Circular Dichroism Spectroscopy of Lysozyme-Silver Nanoparticles Complex

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ABSTRACT

Plasmonic nanoparticles and their interaction with biomolecules has received great deal of interest, mainly due to their potential utility in drug nanocarrier systems. Assuming that a hybrid system of biomolecule and nanoparticles manifests improved functionality without severe structural perturbations, conformation of lysozyme as a model protein was monitored upon interaction with silver NPs, using Circular dichroism spectropolarimetry. Complex formation was evidenced by Fourier transform infrared spectroscopy. Analysis of Far-UV CD spectra showed the protein maintained its typical conformation with increase in the characteristic bands of α-helical structure at 208 and 222 nm. Kinetic investigations revealed that lysozyme almost retained its original catalytic activity after interaction, with decrease in the enzyme-substrate binding affinity. Results of this effort highlight utilization of plasmonic nanoparticles for versatile applications in the emerging drug delivery strategies.

Keywords: silver nanoparticles, lysozyme, circular dichroism spectroscopy, structural perturbation

1 INTRODUCTION

Silver nanoparticles have attracted great deal of interest amongst nanobiotechnologists, owing to promising applications in a wide variety of areas. Such level of interest arises from enhanced optical, and antibacterial properties amongst their nano scale counterparts, as well as high potency in biomedical and biosensing approaches. Today, designing hybrid systems of nanoparticles and biomolecules with enhanced properties has paved the way for future delivery strategies. Pioneers in this field have made great efforts to investigate bioavailability, biocompatibility, self-assembly, functionalization and bioconjugation of nanoparticles with variety of biomolecules. Although such efforts are of great value, it should be noted that surface adsorption property of both biomolecules and nanoparticles might induce severe structural perturbations. Despite the potential utility of these hybrid systems, such probability is noteworthy [1, 2]. Previous studies have shown that protein adsorption on a solid surface is an ensemble of many events such as serious conformational change, transformation into a molten-globule-like intermediate state and aggregation; leading to loss of biological activity and altered immune response [3-5]. On the other hand, in many cases the nanoparticles are capable of improving structural and functional propertiesof biomolecules. Such facts bring the essence of conducting a series of fundamental researches into notice, before in-vitro/ in-vivo exploitation of any nano-based system.

Similar to silver NPs, lysozyme has been extensively studied due to its remarkable biocidial effect. Having well-known structure, function, dynamics and physico-chemical properties, lysozyme hydrolyzes the mucopeptide cell wall of certain microorganisms [6]. Another important aspect of lysozyme relies on its ability to deliver drugs such as captopril [7] , naproxen [8] and puerarin [9]. Herein, we report interaction of AgNPs with lysozyme to monitor possible changes in biomolecule's conformation, using Circular dichroism spectroscopy (CD). As a sensitive analytical technique, CD enables investigation of trace conformational changes in the biomolecule's structure. Kinetic studies were also conducted to monitor the lytic function of lysozyme upon interaction.

2 EXPERIMENTALS

2.1 Materials

Chicken egg white lysozyme, silver nitrate ($AgNO_3$), sodium borhydride ($NaBH_4$) and M. luteus cells were procured from Sigma. Anhydrous sodium dihydrogen phosphate (NaH_2PO_4) and di sodium hydrogen phosphate (Na_2HPO_4) were purchased from Merck.

2.2 Synthesis of Silver Nanoparticles

Silver nanoparticles (AgNPs) were prepared in deionized water, as previously described by Solomon et al. Typically, AgNPs were synthesized through drop wise addition of silver nitrate (1 mL, 1.0 mM) to ice cold sodium

borhydride solution (3 mL, 2.0 mM), with vigorous stirring. Careful optimization of synthesis conditions including appropriate quantities of reagents, stirring time and coldness of $NaBH_4$ solution are critical prerequisites to obtain stable nanoparticles.

2.3 Nanoparticle Characterization

Silver nanoparticles were characterized by monitoring surface plasmon apsorption band in the visible range, using UV–Vis spectrophotometer (Cary 100). Particle size, morphology and distribution was analyzed by scanning electron microscopy (SEM). Fresh samples were sonicated and dried for labeling with a gold monolayer (sputter coating). Samples were imaged by KYKY-EM3200 electron microscope at the accelerating voltage of 26 kV.

Silver contents of nanoparticles were determined by ICP-AES spectrometer. Prior to analysis, nanoparticles were centrifuged at 20000 rpm for 60 minutes. The pellet was digested up to 2 mL with 1% (v/v) HNO_3. Given the average number of atoms per nanoparticle by SEM, and known number of silver atoms in the solution, concentration of the nanoparticles was calculated.

2.4 Nanoparticle-Biomolecule Interaction

A fixed concentration of lysozyme (400 $\mu g.mL^{-1}$ in phosphate buffer 0.02 M, pH 6.2) was interacted with various concentrations of AgNPs at the ambient temperature. Protein concentartion was calculated using Bradford protocol [10]. Samples were then incubated at the same conditions for 3 hours prior to characterization.

2.5 Fourier Transform Infrared Spectroscopy

Samples of protein and protein- nanoparticle complex were made into a dry powder by lyophilizer (LYSFME-Snijders scientific). FTIR spectra were recorded on a NICOLET IR 100 spectrophotometer. Samples were mixed with KBr powders (1:3) and made into thin plates prior to analysis. Data was collected in the range of 800-3800 cm^{-1}.

2.6 Circular Dichroism Spectropolarimetry

Secondary structure of lysozyme was studied by circular dichroism spectropolarimetry (Jasco-715 CD). The instrument was calibrated with D-10-camphorsulphonic acid and Far UV-CD region was scanned (200-250 nm). Results were recorded in terms of mean residue ellipticity [θ], in deg. $cm^2.dmol^{-1}$, as depicted from following equation :

$$[\theta] = 100\ [\theta]\ M_w / cln \qquad (1)$$

Where, θ is the measured ellipticity in degrees, c is the protein concentration in g/L, l is the path length in cm, Mw is the molecular weight of the protein and n is the number of amino acid residues. Data was smoothed and analyzed by Jasco software, after subtracting the buffer contribution from the original protein spectrum.

2.7 Lytic Activity of Lysozyme

Kinetics of lysozyme was investigated as described by Shugar et al [11]. The rate of decrease in the substrate turbidity at 450 nm at pH 6.2 and 25 °C could be directly correlated with lytic activity of enzyme. Lysozyme (30 µL, 20 µg mL^{-1}) was added to *Micrococcus lysodeikticus* cells (M. *luteus*) solution (1ml, 300µg ml^{-1}). The misxture was immediately used for enzymatic assay by UV/Vis spectrophotometer. Kinetic parameters were extraxted from Michaelis-Menten curves using GraphPad Prism software.

3 RESULTS & DISCUSSION

3.1 Characterization of AgNPs

Fiure 1 shows characteristic SPR band and SEM image of AGNPs. Interaction of polarized light with boundary of a metal surface confined to nanoscale results in collective oscillation of electrons in the conduction band, known as Localized Surface Plasmon Resonance (LSPR). Sensitivity of LSPR to trace changes in the local refractive index enables exploitation of plasmonic nanoparticles in wide variaty of biomedical applications, ranging from bio sensing to monitoring hybrid systems of drug-nanocarrier. The nanoparticles exhibit a sharp SPR band at 392 nm, with FWHM of 50-60 nm (Figure 1). SEM image depicted formation of monodisperse AgNPs, with spherical morphology and average size of 32 nm.

3.2 FTIR of AgNPs-Lysozyme Complex

FTIR spectra of lysozyme in the presence and absence of AgNPs are shown in Figure 2A. Amonsgt different vibrational bands that contribute to peptide groups, amide I and amide II bands provide useful information in the infrared spectrum. The amide I vibration (1600–1700 cm^{-1}) arises mainly from C=O stretching vibration and can be directly correlated with the backbone conformation of protein. Potential energy for the amide II band (1500-1600 cm^{-1}) derives mainly from the in-plane N-H bending, and the rest appearing due to C-N and C-C stretching vibrations. Monitoring the intensity changes in such characteristic regions could be correlated with amount of protein adsorption [12]. According to Figure 2, shift in the amide II region from 1643 to 1689 cm^{-1} with loss of intensity clearly shows that lysozyme has been adsorbed on AgNPs.

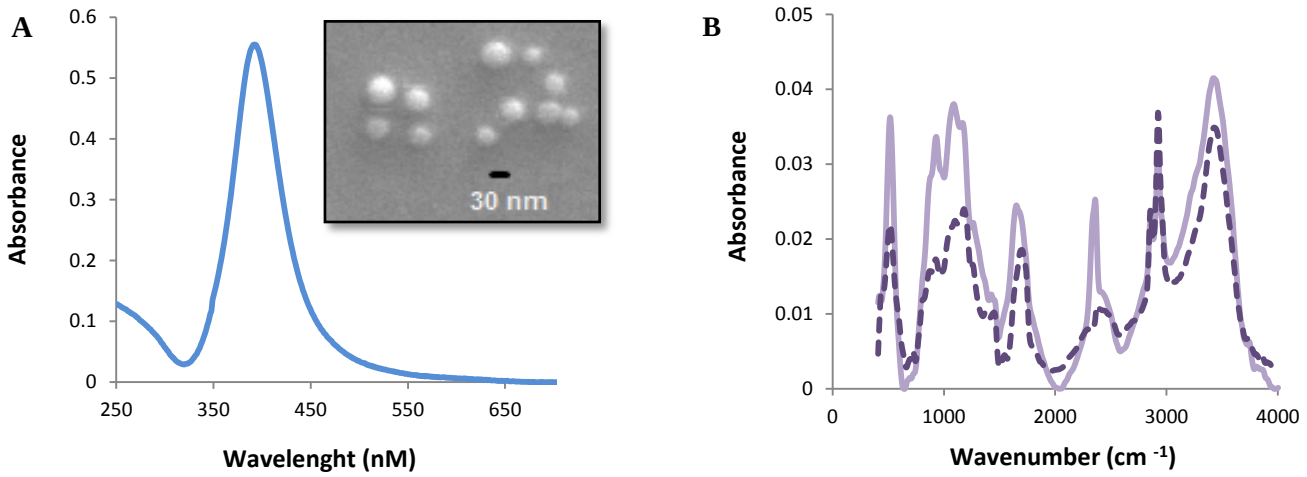

Figure 1: (A) UV–Vis spectrum of AgNPs with corresponding SEM image. (B) FT-IR spectra of lysozyme (solid line) and its complex form with AgNPs (dashed line).

3.3 Secondary Structure of Lysozyme

Circular Dichroism spectroscopy is employed for determination of protein secondary structure. Dichroism is often expressed as the property possessed by chiral molecules of absorbing light to different extents, dependent upon the polarization form of the incident beam. Difference in the absorbance of right and left circularly polarized light in the UV region serves to give remarkable information on conformational changes of biomolecule [13]. Figure 2B shows Far-UV-CD spectra of lysozyme, before and after interaction with two concentrations of silver nanoparticles. Data was smoothed and analzsed by Jasco secondary structural analysis software.

Analysis of Far-UV-CD spectra showed that in the presence of silver nanoparticles conformation of the biomolecule has undergone alterations in terms of intensity of characteristic bands of α-helix at 208 and 222 nm (Figure 2B). Higher negative value of the bands at the specified wavelengths indicates an increase in the helical content of lysozyme upon treatment with nanoparticles. Once the protein is interacted with various concentrations of AgNPs (i.e. 2 and 4 nM), it acquires more regular conformation, leading to higher degree of compactness. A glance at Figure 2 shows that higher concentrations of silver nanoparticles (4 nM) did not induce more conformational changes in the protein's structure.

The α-helical content was further calculated on the basis of the mean residue ellipticity at 222nm, with respect to 100% α-helix (equal to of -39500 deg .cm^2 .dmol^{-1}) [14]. This analysis yields a value of 21.4 % α-helix for bare lysozyme, growing to 24.5% and 24.6% upon interaction with the specified concentrations of AgNPs. Therefore, it could be concluded that secondary structure of lysozyme has undergone negligible alterations upon interaction with both concentrations of silver nanoparticles.

3.4 Lytic Activity of Lysozyme

When conformational changes occur in a typical enzym's structure, its activity might undergo infavourable changes, and at times, the enzyme could totally lose its biological activity. As far as negiligible alterations are concerned, it is expected that lysozyme would depict its natural catalytic activity upon interaction. Therefore, kinetic investigations were carried out to make an analytical assessment on lyozyme activity on binding to AgNPs. Michaelis–Menten curves of the enzyme were plotted versus different concentrations of substrate. Kinetic parameters were extracted using GraphPad Prism software. Figure 3 represents the Michaleis-Menten curve of lysozyme, in the presence and absence of silver nanoparticles. According to the Figure, maximum rate of lysozyme activity at saturating substrate concentrations (V_{max}) showed changes. The value of 5564 (U.mg^{-1}) was calculated for the enzyme in its free state, which decresaed to 5437 and 5014 (U.mg^{-1}), upon interaction with 2 nM and 4 nM of silver nanoparticles, respectively. Although there has been reduction in the V_{max} value, the enzyme complex maintained high level of its catalytic activity, being 97% and 90% with respect to its free state. Comparison of K_m parameter of the enzyme in complex state showed that the value has increased (49 and 56 μg.mL^{-1}), compared to that for native lysozyme (i.e. 25 μg.mL^{-1}). Increase of this value reveals that the enzyme has less affinity for its substrate in the presence of nanoparticles. Possibly, the nanoparticle approaches to the enzyme's catalytic site. The active site of lysozyme contains two catalytic residues, Glu35 and Asp52, which lie in a cleft to the vicinity of the largest pocket, harboring the substrate binding site [15]. Such large depression on the protein surface could provide opportunity for the protein to approach AgNPs. However, the enzyme still performs its catalytic function upon the interaction.

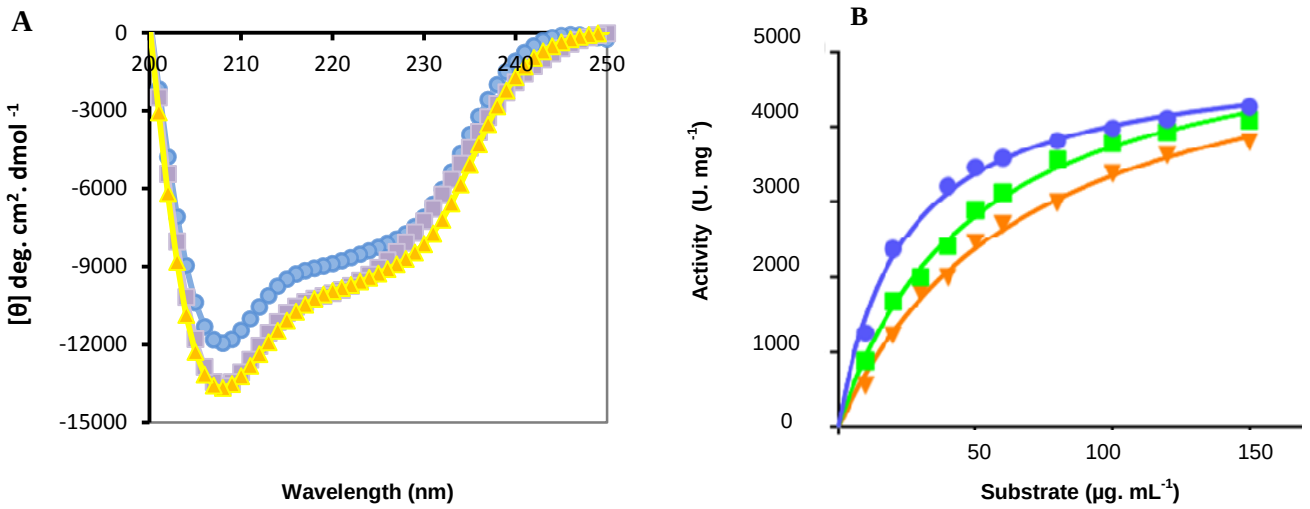

Figure 2: (A) Far-UV CD spectra and (B) Michaelis-Menten plots of lysozyme. Symbols represent (●) for bare lysozyme, (■) for lysoyzme-AgNPs (2 nM) and (▲) for lysoyzme-AgNPs (4 nM), respectively.

CONCLUSION

In this paper, the effect of silver nanoparticles has been studied on conformation and catalytic activity of lysozyme. Analysis of secondary structural content of lysozyme showed increase of compactness. However, the protein maintained high degree of its typical conformation. Kinetic studies revealed that the enzyme almost retained its native lytic activity even after interaction with AgNPs. Although the biomolecule did not experience unfavorable perturbations from structural and functional point of view, the hybrid system shows less affinity for enzyme-substrate binding. It could be assumed that silver nanoparticles approach the biomolecule from its catalytic site, decreasing the opportunity of enzyme-substrate binding. This effort highlights the possibility of designing hybrid systems of plasmonic nanoparticles and biomolecules for the emerging drug delivery strategies.

REFERENCES

[1] T. Tohidi Moghadam, B. Ranjbar, K. Khajeh, S. M. Etezad, K. Khalifeh, M. R. Ganjalikhany, International Journal of Biological Macromolecules, 49, 629-636, 2011.

[2] T. Tohidi Moghadam, B. Ranjbar, K. Khajeh, International Journal of Biological Macromolecules, 51, 91-96, 2012.

[3] A. E. Nel, L. Mädler, D. Velegol, T. Xia, E. M. Hoek, et al. Nature Materials, 8, 543-557, 2009.

[4] A.Vertegel, R. W. Siegel, J. S. Dordick, Langmuir, 20, 6800-6807, 2004.

[5] S. Chakraborty, P. Joshi, V. Shanker, Z. A. Ansari, S. P. Singh, P. Chakrabarti, Langmuir, 27, 7722-7731, 2011.

[6] T. Yang, Z. Li, l. Wang, C. Guo, Y. Sun, Langmuir 23, 10533-10538, 2007.

[7] J. Prakash, A. M. van Loenen-Weemaes, M. Haas, J. H. Proost, D. Meijer, et al. Drug Metabolism and Disposition, 33, 683-688. 2005.

[8] E. J. Franssen, R. G. van Amsterdam, J. Visser, F. Moolenaar, D. de Zeeuw, D. K. Meijer, Pharmaceutical Research, 8, 1223-1230, 1991.

[9] Z. Wang, L. Daojin, J. Jin, Molecular and Biomolecular Spectroscopy, 70, 866-870, 2008.

[10] M. M. Bradford, Analytical Biochemistry, 72, 248-254, 1976.

[11] D. Shugar, Biochimica et Biophysica Acta, 8, 302309, 1952.

[12] W. K. Surewicz, H. H. Mantsch, & D. Chapman, Biochemistry, 32, 389-394, 1993.

[13] B. Ranjbar, P. Gill, Chemical Biology & Drug Design, 74, 101-120, 2009.

[14] B. de Foresta, L. Tortech, M. Vincent & J.Gallay, European Biophysics Journal, 31, 185-197, 2002.

[15] S. Chakraborti, T. Chatterjee, P. Joshi, A. Poddar, B. Bhattacharyya, S.P. Singh, V.Gupta, P. Chakrabarti, Langmuir, 26, 3506–3513, 2010.