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Structural studies on the interaction of nano-SiO_2 with lysozyme

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Abstract

The interaction between nano-SiO_2 and lysozyme was investigated by the method of UV-Visible detection and fluorescence spectroscopic techniques. The thermal denaturation of lysozyme has been investigated in the presence and absence of nano-SiO_2 over the temperature range (293-373) K in different buffers and pH values, using temperature scanning spectroscopy. The presence of nano-SiO_2 caused the destabilization of lysozyme resulting in a decrease in the temperature of unfolding with an increase in nano-SiO_2 concentration.

1. Introduction

The attachment of proteins onto nanoscale materials is becoming more common with the greater availability of inorganic nanoparticles and pillars, functionalized organic polymers, and carbon nanotubes [1-3]. In the case of nanoparticles, recent studies have opened the door to a host of new biomolecular conjugates with applications as diagnostics and sensors, drug delivery, and biocompatible and "smart" materials [4, 5]. Nanoparticles possess unique properties due to the extremely large surface area per unit volume and therefore can be used as biosensors [6, 7]. The nanoparticle surface can be functionalized through immobilization of enzymes for sensor applications in food pathogen detection, pesticide detection and food quality control. Among the different methods of immobilization, adsorption of enzymes onto the nanoparticle surface is the simplest. There has been increasing interest in the development of biosensors for detection of food pathogens and food quality. Biosensors obtain their specificity

from a biological binding reaction which may be derived from any of a wide range of interactions, specificity, and affinities including antigen/antibody, enzyme/substrate, receptor/ligand, energy transducer systems. Enzyme/substrate reactions currently lack the spectrum of antigen/antibody systems, yet they possess inherent advantage in that they rapidly eliminate the target, release the product, and auto-regenerate the binding site with minimal loss of affinity and specificity over a large number of cycles. It is believed that immobilization of various enzymes onto nanoparticles will result in a much faster and accurate assay. Immobilization of enzymes by adsorption, however, may result in partial loss of enzymatic activity. It is important, therefore, to characterize the unfolding of proteins/enzymes at surfaces in order to arrive at rational methodology for the development of protein microarrays and nanoparticles-based sensors [8]. Proteins, including lysozyme, horseradish peroxidase, catalase, and trypsin, adsorb strongly to SiO_2 nanoparticles (sizes ranging from 9 to 40 nm). In the process, these proteins undergo a partial loss of structure and generally a significant loss in enzyme activity. Thus, it should be expected that particle size plays a key role in modulating protein structure and function. However, no systematic study has been performed to date on the effect of nanoscale particle size on the structure and function of adsorbed proteins independent of nanoparticles chemistry [9]. Lysozyme (model protein with dimensions (4.5 × 3.5 × 3.5 nm)) is an enzyme which hydrolyzes the polysaccharides found in many bacterial cell walls [10]. Lysozyme, which occurs naturally in egg white, human tears, saliva, and other body fluids, is capable of destroying the cell walls of certain bacteria and thereby acting as a mild antiseptic, a feature that was discovered serendipitously in 1922 by Alexander Fleming. It has been extensively used kill bacterial cells in water due to the generation of reactive oxygen species [11, 12]. Antibacterial agents may be artificial synthetic drugs or natural secreted substances. Lysozyme is a small spheroidal hydrolase responsible for breaking down the polysaccharide walls of Gram-positive bacteria and those of some Gram-negative bacteria. It is a natural antibacterial protein found in saliva as well as in egg white and mucus. Hence, it is reasonable and logical to think of utilizing the salivary lysozyme as an antibacterial agent [13]. Lysozyme is a compact globular protein with 129 residues, consisting of five α helices, a three stranded antiparallel β sheet, and a large amount of random coil and β turns [14-16]. Also its structure is stabilized by four disulfide bonds [17], with most of the cysteins located in the α helices. The enzyme has an approximately ellipsoidal shape, with a large cleft in one side forming the active site which can bind six carbohydrates to execute its effective catalyst function [8]. The folding pathway for lysozyme has been well characterized and shown to consist of two folding phases. In the first fast folding phase, major parts of α-domain and the β-domain are formed. The second slow folding phase results in the complete formation of secondary structure and native tertiary structure in less than 1 s. In addition, four disulphide bonds are also formed. However, unfolding of lysozyme on surfaces is a much slower process lasting several hours. Previous investigations have been employed tryptophan fluorescence to characterize the change in the tertiary conformation of proteins using the extent of blue shift of the emission spectrum [18]. The secondary structure of proteins was monitored using Fourier Transform Infrared Spectroscopy (FTIR) and Circular Dichroism (CD) [19-21]. Hydrophobic and electrostatic interactions between the protein and adsorbing surface have been shown to influence the changes in secondary structure [19, 20]. Adsorption onto silica nanoparticle was shown to result in a greater loss of α helix content of lysozyme for larger surface coverage [9]. The change in secondary structure of lysozyme upon adsorption onto PTFE was found to exhibit an initial fast conversion of α helix to β sheet within a few minutes followed a much slower conversion [22]. We have investigated the stability of tertiary conformational changes of lysozyme on silica nanoparticle surfaces using tryptophan fluorescence, UV-Vis and Tm studies. The tertiary conformational changes were shown to depend on surface packing density, pH and ionic strength. The silica nanoparticles have similar surface chemistry (hydrophilic OH-terminated surface), which ensures that any difference in interaction with protein molecules is

primarily due to the effect of nanoparticle size [9]. In this work, nano-SiO_2 was selected as being representative of nanomaterials and lysozyme as representative for enzyme.

2. Materials and methods

2.1. Instruments and material

The absorption spectra of all liquids/solutions were recorded with a Model Ultrospec 4000, UV-Visible spectrophotometer equipped with a thermostatic cell holder. The fluorescence spectra of solutions were recorded with a Fluorescence Spectrophotometer (Shimadzu RF-5301PC Fluorescence Spectrophotometer). A scanning electron microscope (SEM) was used to measure the size and shape of the nano-SiO_2. Nano-SiO_2 (size 10 nm) was used without further modification. It was suspended in deionized water and mixed ultrasonically for 3 times in 10 min before use. Egg white lysozyme (0.1 mg/ml; purchased fromSigma) was dissolved in deionized water and stored at less than 4 °C. Different buffer solutions and different pH was prepared to adjust the acidity of the solution. An electrolyte solution (1.5 M NaCl) was prepared to adjust the ionic strength of the solution.

2.2. Spectrophotometry determination of the nano-SiO_2–lysozyme interaction

All studies were carried out in quartz cells containing 0.1 mM lysozyme and different concentration nano-SiO_2 suspension. After 10 min the solid with adsorbed lysozyme was separated by centrifugation of a suspension at 12,000 rpm (twice, 10 min). The absorbance of the supernatant was measured at 280 nm against water by UV–Vis spectrophotometry. Simultaneously, a reagent blank without lysozyme was performed according to the same procedure. With the above procedures, a series of suspensions was prepared, initially containing 0.1 mg/ml lysozyme, different concentration nano-SiO_2. The absorption spectra of the suspension were measured between 210 and 350 nm against water by spectrophotometry.

2.3. Fluorescence measurement

All studies were carried out in quartz cells containing 0.1 mM lysozyme and different concentration nano-SiO_2 suspension. After 10 min, the solid with adsorbed lysozyme was separated by centrifugation of a suspension at 12,000 rpm (twice, 10 min). The fluorescence spectrum of each suspension was measured, where the excitation wavelength was at 280 nm and the emission wavelength was between 290 and 450 nm (using 5 and 3 nm of slit width). Besides, the solid with adsorbed lysozyme was separated according to the method noted above and then the fluorescence spectrum of the supernatant was determined. Simultaneously, a reagent blank without lysozyme was performed according to the same procedure.

2.4. Thermal stability of lysozyme

All studies were carried out in quartz cells containing 0.1 mM lysozyme and different concentration nano-SiO_2 suspension. After 10 min, the solid with adsorbed lysozyme was separated by centrifugation of a suspension at 12,000 rpm (twice, 10 min). The UV–Vis spectrum of the protein in the wavelength was set at 280 nm was and the absorbance in the absence and presence of different amounts of nano-SiO_2 were recorded.

3. Results and discussion

The choices of lysozyme and silica for this study were dictated by the following considerations. First, the values of pI for silica colloids and lysozyme are ~3 and ~ 11, respectively and, therefore, strong Coulombic interaction could be anticipated between the negatively charged silica and positively charged lysozyme over the wide pH range of this study [9, 24]. Second, the structure of lysozyme and unfolding behavior of dissolved lysozyme are well characterized. Finally, at moderate pH values it has a high thermal stability which originates in part from four disulfide bonds. Thus, protein-surface interactions are not expected to alter the native structure of lysozyme drastically, which allows one to study the thermal denaturation of lysozyme even in the adsorbed state [23].

3.1. Determination of particle size of nano-SiO_2

Fig. 1 shows the SEM picture of nano-SiO_2. From SEM measurement we observed that nano-SiO_2 particles have spherical in shape and the average particle diameter is about 10 nm.

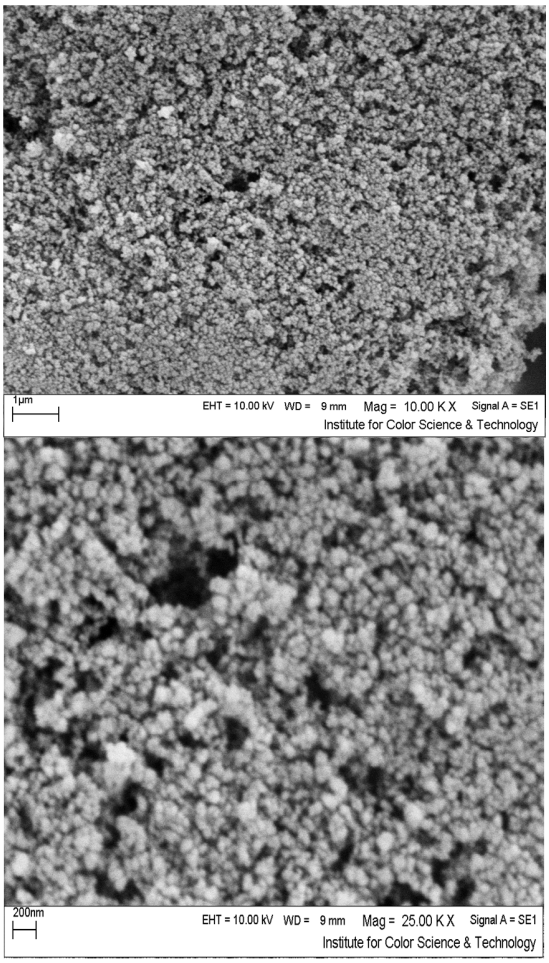

Fig. 1. SEM images of nano-SiO_2.

3.2. Interaction of lysozyme with nano-SiO_2 particle

In the pH range of 4.5 to 11, lysozyme is positively charged. Since the silica nanoparticle is negatively charged, the electrostatic interaction between the protein and nanoparticle surface should promote unfolding [8]. The light absorption of the lysozyme-nano-SiO_2 suspension is shown in Fig. 2. The absorbance (A) of the liquid increased with an increase in the nano-SiO_2 concentration from 0 to 2 mg/L without change in the wavelength of peak at 280 nm. This observation is due to the formation of ground state complex (lysozyme…..SiO_2). It is likely that lysozyme…..SiO_2 complex has higher extinction co-efficient than the unabsorbed state but has absorption maximum at the same position i.e. 280 nm [28].

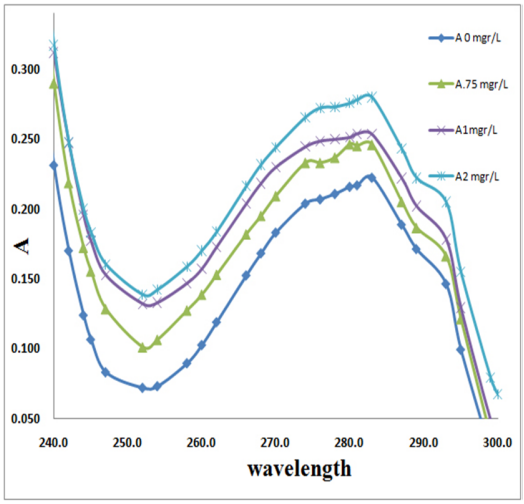

Fig. 2. Absorbance (A) lysozyme-nano-SiO_2 suspension at pH = 7.5.

The results from the absorption study indicated that there is an interaction between SiO_2 NPs and lysozyme via ground state complex formation. Obviously, nano-SiO_2 particle surfaces carry lots of negative charges because its isoelectric point is at pH 3. In contrast, lysozyme with 18 basic amino acid residues carries lots of positive charges in a neutral media (the isoelectric point is at pH 11) [20]. At first, the adsorption of lysozyme occurred by the electrostatic attraction when lysozyme is mixed into nano-SiO_2 particle suspension. When the distance between lysozyme and SiO_2 is short enough, the hydrogen bond will form between SiO_2 and the polar side chains of amino acid residues. Thus, the combination of non-covalent electrostatic interactions and hydrogen bonds led to the firm binding of lysozyme on SiO_2 particle. In the presence of colloidal SiO_2, NPs the absorbance of lysozyme is increased markedly, without change in the wavelength of peak at 280 nm. This observation is due to the formation of ground state complex (lysozyme ….SiO_2). It is likely that the lysozyme ….SiO_2 complex has higher extinction co-efficient than the unabsorbed state but has absorption maximum at the same position, i.e. 280 nm. This may be the reason for increase in the absorbance of lysozyme in the presence of colloidal SiO_2 NPs as supported by the similar observation made earlier. The results from the absorption study indicated that there is

an interaction between colloidal SiO_2 NPs and lysozyme existing via ground state complex formation. After lysozyme is added, the SiO_2–lysozyme particles become colloidal and conjointly global from the independent regular globes.

3.4. Fluorescence analysis of the lysozyme-nano-SiO_2 interaction

There are six Trp residues in hen egg white lysozyme, but only two of them, Trp62 and Trp108, appear to dominate the fluorescence spectrum [29, 30]. Emission of lysozyme is dominated by tryptophan residue, which absorbs at the longest wavelength and displays the largest extinction coefficient. Energy absorbed by phenylalanine and tyrosine is often transferred to the tryptophan residues in the same protein [31]. Fig. 3 (a, b, c) shows the effect of increasing concentration of colloidal SiO_2 NPs on the fluorescence emission spectrum of lysozyme, which resulted in the quenching of its fluorescence emission. In order to further investigate the interaction of lysozyme with nano-SiO_2 particles, the fluorescence spectra of the nano-SiO_2-lysozyme suspensions are determined Fig. 3 (a, b, c). The fluorescence intensity of the liquid decreased sharply with increasing nano-SiO_2. The result of fluorescence spectroscopy indicated that the structure of the Trp residue environments was altered. An additional reason is that the side groups of tryptophan residues: W108, W63, and W62 located in binding site may bind to SiO_2 particles via the N– H···O hydrogen bonds owing to the twist and deformation of lysozyme on SiO_2 particles. With increasing concentration of nano-SiO_2 a red shift is found for all samples indicating an unfolding of the lysozyme structure. The environment of the Trp residues becomes more polar due to neighboring water molecules, and the re-orientational relaxation of which after fluorescence excitation and emission leads to a Stokes shift of the fluorescence spectrum [32]. This observation suggests that in the unfolded state of SiO_2-lysozyme the Trp residues are less accessible to water molecules and are probably in close contact with the silica surface [32].

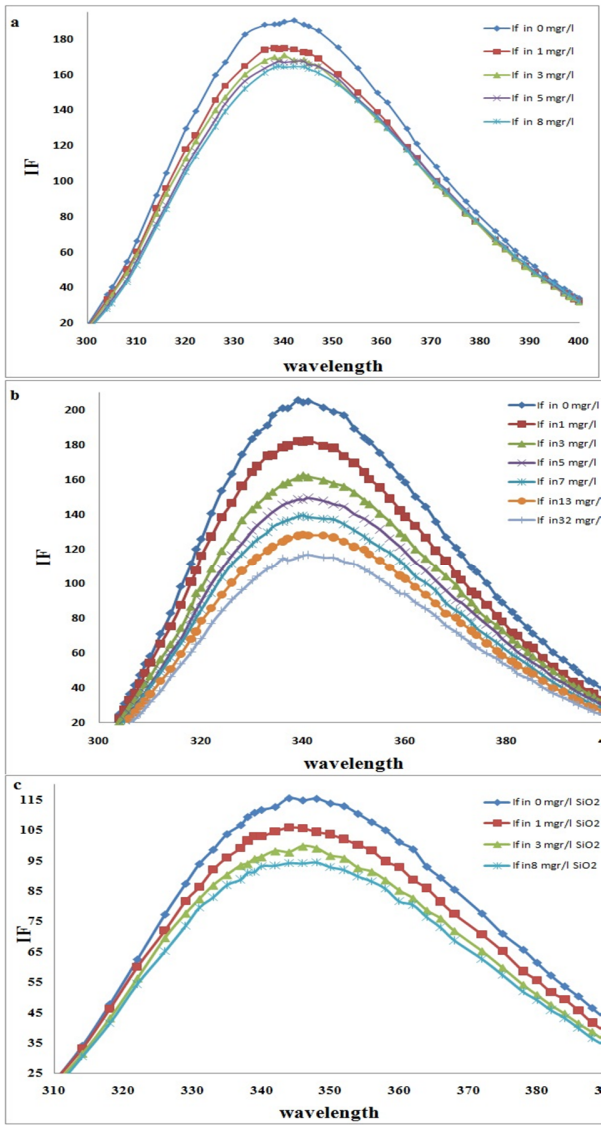

Fig. 3. Fluorescence quenching of lysozyme, λexi = 280 nm in the presence of various concentration of SiO_2 at (a) pH = 2.5, (b) pH = 7.25, (c) pH = 11.

3.5. Thermal stability of lysozyme

Thermal denaturation curves for lysozyme in the presence and absence of nano-$SiO_2$are shown in Fig. 4. The fraction of the denatured protein, Fd, was calculated using the relation [33, 34]:

$$F_d = \frac{Y_{obs} - Y_N}{Y_D - Y_N}$$

Where Y_N and Y_D are respectively the optical properties (absorbance) of the native and denatured molecules under the conditions in which Y_{obs} has been determined. It is obvious that

increasing the concentration of nano-SiO_2 decreases the stability of lysozyme to thermal denaturation. The thermal denaturation of small globular proteins closely approaches a two-state, mechanism [33, 34]. By assuming a two-state mechanism, the difference in free energy between the folded and unfolded conformation, $\Delta G_D°$, can then be calculated using:

$$\Delta G_D° = -RT\ln\left[\frac{Fd}{(1-Fd)}\right] = -RT\ln\left[\frac{Yobs-YN}{YD-Yobs}\right]$$

Where R is the gas constant and T is the absolute temperature. The free energy of denaturation, $\Delta G_D°$, as a function of temperature for lysozyme in the presence and absence of nano-SiO_2 is shown in Fig. 5. These results can be used to determine T_m at which $\Delta G_D° = 0$. It is clear that on increasing the concentration of nano-SiO_2 the curves shift to lower temperatures (Fig. 5). Lysozyme T_m at various concentrations of SiO_2 is tabulated in Table 1. As indicated in Table 1, by increasing the concentration of SiO_2, T_m of lysozyme decreased.

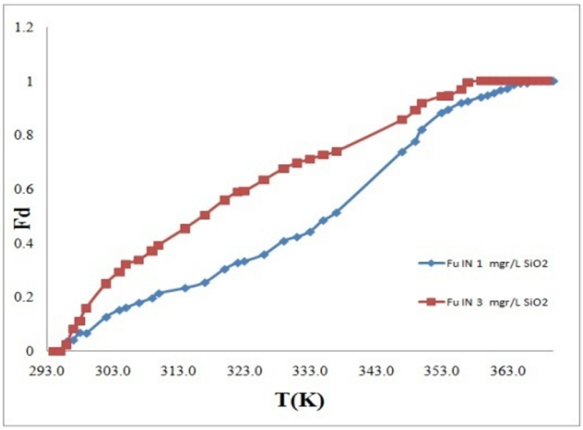

Fig. 4. Thermal denaturation curve of lysozyme in the presence of nano-SiO_2 at pH= 7.25.

The activity of a protein with a particular function depends on its specific conformation, that's to say, the covalent and non-covalent interactions among its amino acid residues. When a certain compound is added to a protein solution, the internal non-covalent interactions of the peptide chain may be altered or even destroyed. While binding of proteins to planar surfaces often induces significant changes in secondary structure, the high curvature of nanoparticles can help proteins to retain their original structure. However, study of a variety of nanoparticle surfaces and proteins indicates that the perturbation of protein structure still happens to varying extents.

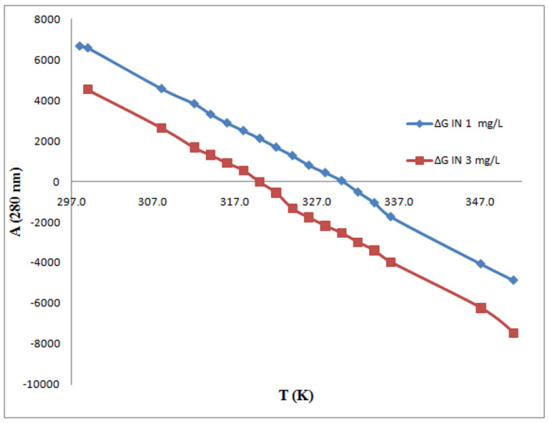

Fig. 5. ΔGD° curves for lysozyme as a function of temperature at pH = 7.25.

Table. 1. Tm values at various nano-SiO_2 concentrations.

Nano-SiO_2 (mg/L)	pH=2.5	pH=4.5	pH=7.25
	T_m °C	T_m °C	T_m °C
1	58	61	56
3	50	59	47
5	49	56	45

The unfolding kinetics of lysozyme or β-lactoglobulin when adsorbed onto silica nanoparticles shows that upon adsorption, the proteins show a rapid conformational change at both secondary and tertiary structure levels [25, 26]. The adsorption of lysozyme on SiO_2 particles decreases as the pH decreases. This agrees well with previous observations of predominantly coulombic lysozyme adsorption onto negatively charged silica, where it was proposed that lysozyme adsorption was strongly influenced by increased protein-protein electrostatic repulsion at a lower pH, when lysozyme molecules bear a higher positive charge (+8 at pH 8.0 versus +10 at pH 4.0) [27]. However, such repulsion would only be significant at high protein loadings and would likely be compensated by stronger protein-silica attraction. An alternative explanation is the pH-

induced change of *ú* potential for silica, 26 which results in decreased Coulombic attraction between lysozyme and silica at lower pH [9]. Nezu et al. reported that electrostatic interaction is the main mechanism controlling the adsorption of lysozyme to SiO_2 [13]. The results presented in this work indicate interactions between lysozyme and silica nanoparticles. α-helix content is lost for protein adsorbed on silica nanoparticles. As the α-helix content decreases, enzyme activity decreases. The active site of lysozyme is located at the opposite side of the positively charged patch [9, 35]. Thus, we may speculate that the first structural changes upon adsorption result in only moderate loss in activity because the structural perturbations are somewhat distant to the active site. More significant perturbation, however, occurs closer to the active site as the native α-helix content is further lost [9]. Lysozyme structure and function upon adsorption onto silica nanoparticles is strongly dependent upon the size of the nanoparticles. Less significant perturbation of lysozyme secondary structure is observed when the protein is adsorbed onto smaller nanoparticles under otherwise similar conditions [9]. However, in the presence of nanoparticles, the α helix content decreased while the β sheet content increased [8]. The secondary conformational changes of lysozyme upon adsorption lead to a loss of α helical content with a corresponding increase in β sheet and random coil. The rapid unfolding of lysozyme upon adsorption onto nanoparticles surface is believed to be due to the rapid decrease in the free energy of adsorbed protein molecule. Lysozyme has two domains, namely, α and β domains. A rapid decoupling of these two domains upon adsorption would lead to a strong disruption of the tertiary structure with an immediate loss of α helix in α domain with a corresponding increase in β turn and random coil [36]. This is quite unstable since it would result in the exposure of most of the hydrophobic amino acid residues from the interior of the molecule to the solvent in a very short time. Therefore, a 'molten globule-like' structure is formed in which α helix is transformed to β-sheet, thereby resulting in a refolding phase [37]. However, this intermediate structure is not stable on the silica surface because a collapse of these two domains may lead to a change in the surface charge distribution and therefore a further reorientation of the molecule leading to further unfolding such that there is largest contact of the positively charged patch to the negative surface.

4. Conclusion

The interaction between nano-SiO_2 and lysozyme has been studies by UV-Visible and fluorescence spectroscopic measurement. The results indicated clearly that nano-SiO_2 quench the fluorescence of lysozyme through complex formation. The result of fluorescence spectroscopy indicated that the structure of the Trp residue environments was altered. The effect of nano-SiO_2 on lysozyme stability has also been studied. Increasing the concentration of nano-SiO_2 decreases the stability of lysozyme to thermal denaturation

References

1. R. Michel, I. Reviakine, D. Sutherland, C. Fokas, G. Csucs, G. Danuser, N.D. Spencer, M. Textor, Langmuir, 18 (2002) 8580-8586.
2. W.J. Parak, D. Gerion, T. Pellegrino, D. Zanchet, C. Micheel, S.C. Williams, R. Boudreau, M.A.L. Gros, C.A. Larabell, A.P. Alivisatos, Nanotechnology, 14 (2003) R15.
3. N.W.S. Kam, T.C. Jessop, P.A. Wender, H. Dai, Journal of the American Chemical Society, 126 (2004) 6850-6851.
4. T.J. Webster, L.S. Schadler, R.W. Siegel, R. Bizios, Tissue engineering, 7 (2001) 291-301.
5. C.M. Niemeyer, Nanoparticles, Angewandte Chemie International Edition, 40 (2001) 4128-4158.
6. Z. Mei, H. Chen, T. Weng, Y. Yang, X. Yang, European journal of pharmaceutics and biopharmaceutics, 56 (2003) 189-196.
7. H. P. Zobel, F. Stieneker, S. Atmaca-Abdel Aziz, M. Gilbert, D. Werner, C. R Noe, J. Kreuter, A. Zimmer, European journal of pharmaceutics and biopharmaceutics, 48 (1999) 1-12.
8. X. Wu, G. Narsimhan, Biochimica et Biophysica Acta (BBA)-Proteins & Proteomics, 1784 (2008) 1694-1701.
9. A. A. Vertegel, R.W. Siegel, J.S. Dordick, Langmuir, 20 (2004) 6800-6807.
10. R. F. Powning, W.J. Davidson, Comparative Biochemistry and Physiology Part

B: Comparative Biochemistry, 45 (1973) 669-672, IN613-IN614, 673-681.
11. A. Kathiravan, M. AshaJhonsi, R. Renganathan, Journal of Luminescence, (2011).
12. Z. Xu, X. W. Liu, Y. S. Ma, H.W. Gao, Environmental Science and Pollution Research, 17 (2011) 798-806.
13. T. Nezu, T. Masuyama, K. Sasaki, S. Saitoh, M. Taira, Y. Araki, Dental materials journal, 27 (2008) 573-580.
14. A. G. Amit, R.A. Mariuzza, S.E. Phillips, R.J. Poljak, Science, 233 (1986) 747.
15. J. C. Cheetham, P. J. Artymiuk, D.C. Phillips, Journal of molecular biology, 224 (1992) 613-628.
16. L. N. Johnson, D. C. Phillips, Nature, 20 (1965) 761.
17. A. G. Murzin, S.E. Brenner, T. Hubbard and C. Chothia, SCOP: , Journal of molecular biology, 247 (1995) 536-540.
18. V.K. Sharma, D.S. Kalonia, Journal of pharmaceutical sciences, 92 (2003) 890-899.
19. M. H. Baron, M. Revault, S. Servagent-Noinville, J. Abadie, H. Quiquampoix, Journal of colloid and interface science, 214 (1999) 319-332.
20. S. Noinville, M. Revault, M.H. Baron, Biopolymers, 67 (2002) 323-326.
21. P. Billsten, P. O. Freskgrd, U. Carlsson, B.H. Jonsson, H. Elwing, FEBS letters, 402 (1997) 67-72.
22. J. Wang, J. McGuire, Journal of colloid and interface science, 185 (1997) 317-323.
23. C. Czeslik and R. Winter, Phys. Chem. Chem. Phys., 3 (2000) 235-239.
24. P. Billsten, M. Wahlgren, T. Arnebrant, J. McGuire, H. Elwing, Journal of colloid and interface science, 175 (1995) 77-82.
25. L. Shang, Y. Wang, J. Jiang, S. Dong, Langmuir, 23 (2007) 2714-2721.
26. L. Fei, S. Perrett, International journal of molecular sciences, 10 (2009) 646-655.
27. T.J. Su, J.R. Lu, R.K. Thomas, Z.F. Cui, J. Penfold, Langmuir, 14 (1998) 438-445.
28. B. S. S. Farhadiana, M. Salavati-Niasari, R. Amooaghaeia, Nanostructures, 1 (2012) 95-103.
29. E. Nishimoto, S. Yamashita, A.G. Szabo, T. Imoto, Biochemistry, 37 (1998) 5599-5607.
30. T. Imoto, L.S. Forster, J.A. Rupley, F. Tanaka, Proceedings of the National Academy of Sciences, 69 (1972) 1151.
31. J. R. Lakowicz, G. Weber, Biochemistry, 12 (1973) 4161-4170.
32. J. R. Lakowicz, B.R. Masters, Journal of Biomedical Optics, 13 (2008) 029901.
33. P. L. Privalov, Stability of proteins, Academic Press, 1979.
34. B. Shareghi, M. Arabi, Iranian Journal of Science & Technology, 32 (2008).
35. S. M. Daly, T.M. Przybycien and R.D. Tilton, Langmuir, 19 (2003) 3848-3857.
36. A. Sethuraman, G. Vedantham, T. Imoto, T. Przybycien, G. Belfort, Proteins: Structure, Function, and Bioinformatics, 56 (2004) 669-678.
37. M.A. Williams, J.M. Thornton, J.M. Goodfellow, Protein engineering, 10 (1997) 895.
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