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Bacterial target-specific photocatalyst for the enhancement of antibacterial property to targets

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A B S T R A C T
A bacterial target-specific titanium oxide (TiO₂) photocatalyst was developed for the enhancement of selective inactivation of targeted bacteria. An antibacterial composition comprising TiO₂ particles immobilized with a bacterial-specific antibody having affinity to bacteria of interest was prepared via a carbodiimide hydrochloride/N-hydroxysulfosuccinimide (EDC/NHS) coupling reaction between polyacrylic acid (PAA) coated TiO₂ and an antibody. As a model case, an antibody to Escherichia coli was conjugated with the PAA-coated TiO₂ (TiO₂-Ab). We evaluated the enhancement of the antibacterial effect of TiO₂-Ab against target E. coli, compared with its effect on other bacteria that lack affinity for the antibody used. The TiO₂-Ab inactivated approximately 90% of the E. coli at 15 min, whereas the raw TiO₂ inactivated approximately 20% of the E. coli after the same period of time under UV irradiation. The TiO₂-Ab did not show an enhanced antibacterial effect against non-target bacteria. We infer that close contact between TiO₂ particles and E. coli, as a result of the specificity of the antibody, can enhance the direct transfer of reactive oxygen species (ROS) generated by TiO₂ particles to the cellular surface under UV irradiation and result in rapid and efficient inactivation of the targeted bacteria. The strategy presented here will facilitate the combination of other receptors and TiO₂ particles for the preparation of highly selective and photocatalytic composites to prevent or remediate contamination by unwanted bacteria in a wide variety of natural and man-made systems.

1. Introduction

Human beings are frequently at risk of infection from a bacteria, fungi and viruses, through various routes of contact (nose, mouth, and skin) from the living environment. Infection by microorganisms can result in serious problems such as food poisoning, enteritis, and Severe Acute Respiratory Syndrome (SARS) [1–3]. Numerous antimicrobial methods, such as filtration [4,5], thermal treatment [6,7], antibiotic agents [8,9], disinfectants [10], and ultraviolet (UV) irradiation [11,12], have been studied and evaluated for their effectiveness against different microorganisms under varying environmental conditions. Interestingly, however, these studies have been mostly dedicated to the non-selective inactivation of microorganisms. Most methods for microorganism sterilization are non-specific, and therefore, useful microorganisms are killed alongside target microorganisms.

Titanium oxide (TiO₂), a photocatalyst, has been widely used in environmental fields due to its high chemical stability, excellent oxidation capability, good photocatalytic activity, low-cost of production, and non-toxicity [13,14]. In addition, TiO₂ photocatalysis has been intensively applied to the inactivation of a broad spectrum of microorganisms [15–17]. It has already been demonstrated that reactive oxygen species (ROS) such as hydroxyl radicals (OH·) and superoxide anions (O₂•−), produced by the photocatalytic activity of TiO₂ under UV irradiation, are reactive with microorganisms and can kill or deactivate bacteria, viruses, and cells more efficiently than UV alone [17–19]. Recently, this enhanced photocatalytic and antibacterial activity was demonstrated using TiO₂ particles loaded with metal oxides (Ag, Au, or Cu) or graphene under visible light [20–23]. However, previous studies of TiO₂ and modified TiO₂ photocatalysts showed no selectivity for target microorganisms where ROS were applied to such processes [12,17–25]. A small number of studies have been conducted on the bioconjugation of TiO₂ with DNA, biotin, or antibodies; however, these studies did not focus on the selective inactivation of bacterial targets [26–29]. In the present work, we prepared bacterial target-specific TiO₂ particles designed to enhance their antibacterial effect and to target and inactivate specific species of bacteria. We prepared TiO₂ particles conjugated with a bacteria-specific antibody (Escherichia coli polyclonal antibody, Ab₁) and evaluated whether the composite displayed enhanced selective antibacterial performance in

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targeting E. coli. The specificity of TiO$_2$-Ab$_E$ was verified by measuring its effect on other bacteria not bound by the antibody, such as Staphylococcus epidermidis, Klebsiella pneumoniae, Enterobacter aerogenes, Citrobacter freundii and Bacillus subtilis.

2. Materials and methods

2.1. Materials

TiO$_2$ (P-25, composition: 75% anatase and 25% rutile, surface area: 50 m$^2$/g) was purchased from the Degussa Company (Germany). Polyacrylic acid (PAA) was purchased from Wako Pure Chemical Industries, Ltd. (Japan). N,N-dimethylformamide (DMF), acetone, ethanol, ethanolamine, and 1-ethyl-3-[3-dimethylaminopropyl] were purchased from Sigma–Aldrich (St. Louis, MO USA). Carbodiimide hydrochloride (EDC), N-hydroxysulfosuccinimide (Sulfo-NHS), 2-(N-morpholine)ethanesulfonic acid (MES), sodium phosphate (PBS), and anti-E. coli polyclonal antibody (Rabbit IgG) were purchased from Thermo Fisher Scientific Inc. (Rockford, IL, USA). Carboxylic acid Dyal M-270 magnetic beads were purchased from Invtrogen (Carlsbad, CA USA). Methylene blue was purchased from Samchun Pure Chemical (Korea); E. coli (KCTC 2571), S. epidermidis (KCTC 1917), K. pneumoniae (KCTC 2208), E. aerogenes (KCTC 2190), C. freundii (KCTC 2006), and B. subtilis (KCTC 1022) were purchased from the Korean Collection for Type Culture (KCTC). Nutrient broth and nutrient agar were purchased from Becton & Dickinson, Co. (Franklin Lakes, NJ, USA).

2.2. Preparation of PAA modified TiO$_2$ particles

TiO$_2$ particles (0.1 g) were dissolved in 20 ml of DMF solution and 2 ml of DMF containing 100 mg/ml of PAA was added, followed by mixing. The mixed solution was incubated for 5 h at 150 ºC and then cooled to room temperature. Acetone (38 ml) was added to the cooled solution, which was then incubated for an additional 1 h at room temperature. The particles were recovered by centrifugation (4000 rpm) for 20 min at room temperature. The recovered PAA-coated TiO$_2$ particles (TiO$_2$-PAA) were washed with 20 ml of ethanol and then centrifuged (4000 rpm) for 20 min at room temperature. The TiO$_2$-PAA particles were dried for 24 h at room temperature. The dried TiO$_2$-PAA particles were solubilized using 2 ml of MES buffer (100 mM, pH 5.9) up to 0.05 g/ml for further usage. The surface of the TiO$_2$, TiO$_2$-PAA, and TiO$_2$-Ab$_E$ was analyzed using an Infinity Gold Fourier transform infrared (FTIR) spectrometer (Thermo Mattson) at room temperature.

2.3. Antibody conjugation with TiO$_2$-PAA particle and magnetic beads

Suspended TiO$_2$-PAA particles (0.05 g/ml) were mixed with 1 ml of MES buffer (100 mM, pH 5.9) containing sulfo-NHS (20 mM) and EDC (80 mM) solution and then incubated for 1 h with shaking (200 rpm) at room temperature. The treated TiO$_2$-PAA particles were recovered by centrifugation (4000 rpm) for 20 min at room temperature and then re-suspended with 1 ml of MES buffer (100 mM, pH 5.9). The suspended TiO$_2$-PAA particles were mixed with 50 µl of antibody (4–5 mg/ml) and incubated overnight at 4 ºC. Subsequently, the activated carboxyl residues were blocked using 0.5 ml of 0.1 M ethanolamine solution, and the mixture was incubated for 30 min at 4 ºC. The antibody-conjugated TiO$_2$ (TiO$_2$-Ab$_E$) particles were recovered by centrifugation (4000 rpm) for 10 min at room temperature and then washed twice using 1 ml of PBS (pH 7.0) buffer. The washed TiO$_2$-Ab$_E$ particles were re-suspended with 2 ml of PBS (pH 7.0) buffer up to 0.05 g/ml and stored at 4 ºC until use.

For conjugation of the antibody with the magnetic beads, 20 µl of carboxylic acid magnetic beads (2 × 10$^8$ particles/ml, 30 mg/ml) were washed three times using MES buffer (25 mM, pH 5.0) and then mixed with 180 µl of MES buffer (25 mM, pH 5.0) containing sulfo-NHS (115 mM) and EDC (130 mM). The mixture was incubated for 1 h with shaking (250 rpm) at room temperature and washed three times using MES buffer (25 mM, pH 5.0). The washed magnetic beads were re-suspended using 200 µl of MES buffer (25 mM, pH 5.0) and mixed with 20 µl of E. coli polyclonal antibody (4–5 mg/ml). The mixture was incubated overnight at 4 ºC after incubating for 1.5 h with shaking (200 rpm) at room temperature. Subsequently, the activated carboxyl groups were blocked with 180 µl of 0.1 M ethanolamine solution and the mixture was incubated for 30 min at room temperature. The antibody-conjugated magnetic beads were separated using magnet for 10 min at room temperature and washed three times using autoclaved deionized water. Finally, the antibody-conjugated magnetic beads were re-suspended with 80 µl of autoclaved deionized water up to 0.15 g/ml.

To confirm the binding of E. coli with TiO$_2$-Ab$_E$, the antibody-conjugated magnetic beads and TiO$_2$-Ab$_E$ particles were mixed with or without E. coli. The E. coli culture (1 × 10$^6$ colony-forming units (CFU)/ml) was washed using PBS buffer (pH 7.0), re-suspended with 200 µl of PBS buffer (pH 7.0), and then incubated for 10 min at 37 ºC with shaking (200 rpm) after mixing with 10 µl (~4 × 10$^6$ particles) of antibody-conjugated magnetic beads. Subsequently, 100 µl of TiO$_2$-Ab$_E$ particles (1 mg/ml) were added and the mixture was incubated for 20 min with shaking (200 rpm) at 37 ºC. The magnetic beads were washed three times using autoclaved water, and then separated using magnets. Finally, the mixture was dried for 24 h at room temperature. The surface morphology of particles was observed using a field emission scanning electron microscope (FE-SEM; S-4100; Hitachi).

2.4. Bacterial culture and investigation of antibacterial activity of particles

All bacteria used in this study were cultivated at 37 ºC in nutrient broth medium (5 g of peptone, 3 g of beef extract in 1 L of distilled water) and all bacteria were cultured at a density of approximately 1 × 10$^6$ CFU/ml. Cultured bacteria were recovered by centrifugation (4000 rpm) for 20 min at room temperature and then washed twice using PBS buffer (pH 7.0) and used for the antibacterial activity test. During the antibacterial activity test, 100 µl of the bacterial samples were removed at each time point, diluted with autoclaved water, coated on a nutrient agar plate and incubated for 17 h at 37 ºC to count CFUs. First, we tested the antibacterial activity of the TiO$_2$ and TiO$_2$-Ab$_E$ particles. E. coli suspension (5 ml, 1 × 10$^5$ CFU/ml) was prepared in glass vials and mixed with 0.5 mg of raw TiO$_2$ or TiO$_2$-Ab$_E$ particles. Before UV irradiation cell suspensions were incubated for 15 min in a shaking incubator (250 rpm) in order to allow the TiO$_2$ or TiO$_2$-Ab$_E$ particles to bind with the E. coli before the UV lamp was turned on. A UV lamp (15 W, with a spectral maximum at 365 nm; Vilber Lourmat; France) was used to irradiate the cell suspensions in a shaking incubator (250 rpm) at room temperature. The distance between the UV lamp and the glass vials was 10 cm and the UV intensity was 0.7 W/cm$^2$. During the UV irradiation 100 µl of cell suspension was sampled to measure CFUs at each time point (0, 15, 45, 75, 115, and 135 min). To test for possible photodegradation and loss of photokilling activity of the TiO$_2$-Ab$_E$ particles, exposed under UV irradiation (15 W UV lamp, 365 nm, 0.7 W/cm$^2$) for 0 min, 30 min, 60 min or 120 min prior to use for photokilling experiment. Then, the photokilling activity of each particle type was tested against E. coli. UV irradiation was performed as previously described. CFUs were measured after 10 min of UV irradiation.
The effect of varying concentration of TiO$_2$ or TiO$_2$-Ab$_E$ was also investigated. The E. coli suspension (2 ml, 1 × 10$^8$ CFU/ml) was transferred to glass vials and TiO$_2$ or TiO$_2$-Ab$_E$ particles were added to the cell suspension at a final concentration of 0.01–0.5 mg/ml. The UV irradiation was performed as previously described, and the CFUs were measured after 20 min of UV irradiation.

The specificity of the photokilling effect of TiO$_2$-Ab$_E$ was evaluated using E. coli, S. epidermidis, K. pneumoniae and E. aerogenes. For this experiment, we used a black box reactor containing a quartz vial, a magnetic stirrer and two 4 W UV lamps (max spectrum: 352 nm; Sankyo Denki; Japan) inside a box sealed to prevent entry of natural light. The UV intensity was 0.17 W/cm$^2$. The raw TiO$_2$ or TiO$_2$-Ab$_E$ (final concentration of 0.1 mg/ml in mixture) was added to 20 ml of cell suspension (1 × 10$^8$ CFU/ml) in a quartz vial. Prior to UV irradiation, suspensions were magnetically stirred for 15 min in the dark, and stirring continued during UV irradiation. Aliquots of cell suspensions (100 μl) were moved from the quartz vial to measure CFUs at various time points during the UV irradiation process (0, 5, 20, 35, and 50 min). The specificity of the particles was also verified using two species of bacteria, C. freundii and B. subtilis; CFUs of these two species were measured after 5 min of UV irradiation and compared with the previously tested bacteria.

The selective photokilling effect of TiO$_2$-Ab$_E$ was tested using mixture of two species of bacteria, E. coli and S. epidermidis. Colony morphology differed so markedly between E. coli and S. epidermidis that their cell survival percentages could be easily measured using a colony counting method on nutrient agar plates. Before UV irradiation of the mixture, 15 min incubation was allowed for binding of TiO$_2$-Ab$_E$ to its target bacteria. The cell survival percentage of each bacterial species was checked after 5 min of UV irradiation. Culture suspensions of E. coli (10 ml, 1 × 10$^8$ CFU/ml) and S. epidermidis (10 ml, 1 × 10$^8$ CFU/ml) were mixed in a quartz vial, and TiO$_2$ or TiO$_2$-Ab$_E$ particles (final concentration of 0.1 mg/ml in mixture) were added. UV irradiation was administered in a black box reactor and CFUs were measured after 5 min. All experiments were repeated at least three times and average values with error bars are presented.

3. Results and discussion

3.1. Synthesis and characteristics of particles

Fig. 1 shows the steps required for preparation of the TiO$_2$-Ab$_E$ particles. First, we modified the TiO$_2$ with a thin layer of PAA to address a carboxylic group, which can involve the conjugation of antibody with TiO$_2$ particles via EDC/NHS coupling reaction, and to prevent aggregation of TiO$_2$ in aqueous solution [30,31]. To confirm the coating of TiO$_2$ with PAA, raw TiO$_2$ and PAA-coated TiO$_2$ were suspended in deionized water. Raw TiO$_2$ particles were precipitated after a few minutes, whereas PAA-coated TiO$_2$ particles remained homogeneously suspended in water for several hours. These results indicate that the ionized carboxylic groups on the PAA-coated TiO$_2$ altered the surface characteristics of raw TiO$_2$ from hydrophobic to hydrophilic. Furthermore, FT-IR data confirmed the generation of carboxyl groups on the PAA-coated TiO$_2$ particles and the generation of an amide bond from the antibody attached to TiO$_2$-PAA (Fig. S1).

To explore the capability of TiO$_2$-Ab$_E$ to specifically bind to E. coli, we used E. coli antibody-conjugated magnetic beads as E. coli capturing modules, and an additional sample of TiO$_2$-Ab$_E$ was mixed with or without E. coli. We expected to observe aggregation of magnetic beads and TiO$_2$-Ab$_E$ particles in the presence of E. coli because E. coli should act as a linker between antibody-conjugated magnetic beads and TiO$_2$-Ab$_E$ particles. Fig. 2 shows the SEM images of the magnetic beads conjugated with E. coli antibody.
in the presence and absence of *E. coli*. No interaction was observed between the TiO₂-AbE particles and the magnetic beads in the absence of *E. coli*, whereas a sandwich complex of magnetic beads and TiO₂-AbE particles was observed when *E. coli* was present.

### 3.2. Inactivation of *E. coli* by raw TiO₂ or TiO₂-AbE particle

On the basis of the observed ability of TiO₂-AbE to bind to *E. coli*, we compared the photocatalytic activity of raw TiO₂ and TiO₂-AbE particles in terms of their antibacterial function. Fig. 3 shows the antibacterial effect of TiO₂ and TiO₂-AbE particles. TiO₂-AbE inactivated *E. coli* was much more strongly than raw TiO₂ particles. The TiO₂-AbE particles killed approximately 90% of *E. coli* at 15 min, whereas the raw TiO₂ particles killed 20% of the bacteria after the same period of time. To achieve a similar degree of cell inactivation (~95%), the TiO₂-AbE solution required 45 min, whereas the raw TiO₂ solution required 75 min, and the UV irradiation-only sample (no TiO₂) required 135 min. Thus, TiO₂-AbE has more rapid and more reliable antibacterial properties than raw TiO₂ particles or only UV irradiation alone. The antibacterial activity of target-specific TiO₂-AbE to *E. coli* was substantially enhanced compared to TiO₂ synthesized using the vapor condensation method or vanadium pentoxide-loaded TiO₂, which were synthesized for the enhanced catalytic activity of P25-TiO₂ and did not have bacterial target specificity [32,33].

Based on the results of the TiO₂-AbE and *E. coli* binding assay, we expect that close contact of TiO₂-AbE with *E. coli* (Fig. 2 and Fig. S2), via antibody–antigen interaction before or after UV irradiation, can enhance the antibacterial effect. TiO₂ photocatalysis inactivates microorganisms mainly by production of ROS and photons [19,34]. ROS usually damage external structures of microorganisms such as cellular outer membranes [35]. Accordingly, considering the short lifespan of ROS in aqueous solutions, we can expect that close surface contact of TiO₂ with *E. coli* would enhance the direct transfer of ROS, which is considered to be a major factor for rapid efficient killing of bacteria.

Furthermore the effect of the TiO₂ concentration was also investigated under 20 min UV exposure after addition of particles. For all concentrations, samples exposed to TiO₂-AbE showed lower cell survival percentages than those exposed to TiO₂ (Fig. 4).

It is possible that the PAA coating on the TiO₂ particles could block the release of ROS, and we therefore tested the photocatalytic activity of raw TiO₂ and TiO₂-PAA particles by comparing their photodegradation of methylene blue (MB) under UV irradiation. Raw TiO₂ particles degraded MB more strongly than TiO₂-PAA or TiO₂-AbE particles (Fig. S3). This result can be interpreted as indirect evidence that the PAA surrounding the TiO₂ particles prevented the release of ROS. Considering the possible reduction in the photocatalytic activity of TiO₂-PAA or TiO₂-AbE compared to raw TiO₂ particles indicated by the MB degradation experiment, the superior antibacterial performance of TiO₂-AbE can be explained by the close contact of the TiO₂ particles with the surface of target bacteria, in spite of the poor photodegradative performance of TiO₂-PAA (Fig. S2).

UV irradiation or ROS generation may also degrade the antibody on the surface of the TiO₂, which may decrease the antibacterial activity of TiO₂-AbE. The enhancement in TiO₂-AbE’s photokilling activity decreased as UV exposure prior to use increased. The TiO₂-AbE particles exposed to UV for 120 min showed a 15% decrease in activity compared to non-exposed particles (Fig. S4). This may be due to loss of function of the antibody on the TiO₂ surface. Nevertheless, the functional activity and specificity to *E. coli* of TiO₂-AbE were not seriously diminished after even 120 min of UV exposure, and remained superior to raw TiO₂.

### 3.3. Specific photokilling effect of TiO₂-AbE

Next, we investigated the enhanced photokilling effect of TiO₂-AbE on the target bacterium. First, we investigated the antibacterial effect of TiO₂-AbE on *E. coli*, as well as *S. epidermidis*, *K. pneumoniae* and *E. aerogenes*, which have no affinity with the *E. coli* specific antibody used. As shown in Fig. 5, no obvious changes were observed in cell survival percentages of *S. epidermidis*, *K. pneumoniae*, or *E. aerogenes* when raw TiO₂ or TiO₂-AbE was mixed with each culture.

**Fig. 3.** Inactivation of *E. coli* by TiO₂ or TiO₂-AbE particles. Before UV irradiation the cell and TiO₂ or TiO₂-AbE mixture was incubated for 1 h. ‘0 min’ denotes the starting point of UV irradiation.

**Fig. 4.** Concentration dependency of antibacterial effect of TiO₂ or TiO₂-AbE on *E. coli* after 20 min of UV irradiation.

**Fig. 5.** A comparison of cell survival ratios of (a) *E. coli*, (b) *S. epidermidis*, (c) *K. pneumoniae* and (d) *E. aerogenes* as a function of the UV illumination time in the presence of TiO₂-AbE or TiO₂ particles.
under UV irradiation. Only *E. coli* showed a difference in cell survival percentage, and TiO$_2$-Ab$_2$ killed *E. coli* more rapidly than raw TiO$_2$ particles. We compared the cell survival ratio of each species of bacteria after 5 min UV irradiation, including two additional bacteria, *C. freundii* and *B. subtilis*, with TiO$_2$ or TiO$_2$-Ab$_2$. Only *E. coli* showed a relatively decreased cell survival percentage in the presence of TiO$_2$-Ab$_2$ compared to raw TiO$_2$ particles (approximately 30% difference) after 5 min of UV treatment, while the other five species of bacteria showed no significant difference in cell survival percentage (Fig. S5).

We further tested the enhanced photokilling effect of TiO$_2$-Ab$_2$ under conditions of bacterial co-existence, using *E. coli* and *S. epidermidis*, and then added raw TiO$_2$ or TiO$_2$-Ab$_2$ particles to the bacterial mixture. As shown in Fig. 6, addition of TiO$_2$-Ab$_2$ resulted in a 55% reduction in the cell survival percentage of *E. coli* whereas addition of raw TiO$_2$ resulted in a 30% reduction. The cell survival ratio of *S. epidermidis* did not show any difference after 5 min of UV irradiation with either raw TiO$_2$ or TiO$_2$-Ab$_2$. The enhanced inactivation of target bacteria in a mixed population implied that TiO$_2$-Ab$_2$ binds specifically to its target, *E. coli*, in a mixed bacterial population, with little non-specific binding to non-target bacteria. Thus, accumulation of TiO$_2$ particles around the target cell surface enhanced the inactivation of target bacterial cells in a mixed population.

4. Conclusions

We prepared TiO$_2$ particles conjugated with a bacterial antibody and measured the enhancement of their bacteria-specific photokilling activity under short periods of UV exposure. In the presence of UV, TiO$_2$-Ab$_2$ enhanced the photokilling of *E. coli* specifically and had no significant photokilling effect on non-target. We suspected that the close contact of TiO$_2$ particles with bacterial cells, due to the high binding affinity of the conjugated receptors to their targets, may enhance ROS transfer to the bacterial surface, resulting in the enhancement of photokilling activity. Although the oxidation ability of PAA-coated TiO$_2$ was weaker than that of raw TiO$_2$, TiO$_2$-Ab$_2$ also inactivated *E. coli* more efficiently than non-target bacteria in a mixed population. The strategy presented in this study will facilitate the combination of other receptors with TiO$_2$ particles for preparation of highly selective and photocatalytic composites or antibacterial coating materials to remediate a wide variety of natural and man-made systems from contamination by unwanted organisms.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.apcatb.2013.11.038.

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