Article

Broad Spectrum Microbicidal Activity of Photocatalysis by TiO₂

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Abstract: Photocatalytically active titanium dioxide (TiO₂) is widely used as a self-cleaning and self-disinfecting material in many applications to keep environments biologically clean. Several studies on the inactivation of bacteria and viruses by photocatalytic reactions have also been reported; however, only few studies evaluated the spectrum of the microbicidal activity with photocatalysis for various species. There is a need to confirm the expected

effectiveness of disinfection by photocatalysis against multidrug-resistant bacteria and viruses. In this study, microbicidal activity of photocatalysis was evaluated by comparing the inactivation of various species of bacteria and viruses when their suspensions were dropped on the surface of TiO$_2$-coated glass. Gram-positive bacteria, e.g., methicillin-resistant *Staphylococcus aureus*, vancomycin-resistant *Enterococcus faecalis*, and penicillin-resistant *Streptococcus pneumoniae*, were easily inactivated by photocatalysis, whereas some gram-negative bacteria, e.g., *Escherichia coli* and multidrug-resistant *Pseudomonas aeruginosa*, were gradually inactivated by photocatalysis. Influenza virus, an enveloped virus, was significantly inactivated by photocatalysis compared with feline calicivirus, a non-enveloped virus. The effectiveness of microbicidal activity by photocatalysis may depend on the surface structure. However, they are effectively inactivated by photocatalysis on the surface of TiO$_2$-coated glass. Our data emphasize that effective cleaning and disinfection by photocatalysis in nosocomial settings prevents pathogen transmission.

Keywords: photocatalysis; disinfection; drug resistant bacterium; virus; UV-A; TiO$_2$

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1. Introduction

Infectious diseases caused by antimicrobial resistance bacteria are becoming one of the major problems during use of antibiotics worldwide [1,2]. Survival of these microbes on environmental surfaces and medical devices leads to nosocomial disease transmission and is thought to contribute to the 90,000 annual deaths from nosocomial pathogens [3–5]. Nosocomial infections are frequently associated with resistant organisms, including methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococcus* spp. (VRE), penicillin-resistant *Streptococcus pneumoniae* (PRSP), *Serratia marcescens*, multidrug-resistant *Acinetobacter baumannii* (MDRA), and multidrug-resistant *Pseudomonas aeruginosa* (MDRP) [6,7]. MRSA and PRSA are also a prevalent cause of community-acquired infection [8]. Currently, new resistance mechanisms, e.g., the New Delhi metallo-β-lactamase-1 (NDM-1), have emerged among several gram-negative bacteria and spread worldwide [9]. Viable pathogens of these organisms in the environment have a significant role in nosocomial transmission, especially for immunocompromised patients.

Furthermore, viruses such as the influenza virus (IFV) and norovirus (NoV) spread across the world [10,11]. These viral species can be transmitted to susceptible hosts via contaminated inanimate surfaces. Control of viral contamination is important to prevent nosocomial transmission. Respiratory and enteric viruses have been reported to survive for days on surfaces, and viral infections are due to transfer from these surfaces to susceptible persons [12–14]. IFV and NoV can be transmitted by direct and indirect contact with contaminated surfaces and/or aerosol and is linked to the outbreaks in hospitals and long-term care facilities [15,16].

These infectious diseases are a threat to human health, and indeed, outbreaks and serious clinical cases have occurred [17]. Effective disinfection of healthcare equipment and surfaces is critical for preventing transmission of potential pathogens [18]. Therefore, new antibacterial and antiviral materials or new countermeasures against such microbes are urgently required to disinfect the surface.

Photocatalytically active titanium dioxide (TiO_2) is widely used as a self-cleaning and self-disinfecting material in many applications to keep environments biologically clean [19–21]. Photocatalysis mainly uses a semiconductor such as TiO_2 which can absorb UV light ($k < 400$ nm) and can photo-stimulate redox reactions on its surface producing Reactive Oxygen Species (ROS) such as hydroxyl radical ($\cdot OH$), superoxide radical ($\cdot O_2^-$) [22] and singlet oxygen ($^1O_2$) [23], and the production of them may contribute to the biocidal activity with strong oxidative activity and destroy organic compounds [24].

This photocatalytic oxidizing power has been applied for the removal of several toxic substances from water and air [25–27]. Many photocatalytic disinfection studies in which TiO_2 was used against bacteria and fungi have been reported [28,29]. Many products that utilize TiO_2 photocatalysis have been commercialized in many countries. For example, TiO_2-coated tiles have been used as self-cleaning and antibacterial materials [30]. While we have reported the virucidal activity of photocatalysis for IFV [31] and bacteriophages [32,33], few studies have addressed viruses [34]. Recently non-TiO_2 photocatalysts (*i.e.*, ZnO and ZnS) reported as significant progress archived in development were also observed their antimicrobial activity [35,36].

Thus, photocatalysis is expected to be applied by antimicrobial materials for many harmful pathogens. To use the superb photocatalytic effect of TiO_2 is a conceptually feasible technology for this material, and its production at industrial scale is easy and inexpensive. However, there is no study comparing the disinfecting activity of photocatalysis for bacteria and virus using typical TiO_2 sample in the same condition, and the effect on drug-resistant bacteria has also not been demonstrated. This study was designed to elucidate the antimicrobial spectrum and the microbicidal activity of photocatalysis on different bacteria species which were bacteria (*i.e.*, gram-positive cocci and gram-negative rods included drug-resistant bacteria) and viruses (*i.e.*, enveloped and non-enveloped viruses).
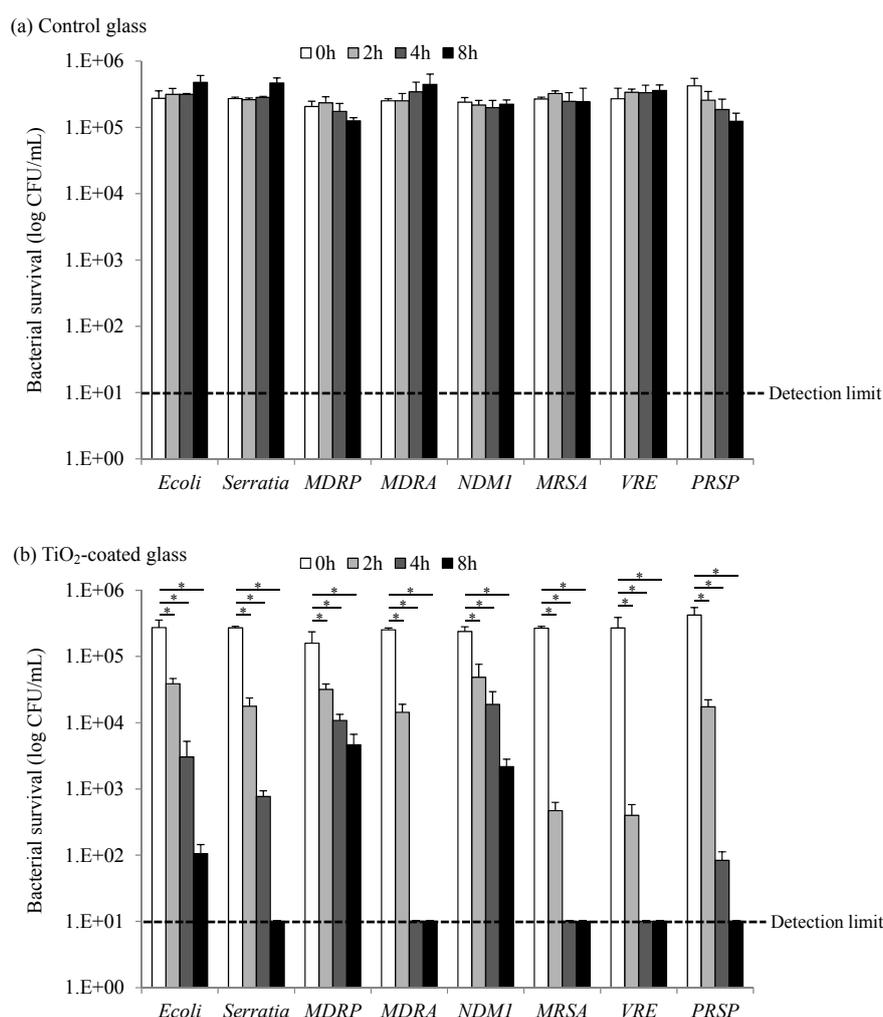
2. Results and Discussion

2.1. Bactericidal Activity of Photocatalysis by TiO₂-Coated Glass

We investigated the bactericidal activity of photocatalysis against different bacteria species that cause an infectious disease at clinical sites. The tested antimicrobial-resistant bacteria species cause opportunistic infections by contact infection and it is very important to cleanse them in a clinical environment. Tested bacteria species on control glass were exposed to 0.1 mW/cm² UVA; however, significant inactivation was not observed, it means the strains were stable under the condition (Figure 1). Photocatalytic inactivation of different bacteria species was evaluated with TiO_2-coated glass under UVA light intensity of 0.1 mW/cm². All bacteria species could be inactivated by treatment with TiO_2 under UV irradiation in a time-dependent manner. We found that TiO_2 exhibited a significantly better performance to reduce the number of surviving bacteria when compared with unirradiated (0 h) (Figure 1, $p < 0.05$). Viable bacterial counts of gram-positive cocci (GPC) such as MRSA, VRE, and PRSP were reduced; the log reduction value (LRV) of them was 1.4–2.8 logs after 2 h, respectively. The number of gram-negative rods (GNRs) such as *Escherichia coli*, *S. marcescens*, MDRP, MDRA, and NDM-1 producing *Klebsiella pneumoniae* gradually decreased, the LRV was

between 0.8 and 1.5 logs after 2 h. It is interesting to note that there was a significantly different bactericidal activity between GPC and GNR. This finding suggests differences in the mode of antimicrobial action.

Figure 1. Photocatalytic inactivation of different bacteria with (**a**) control glass or (**b**) TiO$_2$-coated glass under black-light illumination (UVA light intensity of 0.1 mW/cm^2). The data shown in the figure are the average values of three experiments. Error bars: standard deviations of three replicate experiments. * $p < 0.001$, 2 h, 4 h, 8 h *vs.* 0 h exposed to 0.1 mW/cm^2 UVA.

In particular, GPCs of MRSA, VRE, and PRSP were significantly decreased, *i.e.*, number of surviving cells. They were inactivated below the detection limit (<1 log$_{10}$ colony-forming units/mL) within 4 h. Many MRSA infections occur with a higher incidence rate in hospitals, healthcare facilities, or long-term care facilities. MRSA is transmitted by contact with a colonized carrier (someone carrying the infection on the skin but who is not infected), or a contaminated object, e.g., clothing and environmental surfaces. The bactericidal activity of photocatalysis would inactivate MRSA effectively at environmental surfaces in hospitals and could prevent the spread of nosocomial infections. The effectiveness of the bactericidal activity by photocatalysis could be one of the infection control strategies for preventing environment-associated infections in healthcare facilities. VRE and

PRSP could also be inactivated by photocatalysis and it can be expected to prevent their spread as MRSA.

Except for MDRA, the number of surviving cells of GNRs gradually decreased compared to that of GPCs under UV irradiation with TiO_2. The number of cells of MDRAs significantly decreased as the number of GPCs reached a value below the detection limit within 4 h. The number of cells of all tested GNRs decreased to below the detection limit after 16 h (data not shown). The difference in the bactericidal activity for GPC and GNR could be associated with their surface layer structure (Figure 2 and Table 1). The structure of *S. aureus*, as a representative GPC, is constituted of thick peptidoglycan layer, which covers more than 50%, usually almost 90%. The peptidoglycan layer in the bacterial cell wall has a crystal lattice structure formed from linear chains of two alternating amino sugars, *N*-acetylglucosamine and *N*-acetylmuramic acid. The structure of *E. coli*, as a representative GNR, consists of a thin peptidoglycan layer (which is much thinner than that in GPCs) and an outer membrane containing lipopolysaccharide (LPS) in its outer leaflet and phospholipids in the inner leaflet.

Figure 2. Structure of gram-positive and gram-negative bacteria: **(a)** Gram-positive bacteria have a thick wall composed of peptidoglycans; and **(b)** gram-negative bacteria have an outer membrane and a thin wall composed of peptidoglycans.
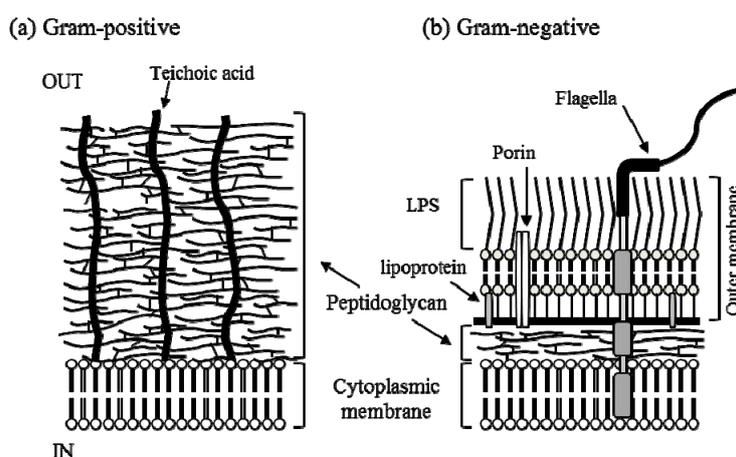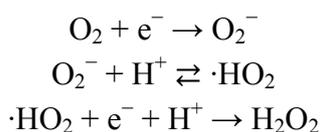

Table 1. Comparative characteristics of gram-positive and gram-negative bacteria.

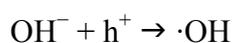| Characteristic	Gram-positive	Gram-negative
Thickness of wall	thick (20–80 nm)	thin (10 nm)
Number of layers	1	2
Peptidoglycan content	>50%	10%–20%
Lipid and lipoprotein content	0%–3%	58%
Lipopolysaccharide content	0	13%

The outer membrane of GNRs is unique due to the high content of lipids. The relative resistance of the cell wall of GNR to dissociation by detergents, disinfectants, and antiseptics is well documented [37,38]. It is proposed that the presence of large amounts of LPS and proteins, together with a very small amount of phospholipids in the outer leaflet of the outer membrane, is the decisive

factor in the effective detergent resistance of this membrane [37]. GNRs are generally more resistant to disinfectants than GPCs due to outer membrane barrier, but the resistant mechanism is not well characterized. Although the microbicidal activity by photocatalysis has not been studied thoroughly and the mechanism of the bactericidal process is still unknown [27,39], there are some suggestions and evidences for the steps leading to cell inactivation. TiO₂ photocatalysis generates various ROS as follows [24].

Reductive reactions:

$$O_2 + e^- \rightarrow O_2^-$$
$$O_2^- + H^+ \rightleftarrows \cdot HO_2$$
$$\cdot HO_2 + e^- + H^+ \rightarrow H_2O_2$$

Oxidative reactions:

$$OH^- + h^+ \rightarrow \cdot OH$$

These ROS can decompose organic compounds and extinguish cellular activity. Recent works attributed that the TiO₂ photocatalytic action induced significant damage in cell membranes by ROS, followed by loss of essential functions such as respiratory activity and bacterial lysis leading to bacterial death [24,25,40]. Sunada *et al*. [41] demonstrated the TiO₂ photocatalytic inactivation of *E. coli* endotoxin, which is a constituent of the outer membrane of GNRs. This finding suggests that the cell wall damage might take place prior to cytoplasmic membrane damage. After the outer membrane has been disordered and partially decomposed (Figure 2), ROS gain access to the cytoplasmic membrane, causing the cell to die. However, while the outer membrane protected GNRs, GPCs have a thick peptidoglycan layer instead of an outer membrane; these ROS would presumably easily penetrate the cytoplasmic membrane and attack the cell membrane directly. The difference in their structure may be of decisive influence on the survival abilities of bacteria.
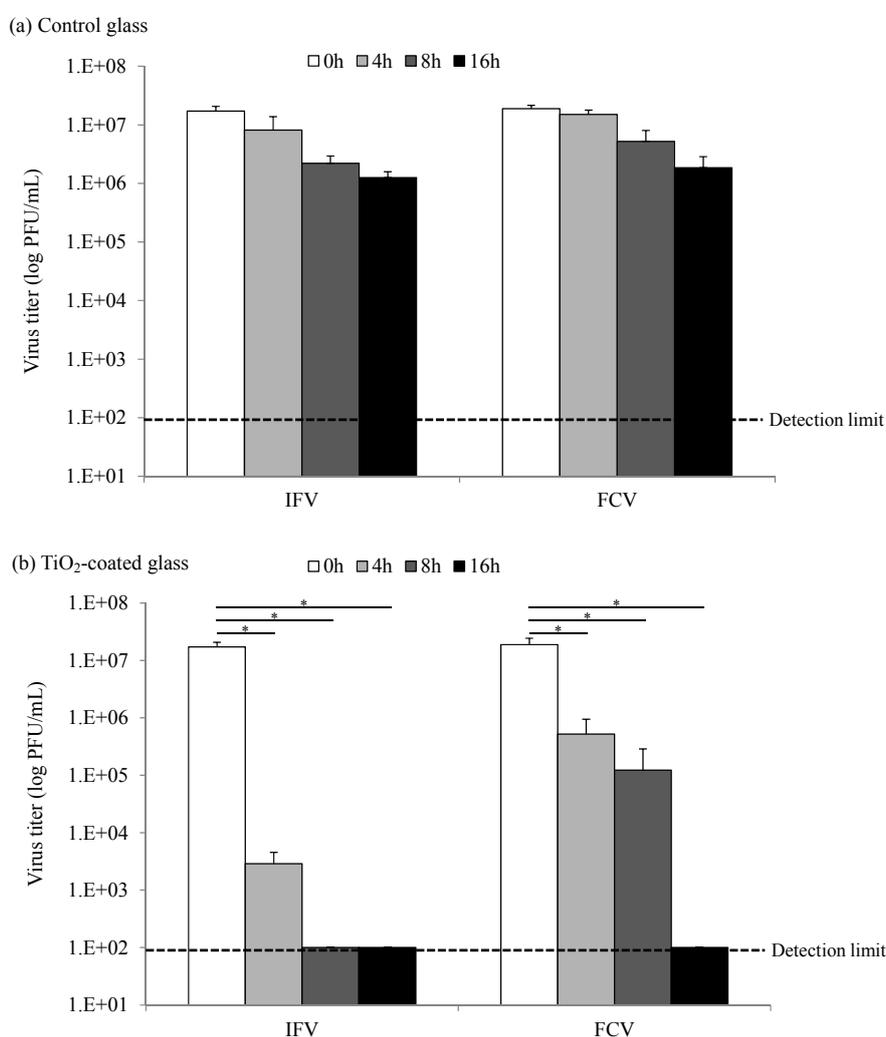
Some GNRs have flagella, which are long filamentous protein structures projected from the cytoplasmic membrane, constituted of microtubules and surrounded by the cell membrane (Figure 2). Bacterial flagella provide mobility for capturing nutrients and avoiding harmful substances to survive [42]. In this study, MDRA is the exceptional strain among these tested GNR bacteria because it has does not have flagella surrounding the cell membrane and it is inactivated by photocatalysis within 4 h. It is suggested that the lack of flagella at the surface structure of MDRA may be part of the reason for higher bactericidal activity by photocatalysis. Alternatively, other structures such as capsule, pilus, and bacterial size and an antioxidative defense system [43] may be associated with the effectiveness of bactericidal activity. Further study is necessary to clarify the mechanism of the photocatalysis reaction to exercise a decisive influence on bactericidal activity by analyzing the effects against the cell membrane surface.

2.2. Virucidal Activity of Photocatalysis by TiO₂-Coated Glass

We investigated the virucidal activity of photocatalysis against IFV and *feline calicivirus* FCV (a surrogate for NoV) using purified and adjusted suspensions. Tested viruses on control glass were also stable under the UVA light intensity of 0.1 mW/cm² (Figure 3). As shown in Figure 3, photocatalytic inactivation of these viruses was evaluated with TiO₂-coated glass under UVA light

intensity of 0.1 mW/cm^2. The viruses were inactivated by the treatment with TiO$_2$ under UV irradiation in a time-dependent manner, as shown for bacteria. The experiments were relatively reproducible and this reduction was statistically significant when compared with unirradiated (0 h) (Figure 3, * $p < 0.001$). The LRV of IFV at 4 h was 3.6 and the titer decreased to below the detection limit within 8 h, while the LRV of FCV at 8 h was only 1.7. The FCV titer decreased to below the detection limit after 16 h. It was concluded that the FCV titer was insignificantly reduced by the photocatalysis effect.

Figure 3. Photocatalytic inactivation of influenza virus (IFV) and feline calicivirus (FCV) with (**a**) control glass or (**b**) TiO$_2$-coated glass under black-light illumination (UVA light intensity of 0.1 mW/cm^2). The data shown in the figure are the average values of three experiments. Error bars: standard deviations of three replicate experiments. * $p < 0.001$, 4 h, 8 h, 16 h *vs.* 0 h exposed to 0.1 mW/cm^2 UVA.

These data demonstrate that IFV is faster inactivated by photocatalysis than FCV. The low virucidal activity of photocatalysis against the non-enveloped virus was not surprising considering previous studies on biocides. IFV, an enveloped virus, consists of a phospholipid bilayer membrane with glycoprotein spikes surrounding the capsid. FCV, a non-enveloped virus, has a capsid that consists of a protein and lacks an envelope that surrounds it. Although enveloped viruses are effectively eliminated by solvent/detergent treatment, non-enveloped viruses are not affected by this treatment [44].

Non-enveloped viruses, without a lipid coat, are resistant to most of the viral inactivation methods that have been described to date [45,46]. These data indicate that the difference in the effectiveness to enveloped and non-enveloped viruses by photocatalysis is the same as that for disinfectants. It is suggested that the virucidal activity of photocatalysis may depend on the viral envelope. Previously, we demonstrated that IFV decreased the infectability by damaging their constituent proteins prior to nucleic acid degradation with photocatalysis reaction [31]. The photocatalytic action of TiO_2 may promote peroxidation of phospholipid components of the enveloped membrane, inducing significant damage in membranes as mode of inactivation that is observed for bacteria. The phospholipid content may influence the virucidal activity of photocatalysis. Further study is required to clarify the mechanism of virucidal activity by evaluating other viruses. Although there was a difference in an effect caused by IFV and FCV, TiO_2 is a potential, environment-friendly antivirus agent.

3. Experimental Section

3.1. Bacteria Strains

Drug-resistant and clinically pathogenic strains of GPC and GNR were used as reference strains to evaluate the bactericidal activity by photocatalysis. They were donated from other researchers or purchased from ATCC (American Type Culture Collection, Manassas, VA, USA). They included the GPC strains MRSA ATCC 43300, VRE ATCC 51575, PRSP ATCC 700677, and the GNR strains *E. coli* ATCC 8739, *S. marcescens* ATCC 13880, MDRA, MDRP, and NDM-1 producing *K. pneumoniae* BAA-2146. MDRA and MDRP were isolated from a hospital in Japan and they were identified to be resistant to ciprofloxacin (minimum inhibitory concentration: MIC $\geq$ 4 mg/mL), imipenem (MIC $\geq$ 16 µg/mL), and amikacin (MIC $\geq$ 64 µg/mL). The profiles of these strains are listed in Table 2.

Table 2. Profiles of the organisms tested in this study.

Species	Structure	Types of Infections [a]
E. coli	Gram-negative rod	Diarrhea, UTI, food poisoning, sepsis
S. marcescens	Gram-negative rod	Nosocomial infections, Pneumonia, UTI, and wound infections
MDRA	Gram-negative rod	Nosocomial infections, Pneumonia, UTI, VAP
MDRP	Gram-negative rod	Nosocomial infections, Pneumonia, UTI, VAP, Bacteremia, cystic fibrosis
NDM-1 producing K. pneumoniae	Gram-negative rod	Nosocomial infections, Pneumonia, UTI, Bacteremia
MRSA	Gram-positive cocci	Nosocomial infections, skin infections, food poisoning, pneumonia, meningitis, bacteremia
VRE	Gram-positive cocci	Nosocomial infections, UTI, meningitis, bacteremia
PRSP	Gram-positive cocci	Community-acquired pneumonia, bacteremia, bacterial meningitis
IFV	Enveloped virus	Respiratory tract infections
FCV	Non-enveloped virus	Respiratory infection in cats

[a] UTI, Urinary tract infection; VAP, Ventilator Associated Pneumonia.

The strains except PRSP were cultivated twice consecutively on nutrient agar plates (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) while PRSP was cultivated on 5% sheep blood agar plates at 37 °C for 18–20 h. Subsequently, the live cells were suspended in 1/500 nutrient broth solutions, which contained 6 mg of meat extract, 20 mg of peptone, and 10 mg of NaCl per liter (pH 7.0), and then diluted to approximately 10^7 colony-forming units per milliliter. All reagents and concentrations used were prepared in accordance with the JIS R 1702 standard method [47].

3.2. Virus Strains

The human IFV A/PR8/H1N1 strain and FCV strain F-9 ATCC VR-782 were used as reference strains to evaluate the virucidal activity by photocatalysis. They were purchased from ATCC. IFV was propagated by using 11-day-old embryonated chicken eggs and purified by using previously described methods [43]. FCV was propagated by using confluent layers of Crandell-Reese feline kidney (CRFK) cell cultures. The harvested samples were clarified and concentrated by a combination of depth and membrane filters (microfiltration with 0.45-μm polypropylene mesh and cross-flow polysulfone hollow-fiber ultrafiltration with a 100-kDa cut-off, GE Healthcare, Japan) [48]. Subsequently, the virus was purified by sucrose density gradient centrifugation using a linear sucrose gradient of 30%–70% [49]. Each individual experimental sample was adjusted to 0.1 mg/mL protein with phosphate-buffered saline, and 0.1 mg/mL of bovine serum albumin was added as stabilizing agent [50,51]. The virus titer of IFV and FCV was determined by using the plaque technique on confluent layers of Madin-Darby canine kidney (MDCK) cell or CRFK cell cultures grown in 12-well culture plates, respectively, as described before[49,52].

3.3. TiO$_2$-Coated Glass

TiO$_2$-coated glass (T3 sample, 50 mm × 50 mm) was obtained from TOTO Ltd., (Fukuoka, Japan) by employing a spin-coating process with TiO$_2$ (obtained from Ishihara Sangyo Kaisha, Ltd., Osaka, Japan) and heat treatment at 150 °C. As mentioned previously, the T3 sample was used as test sample for the preparation of ISO27447:2009(E) [53] and JIS R 1702 [47] for evaluating the photocatalytic antibacterial activity. The phase space of this sample was smooth, the thickness was approximately 200 nm, and the specific surface area was 300 m^2/g. Non-TiO$_2$ coated glass was used as the control. The T3 sample and control glass plates were washed with sterilized water and then sterilized by UV exposure overnight or wiped with ethanol.

3.4. Photocatalytic Reaction

The photocatalytic inactivation of bacteria and virus was investigated by applying the test method for the evaluation of the antibacterial effect and the test method used in our previous study for the evaluation of the antivirus effect [31]. Overhead illumination by long-wavelength UV (UV-A) light was provided by using tubular 20-W black light fluorescent lamps (FL20S Black Light Blue (BLB); λ = 352–368 nm; Toshiba, Japan). Illumination conditions of 0.1 mW/cm^2 were adjusted by changing the horizontal distance between the samples and lamp. UV-A intensity was analyzed by using an integrating actinometer for photocatalysis (C9536–01/H9958; Hamamatsu Photonics Co. Ltd.,

Hamamatsu, Japan). All experiments were performed in a light-tight box to prevent any influence of indoor light and sunlight.

The bacterial or viral suspension (100 μL) was dropped onto the T3 sample or control grass and equally distributed by covering it with adhesive film (VF-15, Kokuyo Co. Ltd., Osaka, Japan, 40 mm × 40 mm). The sample was then illuminated with UV-A irradiation. The photocatalytic reaction utilized light intensity in the range of 0.1 mW/cm^2 at the sample surface, and the UV-A irradiation time was 0–8 h. After illumination for a certain period of time, the sample was immersed and washed with 10 mL of soybean-casein digest broth, which contained lecithin and polysorbate. The collected viable bacteria or virus cells were serially diluted in phosphate-buffered saline and evaluated by using the colony formation method or plaque assay, respectively. All experiments were repeated independently more than three times, and the average titer was determined. Statistical analysis was performed by the Bonferroni test using SPSS software (SPSS Inc., Chicago, IL, USA). $p < 0.05$ was considered statistically significant. All reagents and concentrations used were prepared in accordance with ISO27447:2009(E) [53] and JIS R 1702 [47], and with minor modifications for the concentrations of viruses as previously reported [31].

4. Conclusions

We have demonstrated broad-spectrum biocide efficacy of photocatalysis against both bacteria (GPC and GNR, including drug-resistant bacteria) and viruses (enveloped and non-enveloped virus). GPC such as MRSA and VRE were easily inactivated by photocatalysis, whereas some GNRs such as *E. coli* and MDRP were gradually inactivated by the photocatalysis. The difference in the bactericidal activity against GPC and GNR could be associated with the structure of their surface layer. IFV, an enveloped virus, was significantly faster inactivated by photocatalysis compared with FCV, a non-enveloped virus. These data indicated that the virucidal activity of photocatalysis may depend on the presence or absence of a viral envelope.

The LRV of representative species induced in the presence of the tested photocatalyst were found to decrease in the following order (LRV of the species at 4 h): MRSA (4.4) > IFV (3.6) > *E. coli* (2.1) > FCV (1.3). Photocatalysis has broad-spectrum microbicidal activity and the effects were found to be in the following order: GPC > enveloped virus > GNR > non-enveloped virus. Although there was a difference in the effect due to the surface structure, TiO_2 is a potential, environmentally friendly microbicidal agent.

Photocatalysis would reduce the risk of contact infection spreading within the environmental surfaces at facilities, e.g., as measure against healthcare-associated infections at hospitals or healthcare facilities, and prevention of the spread of community-acquired infections at airports or day-care centers. Furthermore, because of the high level and broad-spectrum biocide efficacy of photocatalysis, it is considered a valuable biocide method for use in various areas, e.g., sewage disposal treatment systems and air purification systems [54].

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Conflict of Interest

The authors declare no conflict of interest.

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