Sol–Gel Preparation of Blood-Compatible Titania as an Adsorbent of Bilirubin

Takuji ASANO,* Shinji TAKEMOTO, Kanji TSURU, Satoshi HAYAKAWA,
Akiyoshi OSAKA and Seisuke TAKASHIMA*

Biomaterials Lab., Faculty of Engineering, Okayama University, 3–1–1, Tsushima-Naka, Okayama-shi 700–8530
*Co-operative Research Center, Okayama University, 5302, Huga, Okayama-shi 701–1221

This study concerns with the adsorption of pathogenic substances on highly blood-compatible titania and the adsorption mechanism. Titania was prepared through a sol–gel procedure by hydrolyzing tetraethylorthotitanate and calcining up to 705°C for 3 h. Pore size distribution and surface charge density were measured as a function of calcining temperature. Adsorption of bilirubin, as a typical pathogenic substance, as well as albumin on the titania powder was studied. Bilirubin was adsorbed either in 4 to 8-nm pores or on the surface with a higher positive charge. Thus, either entrapping bilirubin in the pores or electrostatic interaction between bilirubin and titania surface was effective for the adsorption of bilirubin. The titania powder adsorbed a greater amount of bilirubin than the anion-exchange resin used in clinics, hence they have potential for blood purification therapy.

Key-words: Titania, Sol–gel, Bilirubin, Adsorption, Mechanism, Electrostatic interaction, Surface charge density, Pore size distribution

1. Introduction

Bilirubin (molecular weight: 584) is one of the pathogenic substances. It has a plane-like structure with a π conjugation throughout the heterocyclic rings. Figure 1 shows the molecular structure with two groups, carboxylic (–COOH) and imide (–NH) which play key roles in interacting electrostatically with other substances under certain circumstances. Bilirubin is a metabolite of the heme from hemoglobin in blood. A considerable fraction of bilirubin is present in blood in association with albumin due to the two groups. They are denoted as indirect bilirubin. When present in the form of free molecules, they are denoted as direct bilirubin. Healthy blood contains 2–15 g/m² bilirubin in total, where the concentrations of direct and indirect bilirubin are 1–3 g/m² and 1–8 g/m³, respectively. The molecular size of the indirect form is as large as albumin: 14 nm long and 4–5 nm in diameter. Disorders in successive metabolism cause bilirubin to accumulate in blood and, in consequence, the total bilirubin content in blood sometimes becomes several or several tens of times as much as that in healthy blood. This causes hyperbilirubinemia such as cholangiitis, organophospholasia and neuropathy involving severe malfunction of organs.

Blood purification is the only therapy currently applicable to curing hyperbilirubinemia. Some adsorbents are employed at present, such as anion-exchange resin (AER) coated with polyhydroxymethacrylate or active carbon (AC) coated with polyhydroxymethacrylate. Recently, a few new materials have been proposed as adsorbents of bilirubin, including amine-containing cross-linked chitosan resins, poly (ethylene oxide)-modified chitosan microspheres, polyethylene-coated resin, and polylysine-immobilized chitosan beads. They are classified into two groups with respect to the adsorption mechanisms: (1) physical adsorption: bilirubin molecules are entrapped in the pores of adsorbents, (2) chemical adsorption: electrostatic interactions between bilirubin and the adsorbent surface. Accordingly, the entrapping mechanism is effective for AC and the electrostatic interactions are effective for the resin type adsorbents. Therefore, both pore size distribution and surface charge density are the essential factors for designing new bilirubin adsorbents.

Pore size distribution and the surface charge of ceramics heavily depend on synthesis methods and conditions. Recently, Takashima et al. prepared calcium phosphate, silica-alumina composite, and titania and examined their blood compatibility in terms of partial thromboplastin time, prothrombin time, and the amount of fibrinogen. They pointed out that titania calcined at appropriate temperatures definitely exhibited excellent blood compatibility among those ceramic materials. This suggests that titania is an excellent candidate for adsorption of bilirubin. In the present study, blood-compatible titania was prepared through the sol–gel procedure and calcination at moderate temperatures. The pore size distribution and surface charge density were studied as a function of the calcining temperature and pH of the hydrolyzing water, using HCl or NH₄OH as the catalysts. The correlations between bilirubin adsorption and the physico-chemical characteristics of the titania powder were examined. Moreover, the mechanisms of bilirubin adsorption were discussed.

Fig. 1. Chemical structure of direct bilirubin.
2. Experimental

2.1 Preparation of titania and physico-chemical characterization

Reagent grade tetraethylorthotitanate (Tokyo Kasei Kogyo, Tokyo) was added to water drop-wise and hydrolyzed at room temperature under stirring in a glove box filled with nitrogen. The value of pH of the hydrolyzing water was 5.8. It was also hydrolyzed with HCl and NH₄OH solutions with pH adjusted to 1 and 11, respectively. The suspensions were kept stirring for 3 h for aging while their pH values were kept constant at 1, 5.8 and 11 throughout the reaction and aging. Then they were washed well with distilled water at the point of filtration, and the residues were dried at 40°C for 4 days. The dry cakes were crushed and the titania powder granules of 150 to 212 μm in size were obtained by sieving. The powders were then heated at a rate of 3°C/min to predetermined temperatures ranging from 245°C to 705°C and kept for 3 h at each temperature. Synthesizing and heating processes were carried out in a glove box filled with nitrogen. The titania powder obtained from the hydrolysis at 1, 5.8 and 11 in pH were designated as powder A, N, and B, respectively. Here, A, N, and B stand for acid, neutral, and basic conditions for hydrolysis, respectively.

The titania powder was characterized by X-ray diffraction (XRD), surface charge density, pore size distribution, and infrared spectra. XRD profiles were taken with a RAD-IIIC (Rigaku, Osaka) with Cu Kα radiation (30 kV and 15 mA). Specific surface area (SSA) was derived from multi-point BET analysis of nitrogen gas adsorption isotherms, while pore size distribution was derived from BJH analysis. The nitrogen gas adsorption isotherms for this titania powder were taken with a Micromeritics GEMINI® 2375 machine (SHIMADZU, Kyoto). Potentiometric titration was employed to derive the surface charge density of the titania powder. Fourier transform infrared (FT-IR) spectra were measured by the KBr method (FT-IR3000, JASCO, Tokyo).

2.2 Protein adsorption characteristics

Each titania powder granule (0.1 g) was suspended in a 1-cm³ saline solution in a 5-cm³ polyethylene bottle with a cap that had been autoclaved for 20 min at 121°C. Mixed protein solutions were prepared (pH 7.20, 37°C) that contained 30 kg/m³ of bovine serum albumin (BSA) and 30–400 g/m³ of bilirubin. Then, each bilirubin-BSA mixed protein solution (3 cm³) was added to each of the autoclaved suspensions kept in the bottle. The polyethylene bottles were tightly capped, covered with aluminum foil for shielding light, laid horizontally in a water bath, and incubated at 20°C or 37°C under shacking for 3 h so that the mixed-protein solution should be in good contact with the titania powder. After incubating, the concentration of bilirubin remaining in the solution was measured by the diazo reaction procedure. The concentration of BSA remaining in the solution was also measured by the bromocresol-green method. The amounts of direct bilirubin, indirect bilirubin, total bilirubin, and BSA adsorbed on the titania powder are hereafter denoted as $\Delta W_{\text{Dr}}, \Delta W_{\text{Inh}}, \Delta W_{\text{Tot}}$, and $\Delta W_{\text{BSA}}$, respectively. The values of $\Delta W_{\text{Dr}}, \Delta W_{\text{Inh}}, \Delta W_{\text{Tot}}$, and $\Delta W_{\text{BSA}}$ for AER were also measured in the same way. The results for the three samples gave an average value and standard deviation. The significance of the difference among those average values was statistically evaluated by the one-way analysis of variation (ANOVA).

3. Results

3.1 Characterization of the titania powder

Figure 2 shows XRD patterns of the titania powder calcined at various temperatures. All the XRD peaks were assignable to anatase or rutile. One may find that the titania powder, after heating at lower temperatures, consisted predominantly of anatase but transformed to rutile at higher temperatures. Note that as-dried A already involved rutile, though with lower crystallinity, whereas rutile was not detected in N or B until they were heated at 550°C and 705°C, respectively. This indicated that higher pH for the hydrolyzing solution increased the temperature of anatase-rutile transition or retarded the transformation. The specific surface area (SSA) for the titania powder calcined at 245°C was about 100 m²/g for A and about 200 m²/g for N and B. SSA decreased with increasing the calcining temperature until it reached about 55, 100 and 90 m²/g for A, N, and B, respectively, when they were calcined at 485°C. Then, SSA

![Fig. 2. X-ray diffraction patterns of the titania powder calcined at various temperatures. Powder A, N, and B were hydrolyzed under acidic, neutral, and basic conditions, respectively (see text).](image-url)
for all the powder abruptly decreased over 550°C to their minimum values at 705°C: 1, 0.5 and 1.5 m²/g for A, N, and B, respectively. Figure 3 shows pore size distribution profiles for the titania powder calcined at various temperatures. The pore volume of A was smaller than that of N and B. The peak position on the pore size distribution curves of all the titania powder shifted to the larger pore size as the calcining temperature increased, that is, the mean pore sizes increased due to calcining. It is common on heating porous ceramics that the smaller pores are the first to collapse and this collapse leads to a decrease in SSA and pore volume, as well as an increase in the average pore size. Figure 4 shows the surface charge density (SCD) at pH 7.20 and 25°C plotted as a function of the calcining temperature. SCD of the titania powder calcined below 550°C remained constant at about 0 C/m² but it increased abruptly at 550°C. It reached 15 C/m² at 705°C for A and N while it reached 5 C/m² for B. Figure 5 shows the IR spectra of N calcined at various temperatures. A peak at 800 cm⁻¹ was assigned to Ti-O stretching, and the peaks at 1620 cm⁻¹ and 3360 cm⁻¹ were assigned to H₂O deformation and O–H stretching of the hydroxyl groups, respectively. This confirms that the –OH groups and surface H₂O molecules were eliminated with heating. Similar spectra were obtained for the other powder.

3.2 Bilirubin adsorption on the titania powder

Figure 6 shows the values of ΔW_Dir, ΔW_Ind, and ΔW_Tot adsorbed on the titania powder (μg/m²) at 37°C. Note that ΔW_Dir, ΔW_Ind, and ΔW_Tot for all titania powder increased abruptly at 550°C as found for SCD in Fig. 4. That is, when calcined at 705°C, the titania powder adsorbed a much greater amount of bilirubin than that calcined below 550°C. In Fig. 6(a), ΔW_Dir of the titania powder was less than 10 μg/m² below that for AER in the range of <550°C, and the difference among the powder granules had no statistical significance. In contrast, ΔW_Dir increased up to 60–70 μg/m² and exceeded that for AER when the powder was calcined above 600°C. Similar changes were found for ΔW_Ind and ΔW_Tot in Fig. 6(b) and (c), respectively. Yet, Fig. 6(c) indicates that ΔW_Tot for N and B was larger than that for A in the range of 330°C and 550°C, and the difference was statistically significant. In the range of >700°C, ΔW_Tot for B was smaller than A and N, and the difference was statistically significant. In summary, the present titania powder calcined above 600°C was better than AER in the adsorption of direct bilirubin (ΔW_Dir, Fig. 6(a)) and powder A and N calcined above 700°C was not less effective than AER in adsorbing the total bilirubin per area (ΔW_Tot, Fig. 6(c)).
It is commonly accepted in gas adsorption that the heat of adsorption is dependent on the mechanisms, like chemical adsorption and physical adsorption. By analogy with the gas adsorption, the change in the heat of adsorption for the present case can be correlated to the adsorption mechanisms. Thus, it is useful to derive the heat of adsorption for the present titania powder. The adsorption of bilirubin is an equilibrium process, hence it is dependent on both the initial concentration of bilirubin and temperature. Bilirubin was adsorbed on N at 20°C and 37°C. Application of a Langmuir-type equation to the relation between the initial bilirubin concentration and the amount of bilirubin adsorbed leads to the saturated equilibrium amount of adsorbed bilirubin \( (\Delta W_{\text{Tet}, T})_0 \), where \( T \) is the temperature of the adsorption experiment. Moreover, the Clausius–Clapeyron Eq. (1) describes the temperature dependence of the saturated equilibrium amount of adsorbed bilirubin:

\[
(\Delta W_{\text{Tet}, T})_0 = \Delta H_{\text{ads}}/RT + C
\]

Here \( \Delta H_{\text{ads}} \) is the heat of bilirubin adsorption, and \( C \) is the integration constant. One can derive \( \Delta H_{\text{ads}} \) assuming it remains constant during adsorption. In the present study, Eq. (1) was applied to the bilirubin adsorption data at 20°C and 37°C for N, and \( \Delta H_{\text{ads}} \) was derived and plotted in Fig. 7 as a function of the calcining temperature. \( \Delta H_{\text{ads}} \) was about 20 kJ/mol for N calcined below 330°C, while it increased to about 28 kJ/mol when N was calcined above 485°C.

**4. Discussion**

According to Nakashima et al.,\(^5\), the adsorption of some pathogenic substances on AC was dependent on several factors, such as molecular weight, size, and conformation of the substance, as well as the physical properties of AC. They proposed that, when those characteristics of the pathogenic substance matched well with the physical properties of AC, the adsorption of pathogenic substances easily proceeded. The molecular size of direct bilirubin suggests that the pores of about 1 nm in size are adequate for trapping the direct bilirubin molecules. Unfortunately, Fig. 3 indicates that the present titania powder included a very small amount of pores of that size. In contrast, they had a large volume of pores in the range of >2 nm in diameter which could entrap indirect bilirubin. Therefore, if the molecular entrapment mechanism is effective, the titania powder absorbs indirect bilirubin molecules in those pores. Thus, it is suggested that \( \Delta W_{\text{ind}} \) is strongly correlated to the pore characteristics of the titania powder. Note that some part of bilirubin in the mixed protein solutions would get associated with BSA during the incubation and form indirect bilirubin. Then, let us examine the correlation between \( \Delta W_{\text{ind}} \) and the volume of the pores appropriate to accommodate indirect bilirubin molecules. One may think that not all pores are appropriate for entrapping the indirect bilirubin molecules and that the molecules ‘prefer’ pores of some adequate size. Entrapment in pores which are too big is like adsorption on a plane surface while entrapment in pores of appropriate size causes much physical interaction between the molecule and the pore wall. Thus, from the molecular size of indirect bilirubin, one may assume that pores of 4 to 8 nm in diameter are adequate. In this respect, the fractional pore volume in the range of 4–8 nm \( \Delta V_{4/8} \) should be a good factor to describe the adsorption of indirect bilirubin on the titania powder. So, \( \Delta W_{\text{ind}} \) is plotted as a function of \( \Delta V_{4/8} \) in Fig. 8. It must be emphasized that the plots of \( \Delta W_{\text{ind}} \) for all the titania powder fell around a line. That is, regardless of the calcining temperature or the conditions of hydrolysis, \( \Delta V_{4/8} \) seemed to be the single factor to control \( \Delta W_{\text{ind}} \). In other words, the mechanism of entrapment was significant for adsorbing indirect bilirubin. When a fractional pore volume other than \( \Delta V_{4/8} \) was examined, no such linear relation could be obtained. However, it should also be noted...
that a finite amount of bilirubin was adsorbed on the powder having the smallest values of $\Delta V_{(4.8)}$. This strongly suggests that another mechanism is also at work.

Electrostatic interaction is important for bilirubin adsorption on natural polymers and synthetic resins. According to Faenza et al.,2) chemical bonds were established between the carboxyl groups of bilirubin and positively charged sites on an anion-exchange resin after chloride ions were released from the resin. Zhu et al.11) reported that bilirubin was adsorbed on polylysine-coated resin beads due to the interaction between the carboxyl groups of bilirubin and lysine on the beads under pH 7.8. That is, the carboxylic groups of bilirubin were negatively charged and the amino groups of lysine were positively charged. Moreover, Chandy and Sharma12) reported a similar electrostatic interaction between bilirubin and polylysine-immobilized chitosan beads. Regarding oxides, the hydroxyl groups play a crucial role in the surface charge of titania and hence in the electrostatic interaction mechanism. When titania is soaked in a saline solution, the hydroxyl groups or the surface Ti–O bonds are modified to yield TiO(OH)$_2$-, O–Ti$^4+$–O, Ti–OH$^-–$Ti, or Ti–O$^-$. Accordingly, the surface is charged either positively or negatively. Heating at higher temperatures induces condensation of the hydroxyl groups and eliminates them, i.e., the number of hydroxyl groups decreases and that of the Ti–O–Ti increases as confirmed previously in Fig. 5. As a result, the surface of titania is positively charged. The point of zero-charge for albumin is around 5.8 in pH, hence albumin is negatively charged in the present bilirubin–BSA solution. In addition, the dissociation of the carboxyl groups (–COOH) of the bilirubin molecules into –COO$^-$ groups and protons gives bilirubin negative charges. Thus, regardless of the direct or indirect form, bilirubin was negatively charged and hence was adsorbed on the positively charged sites of the titania powder due to the electrostatic interactions. Figure 4 shows the presence of only a trace amount of negative charge on all titania powder except that significant positive charges were detected for A and N calcined at 705°C for 3 h. Thus, comparing Figs. 4 and 6, one derives that the electrostatic interaction mechanism for the adsorption of indirect bilirubin and direct bilirubin primarily contributes to the titania calcined at 705°C.

Therefore, these results suggest that electrostatic interaction is the primary mechanism for the titania powder calcined at higher temperatures, while the entrapping mechanism is effective for powder calcined at lower temperatures.

Figure 8. Amount of indirect bilirubin $\Delta W_{\text{bil}}$ adsorbed on the titania powder (mg/g) calcined at various temperatures as a function of the fractional pore volume $\Delta V_{(4.8)}$ in the range of 4–8 nm.

Figure 9. Ratio of the amount of indirect bilirubin adsorbed on powder N to that of bovine serum albumin ($\Delta W_{\text{bil}}/\Delta W_{\text{BSA}}$) as a function of calcining temperature. $\Delta W_{\text{bil}}/\Delta W_{\text{BSA}}$ is a measure of selectivity.

Akazawa and Kobayashi21) thermochemically discussed the adsorption of albumin on hydroxyapatite in terms of the Clausius–Clapeyron equation, and derived 20–35 kJ/mol for the heat of albumin adsorption on hydroxyapatite. Their values were of the albumin–apatite electrostatic interaction energy because it had been proposed22) that an electrostatic mechanism was effective for a protein to be adsorbed on hydroxyapatite. The same calculation was conducted for the heat of bilirubin adsorption on N and plotted in Fig. 7 as a function of the calcining temperature: the heat of adsorption remained in the range of 20 to 30 kJ/mol. Careful examination would derive a tendency for it to increase from 20 to 28 kJ/mol with calcining around 500°C. Therefore, it is concluded that the titania calcined at higher temperatures had a greater affinity to bilirubin due to the electrostatic interaction. That is, the driving force of bilirubin adsorption on titania calcined at higher temperatures was dominantly the energy of electrostatic interaction between the positive charge or the negative charge of titania and the imide or carboxyl groups. In contrast, physical interaction or entrapment was effective for powder calcined at lower temperatures. Incidentally, it is generally accepted, as described in the introduction section, that most of the bilirubin molecules, above 80%, are present as indirect bilirubin, or they are combined with albumin. Thus, the electrostatic interaction between albumin and the titania powder primarily contributed to the heat of adsorption derived above for N. So, the heat of adsorption for albumin on the titania powder was comparable to that on the hydroxyapatite reported by Akazawa and Kobayashi.21)

Figure 9 shows the ratio of indirect bilirubin to albumin ($\Delta W_{\text{bil}}/\Delta W_{\text{BSA}}$) adsorbed by N in a bilirubin–BSA standard solution containing 30 kg/m$^3$ of BSA and 168 g/m$^3$ of bilirubin. The $\Delta W_{\text{bil}}/\Delta W_{\text{BSA}}$ for titania calcined at 245°C and 330°C was larger than that for titania calcined above 485°C. Consequently, the titania calcined at low temperatures had more excellent selectivity for indirect bilirubin than the titania calcined at high temperatures. Titania can remove the pathogenic substance, bilirubin, without adsorbing an essential protein, albumin.

For the practical application of titania as an adsorbent for blood purification, the powder is packed in a housing with a finite volume through which blood flows. Thus, it is important to evaluate the residual fraction of bilirubin in blood.
after blood purification or the amount of bilirubin adsorbed per unit volume of the randomly packed titania powder. The residual fraction of bilirubin in bilirubin–BSA solutions after being in contact with 0.1 g of N for 3 h at 37°C was derived from the present data. It is plotted in Fig. 10 as a function of the calcining temperature. Here, the powder with the smaller values of the residual fraction was better as adsorbents. It is indicated that N calcined at higher temperatures adsorbed lesser amounts of bilirubin. In contrast, the solution after being in contact with the titania calcined at 330°C retained the least amount of bilirubin (the powder adsorbed about 75% of the bilirubin). Moreover, Fig. 11 shows the amount of bilirubin adsorbed on the unit volume of titania. The titania calcined below 485°C adsorbed no less bilirubin than AER. Among all, titania calcined at 330°C adsorbed about three times as much as AER. Therefore, if the titania is used as an adsorbent for blood purification, it could preferably adsorb bilirubin to albumin. That is, the present titania powder suppresses the bilirubin concentration in blood to lower levels, hence it is applicable as an effective adsorbent for bilirubin in clinics.

5. Summary

Titania powder was synthesized by hydrolysis of tetraethylorthotitanate in pH 1, 5.8 and 11. SCD of powder A, N, and B were similar. When the titania powder calcined at higher temperatures was in contact with solutions of pH 7.20, the surface was charged to positive. Moreover, the pore volume of A was smaller than that of N and B. Bilirubin adsorption was dependent on two factors; molecular entrapment and electrostatic interaction. Titania has over twice as large the bilirubin adsorption ability as the anion-exchange resin. Titania is useful material as a bilirubin adsorbent in clinics.

Acknowledgment This study was supported by the Medical Engineering-Associated and Applied Research Project of the Industry Development Foundation of Okayama Prefecture of Japan.

References