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Sorption of Bovine Serum Albumin on Nano and Bulk Oxide Particles

Lei Song
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SORPTION OF BOVINE SERUM ALBUMIN ON NANO AND BULK OXIDE PARTICLES

A Thesis Presented
by
LEI SONG

Submitted to the Graduate School of the University of Massachusetts Amherst in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

May 2009

Department of Plant, Soil and Insect Science
SORPTION OF BOVINE SERUM ALBUMIN ON NANO AND BULK-OXIDES

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by

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My special thanks go to my parents, Yaoze Song and Fengying Shi, for their trust, encouragement, and love at all times. This work would not have been possible without them, and I am appreciative for the many sacrifices that they have made along the way.
ABSTRACT

SORPTION OF BOVINE SERUM ALBUMIN ON NANO AND BULK-OXIDES

FEBRUARY, 2010

LEI SONG, B.A., OCEAN UNIVERSITY OF CHINA
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Directed by: Professor Baoshan Xing

Manufactured oxide nanoparticles (NPs) have large production and widespread applications, which will inevitably enter the environment. NPs can interact with proteins in living beings due to the fact that NPs can transport into blood or across cell membranes into cells. Conformational change of protein molecules after sorption on oxide NPs has been reported. Therefore, it is important to understand the adsorption mechanism of protein onto oxide NPs surfaces. Although few works have reported protein adsorption behaviors, a general systematic comparison of the effects of particle size and surface groups on protein adsorption by widely studied NPs still needs to be made. Moreover, the relationship between adsorption maxima, which are related to protein conformational change and particle toxicity, and protein conformational change has not yet been studied. Therefore, in this work, the adsorption behavior of bovine serum albumin (BSA) protein on three types of nano oxide particles (viz., TiO₂, SiO₂, and Al₂O₃) was investigated in order to explore their interaction mechanisms, compared with that on regular bulk particles (BPs). The BSA adsorption maxima on oxide particles were regulated by the
surface area of oxide particles. BSA adsorption was primarily induced by electrostatic attraction and ligand exchange between BSA and oxide surfaces. Surface hydrophilicity, surface charge and aggregation of oxide particles also affected their adsorption of BSA. Calculations suggested that a multilayer of BSA covered $\alpha$-Al$_2$O$_3$, and single layer covered the other oxide particle surfaces. Primary structures of BSA molecules were adsorbed and changed on surfaces of oxide particles.
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CHAPTER 1
GENERAL INTRODUCTION

1.1 Overview

Inorganic nano-oxides, such as TiO$_2$, SiO$_2$, and Al$_2$O$_3$, have extensive applications in pigments, sunscreens, food colorants, wastewater treatment reactors, semiconductors, electrical insulators, and biomedical areas [1, 2]. With their widespread applications, serious concerns have been raised over their health risks once they are released into the environment. Potential toxicological concern comes from the fact that NPs can be transported into blood or across cell membranes into cells [3, 4]. Proteins are widely found in blood and cells of all living organisms with various cellular functions, such as biochemical catalysts, energy sources, molecular messengers, structural components and transport vehicles [5]. Therefore, NPs may interact with proteins in blood or cytosplasmic proteins. Protein adsorption on a solid surface may induce changes in their structures and functions, even the entire protein molecules [6, 7]. Hence, protein adsorption could result in adhesion, proliferation, and differentiation of cells, as well as affecting foreign body response and inflammatory processes [8, 9]. Previous studies have reported the conformational changes of protein molecules after they interacted with oxide NPs. For example, adsorption of fibrinogen protein on TiO$_2$ NPs resulted in an increasing content of β-sheet structure and a decrease of α-helical structure content [10]; the secondary structure conformational changes of human carbonic anhydrase protein after adsorbed on SiO$_2$ NPs was also reported [11]; and BSA protein “side-on” adsorption mode on Al$_2$O$_3$ NPs surfaces, instead of “end-on” mode was proposed [12]. An understanding of the
adsorption mechanism of protein on NPs could help to explore the protein conformational changes on NPs and, furthermore, biological response of living beings to the NPs [13].

In this study, therefore, we investigated the adsorption behaviors of BSA molecules on both bulk- and nanoscaled colloidal TiO$_2$, SiO$_2$ and Al$_2$O$_3$ particle surfaces, in order to explore the effects of their particle sizes and surface groups on protein adsorption. Techniques, such as N$_2$ adsorption-desorption analysis, transmission electron microscopy (TEM), Zeta potential analysis, elemental analysis and Fourier transform infrared (FTIR) spectroscopy were employed to characterize the particle sizes and surface groups of selected oxide particles. Serum albumin protein (BSA) was examined in this study. BSA is the major soluble protein in plasma of the circulatory system and acts in transport and deposition of endogenous and exogenous substances in living beings [14, 15]. Owing to its physiological properties, purification, and stability in biochemical reactions, BSA is widely used as a model globular protein.

1.2 Nanotechnology and Nanomaterials

According to the nanotechnology white paper of Environmental Protection Agency (EPA) [16], nanotechnology is generally defined as: “research and technology development at the atomic, molecular, or macromolecular levels using a length scale of approximately one to one hundred nanometers in any dimension; the creation and use of structures, devices and systems that have novel properties and functions because of their small size; and the ability to control or manipulate matter on an atomic scale.”
Nanomaterials are the materials whose dimensions are reduced to the nano scale. Nanotechnology has the potential applications to build new products atom-by-atom and molecule-by-molecule by self-assembly or molecular assemblers [12]. Commercial applications of nanomaterials currently available or soon to appear in many sectors of world economy, include consumer and health care products, transportation, energy and agriculture [13]. To be specific, the consumer nanomaterials products include coatings, computers, clothing, cosmetics, sports equipment and medical devices (Table 1) [16].

<table>
<thead>
<tr>
<th>TABLE 1. Examples of Products that Use Nanotechnology and Nanomaterials.</th>
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<tr>
<td><strong>Health and Fitness</strong></td>
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<tr>
<td>Wound dressing</td>
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<td>Pregnancy test</td>
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<td>Toothpaste</td>
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<td>Golf club</td>
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<td>Tennis racket</td>
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<tr>
<td>Skis</td>
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<td>Antibacterial socks</td>
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<td>Waste and stain resistant pants</td>
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<tr>
<td>Cosmetics</td>
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<tr>
<td>Air filter</td>
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<tr>
<td>Sunscreen</td>
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<td><strong>Electronics and Computers</strong></td>
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<td>Computer displays</td>
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<td>Games</td>
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<tr>
<td>Computer hardware</td>
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<tr>
<td><strong>Home and Garden</strong></td>
</tr>
<tr>
<td>Paint</td>
</tr>
<tr>
<td>Antimicrobial pillows</td>
</tr>
<tr>
<td>Stain resistant cushions</td>
</tr>
<tr>
<td><strong>Food and Beverage</strong></td>
</tr>
<tr>
<td>Non-stick coatings for pans</td>
</tr>
<tr>
<td>Antimicrobial refrigerator</td>
</tr>
<tr>
<td>Canola oil</td>
</tr>
<tr>
<td><strong>Other</strong></td>
</tr>
<tr>
<td>Coatings</td>
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<tr>
<td>Lubricants</td>
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</table>

Source: Woodrow Wilson Center Consumer Products Inventory.

The EPA nanotechnology white paper [16] sorts nanomaterials into four types:
(1) Carbon-based materials. The compositions of these nanomaterials are mostly carbon with common forms of a hollow spheres, ellipsoids or tubes. Spherical and ellipsoidal nanomaterials are commonly called fullerenes, and cylindrical ones are named nanotubes.

(2) Metal-based materials. Quantum dots, nano-gold, nano-silver and metal oxides (e.g. titanium dioxide) are included in this type of nanomaterials.

(3) Dendrimers. These are nano sized polymers that are built from branched units, which are numerous and can be tailored to perform specific chemical functions.

(4) Composites. These nanoparticles can combine with other nanoparticles or with larger, bulk-type materials.

Nanotechnology also has an increasing potential for risk assessment, management, and prevention. Applications of nanomaterials can detect, prevent, and remove pollutants directly, as well as through indirectly designing cleaner industrial processes and creating environmentally responsible products [16]. However, the impacts of nano materials and nano products on human health and the environment are still unanswered. As the amount of nanomaterial products is increasing rapidly and, therefore, more prevalent in the environment, scientists are thus in urgent need of estimating the impacts of nanomaterials and balancing the advantages and drawbacks of nanotechnology to the environment and society.

1.3 Characteristics of Serum Albumin Protein

Proteins make up to half of cell dry mass [5], among which the albumin proteins are a major constituent of blood serum and play a significant role in biocompatibility [14].
Much of the polypeptide backbone of proteins can fold into stereotypical configurations (secondary structure), including \( \alpha \)-helices, \( \beta \)-sheets, \( \beta \)-barrel, reverse turns and omega loops [17]. The secondary structures of albumin proteins are \( \alpha \)-helices, \( \beta \)-sheets and turns [18, 19]. Polypeptides bound to the \( \alpha \)-carbons using highly favored rotational angles and tight packing of atoms in the core of the helix are called \( \alpha \)-helices; groups of hydrogen-bonded \( \beta \)-strands are called \( \beta \)-sheet [17]. Albumin is classed as a soft protein due to its relatively flexible structure, which can readily undergo conformational changes [14]. The three dimension image of serum albumin molecule is shown in Figure 1.

BSA protein, which is widely used as a model globular albumin protein, has high purification and stability in biochemical reactions [20]. BSA is composed of 604 peptide units with a molecular weight of 66462 g/mol [15, 21]. Generally, the BSA molecule consists of 55 – 65% \( \alpha \)-helices according to various methods of measurement, 21% \( \beta \)-sheet, and the rest are turns [18, 19, 22]. However, the components of BSA secondary structures vary slightly over a range of pH [23]: in the pH range 4.3 to 8.0, BSA keeps a triangular or heart-shaped structure [21] with the normal form, which comprises about 53\% \( \alpha \)-helix structure and the remainder being \( \beta \)-sheet and turns, but at pH below 4.3 the molecule unfolds into the fast form with 45\% \( \alpha \)-helix. At pH below 2.7, further unfolding to the expanded form was observed, with 35\% \( \alpha \)-helix [24]. It is considered that the reduction of \( \alpha \)-helix structure results from unfolding of the domains with consequent loss of intradomain helicity [21]. A gradual change with increasing pH occurs when pH is above 7, which is complete above pH 8 with the molecule adopting the basic form which has 47\% \( \alpha \)-helix [25].
FIGURE 1. Three dimension image of serum albumin molecule. $\alpha$-helix structure colored in red; loop in green. (A) Side view, (B) Front view. Figure from Protein Database.
2.1 Cytotoxicity of Nano Oxide Particles

Engineered oxide nanoparticles have been widely investigated due to their extensive applications in decade and widespread potential commercial uses including hazardous waste treatment, electronics, pharmaceutics, energy storage, industrial catalysis, solid lubricants, and supporting carriers [26-32]. These engineered nano-oxide particles will enter the environment ultimately; so that detailed knowledge of the effects of oxide NPs to organisms is of great interest. The most widely used test organisms for cytotoxicity of NPs are cells and bacteria [2, 33-35]. In this review, previous research on the cytotoxicity of TiO$_2$, SiO$_2$, and Al$_2$O$_3$ will be discussed separately for cells and bacteria.

2.1.1 Cytotoxicity to Cells

The cell, which is the structural and functional unit of all known living organisms, is the smallest unit of an organism that is classified as living [36]. Therefore, cells are widely employed in the cytotoxicity studies of oxide NPs. Lots of studies have reported the cytotoxicity of oxide NPs, which are listed below.

Endothelial cells line the inner surface of blood vessel, which would potentially come into direct contact with these NPs [33]. A cell proliferative activity impairment and a pro-
inflammatory stimulation were reported when endothelial cells were exposed to SiO\textsubscript{2} NPs, which indicates chronic inflammation [33]. Furthermore, TiO\textsubscript{2} (20 – 160 nm) and SiO\textsubscript{2} (4 – 40 nm) NPs are found to be internalized in the cell by vacuoles [33].

Cytotoxicity of homogeneous and weakly aggregated TiO\textsubscript{2} NPs were evaluated by mouse fibroblast cells in aqueous solution [34]. The cells became round and shrank, which formed aggregates and could be easily rinsed off, as the concentration of TiO\textsubscript{2} NPs increased. The transmission electron microscopy (TEM) analysis indicated that the number of lysosomes increased and some cytoplasmic organelles were damaged in a cell-culture medium containing 300 mg/L TiO\textsubscript{2}. A hypothesis on incorporation of TiO\textsubscript{2} NPs into cells was also proposed: TiO\textsubscript{2} NPs were endocytosed from the extracellular fluid when cells are exposed to TiO\textsubscript{2} NPs; then, a portion of the plasma membrane is invaginated and pinched off to form a membrane-bound vesicle, which contains the TiO\textsubscript{2} NPs fusing with lysosomes to form secondary lysosomes [34]. These uncontrolled lysosomes lead to the damage and destruction of organelles, such as discontinuity of the endoplasmic reticulum and disappearance of cell organelles [34].

With the discovery of the properties of TiO\textsubscript{2} as a photocatalytic compound applied in waste water disinfection [37] and photodynamic therapy of certain cancers [38], many studies of the phototoxicity and photogenotoxicity of TiO\textsubscript{2} have been performed [39-41], which distinguishes TiO\textsubscript{2} NPs from SiO\textsubscript{2} and Al\textsubscript{2}O\textsubscript{3} NPs. Among those studies, many published results have evaluated the harmfulness of TiO\textsubscript{2} NPs, including cytotoxicity, apoptosis and inflammation responses, in various cell types such as mesenchymal stem cell [42], lymphoblastoid cells [43], alveolar epithelial cell of the lung [44, 45], alveolar macrophages [46], phagocytes [47], osteoblast [48], mouse fibroblast cells [34], human
bronchial epithelial cells [49] and cellular microtubule protein [3]. Destruction of cells by oxidative stress and reactive oxygen species (ROS), mainly hydroxyl radicals, superoxide ions, and hydrogen peroxide in aqueous phase [50-52], was observed when cells were exposed to TiO₂ NPs and UV radiation, as the ROS could impair the cell membrane architecture through lipid peroxidation [50, 53, 54]. The generation of ROS has been reported: electrons of TiO₂ particles are excited by light energy and then move from the valence band to the conduction band; thus, positive holes are generated at the valence band and react with water to generate hydroxyl radicals in the vicinity of the TiO₂ surfaces [55].

On the one hand, a positive correlation between photocatalytic ROS production and antibacterial activity has been reported [56], which will be discussed later in the bacteria part (paragraph 1.4.2). On the other hand, conflicting data emerged on whether TiO₂ NPs are harmful to cells in the absence of photo-activation from UV radiation. Despite previous studies suggesting the harmlessness of TiO₂ NPs on animal and human cells in the absence of UV radiation [57-59], a steady increase in harmfulness studies of TiO₂ NPs is emerging: TiO₂ NPs (75 nm) in darkness can induce significant cytotoxicity on human bronchial epithelial cells, possibly through apoptotic pathways [49]. Characteristic apoptotic bodies within nuclei were clearly observed after TiO₂ NPs exposure treatment in darkness, while phosphatidylserine translocation, another apoptosis characteristic, from inner to outer leaflet of the cell membrane was revealed, which further corroborated TiO₂-induced cytotoxicity [49]. Similar investigations on TiO₂ cytotoxicity reported that the influence of 40 nm TiO₂ NPs (without UV) on the neuroblatoma-2A cell line lead to significant reduction in cellular viability that increased with TiO₂ concentrations [60].
Studies of cytotoxicity of alumina, which is estimated to account for approximately 20% of the 2005 world market of NPs [61], are less abundant than are studies for the other two particles. Both porcine pulmonary artery endothelial cells and human umbilical vein endothelial cells showed increased mRNA and protein expression of vascular cellular adhesion molecule-1, intercellular adhesion molecule-1, and P- and E-selectins, when exposed to alumina at various concentrations and time [62]. Furthermore, human endothelial cells expressed increased adhesion of activated monocytes when treated with alumina NPs [62]. No significant increases in ROS production were observed, suggesting that certain metal oxide NPs (like Al₂O₃ NPs) cannot significantly promote ROS upon internalization into cells [47, 62-64].

Impairment of cellular organelles and inflammation of cells by contact with oxide NPs are abundantly reported, which evidently indicate the cytotoxicity of these NPs. Although conflicting results of TiO₂ NPs toxicity have been reported, the dispute is only about whether UV plays a role in the damage of cells by TiO₂. Sufficient results of cytotoxicity of oxide NPs call for further studies on the mechanism of cytotoxicity, which is one of the initial incentives of this work. Before exploring the literature on the toxicity mechanism, studies of another important group of organisms, bacteria, will be reviewed first.

2.1.2 Cytotoxicity to Bacteria

Bacteria, which are single-cell organisms, are also good test models with which to study the toxicity of NPs and to examine how the NPs affect the cell function. Moreover,
bacteria perform many critical roles in ecosystem function and productivity. Therefore, the toxicity of NPs to bacteria has caused many concerns. Several important studies on this subject are reviewed below, which will give us a general view about the toxicity of oxide NPs to widely tested bacteria.

Eco-toxicity of water-suspended nanosized TiO$_2$ and SiO$_2$ was investigated by using Gram-positive *Bacillus subtilis* and Gram negative *Escherichia coli* as test organisms [2]. The antibacterial activity of these two photosensitive NPs increased with increasing particle concentration; and TiO$_2$ NPs are more toxic than SiO$_2$ at the same concentration [2]. Moreover, the Gram-positive *B. subtilis* was more sensitive to these NPs than Gram-negative *E. coli*, which could be attributed to the ability of *B. subtilis* to form spores and its cell wall structure [65]. The antibacterial activity of TiO$_2$ towards both bacterial species was significantly greater in the presence of light than in the dark [2].

Toxicity of Al$_2$O$_3$, SiO$_2$, and TiO$_2$ NPs to *B. subtilis, E. coli* and *Pseudomonas fluorescens* was examined and compared to that of their respective bulk counterparts [35]. It was reported that all NPs except TiO$_2$ showed higher toxicity than their bulk counterparts, indicating the particle size did cause a toxicity difference: Al$_2$O$_3$ and SiO$_2$ NPs were toxic to the tested bacteria, while their BPs showed no or lower toxicity; TiO$_2$ NPs did not affect bacterial populations, which was different from the previous results [2]. The TEM images (Figure 2) provide intuitive evidence for the difference of toxicity of these NPs: Al$_2$O$_3$ and SiO$_2$ NPs were coating the whole bacterial cells, while TiO$_2$ NPs were rarely coated on bacterial surfaces [35].
FIGURE 2. TEM images revealing attachment of NPs to the surface of P. fluorescens: Al₂O₃ (A), SiO₂ (B), TiO₂ (C), ZnO (D). Figure from Jiang et al. [35].

Early in 1985, Matsunaga et al. [66] reported that Lactobacillus acidophilus, Saccharomyces cerevisiae, and Escherichia coli were completely sterilized when incubated with TiO₂ particles under metal halide lamp irradiation for 60 – 120 min. However, this work lacked more extensive data on the bactericidal properties of TiO₂ photocatalysts. Therefore, a more systematic comparison on the bactericidal activity of TiO₂ particles was conducted on the irradiation of Escherichia coli and TiO₂ (anatase) with UV-visible light of wavelengths longer than 380 nm [56]. At a dose of 1 g/L TiO₂
under constant illumination, complete killing of the bacteria was apparent in less than 30 min, while no significant decrease in the bacteria population was observed without illumination. Moreover, the proportion of surviving bacteria decreased with increasing O\textsubscript{2} composition of the gas flowing in the suspension, as well as with an increase in the TiO\textsubscript{2} dose. This also suggested that ROS generated by TiO\textsubscript{2} was responded for the bactericidal activity [56]. Another interesting result was reported in that the decrease of \textit{Escherichia coli} continues in the presence of TiO\textsubscript{2} without illumination, and no regrowth was observed within the following 60 hours, suggesting that the induced injuries on the bacteria are irreversible [65]. This “residual disinfecting effect” of the photocatalytic process of TiO\textsubscript{2} was viewed as the most interesting post-irradiation event.

Since cytotoxicity of oxide NPs to living cells and bacteria have been widely reported, current studies are focused on the cell-damage mechanism, which investigate the components of cells or bacteria, such as DNA [43, 67] and proteins [14, 68, 69]. Before reviewing the existing studies of protein sorption onto NPs, an important effect, aggregation of NPs, will be discussed first, because the aggregation effect could greatly influence the cytotoxicity of oxide NPs.

### 2.1.3 Impact of Particle Size to Cytotoxicity

The impact of particle size can be separated into two types: aggregated-particle-size effects and original-particle-size effects. The impact of aggregation of NPs is significant in evaluating the cytotoxicity of NPs [34, 70-72]. Generally, when NPs aggregate into micro-scale structures or precipitates, it is difficult to evaluate the size and dosage
impacts of NPs with respect to cytotoxicity. Cells have to interact with micro-scaled particles or precipitate, which are all larger than any cellular components in size and cannot be easily engulfed by cellular membranes, instead of mono-dispersed NPs [34]. Therefore, the cytotoxicity of NPs was attributed to their physicochemical characteristics (i.e. aggregation) other than the single particle size [34]. But for some studies, whose objectives did not include the aggregation influence, weakly aggregated NPs were used in order to reduce the effect of aggregation [34].

Adams et al. compared the antibacterial activity of different types and sizes of advertised TiO$_2$ and SiO$_2$ NPs for *B. subtilis* and *E. coli* [2]. No significant difference in toxicity among these NPs was observed, which was mainly due to the aggregation of particles in suspension leading to similar particle-size [2]. Although other external factors, including light intensity, surface chemistry, particle morphology and bacteria concentration can also influence the antibacterial activity of those NPs as they reported [2], the influence of these external factors will be neglected if all the samples are performed under the same experimental conditions (e.g. under the same light intensity and bacterial concentration). Therefore, aggregation of particles in suspension should be considered with priority.

The impacts of original-particle-size were mostly considered in weakly or rarely aggregated NPs or experimental conditions. For example, cytotoxicity of SiO$_2$ NPs was reported depending on the size of NPs [73]: SiO$_2$ NPs were found to exhibit size-dependent cytotoxicity toward *Chlorella kessleri* alga, when the 50% inhibitory concentration value was compared for the diameter of 5 nm, 26 nm and 78 nm NPs [73]. The smaller SiO$_2$ NPs exhibited stronger cytotoxicity. Enlargement of the cell body was
reported, which is due to the presence of SiO$_2$ NPs that obstructed cell division. Coagulation of cells with incomplete division was also observed, while several amorphous structures appeared in the cells that were exposed to 5 nm SiO$_2$ NPs [73]. It is hypothesized that smaller SiO$_2$ NPs could enter cells more easily than larger ones, and cause more severe damage to cells. So et al. [74] also reported higher toxicity of SiO$_2$ NPs (30 nm) on mouse liver by comparing a nano-sized silica-particle-fed group of mice with a micron-sized particle (30 µm) fed group. However, no other significant difference was observed on the health of mice at a feeding amount of 140 g silica/kg mouse.

### 2.2 Protein Adsorption and Conformational Change

As we mentioned in the beginning of this thesis, the toxicological concern of NPs comes from the fact that NPs can be transported in blood and can cross cell membranes into cells [3, 4]. Therefore, NPs may interact with proteins in blood or with cytosplasmic proteins. Proteins that adsorbed on the surface of NPs may undergo changes in their structures and functions, even the entire protein molecules [6, 7], which could result in adhesion, proliferation, and differentiation of cells, as well as provoking foreign body response and inflammatory processes [8, 9]. At this point, studies on protein adsorption and conformational change caused by oxide NPs play an important role in the cytotoxicity-evaluation of NPs to living beings. The objectives of this work are to understand the protein-adsorption behavior and conformational-change mechanisms. Previous studies, both their discovery and shortcomings, are discussed below.
2.2.1 Protein Adsorption

Generally, sorption of protein molecules is governed by Columbic forces, van der Waals forces, hydrophobic interactions, and the sorbate conformational stability, while the surface area provides the possible sorption spaces for protein adsorbed on particle surfaces [75, 76]. Three theories of protein adsorption have been proposed in the literature: random sequential adsorption (RSA), diffusion random sequential adsorption (DRSA), and Lattice theory [77-79].

RSA theory assumes that protein molecules are hard spheres and protein adsorption is an irreversible process [77]. Protein molecules adsorb onto random positions of the solid surface sequentially providing a monolayer covering the particle surfaces. This process would continue until the surfaces of particle are fully covered [77]. The random-position adsorption is often not practical because the surfaces of particle are not even, either physically or chemically. DRSA theory comprises three aspects: simulation procedure, static aspects and dynamic aspects, which considers the diffusion adsorption process of protein molecules onto surfaces, the conformational structure of the adsorbed protein, and dynamic nature of the adsorption process, respectively [78]. The advantage of this model is that it considers the interaction between the adsorbed and diffusing protein molecules, which can be applied to explain the adsorption behaviors of globular proteins on solid surfaces [78]. Lattice theory is a mathematical approach which is applicable to rigid proteins that undergo orientational changes upon adsorption [79]. According to this theory, a protein is modeled as a rod shape, which can be adsorbed in two surface states, side-on and end-on (Figure 3). In end-on state, the protein is weakly bound to the surface,
while in the side-on state the bonding is more firm [79]. The surface-exclusion effect requires that any site of lattice on the surface may only be occupied once.

**FIGURE 3.** Three dimensional illustration of two adsorbed rod proteins with side-on and end-on mode.

Based on an examination of the literature, some characteristics of protein adsorbed on oxide NPs can be concluded:

(1) Electrostatic interaction is one of the main driving forces, and it is related to solution pH [80-82]. The electrostatic interaction between the positive charges of a protein and the negative sites of a particle surface was reported as one of the driving forces of fibronectin protein adsorption on TiO$_2$ particle surfaces [80]. Even when the overall charges of both protein and TiO$_2$ surfaces have the same sign, electrostatic
attraction can still drive the adsorption. It is because the different regions of protein surface carry different signs of charge, either positive or negative charge, which could attract oppositely charged particle surfaces [80, 81]. The surface charges of both protein and oxide particle are largely dependent on the pH of solution. For example, human serum albumin (HSA) and TiO$_2$ are both positively charged at pH below 4.7, and both are negatively charged at pH above 6.0 [82]. In the pH range of 4.7 to 6.0, the overall surface charge of HSA is negative, while that of TiO$_2$ is positive [82]. The HSA adsorption at pH below 4.7 or above 6.0 is lower than that in pH range of 4.7 to 6.0, which is due to the electrostatic repulsion between like charges [82].

Then, at what pH does the protein adsorption reach maximum? Compared in the wide-tested pH range, the maxima adsorption of protein reaches at protein isoelectric point (IEP) [82]: a compact protein monolayer with minimum lateral protein-protein interaction and maximum protein adsorption is formed on TiO$_2$ surface [82]. Beyond the IEP of protein molecules, there are two possible situations: surfaces of particles and protein molecules carry the same sign or reverse sign. In the pH region where TiO$_2$ and protein surfaces carry the same sign, electrostatic repulsion between the two surfaces leads to less protein adsorption onto particle surfaces [83]; in the pH region where the two surfaces carry opposite charges, electrostatic attraction weakens the stability of protein structure, causing the protein to unfold and to spread out on TiO$_2$ surface, and thus allowing a small amount to saturate the particle surfaces [84, 85].

Previous studies agree well with the explanation above [82, 86-88]. For example, adsorption of chicken egg lysozyme on SiO$_2$ NPs decreased as the pH dropped [87], which is due to the protein-protein electrostatic repulsion at a lower pH when lysozyme
molecules bear higher positive charges [86]. However, the repulsion would only be important at high protein adsorptions and would likely be compensated by stronger protein-silica attraction [87]. Another explanation is that the pH induces the change of zeta potential for SiO₂, which leads to decreased Coulombic attraction between lysozyme and SiO₂ at lower pH [88].

In sum, particle-surface charge, which largely depends on the pH of aqueous solutions, can influence protein adsorption. Therefore, particle-surface charge is one of the important factors that should be considered in protein adsorption studies. One task in this work is to compare the protein-adsorption behaviors of nano- and bulk-sized oxide particles, which is a more complicated adsorption system than studied in previous works. The influence of surface charge on the protein adsorption within a large diameter range of oxide particles is unclear. For example, effects of surface charge may be compensated by other factors, such as surface functional groups and surface area. Surface charge was examined through the Zeta potential of particle. Hence, Zeta potential of oxide particles was examined in this work. Moreover, most oxide particles have abundant hydroxyl groups on their surfaces [89], which may change the solution pH upon contact. Therefore, pH adjustment is required during protein adsorption in order to keep a neutral pH. The pH adjustment may influence the surface hydrophilicity of oxide particles, which may affect the protein-adsorption behaviors as well. It is unknown whether the protein-adsorption maxima have linear-relation with the amount of hydroxyl groups on particle surfaces. These questions will be addressed in the next chapter.

(2) Protein adsorption can be reduced by certain ions. The adsorption of BSA onto TiO₂ surfaces was reduced in the presence of calcium and phosphate ions (Hanks’
balanced salt solution) [90]. It is because a stable, hydrophilic film induced by these ions is formed on TiO$_2$ surface that inhibits the protein adsorption [90]. Phosphate is widely used in biological experiment as the pH buffer. Most of previous studies of protein adsorption used phosphate in buffer [11, 90-92], which could affect the protein adsorption by changing the surface hydrophilicity of oxide particles. Therefore, deionized water was used instead of phosphate buffer in this work, in order to diminish the influence of phosphate ions on protein adsorption. Protein adsorption in deionized water is rarely reported. Protein-adsorption behaviors is unknown without the influence of phosphate ions either.

HSA adsorption on TiO$_2$ surface was independent of the ionic strength of a NaCl solution [82], which could be explained as neither Na$^+$ nor Cl$^-$ ions can interact with the groups on TiO$_2$ surface and form a hydrophilic film. This result could help us choose the salt ions that could help maintain the protein structure and pH, without impacting the protein adsorption.

(3) Protein adsorption is influenced by the curvature of NPs. Vertegel et al. reported that greater adsorption of chicken egg lysozyme on SiO$_2$ NPs was observed with larger diameter SiO$_2$ NPs [87]. Deducing from the results of Vertegel, one question is whether SiO$_2$ BPs adsorb more protein molecules than NPs. This question may affect the cytotoxic evaluation of oxide NPs. However, previous studies seldom made this comparison. Also, Vertegel’s work was performed in phosphate buffer. The impact of phosphate ion on protein adsorption was not excluded in their comparison study. It was a competitive sorption between protein molecules and phosphate ions, which may
influence the single protein-particle adsorption behaviors. Therefore, one of the tasks in this work was to compare the protein adsorption between NPs and BPs in deionized water.

Furthermore, Vertegel reported that the protein-adsorption models on these SiO$_2$ NPs were different: molecular complexes (stoichiometric protein-silica conjugates) were formed for the protein adsorption on the 4 nm SiO$_2$ with a protein monolayer on the surface; the true adsorption behavior was observed on 20 and 100 nm SiO$_2$ with a protein monolayer and multilayer formed on the surface, respectively [87]. It is unknown whether the protein-adsorption models (monolayer or multilayer adsorption) on oxide NPs and BPs are different. Calculations will be employed to answer these questions.

### 2.2.2 Protein Conformational Changes

Generally, when protein adsorbed on solid surfaces, the binding is optimized by a change in molecular conformation [14, 69]. Besides, albumin interacts more strongly with hydrophobic than with hydrophilic surfaces [68]. Depending on the adsorbing surface, the induced conformational changes could or could not be reversible on desorption [68]. Previous studies [10, 87, 93] had reported conformational changes of protein molecules adsorbed on oxide NPs. As different oxide NPs carry different surface physichemical properties, review of previous studies is seperated by the types of oxide NPs.

#### 2.2.2.1 TiO$_2$


An increasing content of \(\beta\)-sheet and a decrease of \(\alpha\)-helical structure content for fibrinogen protein, and an increasing content of \(\beta\)-sheet and a decreasing content of random coil structure for fibronectin protein were reported after the protein molecules adsorbed on to TiO\(_2\) NP surfaces by Raman spectroscopy [10]. In the Raman spectra of the adsorbed proteins, a characteristic band was assigned to an interaction between TiO\(_2\) NPs and the carboxylate groups of the protein side-chains [10]. Amide I band of the adsorbed fibrinogen spectrum shifted toward higher wavenumbers in comparison to the original bulk protein spectrum, which was due to conformational changes during the adsorption process. A decrease of the peak area of the multiplet of CH\(_3\) and CH\(_2\) deformation modes of the adsorbed fibrinogen was also observed, as compared to the spectra of original bulk fibrinogen [10].

Secondary structural changes of fibrinogen molecules adsorbed on TiO\(_2\) surfaces was reported, with two consecutive steps occurred during the adsorption: firstly, the fibrinogen molecules were adsorbed onto the surface, and then the rearrangement of adsorbed fibrinogen or multilayer adsorption occurred [93]. This hypothesis has been proved by the observation that the \(\alpha\)-helix content of adsorbed fibrinogen obviously decreased and was mainly transformed to \(\beta\)-sheet, while the \(\beta\)-turn and random coil contents were less changed, when the proteins adsorbed on TiO\(_2\) surfaces [93]. Furthermore, the chemical bonding process has been studied: (1) the TiO\(_2\) particle surface is non-charged at around pH 5 [94], so under their experimental pH of 7.4, the predominant TiO\(_2\) surface groups are Ti\(_2\)=O\(^-\) and Ti–OH, with few Ti\(_2\)=OH; and the main protein functional groups are R–COO\(^-\) and R–NH\(_3^+\) [82, 95]. (2) Electrostatic interaction occurs between these groups on the surfaces of both TiO\(_2\) and protein:
Ti−OH\(^+\) : NH\(_2\)−R (electrostatic interactions)

Ti\(_2\)=O\(^-\) +NH\(_3\)−R (electrostatic interactions)

Ti−OH…−COO−R (hydrogen bonding interaction) [93].

Chemical bonding between BSA protein and TiO\(_2\) particles would be similar as the bonding between fibrinogen and TiO\(_2\), but may not be the same. Therefore, we studied how BSA bonds to TiO\(_2\), and to other oxide particles as well.

2.2.2.2 SiO\(_2\)

Beside TiO\(_2\) NPs, conformational changes of protein adsorbed on SiO\(_2\) and Al\(_2\)O\(_3\) NPs were also reported. Adsorption, as well as protein structure and functions, of chicken egg lysozyme on SiO\(_2\) NPs of various diameters are strongly dependent on the size of the nanoparticles (Figure 4) [87]. Greater loss of α-helix structure, which is consistent with a decrease of lysozyme activity, although not linearly, was observed with larger NPs that shows stronger adsorption under the same conditions[87]. Studies of lysozyme adsorbed onto flat SiO\(_2\) surfaces indicate that the largest charged patch on the protein surface would contact with the negatively charged SiO\(_2\) surface, when protein molecule is in its thermodynamically most favorable conformation [96]. The active site of lysozyme is located at the opposite side of the positively charged patch [97]. Therefore, the initial conformational changes upon adsorption result in moderate loss in activity, which is because the conformational perturbations are somehow distant to the active site of protein [87]. Then, more perturbation occurs close to the active site when the native α-helix
content is further lost [87]. Similar results on the research of human carbonic anhydrase I (HCAI) protein adsorbed onto SiO$_2$ NPs of various diameters (6 nm and 15 nm) were also reported [11]: bigger SiO$_2$ NPs form larger protein-particle interaction surface area that cause greater protein secondary-structure conformational change (Figure 4). However, the protein tertiary structure is independent with the curvature of SiO$_2$ particles [11].

If bigger SiO$_2$ NPs lead to greater protein secondary-structure conformational change than smaller SiO$_2$ NPs, will oxide BPs cause more conformational change than NPs? Therefore, one of the tasks in this work is to understand the influence of particle size to protein conformational change.

![Figure 4](image_url)

**FIGURE 4.** Schematic illustration of the relation between particle curvatures and the protein secondary structure modification.

2.2.2.3 Al$_2$O$_3$
In deionized water (pH = 7) condition, the surface charges of BSA protein can be divided into three domains: domain I is -7.8 mV, domain II is -9.0 mV, and domain III is -1.3 mV (Figure 5) [98]. Based on the calculated net charges of the three domains of BSA molecule, the adsorption mechanism of BSA onto Al₂O₃ NPs surfaces is proposed as a multistep process: at first, BSA protein forms a monolayer on Al₂O₃ surface by side-on (domain I and domain II adsorbed on Al₂O₃ surface, Figure 6) adsorption mode because the net charge of domain I + II is -16.8 mV in total, which is the most negatively combined charge compared with domain domain I + III (-9.1 mV) or domain II + III (-10.3 mV); then, with the increase of protein concentration, BSA dimmers (two BSA molecules bonding, Figure 6 and 7) form on the surface instead of the end-on mode (Figure 6) [98]. This is because the protein end-on mode induces more BSA/Al₂O₃ surface interactions that are not favored because of the asymmetric charge distribution of the protein [98]. End-on mode is one of the proposed albumin-protein-adsorption modes, which hypothesized the albumin protein adsorbed on particle surfaces by only one domain, instead of two domains (Figure 6, middle).
FIGURE 5. Three domains of BSA protein molecule. The net charge of the three domains is: domain I -7.8, domain II -9, domain III -1.3.

FIGURE 6. Proposed BSA adsorption model of side-on adsorbed mode (left), end-on adsorbed mode (middle), and dimmer mode (right) on Al₂O₃ particle surface from Rezwan et al. [98]

FIGURE 7. Human serum protein (HSP) dimmer models from the protein data bank [99] showing a side view (left) and a bottom view (right). BSA can also form similar dimmers.
The single-headed arrow indicates the viewing direction of the bottom view. The size of the protein monomer is about $9 \times 5.5 \times 5.5$ nm.

There is a point of zero charge (PZC) of alumina surface shift from the PZC of alumina (pH=9) to the isoelectric point (IEP) of BSA (pH=5) with the protein adsorption on alumina surface [98]. After the first layer of BSA protein molecules fully covers the alumina surface, the pH of surface charges of the $\text{Al}_2\text{O}_3$ reaches the IEP of BSA (pH=5) and cannot be shifted even more protein is adsorbed. Therefore, at pH 7, the surface charge of $\text{Al}_2\text{O}_3$ shifts from positive (PZC of $\text{Al}_2\text{O}_3$ is pH 9) to negative (IEP of BSA is pH 5) with the process of BSA adsorption. Since the surface charges of first layer are negative, the formation of dimmer is based on protein/protein interaction through of hydrogen bonds, disulfide bonds, and hydrophobic effects, and cannot be due to electrostatic interaction with the $\text{Al}_2\text{O}_3$ particle surface (surface charges of both protein and particle are negative at pH 7) [100].

*Side-on* and *end-on* adsorption modes on $\text{Al}_2\text{O}_3$ particle surfaces have been well studied. Can these adsorption modes be used on $\text{TiO}_2$ and $\text{SiO}_2$ particle surfaces, and their BPs as well? All these questions listed above will be discussed in this study.

### 2.3 Hypotheses

From the above review, we have several hypotheses:
(1) Electrostatic interaction, which is caused by surface charge, plays an important role in BSA protein adsorption. But it will be partially compensated by other factors, such as surface area and surface chemical groups.

(2) NPs will adsorb more BSA than BPs. However, the sorption also is likely to depend on the specific surface area of each particle, which is to say that aggregation of NPs may reduce the specific surface area of NPs, and therefore, lower the BSA-adsorption maxima below the maxima of BPs. Multilayer- and monolayer-protein adsorption may also occur on the surfaces of Al₂O₃ and other oxide particles, respectively, based on previous reports.

(3) BSA molecules and oxide surfaces are mainly bonded by hydrogen bonds and electrostatic interactions, which lead to changes in the secondary structure of BSA molecules. BPs may elicit more severe conformational changes than NPs because they have smaller curvature than NPs.

2.4 Objectives

The long term goal of this work is to understand the cytotoxicity mechanisms of NPs. In order to accomplish this goal, we have to clearly know the mechanisms via which NPs contact and enter cells, to understand how NPs interact with the organelles in cells, and finally, to evaluate the influence of NPs on the physiological functions of organisms. Therefore, understanding the interaction between oxide NPs and proteins is one of the efforts to better understand how NPs affect living beings.
Specifically, the objectives of this work were:

(1) to determine BSA adsorption behaviors onto TiO₂, SiO₂, and Al₂O₃ NPs and BPs;
(2) to understand the effects of particle size, surface charge and surface groups on BSA adsorption;
(3) to determine the models of BSA adsorbed onto oxide particles: monolayer or multilayer;
(4) to identify the chemical bonding between surface-chemical groups of protein and oxide particles; and
(5) to discuss the factors affecting BSA adsorption on to oxide NPs and BPs, and to make conjectures about the cytotoxicity of oxide NPs in a natural environment.
CHAPTER 3
EXPERIMENTS AND DISCUSSION

3.1 Materials and Methods

3.1.1 Sorbates and Sorbents

BSA lyophilized powder was purchased from Sigma-Aldrich Co. BSA was prepared in deionized water, followed by adding NaN\(_3\) (200 mg/L) as a biocide and adjusting to pH 7.0. The protein concentration assay set for the measurement of protein was purchased from Bio-Rad Co. Nanoscaled SiO\(_2\) (spherical form), TiO\(_2\) (anatase form), \(\alpha\)-Al\(_2\)O\(_3\) and \(\gamma\)-Al\(_2\)O\(_3\) were purchased from Zhejiang Hongchen material technology Co., China. The regular SiO\(_2\) (spherical form) and TiO\(_2\) particles (anatase form) were from Fisher Scientific Co., and regular Al\(_2\)O\(_3\) particles were purchased from Baker Co. All NPs and BPs were used without further treatment.

The TiO\(_2\) group is composed of three structure forms: rutile, anatase, and brookite. The three forms have the same elemental chemistry, TiO\(_2\), but different structures. Anatase is a polymorph with the two other minerals. At about 915 degrees Celsius, anatase will convert to the rutile form. Anatase and rutile share the same properties such as luster, hardness and density, but slight difference of crystal behavior and significant difference of cleavage. More information can be obtained from:

http://ruby.colorado.edu/~smyth/min/tio2.html and
http://mineral.galleries.com/minerals/oxides/anatase/anatase.htm
\( \alpha \)- and \( \gamma \)-\( \text{Al}_2\text{O}_3 \) forms are the most abundant among the several forms of \( \text{Al}_2\text{O}_3 \). \( \alpha \)-\( \text{Al}_2\text{O}_3 \) is the pure form obtained from calcination at high temperature, while \( \gamma \)-\( \text{Al}_2\text{O}_3 \) keeps stable at about 1000 °C.

\( \text{SiO}_2 \) exists in several forms. Spherical \( \text{SiO}_2 \) NPs were used in this work. More description from the supplier can be obtained from http://www.mrmn.com.cn/product_2.htm.

### 3.1.2 Characterization of Oxide Particles

Specific surface area (\( S_{\text{BET}} \)) of all oxide particles was calculated from \( \text{N}_2 \) sorption isotherms by the multipoint BET method. \( \text{N}_2 \) sorption was conducted at 77 K using a NOVA 1000e instrument (Quantachrome). All samples were outgassed at 105 °C for 16h before \( \text{N}_2 \) adsorption. The C and H contents were determined by combusting samples at 980 °C with oxygen using a Perkin-Elmer 2400 CHN Elemental Analyzer (Sheton, CT). The particle size of all samples was visualized by using transmission electron microscopy (TEM, JEOL 100CX, USA) operated at 80 kV. About 100 individual particles for each sample were employed to determine their particle size based on the magnification by TEM. The hydrodynamic diameter and Zeta Potential values were measured by a Zetasizer nano ZS (Malvern Instruments, United Kingdom) with the dynamic light scattering (DLS) technique at 25 °C, using suspensions containing 50 mg/L of solids in solution. The pH of all suspensions was pre-adjusted by KOH and HCl to keep pH = 7.0 ± 0.2. Measurements were performed after shaking the suspensions for 24 h. Selected structural properties of these oxide particles are listed in Table 2. Fourier transform infrared (FTIR) spectra were recorded with a Perkin-Elmer Spectrum One FTIR.
spectrometer (Shelton, CT). Five milligrams of samples were mixed gently with 95 mg of KBr as a background using a pestle and mortar and analyzed. FTIR spectra were recorded from 400 to 4000 cm\(^{-1}\) at 8 cm\(^{-1}\) resolution over 200 averaged scans. The FTIR spectra of adsorbed BSA were obtained by subtracting that of the BSA-free coating oxide particles from that of the BSA-coated oxide particles.

### TABLE 2. Selected Properties of Oxide Particles

<table>
<thead>
<tr>
<th>Particle</th>
<th>Purity(^a) (%)</th>
<th>(S_{BET})(^b) (m(^2)/g)</th>
<th>Diameter(^c) (nm)</th>
<th>Hydrodynamic Diameter (nm)(^d)</th>
<th>C (%)</th>
<th>H (%)</th>
<th>Zeta Potential(^e) (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nano (\alpha)-Al(_2)O(_3)</td>
<td>(\geq 99.99)</td>
<td>4.73</td>
<td>150±5</td>
<td>1147</td>
<td>0.019</td>
<td>0.062</td>
<td>-0.114 ± 9.11</td>
</tr>
<tr>
<td>Nano (\gamma)-Al(_2)O(_3)</td>
<td>(\geq 99.99)</td>
<td>208</td>
<td>60±5</td>
<td>1482</td>
<td>0.190</td>
<td>1.210</td>
<td>22.9 ± 6.37</td>
</tr>
<tr>
<td>Bulk Al(_2)O(_3)</td>
<td>(\geq 98.5)</td>
<td>11.0</td>
<td>429±5</td>
<td>878</td>
<td>-(^f)</td>
<td>-</td>
<td>-23.5 ± 6.69</td>
</tr>
<tr>
<td>Nano TiO(_2)</td>
<td>(\geq 99)</td>
<td>325</td>
<td>50±5</td>
<td>706</td>
<td>0.034</td>
<td>1.370</td>
<td>-11.0 ± 4.17</td>
</tr>
<tr>
<td>Bulk TiO(_2)</td>
<td>(\geq 99)</td>
<td>7.3</td>
<td>285±5</td>
<td>1181</td>
<td>-(^f)</td>
<td>-</td>
<td>-53.8 ± 6.54</td>
</tr>
<tr>
<td>Nano SiO(_2)</td>
<td>(\geq 99.5)</td>
<td>191</td>
<td>30±5</td>
<td>1274</td>
<td>0.043</td>
<td>1.410</td>
<td>-40.9 ± 8.10</td>
</tr>
<tr>
<td>Bulk SiO(_2)</td>
<td>=100</td>
<td>8.4</td>
<td>745±5</td>
<td>1731</td>
<td>-</td>
<td>-</td>
<td>-25.9 ± 7.80</td>
</tr>
</tbody>
</table>

\(^a\)Provided by the supplier. \(^b\) \(S_{BET}\) was calculated from the adsorption-desorption isotherm of N\(_2\) at 77K by multi-point BET method. \(^c\) Determined by high-resolution transmission electron microscopy (TEM). \(^d\) Determined by dynamic light scattering (DLS). \(^e\) The pH was 7.0 ± 0.2. \(^f\) “-” represents unmeasured.
3.1.3 Adsorption Experiments

All adsorption isotherms were obtained using a batch equilibration technique at 25 ± 1°C in 15-mL screw-cap borosilicate glass vials. The sorption experiments were conducted with ten concentration points; each point including a blank sample was run in duplicate. The mass dosage of nanosized TiO$_2$, SiO$_2$, α-Al$_2$O$_3$ and γ-Al$_2$O$_3$, and bulksized TiO$_2$, SiO$_2$ and Al$_2$O$_3$ added in vials was 100, 400, 500, 100, 2000, 4000, and 1900 mg, respectively. Then 13 mL of BSA solution was added to the 15 mL vials, leaving 2 mL of volume for the subsequent pH adjustment. The initial concentrations of BSA in the vials spanned a range of 100-12000 mg/L. All the vials were rotated in an end-over-end shaker (30 rpm) for 72 h (where preliminary tests indicated that apparent equilibrium was reached before 48 h). The pH of each vial was adjusted twice (at 24 h and 48 h) in order to keep the solution at pH = 7.0 ± 0.2. Deionized water was added to bring total volume up to 15 mL in each vial after the second pH adjustment. After centrifugation (12000 rpm for 10 min, based on preliminary experimental data), the protein concentration in the supernatant was measured using the Bradford method [101] at a UV-wavelength of 595 nm.

3.1.4 Isotherm Models and Regression Analysis

Freundlich (FM) and Langmuir (LM) isotherm models were used for data fitting in this work.

Freundlich model:
\[ q_e = K_f C_e^n \quad (1) \]

where \( q_e \) (mg/kg) is the equilibrium adsorbed concentration; \( C_e \) (mg/L) is the equilibrium solution phase concentration; \( K_f \) [(mg/kg)/(mg/L)\(^n\)] is the Freundlich affinity coefficient; \( n \) is the Freundlich exponential coefficient.

Langmuir model:

\[ q_e = Q_0 b C_e / (1 + b C_e) \quad (2) \]

where \( Q_0 \) (mg/g) is the maximum sorption capacity of solute; \( b \) (L/mg) is the adsorption equilibrium constants. All estimated model parameter values were determined by a commercial software package (SigmaPlot 9.0).

### 3.2 Results and Discussion

#### 3.2.1 Characterization of BSA and Oxide Particles

The Zeta potential of BSA in deionized water was -6.64 ± 9.74 mV. Zeta potentials of oxide NPs and BPs are given in Table 2. Only \( \gamma \)-\( \text{Al}_2\text{O}_3 \) NPs had totally positively charge (22.9 ± 6.37 mV), and \( \alpha \)-\( \text{Al}_2\text{O}_3 \) NPs was nearly neutral (-0.114 ± 9.11 mV). Although the mechanism why the surface charges of \( \alpha \)- and \( \gamma \)-\( \text{Al}_2\text{O}_3 \) NPs are different at pH 7.0 cannot be fully understood, it still can be partially explained by their different crystal structures: \( \alpha \)-\( \text{Al}_2\text{O}_3 \) has a octahedral structure, with the oxide ions forming a hexagonal close-packed array and the aluminum ions are distributed symmetrically among the octahedral interstices [102]; but \( \gamma \)-\( \text{Al}_2\text{O}_3 \) has a defect spinel structure with a deficit of aluminum cations [102]. The surface charges of the other oxide particles were negative at pH 7.0 ±
0.2. The \( S_{BET} \) of selected oxide particles follows the sequence: TiO\(_2\) NPs > \( \gamma \)-Al\(_2\)O\(_3\) NPs > SiO\(_2\) NPs > Al\(_2\)O\(_3\) BPs > SiO\(_2\) BPs > TiO\(_2\) BPs > \( \alpha \)-Al\(_2\)O\(_3\) NPs (Table 2). \( S_{BET} \) of oxide NPs was larger than \( S_{BET} \) of their respective BPs except for \( \alpha \)-Al\(_2\)O\(_3\).

The FTIR spectra of oxide NPs and BPs are presented in Figure 8. Among these oxides, nanosized SiO\(_2\), TiO\(_2\), \( \gamma \)-Al\(_2\)O\(_3\), and bulksized SiO\(_2\) and Al\(_2\)O\(_3\) had hydroxyl groups on their surface in large quantity as the relatively high adsorption intensities at 1628 cm\(^{-1}\) and broad absorption bands at 2500 – 3700 cm\(^{-1}\) [103], while \( \alpha \)-Al\(_2\)O\(_3\) NPs and TiO\(_2\) BPs had few hydroxyl groups. Three types of bound hydroxyl groups at 3300, 3480 and 3620 cm\(^{-1}\) can be recognized. Other absorption bands of oxide NPs showed evidence of impurities as was also true of their bulk counterparts [89]. For SiO\(_2\), adsorption bands at around 456, 792, 938 and 1080 cm\(^{-1}\) can be assigned to the Si-O-Si vibration, Si-O-Si bending, Si-OH stretching and Si-O-Si stretching, respectively [89, 103]. Six humps, which were observed in the region of 1400 to 2100 cm\(^{-1}\) of bulksized SiO\(_2\) spectra, can be assigned to adsorbed or bound water (i.e. bending vibrations of adsorbed water at 1640 cm\(^{-1}\)) [104] or noise components of CH\(_2\)=CH\(_2\) or CH=CH\(_2\) [105]. For TiO\(_2\), the broad adsorption bands at 400 – 800 cm\(^{-1}\) can be assigned to the stretching of Ti-O-Ti; the peak at 1390 cm\(^{-1}\) of TiO\(_2\) NPs can be assigned to a titanium-acetate complex, while the peak at 1040 and 1122 cm\(^{-1}\) corresponded to the end and bridging butoxy groups [85]. For \( \gamma \)-Al\(_2\)O\(_3\), the adsorption bands in the region of 400 – 848 cm\(^{-1}\) can be assigned to the stretching of Al-O-Al, while for \( \alpha \)-Al\(_2\)O\(_3\) the stretching of Al-O-Al present two humps at around 456 cm\(^{-1}\) and in the region of 500 – 800 cm\(^{-1}\) respectively. The peak at 1390 cm\(^{-1}\) of \( \gamma \)-Al\(_2\)O\(_3\) and bulksized Al\(_2\)O\(_3\) can be assigned to the symmetric O-C-O stretching vibration of adsorbed carbonate anion on the particle surfaces, while the band at 1510
cm$^{-1}$ of $\gamma$-Al$_2$O$_3$ to the asymmetric O-C-O stretching vibration of adsorbed carbonate anion [83].

**FIGURE 8.** FTIR spectra of (a) nano SiO$_2$, (b) bulk SiO$_2$, (c) nano TiO$_2$, (d) bulk TiO$_2$, (e) nano $\alpha$-Al$_2$O$_3$, (f) nano $\gamma$-Al$_2$O$_3$, and (g) bulk Al$_2$O$_3$.  

Wavelength numbers, cm$^{-1}$
The C and H mass percent content of oxide particles are listed in Table 2. The hydroxyl groups and bound water on the surfaces of oxide particles were mostly oxygenated and volatilized under the calcined temperature of 980 °C [106]. Therefore, H content, which was calculated from the water vapor contents by elemental analysis, of oxide particles can be primarily attributed to the hydroxyl groups on the particle surfaces and surface-bound water. TiO$_2$, SiO$_2$, γ-Al$_2$O$_3$ NPs and SiO$_2$, Al$_2$O$_3$ BPs had abundant hydroxyl groups on their surfaces, while α-Al$_2$O$_3$ NPs and TiO$_2$ BPs had few hydroxyl groups, which is in agreement with the data of FTIR spectra (Figure 8).

3.2.2 Adsorption of BSA by Oxide Particles

Adsorption isotherms of BSA by oxide particles are shown in Figure 9(a) and (b). Most isotherms followed a typical Langmuir type except α-Al$_2$O$_3$, which showed a steep initial slope at low concentration and then reached a plateau at the equilibrium BSA concentration. Adsorption isotherms normalized by $S_{BET}$ of each oxide particle are shown in Figure 9(c) and (d). The largest BSA adsorption was observed for γ-Al$_2$O$_3$ NPs, followed by TiO$_2$ NPs > SiO$_2$ NPs > α-Al$_2$O$_3$ NPs > Al$_2$O$_3$ BPs > TiO$_2$ BPs > SiO$_2$ BPs. This Langmuir type behavior has also been observed in other studies of protein molecules or humic acid adsorbed on those oxide particle surfaces [89, 98]. The BSA adsorption isotherm of α-Al$_2$O$_3$ was fitted better by the Freundlich type ($r^2 = 0.981$) than by the Langmuir type ($r^2 = 0.860$), indicating the adsorption mechanism of α-Al$_2$O$_3$ could be different from other oxides, which is discussed later.
FIGURE 9. (a)(b) Isotherms of BSA adsorption by oxide particles in deionized water. Most isotherms followed a typical Langmuir type except γ-Al₂O₃, which fitted better with the Freundlich model. (c)(d) After normalization by surface area of each oxide particle, the differences of BSA adsorption maxima among these samples were decreased dramatically.

Results of fitting the Langmuir model to adsorption isotherms of BSA on oxide particles are shown in Table 2. According to the Langmuir maximum sorption capacity of
solute \((Q_0)\), BSA adsorption maxima of all oxide NPs were higher than the BPs, especially for nanosized \(\gamma\text{-Al}_2\text{O}_3\) \((Q_0 = 307 \text{ mg/g})\), TiO\(_2\) \((Q_0 = 155 \text{ mg/g})\), and SiO\(_2\) \((Q_0 = 107 \text{ mg/g})\), which was one order of magnitude higher than their BPs \((Q_0 = 25.2, 8.0,\) and 6.0 mg/g, respectively), indicating that the surface area limited the adsorption maxima of BSA among seven oxide particles. BSA adsorption on \(\alpha\text{-Al}_2\text{O}_3\) NPs was the lowest and close to the adsorption on Al\(_2\text{O}_3\) BPs (Figure 9a and 9b), which could be attributed to their equivalent surface area: 4.73 m\(^2\)/g for \(\alpha\text{-Al}_2\text{O}_3\) particles and 11.0 m\(^2\)/g for Al\(_2\text{O}_3\) BPs (Table 2). Furthermore, the regulation of surface area for adsorption maxima of BSA was supported by: (1) after normalization by particle surface area (Figure 9c, 9d, and Table 3), the differences of BSA adsorption maxima among oxide particles decreased dramatically; and (2) after deleting the point of \(\gamma\text{-Al}_2\text{O}_3\) from Figure 10, a significant linear relationship of the other six oxide particles was observed between \(S_{BET}\) and \(Q_0\). However, even after surface area normalization, the significant differences of BSA adsorption maxima (Figure 9c and 9d) were still observed, implying other factors such as surface charge, hydrophilicity and ligand exchange were involved [89]. For example, the highest BSA adsorption was observed for \(\gamma\text{-Al}_2\text{O}_3\), which could be attributed to the surface charge (the only totally positively charged oxide among all samples). Moreover, the maximum sorption capacity of all bulksized particles was slightly higher than that of the respective NPs (except the \(\alpha\text{-Al}_2\text{O}_3\)) after surface area normalization, which is in agreement with other studies [89, 104]. These results indicate: (1) greater protein absorption of NPs was brought by the larger surface area of NPs; (2) as particle sizes increases, the influence of surface area decreases in regulating protein-adsorption
behaviors, which leads to the higher maximum sorption capacity of BPs than that of respective NPs (except the α-Al_2O_3) after surface area normalization.

Electrostatic interaction could be one of the mechanisms explaining the BSA adsorption by oxide particles. According to the relationship of BSA adsorption maxima with $S_{BET}$ of oxide particles in Figure 10, a significant positive linear relationship was observed after deletion of the point for γ-Al_2O_3, which had a significantly high BSA adsorption. This could be explained partly by the strong electrostatic interaction between the positively charged γ-Al_2O_3 particle surface and slight negatively charged BSA molecules (Table 2). In addition, its large surface area (208 m$^2$/g, Table 1) provides more space for protein absorption. Moreover, the maximum sorption capacity of α-Al_2O_3 was significantly higher than the other particles after normalization with surface area (Figure 9c and 9d). This was mainly because the Zeta potential of α-Al_2O_3 was close to the zero point of charge (ZPC), which meant that smaller electrostatic attraction or repulsion resulted in less protein unfolding or less adsorption, respectively [82, 84]. Oliva et al. [82] reported that human serum albumin adsorption maxima occurred at a pH near the isoelectric point (IEP) of protein molecules, forming a compact layer with minimum lateral protein-protein interaction on TiO$_2$ surfaces. When the surface charge of protein molecules and oxide particles has the same sign, electrostatic repulsion between the two surfaces reduced the density of the protein layer covering an oxide surface [83]. When the surface charges of protein molecules and oxide particles have opposite signs, electrostatic attraction weakens the structure stability of protein molecules, resulting in the unfolding of protein molecules and their spreading out on the particle surfaces, which could also reduce the density of protein layers covering oxide surfaces. In this work, the
Zeta potential of BSA molecules was slightly negatively charged, and only the surface charge of $\alpha$-Al$_2$O$_3$ was neutral. Therefore, the density of the protein layer formed on $\alpha$-Al$_2$O$_3$ could be higher than the density of protein layer covering other oxide particles.

FIGURE 10. Positive linear relationship of BSA adsorption maxima with $S_{BET}$ for oxide particles after deleted the point of $\gamma$-Al$_2$O$_3$, which had a significantly-high-BSA adsorption.
TABLE 3. Results of Model Fits to Adsorption Isotherms of BSA on seven oxide particles

<table>
<thead>
<tr>
<th>Adsorbent</th>
<th>Adsorbate</th>
<th>Langmuir Model</th>
<th>Freundlich Model</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$Q_0$</td>
<td>$b$</td>
</tr>
<tr>
<td>nano TiO$_2$</td>
<td>BSA</td>
<td>155</td>
<td>1.59</td>
</tr>
<tr>
<td>bulk TiO$_2$</td>
<td>BSA</td>
<td>8.00</td>
<td>2.92E-3</td>
</tr>
<tr>
<td>nano SiO$_2$</td>
<td>BSA</td>
<td>107</td>
<td>5.38E-4</td>
</tr>
<tr>
<td>bulk SiO$_2$</td>
<td>BSA</td>
<td>5.99</td>
<td>1.44E-3</td>
</tr>
<tr>
<td>α-Al$_2$O$_3$</td>
<td>BSA</td>
<td>38.8</td>
<td>8.70E-3</td>
</tr>
<tr>
<td>γ-Al$_2$O$_3$</td>
<td>BSA</td>
<td>307</td>
<td>1.88E-1</td>
</tr>
<tr>
<td>Bulk Al$_2$O$_3$</td>
<td>BSA</td>
<td>25.1</td>
<td>1.21E-1</td>
</tr>
</tbody>
</table>

*a All parameter values were determined by Sigmaplot. *b $A_{surf}$ normalized $Q_0$.

Moreover, hydrophilicity could also be involved in BSA adsorption by oxide particles. According to the FTIR spectra shown in Figure 8, nanosized TiO$_2$, SiO$_2$ and γ-Al$_2$O$_3$ had hydroxyl groups on their surface in large quantity, while α-Al$_2$O$_3$ had few hydroxyl groups. A good negative linear relationship between surface-area-normalized adsorption maxima of BSA and H contents (Table 2) was observed for nanosized TiO$_2$, SiO$_2$, α-Al$_2$O$_3$ and γ-Al$_2$O$_3$ (only nanosized particles were compared in order to eliminate the effect of particle size, Figure 11). H content could serve as an indicator of surface hydrophilicity because it corresponded with surface-bound water and hydroxyl groups.
which suggested the significant influence of hydrophilicity of oxide particle surfaces on BSA adsorption. Water molecules can bond on surfaces of oxide particles, forming at least one layer of chemically bonded water molecules [107]. Then, physically sorbed water molecules can further cover this bonded-water layer, forming “vicinal” water films with a thickness of approximately 100 nm [108]. BSA molecules are thought to interact with vicinal water but not directly with surfaces of oxide particles during the adsorption process. Therefore, higher H contents would offer reduced accessibility of BSA molecules to oxide surfaces, which is in line with previous studies on the adsorption of hydrophobic organic compounds [109]. The high adsorption maxima of $\alpha$-Al$_2$O$_3$ normalized by surface area could also be explained by its smaller H content than the other NPs.

![FIGURE 11. Negative linear relationship of surface area normalized BSA adsorption maxima with H content of nanosized TiO$_2$, SiO$_2$, $\alpha$-Al$_2$O$_3$ and $\gamma$-Al$_2$O$_3$.](image)

$$y = -5.7953x + 8.5415$$
$$R^2 = 0.9986$$
3.2.3 Characterization of BSA and BSA-Coated Oxide Particles

3.2.3.1 Adsorption Models

According to published data on the conformation of serum albumin [110], the BSA molecule was modeled as a triangular prismatic shell with optimized dimensions of 84 × 84 × 84 ×30 Å in a neutral solution. Based on this model, one mole of BSA molecules occupy 18.668 × 10^6 m^2 of the surface area at most, and 15.175 × 10^6 m^2 of the surface area with the “side-on” mode at least [98]. Therefore, the surface densities of a close-packed BSA monolayer covered on 1 m^2 of particle surface are 4.37 mg/m^2 and 3.56 mg/m^2, respectively. According to the BSA adsorption maxima (Q_0, in Table 3) and the A_{surf} (Table 2), the specific amounts of BSA adsorbed onto the surfaces of oxide particles are 0.48, 0.56, 8.20, 1.48, 1.10, 0.71 and 2.28 mg/m^2 for nanosized TiO_2, SiO_2, α-Al_2O_3, γ-Al_2O_3 and bulksized TiO_2, SiO_2 and Al_2O_3, respectively (Q_0’ in Table 3). The amounts of BSA adsorbed on oxide particle surfaces were significantly lower than the calculated values of monolayer adsorption model except the α-Al_2O_3, indicating the unfolding of protein molecules or strong lateral protein-protein repulsion between protein molecules that covered on particle surfaces [11, 87, 90]. The amount of BSA adsorbed on α-Al_2O_3 surfaces was significantly higher than the “side-on” calculated value, implying multilayer adsorption of protein molecules coated on α-Al_2O_3 surface, which is in an agreement with previous studies [98]. A multilayer mode of α-Al_2O_3 adsorption could well explain why the BSA adsorption isotherm on α-Al_2O_3 surface is fit better with the Freundlich model (r^2 = 0.981) than with the Langmuir model (r^2 = 0.860) in Table 2, because the Langmuir model is for a homogeneous monolayer adsorption [111]. Therefore, the significantly
higher adsorption maximum of $\alpha$-Al$_2$O$_3$ normalized by surface area could also be due to the less surface area each BSA molecule occupied by multilayer mode.

### 3.2.3.2 FTIR Spectroscopy of BSA-Coated Oxide Particles

The FTIR spectra of BSA and bound-BSA on oxide particles are presented in Figure 12. No FTIR spectrum of bound-BSA on SiO$_2$ BPs was obtained due to the insignificant BSA adsorption. The spectra of bound BSA were obtained by subtraction of the IR spectra of pure oxide particles from that of the BSA-coated oxide particles after freeze-drying. Although freeze-drying may change the primitive structure of BSA molecules because of dehydration, the effect of freeze-drying may be negligible for this current work because all IR spectra were processed the same. Peak positions in the FTIR spectra shown in Figure 12 are listed in Table 4 with their assignments based on the published results [112-120]. The peak intensity of band at 1668 cm$^{-1}$ can be assigned to Amide I (C=O stretching vibration), which is the most widely used single band in studies of protein secondary structure, because it represents the backbone of protein molecules [121, 122]. Other major bands of BSA spectra can be assigned in the regions: 1542 cm$^{-1}$ Amide II band (N-H bending vibration mainly, coupled to C=O and C=C stretching), 1456 cm$^{-1}$ (CH$_2$ and CH$_3$ groups), 1401 cm$^{-1}$ (COOH groups), 1306 cm$^{-1}$ ($\alpha$-helix or N-H bending vibration), 1242 cm$^{-1}$ ($\beta$-sheet), 2888 cm$^{-1}$ (CH$_2$ stretching), 2968 cm$^{-1}$ (CH$_3$ stretching), 3070 cm$^{-1}$ Amide A or B (N-H stretching in the Fermi resonance with 2$^*$Amide II overtone) and 3312 cm$^{-1}$ (O-H and N-H stretching vibration).
By comparison with the spectra of BSA molecule as shown in Figure 12, the observation of dramatic peak intensity of bands at 1668 (Amide I) and 1542 cm\(^{-1}\) (Amide II) indicated that the primary structure of BSA molecules adsorbed on oxide particle surfaces. The increase in the ratios of Amide I/Amide II (Table 5), which indicates the conformational change of protein backbone structure, may result from the interaction of N-H group with the hydrophilic film of oxide particle [69]. Strong interactions of BSA COOH group with TiO\(_2\) BPs and NPs, as well as Al\(_2\)O\(_3\) BPs, may be responsible for BSA adsorption due to the dramatically diminishing of the peak at 1401 cm\(^{-1}\) [89]. Significantly \(\beta\)-sheet structural modifications of BSA molecule adsorbed on TiO\(_2\) and SiO\(_2\) NPs surfaces were observed due to the diminishing of the peak at 1242 cm\(^{-1}\). The peak intensity of band at 3312 cm\(^{-1}\) diminished markedly for both TiO\(_2\) NPs and BPs and Al\(_2\)O\(_3\) BPs bound BSA, indicating the weakening or even breaking of hydrogen bonds of O-H and N-H groups [115]. This weakening of hydrogen bonds would reflect the ligand exchange between hydroxyl groups on TiO\(_2\) NPs and BPs with Al\(_2\)O\(_3\) BPs surfaces and BSA carboxyl/hydroxyl functional groups [89]. This ligand exchange was supported by the large decreasing of the peak intensity of hydroxyl groups at 3000 – 3700 cm\(^{-1}\), as well as negative peak of carboxyl groups at 1401 cm\(^{-1}\). In addition, ligand exchange between hydroxyl groups on all oxide particles with BSA COOH groups would occur as shown by the reduction of the COOH/Amide I intensity ratio (Table 5).
**FIGURE 12.** FTIR spectra of BSA (a) and oxide-particle-bound BSA: TiO$_2$ BPs (b), TiO$_2$ NPs (c), SiO$_2$ NPs (d), $\alpha$-Al$_2$O$_3$ NPs (e), $\gamma$-Al$_2$O$_3$ NPs (f) and Al$_2$O$_3$ BPs (g). The differential spectra of BSA-bound oxide particles were obtained by subtraction of the IR spectra of pure oxide particles from that of the BSA-coated oxide particles after freeze-drying.
<table>
<thead>
<tr>
<th>Wavenumber, cm(^{-1})</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1668</td>
<td>Amide I (C=O stretching) [112, 115, 116]</td>
</tr>
<tr>
<td></td>
<td>Amide II (N-H bending vibration mainly, coupled to C=O and C=C stretching) [113, 115, 116]</td>
</tr>
<tr>
<td>1542</td>
<td>CH(_2) and CH(_3) [114, 116]</td>
</tr>
<tr>
<td>1456</td>
<td>COOH [114]</td>
</tr>
<tr>
<td>1401</td>
<td>(\alpha)-helix or N-H bending vibration [117]</td>
</tr>
<tr>
<td>1242</td>
<td>(\beta)-sheet [117]</td>
</tr>
<tr>
<td>2888</td>
<td>CH(_2) stretching [115]</td>
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<tr>
<td>2968</td>
<td>CH(_3) stretching [115]</td>
</tr>
<tr>
<td>3070</td>
<td>Amide A or B (N-H stretching in the Fermi resonance with 2 folds Amide II overtone) [118-120]</td>
</tr>
<tr>
<td>3312</td>
<td>O-H and N-H stretching vibration [115]</td>
</tr>
</tbody>
</table>
### TABLE 5. Ratios of Amide I/Amide II and COOH/Amide I of Oxide-bound BSA

<table>
<thead>
<tr>
<th>Samples</th>
<th>Ratio of Amide I/Amide II</th>
<th>Ratio of COOH/Amide I</th>
</tr>
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<tr>
<td>BSA</td>
<td>0.989</td>
<td>0.878</td>
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<td>bulk TiO$_2$</td>
<td>1.571</td>
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</tr>
<tr>
<td>nano TiO$_2$</td>
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</tr>
<tr>
<td>nano SiO$_2$</td>
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<td>0.420</td>
</tr>
<tr>
<td>nano $\alpha$-Al$_2$O$_3$</td>
<td>1.181</td>
<td>0.412</td>
</tr>
<tr>
<td>nano $\gamma$-Al$_2$O$_3$</td>
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</tr>
<tr>
<td>bulk Al$_2$O$_3$</td>
<td>1.651</td>
<td>0.280</td>
</tr>
</tbody>
</table>

### 3.2.4 Environmental Implications and Prospective

BSA adsorption by oxide particles depended on three factors: (1) larger surface area of particles could provide more space for BSA adsorption; (2) strong interaction (electrostatic attraction or ligand exchange) could induce the BSA adsorption; and (3) lower hydrophilicity on particle surfaces could allow the BSA molecules to approach their surfaces more easily than on highly hydrophilic surfaces.

Primary structures of BSA molecules were adsorbed and changed on oxide particle surfaces, while ligand exchange between hydroxyl groups on all selected oxide particles with BSA COOH groups occurred. High BSA adsorption by oxide NPs was attributed to
their large surface area. The low BSA adsorption by \(\alpha\)-Al\(_2\)O\(_3\) was due to its small surface area, though its surface hydrophilicity was low and its surface charge was neutral.

Implications can be drawn from the results listed above. First, high protein adsorption by oxide NPs (except \(\gamma\)-Al\(_2\)O\(_3\)) suggested that more protein molecules may be damaged in their function and structures by oxide NPs than oxide BPs in vivo, resulting in the potential higher toxicity to cells of living beings. Second, aggregation of oxide particles, which would influence protein adsorption, should be considered in further studies [2].

We did not discuss the effects of aggregation in this work because the main task of this work was to compare the protein-adsorption behaviors of different types of oxide particles (TiO\(_2\), SiO\(_2\), and Al\(_2\)O\(_3\)). Particle aggregation would influence the particle size and actual surface area in water (i.e. available sites), and affect protein adsorption by oxide particles. Therefore, effects of aggregation in protein adsorption would be considered in the future in two aspects: 1) comparing protein adsorption behaviors of the same oxide particles in different aggregated states; and 2) comparing protein adsorption behaviors of different types of oxide particles in a same aggregated size. Aggregated size should be used instead of individual-particle size because oxide particles hardly remain in the individual-particle state in aqueous solution in the presence of electrolytes.

In this work, one question has not been resolved yet: whether the oxide BPs lead to more protein-conformational change than NPs is still unknown. Particle-aggregation should also be involved in answering this question, because bigger particles, with larger surface area on a single particle, will lead to greater protein-conformational change than would be expected for smaller particles. Therefore, the comparison of protein conformational change should also be performed in the same state of aggregation. But
our IR spectra of protein molecules after adsorption indicate only that protein was adsorbed on oxide particles and primary structure of protein molecules changed. Hence, secondary derivative analysis of IR spectra and circular dichroism spectra, both of which could provide quantified information on BSA conformational change, should be employed in the future. Furthermore, it is unknown whether the conformational change of protein molecules is irreversible, when protein molecules are adsorbed on particle surfaces. Irreversibility of protein-secondary structure cannot be examined while protein molecules are still adsorbed on oxide surfaces. Therefore, conformational change of protein molecules should be examined after desorption from particle surfaces. Moreover, since the biological response of living beings depends on the type, amount, and conformation of adsorbed proteins, adsorption of different types of proteins should be examined for their adsorption quantity and conformational changes by NPs [8, 9]. All of these questions should be answered in the future.
REFERENCES


