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Analytical Technique for Label-Free Multi-Protein Detection Based on Western Blot and Surface-Enhanced Raman Scattering

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We have developed a new analytical procedure for label-free protein detection designated "Western SERS", consisting of protein electrophoresis, Western blot, colloidal silver staining, and surface-enhanced Raman scattering (SERS) detection. A novel method of silver staining for Western blot that uses a silver colloid, an excellent SERS-active substrate, is first proposed in the present study. During the process of silver staining, interactions between proteins and silver nanoparticles result in the emergence of SERS of proteins. In the present study, we use myoglobin (Mb) and bovine serum albumin (BSA) as model proteins. From different protein bands on a nitrocellulose (NC) membrane, we have observed surface-enhanced resonance Raman scattering (SERRS) spectra of Mb and SERS spectra of BSA. The proposed technique offers dual advantages of simplicity and high sensitivity. On one hand, after the colloidal silver staining, we can detect label-free multi-proteins directly on a NC membrane without digestion, extraction, and other pretreatments. On the other hand, the detection limit of the Western SERS is almost consistent with the detection limit of colloidal silver staining, and the SERRS detection limit of Mb is found to be 4 ng/band. This analytical method, which combines the technique of protein separation with SERS, may be a powerful protocol for label-free protein detection in proteomic research.

In many proteomics studies, approaches for protein identification are usually based on the combined use of electrophoresis and mass spectrometric (MS) analysis. A protein mixture from cells or tissues is usually separated by electrophoresis and then transferred by electroblotting onto immobilizing membranes, which is called protein blotting, electrophoretic transfer, or Western blot, for the identification of individual or specific classes of proteins by immunochemical detection methods.[1,2] Recently, mass spectrometric peptide fingerprinting of proteins after Western blot on a poly(vinylidene difluoride) (PVDF) membrane called "Western MS", has been proposed.[3−5] Using the MS analysis, one can identify proteins detected by Western blot directly on a PVDF membrane. Moreover, several sensitive stains for membrane-immobilized proteins, such as metal chelate complexes, colloidal gold, and fluorescent dyes, have been described,[6−8] and highly sensitive, colloidal silver staining has also been reported.[9]

Surface-enhanced Raman scattering (SERS) technique has proved to be a very effective analytical tool due to its high sensitivity, high selectivity, and fluorescence-quenching properties.[10−17] SERS or surface-enhanced resonance Raman scattering (SERRS) has been widely used for ultrasensitive chemical analysis down to single molecule detection, and it expands its realm of applications from chemical−biochemical analysis to nanostructure characterization and biomedical applications.[16,17] It is very useful in detecting conformational changes and structural differences regarding preferred orientations of molecules with respect to a metal surface.[18−23] Many studies have been carried out for label-

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free protein detection using SERS or SERRS, such as green fluorescent protein,[24] human insulin and insulin lispro,[25] cytochrome *c*,[26−28] and so on. SERS detection is sensitive enough to differentiate two insulins that differ only in the interchange of two neighboring amino acids[25] and detect antigen−antibody binding.[29] However, proposed methods in these studies were independent of the process of protein separation and purification. Thus, they could obtain SERS spectra only from purified proteins. Therefore, these methods cannot satisfy the demands of high-throughout proteomic research. Direct SERS detection of label-free proteins on blotting membranes after protein blotting has not been reported so far.

The purpose of the present study is to develop a new protocol called "Western SERS" to identify label-free multi-proteins directly on a nitrocellulose (NC) membrane. We have attempted to combine SERS and Western blot by using colloidal silver staining, taking their advantages together. By using the proposed method, we have measured SERRS spectra of myoglobin (Mb) and SERS spectra of bovine serum albumin (BSA) on a NC membrane, and we have also compared these spectra with their corresponding resonance and normal Raman spectra. The present Western SERS has two advantages. Comparing to mass spectrometry, we can detect label-free proteins directly on a NC membrane without the time-consuming procedures of stripping and digestion. Moreover, the detection limit of the Western SERS is almost consistent with the detection limit of colloidal silver staining, and SERS signals do not self-quench, unlike fluorescence. Thus, the new method has great potential for identifying proteomic components or proteins of differential expression in certain proteome.

EXPERIMENTAL SECTION

Materials. Mb (99%, from muscle of bovine) was obtained from Department of Biology, Jilin University, Changchun, P. R. China. BSA (99%), *N*,*N*-methylene bisacrylamide and acrylamide were purchased from Sigma Co., Ltd. Both Mb and BSA were used without further purification. NC membrane was obtained from American Osmonics. Silver nitrate was from Aldrich, and all other chemicals were purchased from Beijing Chemical Reagent Co., Ltd. Triply distilled water was used throughout the present study.

Preparation of Silver Colloid. Colloidal silver was prepared by the aqueous reduction of silver nitrate with trisodium citrate using a modified method of Lee and Meisel,[30] The plasmon absorption maximum of the silver colloid we prepared was located at 415 nm.

Protein Electrophoresis and Western Blot. We chose two kinds of proteins, Mb and BSA, as model proteins. The two proteins were first dissolved in a sample buffer (62.5 mM Tris-HCl, pH 6.8; 10% glycerol), and then, the protein mixture (containing 1 μg/mL BSA and 1 μg/mL Mb) was electrophoresed with the amounts of 4, 6, 8, 10, and 12 μL in a 10% polyacrylamide gel, which was called polyacrylamide gel electrophoresis as given by Guo et al.[31] After that, the proteins were blotted onto a NC membrane by a semidry transfer instrument for 4 h with a semidry transfer buffer (Tris-HCl buffer, pH 10.4). After blotting, the samples were washed by using a Tris-HCl buffer (pH 7.10). In another experiment, BSA (1 μg/mL), with the amounts of 2, 3, 4, 5, and 6 μL, were electrophoresed and then blotted onto another NC membrane followed by the same procedure described above.

Colloidal Silver Staining. The NC membrane on which the proteins were blotted was then soaked in silver colloid for 20 min. Finally, it was washed using a Tris-HCl buffer (pH 7.10) followed by triply distilled water for three times. After that, the NC membrane was dried naturally at room temperature.

Field Emission Scanning Electron Microscope (FE-SEM). Microcosmic structures of the NC membrane inside of protein bands and outside of protein bands were studied with a JEOL JSM-6700F FE-SEM with an accelerating voltage of 3 kV.

SERS Acquisition. SERS spectra were measured with a Renishaw 1000 model confocal microscopy Raman spectrometer equipped with a CCD detector and a holographic notch filter. Radiation of 514.5 nm from an air-cooled argon ion laser was used for the SERS excitation with the power of 5 mW at the sample position. The microscope attachment based on a Leica DMLM system and a 50× objective was used to focus the laser beam onto a spot with ~1 μm in diameter. The typical accumulation time used in this study was 30 s.

RESULTS AND DISCUSSION

We have designed a novel analytical technique for label-free protein detection named Western SERS, and the procedure is described in Scheme 1. After being separated by native polyacrylamide gel electrophoresis (PAGE) or isoelectric focusing electrophoresis, proteins are electroblotted onto a sheet of NC membrane that has the capacity of binding any protein contacts on it. Furthermore, the proteins stick to the membrane at the same positions as they are in the gel. Therefore, we can observe individual protein bands by using colloidal silver staining. Finally, different SERS spectra corresponding to different protein bands can be obtained by using the confocal Raman spectrometer.

New Colloidal Silver Staining for Western Blot. The NC membrane used for protein blotting is a good immobilizing membrane, with the advantages of high capability of protein combination, high sensitivity, and easy use. The coalescent mechanism between the blotting membrane and proteins is not well understood, and probably there are some interactions between them such as a hydrophobic interaction, an ionic interaction, and so on.[32,33]

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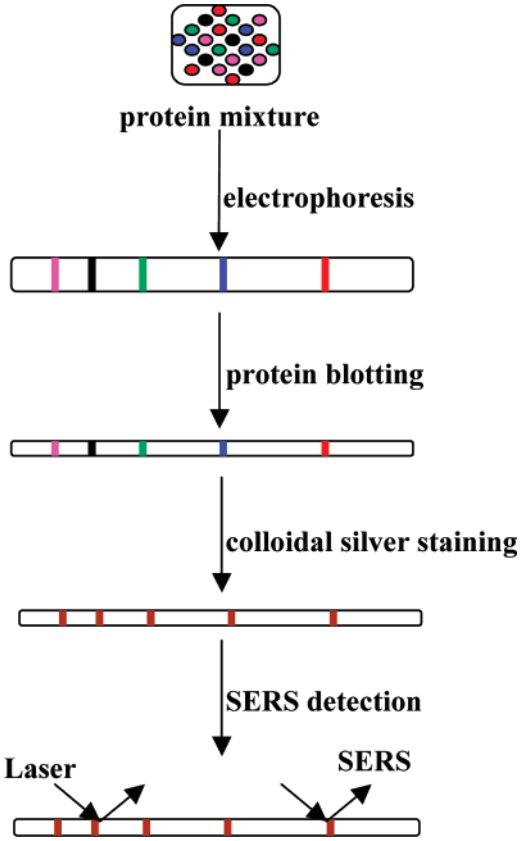
Scheme 1. Newly Proposed Procedure for Protein Detection Based on Western Blot and SERS



We have developed a new method of silver staining for Western blot using a silver colloid, a good SERS-active substrate. The colloidal silver is celadon, and we prepared it by the aqueous reduction of silver nitrate with trisodium citrate.[30] It can be kept at room temperature stably for at least three weeks. From the results of atomic force microscopy and UV−vis measurements (not shown), it has been found that most of the silver nanoparticles are spheroidal, and the plasmon absorption maximum of the colloidal silver is located at 415 nm.

Compared to other methods of silver staining for proteins, the process of colloidal silver staining we used is so easy that, without other reagents, we can just marinate the NC membrane in the silver colloid for 20 min. After that, protein bands appear as expected because of the interactions between proteins and silver nanoparticles. Bands containing different amounts of Mb or BSA (calculated from the amounts of proteins added to the electrophoresis) are obvious in Figure 1a. We made another experiment for investigating the detection limit of the novel colloidal silver staining. Figure 1b indicates that the detection limit of the silver staining is as low as 2 ng/band, which confirms the high sensitivity of this colloidal silver staining. To a certain extent, the amounts of the silver nanoparticles adsorbed on proteins increase with the prolonged time of staining.

Figure 2 shows FE-SEM images of a NC membrane outside (A) and inside (B) the protein bands after colloidal silver staining.

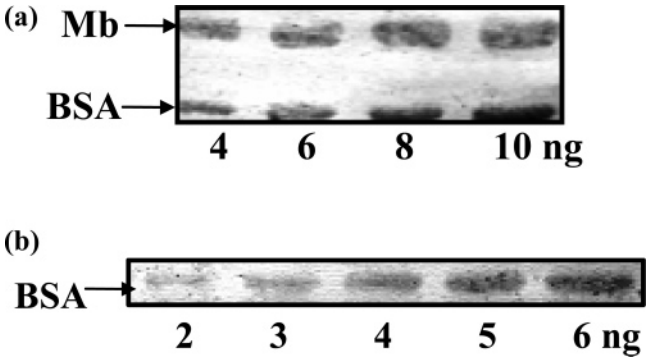


Figure 1. (a) Mb and BSA bands on a NC membrane after colloidal silver staining and (b) BSA bands on another sheet of a NC membrane after colloidal silver staining.

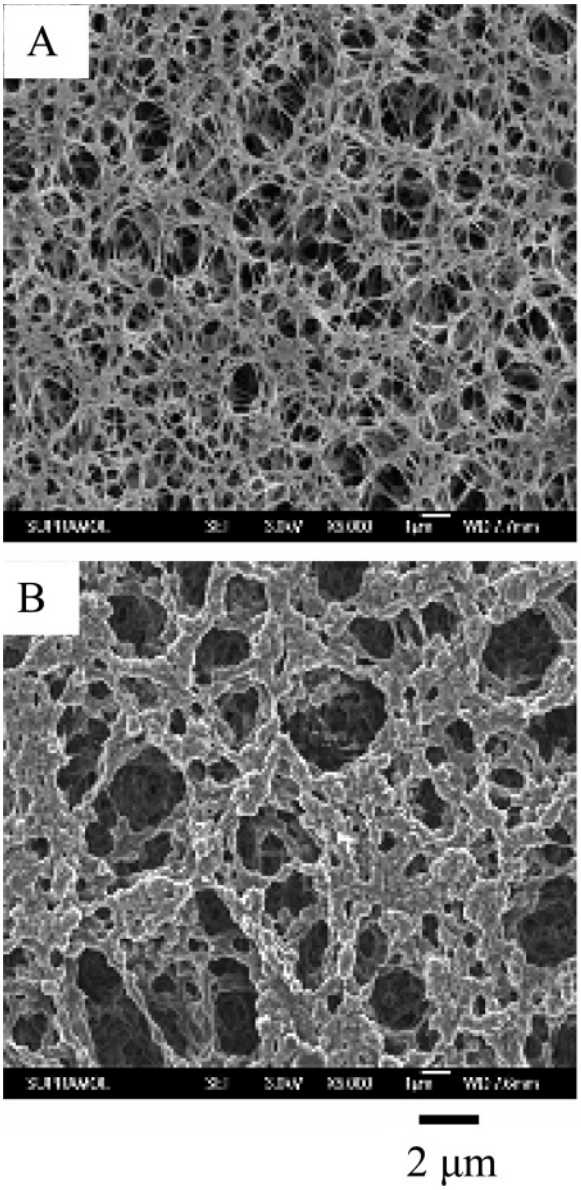


Figure 2. FE-SEM images of a NC membrane (A) outside and (B) inside the protein bands after colloidal silver staining.

Note that one can see the netlike structure of the NC membrane. This structure can increase the adsorption area of NC membrane. This is the reason why it has a high capacity of protein adherence.[33] We have obtained the similar FE-SEM image of the

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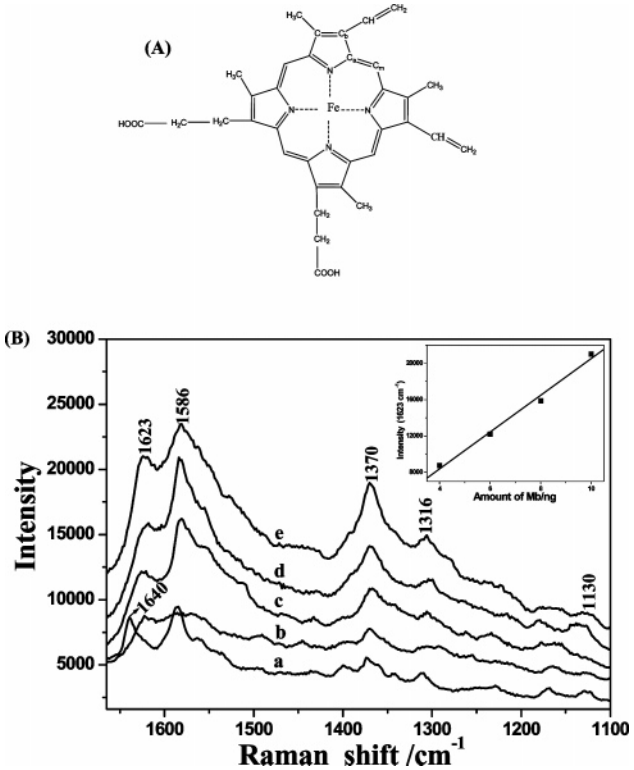

Figure 3. (A) Structure of heme. (B) A resonance Raman spectrum of solid Mb (a) and SERRS spectra of Mb with different amounts on a NC membrane: (b) 4, (c) 6, (d) 8, and (e) 10 ng. The inset shows an intensity of a SERRS peak at 1370 cm^{-1} versus the amount of Mb on a NC membrane.

outside of the protein bands on a NC membrane (Figure 2A) to the one of a blank NC membrane. Probably, little interaction between the NC membrane and silver nanoparticales results in this high sensitivity of the colloidal silver staining protocol. It appears that silver nanoparticals are adsorbed along the net of the membrane where proteins have already adhered, and there are a number of silver aggregates along the net of the NC membrane, which make it possible for SERRS or SERS to emerge.[34]

SERS Detection. A resonance Raman (RR) spectrum provides a sensitive and selective probe for chromophores of proteins and any other biochemical systems. A protein-bound heme gives rise to intense visible absorption bands and thus makes a hemoprotein available for RR studies.[35] Mb, composed of one chain of polypeptide and a prosthetic group of heme, belongs to a hemoprotein family. It plays an important role in binding molecular oxygen for transport.[36] As one of simple proteins, BSA has no protein-bound chromophore, and its SERS-active groups consist of amide groups and aromatic side chains such as tyrosine, tryptophan, phenylalanine, and so on.

Figure 3A shows the structure of the heme group of Mb and (B) a RR spectrum of solid Mb and SERRS spectra of Mb on a NC membrane. We can observe main RR bands of the heme group

Table 1. Band Assignments for the RR and SERRS Spectra of Mb on a NC Membrane[a]

Raman shift (cm^{-1})		
$Mb_{(RR)}$	$Mb_{(SERRS)}$	assignments
1640		ν_{10} ν C_aC_m
	1623	ν C=C
1585	1586	ν_{19} ν_{as} C_aC_m
1399	1399	ν_{20} ν_{as} C_aN
1374	1370	ν_4 ν C_aN
1313	1316	ν_{21} δ_{as} C_mH
1168	1164	ν_{30} ν_{as} C_b-Et
1129	1130	ν_{21} ν_{as} C_aN

[a] δ, deformation; ν, stretching; s, symmetric; as, asymmetric; Et, ethyl.

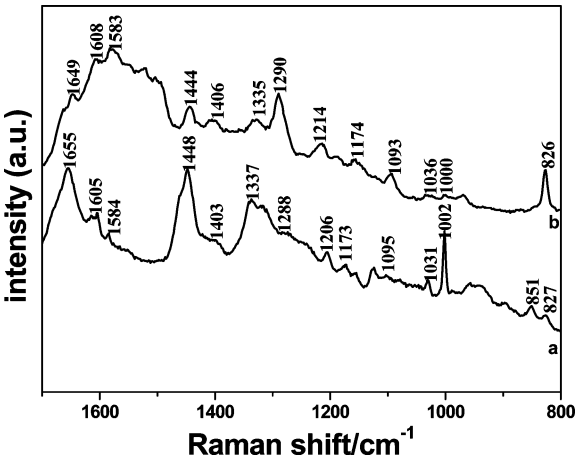

Figure 4. Normal Raman spectrum of solid BSA (a) and a SERS spectrum of BSA on a NC membrane (b).

at 1623, 1586, 1370, 1316, and 1130 cm^{-1}.[37,38] Their RR and SERRS band assignments are listed in Table 1. Compared to the RR spectrum of Mb, there are some significant differences in its SERRS spectra probably because of the interactions between the silver nanoparticles and proteins. In the RR spectrum of Mb (Figure 3B a), one can observe an intense peak at 1640 cm^{-1} assigned to the C_aC_m (ν_{10}) stretching vibration of the heme group. However, this peak is missing in the SERRS spectra of Mb, and instead, a new peak develops at 1623 cm^{-1} due to the C=C stretching vibration. It indicates that the aggregates of silver nanoparticles on which Mb molecules are absorbed can remarkably enhance the intensity of the C=C stretching vibration and that the C_aC_m (ν_{10}) stretching vibration is covered by it in the SERRS spectra. More amounts of Mb molecules absorbed on per unit area of the NC membrane result in stronger SERRS signals. As is shown in the inset of Figure 3B, the peak intensity at 1623 cm^{-1} increases linearly with the amount of Mb absorbed on the NC membrane, and the detection limit of Mb is estimated to be 4 ng. These results indicated the possibility of the present method for quantitative hemoprotein detection.

Figure 4 depicts a normal Raman spectrum of solid BSA and a SERS spectrum of BSA from the band containing 8 ng of BSA (shown in Figure 1a) on a NC membrane. BSA is known to be

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Table 2. Band Assignments for the Normal Raman and SERS Spectra of BSA on a NC Membrane

Raman shift (cm^{-1})		
$BSA_{(Raman)}$	$BSA_{(SERS)}$	assignments
1655	1649	amide I
1605	1608	Tyr or Trp
1584	1583	Phe
1448	1444	δ CH_2
1403	1406	ν_s (COO^-)
1337	1335	Trp
1288	1290	amide III
1206	1214	Tyr or Phe
1173	1174	Tyr
1031	1036	Phe
1002	1000	Phe
851		Tyr
827	826	Tyr

rich in α-helix structure, which can be confirmed by an intense band due to the amide I mode at 1655 cm^{-1} shown in Figure 4a. According to SERS studies for label-free proteins without a prosthetic group, in general, bands due to the amide I mode are relatively weaker than those due to Tyr and Trp residues in SERS spectra, and the SERS spectra of such proteins change markedly with the experimental conditions.[24,25,29] From Figure 4b, one can observe a shoulder band due to the amide I mode (1649 cm^{-1}) and a stronger band arising from the amide III mode (1290 cm^{-1}), which correspond to the α-helix structure.[39] These wavenumbers are slightly different from those in the normal Raman spectrum of BSA. It indicates that there is a slight change in the secondary structure of BSA because of the interactions between BSA molecules and silver nanoparticles. Some strong bands due to Tyr, Phe, and Trp redidues are also observed in the SERS spectrum of BSA (Figure 4b).

Since only the residues near the surface of silver nanoparticels would be enhanced, SERS spectra of adsorbed proteins may not closely resemble the corresponding normal Raman spectra.[40] Note that a band at 1403 cm^{-1} due to the symmetric vibration of the COO^- group is significantly enhanced in the SERS spectrum of BSA, suggesting that the carboxyl groups are also interactive with silver nanoparticles. Table 2 summarizes band assignments for the normal Raman spectrum of BSA and its SERS spectrum on a NC membrane.[40−46] From the results shown in Figure 3, we may be able to conclude that the adsorption of silver nanoparticles results in the slight alteration of the secondary structure of BSA and slight modification of the aromatic amino acid residues, particularly in the parts of BSA that directly interact with silver nanoparticles.

At this stage, we can obtain clear signals only from the 8 ng/band of BSA, and SERS signals of the lower level are much weaker. We cannot get a clear linear relationship between the amount and SERS intensity of BSA. Nevertheless, we believe that this first study of BSA on a NC membrane shows great potential in SERS studies of label-free proteins.

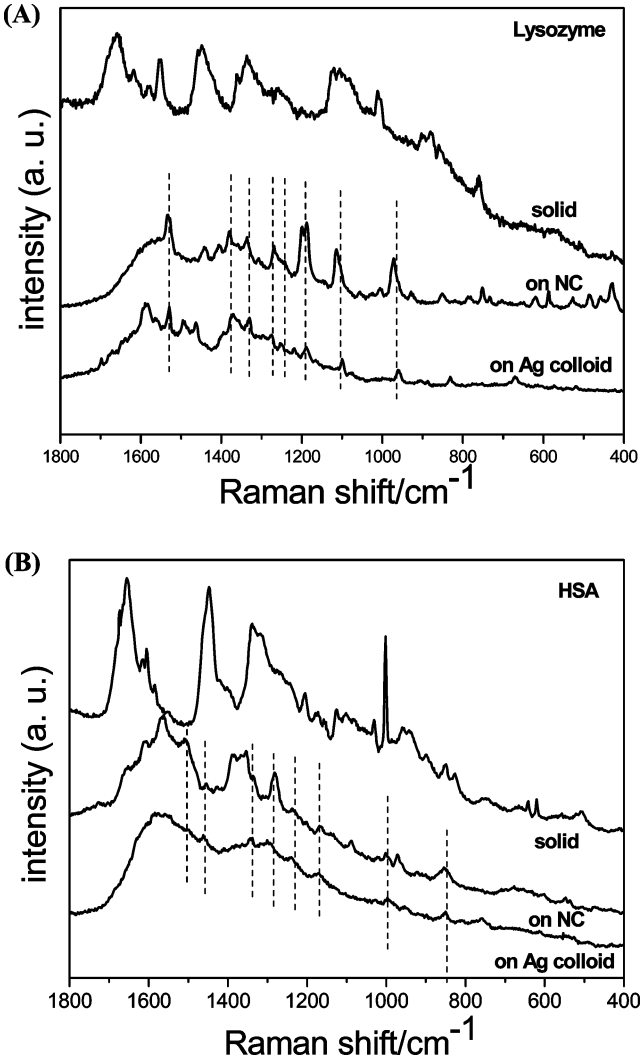

Figure 5. Normal Raman spectra of solid (A) lysozyme and (B) HSA, and their SERS spectra from NC membranes and silver colloids.

Moreover, we obtained SERS spectra of two other proteins (lysozyme and human serum albumin) that do not contain any chromophore on a NC membrane, independently. We dropped the two protein solutions onto a NC memberane, respectively, and then soaked the NC membrane in colloidal silver. Finally, we measured their SERS spectra on the corresponding protein spots. The SERS spectra of lysozyme and HSA (human serum albumin) from silver colloid were also measured. Panels A and B in Figure 5 compare the normal Raman spectra and their SERS spectra. From the results, we can find that though there are significant differences between their normal Raman and SERS spectra, the SERS spectra from NC membranes are similar to those from Ag colloid. It is also noted that the SERS spectra of lysozyme, HSA, and BSA on the NC membranes are clearly different from each other (Figures 4 and 5).

CONCLUSION

By combing the technique of protein separation and SERS, we have developed a simple and effective analytical technique

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called Western SERS for multi-proteins detection by using a new method of colloidal silver staining. The colloidal silver, which is an excellent SERS-active substrate as well as an excellent reagent for protein staining, plays a very important role in the present study. Using this technique, we have detected Mb and BSA on a NC membrane successfully. The detection limit of this method for Mb is as low as 4 ng. From the results, we have found that the NC membrane has little interference with the SERS spectra of proteins in our experimental conditions. Therefore, it seems that NC membranes are very good immobilizing materials not only for protein blotting but also for SERS detection of proteins. Furthermore, the proposed method would also be used in a two-dimensional gel electrophoresis (2D-PAGE)/Western blot system if we could solve the problem of denaturation of proteins on NC membranes. Thus, the proposed SERS detection for label-free proteins holds great potential in proteomic research.

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