Investigation on the toxic interaction of superparamagnetic iron oxide nanoparticles with catalase

Zehua Yu^{a,1}, Hongwei Liu^{a,b,1}, Xinxin Hu^a, Wei Song^a, Rutao Liu^{a,*}

^a Shandong Key Laboratory of Water Pollution Control and Resource Reuse, School of Environmental Science and Engineering, China–America CRC for Environment & Health, Shandong Province, Shandong University, 27# Shanda South Road, Jinan 250100, PR China

^b Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing 100085, PR China

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ABSTRACT

Superparamagnetic iron oxide nanoparticles (SPIONs) have been investigated for various applications in targeted drug delivery and magnetic resonance imaging. Given their clinical relevance, there is a need to understand these particles' potential cytotoxic effects and possible mechanisms of cytotoxicity. Using a variety of spectroscopic techniques, we investigated the interaction of SPIONs with catalase (CAT) in an aqueous environment. Catalase is an important enzyme that protects cells and tissues from oxidative damage by reactive oxygen species (ROS). Therefore, in this work, CAT served as a model protein for examining the physiological effects of SPIONs due to its function in eliminating H₂O₂. Synchronous fluorescence spectroscopy results showed that SPIONs have little effect on tryptophan residues in CAT. Data from circular dichroism (CD) and UV–vis spectroscopies showed that CAT α -helical content decreased from 32.4% to 29.1% in the presence of SPIONs. Moreover, a ca. 10% decrease in CAT activity was observed in the presence of SPIONs at a 20:1 particle:protein ratio. These results show that SPIONs can interact with proteins to alter both their structure and function. Further studies with CAT or other toxicologically relevant enzymes may be used for elucidating the mechanisms of SPION cytotoxicity.

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1. Introduction

Nanotoxicology, as a new area of research, is becoming a new research hotspot gradually. Recently, particular attention has been devoted to study in toxicology of nanoparticles [1]. Due to its unique physical and chemical properties, superparamagnetic iron oxide nanoparticles (SPIONs) has shown prospect in vivo applications such as magnetic resonance imaging, tissue repair, immunoassay, hyperthermia, drug delivery, cell separation, etc. [2–4].

As a targeted drug deliverer for clinical medicine research, the surface engineered SPIONs (i.e. with targeting ligand attached to their surfaces) can be guided to the specified site where the drug is released locally through an external magnetic field. Its magnetic properties depend strongly on the size of the magnetic particles and it offers guidance for applying proper diagnostics modalities to treat diseases in the field of medicine [5–7].

Despite an increasing interest in studying the cytotoxicity of SPIONs [8–11], little work has been carried out which focuses on investigating the interaction mechanism between the SPIONs and

protein at the molecular level. Here, we prepared Fe₃O₄ nanoparticles by co-precipitation of Fe³⁺ and Fe²⁺ in alkaline medium under nitrogen atmosphere [12–14]. And catalase (H₂O₂:H₂O₂ oxidoreductase, EC 1.11.1.6; CAT) is selected as our model functional protein owing to its importance in protecting the cells and tissues from oxidative damage by reactive oxygen species (ROS) [15–19].

In our research, multi-spectroscopic techniques including fluorescence, synchronous fluorescence, UV–vis absorption and circular dichroism were used to investigate the conformation changes for SPIONs binding to CAT. Besides, experiments of CAT inactivation and reactivation under simulative physiological condition were also studied. This study is a reference for further elaborating the adverse impact on CAT induced by SPIONs and is helpful for seeking a clinical control or detoxification methods.

2. Materials and methods

2.1. Reagents

Catalase (CAT, Sigma) was purchased from Shanghai Trading Co., Ltd. H₂O₂ (30%) was purchased from Beijing Sinopharm Chemical Reagent Co., Ltd. (Beijing, China). CAT was dissolved in

* Corresponding author. Tel./fax: +86 53188364868.

E-mail address: rutaoliu@sdu.edu.cn (R. Liu).

¹ These authors contributed equally to this work and share the first author.

ultrapure water to form a 1×10^{-5} mol/L solution for later use. $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$, 25% aqueous ammonia, citric acid, and nitrogen were used for the synthesis of SPIONs, whose stock solution (1.8 mg/L) was prepared by peptizing the synthetic particles in a 500 ml volumetric flask. A 0.2 mol/L mixture of phosphate buffer (mixture of $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ and $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, pH=7.4) was used to control the pH. H_2O_2 was dissolved in ultrapure water and diluted to 0.03% solution.

All the solutions were stored at 0–4 °C and ultrapure water (18.25 M Ω) was used throughout the experiments. And all other reagents were of analytical grade.

2.2. Probing the interaction of SPIONs with CAT

2.2.1. Fluorescence and synchronous fluorescence measurements

Fluorescence measurements were carried out with a 1 cm $\times$ 1 cm quartz cell, using excitation and emission slit widths of 5 nm. Fluorescence spectra were recorded at two different temperatures (293 K and 310 K) on a HITACHI FL-4600 fluorescence spectrophotometer (Hitachi, Japan). The systems were excited at 278 nm, and emission wavelength was recorded from 290 nm to 430 nm at the scanning speed of 1200 nm/min and the PMT (Photo-Multiplier Tube) voltage of 600 V.

The synchronous fluorescence spectra were measured at $\lambda_{\text{ex}}=250$ nm, $\Delta\lambda=15$ nm and $\Delta\lambda=60$ nm. All the test requirements were the same as more of fluorescence measurements.

2.2.2. UV-visible absorption measurements

UV-visible spectra were collected in the range of 190–350 nm. A gradually increased volume of SPIONs solutions and a fixed amount of CAT were added to volumetric flasks in sequence, and then diluted with ultrapure water to 10 mL. The equilibrated solution was poured into 1 cm quartz cells and measured on a SHIMADZU UV-2450 spectrophotometer (Shimadzu, Japan) using SPIONs solutions mixed with buffer as references.

2.2.3. Circular dichroism (CD) measurements

CD spectra measurements were performed over the range of 200–250 nm in 0.5 nm intervals on a J-810 Spectropolarimeter (Jasco, Japan). The scanning rate was adjusted to 200 nm/min. The spectra were measured in a temperature-controlled 1 cm path length cell.

2.2.4. Catalase activity measurements

We defined one unit of CAT activity as the amount of enzyme that decomposes 1 mmol H_2O_2 per minute. The enzymatic activity was measured spectrophotometrically by detecting the decrease in hydrogen peroxide absorption at 240 nm in a 20 mM buffer phosphate medium (pH 7.4) [20,21]. The reaction was initiated by the addition of CAT. The inhibition rate of CAT activity was calculated by the following equation:

$$\text{inhibition rate} = \frac{\Delta A_x}{\Delta A_0} \times 100\% \quad (1)$$

where ΔA_x and ΔA_0 are the reduction of the absorption value at 240 nm of the hydrogen peroxide solution in 30, 60 and 90 min after the addition of CAT with and without SPIONs, respectively.

3. Results and discussion

3.1. Research on quenching mechanism

Fluorescence spectroscopy has been the most popular method to investigate the interaction of proteins and ligands. This

Fig. 1. Fluorescence emission spectra of CAT with different concentration of SPIONs. Conditions: C (CAT) 1.0×10^{-6} mol/L; SPIONs (a)–(g) 0; 2.5; 5.0; 7.5; 10.0; 12.5; 15 ($\times 10^{-6}$ mol/L); PBS 2.0×10^{-2} mol/L (pH=7.40); $T=293$ K.

technology can provide us with all the information about the quenching mechanism, including binding constants and binding sites.

The inner filter effect (IFE) caused by absorption of the excitation and/or emission radiation is often neglected in many studies. For eliminating IFE, we imported the IFE correction equation (1) and obtained a plot (Fig. 1) of fluorescence quenching with increasing concentration of SPIONs corrected for IFE [22]:

$$F_{\text{cor}} = F_{\text{obs}} \times 10^{(A_{\text{ex}} + A_{\text{em}})/2} \quad (2)$$

where F_{cor} is the corrected fluorescence intensity, and F_{obs} is the observed fluorescence intensity; A_{ex} and A_{em} are the sum of the absorbance of protein and ligand at the excitation and emission wavelengths of the fluorescence measurements, respectively.

For a small molecule can quench the intrinsic fluorescence of proteins, the aromatic amino acid residues must be located in or near the binding site. The fluorescence intensity of CAT decreases regularly with an increasing concentration of SPIONs, which indicates the strong interaction between SPIONs and CAT [23].

Quenching mechanisms are classified as static and dynamic quenching, where dynamic quenching results from collision between the quencher and protein. A higher temperature results in larger diffusion coefficients, so the quenching constants will increase with raising temperature. In contrast, increased temperature is likely to result in decreased stability of the complexes and thus lower the value of the static quenching constants [24]. To verify this, the fluorescence quenching data were analyzed according to the Stern–Volmer equation [25]

$$F_0/F = 1 + K_{\text{SV}}[Q] = 1 + k_q\tau_0[Q] \quad (3)$$

where F_0 and F are the fluorescence intensities in the absence and presence of the quencher, respectively. K_{SV} is the Stern–Volmer quenching constant and $[Q]$ is the concentration of the quencher; k_q is the quenching rate constant of the biological macromolecule and the maximum dynamic quenching constant of various quenchers is 2.0×10^{10} mol/l/s [26]; τ_0 is the fluorescence lifetime without quencher. The Stern–Volmer plots for the quenching of CAT by SPIONs at two different temperatures are shown in Fig. 2.

K_{SV} and k_q at two different temperatures are listed in Table 1. The results indicated that the values of quenching constants decreased with increasing temperature and the values of k_q were much greater than 2.0×10^{10} mol/l/s. This analysis implied that the fluorescence quenching was attributed to static quenching.

For the static quenching, results from fluorescence measurements can be employed to estimate the binding constants of the

Fig. 2. Stern–Volmer plots for the quenching of CAT by SPIONs at 293 K and 310 K. Conditions: C (CAT) 1.0×10^{-6} mol/L; SPIONs (a)–(g) 0; 2.5; 5.0; 7.5; 10.0; 12.5; 15 ($\times 10^{-6}$ mol/L); PBS 2.0×10^{-2} mol/L (pH=7.40).

Table 1

The Stern–Volmer quenching constants for the interaction of SPIONs with CAT at 293 K and 310 K.

pH	T (K)	K_{SV} ($\times 10^5$ L/mol)	k_q ($\times 10^{12}$ L/mol s)	R^a	S.D. ^b
7.4	293	0.4419	4.419	0.9943	0.0244
7.4	310	0.4081	4.081	0.9915	0.0276

Table 2

Binding constants and relative thermodynamic parameters of the SPIONs–CAT system.

T (K)	K_A ($\times 10^4$ L/mol)	n	R^a
293	5.46	1.02	0.9901

^a R is the correlation coefficient for the K_A values.

CAT–SPIONs by a different form of the Stern–Volmer equation:

$$\log \left(\frac{F_0 - F}{F} \right) = \log K_A + n \log [Q] \quad (4)$$

where F_0 , F and $[Q]$ are the same as in Eq. (3), n is the number of average binding sites, and K_A is the binding constant. The values of n and K_A at 293 K are shown in Table 2. The number of binding sites n is approximately equal to 1, indicating that there is one binding site in CAT for SPIONs binding during their interaction. The value of K_A we obtained is on the order of 10^4 , indicating that SPIONs can interact with CAT easily.

3.2. Identifications on the conformation changes of CAT

3.2.1. UV–vis spectroscopy

UV–visible absorption spectra can be employed to explore the conformation change of CAT as shown in Fig. 3. The spectrogram contains two absorption peaks: one reflecting the framework conformation of CAT around 210 nm [27], and the other providing us with information of the aromatic amino acids around 280 nm [28]. It can be observed that the absorption peak at 210 nm decreases regularly with a conspicuously red-shift as the SPIONs concentration increases. These results indicated that SPIONs unfolded the skeleton structure of CAT. However the absorption peak at 280 nm was enhanced, which demonstrated that more aromatic acid residues were exposed to the aqueous environment.

Fig. 3. UV–visible absorption spectra of CAT in the presence of SPIONs. Conditions: C (CAT) 1.0×10^{-6} mol/L; SPIONs (a)–(f) 0; 2.5; 5.0; 7.5; 10.0; 12.5 ($\times 10^{-6}$ mol/L); PBS 2.0×10^{-2} mol/L (pH=7.40); $T=293$ K.

In other words, the hydrophobicity of the microenvironment of CAT was decreased [29].

3.2.2. Synchronous fluorescence spectroscopy

To further demonstrate the interaction between CAT and SPIONs, synchronous fluorescence spectroscopy which can analyze the change of the molecular microenvironment in the vicinity of a chromophore was employed. When the D -value ($\Delta\lambda$) between the excitation and emission wavelengths is set as 15 or 60 nm, synchronous fluorescence of CAT gives the characteristic information of tyrosine or tryptophan residues [30], respectively.

The synchronous fluorescence spectra of CAT with various amounts of SPIONs are shown in Fig. 4. The maximum emission position of tyrosine residue (Fig. 4A) remained invariant, while a slight redshift (Fig. 4B, from 280 nm to 284 nm) of maximum emission wavelength of tryptophan residue was observed with the increasing concentration of SPIONs. This phenomenon suggested that the microenvironment around the tryptophan residue of CAT was disturbed by SPIONs, and the tryptophan embedded in the nonpolar hydrophobic cavities were exposed to a more hydrophilic environment [31].

3.2.3. Circular dichroism spectroscopy

The CD measurement takes us nearer to understanding the possible influence of SPIONs binding on the secondary structure of CAT. It can be seen in Fig. 5 that the CD spectra of CAT exhibit two negative peaks in the UV region at 208 and 222 nm, which are characteristic of the α -helical structure of a protein.

The content of α -helix was analyzed with the Jasco secondary structure manager software using the reference CD data Yang.jwr. (Table 3). When the molar ratio of CAT to SPIONs is 1:25, the α -helix decreases from 32.4% to 29.1%. α -Helix is the important characteristic of secondary structure in CAT [32]. The decrease indicates that SPIONs bound to the amino acid residues of CAT loosen the protein skeleton, and change the conformation of protein. The results verified the conclusion obtained from absorption spectroscopy and synchronous fluorescence spectroscopy.

SPIONs in our experiment have a larger particle size compared with CAT. An α -helix is taken as the example to investigate conformation changes in Fig. 6.

The water molecules of microenvironment were supplanted by a small amount of SPIONs. Normally, it is known that the water molecules can access only the amide moieties on the surface of

Fig. 4. Synchronous fluorescence spectra of CAT in the presence of SPIONs. (A) $\Delta\lambda = 15$ nm, specific for Tyr. (B) $\Delta\lambda = 60$ nm, specific for Trp. Conditions: C (CAT) = 1.0×10^{-6} mol/L; SPIONs (a)–(h) 0; 2.5; 5.0; 7.5; 10.0; 12.5; 15; 17.5 ($\times 10^{-6}$ mol/L); PBS 2.0×10^{-2} mol/L (pH = 7.40); $T = 293$ K.

Fig. 5. CD Spectra of CAT in the presence of SPIONs. (a) CAT; (b) SPIONs and CAT. Conditions: C (CAT) 1.0×10^{-7} mol/L; SPIONs (a, b): 0; 2.5 ($\times 10^{-6}$ mol/L); $T = 293$ K.

CAT. However, SPIONs have a significant denaturing impact on CAT with increasing concentration of SPIONs. We find that the amide moieties embedded in the hydrophobic pocket of the protein can

Table 3

The influence of SPIONs on the α -helix content of CAT.

Lines in Fig.5	Molar ratio of CAT to nanoFe ₃ O ₄	α -Helix (%)
a	1:0	32.4%
b	1:25	29.1

Fig. 6. Schematic diagram of the environmental change procedure, taking an α -helix as an example.

Fig. 7. Effect of exposure time and concentration of SPIONs on the activity of CAT. Conditions: C (CAT) = 1.0×10^{-6} mol/L; PBS 2.0×10^{-2} mol/L (pH = 7.40); $T = 293$ K.

encounter water molecules that can be ascribed to the uncurled helical chain.

3.3. CAT enzyme activity measurement

The structural change of a protein is closely related to its biological function [33,34]. The above results indicate that CAT has undergone significant changes in structure and morphology after binding to SPIONs in vitro. The effects of different concentrations of SPIONs on the activity of CAT were investigated. As shown in Fig. 7, the CAT activity decreased with increasing SPIONs concentration ranging from 20 to 50 μ M. The relative CAT activities decreased to $97.6 \pm 1.6\%$, $81.0 \pm 0.6\%$, $79.3 \pm 1.3\%$ and $77.4 \pm 0.8\%$ after treatment with 20, 30, 40 and 50 μ M SPIONs for 30 min, respectively.

The activity of CAT influenced by SPIONs was also investigated at different times. At a molar ratio of 20:1 (SPIONs to CAT), the activities of CAT were reduced to $91.9 \pm 1.4\%$ for 60 min and $88.3 \pm 1.1\%$ for 90 min. Both SPIONs concentration and the reaction time of system have great effects on CAT activity.

4. Conclusions

This work not only illuminates the mechanism of CAT interacting with SPIONs but also provides an approach for the evaluation of protein conformation and function. Based on our spectroscopic results, the SPIONs can loosen the skeleton of CAT and increase the exposure of amide moieties in the hydrophobic pocket. Besides, the conformational changes of CAT influenced the microenvironment of the activity site which resulted in the reduced CAT activity. In order to present an objective evaluation on the toxicity of nanophase materials, experimental studies on nanoparticles with biomolecules in vitro and in vivo (i.e. protein–SPIONs interactions) are being carried out in our laboratory and we are very optimistic on the prospects for the future.

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