Journal of Hazardous Materials 195 (2011) 100–106

Contents lists available at ScienceDirect

Journal of Hazardous Materials

journal homepage: www.elsevier.com/locate/jhazmat

Critical assessment of suitable methods used for determination of antibacterial properties at photocatalytic surfaces

Josef Krýsa [b,*], Eva Musilová [b], Jiří Zita [a]

[a] Institute of Chemical Technology Prague, Department of Inorganic Technology, Technická 5, CZ-166 28 Prague, Czech Republic
[b] Institute of Chemical Technology Prague, Department of Water Technology and Environmental Engineering, Technická 5, CZ-166 28 Prague, Czech Republic

ARTICLE INFO

Article history:
Received 21 April 2011
Received in revised form 29 July 2011
Accepted 4 August 2011
Available online 10 August 2011

Keywords:
TiO_2
Antibacterial activity
ISO 27447:2009(E)
E. coli
E. faecalis

ABSTRACT

This work describes the development of methods necessary for antibacterial effect evaluation on irradiated TiO_2 layers. Two methods using bacteria suspensions and the glass adhesion method (based on ISO 27447:2009(E)) were critically assessed and compared. As test bacteria gram negative *Escherichia coli* and gram positive *Enterococcus faecalis* were employed. The method using 50 cm^3 of bacteria suspension is convenient for testing layers with strong antibacterial effect (prepared from powder photocatalysts). For the evaluation of the antibacterial effect of sol gel layers, the glass adhesion method based on the ISO is more appropriate than the method with 3 cm^3 of bacteria suspension. The reason is that the later does not allow a distinction between the inhibition effect of TiO_2 and UV light itself. Some improvements of the ISO method were suggested, namely the use of gelatinous pills (CCM) of bacteria, using saline solution instead of nutrient broth for bacteria suspension preparation and the application of selective media for bacteria cultivation. Decreasing the light intensity from 0.6 mW cm^{-2} to 0.2 mW cm^{-2} (fulfilling the requirements of the ISO) results in almost negligible effect of UV light itself, thus enabling proper testing of the antibacterial properties of TiO_2 thin films.

1. Introduction

Titanium dioxide (TiO_2) is one of the most popular photocatalysts [1]. In the presence of ultraviolet light (UV-A), TiO_2 in anatase form is capable of decomposing organic compounds and microorganisms on its surface. Due to this ability TiO_2 has high potential in many fields of application, such as medicine [2], architecture and water and air purification [3,4]. So far chlorine is the most common agent for water disinfection. Inhibition of bacteria by chlorine is very fast and efficient. However, it is well-known that chlorine reacts with organic materials (humic substances) producing chloroorganic compounds (e.g. trihalomethanes (THMS)) which are considered to be carcinogenic [5,6]. This has led to the development of alternative methods for water treatment based on the interaction of a photocatalyst with UV light [6–8]. Among the photocatalysts investigated TiO_2 is the most suitable because it is stable, non-toxic and relatively cheap [9–13].

Many different microorganisms are used for antibacterial tests on photocatalytic surfaces namely, *Pseudomonas aeruginosa* [14,18], *Enterococcus faecim* [14], *Candida albicans* [14], *Staphylococcus aureus* [14,19–21], *Bacillus pumilus* [22] and *Bacillus megaterium* [23] but the most commonly used is *Escherichia coli* [14,16,17,23–30].

E. coli belongs to the group of Gram negative thermo tolerant coliform bacteria. Usually it appears in the digestive tract of humans and warm-blooded animals, where it is useful for the host (synthesising vitamins and supporting the overall balance of microorganisms in the intestines by suppressing the growth of harmful bacteria) [31]. *E. coli* usually remains harmlessly confined to the intestinal lumen; however, in a debilitated or immunosuppressed host, or when gastrointestinal barriers are violated, even normal "nonpathogenic" strains of *E. coli* can cause infection. Infections due to pathogenic *E. coli* may be limited to the mucosal surfaces or may disseminate throughout the body. Three general clinical syndromes result from infection with inherently pathogenic *E. coli* strains: (i) urinary tract infection, (ii) sepsis/meningitis, and (iii) enteric/diarrheal disease [32]. *E. coli* is considered as an indicator of faecal contamination and is widely used, not only as a model microorganism for physiological, biochemical and genetic experiments, but also for antibacterial tests of different chemical substances and materials.

There are many papers describing the antibacterial testing of photocatalytic surfaces, but methods and conditions are often different. The most common arrangement is an experimental setup where a drop of bacterial suspension is laid on a glass support covered by TiO_2 layer [14–17]. Another experimental set-up consists

* Corresponding author. Phone +420 220 444 112; Fax: +420 220 444 410.
E-mail address: Josef.Krysa@vscht.cz (J. Krýsa).

0304-3894/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.jhazmat.2011.08.009

in the bacterial suspension placed in Teflon ring placed on titania thin film [18]. The variety of the test conditions and parameters described in the literature requires the creation of a unified system for antibacterial testing. The main reason for such system is so that results obtained on different photocatalytic surfaces and in different laboratories can be easily and clearly compared.

The ISO 27447:2009(E) [33] standard (Fine ceramics (advanced ceramics, advanced technical ceramics) – test method for antibacterial activity of semiconducting photocatalytic materials) was introduced in 2009, because all currently described methods for antibacterial tests of TiO_2 photocatalytic layers have different procedures and conditions. The standard includes selection of suitable microorganisms and determines the conditions of the testing methods such as light intensity, amount of microorganisms and design of apparatus.

The aim of the present work was the critical assessment of several methods used in laboratories worldwide for the determination of antibacterial properties of TiO_2 thin films and their comparison with the ISO standard method. The special attention was given to the question of whether it is really necessary to follow all the conditions mentioned in the ISO standard or if it is possible to adjust some conditions according to the experience and facilities of each laboratory.

2. Experimental

2.1. Chemicals

For bacterial suspension preparation and for bacteria cultivation NaCl (Penta, p.a.), m-FC Agar Base (Himedia), Rosolic acid (Himedia), Slanetz and Bartley Medium (Himedia), NaOH (Penta, p.a.) were used. Titanium(IV) isopropoxide (97%; Sigma-Aldrich) and tetraethyl orthosilicate TEOS (purity 98%; Fluka) were used to prepare the titania and silica in the TiO_2/SiO_2/glass films. Absolute ethanol (p.a. Penta) and ethyl acetoacetate (purity p.a. 99%; Fluka) were utlised as solvents and hydrochloric acid (p.a. 36%; Penta) and nitric acid (p.a. 65%; Penta) were employed as sol–gel catalysts. Evonik-Degussa P25 TiO_2 powder was used for particulate layer preparation.

2.2. Preparation of TiO_2 thin films

The microscope ($75 \times 25 \times 1\,mm^2$) soda-lime glass substrates were first dip-coated (withdrawal speed: $60\,mm\,min^{-1}$) into the SiO_2 sol [34] to form the necessary SiO_2 barrier against metal ion (mainly Na^+) diffusion from the glass substrate into the titania film [35]. The SiO_2 interlayer was calcined at 530 °C for 3 h. The titania layer was then produced by subsequent dip-coating in the titania sol [34]. After the dip-coating process, the titania films were calcined at 530 °C for 3 h. The resulting layers were around 250 nm thick and the amount of titania in each layer was around $0.04\,mg\,cm^{-2}$.

Particulate layers were prepared by sedimentation of ultrasonically pretreated suspensions of P25 TiO_2 (75% of anatase, 25% of rutile, crystalline size around 30 nm, BET surface area around $50\,m^2\,g^{-1}$) on the same glass substrate as for sol–gel layers followed by calcination for 2 h at 300 °C. The amount of photocatalyst deposited on the glass supports was 0.1, 0.2, 0.5 and $1.0\,mg\,TiO_2\,cm^{-2}$.

2.3. Microorganism used

The tested microorganisms were Gram negative (G−) bacterium *E. coli* (CCM 3954) and Gram positive (G+) bacterium *Enterococcus faecalis* (CCM 4224). The pure cultures of bacteria were obtained as gelatinous pills from the Czech Collection of Microorganisms (CCM), Masaryk University, Brno. The pills consist of the lyophilisated form of preserved bacteria (cca 10^8 CFU/ml) and the main composition of the protecting medium is gelatine. The pills must be stored at low temperature (+2 to +8 °C) and used within 3 years. Before each test, it was necessary to dissolve the pill of bacteria for each culture in $9\,cm^3$ of sterile saline solution ($8.5\,g\,dm^{-3}$ NaCl) and cultivate it for 24 h at 37 °C. The bacterial suspension was than diluted with saline solution (10-times dilution method) to obtain the required concentration (CFU/ml) for each test. For the purpose of analysis, the bacterial suspension was diluted several times (10-times dilution method) to obtain the count of 30 colonies to 300 colonies in each Petri dish. To avoid contamination, selective medium m-FC agar for *E. coli* [36] and Slanetz-Bartley for *E. faecalis* [37] were used (selective media were chosen according to the water quality standards). Petri dishes with *E. coli* were than incubated for 24 h at 43 °C and with *E. faecalis* for 48 h at 37 °C. The number of colonies was counted and the results were expressed as the number of colony-forming units per millilitre (CFU/ml).

2.4. 50 cm^3 test

Particulate layers of P25 and sol–gel layers were placed in $50\,cm^3$ of *E. coli* suspension. The scheme of the reactor is shown in Fig. 1A. The incident light intensity was $1.0\,mW\,cm^{-2}$ (SYLVANIA Lynx CFS BLB, maximum at 365 nm) and the initial bacteria concentration was around 1×10^4 CFU/ml. During irradiation a $1\,cm^3$ sample from the reaction solution was taken every 30 min. The sample was then diluted, cultivated and the results of the experiment were recorded as dependence of log(CFU/ml) versus time. To observe the effect of UV light itself, the clear glass substrate (blank) was also tested in $50\,cm^3$ of *E. coli* suspension.

2.5. 3 cm^3 test

In this case the sol–gel TiO_2 sample ($25\,mm \times 30\,mm$) was placed in the small Petri dish (diameter 45 mm). Then the $3\,cm^3$ of bacterial suspension (3.3×10^6 and 2.5×10^4 CFU/ml) was added and the dish was covered by a glass lid to minimize the vaporization (Fig. 1B). The whole system was placed on a platform shaker to insure mixing of the bacterial suspension in contact with TiO_2 surface. In this test, the light intensity was $0.6\,mW\,cm^{-2}$ (BLB Philips TL-D 15W, 300–400 nm, broad maximum at 365 nm). At regular time intervals, $0.1\,cm^3$ of the irradiated cell suspension were taken, diluted and analysed. To see the effect of UV light itself, the clear glass substrate (blank) was also tested in another Petri dish.

2.6. Glass adhesion test

This method is based on the ISO 27447:2009(E) standard, which works with the bacteria (*E. coli*) spread on the test surface ($25 \times 30\,mm^2$) and covered by adhesive glass ($24 \times 24\,mm^2$). This so called "sandwich" was then put in the Petri dish (diameter 45 mm) with wet paper filter and the dish was covered with the cap (Fig. 1C). The volume of the cell suspension was $0.05\,cm^3$ and the concentration of bacteria was within the interval 2.0×10^6 to 8.0×10^6 CFU/ml. In this experimental set-up, the irradiation conditions were the same as in the $3\,cm^3$ test ($0.6\,mW\,cm^{-2}$). After a given interval of time, the cap was removed and the cover glass together with the TiO_2/glass sample were shaken out in $10\,cm^3$ of saline solution, diluted and analysed.

The effect of light intensity on *E. coli* and *E. faecalis* degradation was studied for light intensities in the range 0.2 to $0.6\,mW\,cm^{-2}$. Different intensities of the incident light were achieved by changing the distance of the sample from the light source and also by placing the stainless steel grid in front of the light source. For the

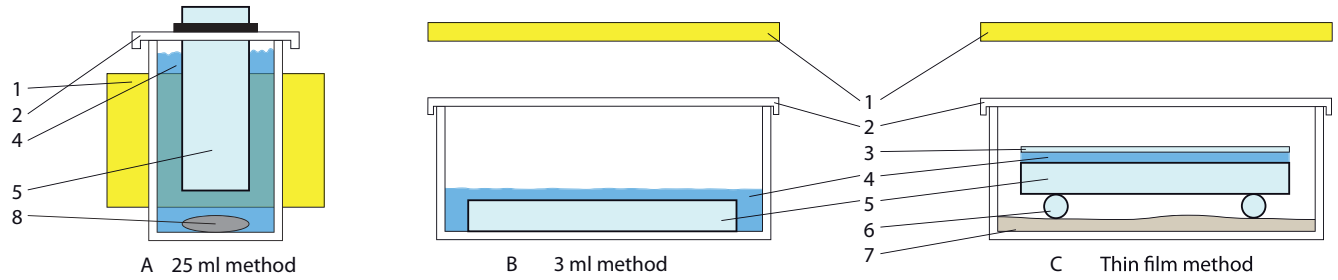

Fig. 1. Schematic diagram of the antibacterial tests arrangement: (A) 50 cm^3 method, (B) 3 cm^3 method, (C) thin film method – according to the ISO standard [33]; 1-light source, 2-moisture preservation glass, 3-cover slide glass, 4-bacterial suspension, 5-TiO_2 sample under test, 6-glass rod, 7-wet filter paper, 8-magnetic stirrer.

distances 24 and 36 cm the light intensities were 0.6 and 0.4 mW cm^{-2}, respectively. When the grid was used the light intensity for the distance of 36 cm was 0.2 mW cm^{-2}.

3. Results and discussion

3.1. Test method

For the determination of the photocatalytic activity of titania layers, photochemical reactors where the TiO_2 layer is placed in the solution of model dye or organic compound are often used. Such types of reactor have been commonly employed in our laboratory for the determination of photoactivity using dye Acid Orange 7 [38]. Our first approach to the antibacterial test was simply to replace the dye solution with bacterial suspension. In Fig. 2 the results of antibacterial test on sol–gel and particulate TiO_2 layers of different titania loading in 50 cm^3 of bacterial suspension can be seen. It is clear that the inhibition effect of the TiO_2 layer on *E. coli* increased as the amount of titania in the layer increased. The advantages of this method and experimental setup are: (i) the effect of UV light irradiation on the inhibition of bacteria in the suspension without TiO_2 layer is almost negligible, (ii) the bacterial suspension is stable in dark even when the TiO_2 layer is present, (iii) samples of bacteria suspension can be taken during the experiment, (iv) only one sample of TiO_2 layer is necessary for the whole experiment (in the glass adhesion test – the ISO standard – one TiO_2 sample is necessary for each point of the CFU dependence on the time of irradiation).

It seems, that the 50 cm^3 method is an ideal test for particulate TiO_2 layers with high activity prepared from powder suspensions.

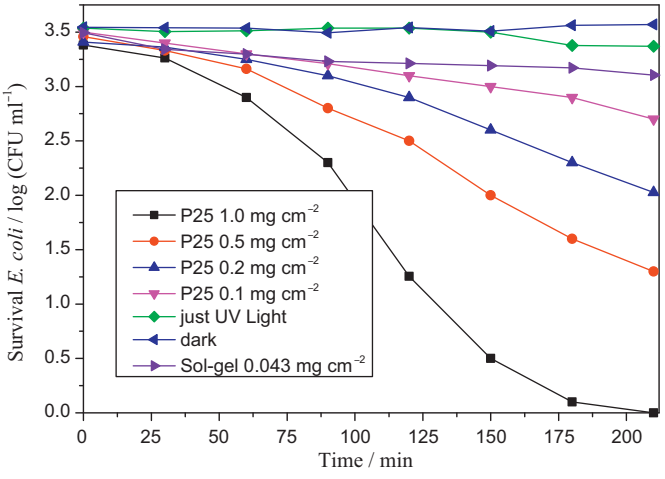

Fig. 2. 50 cm^3 method for *E. coli* antibacterial test. Log scale of surviving bacteria under UV irradiation (1 mW cm^{-2}) for particulate titania layers with different amount of P25 as a function of illumination time. The sol–gel layer is also included (250 nm, 0.043 mg cm^{-2}).

But use of particulate layers in the practical application (antibacterial glasses and tiles) is not favoured due to the low mechanical stability. TiO_2 layers prepared by the sol–gel method have much higher application potential in this field. Sol–gel layers can be applied on various surfaces, such as tiles, glass and metal surfaces and their mechanical stability, compared to powder layers, is much better. On the other hand due to the non-porous structure [39] and much smaller layer thickness (146 nm) than 0.1 mg cm^{-2} particulate film (thickness 800 nm) the resulting photoactivity of sol gel film measured using Acid Orange 7 as model compound is about 8 times smaller than that for particulate film [38]. Thus we can expect the similar behaviour when comparing the antibacterial properties of particulate and sol gel films. In fact, from Fig. 2 it is clear that the antibacterial activity of the sol–gel layer in the 50 cm^3 test is comparable with the antibacterial effect of UV light itself. As a consequence, we had to find and verify different methods for the antibacterial testing of sol–gel layers.

An experimental setup with a drop of bacterial suspension (0.2 cm^3) pipetted onto the coated substrates has been described by Kühn et al. [14]. The volume of the drop in the "drop test" can be smaller, e.g. 0.1 cm^3 [16,17], 0.07 cm^3 [30] or even 0.01 cm^3 [29]. This approach is simple but it has two serious drawbacks. At first, samples with the drop of bacterial suspension were not covered and drying of the drop during irradiation may take place. Secondly, the surface area of TiO_2 film in contact with the bacteria drop is not properly defined. Kikuchi et al. [19] solved the drying problem by placing the TiO_2 sample with the bacterial drop into a Petri dish with a small amount of water and covering it with a glass lid. However the problem with the definition of surface area remained. The above mentioned drawbacks result in a number of discrepancies as is visible, for example, from comparison of the results on TiO_2 layers prepared from Degussa P25. Kühn et al. [14] observed 4 log decrease of *E. coli* CFU after 1 h of irradiation using a 0.2 cm^3 drop (UV light had no effect on the drop of bacterial suspension). Hajková et al. [29] also described a 4 log decrease of *E. coli* CFU after 1 h of irradiation using a 0.01 cm^3 drop, but a 2 log decrease using UV light itself was observed.

As a next step, we eliminated the problem of the ill-defined area and drop drying by creating a new antibacterial test method. Our method defines the size of the tested TiO_2 sample (25×30 mm^2) which fits well into a small Petri dish (diameter 45 mm). Then we put 3 cm^3 of bacterial suspension into the Petri dish to create a thin liquid film above the TiO_2 layer and cover the whole system with a glass lid to eliminate evaporation (Fig. 1B). Using this method we decreased the volume of bacterial suspension from 50 to 3 cm^3 and also the ratio of the irradiated area to the volume was changed from 1:5 (50 cm^3 method) to 1:0.4 (3 cm^3 method). Initially, we tried similar initial bacteria concentration as in the 50 cm^3 test (2.5×10^4 CFU/ml). After irradiation of the system, we expected a faster decrease of bacteria concentration. However the results showed almost no killing of bacteria (Fig. 3). Secondly, we increased the initial concentration to the range recommended in the ISO

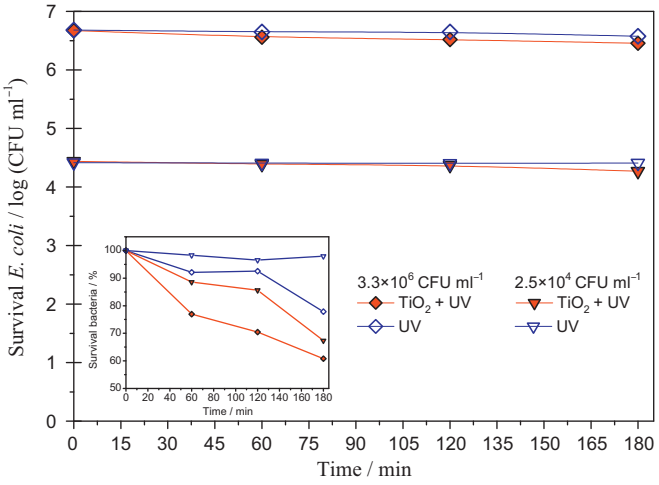

Fig. 3. 3 cm^3 method for the antibacterial test of *E. coli* (two initial concentrations). Log scale of surviving bacteria under UV irradiation (0.6 mW cm^{-2}) for sol–gel titania layer and pure glass substrate as a function of illumination time. Insert diagram shows the percentage of surviving bacteria.

standard [33] (3.3×10^6 CFU/ml). Again the differences between the effects of UV light itself and irradiated TiO_2 layer was not significant. This is possibly due the insufficient contact of bacteria with the TiO_2 layer and the existence of "dead volumes of bacteria suspension" with small or no exchange with the volume of bacteria suspension in contact with TiO_2 layer. The problem of the dead volume was solved by Sunada et al. [24] who placed a cylindrical frame directly on the TiO_2 sol–gel layer and then 1 cm^3 of the *E. coli* suspension was pipetted into it. After 1 h around 50% of the bacteria were killed (only 5% due to UV itself) [24]. If we compare this test (1 cm^3) with our 3 cm^3 test, where we have 35% of killing after 1 h and the same effect of UV (5%), it is clear that, if all the volume of bacterial suspension is in direct contact with the TiO_2 layer, the photocatalytic de-activation of microorganisms is faster. Similarly to Sunada et al. [24], Dunlop et al. [40] used a silicone cylinder placed on a TiO_2 layer and filled this with a 1 cm^3 bacterial suspension of lower concentration (1×10^3 CFU/ml). Even though the experimental setups [24,40] were similar the difference between inhibition efficiency of the TiO_2 + UV light and UV light itself is much smaller in the work of Dunlop et al. [40].

In addition to this observed discrepancy the scale of bacteria concentration used may make comparison difficult. Fig. 3 shows that a percentage scale shows a decrease of viable bacteria, but a log scale suggests negligible antibacterial effect. It seems that a log scale is more suitable for confirmation of photocatalytic inhibition effect of TiO_2 layers, but for evaluation of the effect of UV itself the percentage scale is more useful.

3.2. Critical assessment of the ISO standard – adhesion glass method

In the next step, we adapted our experimental setup according to the ISO [33]. However in our laboratory we are not able to fulfil all the recommendations and requirements of the ISO. In Table 1 we show the differences between the ISO and our own glass adhesion test. The differences are in detail discussed in the following three paragraphs.

At first, according to the ISO standard, *E. coli* (G−) is the species of bacterium recommended for the tests (glass adhesion method), but other types of bacteria can be tested, if necessary. In our work we used *E. faecalis* (G+) as the second test microorganism. The preparation of microorganism suspension according to the ISO is complicated and time consuming (repeated subcultures with one month expiration, many cultivations and dilutions before each experiment). Using the gelatinous pills (CCM) has many advantages: after 24 h the bacterial suspension is ready for the experiment, the concentration of bacteria in the pill is guaranteed, the pill can be stored for 3 years, the purity of bacterium strain is also guaranteed and, finally, it is easy to use.

Secondly, according to the ISO standard, nutrient broth must be used for the preparation of the bacterial suspension. However we think that saline solution is better than nutrient broth because it does not contain organic compounds (meat extract and peptone in nutrient broth) which could also be photocatalytically degraded by the TiO_2 layers during the test and thus slow down the rate of bacteria inactivation. According to the ISO standard, nutrient agar must be used for bacteria cultivation. From a microbiological point of view, this is not the best choice because of possible contamination from the surrounding environment. For this reason we are using selective media in our laboratory.

Finally, according to the ISO standard, the specimen size should be $50 \times 50 \pm 2$ mm^2 and the size of adhesive glass should be $40 \times 40 \pm 2$ mm^2. It is also possible to use a different specimen size but the specimen surface must be covered by adhesive glass of dimension in the range from 400 mm^2 to 1600 mm^2. Our specimen size was 24×30 mm^2 and the area covered by adhesive glass was 576 mm^2. The volume of the cell suspension spread on the specimen was different from that mentioned in ISO standard and was adjusted according to the size of the adhesive glass. The amount of bacteria was the same as the concentration recommended in the ISO standard ($\sim 2 \times 10^6$ CFU/ml).

Fig. 4 shows the results of the adhesive glass test. It is apparent that the difference between the antibacterial effect of the TiO_2 layer and UV light itself is much higher than in the case of the 3 cm^3 test. The explanation is that in the adhesive glass test, the irradiated surface of TiO_2 is in direct contact with bacteria. Comparing the drop test and glass adhesion test the later seems to be more appropriate for TiO_2 thin films. In the present adhesive glass test

Table 1
List of significant parameters of ISO 27447:2009(E) method and their comparison with the glass adhesion method used in our laboratory.

	ISO 27447:2009 (E)	Our laboratory adhesion glass test
Bacterium	*Staphylococcus aureus* (G+) *Escherichia coli* (G−)	*Enterococcus faecalis* (G+) *Escherichia coli* (G−)
Bacteria suspension preparation	Cultivation in nutrient broth	Gelatinous pill
Bacteria cultivation	Nutrient agar	m-FC agar (*E. coli*) Medium Slanetz-Bartley (*E. faecalis*)
Specimen size	$50 \times 50 \pm 2$ mm^2	24×24 mm^2
Sample size covered by adhesive glass	400–1600 mm^2	576 mm^2
Volume of test bacterial suspension	0.15 cm^3	0.05 cm^3
Initial bacteria concentration	6.7×10^5–2.6×10^6 CFU/ml	2×10^6 CFU/ml
Exposure time	4–8 h	3 h
Light source	Fluorescent BLB lamp 300–400 nm	Fluorescent BLB lamp 300–400 nm
UV light intensity	0.001–0.25 mW cm^{-2}	0.2–0.6 mW cm^{-2}

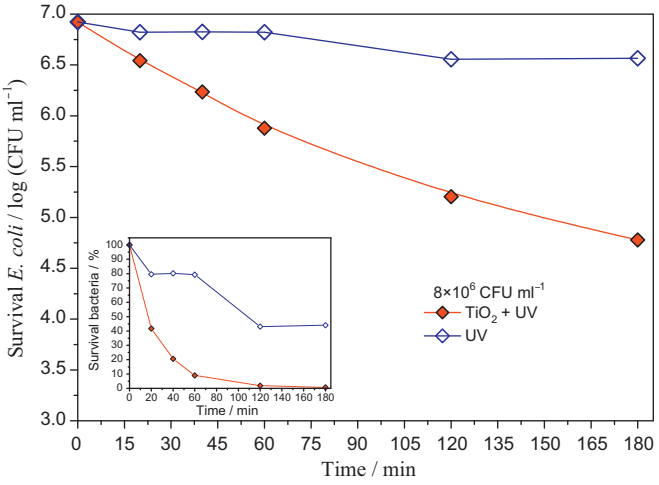

Fig. 4. Adhesion glass method for the antibacterial test of *E. coli*. Log scale of surviving bacteria under UV irradiation (0.6 mW cm^{-2}) for titania sol–gel layer and pure glass substrate as a function of illumination time. Insert diagram shows the percentage of surviving bacteria.

60% of the bacteria were killed after 20 min and after 2 h almost 99% of the surface was disinfected. On the other hand the drop test (100 μl, 10^6 CFU/ml) on sol–gel layers shows inactivation of only 30% of viable bacteria after 3 h [16]

3.3. Influence of light intensity

Light intensity is an important parameter in the antibacterial tests. Firstly, light intensity is one of the rate determining steps in semiconductor photocatalysis. Secondly, UV light itself (especially of low wavelengths) may inactivate bacteria. In the ISO standard fluorescent black light blue (BLB) lamps are recommended (wavelength 300–400 nm, light intensity 0.001–0.25 mW cm^{-2}). However the light sources and their intensities and wavelengths employed in reported antibacterial tests [6,8,10,11,15,21] often vary and, as a consequence, comparison of results is very difficult. For example, Soken et al. [6] describe *E. coli* disinfection using a Ag-TiO$_2$ suspension and a UV light intensity of 5.8 mW cm^{-2} (300 W, 294 nm). Sunada et al. [15] studied the photocatalytic inhibition of *E. coli* on TiO$_2$ thin films by BLB lamp (15 W, 365 nm, 1.0 mW cm^{-2}). Wu et al. [21] used a metal halogen desk lamp to investigate disinfection induced by visible light. The light intensity below 400 nm was less than 0.01 mW cm^2 and the visible light intensity was in the range 1.6 mW cm^{-2} to 0.4 mW cm^{-2} [21].

Ibanez et al. [10] used an UV-A lamp (maximum at 365 nm) for studying the antibacterial effect of TiO$_2$ (P25) suspension on different Gram(−) microorganisms. Because of the high sensitivity of *P. aeruginosa* to UV-A, suspensions of these bacteria were exposed to a lower UV-A intensity, i.e. 1.4 mW cm^2. For other microorganisms (*E. coli*, *Salmonella typhimurium*, *Enterobacter cloacae*) a light intensity of 5.5 mW cm^{-2} was chosen. Benabbou et al. [11] used HPK 125 W light to investigate the disinfection of *E. coli* in TiO$_2$ suspension. Appling an optical filter they were able to work in UVC, UVB and UVA wavelength regions. In the case of UVA light, the intensity varied from 0.48 mW cm^{-2} to 3.85 mW cm^{-2} by virtue of the distance from the light source and the presence of the appropriate grid [11]. It must be emphasized that in all the above mentioned cases, the intensity of the light sources did not fit the interval set by the ISO standard [33].

Fig. 5 shows the results of adhesion glass tests (A – *E. coli*, B – *E. faecalis*) using three different light intensities (0.6, 0.4 and 0.2 mW cm^{-2}). The lowest value, (0.2 mW cm^{-2}) fulfils the ISO standard. It can be seen that the effect of light intensity on *E. coli* and

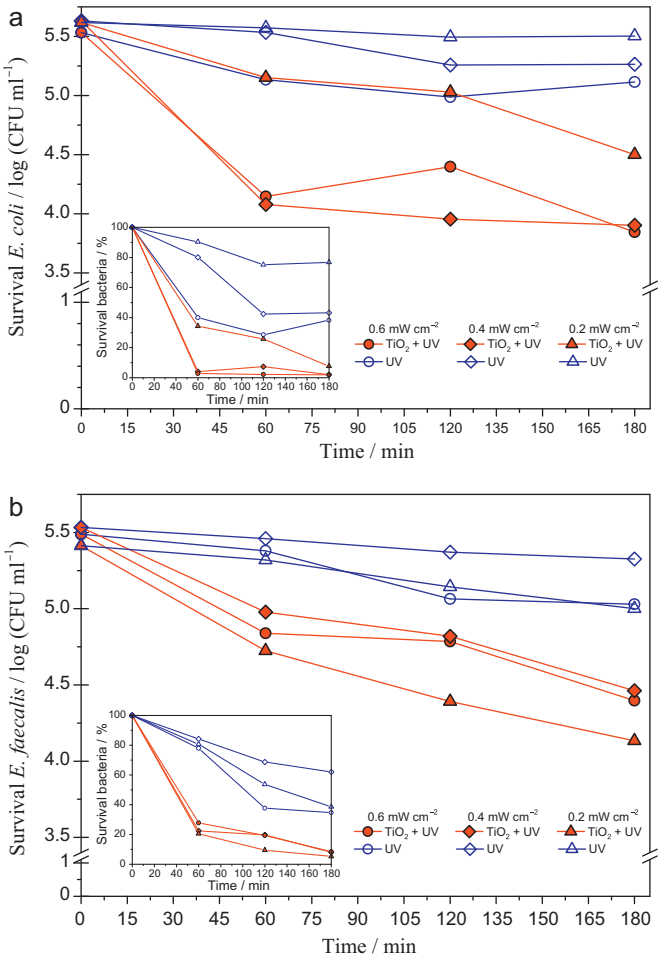

Fig. 5. Log scale of surviving bacteria under UV irradiation for titania sol–gel layer and pure glass substrate as a function of illumination time – adhesive glass method. (a) *E. coli* (initial bacteria concentration – 3.8 × 10^5 CFU/50 μl). (b) *E. faecalis* (initial bacteria concentration – 3.2 × 10^5 CFU/50 μl). Open symbols – UV light itself, full symbols – UV light + TiO$_2$, light intensity 0.6 mW cm^{-2} (○), 0.4 mW cm^{-2} (◊) and 0.2 mW cm^{-2} (△).

E. faecalis inactivation is different. In the case of Gram(−) bacterium *E. coli*, the effect of UV light itself on the bacteria inhibition decreased as the light intensity decreased (Fig. 5A). The percentage of surviving bacteria after 180 min irradiation increased from 38% for the highest light intensity (0.6 mW cm^{-2}) to 77% for the lowest light intensity (0.2 mW cm^{-2}). After 60 min irradiation the difference was even higher: 40% for 0.6 mW cm^{-2} and almost 90% for 0.2 mW cm^{-2}.

In the case of Gram(+) bacterium *E. faecalis*, a decrease in UV light intensity did not have such a definite effect on the bacteria inhibition (Fig. 5B). The percentage of surviving bacteria after 60 min irradiation was around 80% for all UV light intensities. Even after 180 min irradiation the effect of UV light was not as strong as observed in the case of *E. coli*. The effect of UV light on the amount of surviving *E. faecalis* has moved from 35% (highest intensity – 0.6 mW cm^{-2}) to 46% (lowest intensity – 0.2 mW cm^{-2}).

According to our experiments 60 min is the minimum irradiation time necessary to distinguish the antibacterial effect of TiO$_2$ from the effect of UV light itself. In the case of *E. coli* (light intensity 0.2 mW cm^{-2}), we observed 10% inhibition by UV light and 60% by TiO$_2$ layer (after 60 min irradiation). When a higher intensity was used (0.6 mW cm^{-2}), 60% of the bacteria were killed only by UV, but with a TiO$_2$ layer more than 95% bacteria were inactivated (Fig. 5A). This trend (increasing light intensity) is consistent

with recent results of Dunlop et al. [40] who observed, after 40 min irradiation (UVA, 3 mW cm^{-2}), 70% inhibition by UV light and 90% inhibition when TiO_2 layer was applied. A strong effect of UV light itself was also observed by Foster et al. [41] but in the log scale (2 log decrease after 6 h for 2 mW cm^{-2}) while for an order lower light intensity (the ISO) the inhibition due to UV light itself was only 60%.

In the case of *E. faecalis*, for all studied light intensities after 60 min of irradiation 20% of bacteria was inactivated by UV light itself and around 55% was inactivated by using an irradiated TiO_2 layer (Fig. 5B). Thus *E. faecalis* are not as sensitive to UVA light as *E. coli*. On the other hand their inactivation proceeds with similar rate as that of *E. coli*. The results are important because there are few other few other data concerning the photocatalytic degradation of *E. faecalis* on TiO_2. Only Malato et al. [42], in his review, reports that bacterium *E. faecalis* is generally more difficult to disinfect than *E. coli* and Mitoraj et al. [43] confirm this experimentally, but for the case of VIS light irradiation.

4. Conclusions

Gramnegative *E. coli* and gram positive *E. faecalis* were found to be suitable for antibacterial effect evaluation on irradiated TiO_2 layers. It was found that the method using 50 cm^3 of bacteria suspension is convenient for testing layers with strong antibacterial effect (prepared from powder photocatalysts). A decrease in the bacteria suspension volume to 3 cm^3 did not bring the expected result (improvement of the difference between antibacterial effect of irradiated TiO_2 and UV light itself). The possible reason is insufficient contact of bacteria with the TiO_2 layer and the existence of "dead volumes of bacteria suspension" with small or no exchange with the suspension adjacent to the TiO_2 layer. Thus for evaluation of the antibacterial effect of transparent sol gel layers the adhesion glass method based on the ISO standard is the most appropriate. Some parameters stated in ISO 27447:2009(E) can be adapted according to the working conditions used in particular laboratories (sample size, type of microorganism, irradiation time). Furthermore we suggest some improvements: (i) the use of gelatinous pills (CCM) of bacteria leading to simplicity and reproducibility, (ii) the use of saline solution instead of nutrient broth for bacteria suspension preparation, (iii) the application of selective media instead of nutrient agar for bacteria cultivation.

Experiments at three UV light intensities (0.2–0.6 mW cm^{-2}) confirm the inhibition effect of UV light (even at 365 nm) itself. The lowest value of 0.2 mW cm^{-2}, fulfilling the requirements of the ISO standard, and irradiation time 60 min was found to be optimal for testing.

Acknowledgements

The authors acknowledge financial support (project 1M0577) of the Ministry of Education, Youth and Sport of the Czech Republic, the Grant Agency of the Czech Republic (project number 104/08/0435) and the FP7 EU project PILGRIM (No.: 223050). The authors gratefully acknowledge the English correction done by Prof. A.A. Wragg from Exeter University, UK.

References

[1] Q. Li, S. Mahendra, D.Y. Lyon, L. Brunet, M.V. Liga, D. Li, P.J.J. Alvarez, Antimicrobial nanomaterials for water disinfection and microbial control: potential applications and implications, Water Res. 42 (2008) 4591–4602.
[2] M. Yoshinari, Y. Oda, T. Kato, K. Okuda, Influence of surface modifications to titanium on antibacterial activity in vitro, Biomaterials 22 (2001) 2043–2048.
[3] A. Paleologou, H. Marakas, Ni.P. Xekoukoulotakis, A. Moya, Y. Vergara, N. Kalogerakis, P. Gikas, D. Mantzavinos, Disinfection of water and waste water by TiO_2 photocatalysis, sonolysis and UV-C irradiation, Catal. Today 129 (2007) 136–142.
[4] A. Vohra, D.Y. Goswami, D.A. Deshpande, S.S. Block, Enhanced photocatalytic inactivation of bacterial spores on surfaces in air, J. Ind. Microbiol. Biotechnol. 32 (2005) 364–370.
[5] J.A.H. Melián, J.M.D. Rodríguez, A.V. Suárez, E.T. Rendón, C.V.d. Campo, J. Arana, J.P. Peña, The photocatalytic disinfection of urban waste waters, Chemosphere 41 (3) (2000) 323–327.
[6] M. Sökmen, F. Candan, Z. Sümerb, Disinfection of *E. coli* by the Ag-TiO_2/UV system: lipid peroxidation, J. Photochem. Photobiol. A: Chem. 143 (2–3) (2001) 241–244.
[7] R. van Grieken, J. Marugán, C. Sordo, P. Martínez, C. Pablos, Photocatalytic inactivation of bacteria in water using suspended and immobilized silver-TiO_2, Appl. Catal. B: Environ. 93 (2009) 112–118.
[8] N. Lydakis-Simantiris, D. Riga, E. Katsivela, D. Mantzavinos, N.P. Xekoukoulotakis, Disinfection of spring water and secondary treated municipal waste water by TiO_2 photocatalysis, Desalination 250 (2010) 351–355.
[9] J.M.C. Robertson, P.K.J. Robertson, L.A. Lawton, A comparison of the effectiveness of TiO_2 photocatalysis and UVA photolysis for the destruction of three pathogenic micro-organisms, J. Photochem. Photobiol. A: Chem. 175 (2005) 51–56.
[10] J.A. Ibáñez, M.I. Litter, R.A. Pizarro, Photocatalytic bactericidal effect of TiO_2 on *Enterobacter cloacae*: comparative study with other Gram (−) bacteria, J. Photochem. Photobiol. A: Chem. 157 (2003) 81–85.
[11] A.K. Benabbou, Z. Derriche, C. Felix, P. Lejeune, C. Guillard, Photocatalytic inactivation of *Escherischia coli*: effect of concentration of TiO_2 and microorganism, nature, and intensity of UV irradiation, Appl. Catal. B: Environ. 76 (2007) 257–263.
[12] H.-L. Liu, T.C.-K. Yang, Photocatalytic inactivation of *Escherichia coli* and *Lactobacillus helveticus* by ZnO and TiO_2 activated with ultraviolet light, Process Biochem. 39 (2003) 475–481.
[13] D.M.A. Alrousan, P.S.M. Dunlop, T.A. McMurray, J.A. Byrne, Photocatalytic inactivation of *E. coli* in surface water using immobilised nanoparticle TiO_2 films, Water Res. 43 (2009) 47–54.
[14] K.P Kühn, Ir.F. Chaberny, K. Massholder, M. Stickler, V.W. Benz, H.-G. Sonntag, L. Erdinger, Disinfection of surfaces by photocatalytic oxidation with titanium dioxide and UVA light, Chemosphere 53 (2003) 71–77.
[15] K. Sunada, T. Watanabe, K. Hashimoto, Studies on photokilling of bacteria on TiO_2 thin film, J. Photochem. Photobiol. A: Chem. 156 (2003) 227–233.
[16] C.C. Trapalis, P. Keivanidis, G. Kordas, M. Zaharescu, M. Crisan, A. Szatvanyi, M. Gartner, TiO_2(Fe^{3+}) nanostructured thin films with antibacterial properties, Thin Solid Films 433 (2003) 186–190.
[17] O. Akhavan, R. Azimirad, Photocatalytic property of Fe_2O_3 nanograin chains coated by TiO_2 nanolayer in visible light irradiation, Appl. Catal. A: Gen. 369 (2009) 77–82.
[18] P. Amézaga-Madrid, G.V. Nevárez-Moorillón, E. Orrantia-Borunda, M. Miki-Yoshida, Photoinduced bactericidal activity against *Pseudomonas aeruginosa* by TiO_2 based thin films, FEMS Microbiol. Lett. 211 (2002) 183–188.
[19] Y. Kikuchi, K. Sunada, T. Iyoda, K. Hashimoto, A. Fujishima, Photocatalytic bactericidal effect of TiO_2 thin films: dynamic view of the active oxygen species responsible for the effect, J. Photochem. Photobiol. A: Chem. 106 (1997) 51–56.
[20] X. Zhao, Q. Zhao, J. Yu, B. Liu, Development of multifunctional photoactive self-cleaning glasses, J. Non-Cryst. Solids 354 (2008) 1424–1430.
[21] P. Wu, R. Xie, J.A. Imlay, J.K. Shang, Visible-light-induced photocatalytic inactivation of bacteria by composite photocatalysts of palladium oxide and nitrogen-doped titanium oxide, Appl. Catal. B: Environ. 88 (2009) 576–581.
[22] J.C. Yu, W. Ho, J. Lin, H. Yip, P.K. Wong, Photocatalytic activity, antibacterial effect and photoinduced hydrophilicity of TiO_2 films coated on a stainless steel substrate, Environ. Sci. Technol. 37 (2003) 2296–2301.
[23] G. Fu, P.S. Vary, C.-T. Lin, Anatase TiO_2 nanocomposites for antimicrobial coatings, J. Phys. Chem. B 109 (2005) 8889–8898.
[24] K. Sunada, Y. Kikuchi, K. Hashimoto, A. Fujishima, Bactericidal and detoxification effects of TiO_2 thin film photocatalysts, Environ. Sci. Technol. 32 (1998) 726–728.
[25] W. Zhang, Y. Chen, S. Yu, S. Chen, Y. Yin, Preparation and antibacterial behavior of Fe^{3+}-doped nanostructured TiO_2 thin films, Thin Solid Films 516 (2008) 4690–4694.
[26] L. Caballero, K.A. Whitehead, N.S. Allen, J. Verran, Inactivation of *Escherichia coli* on immobilized TiO_2 using fluorescent light, J. Photochem. Photobiol. A: Chem. 202 (2009) 92–98.
[27] N. Baram, D. Starosvetsky, J. Starosvetsky, M. Epshtein, R. Armon, Y. Ein-Eli, Enhanced inactivation of *E. coli* bacteria using immobilized porous TiO_2 photoelectrocatalysis, Electrochim. Acta 54 (2009) 3381–3386.
[28] R.v. Grieken, J. Marugán, C. Sordo, C. Pablos, Comparison of the photocatalytic disinfection of *E. coli* suspensions in slurry, wall and fixed-bed reactors, Catal. Today 144 (2009) 48–54.
[29] P. Hájková, P. Špatenka, J. Krumeich, P. Exnar, A. Kolouch, J. Matoušek, P. Kočí, Antibacterial effect of metal modified TiO2/PECVD films, Eur. Phys. J. D 54 (2009) 189–193.
[30] F.R. Marciano, D.A. Lima-Oliveira, N.S. Da-Silva, A.V. Diniz, E.J. Corat, V.J. Trava-Airoldi, Antibacterial activity of DLC films containing TiO_2 nanoparticles, J. Colloid Interface Sci. 340 (2009) 87–92.
[31] M.T. Madigan, J.M. Martinko, P.V. Dunlap, D.P. Clark, Brock Biology of Microorganisms, 12th edn., Pearson Education Inc., Glenview, IL, USA, 2009, ISBN 978-0321-53615-0.
[32] J.P. Nataro, J.B. Kaper, Diarrheagenic *Escherichia coli*, Clin. Microbiol. Rev. (1998) 142–201.

[33] ISO 27447:2009(E), Fine ceramics (advanced ceramics, advanced technical ceramics) — test method for antibacterial activity of semiconducting photocatalytic materials, first edition 2009-06-01.
[34] J. Zita, J. Krýsa, A. Mills, Correlation of oxidative and reductive dye bleaching on TiO_2 photocatalyst films, J. Photochem. Photobiol. A: Chem. 203 (2009) 119–124.
[35] J. Zita, J. Maixner, J. Krýsa, Multilayer TiO_2/SiO_2 thin sol–gel films: effect of calcinations temperature and Na+ diffusion, J. Photochem. Photobiol. A: Chem. 216 (2010) 194–200.
[36] ISO 9308-1:2000, Water quality – enumeration of *Escherichia coli* and coliform bacteria.
[37] ISO 7899-2:2000, Water quality – detection and enumeration of intestinal enterococci.
[38] M. Zlámal, J. Krýsa, J. Jirkovský, Photocatalytic degradation of Acid Orange 7 on TiO_2 films prepared from various powder catalysts, Catal. Lett. 133 (2009) 160–166.
[39] J. Zita, J. Krysa, U. Cernigoj, U. Lavrencic Stangar, J. Jirkovsky, J. Rathousky, Photocatalytic properties of different TiO_2 thin films of various porosity and titania loading, Catal. Today 161 (2011) 29–34.
[40] P.S.M. Dunlop, C.P. Sheeran, J.A. Byrne, M.A.S. McMahon, M.A. Boyle, K.G. McGuigan, Inactivation of clinically relevant pathogens by photocatalytic coatings, J. Photochem. Photobiol. A: Chem. 216 (2010) 303–310.
[41] H.A. Foster, D.W. Sheel, P. Sheel, P. Evans, S. Varghese, N. Rutschke, H.M. Yates, Antimicrobial activity of titania/silver and titanoa/copper films prepared by CVD, J. Photochem. Photobiol. A: Chem. 216 (2010) 283–289.
[42] S. Malato, P. Fernández-Ibáñez, M.I. Maldonado, J. Blanco, W. Gernjak, Decontamination and disinfection of water by solar photocatalysis: recent overview and trends, Catal. Today 147 (2009) 1–59.
[43] D. Mitoraj, A. Jánczyk, M. Strus, H. Kisch, G. Stochel, P.B. Heczko, W. Macyk, Visible light inactivation of bacteria and fungi by modified titanium dioxide, Photochem. Photobiol. Sci. 6 (2007) 642–648.