Fluorescence Spectroscopy of Lysozyme-Silver Nanoparticles Complex

S. Ashrafpour, T. Tohidi Moghadam, B. Ranjbar

Abstract—Identifying the nature of protein-nanoparticle interactions and favored binding sites is an important issue in functional characterization of biomolecules and their physiological responses. Herein, interaction of silver nanoparticles with lysozyme as a model protein has been monitored via fluorescence spectroscopy. Formation of complex between the biomolecule and silver nanoparticles (AgNPs) induced a steady state reduction in the fluorescence intensity of protein at different concentrations of nanoparticles. Tryptophan fluorescence quenching spectra suggested that silver nanoparticles act as a foreign quencher, approaching the protein via this residue. Analysis of the Stern-Volmer plot showed quenching constant of 3.73 μM^{-1}. Moreover, a single binding site in lysozyme is suggested to play role during interaction with AgNPs, having low affinity of binding compared to gold nanoparticles. Unfolding studies of lysozyme showed that complex of lysozyme-AgNPs has not undergone structural perturbations compared to the bare protein. Results of this effort will pave the way for utilization of sensitive spectroscopic techniques for rational design of nanobiomaterials in biomedical applications.

Keywords—Nanocarrier, Nanoparticles, Surface Plasmon Resonance, Quenching Fluorescence.

I. INTRODUCTION

SILVER nanoparticles offer superior utility in biosensing and medical diagnostics [1], [2], optical labeling [3], hyperthermia and antibacterial applications [4]. They show outstanding photophysical properties, such as tunable fluorescence emission, localized surface plasmon resonance and enhanced FRET efficiency [5]-[7]. Meanwhile, significant adsorption capacity of these nanostructures to bind or carry drugs and biomolecules offers new research opportunities for investigating molecules-nanoparticles binding events [8]. Detailed studies on the absorption processes and binding of nanoparticles to biomolecules at their interface provide in-depth understanding of pharmacological responses for nanobiomaterials. This will set new directions for generation of nanoscale-based systems, with application in variety of areas, spanning from medical implants and bioengineering to drug nanocarrier systems.

For the latter one, a number of criteria such as drug

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circulation time, cell targeting, release, therapeutic index and side effects are all affected by binding properties of drugs to their carriers. Therefore, to fulfill the requirements of such combined systems, structure and function of the overall system should be monitored using sensitive spectroscopic techniques.

Despite the recent active progress on gold nanoparticles, there has been rare information on how biomolecules interact with silver nanoparticles. Previous studies have reported about Surface Enhanced Raman Spectroscopy (SERS) of biomolecules such as lysozyme on Ag electrodes and island films. Those studies indicated that the α-helical conformation is favored for binding to the surface over the random coil or β-sheet conformations, and lysozyme is adsorbed on the silver surface through the disulfide bonds and aromatic amino acid residues [9], [10]. In this effort, fluorescence spectroscopy has been utilized as a convenient technique to study interaction sites of lysozyme-silver nanoparticles complex. High sensitivity of this optical technique makes it a promising tool to understand various pathophysiological steps and enables potential diagnosis of microorganism associated diseases, tissue abnormalities and malignancies [11].

Chicken egg white lysozyme (HEWL) is a single chain polypeptide of 129 amino acids cross-linked with four disulfide bridges. Lysozyme preferentially hydrolyzes the β(1→4) glucosidic linkages between N-acetylmuramic acid and N-acetylglucosamine in the peptidoglycan of certain microorganisms. Recently, the biomolecule's potential ability in killing human immunodeficiency virus (HIV) has been evidenced [12]. With well-known physico-chemical properties and antibacterial activity, the enzyme also shows efficiency in delivery of drug molecules [13].

This effort aims to gain further insight into lysozyme-AgNPs interaction and the extent of stability changes that protein might undergo upon interaction with AgNPs, using fluorescence spectroscopy. In this regard, a series of experiments were conducted to investigate favorable adsorption sites of lysozyme on silver nanoparticles by measuring intrinsic tryptophan fluorescence quenching. To compare stability of the complex with bare lysozyme, spectra of the tryptophan residues were also monitored in the presence of a chemical denaturant.

II. MATERIALS & METHODS

A. Chemicals

Silver nitrate ($AgNO_3$) and sodium borohydride ($NaBH_4$) were procured from Sigma. Anhydrous sodium dihydrogen

phosphate, di sodium hydrogen phosphate and guanidine hydrochloride were purchased from Merck. Deionized water was used throughout the whole experiments. Glassware were thoroughly cleaned with dilute sulfo chromic acid/detergent solution and rinsed with Deionized water.

B. Synthesis of Silver Nanoparticles

Silver nanoparticles (AgNPs) were prepared by chemical reduction method, as previously described by Solomon et al [14]. In a typical experiment, aqueous silver nitrate (1 mL, 1.0 mM) was added drop wise to a large excess of ice-cold NaBH$_4$ solution (3 mL, 2.0 mM), with vigorous stirring. The clear solution turned light yellow when all of the silver nitrate solution was added. Silver contents of nanoparticles were determined using Inductively Coupled Plasma-Atomic Emission Spectroscopy (ICP-AES).

C. Protein-Nanoparticle Interaction

Stock solutions of lysozyme were prepared and concentration was determined using Bradford assay. To initiate the interaction, 200 μL of lysozyme solution (40 μM) was mixed with equal volume of nanoparticles with different concentrations (1-4 nM). Samples were incubated at ambient temperature for two hours.

D. Surface Plasmon Resonance Studies

Formation of silver nanoparticles was studied by UV–Vis spectrophotometer (Cary 100), with spectral resolution of 1 nm in the visible region.

E. Electron Microscopy

Particle size, morphology and distribution were studied by scanning electron microscopy (SEM). Sonicated samples were dried and labeled with a gold monolayer (sputter coating), and imaged by KYKY-EM3200 electron microscope at the accelerating voltage of 26 kV.

F. Dynamic Light Scattering

Size distribution and surface charge are critical parameters on controlling nanoparticles in the biological medium. In this regard, average hydrodynamic diameter and zeta potential of nanoparticles were investigated by Dynamic light scattering (DLS). Measurements were carried out in standard polypropylene quartz cuvettes of 1 cm path length. Samples were excited with a 633 nm wavelength laser. Depending on the size, scattering can be generated by particles introduced into the solvent. Scattered intensities were recorded at a 173° angle in kilo counts per second.

G. Fluorescence Spectroscopy

The emission spectra of protein were recorded on a Perkin-Elmer LS55 spectrophotometer in the wavelength range of 300–400 nm. The excitation wavelength was set at 295 nm. For quenching measurements, data were analyzed using the following Stern–Volmer equation

$$F_0/ F = 1 + K_{SV} \times [Q] \qquad (1)$$

where, F_0 and F represent the steady-state fluorescence intensities in the absence and presence of a quencher, K_{SV} is the Stern-Volmer quenching constant and [Q] is the concentration of the quencher. The fluorescence data can be used to obtain protein–AgNPs binding constant and the number of binding sites.

H. Unfolding Studies of Lysozyme

To monitor the structural changes induced by nanoparticles, denaturation of protein was examined with 8 M guanidine hydrochloride in phosphate buffer (pH 6.2). A series of solutions containing fixed amount of free and interacted lysozyme were incubated with 200 μL denaturant solution of different concentrations. Emission spectra of the protein were recorded two hours after the interaction. All measurements were conducted at room temperature.

III. RESULTS & DISCUSSION

A. Characterization of Silver Nanoparticles

Formation and morphology of silver nanoparticles was studied by monitoring their characteristic surface plasmon resonance peak by UV-Vis absorption spectroscopy and scanning electron microscopy, respectively. The term Surface plasmon resonance (SPR) is the collective oscillation of quantized charge density in a solid/liquid stimulated by the incident light. Such condition is established when the frequency of light photons matches the natural frequency of surface electrons in the conduction band oscillating against the restoring force of positive nuclei. The corresponding resonant peak energies and line widths are highly sensitive to particles size, shape and the dielectric properties of the medium [15]. This characteristic is the basis of many standard tools for measuring adsorption of material onto planar metal (typically gold and silver) surfaces or onto the surface of noble metal nanoparticles. As depicted in Fig. 1, the SPR band of silver nanoparticles exhibits a sharp peak at 398 nm, with FWHM of 50 to 60 nm, consistent with previous studies [14]. Scanning electron microscopy confirmed formation of nanoparticles with spherical morphology and narrow size distribution with average size of 32 nm. DLS yielded a monomodal distribution with hydrodynamic diameters around 22 nm and zeta potential of 23.0 mV.
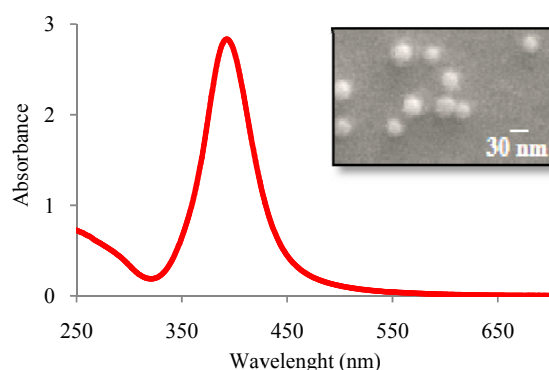

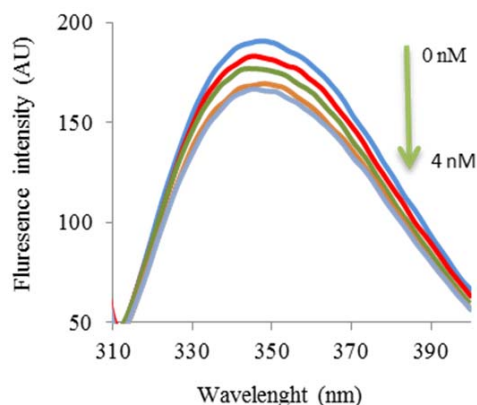Fig. 1 SPR peak and SEM image of AgNPs in aqueous medium

Fig. 2 Quenching of tryptophan fluorescence for lysozyme, in varying concentrations of NPs
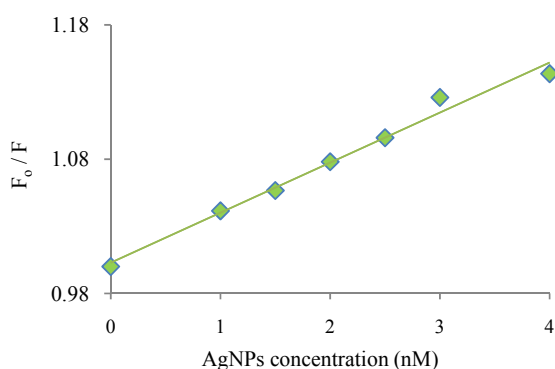

Fig. 3 Stern–Volmer plot. $F_0/F = 0.0373$ [NPs] + 1.0029 (R^2=0.98)

B. Quenching of Trp Fluorescence by Silver Nanoparticles

Fluorescence spectroscopy has been used as a convenient way for investigating protein tertiary structure and its binding behavior. It can provide intimate knowledge on ligand accessibility to Trp residues of protein. Lysozyme molecule contains six Trp residues (28, 62, 63, 108, 111 and 123) with three residues located (Trp62, 63 and 108) in the active site of the enzyme. Trp62 and Trp108 are considered as the most dominant fluorophores. According to Fig. 2, the intrinsic fluorescence of biomolecule experiences steady reduction in its intensity upon increase of nanoparticle's concentration. Therefore, Trp residues are involved in the complex formation. Since Trp 62 and Trp 63 residues are located at the active site between alpha and beta domains, it could be suggested that presence of AgNPs in protein's vicinity might affect the conformation of Trp microenvironment. Spectral shift of 24 nm in the complex form clearly indicates the increment of hydrophobicity around Trp residues. An increase in the helical content of lysozyme and decrease of substrate affinity upon treatment with nanoparticles also indicated that nanoparticles may approach to the enzyme's catalytic site and induce some degree of compactness [16], [17]. Analysis of the Stern–Volmer plot using the intrinsic fluorescence quenching data provided the value of 3.73 μM^{-1} as K_{SV} constant (Fig. 3). The plot showed a straight line, supporting the existence of a single quenching mechanism. Fluorescence intensity data can also be used to obtain the binding constant (Ka) and the number of binding sites (n). When small molecules bind independently to a set of equivalent sites on a macro-molecule, the equilibrium between free and bound molecules is given by the following equation [18]:

$$\text{Log}\,[(F_0 - F)/F] = \log Ka + n \log [Q] \qquad (2)$$

where, Ka is the binding constant and n is the number of binding sites per molecule. The plot of log $(F_0-F)/F$ against log [Q] yields a straight line with slope and intercept of n and Ka, respectively (Fig. 5). From analysis of (2), we obtained n = 0.94 and Ka = 0.04 nM, for Lys and AgNPs. The value of n indicates that there is approximately a single binding site in Lys upon approaching AgNPs. The low affinity of ligand binding represents weak intermolecular forces between lysozyme and AgNPs. Our previous studies on adsorption of Lys onto gold nanoparticles showed higher binding affinity compared to this result [19], [20]. Such difference could be justified by Density Functional Theory (DFT) and molecular dynamic simulation [21]. In vacuo DFT results indicated that water molecules adsorb more strongly to Ag rather than Au surfaces. The expulsion of water molecules in the first solvation layer at the Ag interface presents a free-energy barrier that the adsorbate must overcome to make close contact with silver surface. Presence of this barrier is not seen in a majority of the gold adsorption cases. Therefore, most of the biomolecules favor adsorption onto gold over silver [21].

C. Lysozyme Unfolding Studies

Fluorescence spectroscopy is highly sensitive to the biochemical environment of the fluorophores. In this regard, changes that might be induced in the structure of lysozyme during interaction with AgNPs were explored using a chemical denaturant. Upon addition of GdnHCl, lysozyme experienced unfolding phenomenon (Fig. 4), in which, tryptophan residues became more exposed to the hydrophobic core of the molecule and kept more distance from each other. This could explain enhancement and red-shift of fluorescence emission spectra for the bare protein and its complex to 369 and 367 nm, respectively. Increasing concentration of denaturant led to gradual increase in the fluorescence intensity of biomolecule (data are not shown). Comparison of fluorescence spectra of unfolded bare lysozyme with its nanoparticle-interacted counterpart showed that AgNPs have not induced perturbations in the protein's structure. However, based on our previous report on interaction of lysozyme with gold nanorods, it is worth to mention that nature of the nanoparticles, its morphology and affinity to absorb water molecules are key parameters in induction of structural stability [20], [21]. Therefore, within this range of concentration, AgNPs do not enhance structural stability, as previously seen by gold nanorods [20].
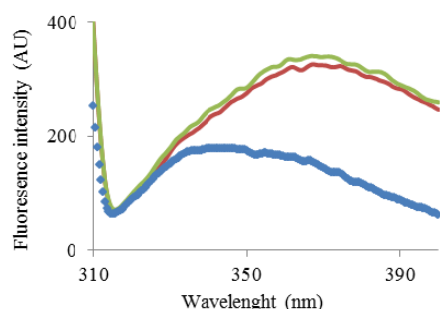
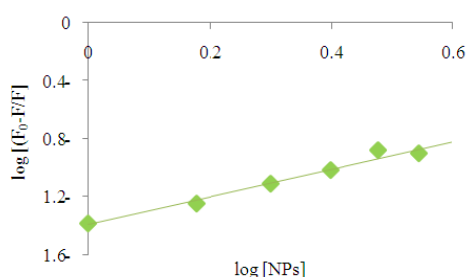Fig. 4 Emission spectra of bare lysozyme (blue), denatured lysozyme (green) and denatured lysozyme–AgNPs (red)

Fig. 5 Plot of log $(F_0-F)/F$ versus log [NPs] to determine binding constant and the number of binding sites ($R^2= 0.97$)

IV. CONCLUSION

Information on protein-nanoparticles interaction and binding parameters is of high significance to tailor hybrid systems of specific function with improved performance. This effort highlights utilization of fluorescence spectroscopy as a sensitive, reliable technique to monitor possible structural changes that a typical biomolecule might undergo upon interaction with nanoparticles of interest. Silver nanoparticles acted as a foreign quencher in the proximity of lysozyme and did not induce structural perturbations, as evidenced by unfolding studies. Moreover, analysis of fluorescence data revealed a single binding site in lysozyme, having low affinity for binding to AgNPs compared to gold nanostructures. These findings encourage in-depth characterizations of protein-nanoparticle interactions prior to design hybrid drug nanocarrier systems.

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