

Spectroscopic Studies on the Interaction of Nano-TiO₂ with Lysozyme

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Abstract

In the present study, the interaction between nano-TiO₂ and lysozyme was investigated by the method of UV-Vis detection and fluorescence spectroscopic techniques. The thermal denaturation of lysozyme has been investigated in the presence *and* absence of nano-TiO₂ over the temperature range (293-373) K in different buffer and pH, using temperature scanning spectroscopy. The presence of nano-TiO₂ caused the destabilization of lysozyme resulting in a decrease in the temperature of unfolding with an increase in nano-TiO₂ concentration.

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1. Introduction

have unique physicochemical properties such as tiny size, large surface area, surface reactivity, charge, shape and media interactions. As a result, the properties of NPs differ substantially from their respective bulk materials of the same composition. However, certain novel properties of NPs could lead to adverse biological effects, with the potential to

create toxicity [1]. More and more nanomaterials are being used in industrial production and daily life because of their unique characteristics [2]. For example, some of them are added in sunscreens to absorb UV light and in toothpaste and paints to give them a white color which lasts for years. Some are used in the electronics industry. Besides, there are also many applications in medicine, sporting equipment, cosmetics, coatings, fuel cells, and other

industries[3]. Nowadays, some nanomaterials are being tried as drug delivery products, and they can help to diagnose diseases as quantum dots that can allow the visualization of cancer cells within the body. With an increasing use of nanomaterials, their human exposure is inevitable. It is important to consider the hazards of these materials. The particle size of nanomaterial is smaller than cells and cellular organelles. It may penetrate these basic blocks, produce physical damage, or induce harmful inflammatory responses. The oxidative stress of nanoparticles can damage lipids, carbohydrates, proteins, and DNA in which lipid peroxidation is considered most dangerous, leading to alterations in cell membrane properties[4, 5]. Moreover, TiO₂ nanomaterial has attracted much more interest in medical fields due to its photo reactivity. It has already been reported that TiO₂ photo catalyst could ips to many respiratory and cardiovascular diseases, such as pneumonia, lung cancer, arteriosclerosis, and myocardial infarction[9]. Recent studies have shown that nanomaterials can cause genotoxicity and cytotoxicity in cultured human cells. There is evidence that nano-TiO₂ can cause inflammation, fibrosis, pulmonary damage, and even DNA damage [10, 11]. The goblet cell hyperplasia and Muc 5ac expression were induced in rats after a single intratracheal instillation of nano-TiO₂. Nano-TiO₂ might enter the human stratum corneum and interact with the immune system. Oxidative DNA damage and increases in the level of cellular nitric oxide were also observed in human bronchial epithelial cells after exposure to nano-TiO₂ [9, 12]. 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 [6]. In recent years, semiconductor titanium dioxide (TiO₂) as photo catalyst has been applied to kill or suppress tumor cells, because TiO₂ particles can react with water molecules adsorbed on the surface of tumor cells to produce hydroxyl (OH) radicals with strong oxidation ability under ultraviolet (UV) light irradiation. Otherwise, TiO₂ particles can be easily engulfed by macrophage cells around blood vessel and fleetly eliminated from normal tissue [7]. The interactions of nanomaterials (often nano-TiO₂ or nano-SiO₂) with functional biomolecules such as proteins, enzymes and DNA are regarded as preconditions for their cytotoxicity and organ toxicity [8]. A large amount of epidemiological and experimental studies indicate that ultrafine particles have close relationship as a model enzyme in studies on enzymology, molecular biology, genetics, protein chemistry, and immunology. The structure of lysozyme which contains a single chain of 129 amino acid residues was characterized by X-ray analysis. Under normal physiological conditions, lysozyme is folded into a compact, globular structure with a long cleft on the surface. It has five α helices, a three stranded antiparallel β sheet, and a large amount of random coil and β turns. Also its structure is stabilized by four disulfide bonds with most of the cysteines 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 [13]. It has many pharmacological functions, such as antiseptic, antiphlogistic, repercussive, antiviral, and antineoplastic actions, as it is effective against gram-positive bacteria cells by hydrolyzing their polysaccharide components. It also improves the human blood circulation and enhances

the human immunity [14]. It is highly expressed in hematopoietic cells where lysozyme is found in granulocytes, monocytes, and macrophages as well as in the bone marrow precursors[15]. Another important function of lysozyme is its ability to carry drugs. Therefore, studies on the interaction between colloidal TiO_2 -NPs and lysozyme indicated that water and solvent molecules not only influence the 3D structure of proteins in solution, but also play a crucial role in their adsorption onto nanomaterial surfaces [16-18]. In this work, nano- TiO_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- TiO_2 . Nano- TiO_2 (size 21 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 from Sigma) 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- TiO_2 -lysozyme interaction

All studies were carried out in quartz cells containing 0.1mM lysozyme and different concentration nano- TiO_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- TiO_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 0.1mM lysozyme and different concentration nano- TiO_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 0.1mM lysozyme and different concentration nano-TiO₂ 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-TiO₂ were recorded.

3. Results and discussion

3.1. Determination of particle size of nano-TiO₂

Fig.1 shows the SEM picture of nano-TiO₂. From SEM measurement we observed that nano-TiO₂ particles have spherical in shape and the average particle diameter is about 21 nm.

3.2. Interaction of lysozyme with nano-TiO₂ particles

The light absorption of the lysozyme-nano-TiO₂ suspension is shown in Fig. 2(a, b) the absorbance (A) of the liquid increases with an increase in the nano-TiO₂ concentration from 0 to 13 mg/L without change in the wavelength of peak at 280 nm.

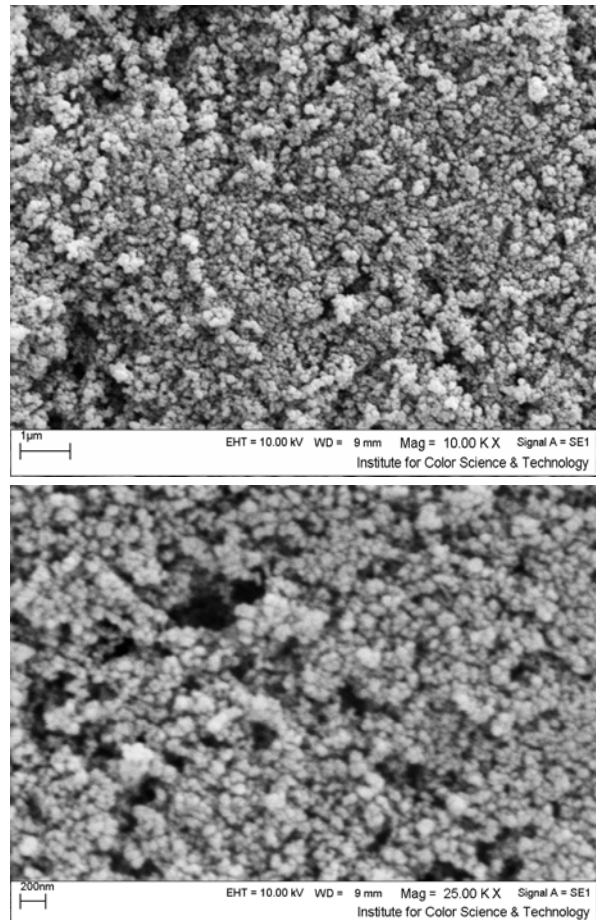


Fig. 1. SEM image of nano-TiO₂

This observation is due to the formation of ground state complex (lysozyme.....TiO₂). it is likely that lysozyme.....TiO₂ complex has higher extinction co-efficient than the unabsorbed state but has absorption maximum at the same position i.e. 280 nm [6]. The results from the absorption study indicated that there is an interaction between TiO₂ NPs and lysozyme via ground state complex formation. Obviously, nano-TiO₂ particle surfaces carry lots of negative charges because its isoelectric point is at pH 6 [19]. 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-TiO₂ particle suspension. When the distance between lysozyme and TiO₂ is short enough, the hydrogen bond will form between TiO₂ and the polar side chains of amino acid residues. Thus, the combination of noncovalent electrostatic interactions and hydrogen bonds led to the firm binding of lysozyme on TiO₂ particle. In the presence of colloidal TiO₂ NPs the absorbance of lysozyme is increased markedly, without change in the wavelength of peak at 280 nm.

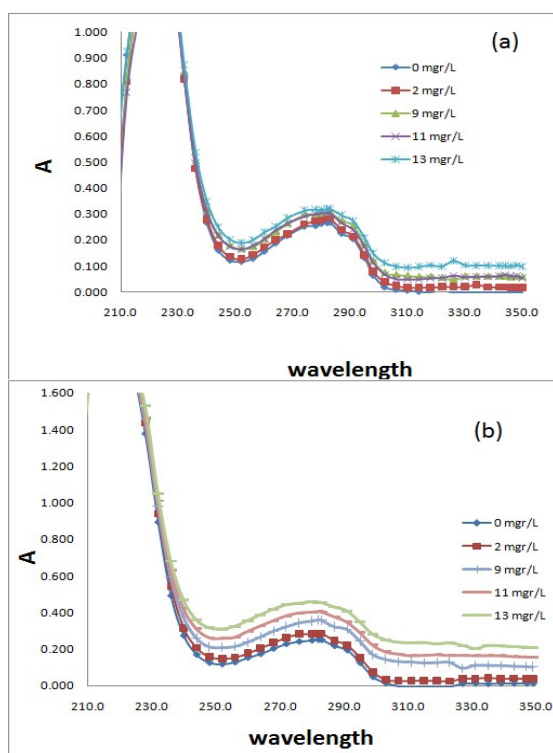


Fig. 2. Absorption spectrum of lysozyme in the presence of colloidal TiO₂ in the concentration range of 0-13 mg/L at (a) pH 2.5, (b) pH 7.25.

This observation is due to the formation of ground state complex (lysozyme ...TiO₂). It is likely that the lysozyme ...TiO₂ 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 TiO₂ NPs as supported by the similar observation made earlier [6, 7]. The results from the absorption study indicated that there is an interaction between colloidal TiO₂ NPs and lysozyme existing via ground state complex formation. After lysozyme is added, the TiO₂-lysozyme particles become colloidal and conjointly global from the independent regular globes.

3.2. Fluorescence analysis of the lysozyme-nano-TiO₂ interaction

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 [21]. Fig.3 (a, b, c) shows the effect of increasing concentration of colloidal TiO₂ NPs on the fluorescence emission spectrum of lysozyme, which resulted in the quenching of its fluorescence emission. This implies that there is a condensation type of interaction between TiO₂ and tryptophan through their carboxyl group [6]. In order to further investigate the interaction of lysozyme with nano-TiO₂ particles, the fluorescence spectra of the nano-TiO₂-lysozyme suspensions are determined Fig. 3 (a, b, c). The fluorescence intensity of the liquid decreases sharply with increasing nano-TiO₂. 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 TiO₂ particles via the N-H...O hydrogen bonds owing to the twist and deformation of lysozyme on TiO₂ particles. When nano-TiO₂ is more than 83 mg/l, a red shift of the

emission peak appears Fig.3 (a, b, c), but this phenomenon is not observed in lower concentrations. It is attributed to the fact that TiO₂ particles have a stronger light absorption or scattering at a short wavelength than that at a long wavelength. Moreover, the presence of nano-TiO₂ particles has not affected the conformation of lysozyme free in the suspension.

3.3. Thermal stability of lysozyme

Thermal denaturation curves for lysozyme in the presence and absence of nano-TiO₂ are shown in Fig. 4. The fraction of the denatured protein, F_d, was calculated using the relation[22, 23]:

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

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-TiO₂ decreases the stability of lysozyme to thermal denaturation. The thermal denaturation of small globular proteins closely approach a two-state, mechanism [22, 23]. By assuming a two-state mechanism, the difference in free energy between the folded and unfolded conformation, ΔG_D^o, can then be calculated using:

$$\begin{aligned} \Delta G_D^o &= -RT \ln \left[\frac{F_d}{(1-F_d)} \right] \\ &= -RT \ln \left[\frac{Y_{obs} - Y_N}{Y_D - Y_{obs}} \right] \end{aligned}$$

Where R is the gas constant and T is the absolute temperature. The free energy of denaturation, ΔG_D^o, as a function of temperature for lysozyme in the presence and absence of nano-TiO₂ is shown in Fig. 5. These results can be used to determine T_m at which ΔG_D^o = 0. It is clear that on increasing the

concentration of nano-TiO₂ the curves shift to lower temperatures (Fig. 5).

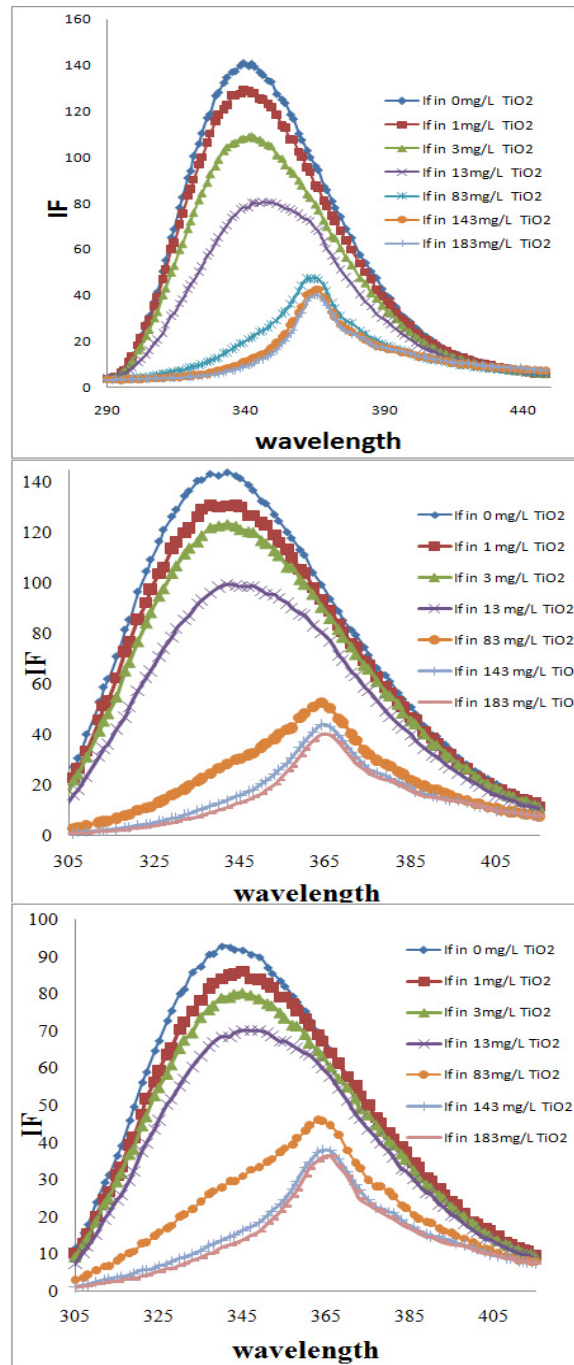


Fig. 3. Fluorescence quenching of lysozyme, $\lambda_{\text{exi}} = 280$ nm in the presence of various concentration of TiO₂ at (a) pH = 2.5, (b) pH = 7.25, (c) pH = 11

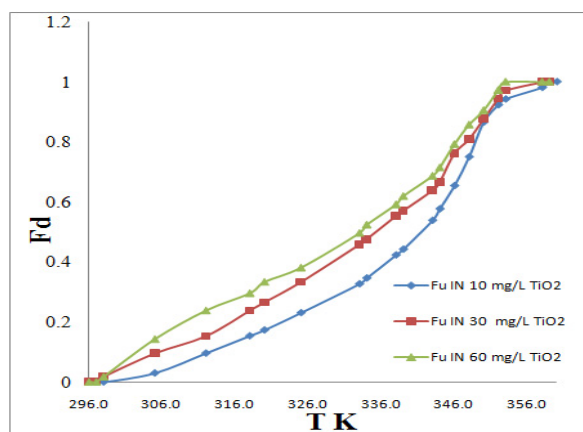


Fig. 4. Thermal denaturation curve of lysozyme in the presence nano-TiO₂ at pH= 7.25.

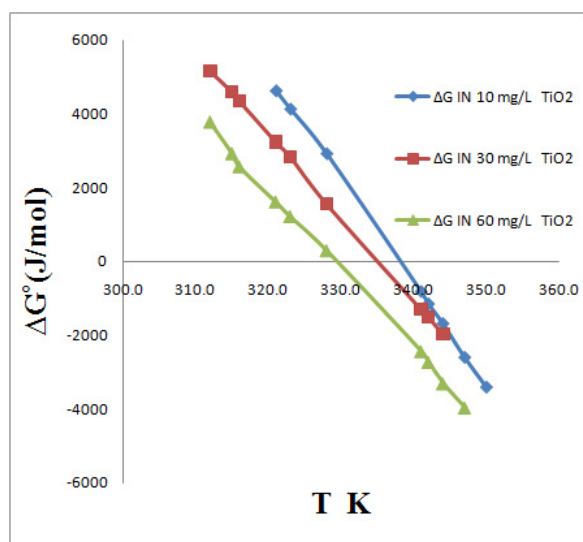


Fig. 5. ΔG_D° curves for lysozyme as a function of temperature at pH = 7.25.

Lysozyme T_m at various concentrations of TiO₂ is tabulated in Table 1. As indicated in Table 1, increasing the concentration of TiO₂ decreases, and T_m of lysozyme. 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.

Table 1. T_m values at various nano-TiO₂ concentrations.

nano-TiO ₂ (mg/L)	pH=2/ 5	pH=4/ 5	pH=7/2 T_m °C
10	45	65	66
30	44	60	62
60	41	48	57

Non-covalent binding is often weak and non-specific but a combination of many non-covalent bonds may alter the conformation and function of the protein. Inevitably, the function of protein would be affected if bond with any chemical substance. TiO₂ has strong effect of binding and -OH can be generated on the surface of particles in aqueous condition [24]. The binding effect and -OH collectively might change the polarity of lysozyme and the structure of active center, leading to the decrease of lysozyme activity.

Lysozyme bound to the TiO₂ surface and then caused the self aggregation of TiO₂ particles. Similar to the small molecule-protein interaction, the interaction of lysozyme with nano-TiO₂ particle may be mainly due to the noncovalent combination of the electrostatic interaction with hydrogen bonds[2]. When the lysozyme is close to the TiO₂ surface, many hydrogen bonds will be formed between TiO₂ and the polar side groups of lysozyme in the N-H...O and O-H...O types. From a change of the particle shape from the regular TiO₂-only globe into the TiO₂-lysozyme colloid, some areas of lysozyme bridge between nano-TiO₂ particles to form bigger particles. Without doubt, the twist and deformation of the lysozyme spatial structure will occur under the traction of TiO₂ particles. The specific conformation of a protein with a particular function results from interactions among its amino acid residues. CD spectrometry is often used to characterize the secondary structure of a protein with β -pleated sheet, β -turn, α -helix, and random coil. From CD change

of the lysozyme in the presence of nano-TiO₂ particles, the fractions of α -helix and β -turn decrease obviously with increasing TiO₂ particles but the β -pleated sheet increases. A possible reason is that lots of polar side groups of lysozyme bind onto TiO₂ particles to cause the twist and deformation of the lysozyme chain so that the inner hydrogen bonds of the helix are destroyed. β -Pleated sheet can induce the formation of amyloid fibrils, a process which plays a major role in pathology [25]. The size of NPs is one of the key parameters that influence the interaction between protein and NPs. The great loss of α helicity was observed for the lysozyme adsorbed onto larger NPs. These results indicate that the size of the NP, perhaps because of the contributions of surface curvature, influences adsorbed protein structure and function [26].

4. Conclusion

The interaction between nano-TiO₂ and lysozyme has been studied by UV-Visible and fluorescence spectroscopic measurement. The results indicated clearly that nano-TiO₂ 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-TiO₂ on lysozyme stability has also been studied. Increasing the concentration of nano-TiO₂ decreases the stability of lysozyme to thermal denaturation.

References

- [1] H. Wang, R.L. Wick, B. Xing, *Environmental Pollution* 157 (2009) 1171-1177.
- [2] Z. Xu, X.W. Liu, Y.S. Ma, H.W. Gao, *Environmental Science and Pollution Research* 17 (2009) 798-806.
- [3] J. Wang, Y. Liu, F. Jiao, F. Lao, W. Li, Y. Gu, Y. Li, C. Ge, G. Zhou, B. Li, *Toxicology* 254 (2008) 82-90.
- [4] G. Bystrzejewska-Piotrowska, J. Golimowski, P.L. Urban, *Waste Management* 29 (2009) 2587-2595.
- [5] M. Heinlaan, A. Ivask, I. Blinova, H.C. Dubourguier, A. Kahru, *Chemosphere* 71 (2008) 1308-1316.
- [6] A. Kathiravan, M. Asha Jhonsi, R. Renganathan, *Journal of Luminescence* (2011).
- [7] J. Wang, J. Wu, Z.H. Zhang, X.D. Zhang, L. Wang, L. Xu, B.D. Guo, H. Li, J. Tong, *Chinese Chemical Letters* 16 (2005) 1105.
- [8] Z. Xu, S.L. Wang, H.W. Gao, *Journal of hazardous materials* 180 (2010) 375-383.
- [9] M.T. Zhu, W.Y. Feng, B. Wang, T.C. Wang, Y.Q. Gu, M. Wang, Y. Wang, H. Ouyang, Y.L. Zhao, Z.F. Chai, *Toxicology* 247 (2008) 102-111.
- [10] R. Dunford, A. Salinaro, L. Cai, N. Serpone, S. Horikoshi, H. Hidaka, J. Knowland, *FEBS letters* 418 (1997) 87-90.
- [11] K. Donaldson, V. Stone, A. Seaton, W. MacNee, *Environmental health perspectives* 109 (2001) 523.
- [12] J.J. Wang, B.J.S. Sanderson, H. Wang, *Mutation Research/Genetic Toxicology and Environmental Mutagenesis* 628 (2007) 99-106.
- [13] X. Wu, G. Narsimhan, *Biochimica et Biophysica Acta (BBA)-Proteins & Proteomics* 1784 (2008) 1694-1701.
- [14] F. F. Chen, Y.N. Tang, S.L. Wang, H.W. Gao, *Amino acids* 36 (2009) 399-407.
- [15] G. Merlini, V. Bellotti, *Clinica chimica acta* 357 (2005) 168-172.

- [16] J. Vörös, *Biophysical journal* 87 (2004) 553-561.
- [17] T. Kopac, K. Bozgeyik, J. Yener, *Colloids and Surfaces A: Physicochemical and Engineering Aspects* 322 (2008) 19-28.
- [18] C. Yongli, Z. Xiufang, G. Yandao, Z. Nanming, Z. Tingying, S. Xinqi, *Journal of colloid and interface science* 214 (1999) 38-45.
- [19] F. Y. Oliva, L.B. Avalle, O.R. Camara, C.P. De Pauli, *Journal of colloid and interface science* 261 (2003) 299-311.
- [20] H. S. Liu, Y.C. Wang, W.Y. Chen, *Colloids and Surfaces B: Biointerfaces* 5 (1995) 25-34.
- [21] J. R. Lakowicz, G. Weber, *Biochemistry* 12 (1973) 4171-4179.
- [22] P. L. Privalov, *Stability of proteins*, Academic Press, 1979.
- [23] B. Shareghi, M. Arabi, *THERMAL Iranian Journal of Science & Technology* 32 (2008).
- [24] R. R. Zhu, S.L. Wang, R. Zhang, X.Y. Sun, S.D. Yao, *Chinese Journal of Chemistry* 25 (2007) 958-961.
- [25] Y. Goto, H. Yagi, K. Yamaguchi, E. Chatani, T. Ban, *Current Pharmaceutical Design* 14 (2008) 3205-3218.
- [26] Z. Wu, B. Zhang, B. Yan, *International journal of molecular sciences* 10 (2009) 4198-4209.