LETTER TO THE EDITOR

Spectroscopic analysis of the interaction between NiO nanoparticles and bovine trypsin

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Today, lots of nanomaterials are produced for commercial purposes. The production and utilization of nanomaterials unavoidably lead to their accumulation in the environment, thereby posing a threat to human and environment (Simonet & Valcárcel, 2009). These compounds go into the human body, soil, or water. There are various types of engineered nanoparticles (ENPs), including the fluorescence (grouping Buckminster fullerenes), the metal ENPs (elemental Au, Fe, etc.), metal oxides (CuO, NiO, Fe2O3, etc.), quantum dots coated with a polymer (CdSe), complex compounds (Co–Zn–Fe oxide), and organic polymers (polystyrene, etc.) (Raghupathi, Koodali, & Manna, 2011). ENPs due to their size that are in the range of 1–100 nm and their very large surface area to volume ratio lead to several electrical, chemical, mechanical, optical, and magnetic properties. The small nanoparticles are adsorbed into the surrounding atoms, and caused to change their behavior and properties (Simonet & Valcárcel, 2009).

Nickel (II) oxide nanoparticles (NiO-NPs) have been extensively studied because of their attributes. NiO-NPs have favorable physiochemical features and are widely used as a photovoltaic material in photo catalysis, absorbents, batteries, gas sensors, fuel cells, electro chromic films, and magnetic materials. Recent developments confirmed that NiO-NP is an interesting cathode material for alkaline batteries and also is an interesting anode material for lithium ion batteries because of its high thermal and chemical stability and low price. Another application of NiO-NPs is a potential gas sensors for H2 and CO and complex compounds and its antibacterial effect, disruption of membranes or membrane potential, oxidation of proteins, genotoxicity, interruption of energy transduction, formation of reactive oxygen species, and the release of toxic constituents (Raghupathi et al., 2011; Simonet & Valcárcel, 2009). The effects of TiO\textsubscript{2}-NPs and Au-NPs on trypsin have already been investigated (Hinterwirth, Lindner, & Lämmerhofer, 2012). In this study, the effect of NiO-NPs on the activity and conformation of trypsin was evaluated for the first time by enzyme kinetic assay and spectroscopic methods including thermal stability, UV–vis absorption spectroscopy, fluorescence spectroscopy, and circular dichroism (CD). This work could be helpful to have better understanding the effect of NiO-NPs on the function and conformation of trypsin. It

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could also provide the information about the effect of NiO-NPs on human body.

Materials and methods

Materials
The bovine pancreas trypsin (Sigma Aldrich Co.) was a crystalline powder. It was dissolved in Tris–HCl buffer (50 mM, pH 8) to form a .1 mg/ml solution. The used NiO-NPs were suspended in deionized water and were mixed by ultrasonic machining for 10 min to prevent the aggregation of nanoparticles. The stoke solution of BAEE (Nα-Benzoyl-L-arginine ethyl esters, 1 mM), as the trypsin substrate, was dissolved in a Tris–HCl buffer (50 mM, pH 8) on the same day. All solutions were stored at a temperature less than 4°C.

Methods

Thermal stability of trypsin
Denaturation curves of trypsin (.1 mg/ml) were made by Pharmacia 4000 UV–vis spectrophotometer in the temperature range of 293–373 K, at 280 nm, and pH 8.0. Temperature was regulated by an external thermostat and the heating rate was 1 K/min.

Enzymatic activity assay
The activity of trypsin (25 µg/ml) was investigated by a UV–vis spectrophotometer at 253 nm, pH 8.0, and the temperature of 37°C, in the absence and presence of different concentrations of NiO-NP (0, 1, 3, 5 and 7 µg/ml). The unit enzymatic activity (U) represented the hydrolysis of 1 µmol of BAEE per minute at 37°C, according to the following reaction:

\[
\text{N-R-benzoyl-L-arginine-ethyl ester} + \text{H}_2\text{O} \leftrightarrow \text{N-benzoyl-L-arginine} + \text{ethanol}
\]

Fluorescence spectroscopy
The fluorescence spectra were measured by a Shimadzu RF-5301 fluorescence spectrophotometer (Japan) equipped with a temperature controller and a 1.0 cm quartz cell. The intrinsic fluorescence of tryptophan residues in trypsin was obtained in the absence and presence of 1, 3, 5, and 7 µg/ml concentrations of NiO-NPs. The fluorescence spectra were recorded with the excitation wavelength at 278 nm and emission wavelengths from 300 to 450 nm. The widths of emission and excitation slits were set at 5.0 and 3.0, respectively. NiO-NP and trypsin solution was equilibrated at two different temperatures (298 and 308 K) for 10 min.

Circular dichroism
The spectra of CD were recorded using an AVIV 215 (USA) at 25°C and a quartz cell of .1 and 1 cm path length for the far UV and near UV regions, respectively. The concentration of trypsin was .8 and 1.6 mg/ml for the far UV and near UV regions, respectively. The suspension trypsin, Tris–HCl and NiO-NP were allowed to equilibrate for 30 min at room temperature. Each spectrum was performed twice and averaged. They were smoothed and converted to the mean residue ellipticity \([\Theta]_{Mr}\) (deg cm²/dmol) by the CDNN program, version 2.1.0.223, using a network trained with 33 complex spectra as the reference set.

Results and discussion

Determination of the particle size of NiO nano particles (NiO-NPs)
NiO nanoparticles were nearly spherical and gray-black with the particle size of 10–20 nm (Supplementary Figure 1).

Thermal stability of trypsin
Thermal denaturation studies of trypsin were recorded with .1 mg/ml concentration of the enzyme in Tris–HCl buffer (pH 8) and in the presence and absence of different concentrations of NiO-NPs. After normalizing the absorbance of native and denatured molecules of trypsin, the mid-point of transition \((T_m)\) of enzyme was obtained. In fact, denaturation data were analyzed by supposing the two-state mechanism between the folded and unfolded states. The fraction of the denatured protein, \(F_{\text{np}}\), was calculated using Equation (1) (Sudha, Chandrasekaran, Sameena, & Israel, 2015):
\[ Y_u = Y - Y_f / Y_u - Y_f \] (1)

where \( Y \) is the observed variable parameter at a given nanoparticles concentration, and \( Y_u \) and \( Y_f \) are, respectively, the absorbance of the unfolded and folded states of molecules under the conditions in which \( Y \) is determined. The changes in \( F_u \) of trypsin were plotted in the absence and presence of NiO-NPs in Figure 1. In this study, the reversible unfolding due to the heat of trypsin occurred with a \( T_m \) of 318 K. As shown, by increasing the concentration of NiO-NPs, the curves were shifted to lower temperature, showing the less stability of trypsin. By assuming a two-state mechanism, the difference in Gibbs free energy between the folded and unfolded conformation, \( \Delta G^\circ \), could be calculated by Equation (2) (Chandrasekaran, Sudha, Premnath, & Enoch, 2015; Sudha & Enoch, 2015; Sudha, Yousuf, Israel, Paulraj, & Dhanaraj, 2016; Sudha et al., 2015):

\[ \Delta G^\circ = -RT \ln[F_u / (1 - F_u)] = -RT \ln[Y - Y_f / Y_u - Y_f] \] (2)

where \( T \) is the absolute temperature and \( R \) is the constant of gas. The free energy of denaturation, \( \Delta G^\circ \), as a function of temperature for trypsin, in the absence and presence of NiO-NPs, is shown in Figure 1. These results could be used to calculate \( T_m \) at which \( \Delta G^\circ = 0 \) (\( T_m \) data are listed in Table 1). As can be seen, \( T_m \) and stability of trypsin were decreased with increasing the concentration of NiO-NPs. It could also be seen that NiO-NPs were effective on the transience of trypsin because it decreased \( T_m \) of trypsin.

| [NiO-NPs] (μg/ml) | \( T_m \) (K) |
|-------------------|------------|
| 0                 | 318.0      |
| 1                 | 317.1      |
| 3                 | 316.2      |
| 5                 | 315.0      |
| 7                 | 314.4      |

### Kinetic parameters of trypsin in the presence of NiO nanoparticles

Temperature influences the enzyme structure and conformational dynamics, but too high or low temperature can denature proteins thereby deactivating their function. In addition, co-solvents can stabilize or destabilize the structure of proteins hence, influence the enzymatic activity. To find whether NiO-NPs can alter the activity of enzyme, the enzyme activity experiments were performed. The effect of NiO-NPs on the trypsin’s activity was determined after the incubation of enzyme for 15 min at different concentrations of NiO-NPs, at pH 8.0, and the temperature of 37°C. The results are listed in Table 2. The activity of trypsin was increased with increasing the concentrations of NiO-NPs. The change in enzyme conformation led to increase the activity of trypsin. It was possible that the binding of NiO-NPs to trypsin led to be the more exposure of the catalytic activity center (Hu et al., 2013).

### Fluorescence quenching of trypsin with NiO-NPs

The fluorescence quenching measurements of proteins were used to study their structure and dynamics such as
binding constants, binding mechanism, and binding sites (Li et al., 2013). Fluorescence is the photon emission, due to the transferring of an electron from a higher energy orbital to a lower one (Vignesh et al., 2015). Molecular interactions such as molecular rearrangement, ground state reaction, and energy transfer can quench the quantum yield of fluorophores. Fluorescence of a protein is mainly due to three fluorophores, Trp, Tyr, and Phe, present in the protein structure. Fluorescence of Tyr is quenched if it is near a carbonyl group, an amino group, and a Trp residue, or when it is ionized and Phe has a low quantum yield. Therefore, Trp is the main fluorophore for the intrinsic fluorescence and trypsin has four Trp residues (Hu et al., 2013; Vignesh et al., 2015). In this study, trypsin solution of a fixed concentration of .1 mg/ml was excited at 287 nm and the emission spectra were recorded between 290 and 450 nm at two temperatures of 298 and 308 K. NiO-NP’s different concentrations were added to the trypsin solution, showing a decrease in fluorescence intensity with NiO-NP increasing concentration (Figure 2). As shown in Figure 2, the emission maximum of trypsin was found at 332 nm. These results indicated that the interaction between NiO-NPs and trypsin changed trypsin conformation and the microenvironment of Trp residues was shifted toward a more hydrophilic environment.

There are two quenching mechanisms that classified as dynamic or static quenching. Dynamic quenching arises from diffusive collisions between fluorophores and the quencher in the excited state. The value of bimolecular quenching constants is expected to be higher with increasing the temperature. Static quenching refers to the formation of the ground state complex between the fluorophores and the quencher. The value of static quenching constants is decreased with increasing the temperature since the stability of the complex is decreased with increasing the temperature. In order to distinguish the fluorescence quenching mechanism, the fluorescence quenching data were analyzed using the Stern–Volmer equation (Equation (3)) (Vignesh et al., 2015):

\[
\frac{F_0}{F} = 1 + K_{sv}[Q] = 1 + k_q \tau_0 [Q] \quad (3)
\]

where \( F_0 \) and \( F \) are the fluorescence intensities in the absence and presence of the quencher (NiO-NPs), respectively, \([Q]\) is the quencher concentration, \(K_{sv}\) is the Stern–Volmer quenching constant, \( \tau_0 \) is the average lifetime of molecule in the absence of quencher (10^{-8} s), and \( k_q \) is the bimolecular quenching rate constant (Vignesh et al., 2015). Figure 3(A) shows the Stern–Volmer plots at two temperatures. There was a good linear behavior within the relative concentration range, indicating single quenching mechanism, either dynamic

| [NiO-NPs] (μg/ml) | \( K_m \) (mM) | \( K_{cat} \times 10^6 \text{ s}^{-1} \) | \( V_{max} \) (s mM^{-1}) | \( K_{cat}/K_m \times 10^6 \text{ mM}^{-1} \text{s}^{-1} \) |
|-----------------|--------------|-----------------|-----------------|-----------------|
| 0               | .88          | 3.1             | 3.33            | 3.52            |
| 1               | .74          | 3.1             | 3.33            | 4.19            |
| 3               | .68          | 3.1             | 3.33            | 4.56            |
| 5               | .61          | 3.1             | 3.33            | 5.08            |
| 7               | .58          | 3.1             | 3.33            | 5.34            |

Figure 2. (A) Fluorescence emission of trypsin in the presence and absence of different concentrations of NiO-NPs (0, 1, 3, 5, 7, 9, 10, 11 μg/ml) at 298 K and (B) at 308 K in 50 mM Tris–HCl buffer.

Table 2. The kinetic values of trypsin at various concentrations of NiO-NPs.
The values of $K_{sv}$ were determined from the slope of these plots at different temperatures. The calculated $K_{sv}$ values are presented at two temperatures of 298 and 308 K in Table 3. The results showed that the $K_{sv}$ values were decreased with increasing the temperature. As a rule, the maximum collisional quenching constant of various quenchers with the biomacromolecules in dynamic quenching was $2.0 \times 10^{10} \text{M}^{-1}\text{s}^{-1}$ (Vignesh et al., 2015). The $k_q$ values obtained from $K_{sv}$ values in the complexes were greater than $2.0 \times 10^{10} \text{M}^{-1}\text{s}^{-1}$ (Table 3). Therefore, the fluorescence quenching process was initiated by the static quenching arising from the formation of the ground state complex between trypsin and NiO-NPs.

**Binding constant and the number of binding sites**

For the static quenching mechanism, when a small molecule binds to a set of equivalent sites on a macromolecule, the number of binding sites ($n$) and the binding constant ($K_a$) can be calculated by Equation (4) (Vignesh et al., 2015):

$$\log\left[\frac{(F_0 - F)}{F}\right] = \log K_a + n \log[Q]$$  \hspace{1cm} (4)

where $K_a$ is the binding constant and $n$ is the number of binding sites. According to Equation (4), a plot of $\log\left[\frac{(F_0 - F)}{F}\right]$ versus $\log[Q]$ could be used to determine the values of $K_a$ and $n$ (Figure 3(B)). The binding data ($K_a$ and $n$) are listed in Table 4. It was obvious that $K_a$ values were decreased with increasing the temperature, causing a reduction in the stability of the trypsin–NiO-NPs complex. The $n$ value was approximately equal to 1, indicating the existence of one binding site on trypsin for NiO-NPs.

**Thermodynamic parameters**

The dependence of the binding constant on temperature indicates that a thermodynamic process is responsible for the formation of the complex. The interaction forces between ligands and proteins include van der Waals forces and hydrogen bonds, as well as hydrophobic and electrostatic forces (Vignesh et al., 2015). The sign and magnitude of $\Delta H^\circ$ and $\Delta S^\circ$ are important for confirming the main force contributing to the binding reaction. The thermodynamic parameters were calculated from the van’t Hoff equation (Equation (5)) (Li et al., 2013):

$$\ln K_a = -\frac{\Delta H^\circ}{RT} + \frac{\Delta S^\circ}{R}$$  \hspace{1cm} (5)

and thermodynamic equation (6):

$$\Delta G^\circ = -RT \ln K_a$$  \hspace{1cm} (6)

where $K_a$ is the binding constant, $R$ is the gas constant ($8.314 \text{ J mol}^{-1}\text{K}^{-1}$), $T$ is the absolute temperature, and $\Delta G^\circ$, $\Delta H^\circ$, and $\Delta S^\circ$ are free energy change, enthalpy, and entropy changes of binding. The thermodynamic parameters are presented in Table 3. The negative values of $\Delta G^\circ$ showed the spontaneous nature of the interaction process. Hence, according to the previous studies, the sign and magnitude of thermodynamic parameters could be associated with various individual kinds of interactions that might take place during protein association.

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Table 3. Stern–Volmer quenching constants of the trypsin-NiO-NPs complexes at pH 8.0.

| $T$ (K) | $K_{sv}$ ($\times 10^8 \text{M}^{-1}$) | $K_q$ ($\times 10^{16} \text{M}^{-1}\text{s}^{-1}$) | $R^2$ |
|---------|---------------------------------|---------------------------------|-------|
| 298     | 4.21                            | 4.21                            | .9945 |
| 308     | 2.73                            | 2.73                            | .9911 |
process. If $\Delta H^\circ > 0$, $\Delta S^\circ > 0$, the main binding force is hydrophobic interaction; if $\Delta H^\circ < 0$, $\Delta S^\circ < 0$, hydrogen bond and van der Waals interaction are the main forces; if $\Delta H^\circ \approx 0$, $\Delta S^\circ > 0$, electrostatic interaction plays the main role in the binding process (Li et al., 2013; Ross & Subramanian, 1981). As shown in Table 4, the positive entropy and low negative enthalpy values indicated that the electrostatic interactions were the main binding force.

### Absorption spectroscopy

A convenient method to diagnose the changes in the structure of a protein is the use of UV–vis absorption measurement. It provides information about the ligand–protein complex formation. Therefore, this study investigated the effect of NiO-NP concentrations on the absorption spectrum of trypsin. There was an absorption peak in absorption curve at 260–300 nm that was related to the aromatic amino acids Trp, Tyr, and Phe (Supplementary Figure 2) (Liu & Liu, 2012). In the present study, with increasing the concentration of NiO-NPs, the intensity of the peak at 280 nm was increased without any shift. The results revealed that NiO-NPs had binding with trypsin and the interaction between NiO-NPs and trypsin caused to increase the hydrophobicity of the microenvironment of trypsin residues (Liu & Liu, 2012). This suggested that NiO-NPs could lead to alter the conformation of enzyme and the environment of Trp because of the formation of the ground state complex. There was a large affinity leading to the strong binding of NiO-NPs to trypsin (Li et al., 2013). Probably, trypsin–NiO-NPs complexes had a higher extinction coefficient than that in the unabsorbed state, but they had the absorption maximum at the same position, i.e. 280 nm. As known, the static quenching can induce the change in the absorption spectrum of fluorophores, but the dynamic quenching does not change the absorption spectrum and affects the excited state of fluorophores. So, this confirmed that the static quenching was the quenching mechanism in the binding process of trypsin with NiO-NPs.

### The secondary and tertiary structure of trypsin as observed by CD studies

The CD spectra provide information about the conformation changes in the secondary and tertiary structure of proteins (Hu et al., 2013). It has been shown that the far-UV CD spectra (200–260 nm) reflect peptide bond absorption and characterize the secondary structure of a protein. The near-UV CD spectra characterize the tertiary structure of a protein due to disulfide bonds and the asymmetric environment of amino acid residues (Hu et al., 2013).

### Table 4. Binding and thermodynamic parameters of trypsin-NiO-NPs interaction at pH 8.0 and two different temperatures.

| $T$ (K) | $K_a$ ($\times 10^7$ M$^{-1}$) | $n$ | $R^2$ | $\Delta H^\circ$ (kJ mol$^{-1}$) | $\Delta G^\circ$ (kJ mol$^{-1}$) | $\Delta S^\circ$ (J mol$^{-1}$ K$^{-1}$) |
|--------|-------------------------------|-----|-------|-------------------------------|-------------------------------|---------------------------------|
| 298    | 3.80                          | .95 | .9993 | $-21.51$                      | $-43.23$                      | 72.91                           |
| 308    | 2.06                          | 1.00| .9985 | $-21.51$                      | $-43.12$                      | 70.19                           |

Figure 4. (A) Far-UV CD spectra and (B) near-UV CD spectra of trypsin in the presence and absence of NiO-NPs (dotted line: 3 $\mu$g/ml and dash line: 7 $\mu$g/ml) in 50 mM Tris–HCl buffer and at pH 8. The Y-axis is the mean-residue ellipticity with the unit of degree cm$^2$/mol.
In order to determine the changes in the secondary structure and confirm the change in the tertiary structure of protein due to the binding of NiO-NPs, the CD spectra of trypsin–NiO nanoparticle complexes were recorded in the presence and absence of NiO-NPs concentrations (Figure 4). There was a global minimum around 208 nm in the far-CD spectrum of the native structure of trypsin, as referred to the α-helix conformation (208 nm) in the crystalline structure of trypsin (Figure 4). CDNN software was used for the analysis and determination of the results of the secondary structure of trypsin, as listed in Table 5. As shown in Table 5, with increasing the NiO-NPs concentration, the contents of secondary structures were changed. The content of α-helix was increased and β-sheet was decreased while the ration of random coil remained invariable. According to these findings, it could be suggested that NiO-NPs might cause little changes in the trypsin secondary structure, especially α-helix formation. Figure 4 shows the near-CD spectra of trypsin. When NiO-NPs were added into the solution, the intensity of trypsin spectra was decreased, it’s because of the intramolecular vibrations that led to the loss of asymmetry in the environment of amino acid residues (Hu et al., 2013). If aromatic residues became more far from each other, the band intensity would be decreased. These results confirmed that the tertiary structure of trypsin was changed with NiO-NPs, which was consistent with the results of absorption and fluorescence spectroscopy.

Conclusions

In this paper, the interaction between trypsin as a model protein and NiO-NPs was investigated to examine thermal stability and enzyme kinetic by UV–vis, fluorescence, and CD spectroscopic techniques. The interaction between trypsin and NiO-NPs was studied by steady-state spectroscopy and the fluorescence spectroscopy. The results showed the change of the Trp residues microenvironment in the enzyme molecule. The intrinsic fluorescence of trypsin was quenched through static quenching mechanism and the Stern–Volmer constants confirmed this model of quenching. The values of the binding constant, the number of binding sites, and the thermodynamic parameters were calculated. This indicated that the electrostatic interaction played a main role in stabilizing the NiO NPs–trypsin complex and the binding process was spontaneous. The thermodynamic parameters also showed that there was one binding site for NiO-NPs. The results of UV–vis absorption and near-CD spectra confirmed the complex formation between the NiO NPs and trypsin, the change in the tertiary structure and environment asymmetry of amino acid residues. The CD spectral studies also revealed that during the binding of NiO-NPs with trypsin, the conformation of the enzyme was changed. The skeletal structure of enzyme was changed and trypsin’s active sites were more exposed. As a result, the activity of trypsin was increased with NiO-NPs, although the thermal stability of enzyme was decreased with the rise of NiO-NP concentrations. To conclude, this study focused on the potential effect of a type of nanoparticles, NiO-NP, on the digestive enzyme of trypsin and the function and structure of it, that show the potential benefits for biochemistry and pharmacology.

Supplementary material

The supplementary material for this paper is available online at http://dx.doi.org/10.1080/07391102.2016.1185041.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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