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Bacterial Biofilm Characterization and Microscopic Evaluation of the Antibacterial Properties of a Photocatalytic Coating Protecting Building Material

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Abstract: Use of photocatalytic paint-like coatings may be a way to protect building materials from microbial colonization. Numerous studies have shown the antimicrobial efficiency of TiO_2 photocatalysis on various microorganisms. However, few have focused on easy-to-apply solutions and on photocatalysis under low irradiance. This paper focuses on (a) the antibacterial properties of a semi-transparent coating formulated using TiO_2 particles and (b) the microscopic investigations of bacterial biofilm development on TiO_2-coated building materials under accelerated growth conditions. Results showed significant antibacterial activity after few hours of testing. The efficiency seemed limited by the confinement of the TiO_2 particles inside the coating binder. However, a pre-irradiation with UV light can improve efficiency. In addition, a significant effect against the formation of a bacterial biofilm was also observed. The epifluorescence approach, in which fluorescence is produced by reflect rather than transmitted light, could be applied in further studies of microbial growth on coatings and building materials.

Keywords: coating; building materials; bacterial growth; proliferation; biofilm; antibacterial

1. Introduction

Since microorganisms are ubiquitous and dispersible from soil and water as well as from air, building materials are permanently exposed to them and may easily become targets for contamination and growth. The requirements' microorganism development, i.e., an energy source, carbon and water, can be satisfied by building materials (organic and/or inorganic) in a large variety of contexts [1–3]. The growth of microorganisms can have various detrimental consequences, including the biodeterioration of materials, which may concern structural and aesthetic properties, and/or the release of aerial contaminants that can be deleterious for human health. Their proliferation on building materials is of growing concern to the scientific community [3–6].

A substantial number of man-made constructions have been reported to be widely contaminated by microorganisms (mainly fungi, bacteria and algae). They include pipes carrying aggressive aqueous media such as sewers [3], moisture-damaged buildings [1,7,8], historical monuments [9], etc. Contaminated building materials are usually visually deteriorated by the activity of fungi and bacteria, which are known to be the most harmful organisms inducing physical and chemical changes in materials.

Microbial contamination of building materials in indoor environments has also been much studied. The degradation of indoor air quality is a growing public health concern worldwide and microorganisms or their components are major polluting agents [2]. Their contribution to the Sick

Building Syndrome (SBS) and Building Related Illness (BRI) is widely emphasized in the literature. Microorganisms may release aerial particles such as spores, allergens, toxins and other metabolites that can be harmful to human health [10–14]. Serious health troubles such as irritations and toxic effects, infections, allergies, etc. have been experienced by building occupants frequently exposed to microbial contaminants [15,16]. Several studies have already reported that indoor building materials can become major sites of microbial growth [13,15,17]. Actually, growth-promoting conditions, i.e., high humidity and presence of nutrients, are easily fulfilled in damp environments such as water-damaged, damp, and/or badly-insulated buildings. Microorganisms grow on interfacial zones (air–substrates, water–air, etc.). Under specific environmental conditions, they develop on surfaces in complex three-dimensional structures, in which adherent cells are embedded within a self-produced matrix of extracellular polymeric substance (EPS). The overall organization, commonly called a microbial biofilm, provides the microorganisms with particular resistance towards physical and chemical aggressions, be they environmental or from disinfection treatments [18–20].

Different solutions have been studied to prevent, stop or at least reduce microbial biofim development on building materials, including core treatments and coatings applied to surfaces. Antimicrobial products can be formulated using biocides [21], metal oxide nanoparticles [22–24] or bio-based products [25]. A substantial amount of literature has been published on the effect of photocatalytic TiO_2 nanoparticles on microorganisms [26–29]. Studies have shown high efficiency of photocatalysis on the viability of a wide variety of microorganisms and on microbial contaminants, including bacteria, endospores, fungi, algae, protozoa, viruses and prions (shown to be the organisms most resistant to disinfectants [30]) as well as on microbial toxins. Photocatalysis needs to be activated by a light irradiation of sufficient energy to create electron–hole pairs. The electron–hole pairs may react with electron donors or acceptors previously adsorbed on the photocatalyst, e.g., O_2, H_2O. The resulting redox reactions lead to the formation of oxide radicals that are highly reactive and degrade organic matter by non-selective action. Additionally, irradiated TiO_2 presents photo-induced superhydrophilicity [31] and may be incorporated in materials to provide self-cleaning properties.

The use of titanium dioxide as a self-cleaning agent in building materials is common for some years now. Photocatalytic concretes have been developed to prevent fouling and biofouling [32,33] or to purify ambient air (VOC, NO_x ...) [34–38]. Regarding bacterial and fungal disinfection, research has mainly focused on the efficiency of photocatalytic TiO_2 particles used alone, powdered or immobilized in the form of thin film coatings by complex processes (usually involving calcination). It seems that only few studies have investigated easy-to-apply devices such as paint-like coatings. In addition, one of the main advantages of such coatings are their passive aspect: once applied to a surface, the photocatalysis is activated by natural light. Furthermore, efficiencies of TiO_2 photocatalysis found in the literature are usually related to very high irradiances (>10 W/m^2) [28,29,39] that are far from the real-world conditions. Only recent studies have begun to investigate on the photocatalytic activity under low irradiances [40].

The main objectives of the present paper were (a) to focus on the antibacterial efficiency under low irradiance of a photocatalytic semi-transparent coating that is easy to apply on existing materials and (b) to carry out microscopic observations (epifluorescence and SEM) of bacterial biofilm development on a coated building materials under accelerated growth conditions.

2. Materials and Methods

2.1. Bacterial Cultivation

The microorganisms the most frequently detected on indoor building materials are (i) fungi genera *Cladosporium*, *Penicillium*, *Aspergillus* and *Stachybotrys*, and (ii) Gram negative bacteria and mycobacteria [2].

In this study, the model bacterium *Escherichia coli* was chosen for methodological reasons, i.e., its relative ease of culture and its high speed of growth. *E. coli* CIP 53126 was obtained from

the Collection of Institute Pasteur (CIP), Paris, France. The strain was preserved at −80 °C in Eugon medium (Biomérieux, Craponne, France) supplemented with 10% glycerol. The *E. coli* strain identification was checked by Gram staining and biochemical characterization (oxydase reaction and Analitical Profile Index 20E) (Biomérieux, Craponne, France). Before each experiment, bacterial cells were pre-cultured on a nutrient agar Petri dish. Colonies were then transferred to trypcase soy agar (TSA) (Biomérieux) and incubated at a temperature of 36 °C for 16 to 24 h. A second culture on TSA was incubated at a temperature of 36 °C for 16 to 20 h prior to the test. For testing, one plastic loop of bacteria was dispersed evenly in a small amount of sterile distilled water and the suspension for inoculation was adjusted to about 10^8 cells/mL using a spectrophotometer (640 nm). A 10-fold dilution of the cell suspension was then prepared to obtain the final concentration of the test suspensions, depending on the type of evaluation (antibacterial activity or resistance to biofilm formation).

2.2. Coatings and Preparation of Supports

2.2.1. Coatings

Two semi-transparent coatings were formulated using water and acrylic resin. The formulation contained 5 wt % TiO_2 dispersion (Kronos type 7454, trial product, KRONOS/Société Industrielle du Titane, Paris, France), 2 wt % acrylic resin and 93 wt % water. A control coating was made of 2 wt % acrylic resin and 98 wt % water. The TiO_2 dispersion contained TiO_2 particles (Kronos VLP7050), some physical characteristics of which are presented in Table 1.

Table 1. Physical characteristics of the VLP5070 (Kronos) TiO_2 particles

Description	VLP5070
TiO_2-Content	>85 %
Crystal modification	anatase
Crystallite size	approx. 15 nm
Specific surface area	>225 m^2/g

2.2.2. Nature and Preparation of Supports

Three types of support were used in this study: glass slides, polycarbonate membranes, and cementitious matrices.

Glass Slides

Preliminary measurements of the antibacterial activity were carried out on glass microscope slides (26×76 mm^2) covered by pipetting the TiO_2 coating or the control coating (without TiO_2). Regarding the coating of glass slides, while drying, the water left an even film of acrylic binder scattered with TiO_2 particles. Although the distribution of TiO_2 was not optimal on these supports, it was rather homogeneous over the entire surfaces of samples.

Polycarbonate Membranes

Isopore hydrophilic polycarbonate membranes (Millipore, 47 mm diameter, 0.4 µm filter pore size, 5%–20% porosity) were also used as supports for the coating. Membranes were placed in sterile Petri dishes, covered by pipetting the coatings and placed under a sterile flow hood for air drying. Then, samples were pre-irradiated with UV light (5 W/m^2) for different durations, from 0 h to 109 h. The role of the pre-irradiation was to increase the antibacterial activity of the coating. It is discussed later in the paper.

Cementitious Supports

Cement paste was chosen as reference building material because of its availability, its ease of casting, and because it is morphologically representative of many building materials (porosity and roughness). The cement paste samples were made of ordinary Portland cement CEM I 52.5R were cast with a 0.55 Water/Cement ratio in cylindrical molds (d = 2.8 cm, h = 1 cm). Samples were demolded after 24 h and stored for 27 days in a moisture chamber (100 %RH, room temperature). It is widely known that the pH of materials is a determining factor in microbial contamination. The pH of unaltered cementitious materials is around 13. In their work on an accelerated laboratory test to study fungal biodeterioration, Wiktor et al. [41,42] showed that, for cementitious materials, a low pH of the surface was essential to increase the bioreceptivity of samples. Recently-cast cementitious materials are thus not so subject to microbial colonization, probably because of their relatively high alkalinity incompatible with microbial growth. Usually, the bioreceptivity of these materials increases over time as carbonation tends to decrease the surface pH, but this phenomenon takes place very slowly (typically, a few weeks in natural conditions). Consequently, accelerated aging of the cementitious mortars was performed. After 28 days, samples were placed in a 2-liter-borosilicate reactor for a leaching operation, as follows: the reactor was continuously supplied with a leaching solution by a peristaltic pump (Masterflex L/S, Cole-Palmer, Vernon Hills, IL, USA). The flow rate was set to 1.4 mL/m in order to renew the reactor content in 24 h. The leaching solution was composed of water and acetic acid (CH_3COOH) 0.12 mol/L and the pH was adjusted to 4.7 with sodium hydroxide (NaOH) at 0.076 mol/L. The operation was carried out for 15 days. Then, samples were removed from the reactor and a pH-paper was applied on their wet surfaces. The pH measured was around 7 for all samples. After this leaching operation, samples were washed with distilled water and stored at 100% RH until tested.

Prior to the experiments, samples were covered with the coatings (TiO_2 coating or control coating) by pipetting 500 µL over the entire surface (6.16 cm^2). They were placed under a sterile flow hood for over 12 h for air drying. Then, samples coated with TiO_2 and control samples coated without TiO_2, were pre-aged by UV irradiation (5 W/m^2) for different durations, from 0 h to 109 h.

2.3. Photocatalysis Activation

The TiO_2 photocatalyst (anatase) is naturally activated by light irradiation in the UV-range. The light intensity, or irradiance, greatly influences photocatalysis efficiency. In the literature, intensity values are usually chosen between 10 and 500 W/m^2 [43,44]. These values are quite different from natural light intensity values, in indoor or outdoor conditions. For example, studies have reported outdoor intensity around 30 W/m^2 on sunny days [36,45,46] and between 5 and 10 W/m^2 on cloudy days [47,48]. The few studies that observed inactivation of microorganisms by photocatalysis with light intensities around 10^{-3} W/m^2 showed much lower inactivation rates [49], confirming the major impact of light intensity on the process. Regarding the resistance to microbial biofilm, a recent study also showed significant efficiency of TiO_2 coatings against the development of microbial biofilms under relatively low irradiance (13 W/m^2) closer to real-world light intensity [40].

UV light in the 325–400 nm range can strongly damage microorganisms and lead to their death. Standards usually recommend the use of low intensities for photocatalytic activity evaluation [50]. The position and the power of the lamp must be chosen so as activate the photocatalyst while making sure to avoid any lethal action of the UV light on bacterial cells. In our experiment, 8 W black light bulbs were chosen (peak wave 365 nm). The distance between lamps and samples was adjusted to obtain 2–4 W/m^2 at the bottom of the Petri dish (irradiance measured with a UV-A radiometer Gigahertz-Optik, GmbH, Türkenfeld, Germany, in the 310–400 nm range).

In order to assess the effect of UV irradiation on the survival of *Escherichia coli*, a drop of a bacterial suspension in phosphate buffer (about 1 mL of a 10^6 CFU/mL suspension) was deposited in a borosilicate Petri dish and irradiated for several hours. Different configurations were tested: irradiation with a borosilicate lid on the Petri dish, irradiation without the lid, and kept in the dark (control).

After the test, bacteria were collected with a recovery solution (9 mL of Soybean Casein Lecithin Polysorbate 80 medium, SCDLP broth, as described in [51]) and numerated (CFU) in TSA after a 24 h incubation at 36 °C . Results are presented in Figure 1.

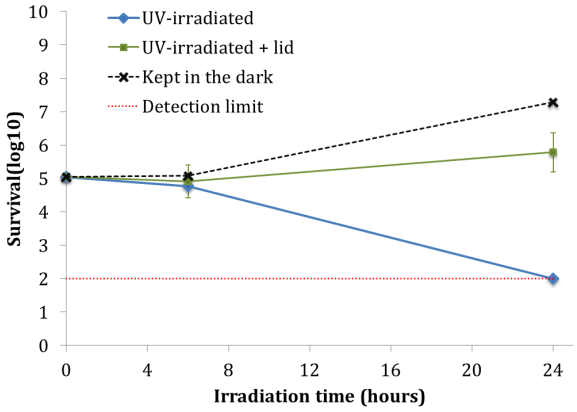

Figure 1. Direct effect of the time of UV irradiation on the survival of *Escherichia coli* CIP 53126 expressed as the log residual viable bacteria, light intensity ≈2.5 W/m^2.

For the three configurations, no significant evolution in the number of CFU was observed in the first 6 h of experiment. Control samples kept in the dark and irradiated in the presence of the borosilicate lid showed an increase of 2 log and 0.75 log, respectively, in 24 h, while no CFUs were detected on samples irradiated without the lid (limit of detection = 2 log). The borosilicate lid allowed UV light to penetrate while keeping the hygrometry constant in the Petri dish and preventing the drop from drying.

2.4. Evaluation of Antibacterial Activity

The antibacterial activity characterizes the ability of a substrate to inactivate bacteria cells, i.e., to kill them or to inhibit their growth. It is a quantitative test that provides a bacterial reduction in terms of Colony Forming Units (CFUs).

The experiment was based on the contact between an antibacterial surface and a bacterial suspension for a given period of time, according to standards JIS Z 2801:2010 [52] and ISO 27447:2009 [50]. The test was carried out on polycarbonate membranes covered with TiO_2 coating, or with control coating, and placed in Petri dishes under a sterile flow hood. Each membrane was inoculated with 0.4 ml of bacterial suspension (cell concentration adjusted between 8×10^4 and 2×10^5 CFU/mL). A transparent sterile film (9–10 cm^2) was carefully placed on the suspension in order to increase the contact area between bacterial cells and coatings [51]. Then, some membranes were UV-irradiated as described earlier (Section 2.3) and others were kept in the dark. The irradiation times were 2 h, 4 h, and 6 h.

After a certain time of contact (t), bacteria were recovered using the SCDLP broth. Quantitative evaluation was performed by CFU counting as described earlier (Section 2.4). The antibacterial activity was then calculated as the difference between the average logarithm of the number of viable bacteria on the control without TiO_2 and the average logarithm of the number of viable bacteria on samples coated with TiO_2:

$$A = \log\left(N_{TiO_2}\right) - \log\left(N_{control}\right) = \log\left(\frac{N_{TiO_2}}{N_{control}}\right), \quad (1)$$

with A: antibacterial activity; N_{TiO_2}: average number of CFU on TiO_2-coated samples; $N_{control}$: average number of CFU on control-coated samples.

2.5. Development of a Bacterial Biofilm under Accelerated Conditions

This experiment was designed to observe the effect of the photocatalytic coating on bacterial growth and spread once coated on a building material. Conditions favorable to microbial growth were chosen to obtain a biofilm very rapidly (24 h).

The test was carried out in aqueous media and in static conditions as previously described [53–55]. The media was chosen so that bacterial development was favored in the form of biofilm without planktonic growth. Direct observations of samples were carried out by epifluorescence microscopy and by scanning electron microscopy in order to highlight the distribution of bacteria.

2.5.1. Biofilm Nutrient Broth

The biofilm nutrient broth used to promote bacterial adhesion and biofilm formation was prepared as described in [53–55]. The composition of the broth is given in Table 2.

Table 2. Composition of the biofilm nutrient broth.

Components	Concentration (g/L)
$FeSO_4, 7H_2O$	0.0005
Na_2HPO_4	1.25
KH_2PO_4	0.5
$(NH_4)_2SO_4$	0.1
Glucose	0.05
$MgSO_4, 7H_2O$	0.02

2.5.2. Procedure

The test was carried out with the cementitious supports covered with TiO_2 coating or with control coating. Cement supports were placed in 6-well culture plates filled with approximately 6 mL per well of biofilm nutrient broth at room temperature. Then, each sample was inoculated with 300 µL of the bacterial test suspension adjusted to about 10^3 CFU/mL . The plates were covered with UV-transparent pyrex lids and incubated at 36 °C . One plate was irradiated with UV light and the other was kept in the dark.

Light intensity on the samples was settled around 3 W/m^2 . In order to avoid planktonic cells (non adherent or released from the biofilm), the wells were washed with sterile distilled water and the biofilm broth was renewed after 4 h, 6 h and 24 h of incubation. Rapid assessments with pH paper was carried out before and after the experiment. The broths of different samples were between 7 and 9. No jump of pH was detected, indicating that the pre-treatment (leaching) of the cementitious supports was efficient. After 24 h, the samples were washed and removed with sterile tweezers and placed in new culture plates. Then, 6 mL of phosphate buffer was added to the wells and the top surface of each sample was gently scraped with a steel spatula. After homogenization of the solution, 1 mL was collected and diluted in sterile distilled water.

2.5.3. Scanning Electron Microscopy

Microstrucutural observations and chemical analyses were performed using a scanning electron microscope (JSM-6380LV, Jeol, Tokyo, Japan) fitted with an Energy Dispersive Spectroscopy detector (XFlash® 3001, Röntec, Berlin, Germany).

2.5.4. Epifluorescence Microscopy

Epifluorescence microscopy was used for rapid assessment of the bacterial colonization of sample surfaces. Following the addition of phosphate buffer at the end of the experiment, 0.75 µL of Syto9 was used as a fluorochrome to stain the bacterial biofilm (Syto9 excitation range: 470–520 nm ; emission ange: 510–540 nm). After 10 min, samples were imaged with a Carl Zeiss Axio Imager-M2

microscope (Carl Zeiss, Oberkochen, Germany) equipped for epifluorescence with an HXP 200 C light source and the Zeiss 09 filter (excitor HP450r HP450200 C light source). Images were acquired with a digital camera (Zeiss AxioCam MRm) every 0.5 mm along the *Z*-axis and the set of images was processed with the Zen (Carl Zeiss) ® software. It should be emphasized that the Syto9 fluorochrome penetrates through damaged membranes as well as through whole membranes. Consequently, the method used here does not differentiate viable and non-viable cells but provides a visualization of global microbial colonization.

3. Results and Discussions

3.1. Antibacterial Activity

The results obtained from the analysis of TiO_2 antibacterial activity on coated glass are summarized in Figure 2.

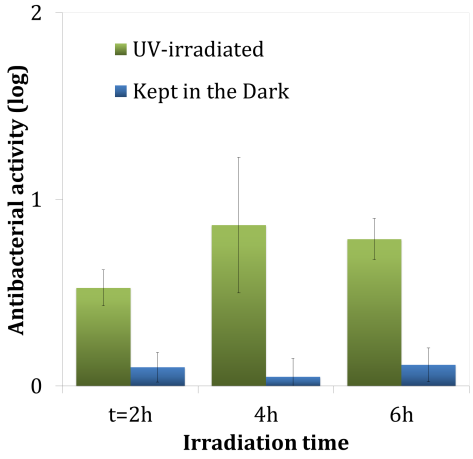

Figure 2. Antibacterial activity on *E. coli* of TiO_2-coating (glass slides), after 2 h, 4 h, and 6 h of contact. Mean ± s.e., $n = 3$.

The TiO_2 coating showed no antibacterial activity in the dark. Irradiation led to lower CFU values and activities were defined at 0.53 ± 0.1 log ($p = 0.241$), 0.96 ± 0.43 log ($p = 0.018$) and 0.79 ± 0.11 log ($p = 0.03$) after 2 h, 4 h, and 6 h of UV irradiation, respectively.

These inactivation values are relatively low when compared to the inactivation values found in the literature on photocatalytic TiO_2 coatings. Many factors strongly impact the microbial inactivation of TiO_2: the inoculum concentration, the nature of aqueous medium of the inoculum, the support material of the coating, the contact between TiO_2 particles and microbial cells, etc. [1–3]. However, two factors are quite often not highlighted: Irradiance and TiO_2 implementation. Regarding irradiance, in the frame of building engineering, it is desired to set up 'passive' antibacterial solutions, meaning that can be activated by low irradiances (daylight or conventional indoor lightning). This is why we chose to work under such values (<5 W/m^2). Regarding the photocatalyst implementation, its optimization often leads to expensive processes or processes that are impossible to implement on building (calcination process generally involved). The most interesting solutions are therefore inclusion in the mass (photocatalytic concrete) or paint-like coatings. One purpose of this study was to emphasize that such photocatalytic paint-like coating presents significant inactivation rates. Even if these values are far from those found in the literature, they are encouraging especially because they are observed in extreme contamination conditions (still far from the contamination that can occur in real conditions).

Nevertheless, these activities are quite a lot lower than those obtained from the commercial TiO_2 powder alone (VLP5070) alone in a previous study [51]: 2.62 ± 0.20 log ($p < 0.001$) after 4 h

and 3.73 ± 0.24 log (p = 0.001) after 6 h of experiments. This can be explained by the presence of organic binder, which can interfere in the photocatalytic disinfection by directly reacting with TiO_2 particles. Various studies have shown that the presence of organic compounds could reduce photocatalytic efficiency [51,56–60]. Moreover, such results could be explained by the encapsulation of TiO_2 particles in the organic binder of the coating substantially reducing the antibacterial efficiency. The encapsulation of particles inside the acrylic binder might hamper the UV incidence on TiO_2 particles, which would reduce photocatalytic reactions.

Figure 3 presents the antibacterial activity of the photocatalytic coating (with TiO_2) on membrane after 4 h of experiment under UV irradiation, for several times of pre-irradiation of the coating (0 h, 48 h, 90 h, and 109 h prior to the test). The antibacterial activity increased with the pre-irradiation time. Samples that had not been pre-irradiated (0 h) showed an antibacterial activity of 0.20 ± 0.06 log (p = 0.016) after 4 h of experiment. The activities increased for samples that had been pre-irradiated for 48 h (0.68 ± 0.06 log) or for 90 h (0.75 ± 0.10 log) with the higher detected activity of 1.22 ± 0.13 log (p < 0.001) for a 109 h pre-irradiation.

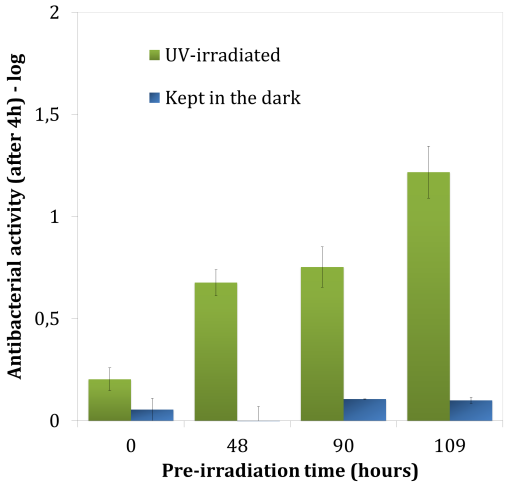

Figure 3. Antibacterial activity of TiO_2-coating on *E. coli* after 4 h of test, depending on the time of pre-irradiation of the coating (3 W/m^2). Mean ± s.e., n = 3.

After a few hours of UV-irradiation, the TiO_2 coating showed significant antibacterial activities that could reflect bactericidal action and/or growth inhibition (bacteriostatic action). Moreover, the antibacterial activity increased significantly after 48 h of pre-irradiation.

A possible explanation for these results may be the degradation of a first layer of the coating that covers most of TiO_2 particles. When samples were irradiated before the test (pre-irradiation), some of the UV beams may have reached encapsulated particles near the surface and activated the photocatalytic process. The organic coating would thus be partially degraded, generating additional porosity in the coating surface. The apparition of this new porosity would favor:

1. The access of UV beams for photocatalytic activation.
2. The access of the pollutants (here bacteria cells) to the particles.
3. Diffusion of the reactive radicals produced by photocatalysis toward pollutants, as shown in Figure 4.

Regarding this phenomenon of self-degradation of the coating, it is important to specify that this study follows previous work on photocatalytic coatings for air purifying [36] and for bacteria inactivation [51]. Coatings' formulation was initially engineered in the thesis of Martinez [48] on air purifying. In his work, TiO_2 coatings included a part of silicates as inorganic binder, promoting adhesion on building materials and sustainability of coatings. In the frame of bacterial inactivation, the presence of alkaline silicates (pH = 11–12) has a major impact on bacteria survival. In order to

prevent overlapping between pH and photocatalysis effects, we decided to use a 'partial' coating, silicate free. Regarding the silicate-based coatings, no degradation phenomenon of the coating was observed over time (same samples were tested after several weeks). It seems possible that only the surface layer of the coating is degraded and that TiO_2 particles will subsequently react with external pollutants or other molecules from ambient air. Further work on pre-irradiation times greater than 109 h would be useful to evaluate if the activity increases, is maintained or decreases.

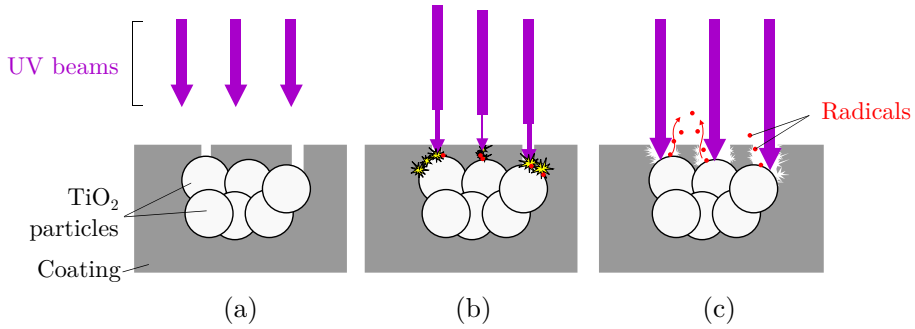

Figure 4. Schematic illustration of the disencapsulation mechanism of TiO_2 particles from acrylic coating. (**a**) starting UV pre-irradiation; (**b**) part of the UV beams reach particles near the surface, activating the photocatalytic reaction: the coating starts to degrade around particles; (**c**) apparition of additional porosity, favoring access of UV beams, diffusion of radicals outside the coating and diffusion of pollutants inside the coating.

The antibacterial activity experiment, carried out under low UV irradiation (2.5–3 W/m^2) confirmed that the efficiency on the bacterial inactivation of the TiO_2 coating increased after 48 h of pre-irradiation. The combined effect of photoinduced hydrophilicity and antibacterial activity of TiO_2 coating suggests an antibacterial action that is effective immediately on contact, providing resistance to bacterial adhesion.

3.2. Developement of a Bacterial Biofilm under Accelerated Conditions

Epifluorescence observations were carried out on the surface of samples after 24 h of experiment. Epifluorescence observation gave the best images of the spread of microbial colonization on samples. Figure 5 shows epifluorescence images of the surface of samples after 24 h of experiment. A light blue color was chosen to represent the coating and red to highlight the bacteria. Images taken on control coating either stored in the dark or exposed to UV irradiation showed bacterial colonization spread over the entire surface of samples (Figure 5a,b).

As can be seen from Figure 5c-1, TiO_2-coated samples that had been irradiated for 24 h showed areas of bacterial colonization spread over the entire surface but with very low density. Only a few red spots were visible in the near-center area of samples, meaning that bacterial cells were somewhat isolated from each other. Figure 5c-2 was taken on the edge area of the sample surface. The figure shows high concentrations of bacterial cells (red spots) surrounding TiO_2 coating (blue areas). It can be clearly seen that bacteria have developed mainly around the coating (directly on cementitious support) and very little on it. This 'repulsive' effect seems to confirm the results from antibacterial activity of the coating. Even under very severe conditions of microbial contamination, the coating was still active and prevented the biofilm development on its surface. These results are also in accordance with a recent study that showed a significant antibacterial effect of TiO_2 coatings against the formation of microbial biofilms under low irradiance [40].

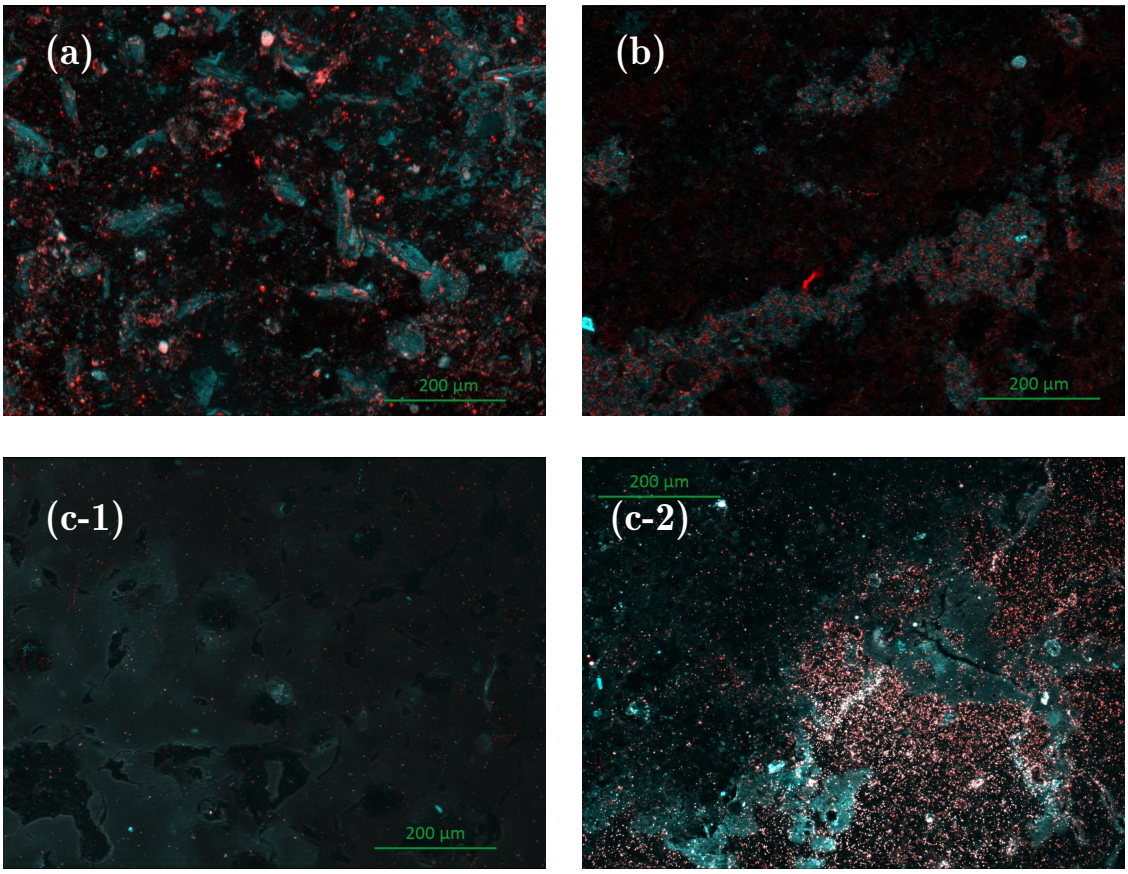

Figure 5. Epifluorescence pictures of surface samples after 24 h of experiment. (**a**) control-coated sample kept in the dark; (**b**) control-coated sample under UV irradiation; (**c**) TiO_2-coated samples under UV irradiation. For TiO_2-coated samples, one image was taken at the center of the sample (**c-1**) and another at the edge (**c-2**).

More tests would be required to validate the methodology. However, the presented images of early trials encourages the use of such device to study microbial growth on coated building materials. Moreover, further experimental investigations could be carried out with bacteria genetically modified to emit their own fluorescence, which would allow the evolution of the microbial colonization of material to be observed in real time, using a time lapse method [61,62]. In addition, the use of a fluorochrome such as propidium iodide, which is able to penetrate through damaged membrane only, would enable live cells, damaged membrane and dead cells to be quantified as previously described by Gregori et al. [63].

Figure 6 shows SEM observations of control-coated samples irradiated with UV (Figure 6a,b). Other samples that have been kept in the dark, i.e., control-coated samples and TiO_2-coated samples, presented the same colonization pattern (as did replicate samples, not shown). On Figure 6a,b, rounded protuberances can be observed, which gather to form a cohesive substance. This substance was not detected on control samples that had not been contaminated (Figure 6c,d). It was likely to be organic-based and produced by bacteria. A similar matrix was observed in the form of a dense network of curli fibers organized as a 'basket'-like structure around the cells [64]. On a smaller scale (Figure 6b), many bacterial cells were visible evolving near the surface or encased by the dense curli network.

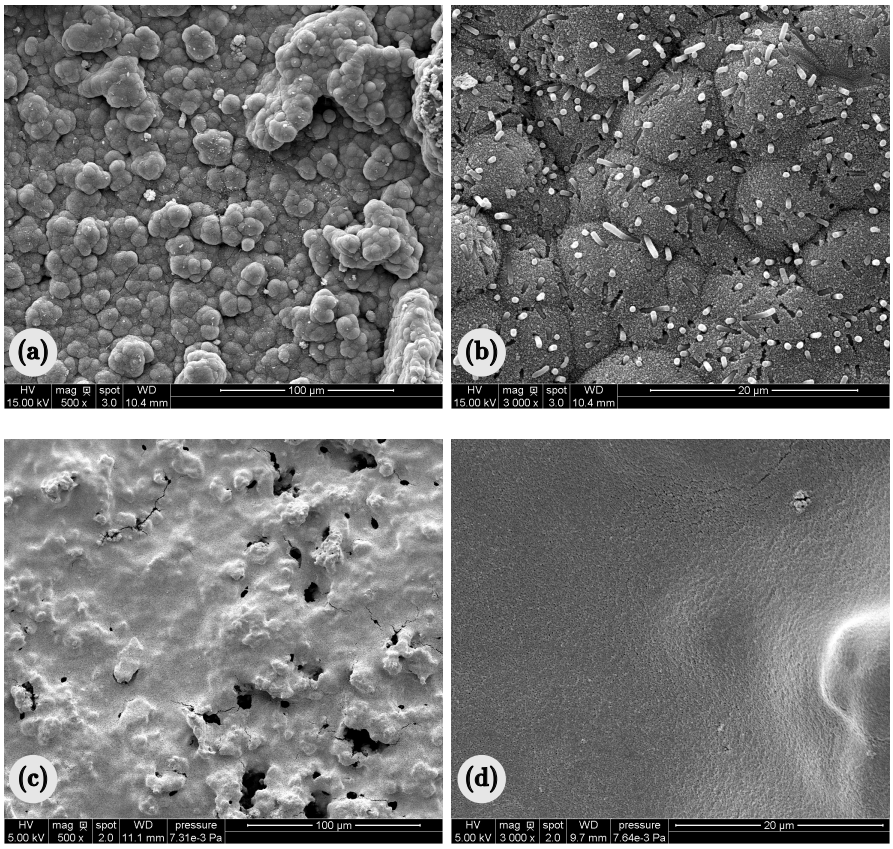

Figure 6. SEM images (secondary electron mode) of samples after 24 h of experiment. (**a**,**b**) SEM images of control samples without TiO_2 ($\times$500 and $\times$3000) showing an organic matrix and ovoid bacteria encased by or near surface of this dense network of curli fibers. (**c**,**d**) SEM images of TiO_2-coated samples that have not been inoculated ($\times$500 and $\times$3000) showing the surface of coating bacteria-free.

Observations of TiO_2-coated samples irradiated with UV light for 24 h of experiment showed colonization patterns. As can be seen from Figure 7, bacteria also colonized the coating in some areas. Figure 7c,d also show that parts of the coating were almost completely covered by the oragnic matrix network.

Damaged bacteria were also detected on the photocatalytic coating that had been UV-irradiated for 24 h. Figure 8a,b show intact bacterial cells from control samples kept in the dark. The natural shape of the bacillus, the classic physical appearance of *E. coli*, is quite visible. Figure 8c,d,e show damaged bacteria that were detected on UV-irradiated TiO_2-coated samples. Several similar clusters of damaged bacteria were found on the coating. The cells were always gathered in clusters. In addition, it can be seen from Figure 8d,e that small aggregates were attached on the cell walls of some bacteria. These particles were obviously submicrometer sized. They were detected only on visibly damaged bacteria. Moreover, such clusters of damaged bacteria were not detected on TiO_2-coated samples kept in the dark nor on control-coated samples. These aggregates thus might be TiO_2 particles that may have participated in the degradation of the cells.

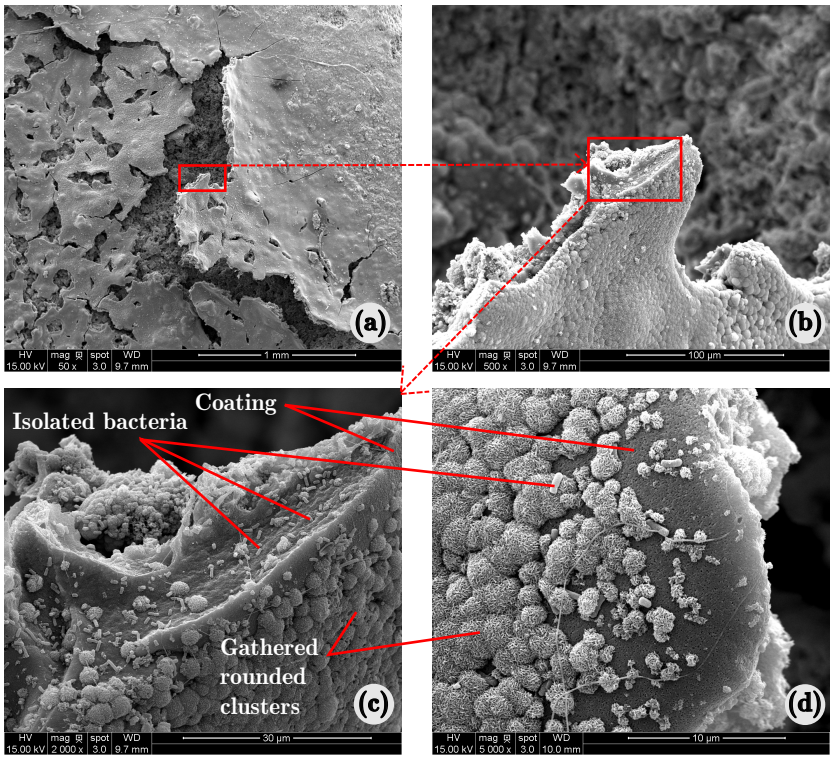

Figure 7. SEM images (secondary electron mode) of TiO_2-coated samples irradiated with UV light for 24 h.

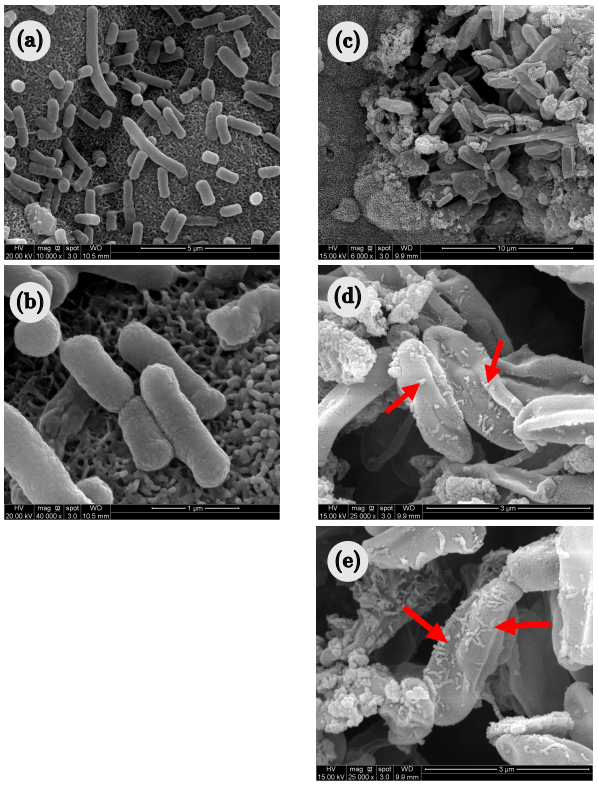

Figure 8. SEM images (secondary electron mode) of intact cells on control-coated samples kept in the dark (**a**,**b**) and of damaged cells observed on TiO_2-coated samples irradiated with UV light for 24 h experiment (**c**,**d**,**e**).

4. Conclusions

In this paper, two types of experiments were carried out in order to evaluate the antibacterial properties of a semi-transparent coating incorporating photocatalytic TiO_2 particles. Experimentation focused on the measurement of: (a) the photoinduced antibacterial activity of TiO_2 coating, and (b) its resistance to the formation of bacterial biofilm under accelerated growth conditions.

The tested coating showed significant effects in terms of antibacterial activity and resistance to biofilm formation under low irradiation (2.5 W/m^2). The antibacterial activity was induced by photocatalysis. SEM analyses confirmed the lethal activity of the coating on bacterial cells, even in favorable conditions of biofilm formation considered as a 'worst case' in comparison to the main conditions of use.

The Epifluorescence approach carried out to evaluate biofilm formation was suitable for TiO_2-coated cementitious supports. Fluorescence observations provided good pictures of the colonization patterns on the surface of samples. This work can be seen as a preliminary study exploring the potential of epifluorescence microscopy in a field in which it is little exploited: microbial contamination of building materials. Further tests are necessary to validate the methodology, e.g., quantitative measurements by chemical or molecular methods. Subsequently, the methodology could be used in studies on microbial growth and spread on coatings and/or on building materials.

Regarding the coating, further research should be undertaken to explore the optimal formulation, the distribution of TiO_2 particles and the application of the coating to building materials. More work should also be carried out to evaluate the antimicrobial properties towards gram positive bacteria, including *Staphylococcus aureus* (also recommended by JIS Z 2801) and molds. The molds will be chosen among the most detected in indoor environments such as *Penicillium*, *Aspergillus*, and *Cladosporium* species.

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Conflicts of Interest: The authors declare no conflict of interest.

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