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Label-free Rapid Semiquantitative Detection of Proteins Down to Sub-monolayer Coverage by Using Surface-enhanced Raman Scattering of Nitrate Ion

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A novel heat-induced SERS method was used to selectively enhance a band of NO_3^- at 1049 cm^{-1} for semi-quantitative detection of lysozyme and insulin down to 10^{-9} and 10^{-8} M, respectively, and bell shape variations of SERS intensities were observed for the concentration dependency of the proteins.

Surface-enhanced Raman scattering (SERS) has recently proved its potential in ultra-sensitive detections of biomolecules.[1–6] However, despite a great number of studies done in this field, label-free detections of proteins which do not contain resonant chromophores, still remain a challenge. Although label-free non-resonant SERS spectra have been obtained for several kinds of proteins,[7–12] no accurate quantitative study of proteins by nonresonant SERS has been reported. To our best knowledge, the only one relative quantitative study was realized by our group.[6] Because proteins without resonant chromophores have, in general, low Raman cross sections, the progress in label-free detections of this class of proteins is rather slow. The major advantages of the method used in the present study exist in its high sensitivity and routine analyses for semi-quantitative study.

We have recently proposed a heat-induced SERS-sensing method for rapid detection of a peptide, glutathione.[13] In this method, 30 μL of glutathione solution was heated on an aluminum pan plate at 100 °C for 3 min to obtain a dry film of glutathione, and then its SERS signal was acquired. Comparing to conventional SERS methods, this protocol enables one to obtain much larger SERS enhancement without loss of vibrational information about an analyte. In the present study, the application of this heat-induced SERS method is extended to protein detection. In the present study, $NaNO_3$ is used as an electrolyte instead of the most commonly used hydrochloride salt. Cl^- binds tightly to silver colloid[4] and thus it is difficult for an analyte to competitively adsorb on the silver surface to yield strong SERS signal.[4,6] First, a citrate buffer containing 6 mM $NaNO_3$ was mixed with a Ag colloid solution. Then, a protein was added to the mixture followed by drying the mixture at 100 °C for SERS measurement. Thanks to this highly sensitive method with combination of large Raman cross section of NO_3^-, semi-quantitative detection of proteins can be achieved down to 10^{-9} M for lysozyme and 10^{-8} M for insulin, respectively.

Figures 1a and 1b compare SERS spectra of lysozyme (10^{-5} M in citrate-reduced silver colloid) measured by use of the heat-induced method and without heat treatment (solution sample). As control in Figure 1c, a normal Raman spectrum of a dry film of 10^{-5} M lysozyme solution is shown. In the SERS spectrum of lysozyme without the heat treatment (its intensity has been magnified by a factor of 3, Figure 1b), there appears several bands due to Trp, Tyr, Phe, disulfide groups (ν(C–S) and ν(S–S)), amide I and amide III modes (see Table 1 in Supporting Information (SI)[22] for detailed band assignments). In the heat-induced SERS spectrum, an intense peak at 1049 cm^{-1} and a series of weaker bands are observed. In normal Raman spectra of solution lysozyme shown in Figures 1d and 1e, there is no peak at 1049 cm^{-1} and this peak also cannot match any bands of citrate anion.[14] The only remaining possibility for the origin of the 1049 cm^{-1} band is NO_3^-. To confirm this speculation, normal Raman spectra of lysozyme solutions containing 0 and 40 mM $NaNO_3$ are compared (Figures 1e and 1f), and the band is found at 1049 cm^{-1}, revealing that it arises from the NO_3^- vibration. This peak has seldom been reported in SERS references; however, Mrozek et al. unambiguously assigned it to the NO_3^- mode in their study of saccharide using electrochemically roughed silver surface.[15] Both their and our work suggest that the dry film methods selectively enhance peak of NO_3^- at 1049 cm^{-1}.

SERS spectra of lysozyme and insulin obtained by the heat-induced method as well as their corresponding normal Raman

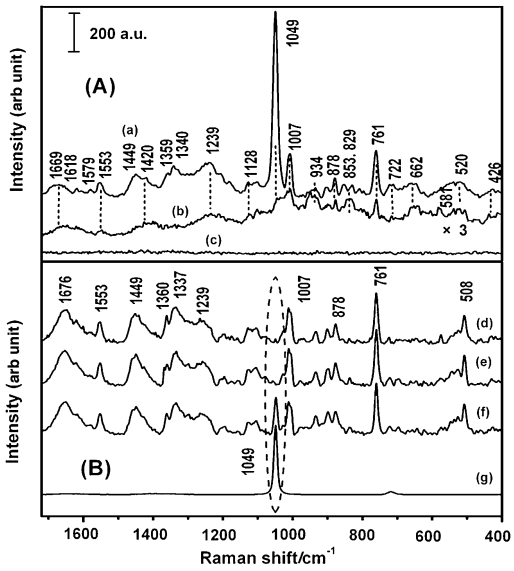

Figure 1. (A)(a) A SERS spectrum of a dry film of the solution containing 10^{-5} M lysozyme and 6 mM $NaNO_3$, (b) a SERS spectrum of a solution containing 10^{-5} M lysozyme and 6 mM $NaNO_3$, and (c) a normal Raman spectrum of a dry film of 10^{-5} M lysozyme solution. (B)(d) A normal Raman spectrum of 7×10^{-3} M lysozyme in pure water; (e), (f) normal Raman spectra of 7×10^{-3} M lysozyme in a 5 mM citrate buffer (pH 4.0) containing 0 mM $NaNO_3$ and 40 mM $NaNO_3$; (g) a normal Raman spectrum of 1 M $NaNO_3$. The intensity bar is applied only for (A) while band intensities in (B) have been adjusted artificially for display.

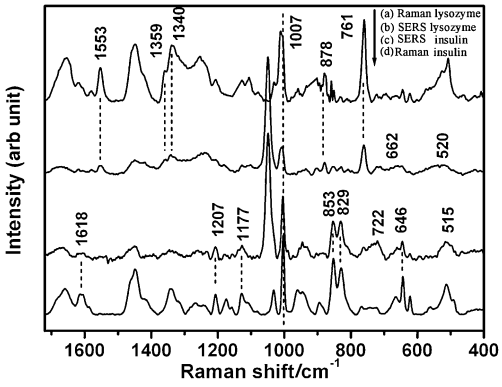

Figure 2. Normal Raman spectra of solid (a) lysozyme and (d) insulin, heat-induced SERS spectra of 10^{-5} M (b) lysozyme and (c) insulin.

spectra are shown in Figure 2, and these band assignments are summarized in Table 1 of SI.[22] Compared to the corresponding intensities in the SERS spectrum of insulin, the band intensities of Tyr residue (1618, 1207, 1177, 853, and 829 cm^{-1}) in SERS spectrum of lysozyme are stronger while the band intensities of Trp residue (1548, 1359, 1340, 878, and 761 cm^{-1}) and disulfide (ν(S–S) and/or ν(C–S), 520–750 cm^{-1}) are weaker. This trend is consistent with the appearance probabilities of the residues (see Table 2 of SI),[22] further confirming the band assignments for the SERS spectra.

The intensity and sharpness of the NO_3^- peak at 1049 cm^{-1} lead us to propose that this band may be suitable for quantitative analysis of the proteins. Figure 3A displays concentration-dependent (10^{-5}–10^{-9} M) SERS spectra of lysozyme. Comparison to previous studies, the results in Figure 3A demonstrate two novelties. First, the detection limit is much lower: down to a submonolayer coverage of 10^{-9} M (the coverage calculation is reported in SI[22]), which is more sensitive than the method proposed before by which the detection limit is 10^{-6} g L^{-1} (equivalent to 7×10^{-8} M).[6] Second, a concentration-dependent curve shows a bell shape rather than a sigmoidal one.[13] Similar results are displayed in Figures 3C and 3D for insulin. The bell shape result for lysozyme (Figure 3B) is supported by UV–visible spectra of mixtures of lysozyme and Ag colloids shown in Figures 4A and 4B. Figure 4A shows that the absorption of UV–visible spectrum decreases with the protein in the range of 10^{-8}–10^{-9} M. On the other hand, in the range of 10^{-5}–10^{-9} M (Figure 4B), there is a minimum absorption at 10^{-7} M, indicating that when the protein concentration is above monolayer coverage, a further increase in protein concentration would cause a negative effect against silver aggregation and decrease the SERS signal. This phenomenon was not found when gluthionine was used as an analyte.[13] The difference leads to the proposal that the crowding of the silver colloid environment is respon-

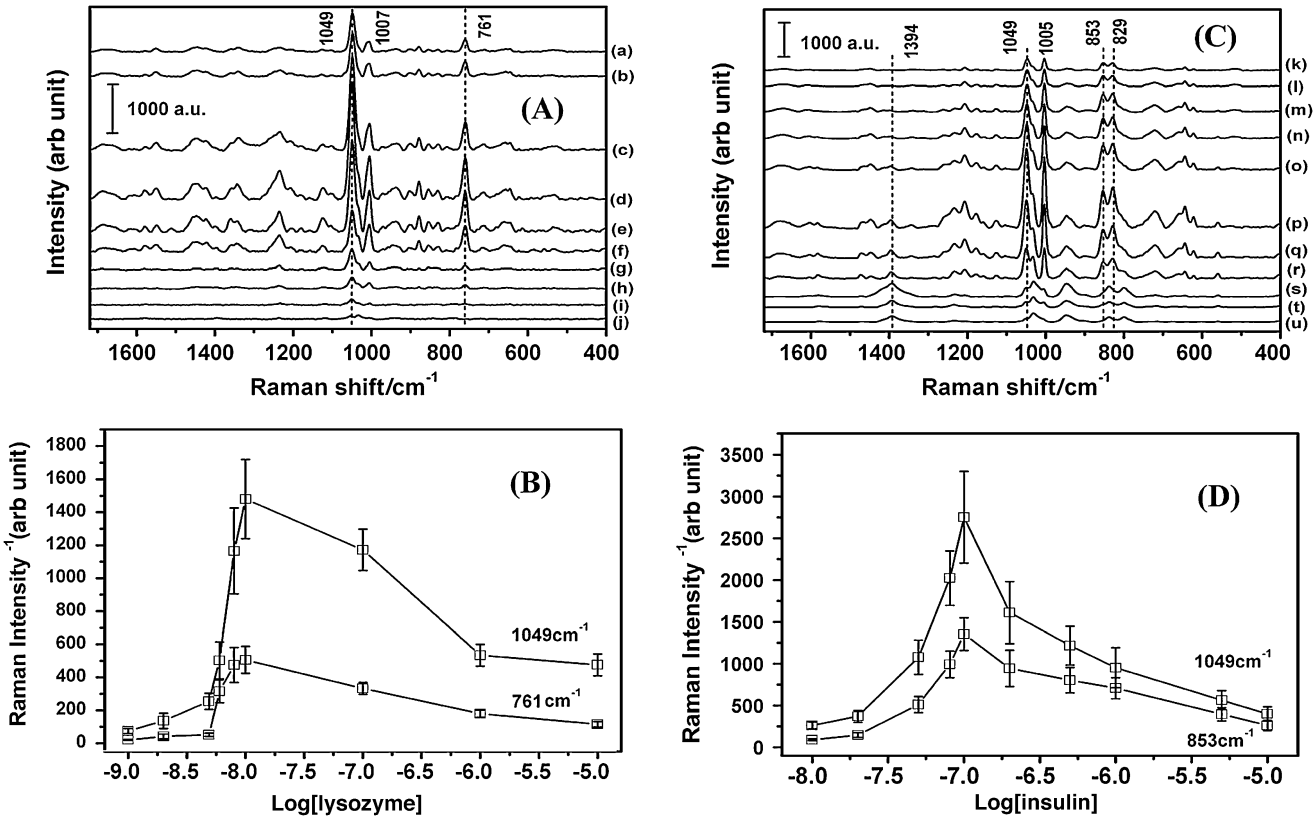

Figure 3. (A) Concentration-dependent (10^{-5}–10^{-9} M) SERS spectra of lysozyme (a) 1×10^{-5}, (b) 1×10^{-6}, (c) 1×10^{-7}, (d) 1×10^{-8}, (e) 8×10^{-9}, (f) 6×10^{-9}, (g) 4×10^{-9}, (h) 2×10^{-9}, (i) 1×10^{-9}, and (j) 1×10^{-10} M; (B) Raman intensity at 1049 (NO_3^- band) and 761 cm^{-1} (Tyr band) of the SERS spectra in (A); (C) concentration-dependent (10^{-5}–10^{-8} M) SERS spectra of insulin (k) 1×10^{-5}, (l) 5×10^{-6}, (m) 1×10^{-6}, (n) 5×10^{-7}, (o) 2×10^{-7}, (p) 1×10^{-7}, (q) 8×10^{-8}, (r) 5×10^{-8}, (s) 2×10^{-8}, and (t) 1×10^{-9} M; (u) 0 M control; (D) Raman intensity at 1049 and 853 cm^{-1} (Trp band) of the SERS spectra in (C).

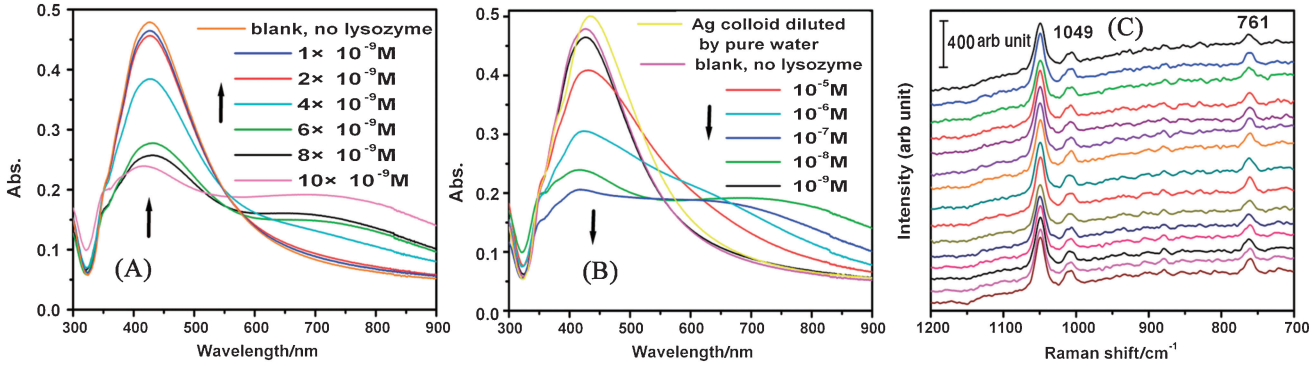

Figure 4. UV absorption spectra of silver colloid measured at 5 min after addition of lysozyme solutions. The final concentrations of lysozyme in silver colloid solutions were (A) 10^{-8}–10^{-9} and (B) 10^{-5}–10^{-9} M; (C) a collection of 15 SERS spectra of lysozyme (10^{-5} M), measured at 15 different points of the sample and averaged to obtain the spectrum (c) in Figure 1. The standard deviation of the spectra intensities at 1049 cm^{-1} is 7.5%.

sible for the silver colloid aggregation. As lysozyme is a large and hard molecule[16] (129 amino acids) compared to the small and soft gluthionine (3 amino acids), it is reasonable to observe that concentrated lysozyme inhibits silver colloid aggregation while the small peptide does not.

Nonlinear curves for quantitative or semiquantitative SERS studies have been found in previous studies.[17–19] In the present work, it shows a bell shape, which means that there are two possible concentrations for a certain SERS intensity. However, dilution of the protein sample can solve the problem. [C_{max}] represents the protein concentration corresponding to the maximum SERS intensity. Dilution of the protein sample with concentration higher than [C_{max}] can induce an increase in SERS intensity while dilution of that with concentration lower than [C_{max}] leads to a decrease in SERS intensity.

The adsorption mechanism of protein and NO_3^- possibly follows the most common co-adsorption scenario, which has been well established as a double-layer adsorption rule.[20] Namely, there is a layer of electrolyte such as citrate and NO_3^- between an adsorbed layer of proteins and Ag colloid. The results in Figure 3 provide some support to this speculation, suggesting that the intensities of protein bands including those at 761 and 853 cm^{-1} are positively correlated with the intensity of the NO_3^- band, which may be evidence for the co-adsorption manner of the protein and NO_3^-. A detailed depiction of the adsorption mechanism of protein and electrolyte was published recently.[21]

Peaks due to acid radicals are usually considered undesirable background in SERS studies, and additional caution should be paid to avoid these peaks. Here, however, we have developed a novel method that can selectively enhance the peak at 1049 cm^{-1} originating from NO_3^- to make it valuable for semiquantitative detection of proteins without any additional resonant effect. This method is simple, rapid (drying time is 210 s), reproducible (Figure 4C), and label-free. The laser power is low (6 mW) and the exposure time is short (20 s), which meets the need for routine analyses. The intensity and the sharpness of the NO_3^- peak suggest its potential in more extended applications, especially those to biomolecules with low Raman cross sections and poor quality spectra by normal SERS protocols. Also, the bell shape indicates the potential of this method in exploring adsorption phenomenon of a protein on a colloidal interface. These studies are ongoing in our group.[22]

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