used Ag NPs without I^- for direct comparison. The spectral feature changed with time, and the signal-to-noise ratio was lower. A characteristic spectrum is shown in Figure 1B, curve e. The spectral feature is much different from the NR spectra of lysozyme but similar to most of the published SERS results.[32,33] The changing spectra may be a result of the interference of the impurities inherited from synthesis of the Ag NPs colloids. The different spectral feature may indicate that the protein has undergone a conformational change upon interaction with Ag NPs.

Parts C and D of Figure 1 show the results for the measurement of the stability, reproducibility, and sensitivity of the Ag IMNPs method. The 10 continuous SER spectra are almost overlapped with each other (Figure 1C), indicating an excellent stability and reproducibility. The detection limit for lysozyme can be as low as 3 μg/mL, as shown in Figure 1D, which is close to the level reported in the literatures.[33,39] It should be pointed out that direct SERS detection of protein with almost identical spectral feature to that of the normal Raman with a high reproducibility has far-reaching significance. First, SERS can provide much higher sensitivity compared with normal Raman spectroscopy. Second, the identical SERS spectral feature to that of normal Raman in both relative peak intensity and frequency indicates that SERS can be used as an important method for both quantitative and qualitative analysis. Third, the excellent reproducibility overcomes the obstacle of SERS and offers SERS as a reliable analytical tool for practical analysis.

One may naturally wonder why the modification of Ag NPs with iodide can provide such a good performance. The point is that the adsorption of iodide will inevitably increase the distance between the protein molecule and the Ag NPs. It is the Ag NPs that provides the enhancement. Therefore, the presence of the iodide layer may more or less decrease the enhancement effect from the viewpoint of the electromagnetic field enhancement. In comparison, in most practical SERS measurements, halide ions with a high concentration (about 0.1 M) are commonly used to induce the aggregation of nanoparticles. In this way, a large number of "hot spots" may be produced in the gap of the nanoparticles in the aggregates. The analytes and the halide ions together with those capping agents may be trapped in the gap of the aggregates producing strong SERS signal. However, due to the addition of halide ions of a high concentration, it may sometimes result in a weaker signal since the halide ions could competitively form a strongly bonded surface layer that repels the analytes adsorbed on the surface, or due to the precipitation of large aggregates.[52] On the other hand, we used I^- ions of a much lower concentration (1 mM) compared with the 0.1 M case and the I^- ions were added to the silver sol before the addition of protein. Therefore, we think in our case, the I^- ions are only used as a competitive adsorption agent to repel the surface impurities on the silver NPs to achieve a clean and uniform surface for the protein detection. It is the protein that acts as the aggregation agent to produce strongly enhanced SERS signal.

Following this assumption, the concentration of iodide ions plays an important role. Ideally the concentration should be just enough to coat as a full monolayer on the Ag surface. We performed ζ-potential measurement to verify the amount of KI on the surface (Supporting Information Figure S2A). The ζ-potential is sensitive to the surface species of NPs. A consecutive change of ζ-potential may be observed when I^- replaces the citrate on the surface until the saturated adsorption of I^-, which can be a good indicator for a full monolayer adsorption. Indeed, the ζ-potential of silver colloid increased from −41.9 to −24.5 mV with the increasing volume of 1 mM KI in the range of 0−20 μL. With the further increase of the volume, the ζ-potential remained unchanged. Therefore, the amount of 1 mM KI to achieve a saturated adsorption was 15−20 μL, which was close to that estimated and used in our experiments (17 μL, here the amount of silver colloid was one-third of that in SERS detection). By contrast, when the amount of KI was insufficient, the SER spectra showed bad signal-to-noise ratio and low reproducibility (Supporting Information Figure S2B), just as the case of bare Ag NPs. Whereas, when the amount of KI was obviously excessive, the SERS intensity decreased, since excessive KI will be adsorbed on the surface of the colloid particles and thus prevent adsorption and detection of incoming analytes,[52] and furthermore, excessive KI will also induce aggregation before the addition of analytes, which will lead to a decrease of the opportunity to trap analytes in the "hot spots".

We also investigated the effect of other halide ions, cation, and Au NPs with our methods. When Ag NPs were modified with Br^- or Cl^-, high-quality SER spectra of protein were also obtained (Supporting Information Figure S3A). Among the

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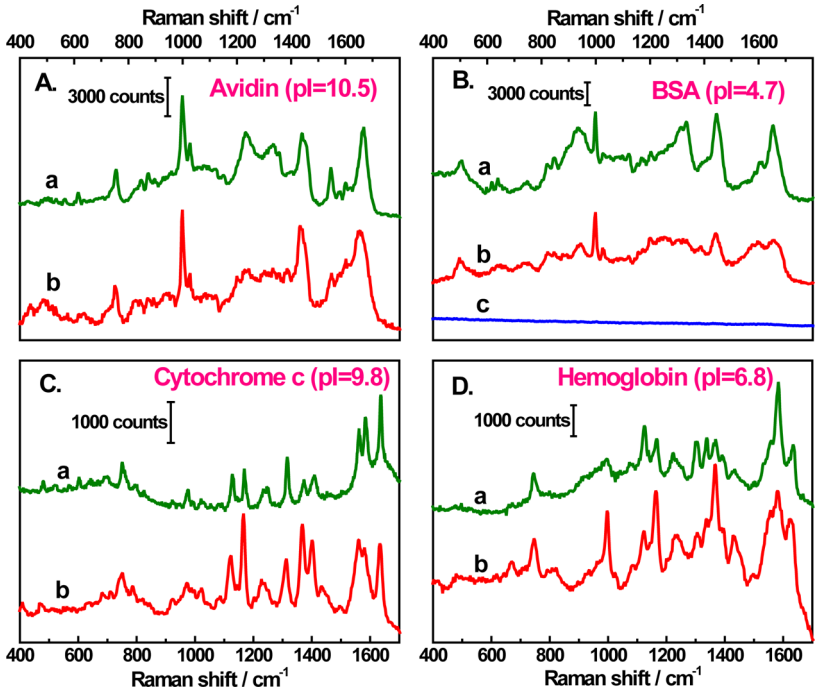

Figure 2. Normal Raman (a) and SER spectra (b) of (A) avidin, (B) BSA, (C) cyt *c*, and (D) hemoglobin. All the SER spectra were obtained with Ag IMNPs at sample concentrations of 300, 300, 3, 30 μg/mL, respectively. The curves c and b in panel B are the SER spectra of BSA recorded before and after Ag IMNPs were aggregated by $MgSO_4$, respectively. The experimental conditions to obtain the normal Raman spectra of avidin, BSA, and Hb solid are 20 mW laser power and 30 s acquisition time.

three halide ions, I^- performed the best, which may be due to its strongest adsorption ability on Ag NP_S, which may displace surface citrate more completely compared with the other halide ions. When we replaced KI with NaI, we did not observe any obvious difference, implying that the cation might not play a decisive role. It is believed that Au NPs are more biocompatible compared with Ag NPs. Therefore, we also used Au NPs for protein detection. Surprisingly, in the literature there are much less reports on the SERS detection of protein using Au NPs, implying the difficulty of such work. Indeed that is what we found for bare Au NPs. However, after I^- modification, we could successfully obtain quite good SER spectra of lysozyme with Au NPs (Supporting Information Figure S3B), and the signal is much weaker than that obtained with Ag NPs. The main reason is that Au has a lower SERS activity compared with Ag. It is particularly interesting to note that the spectral feature is almost identical to that Ag NPs, indicating that the use of Ag will not modify the structure of protein due to the protection of the iodide layer. Especially, for the in vitro detection, the biocompatible issue is not very essential. So we can simply use Ag NPs to produce better signals.

It is still not very clear why so reproducible and reliable SERS signal of proteins can be achieved on Ag IMNPs. Nevertheless, we think at least the following four factors may contribute to the improved results. First, the ζ-potential of Ag IMNPs is −24.5 mV so that proteins with plenty of positive-charged groups on the surface can interact with them via the electrostatic interaction. Second, the strongly adsorbed iodide can replace the existing impurities on the surface of Ag NPs and eliminate the interference from them. Third, iodide modification can avoid the direct chemical interaction between proteins and Ag, reducing the impact of SERS substrate on the native conformation of protein molecules. Last, damage caused by the laser can be reduced to the lowest since all the SERS measurements are conducted in the aqueous solution with a large laser spot. Such a condition is also similar to that of the live cell system, offering unique opportunity for in vivo study of live cells. From the above result, we can conclude that the interfacial property of SERS-active NPs is critically important for a reliable and reproducible SERS detection.

We applied the above method in the detection of four other proteins, including two proteins without chromophore (avidin and BSA) and two hemoproteins (cyt *c* and hemoglobin). Among them, avidin and cyt *c* are basic proteins, whereas BSA and hemoglobin (Hb) are acidic proteins. BSA has been widely used as a protecting agent in the design of SERS probes, because its signal is too weak to interfere with the SERS signal of probe molecules. Different from lysozyme, after the addition of BSA into the Ag IMNPs colloid, no obvious color change has been found, which may indicate that BSA cannot readily induce the aggregation of Ag IMNPs to produce observable signal. To force the aggregation, we introduced an extra aggregation agent (20 μL of 0.01 M $MgSO_4$) into the solution. For other protein, we just directly added protein solution into the Ag IMNPs sol to induce aggregation.

As shown in Figure 2, reproducible SER spectra with a strong signal could be obtained for all the four proteins. All the Raman bands appearing in the normal Raman spectra also showed in the SER spectra. Most interestingly, avidin and BSA, the two proteins without chromophore, gave SER spectra with features (including relative intensity and frequency) similar to that of the respective normal Raman spectra, similar to the case of lysozyme. Both of them show characteristic vibrations of S−S (503 cm^{-1}), Phe (1000 cm^{-1}), Trp (753, 1005, 1549 cm^{-1}), aliphatic residues CH_2 (1446 cm^{-1}), and amide (1658, 1234 cm^{-1}).[32] In contrast, SER spectra of cyt *c* and Hb are dominated by the typical vibration modes of heme, e.g., ν_{15} (749 cm^{-1}, B_{1g}), ν_{22} (1124 cm^{-1}/1120 cm^{-1}, A_{2g}), ν_{10} (1633

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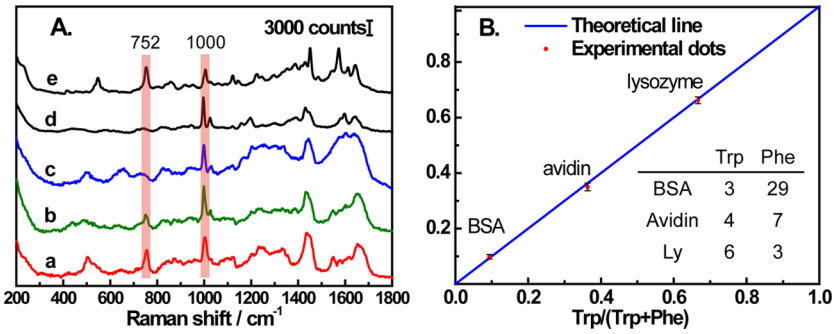

Figure 3. (A) SER spectra of (a) lysozyme, (b) avidin, (c) BSA, (d) Phe, and (e) Trp. All the SER spectra were obtained with Ag IMNPs. (B) Comparison between the experimental values with the theoretical values. The peak intensity ratios of 752−1000 cm^{-1} from the three proteins were normalized by using the signal from Trp. The inset table in panel B is the known number of Trp and Phe residues in each protein molecule.

cm^{-1}/1625 cm^{-1}, B_{1g}), ν_{30} (1164 cm^{-1}, B_{2g}), ν_4 (1366 cm^{-1}, A_{1g}), and etc.[30] Furthermore, their SERS signals are much more sensitive to the experimental condition than those of avidin and BSA, because they are both hemoproteins with resonance Raman effect. By comparing the resonance Raman spectra with that of the SER spectrum, we found that the major difference is on the relative peak intensities, and the peak positions only change slightly. The two weak peaks at 1500 and 1582 cm^{-1} can also be seen in Figure 2. They are the signature of the native six-coordinated low-spin (6cLS) configuration of cyt *c*,[53,54] indicating the native state of cyt *c* has been well-preserved. We observed a shift of the peak at 1375 cm^{-1} in the resonance Raman spectrum to 1366 cm^{-1} in the SER spectrum. These two peaks were attributed to the C_α−N breathing mode of the pyrrole macrocycle (ν_4) of the oxidized and reduced forms of cyt *c*, respectively.[55] They have been used as markers for the redox state of cyt *c*. The oxidized form of cyt *c* was used in our study, and when it interacts with the Ag IMNPs it may be reduced, as has reported by Sibbald et al.[55]

It is well-known that, in conventional SERS measurement, the SERS spectral feature is much different from that of the normal Raman, in both the relative intensity and the peak position. At least the following two reasons may contribute to that: The first is the strong chemical interaction between the molecule and the metal surface, which may lead to a change of the symmetry or even the electronic structure of the molecule. This effect will result in both the change of the relative intensity and frequency shift. The second is the surface selection rule determined by the electromagnetic field on a metal surface. It will selectively enhance the vibrational mode close to the surface and with the polarizability change along the surface normal. In the normal Raman measurement of proteins, one is able to make quantitative and qualitative analysis of high-concentration samples according to their unique fingerprint information by analyzing the relative intensity and frequency. But it becomes complicated in SERS due to the selective enhancement, especially for proteins containing similar amino acid residues. However, the SER spectra obtained with our method are very similar to the normal Raman for molecules without chromophores, especially in the relative intensities. This phenomenon has far-reaching significance for the reliable identification of proteins. We measured the SERS signals (Figure 3A) of some important proteins, such as lysozyme, avidin, and BSA, to seek for the possibility to do qualitative identification. For comparison, we also measured the two amino acids (Trp and Phe) that can give strong SERS signal. It can be seen in Figure 3A that Phe and Trp have a similar Raman cross section. The peak at 752 cm^{-1} is assigned to Trp; the peak around 1000 cm^{-1} is contributed by both Trp and Phe. The SER spectra of the three proteins are dominated by the signals from Phe and Trp, but with very different intensity ratio. Therefore, the peak intensity ratio of 752 to 1000 cm^{-1} can sensitively reflect the relative content of Trp and Phe, which may provide an approach to identify proteins. For example, one BSA molecule contains 29 Phe residues and only 3 Trp residues, and it gives a strong peak intensity at 1000 cm^{-1} (Phe and Trp) but a weak peak at 752 cm^{-1} (Trp). The actual compositions of lysozyme, BSA, and avidin are known, and the relative content of Trp and Phe for them are given in the inset table of Figure 3B. For a more quantitative analysis of the result, we plot the relative Raman intensity of 752/1000 to the ratio of Trp/(Trp and Phe) for the three proteins in Figure 3B. One can see that the relative intensities of 752 and 1000 cm^{-1} were in a surprisingly good agreement with the ratio of Trp to (Trp and Phe). In other words, our proposed Ag IMNPs SERS method can be used as a very simple way to perform label-free identification of proteins with a much better reliability and specificity than traditional identification methods.

In addition to the high spectral specificity and improved sensitivity, SERS also possesses the multiplexing capability due to narrow bandwidths. Multiplex detection is and continues to be a very important direction in analytical chemistry, with the increasing complexity of the samples, especially for analyzing proteins. SERS has been successfully used for the detection of multiple proteins combination with Western blotting,[34] which turns out to be a tedious method as separation steps are needed. Recently, SER spectra of three types of binary protein mixtures have been reported,[56] and the obtained SER spectra were too complicated to assign the contribution of each protein. We applied the Ag IMNPs SERS method for detection of a binary mixture of lysozyme and cyt *c*. As shown in Figure 4, the SER spectrum of the mixture is equivalent to the overlying of the two individual proteins and the contribution of each protein can be easily assigned. The present method offers a reliable and definite way for the identification of binary mixtures, demonstrating its advantages in multiplex protein detection without separation steps. Thus, the proposed SERS detection method for label-free proteins holds great potential in proteomic research and applications in living system.

The dynamic process of protein in a living system is a very important topic in biology. We further tried to monitor the dynamic process of conformational changes of the protein with this method. For this purpose we choose H_2O_2 as a stimulus to induce the protein damage. H_2O_2 is a commonly used oxidant

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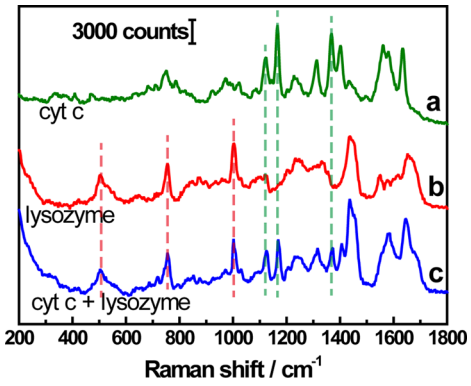

Figure 4. SER spectra of (a) cyt *c*, (b) lysozyme, and (c) mixture of cyt *c* and lysozyme (1.5 μg/mL/15 μg/mL = 1:10). All the SER spectra were obtained with Ag IMNPs. The laser power was 5 mW, and the acquisition time was 30 s.

which can oxidize the reducible groups in the protein molecule such as the disulfide bond. Figure 5 shows the SER spectra of

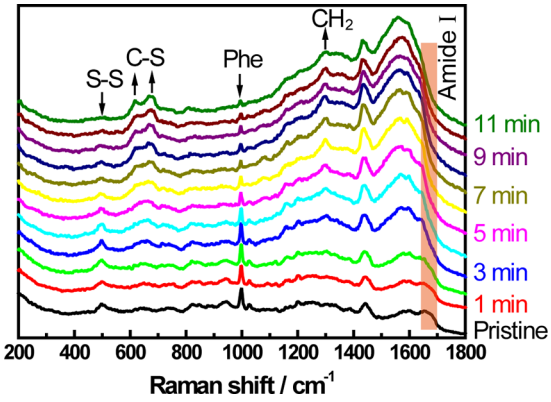

Figure 5. Time-sequence SER spectra recorded after the addition of 5 μL of 1.9 mM H_2O_2 (1:0.025) into the mixture of BSA with Ag IMNPs. Pristine is the original SER spectrum of BSA. All the spectra were collected at a same point, and the time for each SER spectrum is shown in the figure. The laser power was 2 mW, and the acquisition time was 10 s.

BSA during its interaction with H_2O_2. The SER spectra of BSA started to change in 3 min. The peak intensity at 497 (S−S), 997 (Phe), and 1650 cm^{-1} (amide I, α-helix) decreased over time, while some new peaks appeared at 618, 675 (C−S), and 1294 cm^{-1} (CH_2). In the control experiments with Ag IMNPs and BSA but without H_2O_2 (Supporting Information Figure S4A) and Ag IMNPs and H_2O_2 without BSA (Supporting Information Figure S4B) we did not observe any new peaks. These results imply that some changes had occurred in the conformation of BSA, such as the cleavage of the disulfide bond, partial unwinding of the α-helix, and even unfolding. We speculate that BSA was denatured by H_2O_2 under such conditions. This experiment demonstrates that dynamic structural changes of protein can be studied in situ with our method. The effort toward the characterization of protein dynamics in living cells is now ongoing in the lab.

CONCLUSION

With the aim of solving the existing problems of poor reproducibility and low detection sensitivity in the SERS detection of protein, we proposed an iodide-modified Ag nanoparticles method (Ag IMNPs) for label-free detection of proteins. The iodide-coated Ag nanoparticles not only provide a highly enhanced electromagnetic field to increase the Raman signal, but also provide an iodide layer to prevent the direct interaction between the proteins and the metal surface. We have been able to obtain highly reproducible and high-quality SERS signals of five typical proteins (lysozyme, avidin, BSA, cyt *c*, and hemoglobin). The SERS features of proteins without chromophore were almost identical to those of the normal Raman spectra, which allows the qualitative identification of proteins without chromophore using SERS by simply taking the intensity ratio of Raman peaks related to tryptophan and phenylalanine. Unlike previous methods in which proteins were in direct contact with the SERS-active NPs, this method appears to be much more simple but effective and can remarkably improve the reliability, reproducibility, and sensitivity of the label-free SERS detection method. More importantly, native states of the proteins were maintained during the detection processes on account of iodide modification of the surface. These unique features owe to a proper modification of interfacial structure of SERS-active NPs. This method has been successfully applied for label-free multiplex analysis of protein mixture and monitoring the dynamic process during the protein damage stimulated by hydrogen peroxide. Combined with chemometric methods, this method can be used to study more complex systems, such as multiple or complex protein systems. It can also be applied for studying living cell systems and to reveal the interaction between protein molecules, which is now ongoing in the lab.

ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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