Evidence of Conformational Changes in Adsorbed Lysozyme Molecule on Silver Colloids

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Abstract

In this article, we discuss metal-protein interactions in the Ag-lysozyme complex by spectroscopic measurements. The analysis of the variation in relative intensities of SERS bands reveal the orientation and the change in conformation of the protein molecules on the Ag surface with time. The interaction kinetics of metal-protein complexes has been analyzed over a period of three hours via both Raman and absorption measurements. Our analysis indicates that the Ag nanoparticles most likely interact with Trp-123 which is in close proximity to Phe-34 of the lysozyme molecule.

Keywords: Lysozyme, Ag colloids, SERS, optical absorption
I. INTRODUCTION

Metal nanoparticles are finding increasing use in nanomedicine for surface interactions with an array of proteins and/or small molecules. Metal nanoparticles tagged to biomacromolecules are oftentimes used to identify specific antibody-antigen binding sites in cells and tissues [1, 2]. Surface Enhanced Raman scattering (SERS) is an extremely sensitive technique for monitoring the adsorption of species of very low concentration and for characterizing the structure and orientation of the adsorbed species on the rough metal surface or metal colloidal particles [3]. Nonresonance SERS is primarily sensitive to the species attached to the metal surface or present within a few of the metal-dielectric interface as well as to the orientation of the ad-molecule [4]. Thus, it is possible to characterize molecular rearrangement on the metal surface by SERS mechanism. This implies that conformational changes occurring in the protein structure or due to denaturation through possible interactions with metal particles can be detected from a detailed analysis of the SERS spectrum. Modification of the specific interaction sites by covalent or non-covalent methods makes the possibilities of study enormous. In this regard, the interactions of silver nanoparticles with several proteins have been investigated. Some of the proteins studied with silver (Ag) colloids are hemoglobin [5], antirabbit IgG [6], albumin [7] and lysozyme [8–10].

Lysozyme is a small monomeric globular protein consisting of 129 amino acids that has the ability to disrupt many bacterial functions including their membrane structure [11–14]. The protein has $\alpha$-helix and $\beta$-sheet components with four disulfide bonds. It contains six tryptophan (Trp) residues, three of them are located at the substrate binding sites, two in the hydrophobic matrix box, while one is separated from the others [15]. Among them Trp 62 and Trp108 are the most dominant fluorophores, both being located at the substrate binding sites [16]. Lysozyme action on bacteria has been the focus of a lot of research, but interaction studies with Ag colloids have not previously revealed the specific interaction sites on the protein. We have further probed the interactions of lysozyme with Ag nanoparticles to obtain an insight into the specific residues involved in the interaction. This has been investigated by spectral methods and new insights have been obtained in the understanding of how this protein is involved in the interaction with Ag colloids.

Earlier studies on the SERS spectrum of lysozyme molecules adsorbed on Ag electrodes and hydrosols indicate the proximity of the S-S bond and aromatic amino acid residues of
lysozyme molecule on the Ag surface [8–10]. These articles report the orientation of the aromatic amino acid side-chains located on the surface of the metal electrodes. The role of an $\alpha$-helical component of the lysozyme molecule for binding on the metal surface has been shown [8, 9] and a recent study has reported the conformational changes in lysozyme molecule with temperature, while adsorbed on a gold nano-patterned SERS substrate [17]. Thus previous studies indicate the possible regions of interaction of the lysozyme molecule while interacting on the surface of metal colloids. It is necessary to probe the specific region of binding of the protein molecule to the Ag colloidal surface to permit an understanding of which regions of the protein would be free to interact with ligands and/or drugs. For example, the antimicrobial macromolecule formed by a lysozyme-polyphenol complex can be used as a carrier for phenolic antioxidants. Epigallocatechin gallate (EGCG) [18] and Triclosan [19] molecules are known to form complexes with lysozyme via Trp-62 and Trp-63 residues. The knowledge of the specific binding site in Ag-lysozyme complex can provide guidelines to develop nanoparticle tagged lysozyme molecule for drug delivery. It may be noted that the lysozyme is a potential carrier molecule as a renal-selective drug carrier for delivery of the angiotensin-converting enzyme (ACE) inhibitor captopril [20].

II. MATERIALS AND METHODS

Synthesis route for preparing stabilized particles: Ag nanoparticles have been synthesized by chemical routes, using sodium borohydride (NaBH$_4$) as reducing agents. Silver nitrate (AgNO$_3$), sodium borohydride (NaBH$_4$) of analytical reagent grade (SRL, India), were used to prepare the Ag sol. A colloidal silver solution was prepared in deionized water following the method described by Creighton et al. [21]. Essentially, in this chemical route, AgNO$_3$ is reduced by an excess amount of NaBH$_4$. $2.2 \times 10^{-3}$ M AgNO$_3$ was added dropwise to 1 mM NaBH$_4$ at 4 °C. Stirring for 20 minutes was necessary to stabilize the colloidal solution. Later, it was left at room temperature for approximately 1 hour. The excess NaBH$_4$ evaporated and the remaining solution became transparent yellow in color. Colloids generated by above method were stable over a considerable period of time (4-6 weeks). However, all our reported results are either with freshly prepared Ag sol or aged only for a day.

Chicken egg-white lysozyme was obtained from Sigma-Aldrich. In solution, depending on
the pH values, the majority of lysozyme molecules assume different structural forms. The protein has a zero net charge at the isoelectric point (pI). Interactions of neutral protein molecules with water is less favorable than when they are in the charged state. At the pI, the solubility of proteins in water is low and the repulsion between protein molecules is also a minimum. These two factors help in adsorption of the molecules on metal surfaces for pH of the solution close to pI. For lysozyme the value of pI is 10.5. At pH 10.5 the protein is not soluble in water, which would cause problem in comparing the SERS spectrum and normal Raman spectrum of lysozyme molecule. At pH 11 the molecule undergoes rapid hydrolysis \[22\]. Moreover, the pH of untreated metal sol was between 7.0-7.5. For these reasons we have maintained the pH of the lysozyme solution in slightly acidic state, such that the effective pH in the final sol (lysozyme+Ag sol) is close to 7.0-7.5 in all our experiments. 4 mM solution of lysozyme was prepared in deionized water. For SERS measurement the volume ratio of Ag sol to protein solution was maintained at 1:1.

SERS spectra were measured using a 488 nm Argon ion laser as an excitation source using a microRaman spectrometer with 100X objective lens. The spectrometer is equipped with 1200 g/mm holographic grating, a holographic super-notch filter, and a Peltier cooled CCD detector. The laser power on the samples was 5 mW. The unchanged circular dichroism spectra of laser irradiated and un-irradiated samples eliminate the possibility of damage/conformational changes in the protein structure by laser irradiation under the given experimental conditions. The data acquisition time for each Raman and SERS spectrum was 120 sec.

UV-Visible spectra were measured by Spectrascan UV 2600 (Chemito). To study interaction kinetics, measurements were carried out immediately after addition of lysozyme in Ag sol and subsequently after 15 mins, 30 mins, 1 hr, 2hrs and 3 hrs.

For optical absorption measurements the samples were kept in a microcuvette with optical pathlength 1 cm. For SERS measurements a drop of solution was taken on a glass slide and allowed to dry. It took around 10-15 minutes to dry the samples. During kinetic measurements, to investigate the interaction between metal colloids and protein molecules in the solution, drops were deposited after the specific time intervals as given above. Thus, we carried out all measurements after the completion of metal-molecule interaction for the required time, in ambient conditions. All measurements (SERS and optical absorption) and analysis have been carried out in triplicate.
PyMol was used for visualization of the protein conformation. The accessible surface area (ASA) of all the amino acid residues in uncomplexed lysozyme are estimated using NACCESS.

III. EFFECT OF ADDED LYSOZYME MOLECULES ON METAL COLLOIDS

A. Analysis of Plasmon Band of Silver Colloids

All biomolecules do not interact with metal colloids in the same way. Some of them result in agglomeration of the colloidal particles, others do not cause aggregation but modify the surface charge of the particle. To understand the effect of added lysozyme molecules on Ag colloids, we study the plasmon band of Ag particles in the sol on adsorption of the protein molecules. Plasmon band of Ag colloids is shown by black dotted line in Fig. 1(a). Ag sols absorb light at 398 nm, due to the dipolar surface plasmon of small spherical particles of Ag. The average particle size of Ag has been estimated to be $\sim 100$ Å.

Time-dependance of the plasmon band of Ag colloids in the sols is shown in Fig. 1(a). Spectra, recorded just after the addition of protein molecules in the metal sols, are shown by black solid line. Subsequent changes in the spectra with time (after 15 mins., 30 mins, 1 hr, 2 hrs and 3 hrs.) are shown by colored solid lines in the Fig. 1(a). Addition of lysozyme to the sol results in (i) decrease in intensity and (ii) broadening with a (iii) red shift of the plasmon resonance band of Ag particles in the sol. The decrease in relative intensity of the plasmon absorption band in solutions (ratio of maximum intensity of the plasmon band of Ag colloids after addition of protein to that of the Ag colloids), is shown in Fig. 1(b). We observe that the intensity of the plasmon band decays exponentially. The decay time constant of intensity is estimated to be 60 min in the sol. The observed broadening and the red shift of the plasmon band indicate formation of bigger particles in the sol by added lysozyme molecules. Furthermore, the decrease in the intensity of the peak can be explained by precipitation of metal particles from the sol followed by immediate aggregation.

IV. EFFECT OF METAL COLLOIDS ON ADDED LYSOZYME MOLECULES

Analysis of SERS Spectra
Next, we focus on the effect of metal particles on added lysozyme molecules in the sol. Raman spectra and SERS spectra of 4 mM lysozyme in water and in the Ag sol (just after addition) are shown by solid lines and dotted lines respectively in Fig. 2. Appearance of the characteristic feature of the Ag-N vibrational mode at 233 cm$^{-1}$ in the SERS spectrum (shown in the inset of Fig. 2) is a clear signature of metal-protein interaction in the sol via N atom of the ad-molecule. Time variation of SERS spectra of lysozyme in Ag sol over the spectral range 400–900 cm$^{-1}$, 900–1100 cm$^{-1}$ and 1100–1700 cm$^{-1}$ are shown in Fig. 3(a)–(c). The assignments of the prominent bands (indicated by * marks in Fig. 2) are summarized in Table I. To study the interaction of the Ag-lysozyme complex, we have estimated the relative intensity of each feature in the range over 400–1100 cm$^{-1}$ [Fig. 3(a) and (b)] with respect to the intensity of the peak at 877 cm$^{-1}$ (vibration of N$_1$H site of Trp amino acid residues in lysozyme). The methylene $\delta$(CH$_2$) vibration at 1442 cm$^{-1}$ has been used as reference for the spectral features in the range 1100–1700 cm$^{-1}$ [Fig. 3(c)]. Both these peaks (at 877 and 1442 cm$^{-1}$) maintained a constant frequency and were of quite strong intensities. Invariant Raman shift of a band indicates unchanged chemical bonding responsible for these particular vibrational modes over experimental duration.

It was interesting to note that the relative intensity of all vibrational bands of the adsorbed lysozyme molecule do not follow the same trend. A possible explanation for the time-evolution of the spectra can be the conformational changes of the ad-molecules with time, which we discuss in detail below.

A. Vibrational modes of amide bands

Vibrations of peptide backbone in proteins, associated with amide I, mainly involve the C=O stretch with a small contribution of C-N stretching and N-H bending. The same for the amide III region arises predominantly by an in-plane N-H deformation coupled with a C$_\alpha$-N stretch. In lysozyme, amide bands consist of mainly three structural components — three stretches of $\alpha$-helix, antiparallel pleated $\beta$ sheet, in which the polypeptide is hydrogen-bonded between one region to another via a hairpin turn of the chain and random coils, which are folded in an irregular way. Lysozyme is a globular protein with distinctly demarcated $\alpha$-helix and $\beta$-sheet regions. These secondary structural units are connected by random coils. The positions and intensities of the vibrational modes of amides can be used for an empirical estimation of these secondary structures in proteins.

SERS spectrum of amide I band appears between 1610–1700 cm$^{-1}$. The contributions of
the three secondary structures—α-helix, β-sheet and random coil—are expected to occur over the spectral windows 1665-1680 cm$^{-1}$, 1610–1632 cm$^{-1}$ and 1636–1644 cm$^{-1}$, respectively [28]. The amide III band appears over the spectral range between 1230 and 1310 cm$^{-1}$—the signatures of α-helix, β-sheet and random coils are characterized by spectral peaks over the range 1280–1320 cm$^{-1}$, 1235–1242 cm$^{-1}$ and 1250–1260 cm$^{-1}$, respectively [29]. The region 1254–1260 cm$^{-1}$ has been assigned to β-turn [30].

In the measured SERS spectra of lysozyme in Ag sol, the vibrational modes of amide III appear at 1260 cm$^{-1}$, as a shoulder of the strong peak at 1331 cm$^{-1}$ of Trp (Fig. 3). Thus, it is difficult to extract the subtle spectral features of amide III band from spectral analysis. To study the correlation between three secondary structural components of the adsorbed protein molecule, the broad envelope of the amide I band of each spectrum needs to be deconvoluted into the contributions from each of them. Such analysis may also provide information on conformational changes in lysozyme with time upon adsorption on colloidal surface. The time-variation of the vibrational spectra of the amide I band are shown in Fig. 4 by + signs. The deconvoluted and net fitted spectra are shown in Fig. 4 by dotted and solid lines. From the analysis of the spectral profile of amide I band we obtain the following: (i) the spectral feature over the spectral range between 1610 and 1632 cm$^{-1}$ for β-sheet is missing in all spectra and (ii) the relative vibrational intensities of random coils (I$_R$) to α-helix (I$_α$) increases with time (in the inset of Fig. 4).

B. Vibrational modes of amino acid residues

As mentioned before, the sharp SERS spectral line at 877 cm$^{-1}$ corresponds to the vibration of N$_1$H site of Trp residues [31]. The frequency of this band depends on the strength of the H-bond between Trp and other side chain molecule. The same feature is expected to shift by 8 cm$^{-1}$ for non-H-bonding Trp derivatives and lowered by 7 cm$^{-1}$ for H-bonded Trp derivatives. The single sharp peak at 877 cm$^{-1}$ in the Raman and SERS spectra indicates that in the metal-protein complex the Trp-residues do not form any new chemical bond directly with the Ag surface or with other residues. The variation in relative intensities of other vibrational modes of Trp are shown in Fig. 5(a). The intensity of ring breathing mode of Phe-residue at 1004 cm$^{-1}$ first increases and then decreases with time (Fig. 5(b)). The change in δ(CH$_2$) mode of glutamic acid at 1449 cm$^{-1}$ (just next to δ(CH$_2$) methylene vibrational mode at 1442 cm$^{-1}$) is shown in Fig. 5(c). In each plot, the solid line is the guide to the eye.
We did not find any clear signature of Asp and His (histidine), two other amino residues of lysozyme, in the SERS spectra of the molecule.

C. Disulfide bond

There are four pairs of S-S bond in lysozyme. Among these four pairs, two pairs of S-S bonds are near the \( \alpha \)-helix region and two others are in the \( \beta \)-sheet region. The strong SERS spectrum for vibration of S-S bond appears at 505 cm\(^{-1} \) just after addition of lysozyme in the Ag sol. However, the intensity of this band decreases with time (Fig. 5(d)). To check the effect of the reducing environment on the disulfide bond, an additional experiment with lysozyme and NaBH\(_4\) was conducted. The trend in the change of relative intensity of S-S bond (Fig. 5(e)) indicates that reduction of the disulphide linkage by BH\(_4^-\) (note that the Ag colloidal particles are stabilized by BH\(_4^-\) in NaBH\(_4\) stabilized sol) is possible.

Discussion

Here we report the specific amino acid residues of lysozyme molecule which are adsorbed on Ag colloids. Appearance of strong vibrational modes of Trp and Phe residues in the SERS spectra of lysozyme molecule and the similar trend in the change in relative intensities of these modes (Fig. 5(a) and Fig. 5(b)) signify that both these residues are close to the Ag surface and occupying adjacent sites in the adsorbed molecule. A lysozyme molecule contains six Trp residues (Fig. 6). The accessible surface area (ASA) available for these residues are shown in Table II. Three (Trp-62, Trp-63 and Trp-123) of the six Trp residues of a free lysozyme molecule have ASA more than 50 Å\(^2\) and, hence, are likely to be free to bind with a substrate. Trp-62 and Trp-63 are exposed at the edge of the active site cleft and known to interact specifically with a substrate \[29\]. Trp-123 is close to the c-end of the amino acid chain of the molecule. Trp-28, -108, -111 have relatively less ASA and are expected to be burried. Of all three Phe residues (Phe-3, Phe-34 and Phe-38) of lysozyme molecule the ring of Phe-34 is very close to Trp-123 and its ASA is relatively large compared to that of the other two [Fig. 6 and Table II]. Thus, it is most likely that the lysozyme molecules are adsorbed on Ag surface via the red marked region which includes both Trp-123 and Phe-34 residues of the molecule [Fig. 6].

Next we discuss the observed SERS spectra of amide I band. It is to be noted that the ratio of the secondary structure contents i.e \( \alpha \) helix: \( \beta \) sheet: random coil in free lysozyme is expected to be 0.54:0.15:1 [32]. Dominant spectral features of \( \alpha \)-helical component in Fig. 4 indicate that lysozyme is adsorbed on the colloidal Ag surface either in an \( \alpha \)-helical region.
or in a loop with an α-helical element \[33\]. The increase in the intensity ratio \(I_R : I_\alpha\) in the inset of Fig. 4 indicates unfolding of the α-helix or the α-helical turn element to a random coil of adsorbed protein molecule with time. It is interesting to note that the \(\phi,\psi\) angles of Trp-123 in lysozyme are -124.9, -2.5 [red arrow in Fig. 6], that is close to the α-helical region of the Ramachandran plot.

Furthermore, the disulfide bond is expected to be affected by the change in protein backbone conformation \[34\]. Of all four S-S bonds, two of them, (S6-S127) and (S30-S115) are very close to Trp-123. As the turn element, at the arrow in Fig. 6 gets affected due to the interaction with the Ag surface, S6-S127/S30-S115 bond/bonds may break down and this explains the decrease in the intensity of S-S vibrational mode with time (Fig. 5(d)). Furthermore, Glu-7 of lysozyme lies very close to the S6-S127 bond with an ASA corresponding to 82.4 Å\(^2\). If the S-S bond breaks down (Fig. 5(d)), the vibrational mode of Glu-7 is expected to be affected in a similar fashion (Fig. 5(c)). Moreover, it is to be noted that this particular region of the molecule corresponds to the C-terminal end of the protein. Hence, this region of the molecule is more flexible and susceptible to changes in the environment.

Addition of lysozyme in Ag sol results in an immediate agglomeration of the Ag particles. The initial increase in the relative intensity of vibrational bands of amino-acid residues of the molecule with time in Fig. 5(a) and Fig. 5(b) suggest re-orientation of the residues to obtain a low energy configuration from an initial high energy state due to initial favorable electrostatic interactions during change in conformation. The time constant for the unfolding of the α-helix is also estimated to be 45 min (inset of Fig. 4). If we assume only the local electrostatic interaction between the adsorbates and metal colloids, all our results suggest that lysozyme molecules interact with Ag colloids instantaneously; however, it takes longer time for the protein molecule to be stabilized on the metal surface. In general, irreversible laws of thermodynamics are involved in adsorption of proteins on metal surfaces \[35\]. Various time scales are involved in metal-molecule interactions. The fast steps are sometimes reversible (can be probed only by time-resolved experimental measurements) and the relatively slow steps (of time-scale varies from seconds to minutes) result in rearrangements of protein structure by the surface environment and in many cases, can be irreversible \[35\]. We believe that the initial metal-protein interaction via agglomeration of the particles involves the fast process mentioned above and the conformational changes of the lysozyme molecule.
with time corresponds to the latter process. All above conjectures, obtained from spectroscopic measurements, can be further confirmed by studying interaction of Ag-lysozyme complexes with other ligands that are currently underway, for which the interaction sites of lysozyme are already established.

As mentioned earlier that there are few reports in which SERS spectra of lysozyme molecules on Ag colloidal surface have been discussed. Prior knowledge available on the interaction of the Ag-nanoparticles with lysozyme indicates the $\alpha$-helical and random coil components [9]. Our experimental approach identifies the particular Trp residue involved in the interaction. Besides this, the effect of the interaction on the intensities of SERS bands of Phe and disulfide bonds confirm the specific site of the protein involved in the complexation. Conformational changes in the protein molecule on interaction with Ag nanoparticles indicate partial unfolding of the $\alpha$-helix constituted of residues 25-35 of which Phe-34 is a member. Interestingly, it is to be noted that the parts of the protein molecule with $\beta$-sheet comprising of Trp-62 and Trp-63, known to have higher bio-activity, remain unaffected by metal-protein complexation.

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Figure Captions

Figure 1. (a) Kinetic study of the plasmon band of Ag colloids and (b) Time variation in relative intensity of the plasmon band of the Ag upon addition of lysozyme in the sol.

Figure 2. Comparison between normal Raman spectrum of $4 \times 10^{-3}$M lysozyme in water (solid lines) and SERS spectrum of the same in the Ag sol (dotted lines). Inset of the figure shows the characteristic feature of Ag-N vibrational mode.

Figure 3. SERS spectra of Ag-lysozyme complex taken at different time over the range (a) 450-900 cm$^{-1}$, (b) 900-1100 cm$^{-1}$ and (c) 1100-1700 cm$^{-1}$.

Figure 4. SERS spectrum (+ signs) and deconvoluted spectra (dashed lines) of the amide I band. Inset of the figure shows the variation in intensity ratio of random coil to α-helix components of the protein with time.

Figure 5. Variation in intensity of vibrational bands of (a) Trp residues (b) Phe residues (c) glutamic acid (d) S-S bonds of adsorbed lysozyme in Ag sol and (e) S-S bonds of lysozyme in BH$_4$ environment (without metal colloid) with time respectively.

Figure 6. Cartoon of lysozyme molecule and its site of interaction with Ag surface (the region within the red mark. The α-helix element which unfolds upon interaction with metal surface is shown by arrow.
Table Captions

Table I. Assignment of vibrational bands for lysozyme SERS spectra.

Table II. Calculated accessible surface area (ASA) for Trp, Phe and Glu residues in a lysozyme molecule.
FIG. 1: Chandra et al

FIG. 2: Chandra et al
FIG. 3: Chandra et al
FIG. 4: Chandra et al
FIG. 5: Chandra et al
FIG. 6: Chandra et al
| Assignment                                           | peak (cm$^{-1}$) | Reference |
|------------------------------------------------------|------------------|-----------|
| S-S                                                  | 505              | [8, 22]   |
| Phe                                                  | 620              | [25]      |
| Tyr                                                  | 644              | [25]      |
| Trp - indole breathing (W18)                         | 763              | [26]      |
| Trp- N$_1$H site vibration (W17)                     | 877              | [26]      |
| Phe - ring breathing                                 | 1004             | [8]       |
| Trp - ring breathing                                 | 1015             | [8]       |
| Trp- indole ring (C-H deformation)                   | 1338             | [26]      |
| Trp- W7 wagging                                      | 1356             | [26]      |
| $\delta$(CH$_2$)                                     | 1442             | [25]      |
| Glu-$\delta$(CH$_2$)                                 | 1449             | [27]      |
| Trp -indole ring stretching                          | 1540             | [26]      |
| Trp -W2 mode                                         | 1568             | [26]      |
| Phe/Tyr                                              | 1598             | [8]       |
| Amide - I                                            | 1644             | [8]       |
| Trp sequence | ASA (Å²) | Phe-sequence | ASA (Å²) |
|--------------|----------|--------------|----------|
| Trp-28       | 0.12     | Phe-3        | 17.82    |
| Trp-62       | 120.57   | Phe-34       | 64.02    |
| Trp-63       | 48.01    | Phe-38       | 15.33    |
| Trp-108      | 10.59    | Glu-7        | 82.44    |
| Trp-111      | 12.69    | Glu-35       | 34.16    |
| Trp-123      | 55.5     |              |          |