The detection and identification of biologically important molecules has critical importance in several fields such as medicine, biotechnology, and pharmacology. Surface-enhanced Raman scattering (SERS) is a powerful emerging vibrational spectroscopic technique that allows not only for the characterization, but also for the identification and detection of biomacromolecules in a very short time. In this review, efforts to utilize SERS for label-free protein detection and identification is summarized after a short introduction of proteins and the technique.

Index Headings: Surface-Enhanced Raman Scattering; Proteins; Label-Free; Detection; Identification.

INTRODUCTION

Among the biomacromolecules proteins are the most versatile group with a wide range of functional and structural duties in living systems. As an end product of gene expression they undertake numerous responsibilities from catalyzing biochemical reactions to transporting and storing small molecules. Therefore, they show incredible variation in their structure and size. Countless proteins are found in the living world. To identify all proteins in nature with the current technology and level of understanding is inconceivable. It is an extremely difficult task even to study only the human proteome, which deals with the proteins expressed by about 23 000 genes. When differential mRNA splicing, post-translational modifications, and protein complexes are also considered, the complexity of studying proteins can easily be seen. In molecular terms, the diversity in their functions can be attributed to the construction of their molecular structures. They are composed of 20 naturally occurring amino acids extended through the peptide bond. Their enormous structural diversity is the result of the combinatorial assembly of the amino acids to varying lengths. This generates limitless combinations of protein structures. They compose of 20 naturally occurring amino acids extended through the peptide bond. Their enormous structural diversity is the result of the combinatorial assembly of the amino acids to varying lengths. This generates limitless combinations of protein structures. They compose the major part of all living cells and dependent tissues. Proteins are classified into two groups, structural and functional, based on their responsibility in living systems. Structural proteins form support materials such as collagen and elastin in connective tissue and actin and tubulin in cellular cytoskeletons. Functional proteins, which are the subject of this review (also known as globular proteins referring to their general shapes), are responsible for duties such as acting as catalysts and transporting small molecules.

CURRENT PROTEIN DETECTION AND IDENTIFICATION TECHNIQUES

Several physical, chemical, and immunoassay-based methods are routinely used for the detection and identification of proteins. Physical methods requiring the use of a spectroscopic technique such as mass spectroscopy are usually applied after a chromatographic or electrophoretic separation step. Chemical methods, on the other hand, use staining with organic dyes, metal chelates, or colloidal silver after a twodimensional electrophoretic separation step. The modes of immunoassay-based methods are commonly employed for applications from clinical testing to proteomics research when the target protein is already known. In an immunoassay-based approach, a separation step may not be necessary because the capture antibody provides the necessary selectivity.

Although all these approaches are very valuable and critical for ongoing research and clinical testing, the effort to
develop a fast, sensitive, cost effective, and simple protein detection and identification technique is underway. With recent insights into the presence of protein biomarkers for disease diagnosis and prognosis, the need for the development of a more satisfactory detection technique has become apparent.

Spectroscopic techniques such as fluorescence, UV/Vis, IR, Raman, and mass spectroscopy (MS) are best suited for the detection and identification of molecules. However, each of these techniques has certain disadvantages. For example, although fluorescence is a very sensitive technique and can provide information about the concentration-dependent presence of a molecule in a sample, it may not provide fingerprint information for molecular identification. Besides, due to the broad spectral bandwidth, it may not allow the detection of more than one type of molecule in a sample. The vibrational spectroscopic techniques, IR and Raman, and MS can provide fingerprint information that can be used for the identification of molecular species. However, IR spectroscopy suffers from over-sensitivity to water and it is also not suitable for detection at low concentrations. Mass spectroscopy is the most powerful technique of protein detection, however it is a destructive technique and it requires expensive instrumentation and skilled personnel. For proteomic research, a number of different modes of MS are employed. When a separation step such as liquid chromatography (LC) or capillary electrophoresis (CE) is required, electrospray or a mode of electrospray is used as the ionization technique. The mass analyzer is usually a quadrupole or ion trap. Matrix assisted laser desorption ionization (MALDI) or fast atom bombardment (FAB) is used as the ionization technique along with time of flight (TOF) as an analyzer when an online separation system is not used. Due to the extraordinary power of mass spectroscopic techniques, they are unarguably the most important components of proteomic research and protein identification and detection. However, in addition to the disadvantages mentioned above, the difficulty of interpretation of obtained spectra due to the complexity of the molecular structure and reproducibility issues with the ionization methods make the employment of these techniques too complex for easy and effective use.

**WHY LABEL-FREE DETECTION?**

Label-free detection involves the direct use of a property, such as electrochemical, spectroscopic, or physical, of a molecular species. In the indirect detection approach, a reporter molecule covalently attached to the target molecule is used and the concentration of the target molecule is indirectly determined through a signal obtained from the target-label conjugate. Direct detection is highly desired because in indirect detection the labeling step introduces additional uncertainties to the measurement. In a SERS experiment, the requirement that the label molecule and the noble metal structure must be in close contact also introduces further uncertainties. Therefore, label-free detection offers more reliable measurement because the signal originates from the molecular structure itself. In addition, it is highly desirable to be able to identify the components of a mixture without a separation step. Raman spectroscopy has the power of multiplexing due to very narrow spectral bands and SERS can quickly provide information about the presence of specific molecules in a mixture. Hence, there is a significant effort to develop new approaches for label-free detection of biomolecules using SERS.

**SURFACE-ENHANCED RAMAN SCATTERING**

Raman spectroscopy as a vibrational technique can provide very specific information about the chemical structure of a molecule. In addition, narrow spectral bandwidths are much narrower than those of Fluorescence and IR spectroscopy, which may allow for multiplex detection and identification of different molecular components in a sample. However, Raman scattering is a very inefficient process and only $10^{-6}$ of the intensity of the exciting frequency appears as Raman scattering. Due to the inefficiency in scattering, wide acceptance of the technique was delayed until the development of high power laser sources and sensitive solid-state detectors. The real acceptance of the technique gained momentum in 1977 with the discovery that Raman scattering from a molecule brought close to a noble metal surface, such as silver and gold, is extraordinarily enhanced. Because the enhancement phenomenon took place on the surface, the technique was called surface-enhanced Raman scattering (SERS). It is now commonly accepted that the mechanism of this phenomenon has two components: electromagnetic and chemical. The electromagnetic component is related to the excitation of the surface plasmons with the incident light. The role of chemical enhancement component depends on the level of interaction between analyte and noble metal surface. Additional enhancement in Raman scattering can be achieved with the use of a laser wavelength that the molecule of interest can absorb, which is called resonance enhanced Raman scattering (RRS). It is also possible to combine this feature with SERS, which is called surface-enhanced resonance Raman scattering (SERRS).

Since the discovery of the SERS phenomenon, a remarkable effort has been devoted to using the technique in a range of fields from material science to medicine and in a variety of applications. The possibility of achieving as large as $10^{14}$ enhancement, which implies that the detection of single molecules is possible and rivals fluorescence, has contributed to the excitement about the technique in the scientific community.

Although SERS may offer extraordinary sensitivity and may even compete with fluorescence, it is a fact that several parameters must be carefully considered for optimal enhancement and spectral reproducibility. Figure 1 summarizes the components of a standard SERS experiment. As seen, there are a number of parameters influencing the outcome and optimization should be performed based on the nature of the application. Note that the parameters indicated on the figure do not necessarily compete with each other, which outlines the significance of the parameters playing a role in a SERS experiment. Because the ulti-
mate goal is to achieve the highest enhancement in a reproducible manner, selection of a substrate and laser wavelength should be based on the chemical nature of the target analyte. The chemical enhancement component is strongly related to the chemical structure of the molecule and its adsorption level onto the noble metal surface. Small molecules that are rich in electrons are good scatterers while large molecules without moieties carrying sufficient electron density are poor scatterers. For large molecules or molecular structures such as proteins, the nature of the contact with the noble metal surface becomes more critical because only molecular moieties in contact with or in close proximity to the surface contribute to the overall spectrum.

Shortly after the discovery of the SERS phenomenon, the potential of the technique for the characterization of molecules and molecular structures of biological origin began to be explored. The detection, identification, and characterization of a number of biomolecules and biomolecular structures such as proteins, the nature of the contact with the noble metal surface becomes more critical because only molecular moieties in contact with or in close proximity to the surface contribute to the overall spectrum.

SERS AND PROTEINS

SERS has been utilized for protein characterization since the early years of its discovery. Proteins such as Cytochrome c (Cyt c), Hemoglobin (Hb), glucose oxidase, lactate oxidase, and flavoproteins have been thoroughly studied. Perhaps due to the difficulties of obtaining a reasonable and rich spectrum, most of the early reports focused on proteins with a heme group and employed SERRS to benefit from additional resonance enhancement. Silver electrodes or colloidal silver nanoparticles (AgNPs) were used as substrates in almost all of these studies. The interactions and characterization of amino acids, water-soluble peptides, proteins such as lysozyme and bovine serum albumin (BSA), and bacterial membrane proteins such as bacteriorhodopsin have also been studied. The major conclusions of these early studies were that the SERS spectra of peptides and proteins had a short-range character, and that those peptides and proteins were dependent on the degree of adsorption to the surface and the orientation of macro-molecules on the surface to the noble metal substrate. It was also noted that the chemical component of the enhancement mechanism became significant upon adsorption of peptides or proteins to the metal surfaces. The studies conducted with heme-containing proteins revealed that the heme group should interact with the surface plasmons to contribute to the spectrum. Because heme is buried within the protein and performance of SERS depends on the distance of the analyte to noble metal surface, a strategy to sandwich proteins between colloidal AgNPs and gold nanoparticles (AuNPs) was developed by Natan et al. The entrapment of proteins between Ag and AuNPs remedied the drawback of distance dependent issues with the enhancement of the heme group.

There are numerous SERS substrates following several synthesis and fabrication strategies reported in the literature for a range of applications. In the SERS studies of proteins and peptides, colloidal AgNPs or AuNPs, engineered nanoparticles, or surfaces are used as substrates. It is now clear that the substrate choice is application-dependent and one needs to carefully consider several parameters for an optimal outcome from the SERS measurement as discussed earlier. For label-free detection of proteins, the following points arising from the nature of the proteins and SERS should be taken into account: a) nature of the sample containing the protein and knowledge of protein concentration level in the sample, b) size and surface charge of the protein and the presence of functional groups on the protein’s surface that have strong affinity for the noble metal surface, c) choice of SERS substrate (nanostructured surfaces versus nanoparticles), d) molecular or ionic species present on the noble metal surface originating from the preparation of the substrate, and e) detection scheme (assay format). Since the goal is to eliminate reporter molecules and use the intrinsic protein spectrum for the detection, these parameters should be carefully evaluated for a healthy interpretation of the obtained SERS spectrum.

Due to the enormous diversity and functions of proteins in living systems,
The range of 1-3 nm, the protein on the surface of the nanostructure, which is in depth of the surface plasmons on the noble metal surface. Considering the surface and antigen, is closer to the sandwiched between the noble metal target protein because the antibody, biochemical structures and this similar antibody and target protein have similar proteins. For example, in an immunoassay, is the structural similarity of proteins. For example, in an immunoassay, a label molecule as a reporter is used to monitor the molecular level interactions between antibody and antigen. The SERS based immunoassays using a reporter molecule were extensively studied due to the high sensitivity and multiplexing capability of the technique and excellent reports are available in the literature. Although it is rather difficult to monitor the antigen binding to the antibody, the ability to observe antibody-antigen interactions with SERS without using a reporter molecule was shown by Ozaki et al. It was demonstrated that the IgG-anti-IgG complexes on AuNPs could be detected with SERS using a NIR laser. They found that this complex selectively adsorbed onto the AuNPs. Later, Cullum et al. demonstrated that the binding of an antigen to its antibody immobilized on a nanostructured silver surface on silica particles assembled on a glass slide could be observed from the SERS spectra. In that study, they used a substrate constructed by depositing an optimal Ag layer on silica particles in the range of 100–430 nm assembled on a glass slide and removing them from the surface by gently scraping with a sharp object. The antibodies specific for the target protein were immobilized onto the Ag surface through a crosslinker. The prepared silica-Ag-antibody structure was successfully used for the detection of insulin from lysed hybridoma T-cells in the cell culture media. The authors claimed that the additional bands observed on the SERS spectrum upon binding of the antigen to the antibody originated from conformational changes in the antibody after antigen binding. This finding is particularly important because the detection of proteins without a label can be achieved from the spectral changes that occur with antigen binding. In a recent report, it was shown that the binding of an antigen to its antibody could be detected from the change in vibrational modes of a linker molecule with the application of molecular stress to the linker molecule.

Au nanoshells constructed by coating SiO$_2$ nanoparticles with an Au layer were used for the characterization of four different peptides with SERS. The SERS spectra obtained from proteins were compared to their bulk Raman spectra to identify the bands observed on the SERS spectra of peptides. It was concluded that the bands on the SERS spectra of the peptides predominantly originated from the aromatic amino acid side residues and this information could be used for the band assignments on the SERS spectra of proteins and peptides.

Due to the structural similarity of proteins, the technique to use for detection should have the capability of distinguishing small structural differences. There are a number of supporting studies demonstrating this capability. In a report by Chumanov et al., a subtle difference in protein structure was successfully detected with the technique. In that study, the structural stability and redox properties of yeast iso-1-cyt c and its mutant were investigated on a roughened Ag electrode by SERRS. In the mutant form, phenylalanine at position-82 is replaced by histidine. It was shown that SERRS was sufficiently powerful and sensitive enough to observe such a small change in a complex molecule. In another important study, the difference between two protein forms, human insulin and insulin lispro, was detected at a submonolayer density on nanostructured adaptive silver films. Insulin lispro is a recombinant analog of human insulin that is mutated (Lys(B28) Pro(B29)) to prevent its oligomerization into a hexamer state. Because this process significantly reduces solubility and its subsequent adsorption under physiological conditions as a solution lysine and proline on the C-terminus of the B-chain are interlaced. The detection of Cyt c from Saccharomyces cerevisiae and myoglobin at single molecule level using colloidal AgNPs was also reported. Citrate reduced colloidal AgNPs and a 514 nm laser were used for the detection of the protein both in an AgNP suspension and on AgNPs immobilized on a glass substrate.

A pre-concentration step before the detection of very low concentrations of protein biomarkers will be extremely useful. Such a technique for charged molecules before SERS detection was...
demonstrated by Lee et al. The charged analytes were exposed to an electrical field between rod shaped and flat gold electrodes. The negatively charged analyte migrated to the positively charged electrode acting as a SERS substrate. The authors claim that this method increases the detection sensitivity eight orders of magnitude for adenine and this approach can be used for the label-free detection of a number of charged biomolecules including proteins.

The detection of human integrins αVβ3 and α5β1, found in vascular smooth muscle cells, was performed using citrate reduced colloidal AgNPs. Integrins are a group of proteins found on the cell surface that have numerous critical physiological functions such as aiding in blood clotting, blood pressure regulation, and vascular remodeling.

Therefore, their detection is diagnostically important in medical and clinical research. The detection limits for α5β1 and αVβ3 integrins were found to be 30 and 60 nM, respectively. In another study, a mutant form of a 55-residue integrin identified by SERS was used for label-free detection of proteins.

Although these early studies provide valuable information about the potential of the technique, the difficulties of using the technique for protein detection and identification remain. The major problem in the use of SERS for proteins is the diversity of proteins in size, shape, and surface charge. In a series of reports, Ozaki et al. employed several strategies to obtain rich and reproducible SERS spectra for label-free detection of proteins. Western blotting is an analytical technique used to detect proteins in a sample. First, the proteins are separated using gel electrophoresis and then the separated proteins are transferred onto a membrane. In the final step of the procedure, the proteins are stained with labeled target antibodies. After the final step of the Western blotting technique, the membrane can be stained with colloidal AgNPs, and SERS used to identify the proteins on the blot. In the study, myoglobin (Mb) and bovine serum albumin (BSA) were used as model proteins. The detection limit for Mb was found to be 4 ng with the application of SERS.

Surfaces that have been prepared employing different strategies have also been used for label-free detection of proteins. Arrays of gold structures sized 80 to 100 nm and possessing 10–30 nm gap size were prepared with electroplating and electron-beam lithography and used for the detection of proteins. An attomole level detection limit for myoglobin was established with the AgNPs. The temperature gradient study ranging from −65 to 90 °C was performed using lysozyme, ribonuclease B, bovin serum albumin, and myoglobin. It was shown that the changes in conformation of proteins could be observed during the temperature gradient. Uniformly distributed AgNPs on a nanostructured polymer were used as a substrate for label-free detection by addressing the reproducibility of the new substrate. The detection and identification of a dipeptide, Cys–Gly, cytochrome c, coenzyme A, and hemoglobin A were accomplished successfully. Detection limits of 1 ng/mL and 1 μg/mL for dipeptide and cytochrome c respectively were claimed.

The development of a label-free detection and identification method based on SERS is a significant effort in our laboratory. In our studies, we employ citrate reduced AgNPs as SERS substrates and explore the interactions of several proteins with the AgNPs to develop an assay. Some of the results of our studies are briefly summarized here to point out challenges and advantages of the technique for the protein analysis.

To use the colloidal noble metal nanoparticles, the protein sample is simply added into the colloidal-NP suspension. The interaction between NPs and proteins in the suspension is defined by their surface charges and other ionic species present in the suspension. For example, citrate reduced AgNPs possess a negative surface charge due to the adsorbed citrate ions on the surface, and therefore proteins with positive charges such as cyt c and lysozyme strongly interact with the AgNPs and form aggregates. Aggregation is desired for optimal SERS performance because it may help to obtain improved SERS spectra. However, in the case of negatively charged protein such as HSA, the interaction between the AgNPs and protein is very poor due to the negative surface charge of the protein. A strategy to initiate the aggregation of AgNPs using sodium sulfate in the presence of proteins was employed. In that study, lysozyme, ribonuclease B, avidin, catalase, and hemoglobin were used as model proteins and the detection limits for lysozyme and catalase were found to be 5 μg/mL and 50 ng/mL, respectively.

When colloidal noble metal nanoparticles are used as SERS substrates, an aliquot of noble metal nanoparticle and protein containing colloidal suspension is spotted onto a surface and SERS spectrum is acquired from the dried droplet. Although the procedure is quite simple, the distribution of NPs and protein on the droplet area is chaotic and strongly depends on the degree of interaction of protein with the NPs, which depends on the surface charge of NPs and size and charge of the protein. Since the simplicity of sample preparation protocol is crucial for routine analysis, we have been interested in

### Table I. Physicochemical properties of some proteins.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Isoelectric Point (pI)</th>
<th>MW (kDa)</th>
<th>Zeta Potential (mV)</th>
<th>Hydrodynamic Radius (nm)</th>
<th>Property</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA</td>
<td>4.7</td>
<td>66</td>
<td>−15.6 ± 4.2</td>
<td>3.63 ± 0.08</td>
<td>Acidic</td>
</tr>
<tr>
<td>Cytochrome c</td>
<td>9.8</td>
<td>12.3</td>
<td>+22.5 ± 4.4</td>
<td>2.01 ± 0.05</td>
<td>Basic</td>
</tr>
<tr>
<td>Avidin</td>
<td>10.5</td>
<td>66</td>
<td>+27.1 ± 5.1</td>
<td>3.53 ± 0.07</td>
<td>Basic</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>11.4</td>
<td>14.3</td>
<td>+35.9 ± 3.5</td>
<td>2.1 ± 0.06</td>
<td>Basic</td>
</tr>
</tbody>
</table>

TABLE I. Physicochemical properties of some proteins.
Fig. 2. Illustration of the convective assembly process (A), thin films (B) of lysozyme (a) and BSA (b) on glass slide, and white light images of the thin film prepared with BSA under 5x objective (C) and 50x objective (D). [Adapted with permission from Ref. 59, copyright American Chemical Society.]

Fig. 3. SERS spectra of A) avidin, B) BSA, C) cyt c, and D) lysozyme and with decreasing concentrations in AgNP colloidal suspension [Adapted with permission from Ref. 59, copyright American Chemical Society.]
employing colloidal AgNPs as SERS substrates and mixing and spotting onto a surface for sample preparation. However, a well known phenomenon, which is called the “coffee-ring” formation, governs the dynamics in a drying droplet suspension containing the AgNP-analyte. During this process, all species suspended or dissolved in the liquid phase are dragged and jammed at the liquid-solid-air interface. In a droplet of suspension composed of citrate reduced AgNPs and negative charged analytes, this phenomenon is more dominant due to the electrostatic repulsion forces between the AgNPs and the analyte. When an analyte sufficiently interacts with the AgNPs, some level of precipitation of AgNP-analyte on the droplet area is observed. This precipitation is due to the formation of a network-like structure of the NPs and analyte in the suspension. This network of NPs and analyte resist the pulling of the flow toward the droplet contact line. The addition of negatively charged proteins into the colloidal suspension containing AgNPs with negative surface charge results in jamming the majority of the species in the suspension at the droplet edges because the repulsion forces between AgNPs and proteins dominate the behavior in the suspension. Although a level of aggregation of AgNPs is highly desired, their tight packing is not preferred because it causes damping of the electron system of the AgNPs, which results in the quenching of the excitation of surface plasmons. Therefore, it is also important to have a good knowledge of the physicochemical properties of proteins in the system of interest. Table 1 shows some of the physicochemical properties of proteins that may be useful to know for method development.

In an effort to benefit from the dynamics in a droplet for label-free detection of proteins with SERS, we have investigated how to affect the dynamics in a drying droplet to influence or control the aggregation profile. “Convective-assembly” is a process that is used to assemble nanometer and
micrometer size particles at interfaces from a drying and moving droplet. A simple experimental set-up was used to benefit from this process for protein detection. This approach was used to assemble a variety of particles including yeast and bacteria. Figure 2 shows the illustration of the convective assembly set-up and process in our work (A) thin films formed from AgNP-protein (a-lysozyme, b-BSA) (B) white light images of the thin film prepared with BSA under 5x objective (C) and 50x objective (D). First, the reproducibility of the SERS spectra obtained from the thin film was investigated. Several spectra from the strips on x and y axes with 0.5 μm steps were acquired and revealed that the spectra were sufficiently reproducible to enable identification. The main source of variation was the decreasing protein concentration toward the end of the convective assembly. Finally, the detection limit for a number of proteins was predicted using the approach. The SERS spectra with decreasing protein concentration are given on Fig. 3 for Avidin (A), BSA (B), Cyt c (C), and Lysozyme (D). As seen, a detection limit to 0.5 μg/mL can easily be achieved.

In real world applications, almost all analytes are in the form of a mixture. Two approaches can be followed for the analysis of mixtures. Either a purification or separation step before detection or a multiplex detection scheme can be employed. Since SERS is capable of multiplex detection and identification it can be used for the latter. As mentioned earlier, an electrophoretic separation was employed before detection of proteins with SERS. In convective-assembly, the assembly process is controlled by convection during the drying of water and sedimentation. Although citrate reduced AgNPs have wide size variation (20–200 nm), we explored the possibility of separating the AgNP-protein structures with the convective-assembly prior to their detection with SERS.

Other means of influencing the aggregation of AgNPs in a dried droplet were also investigated. For example, inserting a tip into a drying droplet of suspension containing AgNPs and protein prevents the uncontrolled jamming of all species at the droplet edge, which is generally not a favorable region to acquire SERS spectra from proteins. In our most recent study, we demonstrated the effectiveness of drying an AgNP-protein containing droplet suspended from a hydrophobic surface. Figure 4 shows the photography of a droplet suspended from CaF2 surface (A), depiction of the position of AgNP-protein structures at the suspended droplet position (B), and the flow profile during the drying of the droplet (C). As water evaporates from the droplet, the AgNP-protein structures accumulate at the apex of the droplet due to gravity as and finally adhere to the surface as a large aggregate. Figure 5 shows the SERS spectra of A) avidin, B) cytochrome c, and C) lysozyme with decreasing protein concentrations.

The SERS spectra of proteins presented in Figs. 3 and 5 demonstrate the importance of the protocol used for the sample preparation. Typical for any SERS measurement, spectral reproducibility is the major problem. When spectra of the same protein are obtained with the two sample preparation methods, the convective-assembly and suspended-droplet, the main spectral difference is the band at around 1050 cm⁻¹. This band is attributed to the nitrate ions, and is likely due to the co-adsorption of proteins and nitrate ions onto the AgNPs. As Fig. 5 shows, as the concentration of proteins increases, the intensity of this band increases as reported. Although this band is also observed on the SERS spectra of proteins prepared with convective-assembly, it is rather weak. The intensity difference in this band is due to the...
concentration of nitrate ions distributed on the unit area. In the case of the suspended droplet, all species are confined in a much smaller area than that in the convective-assembly. When the spectra of each protein obtained with the same sample preparation method are inspected some spectral variations with the change of protein concentration are also observed. This is due to the variations in the aggregation status of the AgNPs with varying protein concentrations. However, some of the major bands were retained on the spectra and can be used for quantification. Note that the other experimental conditions, laser wavelength and colloidal AgNPs, were the same. This again reveals the importance of the SERS experimental protocol for comparable results. For quantification, linearity in concentration versus spectral intensity is highly desired. However, this relationship for proteins is not always linear and is strongly related to the structural properties of proteins such as size, surface charge, and flexibility.

CONCLUSIONS

The utility of SERS in biological applications has long been of interest in the scientific community. As the understanding of interactions of biomacromolecules and biological structures with noble metal surfaces and nanoparticles is clarified, there is steady progress in the application of the technique for the solution of biomedical, medical, and biotechnology related problems. Developments in the biological applications of the technique have been reviewed periodically. In this review, the efforts to utilize SERS for label-free protein detection and identification have been summarized. Most of the earlier studies focused on understanding the interaction of proteins with the AgNPs or AuNPs and increasing the SERS sensitivity. However, the reproducibility issue has not been fully addressed yet. The superiority of the AgNPs as substrates influences their use as a substrate in SERS experiments but the major drawback of using colloidal AgNPs as substrates is their reproducibility issues. This problem must be fully addressed before the broad use of the technique in label-free protein detection will be practical. It is clear that the use of the technique for biological applications is in its infancy yet. Most of the studies are exploratory in nature and are aimed at trying to understand the potential of the technique. As the number of studies increases, the full potential of the technique will be realized. The combining of the technique with microfluidics could be an area for future studies because separation of proteins from their mixtures can be accomplished with microfluidics before their detection with SERS.

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25. C.D. Keating, K.M. Kovaleski, M.J. Nathan. “Protein/Colloid Conjugates for Surface En-


