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Inactivation of clinically relevant pathogens by photocatalytic coatings

P.S.M. Dunlop[a,*], C.P. Sheeran[a], J.A. Byrne[a], M.A.S. McMahon[b], M.A. Boyle[c], K.G. McGuigan[c]

[a] Nanotechnology and Integrated Bioengineering Centre (NIBEC), University of Ulster, Shore Road, Newtownabbey BT37 0QB, Northern Ireland, UK
[b] Food and Environmental Microbiology Research Group, University of Ulster, Shore Road, Newtownabbey BT37 0QB, Northern Ireland, UK
[c] Department of Physiology and Medical Physics, Royal College of Surgeons in Ireland, 123 St. Stephens Green, Dublin, Ireland

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ABSTRACT

Novel disinfection methods are being sought to provide additional means of protection in a number of areas where disease outbreaks could lead to illness or fatalities. For example, the risk of contamination arising from contact with surfaces and medical devices has received much attention due to the rise in incidence of healthcare acquired infections. It is possible that reducing bio-burden on these sites may supplement the disinfection protocols currently in place and help reduce risk of infection. Photocatalytic surfaces offer promise as innovative and cost-effective biocidal engineering solutions which address these specific problems whilst maintaining stringent health and safety controls.

A method was developed to assess the disinfection efficiency of photocatalytic surfaces allowing (a) determination of pathogen viability as a function of treatment time; (b) assessment of the surface for viable surface bound organisms following disinfection; (c) measurement of the re-growth potential of inactivated organisms. This method was used to demonstrate the inactivation of extended-spectrum beta-lactamase *Escherichia coli*, methicillin resistant *Staphylococcus aureus, Pseudomonas aeruginosa* and *Clostridium difficile* spores using immobilised films of commercial titania nanoparticles. 99.9% reduction in viability (a 3-log kill) was observed for all bacterial cells within 80 min photocatalytic treatment. Complete surface inactivation was demonstrated and bacterial re-growth following photocatalytic treatment was not observed. Greater than 99% inactivation (2.6-log reduction) was observed when the photocatalytic surfaces were challenged with *C. difficile* spores.

The efficacy of photocatalytic disinfection to inactivate *Staphyloccocus epidermidis* cells within a biofilm was also demonstrated, with 3 h treatment rendering 96.5% ± 6 of the biofilm cells on the TiO_2 coated substrate non-viable. Disinfection of cells throughout the 3–4 μm thick biofilm was observed.

1. Introduction

Pathogens can be spread to humans by a number of routes of transmission including air, water, food and through contact with contaminated surfaces. Disinfection strategies are widely practiced to inactivate pathogens and therefore minimise the risk of outbreaks of disease. It is not possible, nor desirable, to create completely sterile environments; however, novel disinfection methods are being sought to provide additional means of protection in a number of areas where disease outbreaks could lead serious illness or fatalities, e.g. food preparation areas, pharmaceutical manufacturing plants and healthcare facilities. The risk of contamination arising from contact with surfaces and medical devices has received much attention due to increased incidence of healthcare acquired infections (HAI). Between 8 and 12% of patients entering UK hospitals contract an infection during their treatment [1]. A rise in the incidence of so-called "super bugs", including methicillin resistant *Staphylococcus aureus* (MRSA) and *Clostridium difficile*, in healthcare facilities across the world has been recorded [2]. In addition to patient trauma and, in extreme cases, fatalities, the annual financial burden attributed to HAI's within the National Health Service in England has been estimated to be £1 billion [3].

Microorganisms, such as *S. aureus, Escherichia coli*, *Pseudomonas aeruginosa* and *C. difficile* spores, can survive for weeks and even months on dry surfaces [4]. Although the complex relationship between environmental pathogen loading and incidence of HAI is not fully understood, a reduction in bio-burden through cleaning, with, or without, disinfectants, is associated with reduced patient infection rates [5]. Surfaces that are frequently touched by hands are thought to provide the greatest risk within healthcare facilities, and those situated in close proximity to patients provide the greatest risk. It is possible that reducing bio-burden on these sites may supplement the disinfection protocols currently in place and further reduce the risk of infection [6].

Antimicrobial agents, such as silver, copper, zinc, antibiotics and biocides, are currently incorporated into and onto the surfaces of

* Corresponding author. Tel.: +44 0 2890368942.
E-mail address: psm.dunlop@ulster.ac.uk (P.S.M. Dunlop).

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a range of medical devices, solid surfaces and paints [7]. However, the efficacy of these products with regards to timely disinfection of bacterial spores [8], the development and the identification of microbial resistance mechanisms to metal ion eluting coatings [9] and the high cost of these products have led to research into alternative solutions. To provide long-term solutions, innovative and cost-effective biocidal engineering solutions are required. Potential solutions must also meet stringent health and safety controls.

Over the past two decades, the inactivation/disinfection of microorganisms using photocatalytic materials has been widely studied. The majority of this work has focused upon the disinfection of a wide range of pathogens suspended in water, comprehensively reviewed by McCullagh et al. [10]; however, recent attention has focused on the development of photocatalytic biocidal, or "self-cleaning", surfaces [11–15].

Photocatalytic disinfection is achieved by the production of reactive oxygen species (ROS) resulting from redox reactions occurring at the surface of photo-excited semiconductor, most commonly titanium dioxide. The proposed mechanism of bacterial inactivation centres on the peroxidation and disruption of lipopolysaccharides and phospholipids within the cell wall and cell membrane, coupled with leakage of cellular components and direct ROS attack of organelles and genetic material [16–22]. Goulhen-Chollet et al. recently reported that the emergence of resistance to photocatalytic treatment is very unlikely given the non-specific nature of ROS attack on the structural proteins found within the outer surface of microbial pathogens [23].

The lack of standard methods to assess the biocidal efficacy of photocatalytic coatings has prevented the direct comparison of published research in this field. In addition to variations in the operational parameters employed during photocatalytic experiments, such as reactor configuration, UV source and intensity of incident photons, a range of pathogens, and experimental techniques to assess the viability of the microorganism following treatment, have been reported. Cushnie et al. highlighted the importance, and implications, of a range of basic microbiological parameters on the observed photocatalytic disinfection [24]. Whilst the recently adopted ISO standard [25] may address a number of these fundamental issues, the following points have not been considered:

(a) Typically, the concentration of the challenge organisms used in photocatalytic disinfection experiments is in the order of 10^6–10^9 colony-forming units per mL (CFU/mL), which is deposited onto small surface areas. This level of contamination is several orders of magnitude above the density of pathogens commonly observed on many surfaces requiring cleaning and therefore represents an unrealistic challenge. For example, Neely and Maley describe that contamination levels of 10^5 CFU/cm^2 could be expected in/on a diabetic would dressing; however, within the environmental vicinity of a patient, a microbial density of 10^2 CFU/cm^2 could be anticipated [26].
(b) The majority of researchers, including the authors, carry out disinfection experiments using laboratory strains of microorganisms, for example *E. coli* K-12. This does provide proof-of-principle, demonstrating that photocatalytic coatings exhibits a biocidal effect, but it does not provide robust evidence related to the efficiency of the coatings when challenged with a diverse range of problematic pathogens typically encountered in, for example, healthcare facilities. In addition, bacterial organisms do not contain the structural components found in microbial spores, cysts and biofilms which have a much greater resistance to disinfection treatments.

In this work, we report the development of robust microbial viability assays designed to assess the efficacy of photocatalytic surfaces towards the disinfection of a range of clinically relevant bacterial cells and spores, at concentrations typically observed in clinical settings. The photocatalytic inactivation of cells within a biofilm was also investigated.

2. Methods and materials

2.1. Preparation of the photocatalytic coatings

Thin films of titanium dioxide were produced by immobilisation of Evonik Aeroxide P25 (1% in methanol) onto 76 mm × 26 mm borosilicate glass substrates (Instrument Glasses, UK) [27]. Substrates were masked to ensure deposition of two circular films, each of 1 cm^2, for bacterial disinfection experiments, or formation of a coating on one half of the slides for biofilm disinfection experiments. Prior to coating, glass substrates were washed in Decon 90 and rinsed three times with distilled water. TiO_2 films were deposited onto glass substrates by dip coating using a withdrawal rate of 0.5 mm s^{-1}. Five layers of titania were coated onto the substrates with each titania layer dried under a current of warm air (45–50 °C). Following coating all films were annealed at 450 °C for 1 h and autoclaved prior to disinfection experiments.

2.2. Preparation of microbial pathogens

E. coli K-12 (*E. coli*) (ACTC 23631), extended-spectrum beta-lactamase *E. coli* (ESBL *E. coli*) (CAH 57, a clinical isolate taken at Craigavon Area Hospital, UK), methicillin resistant *S. aureus* (NCTC 10788), *P. aeruginosa* (NCTC 10662) and *C. difficile* (NCTC 11204) were supplied by the Food Microbiology Research Group, University of Ulster. *E. coli* K-12, ESBL *E. coli*, MRSA and *Pseudomonas* were individually cultured overnight at 37 °C in 10 mL of Luria–Bertani broth without shaking. The suspensions were centrifuged at 5000 rpm, the pellet resuspended in 1/4 strength Ringer's solution and serially diluted to the required cell density (2×10^3 CFU/mL) [28]. *C. difficile* was grown anaerobically in thioglycollate broth for one week at 37 °C. Vegetative *C. difficile* cell suspensions were centrifuged at 5000 rpm and the pellet resuspended in 70% ethanol to induce sporulation. *C. difficile* spores were subsequently collected by centrifugation, resuspended in 1/4 strength Ringer's solution and serially diluted to the required cell density (1×10^3 CFU/mL).

2.3. Photocatalytic disinfection of bacterial cells and spores

Two sterile silicone cell culture chambers (flexiPERM, Greiner Bio-One, USA) were adhered over the circular TiO_2 coatings and onto the uncoated glass substrate, permitting duplicate treatment and control analysis on a single substrate (Fig. 1). Silicone culture chambers were inoculated with 500 μL of test pathogen, typical microbial loading 1–2 × 10^3 CFU/mL per cm^2 surface. Films were exposed to UVA radiation (Sylvania 15 W BLB, 3.0 mW cm^{-2}, peak output 365 nm (Gemini 180, Yobin Yvon, UK)) for a fixed period of time. During exposure the temperature of the bacterial suspension within the silicone chambers did not increase by more than 5 °C and

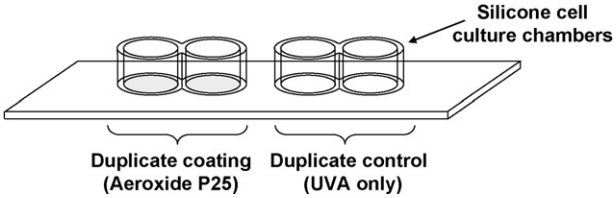

Fig. 1. Schematic representation of the method used to assess disinfection on photocatalytic substrates.

evaporation of the bacterial suspension, assessed by gravimetric analysis, was not observed.

Experiments investigating pathogen viability as a function of UVA exposure time were undertaken. Substrates challenged with bacterial cells were exposed for a total 80 min with individual substrates removed for analysis at 20 min intervals; when using bacterial spores, substrates were exposure for a total of 5 h with individual substrates at hourly intervals. Following exposure, triplicate 100 μL samples were removed for microbial analysis. Control experiments, where uncoated glass substrates were exposed to only UVA radiation, and where the TiO_2 coated and blank substrates were maintained in the dark, were also undertaken. All substrates were analysed in duplicate.

2.4. Analysis of microbial pathogens following photocatalytic disinfection

E. coli K-12, ESBL *E. coli*, MRSA and *Pseudomonas* samples removed following photocatalytic experiments (100 μL) were spread onto LB agar and incubated overnight at 37 °C. *C. difficile* samples (100 μL) were spread onto Braziers agar and grown anaerobically at 37 °C for 48 h. All samples were plated in triplicate. Following incubation, colonies were visually identified and manually counted. Data points on figures show the average number of colony-forming units per mL (CFU/mL); error bars represent standard errors; lines inserted through the data points are not mathematically derived but show the trend within the data series.

To confirm disinfection of the substrate surface following photocatalytic treatment, each silicone culture chamber was filled with cooled molten agar (45–50 °C), LB agar for bacterial cells and Brazier's agar for *C. difficlie* spores, and incubated appropriately. Although individual colonies could be clearly identified following incubation, the contents of the silicone chamber were simply scored as positive, if bacterial growth could be identified, or as negative, if the samples were free from bacterial growth.

2.5. Preparation of biofilm forming organisms

Staphyloccocus epidermidis RP62A (ATCC 35984) was obtained from the American Type Culture Collection and stored at −80 °C. The organism was resuscitated using Brain Heart Infusion (BHI) agar (Oxoid Ltd. UK) and incubated overnight at 37 °C. Stock was also grown overnight at 37 °C on Congo red agar plates, prepared using BHI agar supplemented with 5% sucrose (Sigma, UK) and 0.8 mg/mL Congo red (Sigma, UK), to identify biofilm-positive (black, irregular-shaped, dry colonies) and biofilm-negative (red, smooth colonies) phenotypes. A single biofilm-positive colony was inoculated into 5 mL of BHI broth and incubated overnight at 37 °C, with shaking at 200 rpm. Half coated TiO_2 coated slides were presterilised by autoclaving and placed in a sterile petri dish. BHI broth (10 mL) was added to the petri dish followed by 100 μL of freshly prepared *S. epidermidis* culture. Samples were incubated for 18 h at 37 °C, removed from the growth media and washed three times with sterile deionised water, to remove non-adherent cells.

2.6. Photocatalytic disinfection of biofilm

Duplicate *S. epidermidis* biofilm coated samples were placed in a custom made Perspex cell containing distilled water. Substrates were irradiated through a quartz window using two UVA lamps (PL-S 9W/10, Philips, UK). The UVA intensity incident upon the samples was calculated to be 1.4 mW cm^{-2}, peak output 365 nm (Gemini 180, Yobin Yvon, UK). Substrates were exposed to UVA radiation for 1.5 and 3 h prior to viability analysis. Control samples were maintained in the dark and experiments were carried out in triplicate.

2.7. Analysis of biofilm following photocatalytic disinfection

Biofilm viability was assessed using Live-Dead staining (BacLight Bacterial Viability kit L-13152 Molecular Probes, Netherlands) in conjunction with confocal laser scanning microscopy (LSM510 META Axoplan (Carl Zeiss Ltd., UK) (CLSM). The Live-Dead assay consisted of two nucleic acid stains: SYTO 9 (excitation maximum, 508 nm; emission maximum, 527 nm), a lipophilic membrane permeable cationic stain which labelled viable bacteria with green fluorescence, and propidium iodide (excitation maximum, 536 nm emission maximum, 620 nm), a membrane impermeable anionic stain which labelled membrane-compromised (non-viable) bacteria with red fluorescence. When used alone, SYTO 9 labels both live and dead bacteria green; in contrast propidium iodide penetrates those cells with compromised cell membranes labelling cells red. A ratio of 75:25 SYTO 9:propidium iodide was used in this work.

Microscopy was performed using ×63 magnification objective with a 1.4 numerical aperture. Confocal illumination was provided by either an argon-ion laser (excitation wavelength of 488 nm) fitted with a 505–550 nm band-pass emission filter or a He–Ne laser (excitation wavelength of 543 nm) fitted with a 585–615 nm band-pass emission filter. Images representing 70 μm × 70 μm were acquired using the LSM 5 imaging software. The images of the stained bacteria were segmented using colour thresholding to separate the green and red fluorescence signals. Images were obtained at random from both coated and uncoated parts of the treated substrate. In general, images were acquired towards the surface of the biofilm and thus furthest from the photocatalytic coating. Control samples, not exposed to UVA radiation, were analyzed to negate non-specific staining, signal due to auto-fluorescence and signal cross-over between channels. Images were acquired from at least three frames taken across the sample with a typical cell density of between 800 and 1000 cells per image and were manually processed to determine the percentage viability (ratio of total cells to alive (green) or dead (red) cells. Statistical analysis of the results was carried out using Instat version 3 (Graphpad Software Inc). Data were analyzed by one-way ANOVA with P values of less than 0.05, 0.01 or 0.001 considered to be significant, highly significant or extremely significant, respectively.

3. Results

3.1. Photocatalytic inactivation of bacterial cells and spores

A 99.9% reduction (3-log) in *E. coli* K12 viability was observed following 60 min photocatalytic treatment (Fig. 2). In addition, there was no evidence of bacterial growth on the agar overlaid onto the substrate following 60 min photocatalytic treatment. This demon-

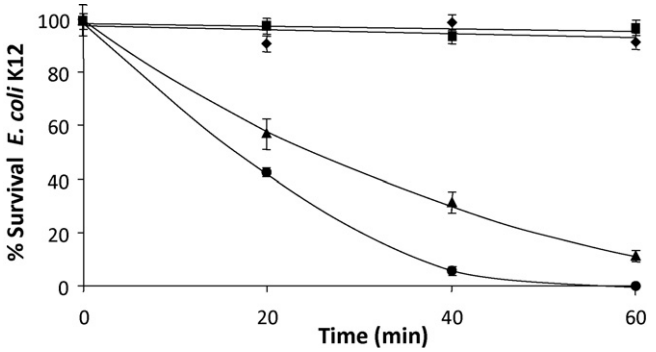

Fig. 2. Inactivation of *E. coli* K12. No treatment (no TiO_2, no UVA) ■; TiO_2, no UVA ♦; UVA only (no TiO_2) ▲; Photocatalysis (UVA and TiO_2) ●.

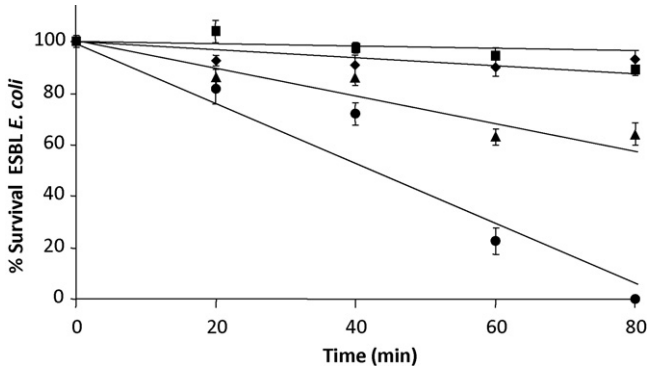

Fig. 3. Inactivation of ESBL *E. coli*. No treatment (no TiO_2, no UVA) ■; TiO_2, no UVA ♦; UVA only (no TiO_2) ▲; Photocatalysis (UVA and TiO_2) ●.

strated complete disinfection of the initial bacterial challenge and confirmed that bacterial re-growth had not taken place, within 24 h. 90% inactivation (1-log) was observed in the UVA only control, i.e. in the absence of the TiO_2 coating. Inactivation was not observed following exposure of *E. coli* K12 cells to the TiO_2 surface in the absence of UVA or in the dark control.

Photocatalytic disinfection experiments using ESBL *E. coli* as the challenge organism followed slower disinfection kinetics than that observed during the inactivation of *E. coli* K12 with 80 min required to achieve 99.9% reduction (3-log) in viable organisms (Fig. 3). At this time point, there was no evidence of bacterial growth following incubation of the agar overlaid substrate. Following 80 min exposure to UVA radiation 46% (0.5-log) inactivation was observed. Significant levels of inactivation were not observed in the control experiments.

Photocatalytic disinfection was demonstrated to be effective for the inactivation of MRSA, a gram positive bacterial organism (Fig. 4). A 99.8% reduction (>2-log) was observed following 40 min photocatalytic treatment, with 99.9% (3-log) observed following 60 min treatment. Bacterial growth was not observed following overnight incubation of agar overlay onto the sample confirming that re-growth of MRSA following photocatalytic treatment had not occurred. Exposure to UVA irradiation alone resulted in ~60% inactivation.

P. aeruginosa was relatively quickly inactivated by exposure to both UVA irradiation and photocatalytic treatment (Fig. 5). A treatment time of 60 min was required to inactivate 99.9% (3-log) of this organism on the TiO_2 coated surface with a 90% (1-log) kill observed in the UVA only experiment. Significant levels of inactivation were not observed in the dark control experiments.

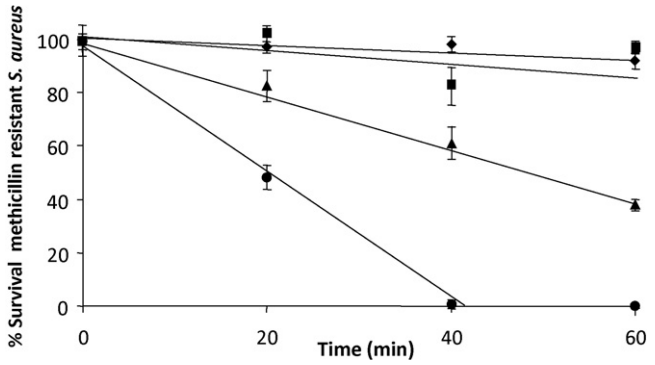

Fig. 4. Inactivation of methicillin resistant *Staphylococcus aureus* (MRSA). No treatment (no TiO_2, no UVA) ■; TiO_2, no UVA ♦; UVA only (no TiO_2) ▲; Photocatalysis (UVA and TiO_2) ●.

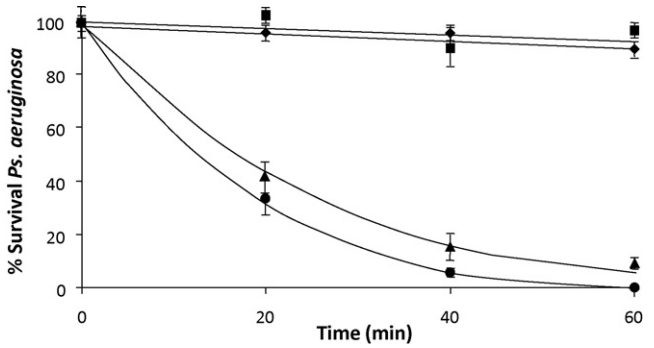

Fig. 5. Inactivation of *Pseudomonas aeruginosa*. No treatment (no TiO_2, no UVA) ■; TiO_2, no UVA ♦; UVA only (no TiO_2) ▲; Photocatalysis (UVA and TiO_2) ●.

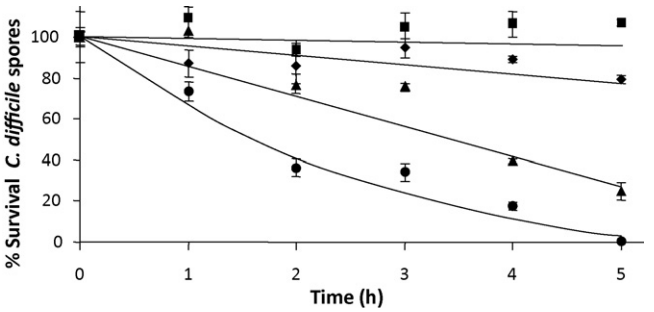

Fig. 6. Inactivation of *Clostridium difficile* spores. No treatment (no TiO_2, no UVA) ■; TiO_2, no UVA ♦; UVA only (no TiO_2) ▲; Photocatalysis (UVA and TiO_2) ●.

The resistance of *C. difficile* spores to photocatalytic treatment warranted a marked increase in exposure time, from minutes to hours (Fig. 6). Five hours photocatalytic treatment was required to achieve 99.7% (>2-log) inactivation. *C. difficile* spores were susceptible to UVA irradiation with 80% of exposed spores rendered non-viable following 5 h UVA exposure. A small decrease in cell density was observed in the control experiments, but this may be due to the difficulty and variability associated with culturing this organism.

3.2. Photocatalytic inactivation of biofilm

S. epidermidis biofilm was uniformly produced across both the TiO_2 coating and the uncoated half of the glass substrate. Confocal laser microscopy images taken during *S. epidermidis* biofilm disinfection are shown in Fig. 7. Exposure of TiO_2 coated substrates to 1.5 h of UVA radiation resulted in a very significant ($P < 0.01$) reduction in viability ($55 \pm 13\%$), in comparison to uncoated samples ($11 \pm 1\%$) (Table 1). After 3 h exposure, $45 \pm 6\%$ of the cells on the uncoated portion were non-viable with $96.5 \pm 6\%$ non-viable on the TiO_2 coated substrate. This result demonstrates that the presence of the TiO_2 coating is extremely significant ($P < 0.001$) when compared to uncoated samples. Disinfection was not observed in the dark control experiments (a decrease in viability of $5.1 \pm 3\%$

Table 1
Staphylococcus epidermidis biofilm cell viability following exposure to photocatalytic and UVA treatment.

Treatment	Exposure time (hours)	Percentage inactivation (%)
UVA-TiO_2	1.5	55 ± 13
	3	97 ± 6
UVA	1.5	11 ± 1
	3	45 ± 6
Dark control (no treatment)	1.5	4.5 ± 3
	3	5.1 ± 3

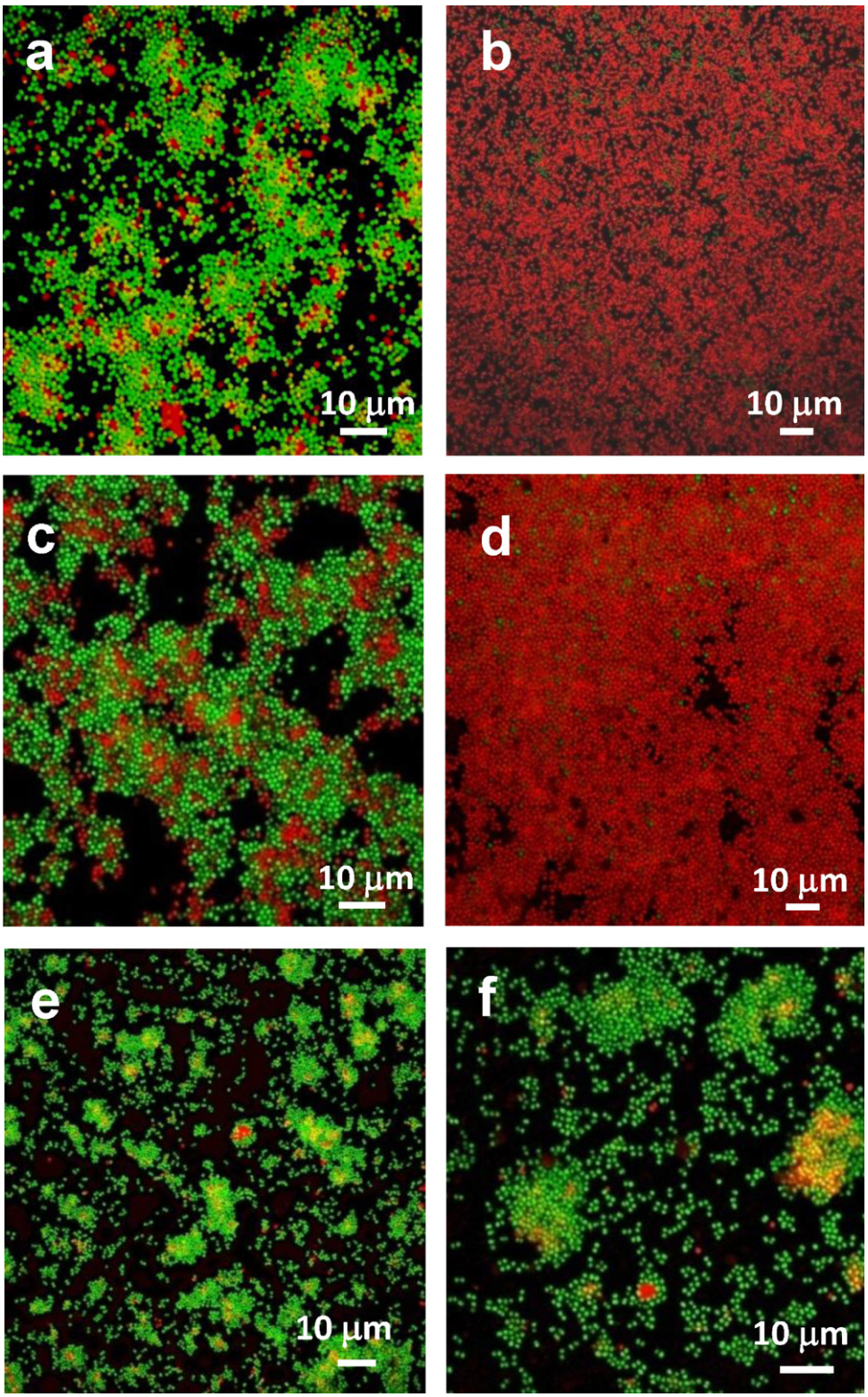

Fig. 7. Fluorescence images of stained *S. epidermidis* cells within a biofilm recorded using confocal laser scanning microscopy. The green and red fluorescence indicate live and membrane-compromised bacteria, respectively: (a) 1.5 h exposure to UVA only; (b) 1.5 h exposure to photocatalytic treatment; (c) 3 h exposure to UVA only; (d) 3 h exposure to photocatalytic treatment; (e) 3 h exposure to TiO_2 in the dark; (f) 3 h no treatment control (no TiO_2, no UVA exposure).

following 3 h). Confocal images acquired at a range of depths, within a thicker section of biofilm, demonstrated a high proportion of cell permeability to the propidium iodide throughout the 3–4 μm biofilm (Fig. 8). Greater inactivation was observed at the titania surface; however, significant inactivation was confirmed at a range of distances within the biofilm and also at the top of the film.

4. Discussion

The reported method, developed to assess the disinfection efficiency of photocatalytic surfaces, not only allows the quantification of viable organisms from samples withdrawn from the suspension above the exposed test substrate, as a function of treatment time,

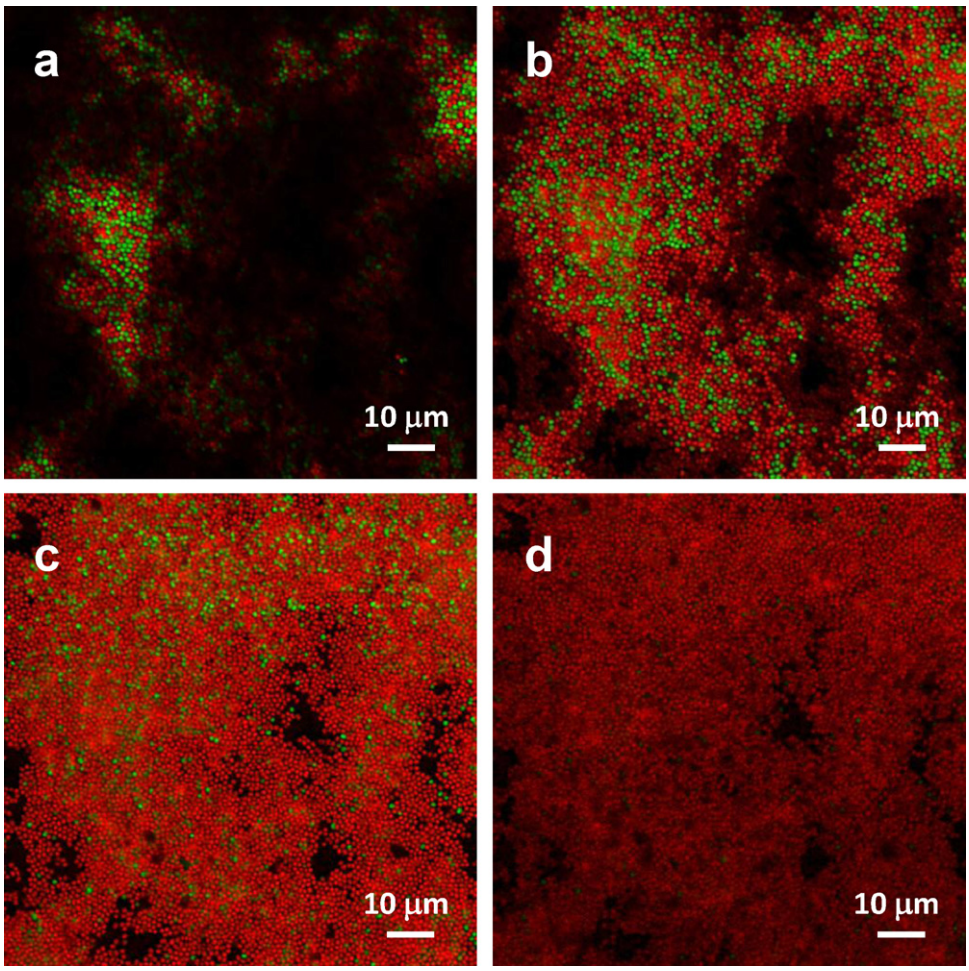

Fig. 8. Fluorescence images of stained *S. epidermidis* cells within a biofilm recorded using confocal laser scanning microscopy. Images were acquired at a series of distances within the biofilm from a substrate exposed to photocatalytic treatment for 3 h: (a) 0.76 μm; (b) 1.52 μm; (c) 2.27 μm; (d) 3.03 μm (0 μm represents the top of the biofilm furthest away from the TiO_2 film).

but also permits examination of the surface following treatment. The latter therefore confirms complete inactivation of the microbial challenge and, in addition, permits examination of the re-growth potential of inactivated organisms remaining on the surface. Inactivation levels of 99.9% (a 3-log reduction) were observed for *E. coli*, methicillin resistant *S. aureus* and *P. aeruginosa*, within 80 min photocatalytic treatment. For *C. difficile* spores, complete photocatalytic inactivation could not be confirmed; however, <99.7% inactivation was still regarded as a significant level of inactivation for an extremely resistant challenge organism.

The results in Figs. 2 and 3 demonstrate that a clinically isolated strain of *E. coli* was more resistant to both UVA and photocatalytic treatment than the model *E. coli* K-12 strain. Model microbial organisms, typically used in teaching, have been genetically selected/modified to ensure they are non-pathogenic. As a result these strains do not possess enhanced resistance mechanisms towards environmental stress or biocide attack. This suggests that caution should be exercised when extrapolating data obtained from experiments using model organisms to application of photocatalytic technology in clinical settings.

Examination of the agar overlaid onto the substrate following disinfection at the final time point confirmed complete surface inactivation for *E. coli* (K-12 and ESBL), methicillin resistant *S. aureus* and *P. aeruginosa*. In addition, bacterial re-growth following photocatalytic disinfection was not observed for these pathogens. Bacterial re-growth following disinfection can be a significant problem and is not considered or examined by the methods currently used to evaluate photocatalytic surfaces. Gelover et al. reported that following photocatalytic disinfection of total coliforms in water samples re-growth was not observed; however, in experiments without the photocatalyst significant levels of re-growth were evident within 24 h [29].

The resistance of pathogens to disinfection treatments can be attributed to the structural components in the outer layers of the microbial cell. Traditionally, microbial susceptibility to antiseptics and disinfectants has been classified based on these differences with descending order of resistance to antiseptics and disinfectants as follows: Coccidian cysts (*Cryptosporidium*) > spores (*Bacillus* sp., C. *difficile*) > gram negative bacteria (*Pseudomonas* sp., *E. coli*) > gram positive bacteria (*Staphylococcus*) [30]. The photocatalytic disinfection of clinical relevant organisms followed a similar pattern with C. *difficile* spores requiring significantly longer treatment that *Pseudomonas* and ESBL *E. coli*. MRSA inactivation required the shortest treatment time with 99.8% inactivation observed following 40 min treatment. We have previously demonstrated that increased photocatalytic treatment time is required to inactivate *C. perfringens* spores [31] and *Cryptosporidium parvum* oocysts [32], in comparison to model organisms and bacterial cells using immobilised titania films.

C. difficile spore inactivation using immobilised photocatalytic material has not been previously reported and demonstrates significant potential for this technology within clinical settings. The

resistance of bacterial spores, including *C. difficile*, to a range of chemical disinfectants commonly used in healthcare facilities is well known and exposure to a number of these agents can promote bacterial sporulation [33]. Due to this inherent resistance, *C. difficile* is now considered to be one of the most important healthcare associated pathogens [34]. Resistance to biocides has been attributed to the complex multi-layer construction of the bacterial spore, which consists of a protoplast (a core of genetic material and low-molecular-weight basic proteins which are rapidly degraded during germination) surrounded by a peptidoglycan cortex and an inner and outer protein spore coat [33].

Other workers have previously examined the efficiency of immobilised photocatalytic films towards the disinfection of a range of microbial pathogens, including clinically relevant organisms. Early work by Kuhn et al. examined the use of P25 coated Plexiglas substrates as light-guides to disinfect *E. coli*, *P. aeruginosa*, *S. aureus* and *Enterococcus faecium* suspensions exposed to UVA radiation [13]. A 6-log reduction in bacterial viability was observed in approx. 60 min. Experiments using *Candida albicans* as a test pathogen demonstrated 2-log inactivation following 60 min treatment. Images acquired via scanning electron microscopy suggested hydroxyl radical damage of the cell wall. Photocatalytic disinfection of MRSA on apatite–tiania coated textiles was reported following 24 h irradiation using a black light blue source [35]. Kubacka et al. report the inactivation of clinical isolates of *P. aeruginosa* and *Enterococcus faecalis* using 280 nm excitation of anatase polymer composites [36].

In a bid to enhance the disinfection rate observed on photocatalytic surfaces, and develop surfaces for specific applications, a number of strategies have been examined. The inclusion of silver nanoparticles within titania films has been reported; however, the biocidal effect of Ag^+ may dominate the observed inactivation kinetics [37,38]. Recently, Mitoraj et al. reported the visible light induced photocatalytic inactivation of *E. coli*, *S. aureus*, *E. faecalis*, *C. albicans* and *Aspergillus niger* on carbon doped and platinum(IV)chloride modified titania in suspensions and on immobilised films [39]. The order of resistance exhibited by the range of microorganisms investigated was related to the inclusion of structural components in the outer layers of the organisms, as previously described. Potential application of photocatalytic technology in healthcare settings was demonstrated by Caballero et al. and Dunnill et al. who described the inactivation of *E. coli* using a commercial photocatalyst (Millennium PC105) and sulphur-doped titania films irradiated by a fluorescent light akin to those commonly found in UK hospitals [40,41].

In addition to disinfection of general surfaces in healthcare environments, contamination of medical devices is a significant problem. Biofilm forming organisms frequent colonise implant and device surfaces resulting in the formation of complex and resistant microbial "communities". Contaminated devices and implants frequently require removal and replacement causing patient discomfort, increased demand on surgical facilities and an additional financial burden on the healthcare system. Biofilms are composed of an extracellular polysaccharide matrix which protects the bacterial cells from the host's defence mechanisms and antimicrobial agents [42]. Furthermore, the altered physiology of cells within a biofilm results in changes in growth rates, which can impair the effectiveness of growth rate-dependent antibiotics. Antibiotic resistant *S. epidermidis* is frequently isolated from implant surfaces [43,44].

The results presented in Fig. 7 demonstrate ROS produced during photocatalysis can inactivate cells within the biofilm structure. Significantly higher rates of photocatalytic disinfection were observed, in comparison to treatment using UVA irradiation. In addition to disinfection at the titania surface, where production of ROS will be highest, Fig. 8 demonstrates inactivation of cells throughout the 3–4 μm structure of the biofilm. We propose that the range of reactive oxygen species generated at the surface of irradiated tiania, including hydroxyl radicals, superoxide radical anion and hydrogen peroxide, contribute to the disinfection of the biofilm cells. Kikuchi et al. investigated the role of a range of ROS during the disinfection of *E. coli* [11]. Addition of increasing concentration of mannitol, a hydroxyl radical scavenger, suppressed the observed level of photocatalytic disinfection. The presence of catalase also reduced the levels of disinfection implying involvement of hydrogen peroxide in the biocidal mechanism. When the bacterial cells and the titania film were separated by 50 μm (using a porous PTFE membrane), disinfection was still observed. The long range biocidal effect was attributed to the production of hydrogen peroxide and the potential to produce additional ROS by photosensitisation of cellular components, such as riboflavin.

Irradiated photocatalytic surfaces have been shown to prevent adhesion of biofilm forming organisms on cement and glass surfaces [45,46]; however, the photocatalytic disinfection of biofilm has not been widely researched. The susceptibility of *P. aeruginosa* (PA01) to photocatalytic treatment using thin films of TiO_2 deposited on glass slides was investigated by Gage et al. [47]. Disinfection of planktonic cells was observed, with a 4-log reduction in viable cells reported following 3 h UVA-TiO_2 treatment; whereas UVA light alone produced a 1-log reduction. For biofilm forming bacteria, photocatalytic treatment did not enhance the inactivation observed using only UVA treatment. A directly comparable study to the research presented in this paper was carried out by Mosnier et al., who report UVA-assisted disinfection of *S. epidermidis* biofilm using 2 μm thick ZnO films deposited onto glass substrates via pulsed laser deposition [48]. Following 2 h exposure 70 ± 12% of the cells in the biofilm were determined to be inactivated; however, photocorrosion of the ZnO films was observed with the possible release of Zn^{2+} ions contributing to the observed disinfection.

5. Conclusion

Conventional methods of manual disinfection within healthcare facilities are laborious, expensive, and due to the introduction of stringent health and safety concerns now require the use of less effective biocidal agents, for example, the use of hypochlorite solutions in many areas is no longer permitted. The results of this work, and others, demonstrate that photocatalysis could play a role in the inactivation of pathogens on surfaces along side regular and effective manual cleaning, and assessment of cleaning.

A method was developed to assess the disinfection efficiency of photocatalytic surfaces allowing (a) determination of pathogen viability as a function of treatment time; (b) assessment of the surface following disinfection to determine the presence of surface bound microorganism; (c) measurement of the re-growth potential of treated/inactivated organisms. This method was used to demonstrate the inactivation of *E. coli*, methicillin resistant *S. aureus, P. aeruginosa* and *C. difficile* spores on immobilised films of commercial nanoparticle titania under UVA irradiation. Inactivation levels of 99.9% (3-log reduction) were observed for ESBL *E. coli* following 80 min photocatalytic treatment. Sixty minutes of photocatalytic treatment was required to achieve 99.9% inactivation of *Pseudomonas* and *E. coli* K12. MRSA inactivation required the shortest photocatalytic exposure time with 99.8% inactivation observed following 40 min. Complete surface inactivation of the bacterial cells used in this study was demonstrated and bacterial re-growth following photocatalytic treatment was not observed. For *C. difficile* spores, <99% inactivation (2.6-log reduction) was observed following 5 h photocatalytic treatment. The efficacy of photocatalytic disinfection to inactivate *S. epidermidis* cells within the biofilm was also demonstrated. Following 3 h UVA exposure 96.5 ± 6% of the

biofilm cells on the TiO_2 coated substrate were shown to be non-viable. The presence of the TiO_2 coating was demonstrated to be extremely significant ($P < 0.001$) when compared to uncoated samples, i.e. inactivation by UVA alone. Disinfection throughout the 3–4 μm thick biofilm was also observed.

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