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Enzyme activity inhibition and secondary structure disruption of nano-TiO_2 on pepsin

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ABSTRACT

In this study, the binding and enzyme activity inhibitory effect of nano-TiO_2 on pepsin was explored compared with micro-TiO_2. Nano-TiO_2 was about 60 nm and micro-TiO_2 was about 200 nm, both round in shape. The activity of pepsin was depressed significantly by nano-TiO_2 comparing to micro-ones. The results of UV spectrometry, HPLC, SDS–PAGE and CD assay proved that micro-TiO_2 has only physical absorption effect on pepsin, but no impairment on primary sequences or secondary structure. However, nano-TiO_2 had coordination interaction with pepsin besides physical binding effect. The secondary structure of pepsin was unfolded with the treatment of nano-TiO_2 at pH 6.5 and pH 3.53, which might consequently affect the β-hairpin loop that protects the active center of pepsin, and then reduce the enzyme activity. Furthermore, the thermodynamic mechanisms of interaction between nano-TiO_2 and pepsin were explored by fluorescence spectrum and ITC analysis. According to the results of thermodynamic analysis, the K value was 3.64×10^6, stoichiometry ($N_{pepsin:nano\text{-}TiO_2}$) was 3.04×10^3, the total ΔH was −2277 cal/mol, ΔS was 22.7 cal/(K mol), therefore the nano-TiO_2–pepsin interaction is spontaneous. The depression of activity and the unfolding of secondary structure of pepsin were resulted from non-covalent reactions, including electrostatic force and hydrophobic binding. This work studied the different inhibitory effects and revealed mechanisms of the interaction between micro/nano-TiO_2 and pepsin, and provided a useful approach for evaluating the health risk of nano-materials on level of proteins.

1. Introduction

Nano-TiO_2 has been widely used in daily necessities, paintings, waste water treatment and various industrial productions owing to its chemical and physical properties, such as large surface area, UV absorption and photocatalysis (Zhou et al., 2005). According to marketing statistics, the production capacity of TiO_2 has reached about 5.3 million tons (http://www.cinkarna.si/en/245), and that amount of nano-TiO_2 has reached 2 million tons annually (Trouiller et al., 2009). Due to the application in the fields of cosmetic, antibacterial containers and even food additives (as white food coloring), humans cannot avoid potential exposures to nano-TiO_2 through skin and gastrointestinal tract. It's well known that matters in nano scale have many special and unknown properties as compared to matters in micro scale. However, as a matter of fact, there is no systemic and recognized safe evaluation for nanotechnology and nano-material till now (Philbert Martin et al., 2006; Schulte and Salamanca Buentello, 2007; Renn and Roco, 2006).

Nowadays, many studies have focused on the toxic effect of nano-TiO_2, mostly on the level of DNA, in vitro and in vivo studies, and it has been found that the biological effect of nano-TiO_2 was different from that of ultrafine TiO_2. TiO_2 ultrafine particles which were non-toxic showed some potential danger when they were made into nanoparticles (Amezaga-Madrid et al., 2003; Wang et al., 2005). The in vitro researches have confirmed that ·OH can be generated by nano-TiO_2 and causes oxidative damage to DNA (Shen et al., 2008). Nano-TiO_2 can be absorbed by alveolar macrophages (Mitsuyasu et al., 2002) and impair the phagocytosis of cells (Renwick et al., 2001), have cyto-/genotoxicity on human lymphoblastoid cells (Wang et al., 2007), and induce genotoxic lung effect (Rehn et al., 2003) and emphysema-like lung injury in mice (Chen et al., 2006). Although the bio-effect of nano-TiO_2 has been widely discussed on the level of DNA, cells and animals, there was little research concerning about the enzyme toxic effect of nano-TiO_2 on proteins. In the level of proteins, many researches focus on the positive effect of TiO_2. For example, it was reported that TiO_2 could be used in protein separation and purification (Sousa et al., 2007), and could improve the activity of hemoglobin under low dose of irradiation (Zhou et al., 2005). However, on the other

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hand, nano-sized TiO_2 could easily enter into the human body and even blood–brain barrier via skin, gastrointestinal tract and mucous membrane due to its ultrafine property (Liu et al., 2009). Potentially a variety of protein would contact with the nanoparticles and be absorbed to the surface of nanoparticles strongly due to the large amount of specific surface area and surface atoms of nanoparticles. It's still unclear what kind of reaction would take place between nanoparticles and proteins.

Following potential oral exposures to TiO_2 nanoparticles caused by the applications of antibacterial containers and food additives, the ingested particulates would transit through the gastrointestinal tract commencing with the esophagus and stomach – with a pH of 1–3. Pepsin is an important protease in stomach and was used as a model to evaluate the health risk of nano-TiO_2 in this work. The inhibitory effect of nano-TiO_2 on the activity of pepsin was determined via Ason method; the binding effect of the particles was detected via UV spectrometry; sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE), high pressure liquid chromatography (HPLC) and circular dichroism (CD) was used to discover whether the decrease of enzymatic activity was caused by the change of the integrity and polarity of pepsin or the change of secondary structure; at last, fluorescent analysis and isothermal titration calorimetry (ITC) assay was used here to determine the thermodynamic mechanism of the inhibitory effect of nano-TiO_2 on pepsin.

2. Materials and methods

2.1. Materials

Anatase nano-TiO_2 was kindly provided by Su-Ping Qian (the Academy of Sciences of China, Shanghai branch). Micro-TiO_2 (99.8%, anatase, CAS No: 1317–70–0), pepsin (from the porcine) and all other chemicals were purchased from Sigma–Aldrich. The samples of both TiO_2 were ultrasonically dispersed in distilled water or HCl solution prior for each experiment.

2.2. Characterization of micro and nano-TiO_2

Particles size of both TiO_2 was observed by TEM (JEOL Ltd., Japan) after ultra-sonicated for 5 min. Size distribution of TiO_2 was analyzed by Laser Particle Size Analyzer (Coulter LS-230, USA). Crystal structure was determined via XRD. The surface area was measured by the BET (Brunauer et al., 1938) method (JW-400 Autosorb, China).

2.3. UV scan

The same volumes of nano/micro-TiO_2 (10, 20, 40, 80, 120, 160 μg/ml) and 400 μg/ml pepsin were mixed together in 30 mM HCl and put in 37 °C water bath for 30 min, and 400 μg/ml pepsin in 30 mM HCl was set as the control. Then the mixtures were centrifuged at 12,000 rpm for 10 min twice to remove TiO_2. Amount of pepsin remaining in the supernatant separated from the reaction system was detected by a UV–visible spectrophotometer (VARIAN, Cary 50 probe).

2.4. Assay of pepsin activity

The enzyme activity was detected by Ason method. Pepsin (200 μg/ml) in 30 mM HCl was mixed with various concentrations of nano/micro-TiO_2 (0, 40, 80, 160 and 200 μg/ml) at 37 °C for 30 min. Then 4 ml of bovine hemoglobin solution was added in. After 10 min, 8 ml of 5% trichloroacetic acid was added to terminate the reaction. The mixture was put there for 5–10 min statically, and then centrifuged at 12,000 rpm for 10 min twice. The supernatant was measured via OD_{275}. The activity of pepsin can be calculated according to OD_{275}. The original activity of pepsin is 1200 U/g, and the activity of treated pepsin was calculated according to the formula:

$$\text{Activity}/_{\text{U/g}} = 1200 \times \frac{\text{OD}_{275}(\text{treated pepsin})}{\text{OD}_{275}(\text{original pepsin})}$$

2.5. Polyacrylamide gel electrophoresis

SDS–PAGE was performed according to the method of Laemmli (1970). Pepsin (10 mg/ml) was treated with 1 mg/ml nano/micro-TiO_2 in 30 mM HCl and put in 37 °C water bath for 30 min. Solutions were mixed at 1:1 (v/v) ratio with the SDS–PAGE sample buffer (0.125 M Tris–HCl, pH 6.8; 4% SDS; 20% glycerol; 10% β-mercaptoethanol) and boiled for 3 min. The samples (15 μL) were loaded on the gel made of 4% stacking and 12.5% separating gels and subjected to electrophoresis at a constant current of 15 mA with a Mini-Protean II Cell apparatus (Bio-Rad). After electrophoresis, the gels were stained with 0.1% Coomassie brilliant blue R-250 in 50% methanol and 7% acetic acid and destained with 7% acetic acid.

Native-PAGE was performed using 12.5% separating gels in a similar manner to the SDS–PAGE, except that the sample was not heated and SDS and reducing agent were not included.

2.6. CD assay

CD spectra were recorded over the range 190–250 nm on a spectropolarimeter. Buffer (1 ml, pH 3.53 and 6.5) was mixed with 1 mg/ml pepsin; 0, 50, 100 and 200 μg/ml nano/micro-TiO_2 were added into four flasks. Samples were allowed to equilibrate for 30 min at 37 °C and then centrifuged at 12,000 rpm for 10 min twice to remove TiO_2. CD spectra were measured in a 1 cm light path cell, and the mean residue ellipticity (MRE) was calculated. Simultaneously, the reagent blank without TiO_2 was measured for correcting the MRE of pepsin. The relative contents of secondary structure forms of pepsin, α-helix, β-pleated sheet, β-turn and random coil were calculated.

2.7. Gradient HPLC analysis

Pepsin (10 mg/ml) was mixed with 1 mg/ml nano/micro-TiO_2 at 1:1 (v/v) ratio. Samples were placed in 37 °C water bath for 30 min, and were centrifuged at 12000 rpm for 10 min twice to remove TiO_2. 5 mg/ml pepsin placed in 37 °C water bath for 30 min was set as the control. Solutions were assessed by HPLC (Agilent 1100 series, Germany) on a C18 column (25 cm × 4.6 mm, 5 μm). The chromatogram was monitored with UV detector at 275 nm. Gradient elution with a flow rate of 0.6 ml/min at 25 °C was as follows: from 65%TFA and 35% Acetonitrile to 35%TFA and 65% Acetonitrile in 20 min.

2.8. Fluorescent analysis

Fluorescent measurements were performed on a HITACHI F-4500 FL fluorescence spectrophotometer connected to a water bath. Protein fluorescence was excited at 295 nm, and the emission was recorded from 200 to 600 nm, and the temperature was set at 27 and 37 °C. The slit widths on both the excitation and emission were set at 10 nm, and the scan speed was 1200 nm/min. For inhibition study, 2 mg/ml pepsin was dissolved in 30 mM HCl. Sample preparation was performed by the addition of various concentrations of nano-TiO_2 to pepsin solutions at 1:1 ratio (v/v). HCl solutions were used as blank solution for all samples.