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Influence of Phosphate on the Adsorption/Desorption of Bovine Serum Albumin on Nano and Bulk Oxide Particles

Lei Song
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Influence of Phosphate on the Adsorption/Desorption of Bovine Serum Albumin on Nano and Bulk Oxide Particles

A Dissertation 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

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May 2012

Department of Plant, Soil and Insect Science
INFLUENCE OF PHOSPHATE ON THE ADSORPTION/DESORPTION OF BOVINE SERUM ALBUMIN ON NANO AND BULK OXIDE PARTICLES

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Lei Song

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ABSTRACT

INFLUENCE OF PHOSPHATE ON THE ADSORPTION/DESORPTION OF BOVINE SERUM ALBUMIN ON NANO AND BULK OXIDE PARTICLES

MAY 2012

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Directed by: Professor Baoshan Xing

This work consists of four sections: 1) the adsorption behavior of bovine serum albumin (BSA) by three types of oxide nanoparticles (NPs), TiO$_2$ (50 ± 5 nm), SiO$_2$ (30 ± 5 nm), and Al$_2$O$_3$ (150 ± 5 nm for α type and 60 ± 5 nm for γ type) in deionized water; 2) phosphate adsorption on these oxide NPs and bulk particles (BPs); 3) influence of phosphate ions on BSA adsorption; and 4) BSA desorption from oxide NPs in phosphate solution. BPs were also used for comparison with NPs. For BSA adsorption in deionized water, the adsorption maxima on oxide particles are controlled by the surface area and hydrogen content, while the adsorption process is primarily induced by electrostatic interaction, hydrophobic interaction, and ligand exchange between BSA and oxide surfaces. With increasing of hydrogen content, the BSA adsorption mechanism switches from a mainly hydrophobic interaction to hydrogen bonding and ligand exchange. Calculations based on surface area and BSA size, suggest that a multilayer of BSA covers α-Al$_2$O$_3$, but only a single layer surrounds the other oxide particle surfaces. BPs lead to greater conformational change of BSA molecules after their adsorption on the surfaces of oxide particles, although NPs adsorbed more BSA than BPs by weight. For phosphate,
the adsorption process is mainly governed by the surface charge of the oxides. Strong electrostatic repulsion can prevent the adsorption of phosphate ions on an oxide surface. Meanwhile, a good linear relationship was observed between surface-normalized BSA adsorption maxima and surface charge of the oxides. For the influence of phosphate ions on BSA adsorption, BSA adsorption is suppressed by phosphate ions, while BSA molecules have no influence on phosphate adsorption. The competition between BSA molecules and phosphate ions is regulated by electrostatic interaction, the hydrogen content of the oxides and oxide surface area (especially micropore surface area). The difference of influence between hydrophobic and hydrophilic interactions on BSA adsorption reduces with the increase of phosphate concentration. Moreover, quantification was employed to calculate the displacing amount of phosphate ions to BSA molecules in competition. The displacing amount of phosphate ions is regulated by micropore surface area, and shows a good linearity with the hydrogen content. For BSA desorption, the BSA desorption hysteresis is observed for SiO$_2$ NPs due to the high aggregation of this type of NPs. The aggregation of NPs can entrap BSA molecules in the closed interstitial spaces, leading to the BSA desorption hysteresis. For $\alpha$-Al$_2$O$_3$ and $\gamma$-Al$_2$O$_3$ NPs, the hysteresis is observed only at low BSA concentration due to the influence of BSA molecules and electrostatic repulsion to the suspension of NPs. For TiO$_2$ NPs, no significant hysteresis is observed because of their low aggregation and strong electrostatic repulsion. Phosphate adsorbed amounts remain unchanged within the adsorption and two-cycle desorption, indicating the entrapped BSA molecules may not bond to the oxide NPs.
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CHAPTER 1
INTRODUCTION

Nanomaterials are materials whose dimensions are found on the nanometer scale. Commercial applications of nanomaterials are currently available or will soon appear in such sectors of the world economy, as consumer products, health care, transportation, energy and agriculture (Table 1) [1]. The Consumer Products Inventory of the Woodrow Wilson International Center for Scholars reported that the nanotechnology consumer products inventory contains 1317 products or product lines as of March 10, 2011. Compared with March 2006, the inventory has grown by nearly 521% (from 212 to 1317 products).

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Because of their extensive production and widespread potential commercial uses, manufactured nanoparticles (NPs) will inevitably enter the environment [2]. According to the Consumer Products Inventory of the Woodrow Wilson, within the 1317 products, 56% (a total of 738 products) are in the category of health and fitness. Safety concerns rise from the deep connection between these new nanomaterials and everyday human life.

An EPA nanotechnology white paper [3] sorts nanomaterials into four types:

(1) Carbon-based materials. The composition of these nanomaterials consists mostly of carbon in its common forms of a hollow spheres, ellipsoids or tubes. Spherical and ellipsoidal carbon nanomaterials are 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 numerous branched units, which can be tailored to perform specific chemical functions.

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

Within all types of nanomaterials, inorganic oxide NPs, such as TiO₂, SiO₂, and Al₂O₃, have extensive applications in pigments, sunscreens, food colorants, wastewater treatment reactors, semiconductors, electrical insulators, and biomedical areas [4]. Potential toxicological concern comes from the fact that NPs can be transported into the blood or across cell membranes into cells [5, 6]. Protein is an important component of all living organisms, and has various cellular functions, such as biochemical catalysts, energy sources, molecular messengers, structural components and transport vehicles [7].
It is believed that NPs are covered by protein molecules immediately in a physiological environment, resulting in a protein corona on the particle surface, and the modifications to protein molecular structures [8-11]. Adsorption of proteins on solid surface in the body may induce changes in their structures and functions, or even in the entire protein molecule itself [10, 11]. Hence, protein adsorption could result in adhesion, proliferation, and differentiation of cells, as well as affecting foreign body response and the inflammatory processes [12, 13].

Previous studies have reported that conformational changes of protein molecules occur 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 [14]. Secondary structure conformational changes of human carbonic anhydrase protein after its sorption on SiO$_2$ NPs was also reported [15]. In addition, a “side-on” adsorption mode of bovine serum albumin (BSA) on Al$_2$O$_3$ NPs surfaces was explored [16]. However, most previous studies were performed in phosphate buffer, which might interfere with the adsorption and conformational change of the protein molecules on NPs surfaces that is caused by possible competitive adsorption between buffer ions and protein molecules. It was reported that BSA adsorption to microcrystalline boehmite (PT-A) was significantly obstructed by the coexistence of phosphate (p < 0.01), yet phosphate adsorption to PT-A was unchanged regardless of the presence or absence of BSA [17]. Adsorbed BSA would also be competitively desorbed from hydroxyapatite coatings in the presence of phosphate ions [18]. These phenomena were explained by the faster diffusion velocity of phosphate, which is in inclined to cover on the surface of sorbent, thereby increasing the electrostatic
repulsion between protein and sorbent [17, 18]. Moreover, the adsorption amount of BSA increased rapidly when NaCl concentration was raised, indicating the effect that electrolyte strength has on protein adsorption [18]. Therefore, competitive adsorption between BSA molecules and phosphate ions onto oxide particle surface seems likely. However, the adsorption mechanism of protein, which is governed by hydrophobic interaction, surface area and surface chemical groups, has not been discussed in depth as of yet [19, 20]. Therefore, a further investigation of all possible mechanisms of competitive adsorption between protein and phosphate is necessary. The knowledge of these results will provide a valuable platform for studies of the toxicity of NPs in vivo, as well as the environmental implications of NPs.

In this work, TiO$_2$, SiO$_2$ and Al$_2$O$_3$ NPs were selected because of their extensive applications in pigments, sunscreens, food colorants, wastewater treatment reactors, semiconductors, electrical insulators, cosmetic additives, and biomedical areas [21-23]. In addition, respective BPs are chosen as a comparison for particle size and surface area influence. BSA was selected as the model protein because it is a major soluble protein in plasma of the circulatory system [24], and transports and deposits endogenous and exogenous substances in living beings [25, 26]. Owing to its physiological properties, purification, and stability in biochemical reactions, BSA is widely used as a model globular protein [27, 28]. Albumin protein is classed as a soft protein due to its relatively flexible structure, which can readily undergo conformational changes [29]. A three dimensional image of the serum albumin molecule is shown in Figure 1. BSA protein has high purification and stability in biochemical reactions [28]. BSA is composed of 604 amino acids with a molecular weight of 66462 g/mol [30, 31]. Generally, the BSA
molecule consists of 55 – 65% α-helices according to various methods of measurement, 21% β-sheet, and the rest are turns [32-34]. However, the components of BSA’s secondary structures vary slightly over a range of pH [35]: in the pH range 4.3 to 8.0, BSA keeps a triangular or heart-shaped structure as the normal form, with 53% α-helix structure and the remainder being β-sheet and turns [31]; once the pH falls below 4.3 the molecule unfolds into the fast form with a 45% α-helix; an even lower pH of below 2.7 causes further unfolding with a 35% α-helix [36]. It is believed that the reduction of the α-helical structure results from an unfolding of the domains, with consequent loss of intradomain helicity [31]. 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 that has 47% α-helix [37].

Figure 1. A three dimensional image of the serum albumin molecule with the α-helix structure colored in red and the loop in green. (A) is the side view; (B) is the front view. This figure is from Protein Database 1AO6, 1999.
CHAPTER 2
LITERATURE REVIEW

2.1 Cytotoxicity of Oxide NPs

Since engineered oxide NPs eventually will enter the environment, detailed knowledge of the effects of oxide NPs on organisms is of great interest. The most widely used test subjects for cytotoxicity of NPs are cells and bacteria [4, 38-40]. Cells, which are the structural and functional units of all known living organisms, are the smallest units of an organism that is classified as living [41]. Bacteria, which are single-celled organisms, are also good test models with which to study the toxicity of NPs and to examine how NPs affect cell function. In addition, bacteria perform many critical roles in ecosystem function and productivity. Therefore, cells and bacteria are widely employed in cytotoxicity studies of oxide NPs, of which there have been many.

2.1.1 Cytotoxicity to Cell

Because endothelial cells line the inner surface of blood vessels, in certain circumstances, they may come into direct contact with NPs [38]. Impairment of cell proliferative activity and chronic inflammation were reported when endothelial cells were exposed to SiO$_2$ NPs in one study [38]. Furthermore, TiO$_2$ (20 – 160 nm) and SiO$_2$ (4 – 40 nm) NPs were internalized in those cells by vacuoles [38].

The cytotoxicity of homogeneous and weakly aggregated TiO$_2$ NPs were evaluated with their effect on mouse fibroblast cells in aqueous solution [39]. The cells were found to become more round and shrinked; and they formed aggregates which could
be rinsed off easily, as the concentration of TiO$_2$ NPs was increased. Transmission electron microscopy (TEM) revealed that the number of lysosomes increased and some cytosplasmic organelles were damaged in a cell-culture medium containing 300 mg/L TiO$_2$. Then, the incorporation of TiO$_2$ NPs into cells was explored. First, TiO$_2$ NPs were endocytosed from the extracellular fluid when cells were exposed to TiO$_2$ NPs. Second, a portion of the plasma membrane was invaginated and pinched off to form a membrane-bound vesicle, containing the TiO$_2$ NPs. Third, these vesicles were fused with lysosomes to form secondary lysosomes [39]. These uncontrolled lysosomes led to damage and destruction of organelles, such as discontinuity of the endoplasmic reticulum and the disappearance of cell organelles [39].

After the discovery of the utility of TiO$_2$ as a photocatalytic compound applied to waste water disinfection [42] and to the photodynamic therapy of certain cancers [43], many studies of the phototoxicity and photogenotoxicity of TiO$_2$ were performed [44-46]. These studies assumed the cytotoxicity, apoptosis and inflammation responses caused by TiO$_2$ NPs on various cell types such as mesenchymal stem cells [47], lymphoblastoid cells [48], alveolar epithelial cells of the lung [49, 50], alveolar macrophages [51], phagocytes [52], osteoblast [53], mouse fibroblast cells [39], human bronchial epithelial cells [54] and cellular microtubule protein [5]. Destruction of cells by oxidative stress and reactive oxygen species (ROS), mainly hydroxyl radicals, superoxide ions, and hydrogen peroxide in aqueous phase [55-57], was observed when cells were exposed to TiO$_2$ NPs and UV radiation. The ROS impaired the cell membrane architecture through lipid peroxidation [55, 58, 59]. The generation of ROS has been studied as well. The electrons of TiO$_2$ particles were excited by light moving them from the valence band to the
conduction band, generating positive holes at the valence band and reacting with water to generate hydroxyl radicals in the vicinity of the TiO\textsubscript{2} surfaces [60].

On the one hand, a positive correlation between photocatalytic ROS production and antibacterial activity has been reported (see below) [61]. On the other hand, conflicting data has emerged on whether TiO\textsubscript{2} NPs are harmful to cells in the absence of photo-activation from UV radiation. Despite previous studies suggesting the harmlessness of TiO\textsubscript{2} NPs for animal and human cells in the absence of UV radiation [62-64], a steady increase in studies reporting the harmfulness of TiO\textsubscript{2} NPs has since emerged. TiO\textsubscript{2} NPs (75 nm) in darkness can induce significant cytotoxicity on human bronchial epithelial cells, for instance, possibly through apoptotic pathways [54]. Characteristic apoptotic bodies within nuclei were clearly observed after exposure to TiO\textsubscript{2} NPs in darkness, while phosphatidylserine translocation, another apoptosis characteristic, was observed from the inner to the outer leaflet of the cell membrane, further corroborating TiO\textsubscript{2}-induced cytotoxicity [54]. Similar investigations on TiO\textsubscript{2} cytotoxicity reported that the influence of 40 nm TiO\textsubscript{2} NPs (without UV) on the neuroblatoma-2A cell line lead to a significant reduction in cellular viability that increased with TiO\textsubscript{2} concentrations [65].

Studies of the cytotoxicity of Al\textsubscript{2}O\textsubscript{3}, which is estimated to account for approximately 20% of the 2005 world market of NPs [66], are less abundant than the 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 Al\textsubscript{2}O\textsubscript{3} at various concentrations over different lengths of
time [22]. Furthermore, human endothelial cells expressed increased adhesion of activated monocytes when treated with Al₂O₃ NPs [22]. No significant increases in ROS production were observed, suggesting that certain metal oxide NPs (like Al₂O₃ NPs) cannot significantly promote ROS formation upon internalization into cells [22, 52, 67, 68]. As shown above, the impairment of cellular organelles and the inflammation of cells by contact with some oxide NPs have been abundantly reported. So do the studies on the cytotoxicity of oxide NPs to bacteria.

2.1.2 Cytotoxicity to Bacteria

The eco-toxicity of water-suspended TiO₂ and SiO₂ NPs were investigated by using Gram-positive Bacillus subtilis and Gram-negative Escherichia coli as test organisms [4]. The antibacterial activity of these two photosensitive NPs increased at higher particle concentrations, with the TiO₂ NPs are more toxic than the SiO₂ NPs at a same concentration [4]. Moreover, the Gram-positive B. subtilis was more sensitive to these NPs than the Gram-negative E. coli., which was attributed to the ability of B. subtilis to form spores and its cell wall structure [69]. The antibacterial activity of TiO₂ towards both bacterial species was significantly greater in the presence of light than in the dark [4].

The toxicity of TiO₂, SiO₂, and Al₂O₃ NPs to B. subtilis, E. coli and Pseudomonas fluorescens was examined and compared to that of their respective bulk counterparts [40]. Al₂O₃ and SiO₂ NPs were toxic to the tested bacteria, while their BPs showed no or lower toxicity, indicating the particle size did cause a toxicity difference. TiO₂ NPs did not affect bacterial populations, which is different from the previous results [4]. TEM images
Figure 2) provide intuitive evidence for the difference in toxicity of these NPs: SiO$_2$ and Al$_2$O$_3$ NPs coated the whole bacterial cell surface, while TiO$_2$ NPs coated very little of the bacterial surface [40].

Early in 1985, Matsunaga et al. [70] reported that Lactobacillus acidophilus, Saccharomyces cerevisiae, and Escherichia coli were completely sterilized when incubated with TiO$_2$ NPs under metal halide lamp irradiation for 60 – 120 min. However, this work lacked more extensive data on the bactericidal properties of TiO$_2$ photocatalysts. Therefore, a more systematic investigation of the bactericidal activity of TiO$_2$ NPs were conducted on the irradiation of Escherichia coli and TiO$_2$ NPs (anatase) with UV-visible light of wavelengths longer than 380 nm [61]. At a dose of 1 g/L TiO$_2$ NPs under constant illumination, complete killing of the bacteria was apparent in less than 30 minutes, while no significant decrease in the bacterial population was observed without illumination. Moreover, the proportion of surviving bacteria decreased with increasing O$_2$ composition of the gas flowing through the suspension, as well as with an increase in the TiO$_2$ dose. This also suggested that ROS generated by TiO$_2$ NPs was responsible for the bactericidal activity [61]. Another interesting result was that the decrease of Escherichia coli continued in the presence of TiO$_2$ NPs without illumination, and no regrowth was observed within the following 60 hours, suggesting that the induced injury of the bacteria was irreversible [69]. This “residual disinfecting effect” of the photocatalytic process of TiO$_2$ NPs was viewed as the most interesting post-irradiation event.
Figure 2. TEM images revealing attachment of NPs to the surface of P. fluorescens: (A) Al₂O₃, (B) SiO₂, (C) TiO₂, (D) ZnO. This figure is from Jiang et al. [40].

Since the cytotoxicity of oxide NPs to living cells and bacteria have been widely reported, current studies focus on the cell-damage mechanism, involving components of cells or bacteria, such as DNA [48, 71] and proteins [29, 72, 73]. Before reviewing the existing studies of protein sorption onto NPs, an important effect, the aggregation of NPs, will be discussed since aggregation could greatly influence the cytotoxicity of oxide NPs.
2.1.3 Impact of Particle Size on Cytotoxicity

The impact of particle size can be separated into two types: aggregated-particle-size effects and original-particle-size effects. The aggregation properties of NPs have a significant impact on the cytotoxicity [39, 74-76]. NPs can aggregate into larger hydrodynamic-diameter bulks. For example, TiO$_2$ NPs showed much poorer dispersion when suspended in cell culture media without any dispersing agents [77]. 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 precipitates, which are all larger than any cellular components in size and cannot be easily engulfed by cellular membranes, instead of mono-dispersed NPs [39]. In this case, the cytotoxicity of NPs was attributed to their physicochemical characteristics (i.e. aggregation) rather than their original particle size [39]. But for some studies, whose focus did not include the aggregation influence, weakly aggregated NPs were used in order to reduce the effect of aggregation [39].

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* [4]. No significant difference in toxicity among these NPs was observed, mainly due to the aggregation of particles in suspension leading to similar effective particle-size [4]. Although other external factors, including light intensity, surface chemistry, particle morphology and bacterial density could also have influenced the antibacterial activity of those NPs as they reported [4], the influence of those external factors was minimized when all the samples were performed under the same experimental conditions (e.g. under the same light intensity and bacterial concentration). Therefore, aggregation of particles in suspension should be explored.
The impact of original particle size was mostly considered in weakly or rarely aggregated NPs. For example, the cytotoxicity of SiO$_2$ NPs was reported to depend on the size of the NPs [21]. 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 [21]. The smaller SiO$_2$ NPs exhibited stronger cytotoxicity. Enlargement of the cell body was reported, which was apparently due to the presence of SiO$_2$ NPs that obstructed cell division. Coagulation of cells with incomplete division was also observed, and several amorphous structures appeared in the cells that were exposed to 5 nm SiO$_2$ NPs [21]. 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. 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 [78].

2.2 Protein Adsorption

As mentioned at the beginning, potential toxicological concern comes from the fact that NPs can be transported into the blood or across cell membranes into cells [5, 6]. Subsequently, NPs may interact with proteins in the blood or cytoplasmic proteins. Protein adsorption on a solid surface may induce changes in its structures and functions, even the entire protein molecule [10, 11]. Hence, protein adsorption can result in adhesion, proliferation, and/or the differentiation of cells, as well as affecting foreign body response and inflammatory processes [12, 13].
The interaction among three items (BSA protein, phosphate ions, and oxide NPs) was studied in this work. In previous studies, any two of the three items have been discussed: BSA adsorption on NPs, BSA bonding to phosphate ions (or anions), and phosphate ions adsorption on NPs. Competitive adsorption of BSA and phosphate ions onto oxide NPs has been discussed only rarely. In this section of our Literature Review chapter, previous work is discussed in the following order: protein adsorption on NPs, protein bonding to anions, phosphate adsorption to NPs, and competitive adsorption between protein and anions.

2.2.1 Protein Adsorption on NPs

Generally, sorption of protein molecules is governed by Columbic forces, van der Waals forces, hydrophobic interactions, and the protein conformational stability, while the surface area provides the possible sorption sites [19, 20]. Three theories of protein adsorption have been proposed in the literature: random sequential adsorption (RSA), diffusion random sequential adsorption (DRSA), and Lattice theory [79-81].

RSA theory assumes that protein molecules are hard spheres and protein adsorption is an irreversible process [79]. Protein molecules adsorb onto random positions of the solid surface sequentially, leading to a monolayer cover on particle surfaces. This process would continue until the surfaces of particles are fully covered [79]. RSA is often not practical because the surfaces of particles are not uniform, 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 [80]. The advantage of this model is that it considers the interaction between the adsorbed and the diffusing protein molecules, which can be applied to explain the adsorption behaviors of globular proteins on solid surfaces [80]. Lattice theory is a mathematical approach which is applicable to rigid proteins that undergo orientational changes upon adsorption [81]. 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 the end-on state, the protein is weakly bound to the surface, while in the side-on state the bonding is firmer [81]. 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-shaped proteins with side-on and end-on modes.

Based on an examination of the literature, some characteristics of protein adsorbed on oxide NPs can include:
(1) Electrostatic interaction is one of the main driving forces, and it is related to solution pH [82-84]. 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 TiO₂ particle surfaces [82]. Even when the overall charges of both protein and TiO₂ surfaces have the same sign, electrostatic attraction can still drive the adsorption. This is due to different regions of protein surface carrying different signs of charge, either positive or negative charge, which could attract oppositely charged particle surfaces [82, 83, 85]. A schematic diagram (Figure 4) of charged side-chains on a protein molecule illustrates that a protein has a potentially very substantial number of positively charged residues at pH levels in the vicinity of 7.0. The surface charges of both the protein and oxide particles are largely dependent on the pH of the solution. For example, human serum albumin (HSA) and TiO₂ are both positively charged at pH below 4.7, and both are negatively charged at pH above 6.0 [84]. In the pH range of 4.7 to 6.0, the overall surface charge of HSA is negative, while the overall surface charge of TiO₂ is positive [84]. 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 [84].

![Figure 4. Schematic diagram of charged side-chains on a protein molecule [85].](image-url)
In the widely-tested pH range, the maximum adsorption of a protein occurs at the protein isoelectric point (IEP) [84]. A compact protein monolayer with minimum lateral protein-protein interaction and maximum protein adsorption is formed on the TiO$_2$ surface [84]. When the pH approaches the IEP of protein, electrostatic effects are not the dominant factor in the adsorption process; instead hydration effects take on that role. The charge of the protein molecule and the degree of hydration decrease, allowing short-range attractive forces to come into play [86]. This results in the protein being directly adsorbed onto the NPs surface by bonding between the carboxyl acid groups on the BSA and the hydroxyl groups on the TiO$_2$ particle surface [87]. Beyond the IEP effect on protein molecules, there is another variable: the particle and protein molecule surfaces may carry the same sign or the 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 [88]; in the pH region where the two surfaces carry opposite charges, electrostatic attraction weakens the stability of the protein structure, causing the protein to unfold and to spread out on the TiO$_2$ surface, and thus allowing a small amount of protein to saturate the particle surface [89, 90]. Wassell *et al.* provided a more specific explanation [86]. At low BSA concentrations, the surface is not saturated and the adsorbed molecules are in random orientations on the surface. At greater than 50% coverage, lateral interactions between the molecules become important, which may order the adsorbed protein, and hence the surface has room for more protein to adsorb [86].

Previous studies also agree with the explanation above [84, 91-93]. For example, adsorption of chicken egg lysozyme on SiO$_2$ NPs decreased as the pH dropped [92],
which is due to the protein-protein electrostatic repulsion at a lower pH when lysozyme molecules bear higher positive charges [91]. However, the repulsion would only be important at high protein adsorption levels and would likely be compensated by stronger protein-silica attraction [92]. Another possible explanation is that the pH induces a change of zeta potential for the SiO₂, which leads to decreased Coulombic attraction between lysozyme and SiO₂ at lower pH [93].

In summary, particle surface charge, which largely depends on the pH of the aqueous solutions, can influence protein adsorption and should be considered in relevant studies. Moreover, most oxide particles have abundant hydroxyl groups on their surfaces [94], which may change the solution pH upon contact and may affect protein-adsorption behavior as well. Therefore, pH adjustment is required during protein adsorption in order to maintain a neutral pH. The relationship between the protein-adsorption maximum and the amount of hydroxyl groups on the particle surfaces was unknown until this point, but it will be discussed below.

(2) Protein adsorption is influenced by the curvature of NPs. Vertegel et al. reported that greater adsorption of chicken egg lysozyme on SiO₂ NPs was observed with larger diameter SiO₂ NPs [92]. Extrapolating from the results of Vertegel, one question is whether SiO₂ 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 evaluated in their comparison study. A competitive sorption may exist between protein molecules and phosphate ions, which may influence the single protein-particle adsorption behaviors. Therefore, one of the
tasks in this work will be to compare the protein adsorption between NPs and BPs in deionized water.

Furthermore, Vertegel et al. 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 [92]. 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 in this work.

(3) Protein adsorption can be reduced or increased by certain ions. Bridging effects of divalent cations, such as calcium and magnesium, can boost protein adsorption on minerals [86, 95]. The surface charge of TiO$_2$ becomes less negative in the presence of calcium ions, which is probably due to calcium bridging between the $–$COO$^-$ group of BSA and acidic hydroxyl group of the titanium [86]. Figure 5 is a schematic diagram depicting electrostatic adsorption of HSA molecules onto a TiO$_2$ surface [86, 95]. Divalent cations of calcium and magnesium are shown bridging the negative albumin molecule and the TiO$_2$ surface. Bridging is impossible in the presence of monovalent potassium cations [86, 95]. However, do Serro et al. reported that the adsorption of BSA onto TiO$_2$ surfaces was reduced in the presence of calcium and phosphate ions (Hanks’ balanced salt solution) [96]. They explained that the stable, hydrophilic film induced by these ions that forms on the TiO$_2$ surface inhibited the protein adsorption [96]. An opposite result might come from adding phosphate ions: it was hypothesized that the Ca$^{2+}$
bridging effect would be diminished with an increase of phosphate ions, the latter having a much higher affinity than the carboxyl groups of BSA for Ca\(^{2+}\) \[77, 97\]. Phosphate is widely used in biological experiments as a pH buffer. Most previous studies of protein adsorption used phosphate buffer \[15, 96, 98, 99\], which could affect the protein adsorption by changing the surface hydrophilicity of oxide particles. More detailed information on this point will be reviewed in the next section.

![Diagram](image)

**Figure 5.** A schematic diagram depicting electrostatic adsorption of HSA to the TiO\(_2\) surface. Divalent cations of calcium and magnesium are seen bridging the negative albumin molecule and the TiO\(_2\) surface. Bridging is impossible in the presence of monovalent potassium cations \[95\].

(4) Hydrophobic and hydrophilic interaction can regulate the BSA adsorption behavior. Generally, the surface area of particles provides sites for adsorption, while hydrogen content, which is an indicator of surface hydrophilicity, represents the amount of hydroxyl groups or chemically bonded water molecules (i.e., hydroxylation layer) on particle surfaces \[10, 13\]. Basically, two types of non-bonded interactions occur in protein adsorption: long-range (or electrostatic) interaction and short-range (including...
hydrogen bonding and hydrophobic) interaction [27]. Previous studies reported that BSA adsorbed more strongly on hydrophobic surfaces due to hydrophobic interaction [28, 29], which is the attraction between the non-polar regions of BSA molecules and the hydrophobic surface [100]. However, opposite results were also published. For example, quantitative and qualitative evaluation of adsorption/desorption of BSA on hydrophilic and hydrophobic surfaces was studied by Jeyachandran et al. [100]. He reported the surface coverage of BSA molecules over hydrophobic and hydrophilic surfaces was the same (35% and 53% respectively) for both 30 minutes and 2 hours incubation time. But the coverage of BSA over a hydrophilic surface increased dramatically to 95% after 12 hours incubation time, while the coverage over a hydrophobic surface remained 48%, indicating the adsorbed BSA molecules are dispersed and are distributed uniformly on hydrophobic and hydrophilic surfaces [100]. This is because BSA molecules formed multiple point interactions with hydrophilic surfaces through hydrogen bonding, which interacted much more strongly than with hydrophobic surfaces, as shown in Figure 6. To be specific, BSA molecules interacted with hydrophobic surfaces through CH$_3$ groups, whereas on the hydrophilic surface, polar COOH groups participated in the interaction [100]. However, unfolding was not considered in Jeyachandran’s work. As seen in Figure 6, to satisfy the preference of hydrophobic interaction, unfolding of BSA molecules could occur when adsorbed on hydrophobic surfaces. Therefore, to fully understand the hydrophobic/hydrophilic interaction and their relation with protein molecule unfolding is attempted in this work.
2.2.2 Protein Bonding to Anions

The ability of crystallized proteins (in particular, serum albumin) to bind numerous organic and inorganic anions has been demonstrated repeatedly [101-104]. Specific ion effects are ubiquitous in protein research. Such effects exhibit a re-occurring trend called the Hofmeister series [105], which is generally more pronounced for anions than for cations. At the beginning, scientists believed that an ion’s influence on macromolecular properties was caused at least in part by ‘making’ or ‘breaking’ bulk water structure. However, more studies reported that the interaction between protein and anion is not through altering the hydrogen bonding network of anion’s first hydration shell [106]. For example, Klotz et al. reported that cationic residue of protein molecules largely determines the anion-binding ability of a protein, but their presence alone is not a sufficient condition for binding [107]. The “binding index” $\Sigma(NH^+) / \Sigma(COO^-) - (\Sigma(OH))$, devised by Klotz et al. and supported by experimental data, predicts whether a given protein would show a measurable affinity for anions [107]. A very strong case may be made for a preferential bond of the type $OH\cdots OOC$ rather than $HO\cdots NH^+$, due to the $OH\cdots O$ bond being stronger than the $O\cdots HN$ bond [107, 108]. The net result of this
interaction would be to decrease the number of COO\(^-\) and OH groups which can combine with the cationic NH\(^+\) loci, and hence to increase the number of free cationic nitrogen atoms, and consequently, the possibility of anion binding by the protein molecules [85, 107]. Figure 7 illustrates some hydrogen bonds between polar residues in a protein molecule and their corresponding dissociation energies, which clearly shows the preferential bond of OH\(\cdots\)OOC [85]. The mechanism of adsorption of phosphate with BSA has been studied previously. Basically, what the studies have shown is that protein molecules form strong complexes with phosphate ions. For example, Klotz et al. found that phosphate ions formed complexes with albumin [104]. Protein molecules can also form strong complexes with other small anions: the order of binding ability is NO\(_3^-\) > Cl\(^-\) > CH\(_3\)COO\(^-\) [104]. Dawra and colleagues reported that iron-induced lipid peroxidation was inhibited with increasing phosphate concentration, and that the effect was more pronounced in the presence of BSA [109]. This effect might be due to the binding of the iron to phosphate-protein complexes [109]. Most recently, Ninham et al. [110, 111] modified the Derjaguin-Landau-Verwey-Overbeek (DLVO) theory successfully and interpreted inter-particle interactions by treating colloid stability in terms of a balance of attractive van der Waals forces and repulsive electrical double-layer forces with a dispersion potential parameter [112]. This DLVO theory has much improved the mechanism of the interaction between protein molecules and anions.
2.2.3 Phosphate Adsorption on Oxides

2.2.3.1 Anion Adsorption

Adsorption mechanisms of anions to oxide minerals have been discovered over the past few decades. For example, Hingston et al. systematically studied the adsorption of anions onto oxide mineral surfaces, and their adsorption mechanism has been widely accepted and cited in related studies [113]. Their mechanism of anion adsorption can generally guide the study of phosphate adsorption. In Hingston’s work, the specific adsorption (ligand exchange) of an anion with fully dissociated acid and with incompletely dissociated acid were discussed [113]. The ligand exchange of the anion of a fully dissociated acid could only occur on a positively charged surface: little specific adsorption was observed at pH values higher than the point of zero charge (PZC) of the mineral surface [113]. Ligand exchange neutralized the positive sites of the surface until
a new zero point was reached. Thereafter, no further adsorption of the specifically adsorbed anion takes place. In addition, no evidence of anion adsorption on a neutral or negative surface has been reported. The ligand exchange of anions involves the formation of a coordination complex at the oxide surface [113]. However, with ligand exchange of incompletely dissociated acid, anion adsorption can occur at pH values higher than PZC. The undissociated acid molecules can be adsorbed as long as protons can be dissociated at the surface. The dissociated protons react with surface OH− groups of neutral sites to form water which is readily displaced by the anion [113].

2.2.3.2 Phosphate Ions Adsorption on TiO₂

Three adsorption processes were reported to take place on the oxide surface: ligand exchange, ion-dipolar interaction, and precipitation [114]. Ligand exchange from the solution is accompanied by an increase of pH, as hydroxyl ions are displaced. Anion molecules can penetrate into the ion-exchanger phase and interact with the dipoles of the titanium dioxide due to the ion-dipole interaction mechanism (Figure 8). Insoluble titanium phosphate precipitates on the surface of titanium dioxide after the dissolution of the sorbent. Additionally, Boehm et al. believe that phosphate is adsorbed on hydrated TiO₂ surfaces through a condensation process that forms Ti-O-PO₃H₂ groups [115]. H₂PO₄⁻, H₂PO₄²⁻ or both may adsorb by an exchange reaction with the basic hydroxyl groups: TiOH + H₂PO₄⁻ = TiH₂PO₄ + OH⁻ [86, 116]. Therefore, the bonding in these groups is of a transitional type between ionic and covalent.
Figure 8. Schematic illustration of ligand exchange and ion-dipole interaction mechanism on TiO$_2$ surface. Hydroxylation of TiO$_2$ surface (left); anion ligand exchange with hydroxyl group on TiO$_2$ surface (middle); ion-dipole interaction of hydroxyl group with anion (right).

2.2.3.3 Phosphate Ions Adsorption on SiO$_2$

The mechanism of phosphate adsorption on silica surface has been studied by Murashov et al. [117]. It is reported that the phosphate groups can form strong hydrogen-bonded complexes with the silanols of the silica surface (Figure 9). The silanol groups can partially deprotonate (pK$_a$ ranging from 6.8 to 7.1 [118]) in neutral to weakly alkaline condition so that the silica surface bears a negatively charged surface in aqueous solutions, which repels the anionic phosphate. Therefore, when positively charged ions (such as sodium ions) are present in solution, negatively charged silica surface and phosphate ions could be neutralized so that the formation of silica-phosphate complexes can be enhanced [117]. In this proposal, all experiments will be performed at pH = 7.0, so that the silanol groups on silica surface will be partially deprotonated. Therefore, it is probable that a negatively charged silica surface (preliminary results show the zeta potential of silica in deionized water and phosphate buffer solution is -30.8 and -41 mV, respectively) will repel negatively charged phosphate ions due to the electrostatic force.
Figure 9. Hydrogen-bond complexes Si(OH)$_4$-H$_2$PO$_4$- (left); partly deprotonated Si(OH)$_4$ group (middle); positively charged ions neutralized Si(OH)$_4$ group (right).

Dalas et al. reported that the adsorption of phosphate onto porous glass and SiO$_2$ particles depends on the ionic strength and experimental temperature [119]. Phosphate uptake is suppressed with increasing ionic strength, showing that electrostatic forces are significant in the adsorption process. Increasing the temperature from 25°C to 40°C resulted in a four-fold increase in the phosphate uptake at equilibrium. The adsorption equilibrium for both porous glass and SiO$_2$ particle surfaces can be established within 10 minutes, irrespective of the initial phosphate concentration in the experiment solution. Therefore, in this proposal, temperature will be kept at room temperature (25°C).

2.2.3.4 Phosphate Ions Adsorption on Al$_2$O$_3$

The ligand exchange reactions of phosphate adsorbed on hydrous alumina has been described by Helmy et al. [120]:

\[
[\text{OH}]_s^- + [\text{H}_2\text{PO}_4]_a^- \leftrightarrow [\text{H}_2\text{PO}_4]_s^- + [\text{OH}]_a^-
\]

\[
[\text{OH}]_s^- + [\text{HPO}_4]_a^{2-} \leftrightarrow [\text{HPO}_4]_s^- + [\text{OH}]_a^-
\]

where $s$ means solid phase and $a$ means aqueous phase. Hydroxyl groups on the alumina surface are displaced into solution so that the pH of the solution goes up, which
is in line with preliminary data obtained during the development of this proposal. Chen et al. [121] studied the adsorption of phosphate ions on alumina and kaolinite over a low phosphate concentration range ($6.2 \times 10^{-3}$ to 6.2 ppm). They reported that divalent cations can increase phosphate adsorption while amino acids and anions have essentially no effect. The phosphate concentration range used in their work is much lower compared with the concentration performed in this work (50 to 1800 ppm).

The adsorption process can also be affected by the electrostatic force, the complexing capacity of phosphate, and the properties of the particle surface. Stumm [122] concluded that the affinity of phosphate for aluminum oxides is affected by the complexing capacity of phosphate, which controls the process of binding to the surface through ligand-exchange reactions and the electrostatic force of the charged surface.

### 2.2.4 Competitive Adsorption between Protein and Phosphate

As mentioned in Chapter 1, most previous work was performed using a phosphate-buffered environment, which may interfere with the adsorption and conformational change of the protein molecules on oxide NPs surfaces caused by possible competitive adsorption between buffer ions and protein molecules. Similar studies have been conducted using other oxides rather than the selected oxide NPs in this work.

The mechanism of interaction between PT-A (microcrystalline boehmite) and phosphate in the presence of BSA has been discussed in a previous study [17]. PT-A was synthesized as an efficient phosphate adsorbent to replace aluminum hydroxide gel. Adsorption isotherms demonstrated that phosphate adsorption to PT-A was unchanged
regardless of the presence or absence of BSA, whereas BSA adsorption to PT-A was significantly obstructed by the presence of phosphate (p < 0.01) [17]. This indicates that phosphate, which is a smaller molecule and has a faster diffusion velocity than BSA, is first adsorbed on the surface of PT-A and increases the negative charge of the surface, and therefore, by the force of electrostatic repulsion, these phosphate ions hinder the adsorption of BSA, which also has a negative charge over the pH region examined [17]. Nitrogen adsorption/desorption isotherms and energy dispersive X-ray analyses demonstrated that phosphate could diffuse into the smaller tunnels of PT-A freely even if the external surface of PT-A was covered with BSA. Moreover, the main site of adsorption of phosphate was in micropores of PT-A, whereas BSA was adsorbed only to the external surface and none entered inside the smaller tunnels consisting of micro- and mesopores [17].

Similarly, Yang et al. employed the quartz crystal microbalance (QCM) technique to study the adsorption/desorption behavior of BSA on nanosized hydroxyapatite coatings [18]. Phosphate ions adsorbed on nanosized hydroxyapatite coatings were reported to prevent BSA adsorption and, adsorbed BSA would be competitively desorbed from hydroxyapatite coatings in the presence of phosphate ions [18]. Competition occurred between BSA and phosphate ions: most of the adsorption sites on hydroxyapatite coatings were occupied by phosphate ions, hindering the adsorption of BSA or desorbing adsorbed BSA, as illustrated in Figure 10 [18].
Yang’s group also reported that the amount of BSA adsorption grew rapidly with increasing NaCl concentration [18]. Such a result can be explained as follows: increasing NaCl concentration reduces the negative surface charge of hydroxyapatite, and thus, the electrostatic repulsion force between hydroxyapatite and BSA is reduced, which enhances the protein surface interaction and facilitates the adsorption of BSA molecules [18]. However, Oliva et al. reported independence of the pH of maximum adsorption of human serum albumin (HSA) onto TiO₂ from the increasing NaCl concentration [84]. Their work indicated that there were no ions competing for the adsorption surface groups nor accumulation of net charge between the adsorbed layer and the surface despite the specific adsorption of Cl⁻ in HSA [84]. This might reflect the different physical and chemical properties of BSA and HSA molecules. Since BSA will be used in this work,
the effects of electrolytes, especially NaCl, will be considered while designing the experiments.

As we reviewed in the *hydrophobic/hydrophilic interaction* section 2.2.1, Jeyachandran *et al.* studied the adsorption/desorption behavior of BSA on hydrophobic/hydrophilic surfaces with IR spectra [100]. BSA molecules first adsorbed on the surface (both hydrophobic and hydrophilic) and no BSA-phosphate complex formed within a 30-minute incubation time. Then, the conformation of the initially adsorbed BSA molecules changed to a stable form that could interact with phosphate ions more easily, so that a BSA-phosphate complex was formed. Conformational change of BSA molecules depended on the concentration of BSA-phosphate solution, incubation time and types of surface: at higher solution concentration or longer incubation time, a stable BSA conformation was more easily formed; and this conformational change preferred the hydrophobic surface over the hydrophilic one [100]. Another study reported that BSA molecules were found to adsorb in a more compact and stable conformation in phosphate buffer than in Hepes buffer or ammonium bicarbonate buffer, because the binding efficiency of phosphate ions to BSA molecules was relatively higher and the unfavorable structural electrostatic repulsion was less than with the other two buffers [123].

All these reviewed works either employed X-ray diffraction, or infrared spectroscopy and quartz crystal microbalance techniques, rather than the batch-equilibration-adsorption technique, so that the detailed adsorption information about isotherms, such as adsorption maxima, affinity and capacity, was not provided. Therefore, some other works that used the batch-equilibration-adsorption technique but not on oxide
NPs are reviewed below, which could provide some inspiration from another point of view.

Carbon nanomaterials, including fullerenes, single-wall carbon nanotubes (SWCNTs) and multiwalled carbon nanotubes (MWCNTs) are novel manufactured materials, having widespread potential applications [124]. Competitive adsorption between different organic compounds on carbon nanotubes (CNTs) was studied in depth. It is because this competitive adsorption is essential for application of CNTs as superior sorbents (i.e. purification of air and water as adsorbents) [125] and also for environmental risk assessment of both CNTs and organic contaminants. Yang et al. [125] studied the competitive adsorption between nonpolar organic compounds (naphthalene) and polar ionic organic compounds, dichlorophenol (DCP) and chloroaniline (PCAN), on 15 nm MWCNTs. Yang et al. created a useful calculation for the analysis of competitive adsorption that will be taken advantage of here. Yang began by following a calculation method created by Lin et al. [126] for obtaining the surface coverage percentage of each organic compound over MWCNTs. Lin used the properties of molecular weight and density of all selected organic compounds in his calculation [126]. Based on the coverage percentage, the adsorption mode of the organic compounds and three surmised types of surface sites of the MWCNTs a model of sorption was obtained, as shown in Figure 11. The heterogenetic nature of the MWCNTs surface is represented here by three different types of adsorption sites based on their hydrophilicity: site I > site II > site III. Site I can be occupied by DCP and PCAN but not by naphthalene due to the hydrophilicity of this type of site. Site II and III can be occupied by DCP, PCAN and naphthalene, but DCP and PCAN has priority to be adsorbed onto site II, while naphthalene has priority to be
adsorbed onto site III. Then, adsorption coefficients \( (K_d) \), as well as adsorption affinity and capacity, of naphthalene, DCP and PCAN in bisolute-system were analyzed and discussed. Since the oxide particles carry hydrophilic groups on surfaces too, the discussion about surface coverage percentage and different types of surface sites in Yang’s work could be utilized. Moreover, as discussed above, the porosity of oxide particles (i.e. micropores, mesopores and macropores) can influence the adsorption of protein molecules and phosphate ions as well [17]. Therefore, the types of adsorption sites of oxide particles were separated in this work by types of pore and by the hydrophilicity of particle surface.

**Figure 11.** Schematic diagram of possible adsorption and competition of naphthalene, DCP and PCAN on MWCNTs surface with different potential adsorption sites: site I, II and III. This figure is obtained from Yang’s work [125].

Yang and colleagues also studied the influence of cationic surfactant on naphthalene sorption on single-walled carbon nanotubes [127]. Yang put forward an
equation for the surface area: \( A_{\text{occupied}} = A_{\text{surf}} - A_{\text{cal}} \), where \( A_{\text{occupied}} \) represents the surface area occupied by adsorbed cetylpyridinium chloride (CPC), \( A_{\text{surf}} \) represents the surface area measured through the adsorption-desorption isotherm of \( \text{N}_2 \) at 77K with the multipoint BET method, and \( A_{\text{cal}} \) represents the calculated surface area by a specific equation using CPC molecular weight. The surface area calculation proposed that adsorption of CPC and naphthalene were regulated by surface area, hydrophobic interaction and \( \pi - \pi \) interaction [127]. In addition, Yang observed an increase in the Freundlich exponential coefficient, \( n \) (\( n \to 1 \)) and proposed that naphthalene partitioned into adsorbed CPC. In these two papers reviewed above, Yang utilized two experimental and calculation methods of surface area: the molecular weight and density method and the adsorption-desorption isotherm of \( \text{N}_2 \) method. Both methods inspired experimental design and analysis of results in this work.

2.3 Protein Conformational Changes

Generally, when protein becomes adsorbed on solid surfaces, the binding is optimized by undergoing molecular conformational change to maximize the favorable interaction between the surface and the solvent [29, 73, 100]. It has been reported that the \( \alpha \)-helical content in the adsorbed BSA molecules is decreased, whereas the \( \beta \)-structure and random coil content is increased. Besides, conformational modification of protein molecules occurs when solution pH changes [85]. The change, however, is not a denaturation process, but a reversible one [85]. Moreover, albumin interacts more strongly with hydrophobic than with hydrophilic surfaces [72]. Depending on the adsorbing surface, the induced conformational changes may or may not be reversible on
desorption [72]. Previous studies [14, 92, 128] reported on the conformational changes of different protein molecules adsorbed on oxide NPs. Chemical bonding between these proteins and NPs might be similar to the bonding between BSA and NPs, which could offer insights helpful in this work. Since the physical and chemical properties of the NPs selected in this work are not the same, the review of previous studies offered below is separated by the types of oxide NPs.

2.3.1 TiO$_2$ Particles

An increasing content of β-sheet and a decrease of α-helical structure content for fibrinogen protein, and an increasing content of β-sheet and a decreasing content of random coil structure for fibronectin protein were reported using Raman spectroscopy after the protein molecules adsorbed on to TiO$_2$ NP surfaces [14]. In the Raman spectra of the adsorbed proteins, a characteristic band was assigned to the interaction between the TiO$_2$ NPs and the carboxylate groups of the protein side-chains [14]. The amide I band of the adsorbed fibrinogen spectrum shifted toward higher wave numbers in comparison to the original bulk protein spectrum, which was attributed 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 spectrum of the original bulk fibrinogen [14].

Secondary structural change of fibrinogen molecules adsorbed on TiO$_2$ surfaces were reported, with two consecutive steps occurring during the adsorption: firstly, the fibrinogen molecules were adsorbed on the surface and secondly, the rearrangement of adsorbed fibrinogen or multilayer adsorption occurred [128]. This hypothesis was
supported by the observation that the α-helix content of adsorbed fibrinogen obviously decreased and was mainly transformed to β-sheet, while the β-turn and random coil contents were less changed, when the proteins adsorbed on TiO$_2$ surfaces [128]. Furthermore, the chemical bonding process was studied: (1) the TiO$_2$ particle surface is non-charged at around pH 5 [129], so under their experimental pH of 7.4, the predominant TiO$_2$ surface groups are Ti$_2$=O$^-$ and Ti−OH, with only a few Ti$_2$=OH; and the main protein functional groups are R−COO$^-$ and R−NH$_3^+$ [84, 130]. (2) Electrostatic interaction occurs between these groups on the surfaces of both the TiO$_2$ and the protein:

\[
\text{Ti−OH}_2^+: \text{NH}_2−\text{R (electrostatic interactions)}
\]

\[
\text{Ti}_2\text{=O}^- + \text{NH}_3^−−\text{R (electrostatic interactions)}
\]

\[
\text{Ti−OH}…−\text{COO−R (hydrogen bonding interaction)} [128].
\]

**2.3.2 SiO$_2$ Particles**

Besides 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 12) [92]. Greater loss of α-helix structure, which is consistent with a decrease of lysozyme activity, although not linearly, was observed with larger NPs that show stronger adsorption under the same conditions[92]. Studies of lysozyme adsorbed onto flat SiO$_2$ surfaces indicated that the most highly charged patch on the protein surface would contact with the negatively charged SiO$_2$ surface, when the protein molecule is in its thermodynamically most favorable conformation [131]. The active site of lysozyme is located on the opposite side from the positively charged patch.
Therefore, the initial conformational changes upon adsorption result in moderate loss in activity, which is because the conformational perturbations are somehow distant from the active site of the protein [92]. More perturbation occurs close to the active site when the native α-helix content is further lost [92]. Similar results from research on human carbonic anhydrase I (HCAI) protein adsorbed onto SiO₂ NPs of various diameters (6 nm and 15 nm) were also reported [15]. Larger SiO₂ NPs produce larger protein-particle interaction surface areas that cause greater protein conformational change (Figure 12). However, the protein tertiary structure is independent of the curvature of the SiO₂ particles [15].

It is interesting that larger SiO₂ NPs lead to greater protein secondary-structure conformational change than smaller SiO₂ NPs, since a conclusion could be extrapolated that bulk particles may cause more conformational change than NPs. However, this extrapolation is probably not accurately true. Therefore, one of the goals of this work is to understand the influence of particle size on protein conformational change.

![Figure 12. Schematic illustration of the relation between particle curvatures and the protein secondary structure modification.](image)
2.3.3 Al₂O₃ Particles

In deionized water (pH = 7), the surface charge 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 13) [16]. Based on the calculated net charges of the three domains of the 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 a side-on (domain I and domain II adsorbed on the Al₂O₃ surface, Figure 14) adsorption mode because the net charge of domains I + II is -16.8 mV in total, which is the most negatively combined charge compared with domains I + III (-9.1 mV) or domains II + III (-10.3 mV). Then, with an increase in protein concentration, BSA dimmers (two BSA molecules bonding, Figure 14 and 15) form on the surface instead of the end-on mode that formed on the surface (Figure 14) [16]. This is because the protein end-on mode induces more BSA/Al₂O₃ surface interactions that are not favored because of the asymmetric distribution of charge on the protein [16]. The end-on mode is one of the proposed albumin-protein-adsorption modes, which hypothesizes the albumin protein adsorbed on particle surfaces by only one domain, instead of two domains (Figure 14, middle).
**Figure 13.** Three domains of BSA protein molecule. The net charge of the three domains is: domain I -7.8 mV, domain II -9 mV, domain III -1.3 mV. This Figure is obtained from Rezwan’s work [16].

**Figure 14.** Proposed BSA adsorption model of *side-on* adsorbed mode (left), *end-on* adsorbed mode (middle), and dimmer mode (right) on Al₂O₃ particle surface. This figure is obtained from Rezwan *et al.* [16].
There is a shift in the point of zero charge (PZC) on the alumina surface from the PZC of alumina (pH=9) to the isoelectric point (IEP) of BSA (pH=5) when the protein adsorbs on the alumina surface [16]. After the first layer of BSA protein molecules fully covers the alumina surface, the pH of the surface charges of the $\text{Al}_2\text{O}_3$ reaches the IEP of BSA (pH=5) and cannot be shifted further even after more protein is adsorbed. Therefore, at pH 7, the surface charge of $\text{Al}_2\text{O}_3$ shifts from positive (the PZC of $\text{Al}_2\text{O}_3$ is pH 9) to negative (the IEP of BSA is pH 5) with the process of BSA adsorption. Since the surface charges of the first layer are negative, the formation of dimers probably results from protein/protein interaction through hydrogen bonds, disulfide bonds, and/or 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) [134].

### 2.4 Desorption of Protein

Adsorption is a dynamic process, which means desorption occurs with adsorption simultaneously. Adsorption maxima cannot be reached until adsorption and desorption reach an equilibrium. Understanding desorption behavior can provide more detailed
information about adsorption sites that we obtained from adsorption analysis, which is another critical task for evaluating the environmental and health impacts of oxide NPs. Previous studies reported on desorption phenomena of BSA molecules, but the mechanism of desorption was not deeply studied. For example, Yang et al. studied the desorption behavior of BSA from nanosized hydroxyapatite coating with quartz crystal microbalance (QCM) technique [18]. BSA molecules were introduced to the hydroxyapatite coatings and left until adsorption equilibrium was reached, as shown in Figure 16 stage I. This process led to a decrease in frequency of QCM because of the adsorption of the BSA. Then the coated surface was rinsed with deionized water three times (stage II). A small increase in frequency was observed during rinsing, which can be attributed to the removal of the weakly bound BSA. Finally, phosphate buffer was applied to the rinsed coatings twice to competitively adsorb with the coated BSA molecules. This process led to an immediate increase in frequency, which indicated that the BSA molecules had desorbed from the nanosized hydroxyapatite coatings. Figure 17 shows a reversed sequence of the introduction of the BSA and the phosphate compared with Figure 16. Phosphate buffer was firstly introduced (stage I), then rinsed three times with deionized water (stage II), and finally applied BSA molecules to the coatings (stage III). No obvious change in frequency was observed after the BSA was introduced, suggesting only rare or no BSA adsorption to the phosphate-pretreated surface. Yang explained the phenomenon as phosphate ions exhibiting a higher affinity to hydroxyapatite coatings than BSA molecules exhibit. Yang reported that “competition occurs between BSA and phosphate ions”, and “most of adsorption sites on hydroxyapatite coatings are occupied by phosphate ions, leading to desorption of
adsorbed BSA or hindering the adsorption of BSA”. The author did not deeply explain the factors governing phosphate higher adsorption affinity and BSA desorption, which should be completed in this work. Also, in stage III of Figure 16, it was not reported whether the adsorbed BSA molecules were totally replaced by phosphate ions after phosphate buffer was introduced twice. This information would help to elucidate the nature of the adsorption sites and the physical-chemical interaction occurring among BSA molecules, phosphate ions and hydroxyapatite coatings.

**Figure 16.** Plot of frequency of QCM change against time. (I) BSA adsorption, (II) rinsing with deionized water, (III) desorption of BSA caused by introduced phosphate buffer. This figure is obtained from Yang’s work [18].

**Figure 17.** Plot of frequency change against time. (I) adsorption of phosphate ions in phosphate buffer, (II) rinsing with deionized water, (III) introduction of BSA. This figure is obtained from Yang’s work [18].
Desorption hysteresis is widely observed for the adsorption of organic compounds, such as pesticides, chlorinated benzenes and polycyclic aromatic hydrocarbons onto soils, sediments, charcoals and CNTs [135-138]. There are two types of hysteresis frequently observed: reversible and irreversible hysteresis [137]. Yang et al. fully discussed the nature of the phenomena and the general mechanisms of how these two types of hysteresis operate [135]. Irreversible hysteresis is defined as the complete desorption that cannot be achieved without intervention in the experiment. Whereas, reversible hysteresis is defined as the complete desorption that can be achieved without any intervention. In the latter case, a closed hysteresis loop in desorption and adsorption branches of an isotherm can be observed [135]. Desorption hysteresis can also be separated into true hysteresis or artificial hysteresis [139]. An artificial hysteresis is caused by “an insufficient time allowed for diffusion equilibrium and/or some auxiliary process such as volatilization of organic compounds, which depend on experimental conditions and can be eliminated” [135]. To avoid artificial hysteresis, abundant time for desorption-diffusion equilibrium in this study is allowed. In addition, the sorbate and the solution used in this work are BSA protein and phosphate buffer, both of which have insignificant volatility. Yang et al. discussed [135] desorption hysteresis of polycyclic aromatic hydrocarbons (PAHs) from fullerene in water, which can provide inspiration for this study. This is because the geometry of fullerene and oxide particles is very close, and BSA molecules can be regarded as organic chemicals. There are two hypotheses that can explain the true hysteresis of porous solids [140]: capillary condensation in mesopores or macropores and pore deformation. Pore deformation is the mechanism that causes the hysteretic desorption of PAHs from fullerene [135]: when the absorbate desorbs from the
adsorbent, pore structure of the adsorbent may not recover to its original state, resulting in a change of the microscopic pathways for adsorption and desorption [137, 139]. Yang suggested that the formation of deformable closed spaces in aggregates is responsible for irreversible hysteresis. This is because fullerene can form closed interstitial spaces either in small aggregates or between small aggregates. Small fullerene aggregates in water cannot be broken down to monomers by extended stirring and even solvent exchange [141-143]. Therefore, the closed interstitial space in small aggregates cannot be used for adsorption. However, the closed interstitial space between small aggregates possibly can become available for adsorption. Figure 18 shows the schematic view desorption hysteresis of PAHs on fullerene. Either the rearrangement of small fullerene aggregates to form new closed interstitial space or the penetration of PAHs into the closed interstitial space may lead to the deformation [136, 144].

Figure 18. Schematic view of PAHs adsorption/desorption on fullerene. Step (1): adsorption; step (2): penetration into space A; step (3): rearrangement leading to opening of space A; step (4): deformation at site B leading to opening of space A; step
(5): rearrangement at site C by combining with another small aggregate; step (6): rearrangement at site C; steps (7), (8) and (9): desorption, showing entrapment of organic molecules in the closed interstitial spaces. The figure is obtained from Yang’s work [135].

2.5 Infrared Spectrum of Protein

Utilizing infrared (IR) spectra provides very useful information for protein adsorption studies. For example, detailed protein conformational change, especially change to the secondary structure, is mainly measured by IR spectrum. As early as 1960, Miyazawa [145] developed the fundamental theory for protein IR spectra conformational analysis. In 1986, nine characteristic vibrational bands or group frequencies arising from the protein amide group were identified by Krimm and Bandekar [146]. Table 2 is obtained from the results of Susi [145], giving the generally accepted nomenclature, the approximate frequencies, and the approximate descriptions of nine characteristic infrared absorption bands. The amide A, amide I, and amide II bands are most frequently used for conformational investigations [147-149]. Among all the bands, the amide I band is almost entirely due to the C=O stretch vibration of the peptide linkages, which constitute the protein backbone structure. The amide I band is widely used in determining the protein secondary structure, because each type of secondary structure corresponds to a unique C=O stretch frequency in the amide I spectrum [150-152]. Miyazawa et al. [152] and Krimm et al. [153] had extensively examined the basic theoretical calculations for all vibrations in the amide I region. Table 3 is from Susi’s results [145], listing the solid-state protein spectra branches that can be observed under laboratory conditions.
Table 2. Characteristic infrared bands of the peptide linkage

<table>
<thead>
<tr>
<th>Designation</th>
<th>Approximate Frequency (cm$^{-1}$)</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>~3300</td>
<td>NH stretching in resonance with (2*amide II) overtone</td>
</tr>
<tr>
<td>B</td>
<td>~3100</td>
<td>C=O stretching</td>
</tr>
<tr>
<td>I</td>
<td>1600-1690</td>
<td>CN stretching, NH bending</td>
</tr>
<tr>
<td>II</td>
<td>1480-1575</td>
<td>CN stretching, NH bending</td>
</tr>
<tr>
<td>III</td>
<td>1229-1301</td>
<td>CN stretching, NH bending</td>
</tr>
<tr>
<td>IV</td>
<td>625-767</td>
<td>OCN bending, mixed with other modes</td>
</tr>
<tr>
<td>V</td>
<td>640-800</td>
<td>Out-of-plane NH bending</td>
</tr>
<tr>
<td>VI</td>
<td>537-606</td>
<td>Out-of-plane C=O bending</td>
</tr>
<tr>
<td>VII</td>
<td>~200</td>
<td>Skeletal torsion</td>
</tr>
</tbody>
</table>

$^a$ Based on model compounds. The table is from Miyazawa et al. [154].

Table 3. Prominent amide I and II branches in the solid state$^a$

<table>
<thead>
<tr>
<th>Conformation</th>
<th>Strongest amide I component</th>
<th>Strongest amide II component</th>
<th>Weak amide I component, ca. 1690 cm$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unordered</td>
<td>1658</td>
<td>1520</td>
<td>-</td>
</tr>
<tr>
<td>ACPS $^b$</td>
<td>1632</td>
<td>1530</td>
<td>1685</td>
</tr>
<tr>
<td>PCPS $^c$</td>
<td>1632</td>
<td>1530</td>
<td>-</td>
</tr>
<tr>
<td>Parallel-chain polar sheet $^d$</td>
<td>1648</td>
<td>1550</td>
<td>-</td>
</tr>
<tr>
<td>A-Helix</td>
<td>1650</td>
<td>1546</td>
<td>-</td>
</tr>
<tr>
<td>Triple helix (polyglycine II)</td>
<td>1648</td>
<td>1558</td>
<td>-</td>
</tr>
</tbody>
</table>

$^a$ Calculated values [148]. $^b$ Antiparallel-chain pleated sheet $^c$ Parallel-chain pleated sheet $^d$ Approximate polarization; not determined by symmetry. This table is from Miyazawa et al. [154].

The interaction of bovine serum albumin (BSA) with soil mineral surfaces has been studied by Quiquampoix et al. [90]. The amide I band can be decomposed to five common components in BSA solution, and two additional components appearing only with BSA adsorbed on montmorillonite surface. The amide I region IR frequencies were regrouped into three classes: $S_1$ (1662 cm$^{-1} + 1619$ cm$^{-1}$), $S_2$ (1681 cm$^{-1} + 1672$ cm$^{-1} +$
1639 cm\(^{-1}\), and \(S_3\) (1655 cm\(^{-1}\) +1633 cm\(^{-1}\)). \(S_1\) is the sum of the components appearing only for the adsorbed BSA; \(S_2\) is the sum of the components corresponding to non-ordered peptide units, appearing in both solution and the adsorbed state; \(S_3\) is the sum of the components corresponding to the classical ordered secondary structure (\(\alpha\)-helices and \(\beta\)-sheets). During the adsorption process, the classical \(S_3\) group could disappear and become transformed into \(S_1\) and/or \(S_2\) groups depending on the pH value. The results achieved by Quiquampoix \textit{et al.} could help in partitioning the amide I band when analyzing IR data in this research.

Tarasevich \textit{et al.} [155] studied the adsorption between BSA and silica surfaces. It is reported that the carbonyl groups of protein macromolecules interact with vicinal hydroxyl groups (3640 cm\(^{-1}\)), while imido groups interact with the individual hydroxyl groups (3750 cm\(^{-1}\)) of a silica surface. Figure 19 shows the difference between the vicinal and individual hydroxyl groups [156]. Individual hydroxyl groups are strong adsorption sites that can pair with lone pair adsorbates (like NH\(_3\)). Vicinal hydroxyl groups are adjacent hydroxyl sites that form strong H bonding to each other but cannot form a hydrogen bond with lone pair adsorbates [156]. However, water molecules are able to bond with these vicinal hydroxyl groups. Also, these water molecules can interact with long pair molecules by hydrogen bonding. Therefore, abundant water can increase adsorption of vicinal hydroxyl groups.

The protein IR spectra indicated that the adsorption of BSA macromolecules results in the dehydration of SiO\(_2\) NPs surfaces and the appearance of additional unfolded \(\beta\)-regions on the adsorbed BSA. However, the \(\alpha\)-helial structure remains undisturbed. In this work, \(\alpha\)-helices and \(\beta\)-regions of BSA protein were examined in order to determine
whether the nanosized particles can cause different results from those obtained with the microscale oxide particles. Due to the small size of the NPs, more vicinal hydroxyl groups are expected. Consequently, the interaction of the carbonyl groups of proteins and vicinal hydroxyl groups on silica surface were studied.

Similar results were obtained by Lundqvist et al. [15]. In research on human carbonic anhydrase I (HCAI) adsorption onto silica NPs, their results indicated that the secondary structure of HCAI protein is strongly influenced by particle curvature. Greater protein diameter leads to larger particle-protein interaction surface area which in turn caused more perturbations of protein secondary structure. However, tertiary structure seemed undisturbed by particle curvature.

\[
\begin{align*}
(a) & \quad \text{Si-O-H-H-N} \\
(b) & \quad \text{Si-O-H} \\
(c) & \quad \text{Si-O-H} \\
(d) & \quad \text{Si-O-H-N} \\
\end{align*}
\]

**Figure 19.** Vicinal and individual hydroxyl groups on a silica surface. (a) Individual hydroxyl groups are strong adsorption site that can pair with lone pair NH\textsubscript{3}; (b) Vicinal hydroxyl groups form strong H bonding to each other and cannot form a hydrogen bond with lone pair adsorbates; (c) water molecules are able to bond with these vicinal hydroxyl groups; (d) lone pair NH\textsubscript{3} can interact with adsorbed water molecule by hydrogen bonding [156].

The study of protein conformational change can provide information relevant for particle cytotoxic evaluation, and can help with studies of adsorption mechanisms.
Therefore, the conformational change of BSA after adsorption was studied as another objective of this work.

2.6 Hypothesis

The surface characteristics of oxide NPs are different from bulk particles in specific surface area, surface chemical groups, dispersing behavior and sorption capacity for organics [157]. Therefore, three different oxide particles (TiO$_2$, SiO$_2$, and Al$_2$O$_3$) with both nano- and bulksize (including two types of Al$_2$O$_3$ NPs) were used in this work. Based on the review offered above, several hypotheses are proposed:

(1) Electrostatic interaction, surface area, surface chemical groups and hydrophilicity (hydrogen content) regulate both phosphate and BSA molecules adsorption.

(2) BSA adsorption maxima of NPs may be higher than BPs by weight. However, after surface area normalization, the sequence of adsorption maxima may be changed because adsorption is likely to be depended on the specific surface area of each particle.

(3) Competitive adsorption between BSA and phosphate ions on oxide particles may occur: BSA adsorption would be hindered with the increasing phosphate concentration.

(4) 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.
(5) BSA desorption hysteresis is related with the types/characteristic of oxides that influence BSA adsorption maxima. Besides, the desorption hysteresis may be related with the affinity or capacity of phosphate adsorption of selected oxides.

2.7 Objectives

(1) to determine BSA adsorption behaviors onto TiO$_2$, SiO$_2$ and Al$_2$O$_3$ NPs and BPs under the competitive impacts of phosphate ions;

(2) to understand the effects of particle size, surface charge, hydrophilicity and other surface chemical groups on BSA adsorption;

(3) to identify the chemical bonding between surface chemical groups of protein, phosphate and oxide particles;

(4) to discuss the factors affecting competitive adsorption of BSA and phosphate ions onto oxide NPs and BPs, and to make conjectures about the cytotoxicity of oxide NPs in a natural environment;

(5) to determine the possible mechanism of BSA desorption, and understand the influence of phosphate ions to BSA desorption; and

(6) to determine the possible different regions or types of adsorption sites over oxide particle surface for BSA molecules.

Detailed information about experimental procedures to reach these objectives is in the next chapter.
CHAPTER 3

EXPERIMENTS

3.1 Materials and Methods

3.1.1 Chemicals and Oxides

Potassium phosphate monobasic (KH$_2$PO$_4$) was prepared in deionized water with phosphate concentration at 1500 mg/L. The pH of phosphate solution was adjusted to 7.0 with HCl and KOH. BSA lyophilized powder (A9647) was purchased from Sigma-Aldrich Co. Nanoscaled SiO$_2$ (spherical form), TiO$_2$ (anatase form), α-Al$_2$O$_3$ and γ-Al$_2$O$_3$ were purchased from Zhejiang Hongshen 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$_3$ (α type) particles were purchased from Baker Co. All NPs and BPs were used without further treatment. A description of the forms of these oxide particles is given below, as do FTIR spectra of these oxides (Figure 20). 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 many similar properties such as luster, hardness and density, but have a slight difference in crystal behavior and a significant difference in 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. α- and γ-Al$_2$O$_3$ forms are the most abundant among the several forms of Al$_2$O$_3$. α-Al$_2$O$_3$ is the pure form
obtained from calcination at high temperature, while γ-Al₂O₃ keeps stable at about 1000 °C. SiO₂ exists in several forms. Spherical SiO₂ NPs will be used in this work. Additional description from the supplier can be obtained from http://www.mrnm.com.cn/product_2.htm.

Figure 20. FTIR spectra of nano TiO₂ (a), bulk TiO₂ (b), nano SiO₂ (c), bulk SiO₂ (d), nano α-Al₂O₃ (e), nano γ-Al₂O₃ (f), and bulk Al₂O₃ (g).
3.1.2 Characterization of Oxide particles

Specific surface area (\(S_{BET}\)) of all oxide particles was calculated from \(N_2\) sorption isotherms by the multipoint BET method. \(N_2\) sorption was conducted at 77 K using a NOVA 1000e instrument (Quantachrome). All samples were outgassed at 105 °C for 16h before \(N_2\) 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 NPs was provided by manufacturer, while the size of all BPs 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 of TEM. Hydrodynamic diameter and \(\zeta\) potential values were measured by a 90 Plus Particle Size Analyzer (Brookhaven Instruments) 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 structure properties of these oxide particles are listed in Table 4. 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 the spectra of pure oxide particles from that of the BSA-coated oxide particles.
Table 4. Selected Properties of Oxide Particles

<table>
<thead>
<tr>
<th>Particle</th>
<th>Purity</th>
<th>Surface Area</th>
<th>Diameter</th>
<th>Hydrodynamic Diameter</th>
<th>C (%)</th>
<th>H (%)</th>
<th>Zeta Potential</th>
<th>D_ratio</th>
<th>H_SA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nano TiO₂</td>
<td>≥ 99</td>
<td>325</td>
<td>171</td>
<td>154</td>
<td>50±10</td>
<td>706</td>
<td>0.320</td>
<td>14.1</td>
<td>0.004</td>
</tr>
<tr>
<td>Bulk TiO₂</td>
<td>≥ 99</td>
<td>7.30</td>
<td>-</td>
<td>-</td>
<td>285±10</td>
<td>1181</td>
<td>0.405</td>
<td>4.14</td>
<td>0.571</td>
</tr>
<tr>
<td>Nano SiO₂</td>
<td>≥99.5</td>
<td>191</td>
<td>65</td>
<td>123</td>
<td>30±10</td>
<td>1274</td>
<td>0.249</td>
<td>42.5</td>
<td>0.007</td>
</tr>
<tr>
<td>Bulk SiO₂</td>
<td>=100</td>
<td>8.40</td>
<td>-</td>
<td>-</td>
<td>745±10</td>
<td>1731</td>
<td>0.250</td>
<td>2.32</td>
<td>0.304</td>
</tr>
<tr>
<td>Nano α-Al₂O₃</td>
<td>≥99.99</td>
<td>4.73</td>
<td>0</td>
<td>4.73</td>
<td>150±10</td>
<td>1147</td>
<td>0.247</td>
<td>7.65</td>
<td>0.013</td>
</tr>
<tr>
<td>Nano γ-Al₂O₃</td>
<td>≥99.99</td>
<td>208</td>
<td>0</td>
<td>208</td>
<td>60±10</td>
<td>1482</td>
<td>0.349</td>
<td>24.7</td>
<td>0.006</td>
</tr>
<tr>
<td>Bulk Al₂O₃</td>
<td>≥98.5</td>
<td>11.0</td>
<td>-</td>
<td>-</td>
<td>431±10</td>
<td>878</td>
<td>0.224</td>
<td>2.05</td>
<td>0.741</td>
</tr>
</tbody>
</table>

a Provided by the supplier. b \( S_{\text{BET}} \) was calculated from the adsorption-desorption isotherm of N\(_2\) at 77K by multi-point BET method. c Determined by high-resolution TEM. d Determined by DLS. e DI represents the zeta potential of selected oxides in deionized water, while Equilibrium represents the average zeta potential value of oxide surface that reached adsorption equilibrium. Both the measurements were performed under pH = 7.0 ± 0.2. f Obtained by hydrodynamic diameter/individual particle diameter. g Specific hydrogen content, obtained by hydrogen content/surface area. “−” represents unmeasured.
3.1.3 Adsorption Experiments

The adsorption experiments of this work consist of three sections: BSA adsorption section, phosphate adsorption section and BSA/phosphate competitive adsorption section. BSA powder and phosphate was prepared in deionized water for the first two sections, while BSA was dissolved in 1500 mg/L phosphate solution for the third section. NaN₃ (200 mg/L) was added into all solution as a biocide, followed by adjusting pH of all solution to 7.0 ± 0.2. All adsorption isotherms were obtained using a batch equilibration technique at 25 ± 1°C in 15 mL screw-cap borosilicate glass vials. The adsorption experiments were conducted with ten concentration points; each point including a blank sample was run in duplicate. The mass dosage of TiO₂, SiO₂, α-Al₂O₃ and γ-Al₂O₃ NPs, and TiO₂, SiO₂ and Al₂O₃ BPs added in vials was 0.1, 0.4, 0.5, 0.1, 3, 4, and 1.9 g, respectively. Then designed amount of BSA and phosphate solution was added to the 15 mL vials simultaneously, left at least 2 mL volume for the following pH adjustment. The initial concentration of BSA in the vials was in the range of 100-10000 mg/L, while the various initial concentration of phosphate was from 20 to 1500 mg/L, because over these ranges of BSA and phosphate, oxides can adsorb 20–85% of BSA, while competitive effects of phosphate can be clearly observed. All the vials were rotated in an end-over-end shaker (30 rpm) for 72h (where preliminary tests indicated that apparent equilibrium was reached within 24h). The pH of each vial was checked and adjusted twice at 24h and 48h in order to keep the solution at pH = 7.0 ± 0.2. Deionized water was filled up to 15 mL in each vial after the second pH adjustment. After centrifugation (12000 rpm for 10 min), BSA concentration in the supernatant was determined by a Total Organic Carbon Analyzer (TOC-L CPH, Shimadzu) using the
NPOC (nonpurgeable organic carbon) method [158]. Phosphate concentration in the supernatant was measured by Quikchem 8500 instrument (Lachat Instruments). All kinds of reagent dye were made freshly following instructions in the instrument manual.

3.1.4 Desorption Experiments

For desorption, 10 mL supernatant was removed and used for BSA/phosphate concentration measurement, and amended by the same volumes and same concentration of respective phosphate solution immediately. The vials were ressealed and shaken for an additional 48 hours. After equilibrium, vials were centrifuged and the concentration of BSA and phosphate in supernatant was measured. The sorbed concentrations of sorbate were calculated by mass difference. The procedures were repeated for a second desorption cycle.

3.1.5 Isotherm Models and Regression Analysis

Freundlich (eq 1), Langmuir (eq 2) isotherm models were used for data fitting in this work.

Freundlich model:

\[ q_e = K_f C_e^n \]  \hspace{1cm} (1)

where \( q_e \) (mg/g) is the equilibrium adsorbed concentration; \( C_e \) (mg/L) is the equilibrium solution phase concentration; \( K_f \) ((mg/g)/(mg/L)^{1/n}) is the Freundlich affinity coefficient; \( n \) is the Freundlich exponential coefficient.

Langmuir model:

\[ q_e = Q_0 C_e / (K_l + C_e) \]  \hspace{1cm} (2)
where $Q_0$ (mg/g) is the maximum volume sorption capacity of solute; $K_r$ (mg/L) is the affinity coefficient. All estimated model parameter values were determined by commercial software (SigmaPlot 9.0).
CHAPTER 4
RESULTS AND DISCUSSION

4.1 Characterization of BSA and Oxide Particles

Zeta Potential value of BSA in deionized water is $-6.64 \pm 9.74$ mV, while the values of oxide NPs and BPs as a function of phosphate concentration are given in Figure 21. In deionized water, $\gamma$-$\text{Al}_2\text{O}_3$ NPs have positive charge (22.5 mV), and $\alpha$-$\text{Al}_2\text{O}_3$ NPs are nearly neutral (-1.51 mV), while the other oxide particles carry negative charge. When the phosphate concentration raises, zeta potential values of TiO$_2$, $\alpha$-$\text{Al}_2\text{O}_3$, $\gamma$-$\text{Al}_2\text{O}_3$ NPs and Al$_2$O$_3$ BPs drop and then reach a plateau when phosphate concentration beyond 150 mg/L. However, zeta potential values of SiO$_2$ NPs and TiO$_2$, SiO$_2$ BPs are approximately independent of phosphate concentration. This may be caused by the electrostatic repulsion between negatively charged oxide surface and phosphate anion prevents the adsorption of phosphate on oxide surface, which is in line with the phosphate adsorption result and will be discussed later. $S_{\text{BET}}$ of selected oxide particles follows the sequence: TiO$_2$ NPs > $\gamma$-$\text{Al}_2\text{O}_3$ NPs > SiO$_2$ NPs > Al$_2$O$_3$ BPs > SiO$_2$ BPs > TiO$_2$ BPs > $\alpha$-$\text{Al}_2\text{O}_3$ NPs (Table 4). $S_{\text{BET}}$ of oxide NPs is larger than $S_{\text{BET}}$ of their respective BPs except for $\alpha$-$\text{Al}_2\text{O}_3$. 


Figure 21. Zeta Potential values of oxide particles as a function of phosphate concentration at pH = 7.0 ± 0.2.

The FTIR spectra of oxide NPs and BPs are presented in Figure 22. Among these oxides, SiO$_2$, TiO$_2$, γ-Al$_2$O$_3$ NPs and TiO$_2$, SiO$_2$ and Al$_2$O$_3$ BPs have 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\(^{-1}\) [159], while \(\alpha\)-Al\(_2\)O\(_3\) NPs and SiO\(_2\) BPs have few hydroxyl groups. Three types of bound hydroxyl groups at 3300, 3480 and 3620 cm\(^{-1}\) can be recognized. Other adsorption bands of oxide particles show the impurity of composition and also their bulk counterparts [94]. 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 [94, 159]. Six humps, which are observed in the region of 1400 to 2100 cm\(^{-1}\) of SiO\(_2\) BPs spectra, can be assigned to adsorbed or bound water (i.e. bending vibrations of adsorbed water at 1640 cm\(^{-1}\)) [160] or noise components of CH\(_2\)=CH\(_2\) or CH=CH\(_2\) [161]. 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 peaks at 1040 and 1122 cm\(^{-1}\) correspond to the end and bridging butoxyl groups [90]. 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 [88].
Figure 22. FTIR spectra of (a) nano SiO$_2$, (b) bulk SiO$_2$, (c) nano TiO$_2$, (d) bulk TiO$_2$, (e) nano α-Al$_2$O$_3$, (f) nano γ-Al$_2$O$_3$, and (g) bulk Al$_2$O$_3$.

The C and H mass percent content are listed in Table 4. The hydroxyl groups and bound water on the surfaces of oxide particles are mostly oxygenated and volatilized under the calcined temperature of 980 °C [162]. Therefore, H content, which is 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$_2$ and $\gamma$-Al$_2$O$_3$ NPs have abundant hydroxyl groups on their surfaces, while $\alpha$-Al$_2$O$_3$ NPs and TiO$_2$, SiO$_2$ and Al$_2$O$_3$ BPs have few hydroxyl groups, which is in an agreement with the data of FTIR spectra (Figure 22).

4.2 Adsorption Section

4.2.1 Adsorption of BSA on Oxide Particles

Adsorption isotherms of BSA by oxide particles are shown in Figure 23A. Results of fitting the Langmuir and Freundlich model to adsorption isotherms of BSA on oxide particles are shown in Table 5. Adsorption isotherms normalized by $S_{BET}$ of each oxide particle are shown in Figure 23B. The largest BSA adsorption is observed for $\gamma$-Al$_2$O$_3$ NPs, followed by TiO$_2$ NPs > SiO$_2$ NPs > $\alpha$-Al$_2$O$_3$ NPs > Al$_2$O$_3$ BPs > TiO$_2$ BPs > SiO$_2$ BPs. This Langmuir type behavior is also observed in other studies of BSA molecules or humic acid adsorbed on those oxide particle surfaces [94].

Table 5. Results of Model Fitted Adsorption Isotherms of BSA on Seven Oxide Particles$^a$

<table>
<thead>
<tr>
<th>Adsorbent</th>
<th>Adsorbate</th>
<th>$Q_0$</th>
<th>$K_L$</th>
<th>$r^2$</th>
<th>$Q_0^{b}$</th>
<th>$K_f$</th>
<th>$n$</th>
<th>$r^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>nano TiO$_2$</td>
<td>BSA</td>
<td>106</td>
<td>26.7</td>
<td>0.996</td>
<td>0.326</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>bulk TiO$_2$</td>
<td>BSA</td>
<td>4.28</td>
<td>845</td>
<td>0.975</td>
<td>0.586</td>
<td>42.1</td>
<td>0.131</td>
<td>0.983</td>
</tr>
<tr>
<td>nano SiO$_2$</td>
<td>BSA</td>
<td>43.0</td>
<td>704</td>
<td>0.991</td>
<td>0.225</td>
<td>0.593</td>
<td>0.540</td>
<td>0.984</td>
</tr>
<tr>
<td>bulk SiO$_2$</td>
<td>BSA</td>
<td>0.790</td>
<td>29.3</td>
<td>0.991</td>
<td>0.094</td>
<td>0.149</td>
<td>0.271</td>
<td>0.976</td>
</tr>
<tr>
<td>$\alpha$-Al$_2$O$_3$</td>
<td>BSA</td>
<td>23.2</td>
<td>0.941</td>
<td>0.995</td>
<td>4.90</td>
<td>13.6</td>
<td>0.081</td>
<td>0.996</td>
</tr>
<tr>
<td>$\gamma$-Al$_2$O$_3$</td>
<td>BSA</td>
<td>246</td>
<td>3.09</td>
<td>0.994</td>
<td>1.18</td>
<td>183</td>
<td>0.042</td>
<td>0.991</td>
</tr>
<tr>
<td>Bulk Al$_2$O$_3$</td>
<td>BSA</td>
<td>18.7</td>
<td>42.2</td>
<td>0.982</td>
<td>1.70</td>
<td>3.81</td>
<td>0.221</td>
<td>0.936</td>
</tr>
</tbody>
</table>

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

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Figure 23. BSA adsorption isotherms by oxide particles in deionized water (A). After normalization by surface area of each oxide particle, the differences of BSA adsorption maxima among these samples were decreased dramatically (B).
According to the Langmuir maximum sorption capacity \( (Q_0) \) (Table 5), BSA adsorption maxima of all oxide NPs are higher than the BPs, especially for nano-sized \( \gamma \)-\( \text{Al}_2\text{O}_3 \) \( (Q_0 = 246 \text{ mg/g}) \), TiO\(_2\) \( (Q_0 = 106 \text{ mg/g}) \) and SiO\(_2\) \( (Q_0 = 43.0 \text{ mg/g}) \), which is one order of magnitude higher than their BPs \( (Q_0 = 18.7, 4.28 \text{ and } 0.73 \text{ mg/g}, \text{respectively}) \). Thus, the surface area regulates the adsorption maxima of BSA among seven oxide particles. BSA adsorption amount on \( \alpha \)-\( \text{Al}_2\text{O}_3 \) NPs \( (23.2 \text{ mg/g}) \) is the lowest, close to the adsorption amount on \( \text{Al}_2\text{O}_3 \) BPs \( (18.7 \text{ mg/g}, \text{Table 5}) \). This can be attributed to their close surface area values, 4.73 \( \text{m}^2/\text{g} \) for \( \alpha \)-\( \text{Al}_2\text{O}_3 \) NPs and 11.0 \( \text{m}^2/\text{g} \) for \( \text{Al}_2\text{O}_3 \) BPs (Table 4). Furthermore, the importance of surface area for BSA adsorption maxima is demonstrated by the fact that the differences of BSA adsorption maxima among oxide particles decreased dramatically after surface area normalization (Figure 23B and Table 5). However, even after surface area normalization, the significant difference in BSA adsorption maxima is still observed, implying other factors such as surface charge, hydrophilicity and ligand exchange could be involved. Surface area normalized adsorption maxima of BPs are much close to those of the corresponding NPs, especially for TiO\(_2\) BPs and \( \text{Al}_2\text{O}_3 \) BPs that are higher than their corresponding NPs. It can be explained by the aggregation of oxide particles in solution. During aggregation process, individual particles form small aggregates first and then big aggregates [163]. Aggregation of spherical oxide particles may result in closed interstitial spaces in its aggregates and the decrease of their exposed effective surface for adsorption [163]. Because of the absence of entering pathway for BSA molecules, closed interstitial spaces in oxide aggregates will not be available for BSA adsorption. \( D_{ratio} \) in Table 4 represents the ratio of particle hydrodynamic diameter to individual particle diameter. \( D_{ratio} \) of NPs
is much higher than that of BPs, indicating that aggregation is greater for NPs than for BPs. Therefore, surface normalized adsorption maximum of NPs is smaller than that of BPs.

Previous published data on the conformation of serum albumin showed that BSA molecule is modeled as a triangular prismatic shell with optimized dimensions of $84 \times 84 \times 30$ Å in a neutral solution [164]. Based on this model, one mole BSA molecules occupy $18.7 \times 10^6$ m$^2$ of the surface area at most, and $15.2 \times 10^6$ m$^2$ of the surface area with the “side-on” mode at least [16]. 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 ($Q_0$, Table 5) and the $S_{BET}$ (Table 4), the specific amounts of BSA adsorbed onto the surfaces of oxide particles are 0.33, 0.59, 0.23, 0.09, 4.91, 1.18 and 1.70 mg/m$^2$ for nano-sized TiO$_2$, SiO$_2$, $\alpha$-Al$_2$O$_3$, $\gamma$-Al$_2$O$_3$ and bulk-sized TiO$_2$, SiO$_2$ and Al$_2$O$_3$, respectively ($Q_0$, Table 5). The amounts of BSA adsorbed on oxide particle surfaces are significantly lower than the calculated values of monolayer adsorption except the $\alpha$-Al$_2$O$_3$, indicating a less compact protein layer caused by strong lateral protein-protein repulsion between protein molecules that covered on particle surfaces [15, 92, 96]. The amount of BSA adsorbed on $\alpha$-Al$_2$O$_3$ surfaces is significantly higher than the “side-on” calculated value, implying multilayer adsorption of protein molecules on $\alpha$-Al$_2$O$_3$ surface, which is in agreement with a previous study [16]. Moreover, the significant higher adsorption maximum of $\alpha$-Al$_2$O$_3$ after normalized by surface area can also be explained by the smaller surface area each BSA molecule occupied via multilayer mode.
Hydrophilicity is one of possible mechanisms regulating BSA adsorption by oxide particles. As illustrated in Figure 24, a significant linear relationship between surface area-normalized adsorption maxima of BSA and hydrogen contents is observed for both NPs ($R^2 = 0.996$, $P < 0.01$) and BPs ($R^2 = 1.000$, $P < 0.01$), indicating that surface area and hydrogen content regulate protein adsorption process. Generally, surface area of particles provides sites for adsorption, while hydrogen content, which is served as an indicator of surface hydrophilicity, represents the amount of hydroxyl groups or chemically bonded water molecules (i.e., hydroxylation layer) on particle surfaces [18, 165]. Basically, two types of non-bonded interactions occur in protein adsorption: long-range (or electrostatic) interaction and short-range interaction (including hydrogen bonding and hydrophobic interaction) [100]. Previous studies reported that BSA adsorbed more strongly on hydrophobic surfaces due to hydrophobic interaction [166, 167]. Therefore, the linear relationship between surface area normalized adsorption maxima and hydrogen content of NPs is negative because higher hydrogen (or hydroxyl) contents can reduce hydrophobic interaction between BSA molecules and oxide surfaces (Figure 24 and 25). However, with increasing hydrogen contents on oxide surfaces, more hydrogen bonds can form, leading to a trend of positive relationship between surface area normalized adsorption maxima and hydrogen content as shown in Figure 24 of BPs (the specific hydrogen contents ($H_{SA}$, Table 4) of BPs is at least 20 folds higher than that of NPs). The more hydrogen bonds form between COOH groups of BSA and OH groups or bonded water molecules on oxide surfaces, the fewer amounts of COOH groups of BSA remains, which are confirmed by the FTIR results (ratio of COOH/Amide I in Table 6). Furthermore, ligand exchange between COOH of BSA and OH of oxide may also occur
after BSA molecules adsorbed by hydrogen bonding [18]. In sum, both hydrophobic interaction and hydrogen bonding could regulate BSA adsorption; with the increase of hydrogen content, the adsorption mechanism switched from mainly hydrophobic interaction to hydrogen bonding and ligand exchange.

Figure 24. Linear relationship of surface area normalized BSA adsorption maxima with H content of oxide particles.

Figure 25. A schematic view of BSA molecules adsorbed on surfaces of oxide particles. Left is BSA adsorbed on least hydrophilic surfaces of oxides, where hydrophobic interaction dominates adsorption process. The middle diagram is BSA adsorbed on less hydrophilic surfaces of oxides, where surface water molecules hindered hydrophobic interaction. The right diagram is BSA adsorbed on more hydrophilic surfaces, where hydrogen bonding dominates the adsorption.
Electrostatic interaction is another mechanism explaining the BSA adsorption by oxide particles. The surface area and hydrogen content of SiO₂ and γ-Al₂O₃ NPs are very close (Table 4). However, the surface normalized adsorption maximum of γ-Al₂O₃ ($Q_o' = 1.18$ mg/g, Table 5) in Figure 23B is distinctly higher than that of SiO₂ NPs ($Q_o' = 0.225$ mg/g, Table 5), indicating a stronger electrostatic interaction between the positively charged γ-Al₂O₃ particle surface and slight negatively charged BSA molecules (Table 4). Although TiO₂ NPs have the largest surface area of all these particles (325 m²/g, Table 4), the surface area normalized adsorption maximum ($Q_o' = 0.326$ mg/g, Table 5) is three times smaller than that of γ-Al₂O₃ (Figure 23B and Table 5), which can also be explained by electrostatic repulsion between the negatively charged surface of TiO₂ NPs and BSA molecules. Moreover, the maximum sorption capacity of α-Al₂O₃ is significantly higher than the other particles after normalization with surface area (Figure 23B). This is mainly because α-Al₂O₃ NPs have nearly neutral charge, which leads to a higher density of protein layer covering on α-Al₂O₃ surfaces [84, 89]. When the surface charge of protein molecules and oxide particles has the same sign, electrostatic repulsion between the two surfaces reduces the density of the protein layer covering on an oxide surface [88]. When the surface charges of protein molecules and oxide particles have opposite signs, electrostatic attraction weakens the structure stability of protein molecules, resulting in a less compact protein layer covering oxide surfaces. In this work, only α-Al₂O₃ NPs’ surface charge is neutral, neither same nor opposite to BSA. Therefore, the density of the protein layer forms on α-Al₂O₃ can be higher than that of protein layer covering on other oxide particles. However, the total adsorption amount of α-Al₂O₃ is lower than the adsorption amount of other NPs because of the small surface area.
FTIR spectra of BSA and bound-BSA on oxide particles are presented in Figure 26. 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 certain parts or levels of BSA molecular structure due to dehydration, the effect of freeze-drying may be negligible for this work because all IR spectra were processed in a same way and the subtraction of the IR spectra process could diminish the influence of freeze-drying. Peak positions in the FTIR spectra (Figure 26) are listed in Table 6 along with their assignments. The peak intensity at 1668 cm\(^{-1}\) can be assigned to Amide I (C=O stretching vibration), which is the most widely used single band in the studies of protein secondary structure [33]. 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\(_2\) scissoring and CH\(_3\) asymmetric bending), 1401 cm\(^{-1}\) (symmetric stretching of COO\(^{\prime}\)), 1306 cm\(^{-1}\) (α-helix or N-H bending vibration), 1242 cm\(^{-1}\) (β-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 folds amide II overtone) and 3312 cm\(^{-1}\) (O-H and N-H stretching vibration).

By comparison with the spectrum of BSA molecules (Figure 26), the observation of dramatically high peaks intensity at 1668 (amide I) and 1542 cm\(^{-1}\) (amide II) indicate the adsorption of BSA secondary structure on oxide particle surfaces. The increase in the ratio of amide I/amide II (Table 7) indicates the conformational change of protein molecular structure, which may result from the interaction of N-H group with the hydrophilic film of oxide particle [34]. The ratio values of BPs are higher than the values of NPs, indicating that the larger particles lead to greater conformational change (Table
6). The possible mechanism can be that bigger particles form larger protein-particle interaction interfacial area, which caused greater conformational change. Among the four NPs, the ratio values of negatively charged TiO\textsubscript{2} and SiO\textsubscript{2} NPs are very close to that of BSA, while the ratio of neutrally charged α- Al\textsubscript{2}O\textsubscript{3} is higher, and the positively charged γ-Al\textsubscript{2}O\textsubscript{3} NPs have the greatest Amide I/Amide II change. This can be explained by electrostatic interaction. When the surface of protein and oxides has the same charge sign, electrostatic repulsion between the two surfaces reduces the accessibility of BSA molecules to oxide surfaces and therefore, limits protein conformational change. When the surfaces of protein and oxides have opposite charge signs, electrostatic attraction weakens the structure stability of protein molecules, enhancing protein conformational change. The reduction of the COOH/Amide I ratio (Table 7) is caused by hydrogen bonding and/or ligand exchange which occurs between BSA COOH groups and hydroxyl groups and/or surface bonded water molecules on all oxide particles. Significant β-sheet structural modifications of BSA molecule adsorbed on TiO\textsubscript{2} and SiO\textsubscript{2} NPs surfaces are observed due to the diminishing of the peak at 1242 cm\textsuperscript{-1}, supported by a previous study [35]. Ligand exchange is demonstrated by the large decrease of the peak intensity of hydroxyl groups at 3000 – 3700 cm\textsuperscript{-1}, as well as negative peak of carboxyl groups at 1401 cm\textsuperscript{-1}.
Table 6. Peak Positions with Corresponding Assignments in the FTIR Spectra of BSA

<table>
<thead>
<tr>
<th>Wavenumber, cm⁻¹</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1668</td>
<td>Amide I (C=O stretching) [7, 8]</td>
</tr>
<tr>
<td>1542</td>
<td>Amide II (N-H bending vibration mainly, coupled to C=O and C=C stretching) [7, 8]</td>
</tr>
<tr>
<td>1456</td>
<td>CH2 and CH3 [8, 9]</td>
</tr>
<tr>
<td>1401</td>
<td>COOH [9]</td>
</tr>
<tr>
<td>1306</td>
<td>α-helix or N-H bending vibration [10]</td>
</tr>
<tr>
<td>1242</td>
<td>β-sheet [10]</td>
</tr>
<tr>
<td>2888</td>
<td>CH2 stretching [7]</td>
</tr>
<tr>
<td>2968</td>
<td>CH3 stretching [7]</td>
</tr>
<tr>
<td>3070</td>
<td>Amide A or B (N-H stretching in the Fermi resonance with 2*Amide II overtone) [11, 12]</td>
</tr>
</tbody>
</table>

Table 7. 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>
</thead>
<tbody>
<tr>
<td>BSA</td>
<td>0.989</td>
<td>0.878</td>
</tr>
<tr>
<td>Nano TiO₂</td>
<td>1.043</td>
<td>0.375</td>
</tr>
<tr>
<td>Bulk TiO₂</td>
<td>1.571</td>
<td>0.246</td>
</tr>
<tr>
<td>Nano SiO₂</td>
<td>1.096</td>
<td>0.420</td>
</tr>
<tr>
<td>Bulk SiO₂</td>
<td>1.348</td>
<td>0.296</td>
</tr>
<tr>
<td>Nano α-Al₂O₃</td>
<td>1.181</td>
<td>0.412</td>
</tr>
<tr>
<td>Nano γ-Al₂O₃</td>
<td>1.291</td>
<td>0.360</td>
</tr>
<tr>
<td>Bulk Al₂O₃</td>
<td>1.651</td>
<td>0.280</td>
</tr>
</tbody>
</table>
Figure 26. FTIR spectra of BSA and oxide-particle-bound BSA. The differential spectra of BSA-bound oxide particles were obtained by subtraction of the FTIR spectra of pure oxide particles from that of the BSA-coated oxide particles after freeze-drying.

After systematically comparing the selected oxide particles, three factors that greatly influenced BSA adsorption on oxide particles in deionized water are: 1) surface area of particles, which can provide space for BSA adsorption; 2) hydrophilicity on
particle surfaces, which can regulate the BSA adsorption mechanism switching from mainly hydrophobic interaction to hydrogen bonding and ligand exchange; and 3) electrostatic attraction, which can affect the BSA adsorption affinity. Among the three factors, surface area and hydrophilicity dominate the BSA adsorption maxima, while adsorption process is primarily induced by hydrophobic interaction and electrostatic interaction between BSA and oxide surfaces. Conformational change of BSA molecules is also observed after the BSA molecules adsorbed on oxide particle surfaces, as supported by the increase in the ratio of amide I/amide II and the decrease in the ratio of COOH/amide I. The discussion in this section provides useful information on adsorption behavior of BSA on oxide particles in the absence of phosphate. The adsorption mechanism that discussed and the data collected in this section can be used to compare the influence of phosphate to BSA adsorption.

4.2.2 Adsorption of Phosphate on Oxide Particles

Phosphate adsorption is only observed with TiO$_2$, α-Al$_2$O$_3$, γ-Al$_2$O$_3$ NPs and Al$_2$O$_3$ BPs, as shown in Figure 27 (For convenience, these four oxides are named P-adsorbing oxides in this study, while the other three oxides are called non-P-adsorbing oxides). Isotherms representing the adsorption of phosphate on these oxides are nonlinear and are well fitted by both Langmuir and Freundlich models (Line P in Table 8). Non-P-adsorbing oxides have no phosphate adsorption detected in this work, which may be caused by electrostatic repulsion between phosphate anions and negatively charged oxide surfaces. It is reported that the interaction of oxides (such as TiO$_2$, SiO$_2$ and Al$_2$O$_3$) with phosphate ions is regulated by ligand exchange, hydrogen bonding, ion-dipolar
interaction and electrostatic interaction [114, 117, 120]. At pH 7.0, the two predominant coexisting forms of phosphate are $\text{HPO}_4^{2-}$ and $\text{H}_2\text{PO}_4^-$ because $pK_a$ of $\text{KH}_2\text{PO}_4$ is 2.15, 6.82 and 12.38. The phosphate anions can interact with hydroxyl groups on oxide surface by ion-dipolar interaction, hydrogen bonding and ligand exchange [114, 117, 120]. Therefore, the surface charge of oxides becomes more negative when phosphate anions adsorb on the surface, as seen in Figure 21. When the phosphate adsorption reaches equilibrium, the electrostatic repulsion between negatively charged oxide surface and phosphate anion prevents the adsorption of phosphate. Therefore, a plateau can be found in both the phosphate-adsorption isotherms and zeta-potential curves of P-adsorbing oxides. However, for non-P-adsorbing oxides, the negative charge carried by these oxides in deionized water at pH 7.0 is strong enough to prevent the adsorption of phosphate. Therefore, the surface charge of these oxides remains constant with increasing phosphate concentration, and no phosphate adsorption is observed. Moreover, the regulation by surface charge of phosphate adsorption is clearly illustrated in Figure 28. A significant linear relationship ($P < 0.01$) between phosphate-adsorption maxima and equilibrium zeta potential of P-adsorbing oxides (Table 4) is observed.
**Figure 27.** Phosphate adsorption isotherms by different oxide particles. The arrows show that phosphate concentrations for $\alpha$-$\text{Al}_2\text{O}_3$ should be read from the right and upper axes.

**Figure 28.** Linear relationship between phosphate adsorption maxima and equilibrium zeta potential of oxide particles.
<table>
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<tr>
<th></th>
<th>Langmuir</th>
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<td>( Q_0 )</td>
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<td>( K_f )</td>
<td>( n )</td>
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<tr>
<td>D1 \textsuperscript{a}</td>
<td>106 ± 1.8</td>
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<td>P1</td>
<td>96.4 ± 1.6</td>
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<tr>
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<tr>
<td>P</td>
<td>18.8 ± 0.5</td>
<td>2.12 ± 0.8</td>
<td>0.993</td>
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<td>P\textsubscript{BSA}</td>
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<td></td>
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<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
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<td>P_{BSA}</td>
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<td>14.4 ± 1.4</td>
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<td>0.34 ± 0.0</td>
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<td>P_{BSA}</td>
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<td>22.3 ± 7.0</td>
<td>0.888</td>
<td>0.29 ± 0.0</td>
</tr>
</tbody>
</table>

a All parameter values were determined by SigmaPlot. b Average concentration of phosphate added initially in competitive adsorption experiments. c Average adsorbed concentration of phosphate after reaching equilibrium. d DI, P1, P2 and P3 represent different phosphate concentration in competitive experiments. Their respective values are listed in the column Initial Phosphate Conc. (mg/L). P represents phosphate adsorption in deionized water. P_{BSA} represents phosphate adsorption with fixed BSA concentration. e “-” represents no measurement.
4.2.3 Competitive Adsorption of Phosphate with BSA on Oxide Particles

4.2.3.1 Competitive Adsorption with Fixed Phosphate Concentrations

Figure 29 shows BSA adsorption isotherms of all selected oxide particles with phosphate as the competitor at different initial concentrations (DI, P1, P2 and P3, Table 6). For P-adsorbing oxides, crosses with corresponding color represent $K_d$ values of phosphate. All isotherms of BSA can be fitted well by both the Langmuir and Freundlich model (Table 8). For P-adsorbing oxides, as discussed above, phosphate ions can adsorb on the particle surface and increase the negative charge on the particle surface, thereby restraining BSA adsorption to particles. However, the BSA adsorption on non-P-adsorbing oxides that does not have phosphate adsorption is also hindered. This is because phosphate may neutralize positively charged domains of BSA molecules and increase the electrostatic repulsion between BSA molecules and negatively charged oxide surfaces. Although the overall surface charge of BSA in deionized water is negative (-6.64 ± 9.74 mV), certain regions of the protein surface can still carry positive charge [82, 83]. With increasing of phosphate concentration in solution, more and more positively charged regions of BSA molecules are neutralized and the overall surface charge of BSA become more negative. Therefore, the adsorption of BSA molecules to oxide particles is hindered by the increasing electrostatic repulsion. In sum, for non-P-adsorbing oxide, phosphate ions can only influence BSA surface charge, while for P-adsorbing oxides, phosphate ions can affect both BSA and oxide surface charge. This explanation is also supported by the more closely spaced BSA isotherms of non-P-adsorbing oxides than those of P-adsorbing oxides. This is because phosphate ions cannot adsorb on the surface of non-P-adsorbing oxides, resulting in fewer influence to adsorption sites of the particle
surface and showing rare change of BSA $K_d$ values with the increasing of phosphate concentration. In addition, the electrostatic repulsion can also explain the decreasing of BSA adsorption maximum with increasing phosphate initial concentrations ($Q_{0-DI} \geq Q_{0-P1} \geq Q_{0-P2} \geq Q_{0-P3}$, Table 8).
The diagrams illustrate the adsorption of BSA (Bovine Serum Albumin) on TiO$_2$ NPs and α-Al$_2$O$_3$ NPs as a function of equilibrium BSA concentration. The adsorbed BSA concentration is shown on the y-axis, while the equilibrium BSA concentration is shown on the x-axis. The data points are accompanied by error bars, indicating the variability in the adsorption process.

Additionally, the diagrams depict the Phosphate $K_d$, L/g, as a function of equilibrium BSA concentration. The Phosphate $K_d$ values are shown on the y-axis, and the equilibrium BSA concentration is again on the x-axis. The data points are represented by various markers, with error bars indicating the spread in the measured values.
**Figure 29.** BSA adsorption isotherms by selected oxide particles with phosphate as the competitor at different initial concentrations: DI, P1, P2, P3 (respective phosphate concentrations were listed in Table 8); Cross (×) with corresponding color represents $K_d$ of phosphate at P1, P2 and P3 respectively.
For P-adsorbing oxides, adsorption coefficients \((K_d = q_e/C_e)\) of phosphate as a function of BSA equilibrium concentration are shown in Figure 29. Phosphate \(K_d\) values at any fixed phosphate initial concentration (i.e. at P1, P2 or P3) remain unchanged, indicating BSA molecules have no influence on phosphate adsorption, which is in agreement with previous work [17, 18]. However, phosphate \(K_d\) values decrease with increasing initial phosphate concentrations, as illustrated in Figure 29 in that all the red crosses are higher than the green ones, and the green crosses are higher than the blue ones. There are possibly two reasons to explain this sequence of phosphate \(K_d\) values. The first reason is the electrostatic interaction between phosphate ions and oxide surface, which has been discussed above. In sum, with higher initial phosphate concentration, stronger electrostatic repulsion occurs, increasing the difficulty for phosphate ions to adsorb on the oxide surface, leading to the smaller \(K_d\) value of phosphate. The second reason to the decrease of phosphate \(K_d\) values is that the availability of adsorption sites on oxide surface is different for phosphate and BSA. Some oxide particles have micropores, which are so small that only phosphate ions can enter freely [17, 94]. BSA molecules can only adsorb onto the external surface and cannot enter into micropores [17]. When the phosphate concentration is relatively low (i.e. at concentration P1), most of phosphate ions enter into micropores and adsorb on their surface rather than adsorbing on the external surface of oxides. This is because phosphate adsorption on micropores surface has an energy advantage over adsorption on external surface that can also be occupied by BSA molecules. TiO\(_2\) NPs are the only particles that have micropores on their surfaces among all P-adsorbing oxide \((S_{micro} = 171 \text{ m}^2/\text{g}, \text{Table 4})\). So that the phosphate \(K_d\) values (P1) of TiO\(_2\) NPs (average value is 6.15 L/g, Figure 29) is the highest of all P-adsorbing
oxides. And when the phosphate concentration is relatively high (i.e. at P3), the micropore surface is fully occupied by phosphate ions. So these ions are forced to competitively take adsorption sites that are occupied by BSA molecules, leading to the reduction of phosphate $K_d$.

In the section 4.2.1 *Adsorption of BSA on Oxide Particles*, the relationship between surface-area-normalized BSA-adsorption maxima and the hydrogen content of selected oxides in deionized water (Figure 24) was discussed. The NPs show a negative trend representing hydrophobic interaction, while BPs show a positive trend indicating hydrophilic interaction and hydrogen bonding. When phosphate ions are added, the relationship between surface-area-normalized BSA-adsorption maxima and the hydrogen content of selected oxides for both NPs and BPs is changed (Figure 30A). Based on preliminary results, TiO$_2$ NPs and BPs, SiO$_2$ NPs, γ-Al$_2$O$_3$ NPs and Al$_2$O$_3$ BPs at phosphate concentration P1 and P2 (100 and 200 mg/L respectively) were chosen (Table 8). Slope values of the selected NPs and BPs at phosphate concentrations DI, P1 and P2 were calculated and listed in Table 9. Slopes of both NPs and BPs are inclined to approach zero as the phosphate concentration increases. Moreover, the linearity of selected oxides ($R^2$ values in Figure 30B) increases with rising phosphate concentration. As shown in Figure 30B, the $R^2$ value of all selected oxides in deionized water is 0.037, indicating a very bad linearity. However, a significant increase of linearity from P1 to P2 can be observed as $R^2$ (P1) = 0.392 and $R^2$ (P2) = 0.837. The inclining-to-zero slopes and increasing linearity indicate that the influence of BSA adsorption unifies with increasing of phosphate concentration. For P-adsorbing oxide, the increasing phosphate concentration leads to growing phosphate coverage over the oxide surface, which may
reduce the influence of hydrophobic and/or hydrophilic interaction that leads to BSA adsorption. Therefore, the dramatic difference of adsorption mechanisms between NPs and BPs in deionized water is diminished with higher phosphate concentration. For non-P-adsorbing oxides, phosphate ions cannot adsorb on their surfaces and hence, may not influence the hydrophobic and/or hydrophilic interaction. It is possibly related to the complexation between BSA molecules and phosphate ions [104], which may change the hydrophilicity of the BSA surface and merits further study.
Figure 30. Surface area normalized adsorption maxima of selected oxides as a function of hydrogen content at different phosphate concentration (DI, P1 and P2). (A) Negative linear relationship of NPs and positive linear relationship of BPs are observed at DI, P1 and P2. (B) Linear relationship of all selected oxides increases with rising phosphate concentration.

Table 9. Slope values of NPs and BPs at the three phosphate concentrations

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<th>NPs</th>
<th>BPs</th>
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</tr>
<tr>
<td>P1 (100 mg/L)</td>
<td>-1.511</td>
<td>0.026</td>
</tr>
<tr>
<td>P2 (200 mg/L)</td>
<td>-0.186</td>
<td>0.011</td>
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</table>

4.2.3.2 Competitive Adsorption with Fixed BSA Concentrations

Adsorption coefficients of BSA (Figure 31), at a initial concentrations of 1.7, 2.0, 0.85, 0.25, 2.0, 2.0 and 2.0 mg/g for TiO$_2$ NPs and BPs, SiO$_2$ NPs and BPs, α-Al$_2$O$_3$, γ-Al$_2$O$_3$ NPs and Al$_2$O$_3$ BPs respectively, decrease significantly with increasing phosphate acting as the competitor. There are two reasons for choosing different initial BSA
concentrations for the different oxides: over the tested range of phosphate concentrations, both BSA and phosphate adsorption can reach equilibrium, and the adsorbed percentage of phosphate is from 20% to 80%, which leads to more accurate results. Phosphate-adsorption isotherms under competition with BSA are also shown in Figure 31. For non-P-adsorbing oxides, the decrease of BSA $K_d$ values is less than the decrease of BSA $K_d$ values on P-adsorbing oxides. Moreover, BSA $K_d$ values for non-P-adsorbing oxides can reach their equilibriums, indicating that phosphate ions cannot totally hinder BSA adsorption on non-P-adsorbing oxides. This is because phosphate ions suppress BSA adsorption on non-P-adsorbing oxides only through increasing the amount of negative charge on BSA surface. BSA-adsorption sites on non-P-adsorbing oxide surface cannot be occupied by phosphate ions. Therefore, BSA adsorption on non-P-adsorbing oxides can only be limited but not totally hindered by phosphate ions. For P-adsorbing oxides, the decrease of BSA $K_d$ values is more significant than the decrease of BSA $K_d$ values on non-P-adsorbing oxides. And, the BSA $K_d$ values of P-adsorbing oxides approach zero, indicating the stronger influence and the totally suppressing effect of phosphate ions on BSA adsorption on P-adsorbing oxides. Although the zero-approaching trend of BSA $K_d$ curve for $\alpha$-Al$_2$O$_3$ NPs in Figure 31 is not very clear, preliminary results show that BSA adsorption on $\alpha$-Al$_2$O$_3$ NPs can be totally suppressed at an initial phosphate concentration of 200 mg/L. Since the equilibrium of phosphate adsorption on $\alpha$-Al$_2$O$_3$ NPs can be reached at a very low phosphate concentration (Figure 31), the tested range of phosphate concentration was designed below 100 mg/L. There are two possible reasons for the total suppression by phosphate ions of BSA adsorption on P-adsorbing oxides. One reason is that phosphate ions can adsorb on both oxide and BSA surface, resulting in a stronger
electrostatic repulsion. The other reason is that phosphate ions can occupy potential BSA adsorption sites on the oxide surface. In addition, an abrupt drop of BSA $K_d$ curves for $\alpha$-Al$_2$O$_3$ NPs, $\gamma$-Al$_2$O$_3$ NPs and Al$_2$O$_3$ BPs is found (Figure 31). This is because these three oxide particles have no micropores and BSA/phosphate adsorption occurs only on the oxide external surface. Significant competition between BSA and phosphate over the oxide surface shows up upon the addition of phosphate ions. As comparison, the decrease of $K_d$ curve for TiO$_2$ NPs, which have large micropore surface area ($S_{micro} = 171 \text{ m}^2/\text{g}$, Table 4), is much more smooth. This is because phosphate ions wait to adsorb on oxide external surface until the phosphate adsorption on micropore surface is saturated, which has been discussed above. The influence of phosphate ions on BSA adsorption is mainly through increasing the electrostatic repulsion between oxide and BSA surface but not through taking up BSA adsorption sites at low phosphate concentration, resulting the smooth $K_d$ curve for TiO$_2$ NPs.

Moreover, phosphate isotherms on P-adsorbing oxides in fixed BSA concentration solutions were obtained and were compared with phosphate isotherms in deionized water (Figure 32). Phosphate adsorption on P-adsorbing oxides stays constant regardless of the presence or absence of BSA molecules, indicating a preferential of adsorption phosphate over BSA adsorption on the oxide surface. This is because phosphate ions, which are smaller than BSA molecules, can adsorb on the micropore and external surface of oxides, leading to increased electrostatic repulsion between the oxide surface and BSA molecules, thus restraining BSA adsorption on the oxide particles, which is in agreement with previous studies [17, 18].
BSA \( K_d \), L/g

- BSA
- Phosphate

\( \gamma \)-Al\(_2\)O\(_3\) NPs

\( \alpha \)-Al\(_2\)O\(_3\) NPs

BSA \( K_d \), L/g

- BSA
- Phosphate

\( \gamma \)-Al\(_2\)O\(_3\) NPs

\( \alpha \)-Al\(_2\)O\(_3\) BPs

Phosphate equilibrium concentration, mg/L

Phosphate adsorbed concentration, mg/g

Phosphate adsorbed concentration, mg/g

Phosphate equilibrium concentration, mg/L
Figure 31. $K_d$ of BSA, as well as phosphate adsorbed concentration, as a function of phosphate equilibrium concentration at initial BSA concentrations of 1.7, 2.0, 0.85, 0.25, 2.0, 2.0 and 2.0 mg/g for TiO$_2$ NPs and BPs, SiO$_2$ NPs and BPs, $\alpha$-Al$_2$O$_3$, $\gamma$-Al$_2$O$_3$ NPs and Al$_2$O$_3$ BPs, respectively.
Figure 32. Phosphate adsorption isotherms in BSA solution and deionized water. Colored isotherms represent phosphate adsorption in deionized water. Non-colored symbols of same shape represent phosphate adsorption in BSA solution.

4.2.3.3 Competition Quantification

For nanosized P-adsorbing oxides (TiO$_2$, $\alpha$-Al$_2$O$_3$, and $\gamma$-Al$_2$O$_3$ NPs), the competitive adsorption of phosphate at fixed BSA concentration was quantified, to determine the number of BSA molecules that were displaced by phosphate ions from oxide surfaces (Table 10). The amount of BSA displaced was calculated as:

$$M = (Q_{0-BSA} - q_{e-BSA})/q_{e-phosphate} \quad (3)$$
where $M$ represents the amount of BSA molecules displaced by phosphate ions (mg/mg); $Q_{0-BSA}$ is the BSA adsorption maximum (mg/g) in deionized water; $q_{e-BSA}$ is the BSA adsorption concentration (mg/g) at a certain phosphate equilibrium concentration $C_e$; $q_{e-phosphate}$ is the phosphate adsorption concentration (mg/g) at the same $C_e$ of $q_{e-BSA}$. Values of $Q_{0-BSA}$, $q_{e-BSA}$ and $q_{e-phosphate}$ were obtained from Figure 31. The zero value of $M$ at the beginning of each column shows that no BSA molecules were displaced by phosphate ions in deionized water solution. Quantification was also employed in analyzing the competitive adsorption of BSA at fixed phosphate concentrations. The amount of BSA molecules displaced by phosphate ions is listed in Table 11.

$$M = \frac{(Q_{0-DI} - Q_{0-Pn})}{q_{e-phosphate}} \quad (4)$$

where $Q_{0-DI}$ represents the BSA-adsorption maximum in deionized water (mg/g); $Q_{0-Pn}$ is the BSA adsorption maximum at phosphate concentrations of P1, P2 and P3 (mg/g). Values of $Q_{0-DI}$, $Q_{0-Pn}$ and $q_{e-phosphate}$ were obtained from Table 8. As illustrated in Table 10, with increasing phosphate adsorbed concentration ($q_{e-phosphate}$), the M values of $\alpha$-$\text{Al}_2\text{O}_3$ NPs and $\gamma$-$\text{Al}_2\text{O}_3$ NPs basically remain constant at an average of 43.1 and 11.8 respectively, while the M value of $\text{TiO}_2$ NPs increased from around 1.59 to 4.96. The constant M values of $\alpha$-$\text{Al}_2\text{O}_3$ NPs and $\gamma$-$\text{Al}_2\text{O}_3$ NPs are because these particles have no micropore but just external surface (Table 4). Thus the adsorption sites on the oxide surfaces for BSA molecules and phosphate ions are evenly distributed. Figure 33 shows a schematic view of the competition between BSA molecules and phosphate ions on three NPs. The competition between BSA molecules and phosphate ions for these adsorption sites is unchanged regardless of phosphate concentration, resulting in the constant amounts of BSA molecules displaced by phosphate ions from $\alpha$-$\text{Al}_2\text{O}_3$ NPs and $\gamma$-$\text{Al}_2\text{O}_3$ NPs...
NPs. TiO$_2$ NPs have large micropore surface area ($S_{\text{micro}} = 171 \text{ m}^2/\text{g}$, Table 4). Most phosphate ions adsorb on micropore surface rather than on external surface at low phosphate concentration, leading to the relatively small amount of displaced BSA (Table 10). With increasing phosphate concentration, the micropore surface becomes saturated and more phosphate ions adsorb on the external surface of the oxides, resulting in an increase in the amount of displaced BSA. Moreover, the average M value for $\alpha$-Al$_2$O$_3$ NPs is 43.1, which is much higher than the M values for $\gamma$-Al$_2$O$_3$ NPs and TiO$_2$ NPs (N = 11.8 and 4.96, respectively). This may be caused by the smaller hydrogen content of $\alpha$-Al$_2$O$_3$ NPs compared to $\gamma$-Al$_2$O$_3$ NPs or TiO$_2$ NPs (0.062%, 1.21%, and 1.37%, respectively, Table 4). As discussed in connection with Figures 24, 25, and 30A, hydrophobic interaction regulates BSA adsorption on $\alpha$-Al$_2$O$_3$ NPs, $\gamma$-Al$_2$O$_3$ NPs and TiO$_2$ NPs surfaces. The smaller hydrogen content of $\alpha$-Al$_2$O$_3$ NPs leads to a stronger hydrophobic interaction between BSA molecules and the $\alpha$-Al$_2$O$_3$ NPs surfaces. Good linearity in the relationship between the amount of BSA displaced and hydrogen content for these three oxides was observed, as shown in Figure 34, indicating the influence of hydrophobic interactions on the amount of BSA displaced, M. In Table 11, with increasing initial phosphate concentration (from DI to P3), the M value of TiO$_2$ NPs increases from around 0.79 to 4.42, while the M values of $\alpha$-Al$_2$O$_3$ NPs and $\gamma$-Al$_2$O$_3$ NPs remain essentially constant at an average of 40.8 and 9.33, respectively. The pattern of change in M values, or lack thereof, for each oxide in Table 10 and 11 is the same. And, the ranges of M values for each oxide in the two Tables are very similar, which can support the discussion about the amounts displaced, above. Moreover, the Freundlich exponential coefficient, n, for $\alpha$-Al$_2$O$_3$ NPs and $\gamma$-Al$_2$O$_3$ NPs increases with rising
phosphate concentration \((n \rightarrow 1\), Table 8\), indicating that higher phosphate competition can increase the linearity of the BSA isotherms on \(\alpha\)-\(\text{Al}_2\text{O}_3\) NPs and \(\gamma\)-\(\text{Al}_2\text{O}_3\) NPs. The increased linearity of BSA isotherms may be a result of increased uniformity of oxide surface with the adsorption of phosphate ions. The uniformity may also indicate the increased partitioning of BSA molecules over phosphate-coated surfaces of \(\alpha\)-\(\text{Al}_2\text{O}_3\) NPs and \(\gamma\)-\(\text{Al}_2\text{O}_3\) NPs. For TiO\(_2\) NPs, the Freundlich exponential coefficient, \(n\), decreases with the rising phosphate concentration \((n \rightarrow 0\), Table 8\), indicating decreased linearity of the BSA isotherms on TiO\(_2\) NPs surface. This is possibly because the large \(S_{\text{micro}}\) of TiO\(_2\) NPs hinders the formation of an evenly distributed phosphate coating over TiO\(_2\) NPs surface.

**Table 10. The displaced amount of BSA molecules by phosphate ions at fixed BSA concentrations\(^a\)**

<table>
<thead>
<tr>
<th>(q_{\text{e-phosphate}}) M</th>
<th>(\alpha)-(\text{Al}_2\text{O}_3) NPs</th>
<th>(q_{\text{e-phosphate}}) M</th>
<th>(\gamma)-(\text{Al}_2\text{O}_3) NPs</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.3</td>
<td>1.59</td>
<td>2.4</td>
<td>42.1</td>
</tr>
<tr>
<td>22.9</td>
<td>1.65</td>
<td>6.9</td>
<td>43.3</td>
</tr>
<tr>
<td>56.6</td>
<td>2.47</td>
<td>13.9</td>
<td>44.9</td>
</tr>
<tr>
<td>99.5</td>
<td>3.37</td>
<td>20.8</td>
<td>41.4</td>
</tr>
<tr>
<td>178.8</td>
<td>3.43</td>
<td>29.8</td>
<td>42.4</td>
</tr>
<tr>
<td>270.2</td>
<td>3.87</td>
<td>38.5</td>
<td>42.1</td>
</tr>
<tr>
<td>461.4</td>
<td>4.10</td>
<td>49.8</td>
<td>43.4</td>
</tr>
<tr>
<td>671.0</td>
<td>4.91</td>
<td>61.5</td>
<td>44.6</td>
</tr>
<tr>
<td>883.6</td>
<td>4.96</td>
<td>76.8</td>
<td>43.5</td>
</tr>
</tbody>
</table>

\(^a\) The fixed BSA concentrations for TiO\(_2\), \(\alpha\)-\(\text{Al}_2\text{O}_3\) and \(\gamma\)-\(\text{Al}_2\text{O}_3\) NPs are 1.7, 2.0 and 2.0 mg/g respectively.
Table 11. The amounts of BSA displaced by phosphate ions at fixed phosphate concentrations

<table>
<thead>
<tr>
<th></th>
<th>TiO$_2$ NPs</th>
<th></th>
<th>α-Al$_2$O$_3$ NPs</th>
<th></th>
<th>γ-Al$_2$O$_3$ NPs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M</td>
<td>n</td>
<td>M</td>
<td>n</td>
<td>M</td>
</tr>
<tr>
<td>D1</td>
<td>0</td>
<td>0.13</td>
<td>0</td>
<td>0.08</td>
<td>0</td>
</tr>
<tr>
<td>P1</td>
<td>0.79</td>
<td>0.13</td>
<td>42.08</td>
<td>0.08</td>
<td>9.40</td>
</tr>
<tr>
<td>P2</td>
<td>3.58</td>
<td>0.05</td>
<td>39.24</td>
<td>0.10</td>
<td>10.03</td>
</tr>
<tr>
<td>P3</td>
<td>4.42</td>
<td>0.06</td>
<td>41.14</td>
<td>0.15</td>
<td>8.55</td>
</tr>
</tbody>
</table>

$^a$ $n$ is the Freundlich exponential coefficient, obtained from Table 8.

Figure 33. Schematic views of the competition between BSA molecules and phosphate ions on: (A) α-Al$_2$O$_3$ NPs and γ-Al$_2$O$_3$ NPs at a low phosphate concentration; (B) α-Al$_2$O$_3$ NPs and γ-Al$_2$O$_3$ NPs at high phosphate concentration; (C) TiO$_2$ NPs at low phosphate concentration; (D) TiO$_2$ NPs at high phosphate concentration.
**Figure 34.** A good linear relationship between displaced amount of BSA and the hydrogen content of $\alpha$-Al$_2$O$_3$ NPs, $\gamma$-Al$_2$O$_3$ NPs and TiO$_2$ NPs.

### 4.2.3.4 Implication and Prospect

Most previous and current biochemical studies on NPs are performed in buffers containing phosphate ions. Consequently, it is important to understand the influence of phosphate ions on BSA adsorption. In this study, phosphate adsorption on oxide particles was found mainly to be governed by the surface charge of the oxide. Phosphate adsorption on oxide particles can be totally hindered if the electrostatic repulsion between phosphate ions and oxide surface is strong enough (i.e. non-P-adsorbing oxides). Phosphate influence on BSA adsorption is through increasing the electrostatic repulsion between BSA molecules and oxide surfaces, and through occupation of BSA-adsorption sites on oxide surfaces. Competition between phosphate ions and BSA molecules on oxide particles with micropores is regulated by micropore surface area through preferential phosphate adsorption on micropore surfaces rather than external surfaces of
oxide particles. Phosphate adsorption cannot be affected by BSA molecules, indicating the dominance of phosphate ions in their competition with BSA molecules. Moreover, with increasing phosphate concentration, the difference between hydrophobic and hydrophilic interaction for BSA adsorption decreases and the regulation by hydrogen content to BSA adsorption is uniformed. Quantification of the competition shows a good linearity between the displaced amount of phosphate and hydrogen content. According to the results in this work, studies of the environmental impact of oxide NPs, especially using *in-vitro* experiments with phosphate buffers, should take the influence of phosphate ions into consideration. Since phosphate ions can suppress BSA adsorption on oxide surface, further study should focus on the desorption of BSA from oxide surface under the influence of phosphate ions, because BSA-coated oxide NPs in vivo are in a liquid environment that is related by metabolism.

**4.3 Desorption Section**

Figure 35 shows the adsorption-desorption isotherms of BSA molecules on TiO$_2$, SiO$_2$, α-Al$_2$O$_3$ and γ-Al$_2$O$_3$ NPs, as well as their respective phosphate adsorbed concentration. All the isotherms are fitted by Langmuir model (Table 12). As illustrated in Figure 35, TiO$_2$ isotherms have no significant adsorption-desorption hysteresis, while SiO$_2$ isotherms present clear hysteresis; α-Al$_2$O$_3$ and γ-Al$_2$O$_3$ isotherms have hysteresis at low BSA concentrations, but the hysteresis is gradually reduced with the increase of BSA concentration. As shown in Table 12, the BSA adsorption maxima ($Q_0$) of TiO$_2$, α-Al$_2$O$_3$ and γ-Al$_2$O$_3$ NPs have no significant difference, while the $Q_0$ values of SiO$_2$ show obvious hysteresis. However, when the BSA equilibrium concentration ($C_e$) is at 100
mg/L, the BSA adsorbed concentration ($q_e$) of SiO$_2$, $\alpha$-Al$_2$O$_3$ and $\gamma$-Al$_2$O$_3$ NPs shows a clear increase, while the $q_e$ values of TiO$_2$ are still very close.
**Figure 35.** Adsorption-desorption isotherms of BSA by oxide particles in phosphate solution, and respective phosphate adsorbed concentration.
Table 12. Parameters of Langmuir Model Fitted BSA Adsorption/Desorption on NPs

<table>
<thead>
<tr>
<th></th>
<th>Langmuir</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$Q_0$</td>
<td>$K_l$</td>
<td>$r^2$</td>
<td>$q_{e(100)}^b$</td>
</tr>
<tr>
<td>TiO$_2$ NPs</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adsorption</td>
<td>50.0 ± 1.6</td>
<td>16.2 ± 3.0</td>
<td>0.973</td>
<td>0.430</td>
</tr>
<tr>
<td>Desorption 1</td>
<td>56.0 ± 4.6</td>
<td>26.0 ± 9.2</td>
<td>0.715</td>
<td>0.444</td>
</tr>
<tr>
<td>Desorption 2</td>
<td>61.0 ± 9.0</td>
<td>36.7 ± 16</td>
<td>0.678</td>
<td>0.446</td>
</tr>
<tr>
<td>SiO$_2$ NPs</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adsorption</td>
<td>23.3 ± 0.2</td>
<td>250 ± 33</td>
<td>0.981</td>
<td>0.067</td>
</tr>
<tr>
<td>Desorption 1</td>
<td>25.9 ± 0.6</td>
<td>136 ± 18</td>
<td>0.985</td>
<td>0.110</td>
</tr>
<tr>
<td>Desorption 2</td>
<td>27.3 ± 0.5</td>
<td>50.9 ± 3.8</td>
<td>0.996</td>
<td>0.181</td>
</tr>
<tr>
<td>α-Al$_2$O$_3$ NPs</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adsorption</td>
<td>9.17 ± 0.2</td>
<td>15.2 ± 2.1</td>
<td>0.977</td>
<td>0.080</td>
</tr>
<tr>
<td>Desorption 1</td>
<td>10.0 ± 0.5</td>
<td>24.5 ± 4.5</td>
<td>0.925</td>
<td>0.080</td>
</tr>
<tr>
<td>Desorption 2</td>
<td>10.1 ± 0.6</td>
<td>9.88 ± 1.9</td>
<td>0.921</td>
<td>0.092</td>
</tr>
<tr>
<td>γ-Al$_2$O$_3$ NPs</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adsorption</td>
<td>103 ± 1.6</td>
<td>58.3 ± 4.3</td>
<td>0.936</td>
<td>0.654</td>
</tr>
<tr>
<td>Desorption 1</td>
<td>106 ± 4.2</td>
<td>49.9 ± 7.7</td>
<td>0.937</td>
<td>0.710</td>
</tr>
<tr>
<td>Desorption 2</td>
<td>108 ± 6.0</td>
<td>27.3 ± 9.2</td>
<td>0.914</td>
<td>0.845</td>
</tr>
</tbody>
</table>

$^a$ All parameter values were determined by Sigmaplot. $^b$ BSA adsorbed concentration ($q_e$) were calculated at $C_e = 100$ mg/L by using the Langmuir equation with respective fitted parameters.

Adsorption-desorption hysteresis is categorized as true hysteresis or artificial hysteresis [139]. Artificial hysteresis is caused by insufficient equilibrium of sorbate and/or auxiliary process (i.e. volatilization of organic compounds) [135]. In this work, sufficient equilibrium time is provided, and neither BSA molecules nor phosphate ions are volatile. Besides, significant hysteresis of BSA molecules is observed for SiO$_2$ but not for TiO$_2$, indicating the observed hysteresis is true. For SiO$_2$ NPs, desorption hysteresis is clearly observed, which is related with the stronger aggregation ($D_{ratio} = 42.5$, Table 4). Yang et al. discussed the desorption hysteresis on fullerene and suggested the possible mechanism is the geometry and aggregation of fullerene [135]. Closed interstitial spaces can be formed either in small aggregate of fullerene or among these small aggregates. Sorbate molecules can penetrate and/or be entrapped in these closed interstitial spaces, leading to the irreversible hysteresis [135]. As discussed above, BSA molecule is modeled as a triangular prismatic shell with optimized dimensions of $84 \times 84 \times 84 \times 30$ Å.
in a neutral solution [164]. Based on these dimensions, the diameter of BSA molecule is 9.70 nm. Meanwhile, the hydrodynamic diameter of TiO\textsubscript{2}, SiO\textsubscript{2}, α-Al\textsubscript{2}O\textsubscript{3} and γ-Al\textsubscript{2}O\textsubscript{3} NPs was measured after the BSA adsorption reached equilibrium in the 1500 mg/L phosphate buffer, as shown in Figure 36. The hydrodynamic diameter data in Table 4 is different from the hydrodynamic diameter data in Figure 36 at the BSA concentration is zero. This is because the hydrodynamic diameter data obtained in Figure 36 was in measured in phosphate buffer with pH adjustment, while the hydrodynamic diameter data obtained in Table 4 was in deionized water without any treatment. For α-Al\textsubscript{2}O\textsubscript{3} and γ-Al\textsubscript{2}O\textsubscript{3} NPs, the hysteresis is observed at the low BSA equilibrium concentration, but reduced with the increase of BSA equilibrium concentration. There are possibly two reasons for these phenomena. The first reason is possibly that the adsorbed BSA molecules can reduce the aggregation and increase the suspension of α-Al\textsubscript{2}O\textsubscript{3} and γ-Al\textsubscript{2}O\textsubscript{3} NPs. For α-Al\textsubscript{2}O\textsubscript{3} and γ-Al\textsubscript{2}O\textsubscript{3} NPs, as shown in Figure 36, the hydrodynamic diameters of these NPs are much higher than the other NPs at low BSA concentrations, indicating very strong aggregation of α-Al\textsubscript{2}O\textsubscript{3} and γ-Al\textsubscript{2}O\textsubscript{3} NPs. Then, a clear drop of hydrodynamic diameter can be observed at low BSA equilibrium concentrations and reach the plateau at around 100 and 500 mg/L respectively. Compared with the adsorption-desorption isotherms in Figure 35, the hysteresis of α-Al\textsubscript{2}O\textsubscript{3} NPs was significantly reduced at about 100 mg/L, while the hysteresis of γ-Al\textsubscript{2}O\textsubscript{3} NPs dramatically abates at about 500 mg/L too, indicating the regulation of BSA concentration to the aggregation of oxide particles. BSA molecules have both hydrophobic groups (i.e. CH\textsubscript{3}) and hydrophilic groups (i.e. COOH) so that the molecules may act as the surfactant to oxide aggregations. This is because surfactants, which are
amphipathic molecules with both hydrophilic and hydrophobic moieties, can form micro-emulsion with the hydrophobic groups on solid surface (i.e. the surface of oxide NPs in this work) [168]. This process can increase the suspension of oxide NPs and thereby reduce the aggregation. The second reason for the reducing hysteresis of $\alpha$-Al$_2$O$_3$ and $\gamma$-Al$_2$O$_3$ NPs is that the electrostatic repulsion between these NPs increases with the phosphate adsorption, as shown in Figure 21 and Table 4. The repulsion can reduce the aggregation of the oxide NPs, leading to less entrapment of BSA molecules by these aggregates. For TiO$_2$ and SiO$_2$ NPs, both of their hydrodynamic diameters have little change with the rise of BSA concentrations. However, TiO$_2$ NPs have no clear hysteresis while SiO$_2$ NPs have obvious hysteresis. This is because TiO$_2$ NPs have less aggregation but bigger particle diameter than SiO$_2$ NPs (the averages of hydrodynamic diameter for TiO$_2$ and SiO$_2$ NPs in Figure 36 are 640 nm and 860 nm, while the particle diameters in Table 4 are 50 nm and 30 nm, respectively). The ratios of hydrodynamic diameter over particle diameter for TiO$_2$ and SiO$_2$ NPs are 12.8 and 28.6 respectively. Therefore, the closed interstitial spaces of TiO$_2$ NPs aggregates may not entrap BSA molecules in the aggregates. For $\alpha$-Al$_2$O$_3$ and $\gamma$-Al$_2$O$_3$ NPs, although their particle diameters are larger than the particle diameter of TiO$_2$ NPs, the hydrodynamic diameters of their aggregates at low BSA concentrations are much higher than that of TiO$_2$ NPs.
Figure 36. Particle diameter of TiO$_2$, α-Al$_2$O$_3$ and γ-Al$_2$O$_3$ NPs as a function of BSA equilibrium concentration.

In addition, as illustrated in Figure 35, the phosphate adsorbed concentrations of TiO$_2$, α-Al$_2$O$_3$ and γ-Al$_2$O$_3$ NPs remain unchanged within the processes of BSA adsorption and two-cycle desorption. TiO$_2$ NPs have no significant desorption hysteresis so that the BSA desorbed sites are taken by phosphate ions, leading to the unchanged phosphate adsorbed concentrations, which is in line with our previous discussion on the influence of phosphate ions to BSA adsorption. However, clear hysteresis is observed for α-Al$_2$O$_3$ and γ-Al$_2$O$_3$ NPs at low BSA concentration, and the phosphate adsorbed concentrations are also unchanged. This might be partly explained as the entrapped BSA molecules are not adsorbed on oxide surface, but freely entrapped in the closed interstitial spaces of the aggregates of NPs. As we discussed in section 4.2.3 Competitive Adsorption
of Phosphate with BSA on Oxide Particles, phosphate ions have priority to adsorb on oxide surface rather than BSA molecules. Meanwhile, phosphate ions are so small that they can enter the micropores of oxides freely [17]. So the aggregations of oxides may not obstruct the phosphate ions to enter into the closed interstitial spaces. Therefore, the entrapped BSA molecules may be replaced by the phosphate ions but still entrapped in the closed interstitial spaces, leading to the unchanged phosphate adsorbed concentrations. A schematic view is shown in Figure 37.

![Figure 37. A schematic view of the BSA adsorption-desorption hysteresis procedure.](image)

In summary, the desorption hysteresis of BSA molecules from the surface of oxide NPs is governed by: 1) aggregation of NPs, which can cause the hysteresis of BSA desorption; 2) BSA equilibrium concentration, which can regulate the aggregation of NPs
and the desorption hysteresis; 3) surface charge of oxide NPs, which can affect the electrostatic repulsion between oxide NPs and thereby influence the aggregation of NPs. BSA desorption hysteresis is observed for SiO$_2$ NPs, while no significant hysteresis for TiO$_2$ NPs. $\alpha$-Al$_2$O$_3$ and $\gamma$-Al$_2$O$_3$ NPs show hysteresis at low BSA concentration and no significant hysteresis at high BSA concentration. The high BSA adsorption capacity and desorption hysteresis on oxide NPs imply the potential damage of oxide NPs to living being, especially to serum albumin protein molecules.
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