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The Effect of Immobilized Anatase Photocatalyst Nanopowder in the UV-A Mediated Killing of *Escherichia coli* B23

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Solar water disinfection (SODIS) is a method using ultraviolet-A radiation and heat for inactivating microbial activity in water. Adding suspended anatase (TiO_2) greatly improves the disinfection efficiency. It is thought that the mechanism of microbial killing by UV-A and anatase may be mediated through the generation of reactive oxygen species (ROS) that damage the microbial lipid membranes. This experiment tested the effects of UV-A radiation with an immobilized anatase photocatalyst on the viability of *Escherichia coli* B23 (*E.coli* B23) in sterile distilled water. The extent of ROS formation was measured through the formation of the lipid peroxidation product malondialdehyde (MDA). Our experiments demonstrated the effectiveness of immobilized anatase on the formation of the lipid peroxidation product MDA, however the formation of MDA did not correlate to decreased viability of *E. coli* B23.

In the developing world, 80% of illnesses are water related (World Vision [http://www.Worldvision.com.au/wvconnect/content.asp?topicID=173]). The U.N. estimates that there are 2.4 billion people in the world that do not have the proper access to sanitation facilities and of that, 1.2 billion people have no access to safe drinking water. Every year, 3.3 million people die from diarrheal diseases related to bacterial infections such as *E. coli*, salmonella and cholera, parasites such as cryptosporidium and giardia, as well as numerous viruses such as rotaviruses and enteroviruses (Schumacher, A.J., U.N. Chronicle [http://www.un.org/Pubs/chronicle/2005/issue2/0205p20.html]).

Solar water disinfection (SODIS) is a simple, sustainable, and energy-efficient method endorsed by the World Health Organization for the disinfection of drinking water. This method relies on heat and ionizing radiation to partially disinfect the water. The ionizing radiation serves to form hydroxyl radicals from the radiolysis of water. These hydroxyl radicals, along with other reactive oxygen species (ROS) are thought to cause cell killing by oxidizing various targets including intracellular Coenzyme A (CoA) and to membrane phospholipids, lipoproteins, and nucleic acids (7).

Anatase, a form of titanium dioxide (TiO_2) is a photocatalytic semiconductor whose properties have been investigated extensively in the areas of solar energy conversion and storage, reductive fixation of carbon dioxide, organic synthesis, and detoxification of organic compounds (8). When excited by solar irradiation, TiO_2 transfers excited electrons onto oxygen acceptors (7).

The purpose of this study was to implement and correlate the enhanced germicidal effects associated with the implementation of an immobilized TiO_2 photocatalyst in UV-A irradiation to the levels of lipid peroxidation. Cell membrane damage was determined by measuring malondialdehyde (MDA) formation, a product of lipid peroxidation.

MATERIAL AND METHODS

Bacterial Strains and Growth conditions: *Escherichia coli* B23 (*E. coli* B23) was obtained from the University of British Columbia Department of Microbiology and Immunology MICB 421 culture collection. Overnight cultures were prepared by inoculating a loopful of *E. coli* B23 into 20 ml of Luria-Bertani broth (5 g/L yeast extract (BD, #0127-01), 10 g/L Tryptone (BD, #298222), 5 g/L NaCl (Fisher, #S671-3), pH 7.0). Cultures were incubated aerobically at 37°C on a rotary shaker (100 rpm) for 18 hours. Bacterial concentrations of the overnight culture were determined by turbidity measurements using the Spectronic 20 spectrophotometer using the approximation that an OD_{460} reading of 1.00 corresponded to a bacterial density of $1x10^9$ cells/ml.

Photocatalytic Reaction: Experimental and control treatments are listed in Table 1. An average of 0.027g of TiO_2 nanopowder (Sigma-Aldrich, #637254) was immobilized onto 6 cm X 5 cm strips of cellophane packing tape (Staples) which were fixed to the bottom of Petri dishes with hot glue (Walmart). 25 ml of sterile distilled water was inoculated with *E. coli* B23 cells to a final concentration of 10^8 cells/ml. Petri dishes were placed on a shaking platform operated at 60 rpm (Model 3520 Orbit Shaker, Labline Instruments) to ensure mixing and even distribution of bacterial cells, and exposed at a distance of 12 cm to two 20 W Sylvania Blacklight bulbs with a peak wavelength at 366 nm. Control Petri dishes were wrapped in aluminum foil to prevent exposure to UV light (see fig. 1).

Samples were taken from the Petri dishes at 0, 15, 30, 60, 90, and 120 minute time points.

TABLE 1: Treatment groups for testing the effects of anatase on the UV-A mediated killing of *E. coli* B23:

Treatment	Blank Control	UV Control	Experimental Group	Anatase Control
Anatase Treatment	-	+	+	-
UV Treatment	-	-	+	+

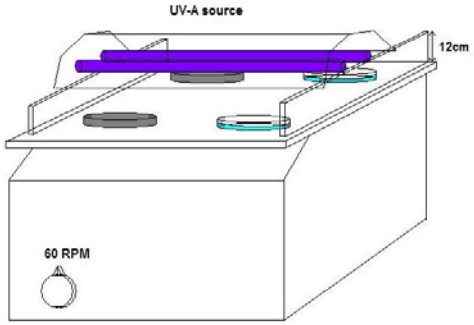

FIG 1. UV-A irradiation setup. Four petri dishes, two wrapped with aluminum foil, were placed on a shaking platform 12 cm below the UV-A light source.

Determination of Lipid Peroxidation: Malondialdehyde (MDA, Caymanchem, #10009202) formed by peroxidation of cellular lipid was used as an index of lipid peroxidation. MDA was quantified based on its reaction with thiobarbituric acid (TBA). At low pH and elevated temperature, MDA participates in nucleophilic addition reactions with thiobarbituric acid (TBA), forming a pink MDA-TBA adduct (4). The amount of MDA-TBA formed was then quantified using absorbance measurements at 532nm and bacterial survival was determined. At each time point, 1 ml of the experimental sample was mixed with 0.5 ml of 40% (wt/vol) trichloroacetic acid (TCA, Sigma, #116114), and subjected to centrifugation at 11,000xg for 8 minutes to ensure the complete removal of cells and solid material from the sample. 2 ml of freshly made 0.67% (wt/vol) thiobarbituric acid (TBA, Sigma,#T5500) was then added to the supernatant. The samples were incubated in a boiling water bath for 60 minutes, cooled, and the absorbance was measured at 532nm using the Spectronic 20 spectrophotometer. Concentrations of MDA formed were determined by comparing the samples to a standard curve prepared reacting known concentrations of MDA with TBA to form the chromogen complex.

Enumeration: To examine the bactericidal effect of TiO_2 under UV-A illumination, bacterial growth was monitored during the time course of exposure by the spread plate method. At each time point, 1 ul was sampled from each experimental group, and duplicate plates were plated at a final plated dilution of 10^{-6}. Plates were incubated aerobically for 15 hours at 37°C.

RESULTS

Decline in *E. coli* growth was not observed with increasing periods of UV-A and anatase exposure. In order to study the killing efficiency, *E. coli* population changes were monitored through a 120 min time course. However, the large standard deviation and overlapping error bars (95% confidence interval) have made it difficult to predict the trend of bacterial population fluctuation along the time course between the different experimental conditions (fig. 2). The overall trend in bacterial growth in all four treatment groups is erratic and unreliable, showing no significant reduction in viability. However, significant bactericidal effects were observed in the UV-A only group at 15 min compared to the anatase only group. In addition, the combined condition (UV-A and anatase) at 30 min shows significant reductions in bacterial survival compared to the control group. This is characterized by a lower bacterial population at 30 min with a confidence interval clearly out of range from the control condition. After 30 min, however, the differences in bacterial cell counts of the combined condition was not statistically significant as evidenced by the overlapping confidence intervals observed in the three time points after 30 min (fig. 2).

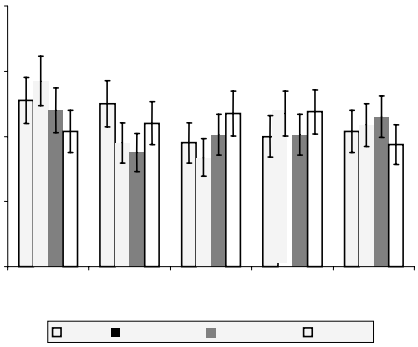

FIG 2. Effects of UV-A light and anatase exposure on the observed survival for *E. coli* B23 in sterile distilled water. Error bars represent 95% confidence intervals

Immobilized anatase enhances levels of UV-A induced lipid peroxidation of *E. coli*. Since the bactericidal effect of UV-A irradiation is believed to be caused by membrane damage (7), we examined production of MDA, a product of lipid peroxidation as an index of such damage. A rough trend line (fig. 3) showed that the MDA level peaked at around 60 min for groups treated with anatase alone or with both anatase and UV light. The latter group displayed a more significant increase in MDA formation than the former. After 60 min, the MDA level of both groups drops. For the control and the UV-A light alone treatment groups, the changes in MDA were not significant over the timecourse of the experiment. Thus, the formation of MDA was enhanced to a greater degree by the presence of anatase alone than the presence of UV-A light alone. However, UV-A light in the presence of anatase seemed to be enhance the levels of lipid peroxidation.

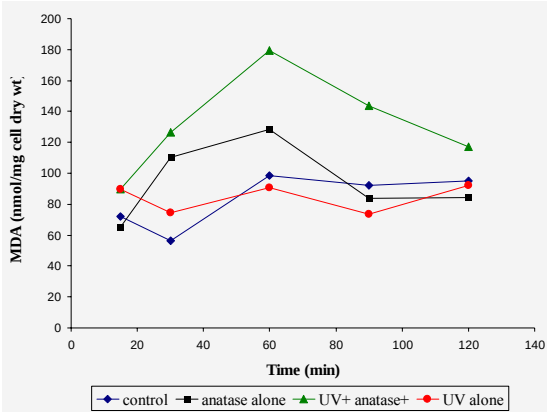

FIG 3. Effects of UV-A light and anatase exposure on formation of MDA in *E. coli* B23 by lipid peroxidation.

DISCUSSION

Contrary to previous investigations (5, 6) our viability study showed no significant reduction in cell viability following exposure to UV-A radiation in the presence or absence of anatase (Fig. 2). The initial steep drop observed in the experimental groups with anatase may be reflective of bacterial death due to osmotic shock in combination with a lag in bacterial growth due to the change in growth conditions (ie. cells were removed from a nutrient rich environment and introduced to distilled water). Figure 2 also shows reduced viability of bacteria exposed to anatase in the dark compared to bacteria cultured under similar conditions lacking the photocatalyst during the first hour of incubation. This observation was reported by other investigators where it was attributed to clumping of bacteria to anatase particles so that numbers were underrepresented in the plate count assay (6). Despite all the variation in cell counts between the four experimental conditions throughout the experiment, the viable cell counts at the end of the two hour incubation period were all within 10% of each other. The large variation in the viable plate count results over the two hours may have been caused by the inherently large degree of error in the small sampling volume (1 µL from each plate diluted into ~10 mL). Thus these results might not accurately reflect the trends in bacterial killing from each of these experimental conditions and must be considered inconclusive. This variation would also mask any potential bacterial growth in our control sample, since the growth would be slow because of the room temperature, limited aeration and minimal nutrients in the water.

An inherent flaw in the design of this experiment may have caused the absence of significant cell death in the samples with anatase. In an attempt to maximize the catalytic surface exposed to UV illumination, the anatase used in this experiment was the nanopowder form. However, studies have shown that overall kinetics of photocatalysis depend on two contradicting properties of the catalyst: large surface area and high crystallinity (8). Thus lack of crystalline structure in the nanopowder form of anatase may have significantly hindered its photocatalytic properties and significantly reduced its bactericidal activity, even though it did cause lipid peroxidation (Fig. 3). The MDA measurements showed a significant time dependent increase in the amount of lipid peroxidation per mg of bacterial dry weight in both anatase conditions, that peaked then dropped steadily thereafter. This trend has been documented previously (6, 10). The decline in the amount of MDA has been attributed to the photocatalytic oxidation of MDA (6). Further more, MDA is known to be quite reactive and capable of forming adducts with DNA and proteins, accounting for its decline (1, 9). The fact that samples incubated in the dark in the presence of anatase also displayed a steep initial increase in MDA formation might be due to activation of anatase to ambient light conditions prior to the start of the experiment. This potential activation may have primed the reducing activity of the catalytic surface, leading to an initial surge in MDA formation. The absence of the anatase photocatalyst resulted in significantly less formation of MDA regardless of exposure to UV-A light, thus confirming the catalytic role of TiO_2 in the generation of reactive oxygen species and lipid peroxidation.

The slight zig-zag pattern observed in the levels of MDA produced in UV alone and control samples may be a byproduct of normalization. Values reported in Figure 3 were normalized to the dry weight of viable bacteria (obtained from the viability study). As there was a large degree of uncertainty in the bacterial counts, this large degree of uncertainty would be reflected in the normalized results for the MDA-TBA assay.

The results of the viability study at first may appear to contradict the results of the MDA-TBA assay, as one showed no significant difference in bacterial survival, while the other showed a significant increase in lipid peroxidation upon inclusion of anatase in the solar water disinfection apparatus. However, it is important to note that the MDA-TBA assay is used to measure the extent of membrane damage (in the form of lipid peroxidation) inflicted upon the bacteria, and not the rate of bacterial killing. It is also important to

note that membrane damage does not imply cell death, as bacteria possess the ability to repair membrane damage (3). Furthermore, the discrepancies between the viability study and the MDA-TBA assay could be attributed to inherent shortcomings in the MDA-TBA assay (4). This assay does not measure MDA formation directly, but rather, correlates the amount of MDA present to the amount of pink chromogen (MDA-TBA adduct) produced upon reaction of MDA with TBA. However, TBA is intrinsically non-specific for MDA, and various other lipid peroxidation products as well as nonlipid materials give false positive results (4).

In our study we observed the effects of UV-A on the survival of *E. coli* B23 in the presence and absence of anatase. Although we demonstrated that the presence of anatase did have an appreciable effect on the extent of lipid peroxidation as measured by our MDA-TBA assay, it did not demonstrate any conclusive enhancement of UV-A killing. Due to limitations in time, we were not able to further explore the reasons for our unexpected trends in bacterial counts that have resulted in inconclusive support for our hypothesis. It would have been ideal to have had the time to find a method to optimize the anatase immobilization onto a surface for use in our experiment. It was found that the cellophane tape used in this experiment had some electrostatic effects with the anatase nanopowder used, repelling some off if pressure was not applied. As well, with the sticky medium that the tape is, it was difficult to determine how evenly spread the immobilized anatase was on the surface.

FUTURE EXPERIMENTS

Future experiments could investigate the effectiveness of different anatase immobilization techniques. One method would be to involve the use of a Sol-Gel method for affixing anatase onto the surface. The Sol-Gel would be formed by obtaining a solution of titanium alkoxide dispersed into a solvent, then subjecting this solution to a series of hydrolysis and condensation reactions so that an interconnected network of inorganic TiO_2 (metal-oxo) polymers would result, forming a gel (2). A rigid, heat-resistant material such as a glass slide could be used in this process to obtain an even coating of TiO_2 to eliminate some of the variability in this experiment. Although in theory, anatase is the only photocatalytically active form of TiO_2, it has been shown that a 75% anatase and 25% rutile (photocatalytically inactive) formulation of TiO_2 (Degussa P-25) has been more effective than that of pure anatase (6). Thus, the introduction of several different TiO_2 formulations into our experimental protocol would allow for the comparison of relative ROS production efficiencies and their germicidal effects.

To address the fact that the MDA-TBA assay is a non-specific, indirect method of quantitating MDA formation from lipid peroxidation more direct methods of detecting MDA can be applied. One simple and direct method for quantifying MDA makes use of the low molecular weight of MDA, allowing it to be separated on a size-exclusion column such as Sephadex G-10 (4). Purified MDA can then be quantified using UV spectrophotometry (4)

Since even the control group did not demonstrate appreciable bacterial growth, it was postulated that the osmotic shock subjected to the cells after inoculation into the sterile distilled water may have affected cell membrane integrity. To confirm this and rule out the possibility of random error due to the small sampling volume, a larger volume should be sampled at each time point in future experiments. As well, in order to compensate for the great variability seen in the results, future experiments should include several replicates.

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