Optimization of amino group density on surfaces of titanium dioxide nanoparticles covalently bonded to a silicone substrate for antibacterial and cell adhesion activities

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Abstract: A composite consisting of titanium dioxide (TiO2) particle, the surface of which was modified with amino groups, and a silicone substrate through covalent bonding at their interface was developed, and antibacterial and cell adhesion activities of the composite were evaluated. The density of the amino groups on the TiO2 particle surface was controlled by the reaction time of the modification reaction. The degradation rate of CH3CHO in the presence of the TiO2 particles under UV irradiation decreased with an increase in the amino group density on the TiO2 surface. On the other hand, the number of L929 cells adhering on the TiO2/silicone composite increased with an increase in the amino group density. From the above two results, the optimum density of amino groups for both photoreactivity and cell adhesiveness was estimated to be 2.0–4.0 molecules/nm2. The optimum amino group-modified TiO2/silicone composite sheet (amino group density, 3.0 molecules/nm2) showed an effective antibacterial activity for Escherichia coli bacteria under UV irradiation. © 2005 Wiley Periodicals, Inc.

Key words: titanium dioxide; antibacterial activity; composite; covalent bonding; cell adhesion

INTRODUCTION

Since Fujishima et al.1 reported water cleavage on photoexcited titanium dioxide (TiO2) electrodes, TiO2 has attracted great interest in environmental fields such as air and water purification, because the electron and hole created by photoexcitation of TiO2 can reduce or oxidize several chemical species adsorbed on the TiO2 surface.2–5 The antibacterial activity or cytotoxicity, which is expected to be applicable to biology and medicine, of photoexcited TiO2 has also been shown.6–8 In addition, the effect of TiO2 particles on animals has been investigated from the viewpoint of genetic toxicity; Bischoff et al.9 and Bernard et al.10 reported the nontoxicity of TiO2 particles to animals.

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substrate and to promote cell adhesion on TiO₂ particles, the surfaces of TiO₂ particles were modified with an amino group-terminated silane coupling agent. Although the composite showed good cell adhesiveness, the photoreactivity was approximately 30 times lower than that of the original TiO₂, which should be due to high coverage of the TiO₂ surface by amino groups.

In this article, in order to develop a TiO₂/silicone composite having both antibacterial and cell adhesion activities, the amino group density on the TiO₂ particle surface was optimized. Antibacterial activity test of the composite was additionally conducted in order to evaluate the functionality of the amino group-modified TiO₂/silicone composite.

MATERIALS AND METHODS

Materials

Anatase TiO₂ particles with an inter-diameter of 200–300 nm and a specific surface area of 5.0 m²/g were kindly donated by Ishihara Sangyo Co., Ltd. (Osaka, Japan). A silicone sheet (Shin-Etsu Polymer Co., Tokyo, Japan) with a thickness of 0.3 mm was purified by methanol with a Soxhlet extractor. Acrylic acid (AAc; Nacalai Tesque, Inc., Kyoto, Japan) was purified by vacuum distillation. γ-Aminopropyltriethoxysilane (γ-APS; Shin-Etsu Chemical Industries Co., Tokyo, Japan) was used without further purification.

Introduction of amino groups on the surfaces of the TiO₂ particles

TiO₂ particles, 5.0 g, after drying at 120°C for 24 h were added to 150 mL of anhydrous toluene in a 300-mL threenecked flask equipped with an inlet of N₂, a reflux condenser, and a half-moon type stirrer. After the temperature of the mixture was raised to 30°C, γ-APS was injected into the mixture, and it was stirred at 30°C for different reaction periods. After the reactions, the TiO₂ particles were washed with toluene and acetone by centrifugation to remove any unreacted silane coupling agent and dried at 60°C for 24 h. The density of amino groups on the surfaces of the TiO₂ was calculated from the specific surface area of the particles and the weight percentage of carbon atoms in the modified particles. The weight percentage of carbon atoms was measured with an elemental analyzer (EMIA-110; Horiba Ltd., Kyoto, Japan), assuming that all of the amino groups existed on the particle surfaces.

Photoreactivity of the amino group-modified TiO₂ particles

The photoreactivity of the amino group-modified TiO₂ was evaluated by the degradation rate of CH₃CHO. The initial concentration of CH₃CHO in a Pyrex reaction vessel (760 mL) was fixed at approximately 300 ppm in air. UV/Vis light (wavelength, >300 nm) was irradiated with a Xe lamp (HX-500; Wacom Electric Co., Ltd, Tokyo, Japan) at 2000 μW/cm² onto the samples (0.07 g) after the adsorption equilibrium of CH₃CHO in the reaction vessel had been achieved. The concentration of CH₃CHO was determined by a gas chromatograph (GC-8AFT, Shimadzu Co., Kyoto, Japan) equipped with an f.i.d. column Shincarbon A. The rate constant for the degradation of CH₃CHO was calculated from the first-order rate equation.

TiO₂/silicone composite

First, graft-polymerization of AAc was conducted on the surface of the silicone sheet. The silicone sheet was initially treated by corona-discharge to donate radicals on the surface. The sheet was immersed into a 10 wt% AAc aqueous solution in 50-mL thick-walled tubes, and the tubes were subsequently degassed and sealed. Polymerization was conducted at 60°C for 30 min, and the poly(AAc)-grafted silicone sheet was rinsed with a great deal of hot water to remove homopolymers adsorbed physically. Surface-treated sheets possessing a poly(AAc)-grafted density of 10–20 μg/cm² were used in this study.

In order to adsorb the modified TiO₂ particles onto the poly(AAc)-grafted silicone sheet, 0.2 g of the particles were suspended in 50 mL of water, and the sheet was soaked in the suspension for 1 h at room temperature. After the adsorption, the sheet was heated at 180°C for 2 h in a vacuum for a reaction between the amino groups on the TiO₂ particles and the carboxyl groups on the poly(AAc)-grafted silicone sheet. The composite was washed by using an ultrasonic generator for 3 min (output, 20 kHz; 35 W) in water to remove the particles physically adsorbed on the sheet. In the case of the original TiO₂ particles, the ultrasonic cleaning was not conducted because all of the particles were just physically adsorbed on the sheet. The surface of the composite was observed by scanning electron microscopy (SEM; JSM-6301F, JEOL Ltd., Tokyo, Japan), and the surface-coverage ratio by TiO₂ particles was determined from SEM images. In following experiments, the TiO₂/silicone composite sheets, whose surface-coverage ratio by TiO₂ particles were 50–60%, were used (Fig.1).

Cell adhesion

L929 mouse fibroblast cells were placed onto the TiO₂/silicone composite in 24-well multilpates at 1 × 10⁵ cells/well in a culture medium consisting of an α-minimum essential medium (α-MEM; Gibco Laboratories Inc.) and 10% fetal bovine serum, and incubated at 37°C for 24 h. For SEM observation, the cells were dehydrated with aqueous ethanol (30–100%) and 100% n-butanol for 5 min at room temperature step by step. The samples were subsequently lyophilized and coated with gold. The number of L929 cells on the sample substrate was counted from SEM images. As a control sample, the poly(AAc)-grafted-silicone sheet, on
which the original TiO$_2$ particles were physically adsorbed, was used.

**Cytotoxicity assay**

A cytotoxicity assay for the TiO$_2$/silicone composite was conducted as follows. One gram of the composite was cut into small pieces and added into 10 mL of cell culture medium (α-MEM with 10% fetal bovine serum), and the mixture was incubated at 37°C for 24 h in darkness. After L929 cells (initial number, 1 x 10$^4$ cells/well) were precultured at 37°C for 24 h, the culture medium was replaced by the medium exposed to the samples, and the cells were further incubated for another 24 h at 37°C. The number of cells was counted with a hemacytometer after trypsinization and dilution. As a control, the same procedure was conducted with a nontreated medium.

**Antibacterial activity**

_E. coli_ bacteria (NBRC 3301 strain; Biological Resource Center, Biotechnology Center, National Institute of Technology and Evaluation, Chiba, Japan), precultured at 37°C for 16 h, were washed by centrifuging at 4000 rpm, resuspended, and diluted to 1 x 10$^7$ cells/mL with a physiological salt solution. The composite sheets were placed in 24-well multiplates, and then 1 mL of the _E. coli_ suspension was pipetted into each well. This system was irradiated with a 4-W back-light bulb (wavelength, 360 nm; 470 μW/cm$^2$; FL15 BL-B; National Panasonic) at 37°C for 2 h. After the irradiation, 100 μL of each _E. coli_ suspension was pipetted out and incubated in a nutrient agar medium at 37°C for 16 h, and the number of viable bacteria was counted. As a control test, the above procedure was conducted without the composite sheet or without UV irradiation.

**Statistical analysis**

Data resulting from cytotoxicity assays and antibacterial activity tests are presented as means ± SD for mean (N = 4). Statistical comparisons were performed with the use of a Student’s _t_ test. The level of statistical significance was defined as _p_ < 0.05.

**RESULTS AND DISCUSSION**

The TiO$_2$ particles were chemically modified with an amino group-terminated silane coupling agent at 30°C for different reaction time. In the previous article, modification with amino groups was confirmed by FT-IR. Figure 2 shows the density of amino groups on the surfaces of the TiO$_2$ particles after modification at 30°C for different reaction times. The density of the amino groups was determined from the specific surface area of the TiO$_2$ particles and the carbon content measured by elemental analysis. In order to determine the amino group density, the nitrogen content was not used because it was below the detection limit. The density of the amino groups incorporated on the particle surfaces drastically increased within 1 h, gradually increased with an increase in the reaction time, and then almost reached a plateau at about 5 molecules/nm$^2$. Judging from the fact that the theoretical density of the OH groups on the anatase TiO$_2$ surface is 12–14 molecules/nm$^2$, which is estimated from lattice constant, and that each silane coupling agent reacts with less than three OH groups, it is estimated...
that the modified TiO$_2$ particle surface consisted of not only an amino group-donated surface but also an intact TiO$_2$ surface, at least in the case of an amino group density of $\tilde{\text{4}}$ molecules/nm$^2$.

The photoreactivity of the modified TiO$_2$ was evaluated by the degradation rate of CH$_3$CHO. In the previous article, although the anatase phase of the TiO$_2$ particles did not change after the reaction between the silane coupling agent and the OH groups on the outermost surface of the particle, the photoreactivity became approximately 30 times lower than that of the original TiO$_2$, which should be due to high coverage of the TiO$_2$ surface by silane coupling agents. The high coverage in the previous study should be due to crosslinking reactions among the silane coupling agents at a higher reaction temperature (120°C) than that of this study (30°C).

Figure 3 shows the relationship between the density of amino groups on the TiO$_2$ particle surfaces and the rate constant calculated from the decrease of CH$_3$CHO concentration by UV irradiation (wavelength, >300 nm; 2000 $\mu$W/cm$^2$; 2 h).

The cytotoxicity assays were conducted with the poly(AAc)-grafted silicone sheet, on which the original TiO$_2$ particles were physically adsorbed, and the amino-group-modified TiO$_2$/silicone composite (amino group density, 3.0 molecules/nm$^2$). Figure 5 shows the results. The number of L929 cells after incubation at 37°C for 24 h (total incubation period, 48 h) in the medium, which was exposed to each sample at 37°C for 24 h in darkness, was not statistically significant compared with that after incubation in the nontreated medium (control). This indicates that the original TiO$_2$/silicone and the amino-group-modified TiO$_2$/silicone composite sheet had no cytotoxicity to L929 cells.

Figure 4 shows the FT-IR spectra of the original TiO$_2$ and the amino group-modified TiO$_2$ particles (amino group density, 3.0 molecules/nm$^2$) before and after UV irradiation (wavelength, >300 nm; 2000 $\mu$W/cm$^2$; 2 h). Each sample was heated at 120°C for 24 h before the FT-IR measurement to remove adsorbed water. In the spectrum of the original TiO$_2$ [Fig. 4(a)], the band at 3692 cm$^{-1}$ is attributed to the OH stretching vibration of the bridge-OH terminal groups on the outermost surface of TiO$_2$. The peaks at 3300 and 1650 cm$^{-1}$ depend on adsorbed H$_2$O on the TiO$_2$. In the spectrum of the TiO$_2$ after modification with $\gamma$-APS [amino group density, 3.0 molecules/nm$^2$; Fig. 4(b)], the intensity of the band at 3692 cm$^{-1}$, attributed to the bridge-OH terminal groups of TiO$_2$, decreased, which corresponded to that reported in the previous article, indicating the reaction of the OH groups with the silane coupling agent. An additional peak is present with respect to the original TiO$_2$ spectrum at 2928 cm$^{-1}$, indicating C—H stretching of the organic compound. As compared to the spectrum of the amino group-modified TiO$_2$ before UV irradiation, that after UV irradiation [Fig. 4(c)] in air did not change, which indicates that the covalent bonding did not cleave under the UV irradiation conditions within 2 h (wavelength, >300 nm; 2000 $\mu$W/cm$^2$). This result might be due to the slightly larger bonding energy of Si—O (369 kJ/mol) compared to that of C—C (350 kJ/mol) or C—N (291 kJ/mol) and also due to multicoupling between the silane coupling agent and the OH groups on the TiO$_2$ particles.
In order to evaluate the cell adhesion activity of the TiO2/silicone composite, L929 cells were scattered and incubated on the composite sheet for 24 h at 37°C. Figures 6 and 7 show, respectively, SEM photographs and the number of the L929 cells adhering on the control sample [the poly(AAc)-grafted silicone sheet, on which the original TiO2 particles were physically adsorbed, and the amino-group modified TiO2/silicone composite sheets (the amino group density, 3.0 molecules/nm²)]. Initial number of cells, $1 \times 10^4$ cells/well. Error bars represent standard deviations of quadruplicates.

In order to evaluate the cell adhesion activity of the TiO2/silicone composite, L929 cells were scattered and incubated on the composite sheet for 24 h at 37°C. Figures 6 and 7 show, respectively, SEM photographs and the number of the L929 cells adhering on the control sample [the poly(AAc)-grafted silicone sheet, on which the original TiO2 particles were physically adsorbed] and the composite sheet with the TiO2 having different amino group densities. In the case of the control sample shown in Figure 6(a), few cells adhered. Judging from the cytotoxicity assay shown in Figure 5, the lack of cell adhesion on the control sample was not due to the cytotoxicity of the TiO2/silicone, but high hydrophilicity of the original TiO2 surface. On the other hand, the cells dramatically adhered on the composite surface compared with the control sample, and the number of cells adhering on the composite sheet increased with the increase in the amino group density (Fig. 7). This is because cationic groups such as amino groups promote initial cell adhesion and growth. In photographs with higher magnification [Fig. 6(b’,c’)], the cells elongated their needlelike

![Figure 5](image1.png)

**Figure 5.** The number of L929 cells after incubation at 37°C for 24 h (total incubation period, 48 h; see Materials and Methods) in the medium exposed to the poly(AAc)-grafted silicone sheet, on which the original TiO2 particles were physically adsorbed, and the amino-group modified TiO2/silicone composite sheets (the amino group density, 3.0 molecules/nm²). Initial number of cells, $1 \times 10^4$ cells/well. Error bars represent standard deviations of quadruplicates.

![Figure 6](image2.png)

**Figure 6.** Relationship between the amino group density on the TiO2 particle surfaces and the number of L929 cells adhering on the TiO2/silicone composite sheets after incubation in 24-well multiplates ($1 \times 10^5$ cells/well) at 37°C for 24 h.

![Figure 7](image3.png)

**Figure 7.** SEM photographs of L929 cells adhering on amino-group modified TiO2/silicone composite sheets after incubation in 24-well multiplates ($1 \times 10^5$ cells/well) at 37°C for 24 h. The amino group density on TiO2 particle surfaces (molecules/nm²): (a,a’) 0; (b,b’); 2.1; (c,c’) 3.0. (a,b,c) low magnification; (a’,b’,c’) high magnification.
The antibacterial activity of the optimum amino group-modified TiO2/silicone composite (amino group density, 3.0 molecules/nm2) was estimated from survival ratio of E. coli bacteria on the composite sheet after UV irradiation (wavelength, 360 nm; 470 μW/cm2; 2 h), which is shown in Figure 8. In the case of the control test after UV irradiation, the direct antibacterial effect of UV rays was slightly observed. On the other hand, in the case of the composite sheet, 29% decrease in the number of E. coli bacteria was observed before UV irradiation, and the number of bacteria decreased significantly after UV irradiation (72%). These results indicate a bacteria adsorption onto the optimum amino group-modified TiO2/silicone composite and effective antibacterial activity by photoexcited TiO2 under UV irradiation. It is expected that a completely destroying of bacteria will be obtained by optimizing the surface area of the composite sheets and UV irradiation conditions such as wavenumber, intensity, and irradiation time of UV light. It is worth pointing out that the covalent bonding was not cleaved under the UV irradiation (wavelength, >300 nm; 2000 μW/cm2; 2 h) as shown in Figure 4. Therefore, the composite developed here is expected to show antibacterial activity under UV irradiation while maintaining tissue adhesiveness.

In conclusion, the amino group density on the TiO2 particle surface was optimized in order to develop a composite consisting of amino group-modified TiO2 particles and a flexible silicone sheet through covalent linkage, having both photoreactivity and cell adhesiveness. The photoreactivity of the TiO2 particles decreased with an increase in the amino group density on the TiO2 particle surfaces. On the other hand, the number of L929 cells adhering on the composite sheet increased with an increase in the amino group density. Based on the above results, the optimum density of amino groups for both photoreactivity and cell adhesiveness was estimated to be 2.0-4.0 molecules/nm2, and a composite consisting of TiO2 particles having that optimum density was developed. Irradiation of UV light onto E. coli on the optimum amino group-modified TiO2/silicone sheet showed an effective antibacterial activity of the composite. The composite developed here could be utilized for elastic percutaneous or subcutaneous devices having good tissue adhesiveness and an antibacterial effect.

References

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