

Binding of Human Serum Proteins to Titanium Dioxide Particles *In Vitro*

Mazen S.K. ZAQOUT, Tomoyuki SUMIZAWA, Hideki IGISU, Toshiaki HIGASHI and
Toshihiko MYOJO

Institute of Industrial Ecological Sciences, University of Occupational and Environmental Health, Japan

Abstract: Binding of Human Serum Proteins to Titanium Dioxide Particles *In Vitro*: Mazen S.K. ZAQOUT, et al. Institute of Industrial Ecological Sciences, University of Occupational and Environmental Health, Japan—Objectives: To determine the capacity of human serum proteins to bind to titanium dioxide (TiO₂) particles of different polymorphs and sizes. **Methods:** TiO₂ particles were mixed with diluted human serum, purified human serum albumin (HSA) or purified human serum gamma-globulin (HGG) solutions. After incubation at 37°C for 1 h, the particles were sedimented by centrifugation, and proteins in the supernatant, as well as those bound to the particles, were analyzed. **Results:** The total protein concentration in the supernatant was lowered by TiO₂, whereas the albumin/globulin ratio was elevated by the particles. Incubation with TiO₂ also lowered the immunoglobulin, pre-albumin, beta2-microglobulin, ceruloplasmin and retinol-binding protein levels, but not ferritin levels, in the supernatant. After sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), proteins in the supernatant, especially HGG, were observed to decrease, while those released from the particles (after adding 1% SDS and heating) increased, depending on the dose of TiO₂. Purified HGG and HSA were also bound to TiO₂, although the former appeared to have a higher affinity. All the proteins tested showed the highest binding potency to the amorphous particles (<50 nm) and the lowest to the rutile particles (<5,000 nm), while binding to anatase particles was intermediate. The affinity to the larger anatase was higher than that to smaller anatase particles in most cases. **Conclusions:** Human serum proteins, including the two major

components, HSA and HGG, are bound by TiO₂ particles. The polymorph of the particles seems to be important for determining the binding capacity of the particles and it may affect distribution of the particles in the body.

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Key words: Albumin, Gamma-globulin, Nanoparticle, Nanotoxicology, Serum protein, Titanium dioxide

Because of its brightness and very high refractive index (n=2.7), titanium dioxide (TiO₂) is extensively used as a pigment to provide whiteness and opacity to paints, coatings, plastics, and paper¹. When irradiated by light, TiO₂ can degrade almost any organic compound (photocatalytic effect) and water is attracted to TiO₂-treated surface (superhydrophilicity). Due to these properties, TiO₂ is used to manufacture “air-cleansing panels” and “self-cleaning” tiles². Because of its many applications, TiO₂ accounts for 70% of the total production volume of pigments, and worldwide production in 2004 was 4.4 million tons¹.

Two cohort studies undertaken in the USA^{3, 4} and a population-based case-control study in Canada⁵ revealed no increase in the risks of lung cancer associated with exposure to TiO₂. Nevertheless, a large cohort study in six European countries indicated a statistically significant increase in the occurrence of lung cancer among workers in the TiO₂ production industry⁶. In rats, inhalation or intratracheal instillation of TiO₂ has been shown to produce pulmonary tumors^{1, 7}. Hence, the International Agency for Research on Cancer has concluded that while there is inadequate evidence of the carcinogenicity of TiO₂ in humans, there is sufficient evidence from studies in experimental animals, and has thus classified titanium dioxide as a Group 2B compound (possibly carcinogenic to humans)¹.

In general, however, TiO₂ has been regarded as inert and is added to cosmetics, toothpaste, drugs and foods⁸. It has

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Correspondence to: H. Igisu, Department of Environmental Toxicology, Institute of Industrial Ecological Sciences, University of Occupational and Environmental Health, Yahata-Nishi-Ku, Kitakyushu, Fukuoka 807-8555, Japan
(e-mail: igisu@med.uoeh-u.ac.jp)

often been used as a negative control in experiments on the effects of particles or fibers in cells and animals^{9, 10}. Contradicting this assumption, we previously demonstrated that TiO₂ can hemolyze washed human erythrocytes *in vitro*¹¹, and showed that the hemolytic potential varies according to the type of polymorph being examined. However, the addition of plasma could abolish hemolysis, and not only human serum albumin (HSA) but also human gamma-globulin (HGG) suppresses hemolysis induced by the anatase form of TiO₂, which showed the highest hemolytic potential among the particles tested¹¹. This was in clear contrast to the hemolysis caused by pentachlorophenol, which was completely suppressed by HSA but not at all by HGG (<0.5%)¹².

Several experiments have indicated that HSA or bovine serum albumin binds to TiO₂¹³⁻¹⁸, but little attention has been paid to HGG or other serum proteins. To our knowledge, Ellingsen's study is the only study to have examined the binding of IgG and pre-albumin, as well as that of HSA, to TiO₂. However, this previous study did not undertake any quantitative analysis of the proteins. Moreover, the effects of polymorphs of TiO₂ on the interaction with serum proteins have not been reported. We therefore examined the binding of numerous serum proteins to TiO₂ particles of different polymorphs and sizes.

Materials and Methods

Materials

Anatase TiO₂ (primary size <25 nm, 99.7%) and holotransferrin (human) were purchased from Sigma-Aldrich Japan (Tokyo). Other forms of TiO₂ (>99.9%) and purified human serum proteins (albumin and γ -globulin, >95%) were supplied by Wako Pure Chemicals (Osaka, Japan). Papain, 2X crystallized, was obtained from MP Biomedicals Japan (Tokyo). The cocktail set III (protease inhibitor) was obtained from Merck-Calbiochem Japan (Tokyo). The DC Protein Assay kit, gels (Ready Gel J 10%) for the sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), Coomassie G-250 stain, and Precision Plus protein standards were obtained from Bio-Rad Japan (Tokyo). All other chemicals were of analytical grade.

Blood was obtained from healthy males (36, 40 and 49 yr old) into Terumo Venoject tubes containing no additives. The blood was left at room temperature for 30 min, then serum was separated by centrifugation at 1,000 \times g for 15 min.

Specific surface area and zeta potentials of the particles

Based on the BET theory¹⁹, the specific surface areas were measured by N₂ adsorption onto the samples using a Quantachrome Instruments NOVA-1200 in the Ube Scientific Analysis Laboratory (Ube, Japan). The particle diameters of the TiO₂ samples were estimated, assuming

all the particles had the same spherical shape and size. The diameter D was calculated as: $D=6/S\rho$, where S was the specific surface area measured by the BET method and ρ the density of the sample (g/cm³), which was assumed to be 3.9 (anatase and amorphous) or 4.2 (rutile particles).

The zeta potentials were determined by the electrophoretic light scattering method using a Beckman-Coulter DelsaNano C in the Ube Scientific Analysis Laboratory (Ube, Japan).

Reaction

Two buffer solutions were used for the reactions. Buffer I contained 30 mM Tris-HCl, pH 7.4, 120 mM NaCl, 5 mM KCl and 2 mM MgCl₂, and Buffer II contained 10 mM phosphate buffer, pH 7.4, 138 mM NaCl and 2.7 mM KCl.

The reaction mixture (1 to 3 ml) contained serum (1 to 50%), purified HSA (0.1%), HGG (0.1%) or papain-digested HGG (0.0125%) and buffer containing TiO₂. Two series of TiO₂ concentrations were used: 0, 0.3, 1.5, 3 and 6 mg/ml, and 0, 15, 30 and 50 mg/ml. These were chosen based on the results of several preliminary experiments and on our previous results¹¹. The second series was comparable with the concentrations used by Horie *et al.*²⁰. After the mixture was incubated at 37°C for 60 min with gentle shaking, the mixture was centrifuged at 20,000 \times g for 30 min to separate the supernatant (supernatant I) and the particles. Supernatant I was clear on visual inspection.

To desorb proteins from the particles, the particles were washed (adding 1 ml of the buffer and centrifuging as above) 3 times, and then treated with 1 ml of the buffer containing 1% SDS at 100°C for 5 min. The particles were again removed by centrifugation and the supernatant (supernatant II) was separated and retained.

Determination of total serum protein, and proteins other than albumin and globulin

The total serum protein and serum proteins other than HSA and HGG were measured by conventional automated methods at SRL Laboratories (Tokyo). Total protein was measured by the Biuret method with a JEOL JCA-BM8060. Electrophoresis (to determine the albumin/globulin ratio on cellulose-acetate membranes) was performed with an Olympus AES630. Serum levels of IgA, IgM and IgG were determined by turbidometric immunoassay with a JEOL JCA-BM1650. Pre-albumin, retinol-binding protein, and ceruloplasmin were assessed by immunonephelometry with a Boehringer Nephelometer Analyzer II. The beta2-microglobulin level was examined by latex immunoassay, and the transferrin level was determined by turbidometric immunoassay with a JEOL JCA-BM8000. The ferritin levels were examined by chemical luminescence enzyme immunoassay with a

Table 1. TiO₂ particles used in the experiments

	Crystal structure (size)	Surface area* (m ² /g)	Particle size** (nm)	Zeta potential*** (mV)	
				in Buffer I	in Buffer II
I	Amorphous (<50 nm)	110	14	-0.11	-28.18
II	Anatase (<5,000 nm)	53.2	29	4.8	-25.56
III	Anatase (<25 nm)	116	13	-2.93	-21.78
IV	Rutile (<5,000 nm)	6.31	226	-4.66	-23.75

The crystal structure and size were provided by the manufacturer (Particles I, II and IV from Wako, and III from Sigma-Aldrich). According to the manufacturer, the purity of particles (%) was 99.9 (particles I, II and IV), and 99.7 (particle IV). *Measured by N₂ adsorption (BET method). **Estimated from the BET surface area, assuming the density of the particles (g/cm³) to be 3.9 (particles I, II and III) or 4.2 (particle IV). ***Determined by electrophoretic light scattering. Buffer I contained 30 mM Tris-HCl, pH 7.4, 120 mM NaCl, 5 mM KCl and 2 mM MgCl₂, and Buffer II 10 mM phosphate buffer, pH 7.4, 138 mM NaCl and 2.7 mM KCl.

FujiRebio Lumipulse PrestoII.

SDS-PAGE and measurement of HSA and HGG

Before applying to SDS-PAGE, supernatant I was diluted with buffer and 16 μ l of the diluted supernatant was mixed with the sample buffer (4 μ l) containing 5% SDS. Supernatant II was loaded onto the gels without addition of any sample buffer.

The concentrations of HSA and HGG in supernatant I were measured spectrophotometrically (optical density at 280 nm) using a Beckman-Coulter DU640 spectrophotometer. HSA and HGG desorbed from the particles (in Buffer II) (supernatant II) were assayed using the DC Protein assay kit on a Bio-Rad 680 microplate reader, following the manufacturer's instructions, with the exception of increasing volume of the samples to 30 μ l.

Papain-digestion of gamma-globulin

The method developed by Porter²¹⁾ was used to digest gamma-globulin with slight modifications. Five hundred mg of HGG was dissolved in 40 ml of 0.1 M phosphate buffer, pH 7.0 containing 2 mM EDTA. Next, 5 mg of papain, which was activated in 0.3 ml of 10 mM cysteine at 37°C for 10 min, was added and incubated at 37°C for 2 h. The reaction was terminated by the addition of 0.5% Cocktail Set III.

Hill plot and statistical analyses

When appropriate, the results were analyzed by Hill plot, i.e., plotting log (P/(1-P)) against log (X), where P is the relative protein concentration (assuming the total protein as 1) and X the concentration of TiO₂. Regression analysis was carried out using Microcal Origin (version

6.0J, Microcal Software, Northampton, MA, USA). Dunnett and Bonferroni tests following ANOVA were performed using GraphPad Prism (version 2.01 and 5, GraphPad Software, San Diego, CA, USA).

Unless otherwise stated, all data are expressed as means \pm SD. All experiments were carried out at least three times.

Results

BET surface area and Zeta potentials of the particles

Estimated particle size did not differ as greatly as the nominal size suggested between the anatase particles (Table 1) but the difference in the zeta potentials was clear. The zeta potentials differed greatly between buffers I and II.

Total serum protein and albumin/globulin ratio

For the reaction mixture, we first used Buffer I because it was used in our previous experiments¹¹⁾. When the diluted serum was incubated with TiO₂, the total protein in the supernatant decreased dose-dependently, and the decrease was most pronounced when the serum was incubated with amorphous particles (Fig. 1, top). On the other hand, the albumin/globulin ratio obtained by electrophoresis on cellulose-acetate membranes showed that the ratio increased, depending on the dose of TiO₂, and that the increase was highest when the serum was incubated with amorphous particles, followed by anatase particles (Fig. 1, bottom). Thus, it appears that serum proteins bound to TiO₂, but that not all proteins were bound equally to the particles, i.e., more globulin was bound than albumin.

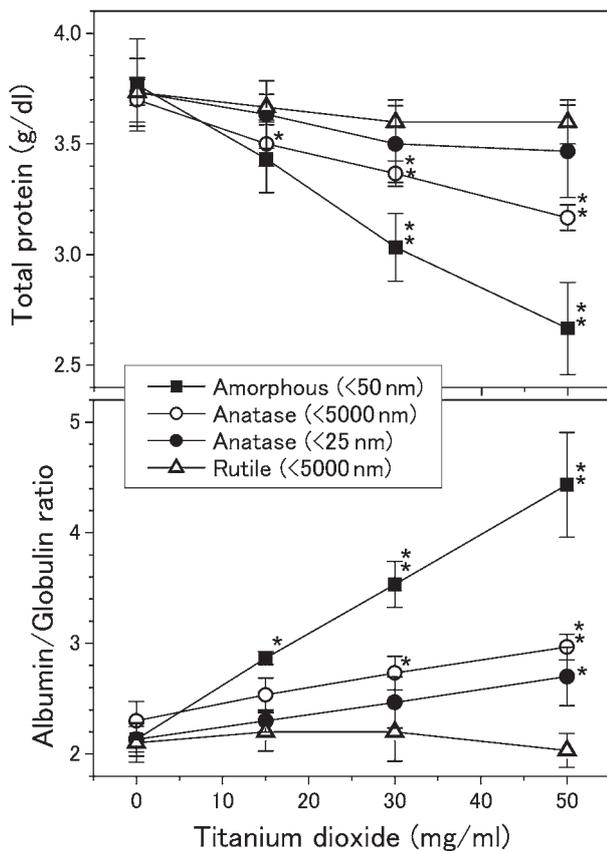


Fig. 1. Total protein (top) and the albumin/globulin ratio (bottom) in the supernatant (supernatant I) after diluted serum was incubated with TiO_2 particles (mean \pm SD). Serum obtained from healthy males ($n=3$) was diluted to 50% with Buffer I containing TiO_2 . After incubating at 37°C for 1 h, the particles were removed by centrifugation at $20,000 \times g$ for 30 min. The total protein was measured by the Biuret method, and the albumin/globulin ratio was determined by electrophoresis on cellulose acetate membranes. Asterisks indicate statistically significant differences compared to the control (no TiO_2), * $p<0.05$ and ** $p<0.01$ (ANOVA followed by Dunnett's test). Comparing polymorph (at 50 mg/ml) effects on total protein, statistically significant differences were found between the amorphous and all other forms ($p<0.001$), between anatase (<5,000 nm) and rutile ($p<0.01$), and between anatase (<5,000 nm) and anatase (<25 nm) ($p<0.05$) (ANOVA followed by Bonferroni's test). For the albumin/globulin ratio, only the effect of the amorphous for was statistically significantly different from those of other forms ($p<0.001$) (ANOVA followed by Bonferroni's test).

Changes in serum proteins other than HGG and HSA

The concentration of ferritin in the supernatant did not change with increasing concentrations of TiO_2 (Fig. 2). Transferrin and IgA were decreased slightly by amorphous particles, but not by other particles. In contrast, IgM, IgG

and pre-albumin were all clearly decreased by all but rutile forms of TiO_2 particles (Fig. 2). Similarly, beta2-microglobulin, ceruloplasmin and retinol-binding protein were decreased by the amorphous and anatase forms of TiO_2 (data not shown).

SDS-PAGE analysis of serum proteins

To confirm the above findings, we attempted to analyze the proteins by SDS-PAGE. Desorbing the proteins from the particles was extremely difficult when the reaction mixture was prepared using Buffer I (later, we found that adding higher concentrations of SDS (4%) and 8 M urea resulted in release of the proteins, but that the protein release was not quantitatively achieved). We therefore used Buffer II to desorb the proteins, and found that proteins were desorbed from the particles by relatively mild treatment (1% SDS and heating). The amount of protein found increased with the concentration of TiO_2 (Fig. 3). Consistent with the results shown in Fig. 1 and 2, the amount of proteins bound to TiO_2 was the largest when the amorphous particles were incubated and the smallest when the rutile particles were incubated.

Changes of purified HGG and HSA

When purified HGG and HSA (1 mg/ml) were incubated with TiO_2 in Buffer I or II, both HGG and HSA decreased in the supernatant in a TiO_2 dose-dependent manner (Fig. 4). Here again, as shown in Fig. 1–3, the potency of the particles to lower the protein concentration in the supernatant was in the order of amorphous > anatase > rutile. The decrease in HGG was always larger than the decrease in HSA except for the decrease with the amorphous form in Buffer II (C vs. D). The decrease in HGG, as well as the decrease in HSA, was greater in Buffer I than in Buffer II (A vs. C, and B vs. D) (Fig. 4).

After incubation in Buffer II, the concentrations of HGG and HSA desorbed from TiO_2 particles (in 1% SDS for 5 min at 100°C) were elevated in a dose-dependent manner (Fig. 5). The amount of protein desorbed was greatest with the amorphous, followed by the anatase and the rutile particles.

Binding of native HGG and papain-digested fragments

By treating HGG with papain, a mixture of native HGG, and its fragments (Fab and Fc) were obtained. After adding TiO_2 and incubating with the proteins in Buffer II, proteins in the supernatant and those desorbed from the particles were applied to SDS-PAGE (Fig. 6). No clear difference was observed between the native HGG and fragments.

Discussion

HSA and HGG are the two most abundant proteins present in plasma. Their concentrations in the plasma of healthy subjects are 3.5–5.5 g/dl and 0.7–1.7 g/dl,

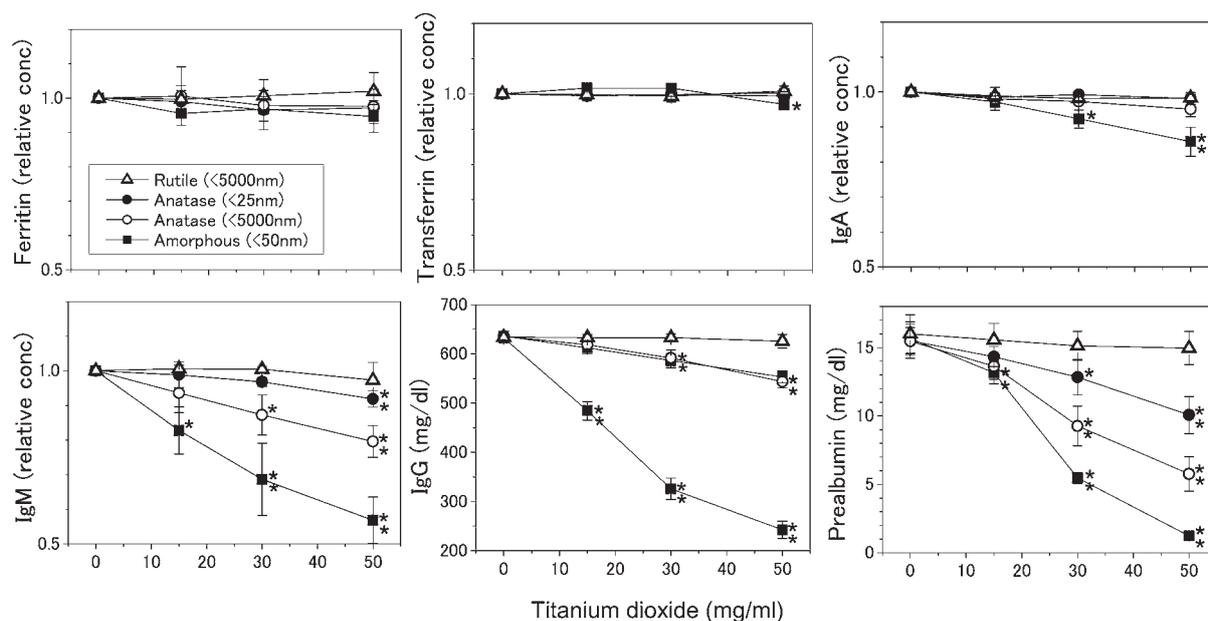


Fig. 2. Ferritin and other serum proteins in the supernatant (supernatant I) after diluted serum was incubated with TiO₂ particles (mean ± SD). Serum obtained from healthy males (n=3) was diluted to 50% with Buffer I containing TiO₂. After incubating the samples at 37°C for 1h, the particles were removed by centrifugation at 20,000 × g for 30 min. Since inter-individual differences were large with regard to the concentrations of ferritin, transferrin, IgA and IgM, these values are expressed as the relative concentration (ratio to samples incubated without TiO₂). Asterisks indicate statistically significant differences compared to the control (no TiO₂), **p*<0.05 and ***p*<0.01 (ANOVA followed by Dunnett’s test). Except for ferritin, the effect of amorphous particles (at 50 mg/ml) was significantly different from other forms (*p*<0.001) (ANOVA followed by Bonferroni’s test). Significant differences were also observed between anatase (<5,000 nm) and anatase (<25 nm) for IgM (*p*<0.05) and for prealbumin (*p*<0.001), between anatase (<5,000 nm) and rutile for IgM, IgG and prealbumin (*p*<0.001), and between anatase (<25 nm) and rutile for IgG (*p*<0.001) and on prealbumin (*p*<0.001).

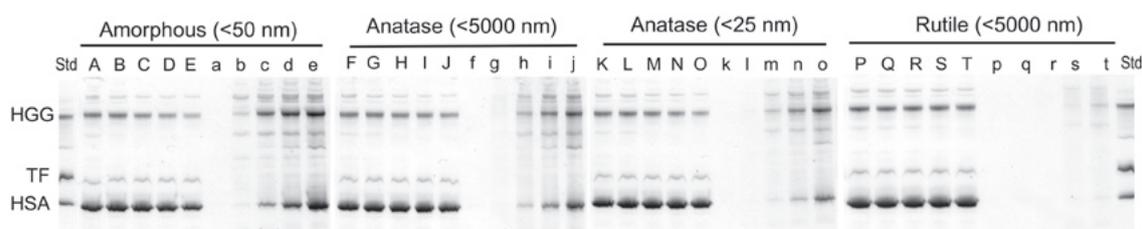


Fig. 3. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (on Ready Gel J 10%) of free serum proteins and those bound to TiO₂ particles. Serum diluted to 1% with Buffer II was incubated with TiO₂ at 37°C for 1 h. The particles were sedimented by centrifugation at 20,000 × g for 30 min. Sixteen microliters of the diluted (5 times with the buffer) supernatant (supernatant I) was mixed with 4 μl of the sample buffer containing 5% SDS and loaded onto the gel (A to E, F to J, K to O, and P to T). The dose (mg/ml) of the particles was; 0 (A, F, K, P), 0.3 (B, G, L, Q), 1.5 (C, H, M, R), 3 (D, I, N, S) and 6 (E, J, O, T). The sedimented particles were washed (suspended and centrifuged) with the buffer 3 times, and then treated with 1 ml of 1% SDS for 5 min at 100°C to desorb proteins. After removing the particles by centrifugation as described above, 16 μl of the supernatant (supernatant II) was mixed with 4 μl of buffer containing 1% SDS and loaded onto the gel (a to e, f to j, k to o, and p to t). A mixture of HGG, transferrin (TF), and HSA was loaded onto the Std lanes. The gels were stained with Coomassie Brilliant Blue.

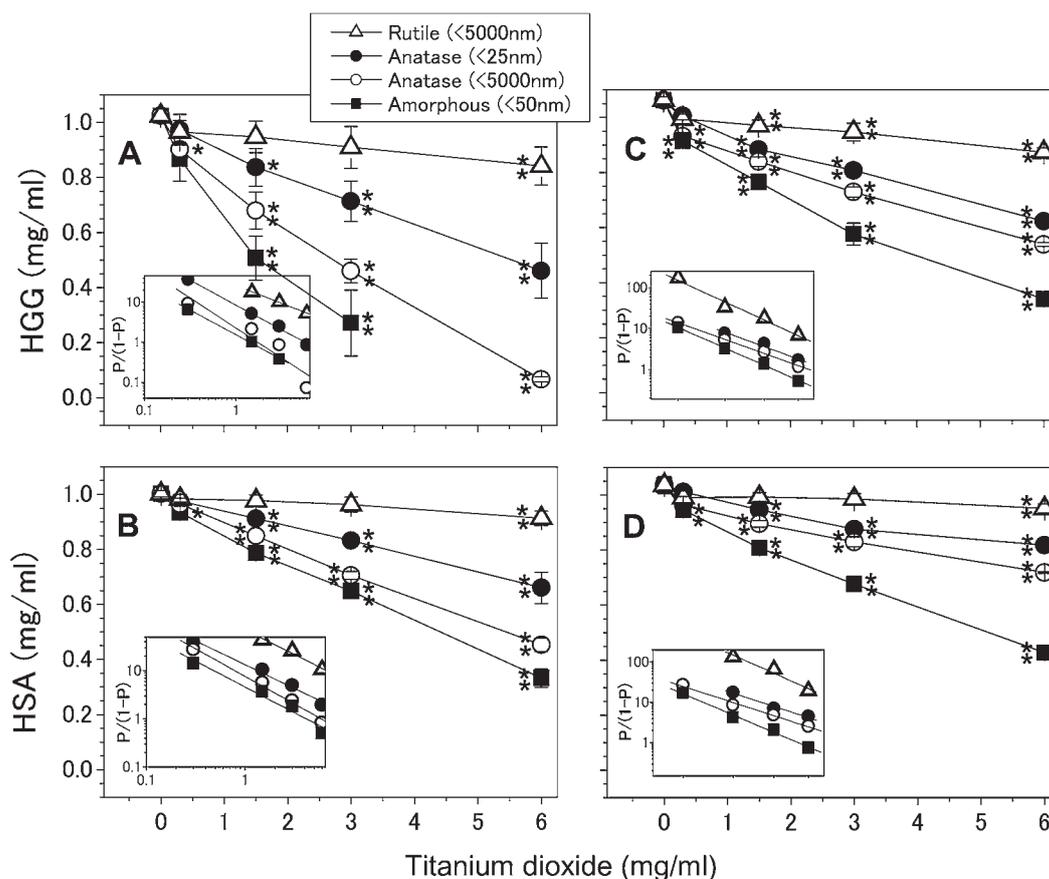


Fig. 4. Human serum gamma-globulin (HGG) and human serum albumin (HSA) in the supernatant after incubation with TiO_2 (mean \pm SD, $n=3$). TiO_2 particles were incubated with 1 mg/ml of purified HGG or HSA in Buffer I (A and B) or Buffer II (C and D) at 37°C for 1 h. After the particles were removed by centrifugation ($20,000 \times g$ for 30 min), the protein concentrations in the supernatant (supernatant I) were measured by spectrophotometry (optical density at 280 nm). Asterisks indicate significant differences from the control (no TiO_2), * $p<0.05$ and ** $p<0.01$ (ANOVA followed by Dunnett's test). The results were re-plotted according to the Hill equation, and are shown in the inset. The estimated concentrations (mg/ml) of amorphous, anatase (<5,000 nm), anatase (<25 nm), and rutile particles to decrease the protein concentration by 50% on Hill plots were 1.4, 1.7, 5.7, and 40.4, respectively, in A; 4.2, 5.8, 13.8, and 62.7 in B; 7.3, 8.3, 8.9, and 10.6 in C; 6.7, 8.7, 9.9, and 12.1 in D. Comparing the effects of polymorphs (at 6 mg/ml), significant differences were found between the amorphous and all other forms, between the anatases and rutile, and between the anatases ($p<0.001$). (ANOVA followed by Bonferroni's test).

respectively, whereas the concentration of total protein is 6.7–8.6 g/dl²²). HSA is synthesized in the liver, and has a good binding capacity for various substances. In fact, HSA adducts of chemicals can be used as markers in biological monitoring²³. In contrast, HGG is made up of immunoglobulins produced by B lymphocytes, and is important in immune defense mechanisms. However, HGG does not seem to play a major role in protecting organisms from non-living hazardous materials. For instance, whereas HSA completely suppressed the effects of pentachlorophenol¹² and galactosylsphingosine²⁴, HGG

did not²⁴, and the binding of HGG to these “toxic” chemicals, was much weaker (or completely lacking) in comparison to that of HSA^{12,25}.

However, in our experiment using purified proteins, the amount of HGG bound to TiO_2 particles was not less than the amount of bound HSA in Buffer II. In Buffer I, the decrease in HGG in the supernatant after incubation with TiO_2 particles was even greater than that of HSA, indicating that HGG has higher affinity for TiO_2 than HSA. When diluted human serum was incubated with TiO_2 particles, an increase in the albumin/globulin ratio was

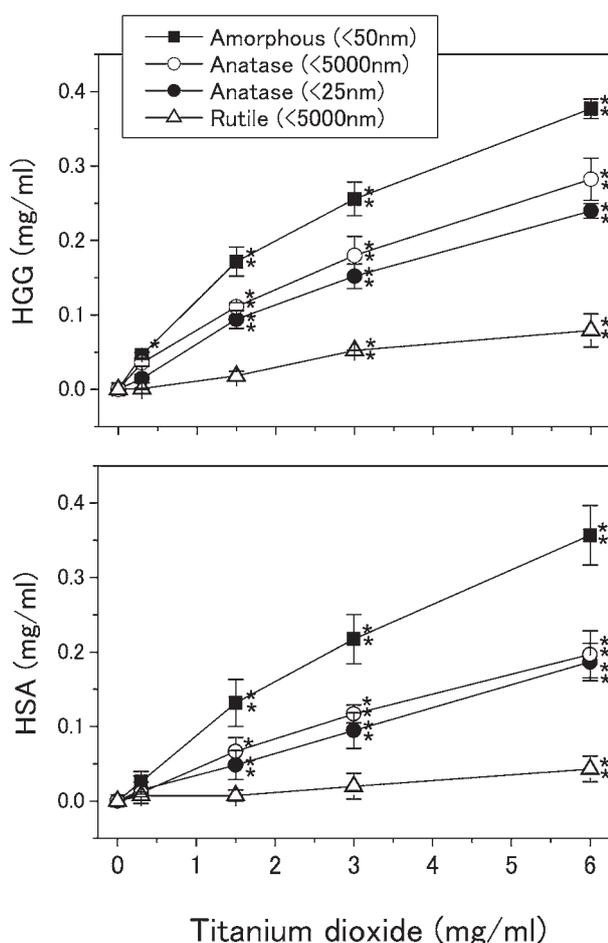


Fig. 5. Human serum gamma-globulin (HGG) and human serum albumin (HSA) released from TiO₂ (mean \pm SD, n=3). TiO₂ particles were incubated with 1 mg/ml of purified HGG or HSA in Buffer II for 1 hr at 37°C and sedimented by centrifugation (20,000 \times g for 30 min). To desorb proteins, after washing with the buffer 3 times, the particles were treated with 1 ml of the buffer containing 1% SDS at 100°C for 5 min. The particles were removed by centrifugation, and the concentrations of the desorbed proteins (in supernatant II) were determined by the DC Protein Assay (microplate assay protocol). Asterisks indicate significant differences from the control (no TiO₂), * p <0.05 and ** p <0.01 (ANOVA followed by Dunnett's test). The levels of HGG released from the particles (6 mg/ml) were significantly different between amorphous and all other forms (p <0.001), between the anatase and rutile (p <0.001), and between two of the anatase forms (p <0.01). The levels of released HSA level were significantly different between the amorphous and all other forms (p <0.001), between anatases and rutile (p <0.001), but not between the two anatase forms (ANOVA followed by Bonferroni's test).

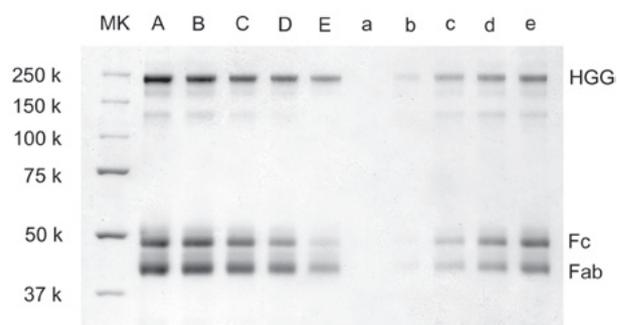


Fig. 6. Papain-digested human serum gamma-globulin (HGG) incubated with TiO₂. A mixture (1 ml) of native HGG and its fragments (Fab and Fc) (125 microgram in total) was incubated in Buffer II with TiO₂ (amorphous form) at 0 (A), 0.3 (B), 1.5 (C), 3 (D) and 6 (E) mg/ml. Sixteen microliters of the supernatant (supernatant I) was mixed with 4 μ l of the sample buffer containing 5% SDS before application onto the gels. After the desorption was carried out as described in the legend for Fig. 5, 16 μ l of the samples (supernatant II) was mixed with 4 μ l of the buffer containing 1% SDS and loaded onto the gel (a to e). Five microliters of Precision Plus protein standard (BioRad) was loaded onto the "MK" lane.

observed in the supernatant (determined by electrophoresis on cellulose-acetate membranes), confirming the changes of HGG and HSA observed in SDS-PAGE. All of these data are consistent with our previous observation that the addition of plasma abolished hemolysis caused by TiO₂ *in vitro*, and that both HSA and HGG completely suppressed hemolysis induced by anatase particles¹¹.

Binding of serum proteins to TiO₂ particles is not limited to HGG and HSA, and several less abundant proteins also exhibit binding to TiO₂. It is worth noting that some of these proteins (or their components), i.e., immunoglobulin light chain or heavy chain, pre-albumin, and beta2-microglobulin are precursor of amyloid, which is believed to be a cause of disorders such as Alzheimer's disease, transmissible prion disease and familial neuropathy²⁶. On the other hand, it has been reported that TiO₂ particles (20 nm in diameter, 80% anatase plus 20% rutile form) promoted beta-amyloid fibrillation *in vitro* by shortening the nucleation process²⁷. Hence, the interaction of serum protein(s) with TiO₂ particles may have pathogenetic significance in some neurodegenerative disorders.

Since several serum proteins showed similar binding potency, it appears that some common structure(s), rather than the specific functional structures in the protein are responsible for binding to TiO₂ particles. Indeed, with regard to HGG, there were no differences in the binding among Fab, Fc and the native form of the protein, suggesting that the antigen-binding sites do not play a major role in the binding. On the other hand, the binding

potency differed among the polymorphs of the particles. Moreover, the binding potency of the particles is completely different from that seen for hemolysis¹¹. While the anatase form is the most potent, and the amorphous form the weakest, in causing hemolysis, the amorphous form is the most potent in binding serum proteins, followed by the anatase form, with the rutile form showing much smaller binding potency. Thus, the mechanism of the interaction between TiO₂ particles and serum proteins seems to be different from that mediating the interaction of the particles with erythrocytes.

The present study has limitations because we were not able to determine the concentrations of TiO₂ particles in solution or their size distributions. Nevertheless, the results of the zeta potential as well as those of the BET surface area may provide some clues about the possible mechanism of the interactions between TiO₂ particles and serum proteins.

The zeta potentials of all particles examined were smaller in Buffer I than in Buffer II, indicating that the particle suspensions were less stable (particles tend to aggregate) in Buffer I. Hence, to some extent, the differences in particle behavior in Buffers I and II may be explained by their zeta potential. However, the zeta potentials do not account for the difference between the polymorphs, because they showed no relationship with the apparent binding potency to TiO₂ particles of different polymorphs in each buffer. Whereas the amorphous and smaller anatase particles showed clear differences in their binding to HSA, HGG, immunoglobulins and pre-albumin, the surface area did not differ greatly (110 vs. 116 m²/g), nor did the particle size (14 vs. 13 nm). Furthermore, in most cases, larger anatase particles showed higher affinity than the smaller anatase particles for proteins. These indicate that the surface area of TiO₂ particles is not the major determinant of their binding to serum proteins. Although the mechanism is still obscure, the present results indicate that the polymorph can greatly affect the interaction of TiO₂ particles with serum proteins.

Lung specimens from workers employed in processing TiO₂ contained higher amounts of the compound compared to lung specimens from a general autopsy population, and the particles were present in the lymphatic system of the workers²⁸. Moreover, in the intestine, TiO₂ particles were observed in macrophages within gut-associated lymphoid tissues of all the human specimens examined²⁹. These findings suggest that the TiO₂ particles are cleared via the lymphatic system, and that particles enter into the systemic circulation. Indeed, in rats exposed by inhalation to TiO₂ particles for 2 yr, dose-dependent deposition of particles was found in the liver and spleen, and an electronmicroscopic examination indicated that particle-laden macrophages had entered the blood or lymphatic vessels in the lymph nodes and then passed into the general circulation³⁰. When a large amount (5 g/kg body weight) of TiO₂ (25, 80 and

155 nm in diameter) was given to mice by a single oral gavage, the concentrations of the particles (mainly those 25 and 80 nm in diameter) were elevated in several organs including the kidneys, liver and spleen²⁹. Thus, TiO₂ particles can be transported by the blood to remote organs. However, Wang *et al.*³¹ found no increase of TiO₂ in erythrocytes. Hence, leukocytes (macrophages) and/or serum appear to play an important role in transporting TiO₂. When their abundance is taken into consideration, HSA and HGG are important, especially if the particles are in the amorphous or anatase form. In addition, protein(s) bound to TiO₂ may work as an "opsonin", attracting macrophages, thus resulting in even greater accumulation within the lymphatic system.

The present results show that proteins incubated in Buffer I were much more difficult to desorb from the particles than those incubated in Buffer II. At present, it is not clear whether or not conformational changes took place in the proteins bound to TiO₂ in Buffer I, but our findings indicate that the environment in which the proteins interact with the particles can greatly influence the properties of the adsorbed substance. Further studies are needed to examine if the proteins (or their fragments) binding to TiO₂ particles may work as nuclei for the further deposition of protein-related materials such as amyloid, and if the particles binding to the protein affect the physiological function(s) of the protein through conformational changes.

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