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Bacterial inactivation in water, DNA strand breaking, and membrane damage induced by ultraviolet-assisted titanium dioxide photocatalysis

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ABSTRACT

The effects of UV-assisted TiO_2-photocatalytic oxidation (PCO) inactivation of pathogenic bacteria (*Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella typhimurium*) in a liquid culture using different domains of UV irradiation (A, B and C) were evaluated. Structural changes in super-coiled plasmid DNA (pUC19) and genomic DNA of *E. coli* were observed using gel electrophoresis to demonstrate the photodynamic DNA strand breaking activity of UV-assisted TiO_2-PCO. Membrane damage in bacterial cells was observed using both a scanning electron microscope (SEM) and a confocal laser scanning microscope (CLSM). Both UVC-TiO_2-PCO and UVC alone resulted in an earlier bactericidal phase (initial counts of approximately 6 log CFU/mL) in 60 s and 90 s, respectively, in liquid culture. UVC-TiO_2-PCO treatment for 6 min converted all plasmid DNA to the linear form; however, under UVC irradiation alone, super-coiled DNA remained. Prolonged UVC-TiO_2-PCO treatment resulted in structural changes in genomic DNA from *E. coli*. SEM observations revealed that bacteria suffered severe visible cell damage after UVC-TiO_2-PCO treatment for 30–60 min. *S. typhimurium* cells showed visible damage after 30 min, which was confirmed using CLSM. All treated cells were stained red using propidium iodide under a fluorescent light.

1. Introduction

Contaminated water is one of the biggest sources of potentially hazardous microorganisms that can cause severe health problem for humans either by direct consumption or through use in washing of food materials and food contact surfaces (Brassard et al., 2011). Hence, there is a continuous need of developing innovative water disinfection methods (Malato et al., 2009). Use of the titanium dioxide photocatalytic oxidation reaction (TiO_2-PCO) for removal of organic materials and pollutants in water and wastewater has been well-documented. Applications of the TiO_2-PCO reaction are increasing as a disinfection method; however, in comparison to widely used chemical disinfectants, such as alcohol, iodine, and chlorine, disinfection using TiO_2-

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PCO methods is still in the developmental stage (Ye et al., 2010). Comparative studies regarding photocatalytic effects on different bacteria using different types of UV light alone and in combination with TiO_2-PCO are needed. The synergistic effects of ultrasound on the photo-degradation of different chemical compounds in *Escherichia coli* have been reported (Dadjour et al., 2005). Some studies have indicated that TiO_2 can catalyze production of hydroxyl radicals and other oxidizing agents, such as hydrogen peroxide, super oxides, conduction band electrons, and valence band holes on the surface of TiO_2 in photochemical reactions (Dadjour et al., 2006). Such reactive oxygen species (ROS) can disrupt and damage cell functions and structures (Kim et al., 2005). Other researchers have reported that ROS, including hydroxyl radicals, can cause DNA and cellular membrane damage (Ashikaga et al., 2000). However, we still do not understand if such effects are required to cause bacterial inactivation and weather inactivation is caused due to DNA damage or due to other kinds of oxidative changes in cellular organs. Irradiation of TiO_2 with UV light ($\lambda < 400$ nm) results in promotion of an electron from the valence band to the conduction band, leaving a positive hole in the valence band. These holes can migrate to the particle–solution interface where they can oxidize hydroxyl ions or water to form hydroxyl radicals. These photo-generated electrons must be removed from the conduction band in order to maintain electrical neutrality; hence the dissolved oxygen acts as electron acceptor. Reduction of dissolved oxygen generates additional reactive oxygen species including superoxide radical anion ($O_2^{\cdot-}$), hydroperoxyl radical ($HO_2^{\cdot}$) and hydrogen peroxide (H_2O_2). These species can attack organic and inorganic species present in the water, directly (Mills and Le Hunte, 1997; Dunlop et al., 2008). But the direct attack by hydroxyl radicals may only be partly responsible for the bacterial disinfection. There are also possibilities of a secondary indirect pathway in which H_2O_2 produced by the photocatalytic reaction may also feed into an *in vivo* Fenton reaction resulting in OH radical formation within the target organism. The iron-catalysed Haber–Weiss reaction ($H_2O_2 + O_2^{\cdot-} \rightarrow O_2 + OH^- + OH^{\bullet}$) can cause production of OH radicals in the presence of H_2O_2 and $O_2^{\cdot-}$ (Dunlop et al., 2008). The radicals produced either directly from photocatalysis or through indirect ways, can cause different inactivation effects through damaging cellular parts. We used DNA as a molecular target for oxygen radicals in order to evaluate the time needed for a UV-assisted TiO_2-PCO reaction to cause structural changes in DNA. Investigators have suggested that ROS attack the base and sugar moieties, resulting in multiple effects, including 1) single- and double-strand breaking in the backbone, 2) adduction of the base and sugar groups, 3) cross-linking to other molecules, and 4) causing lesions that block replication (Sies and Menck, 1992; Sies, 1993). Once damaged, super-coiled circular plasmid DNA transforms to the linear form, which migrates slower than intact super-coiled plasmid DNA during agarose gel electrophoresis. The ratio of linear to super-coiled plasmid DNA can be used as an index for quantification of the DNA damage induced by photocatalysts (Yang and Wang, 2008). Scanning electron microscopy can be used to study the cell structure of bacteria; however, cell membrane permeability needs to be investigated after TiO_2-PCO treatment of pathogenic bacteria. Flow cytometry with propidium iodide can be used for this study (Liao et al., 2010). More direct and precise observations of cell staining to distinguish intact cells from cells with damaged membranes can be made using confocal laser scanning microscopy (CLSM), which has become widely established as a valuable tool for ultra-structural analysis of fluorescently labeled cellular elements (Lopez-Amoros et al., 1997). Sunnotel et al. (2010) used propidium iodide as vital dye for studying the photocatalytic inactivation of *Cryptosporidium parvum* on nanostructured titanium dioxide films. Dunlop et al. (2010) used CLSM to verify membrane-compromised *Staphylococcus epidermidis* within a biofilm after photocatalytic disinfection. More studies using CLSM are required to investigate cellular changes in UV-assisted TiO_2-PCO treated microorganisms. Moreover, the membrane fluidity of microorganisms also needs to be investigated after photocatalytic treatment (Liao et al., 2010).

The objective of this study was to analyze the effects of UV-assisted TiO_2-PCO treatments for inactivation of bacterial pathogens (*E. coli* O157:H7, *Listeria monocytogenes*, and *Salmonella typhimurium*) in water. Structural changes in DNA, morphological damage to bacterial cells, and changes in cell membrane permeability induced by UV-assisted TiO_2-PCO treatment were also investigated.

2. Materials and methods

2.1. Preparation of bacterial cultures

E. coli (ATCC 25922), *L. monocytogenes* (KCCM 40307), and *S. typhimurium* (ATCC 14028), obtained from the American Type Culture Collection (Rockville, MD, USA) and the Korean Culture Center of Microorganisms (Seoul, Korea), were used for inactivation experiments. Cells were grown in nutrient broth at 37 °C with shaking, and harvested by centrifugation at 4000× *g* for 10 min, then washed with saline water (0.85% NaCl solution). Transformed *E. coli* TOP10 cells with pUC19 DNA (2686 bp) were incubated in Luria-Bertani (LB) medium at 37 °C for 14–16 h with 200 rpm shaking. The culture medium was then refreshed and incubation continued with vigorous aeration at 37 °C for 14–16 h.

2.2. Reactors for photocatalytic experiments

Photocatalytic oxidation (PCO) experiments for monitoring antibacterial and DNA strand breaking activities were carried out in lab-scale UV-TiO_2-PCO reactors, as shown in Fig. 1A and Fig. 1B, respectively. The reactor for UV antibacterial experiments consisted of a stainless-steel chamber with a 3.5 L working volume and a UV lamp surrounded by either a TiO_2-coated quartz tube (diameter 36 mm and length 570 mm) or a quartz tube without a TiO_2 coating. A magnetic stirring bar was placed in the bottom of the reactor to allow for sufficient mixing. The reactor used for studying the DNA breaking activity consisted of a cap, a UV lamp, and a quartz tube with an internal diameter of 25 mm and a height of 50 mm $TiCl_4$ was used as a source of titanium for preparation of TiO_2 thin films. A TiO_2 solution was prepared by dissolving $TiCl_4$, HCl, $2(NH_4)HCO_3$, and H_2O_2 in distilled water. This solution was deposited on the surface of a quartz tube and completely dried at 250 °C for 24 h (Cho et al., 2007). The specifications of the different UV lamps used in this study are given in Table 1. The control experiments were also performed using TiO_2 alone without UV illumination.

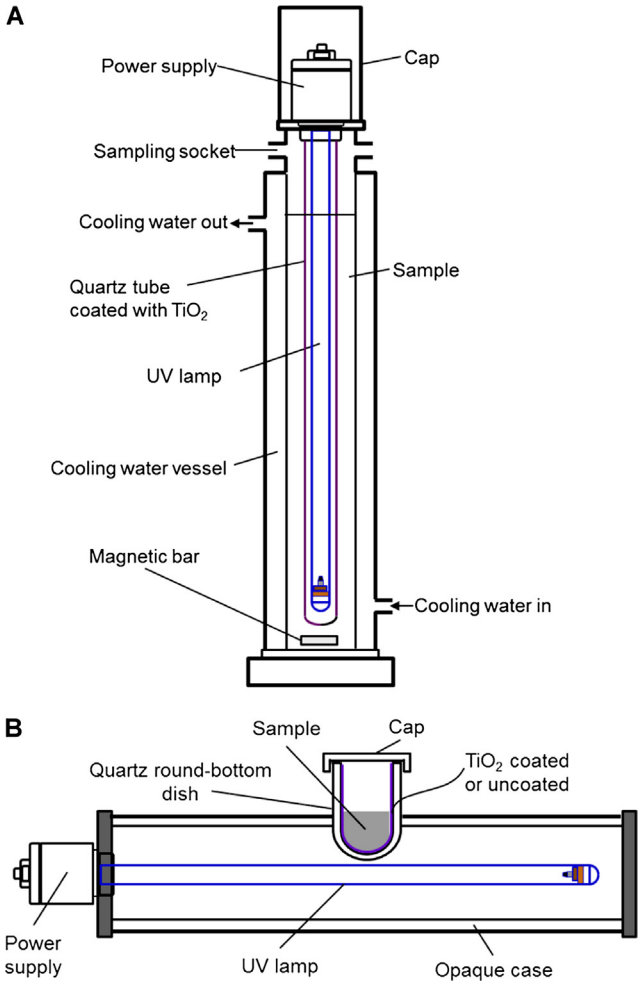

Fig. 1 – Schematic diagram of the lab-scale UV-assisted TiO_2-PCO reactor for antibacterial (A) and DNA strand breaking (B) activities.

2.3. *Inactivation of pathogenic bacteria in liquid culture*

The initial bacterial population was set at approximately 6 log CFU/mL in 3 L of water containing 0.85% NaCl. A 10 mL sample of the treated solution was withdrawn from the reactor at 30 min intervals for 180 min for both the UVA alone and the combination with TiO_2 photocatalysis analyses. When UVB and UVC were used, samples for microbial analyses were taken at 1 min intervals for 8 min, and 10 s intervals for 90 s, respectively. These experimental conditions were selected based on our preliminary trials and results of previous studies (Cho et al., 2007). Solutions were enumerated using the pour plate method on nutrient agar, and incubated at 37 °C for 24 h to obtain counts of viable bacteria. Counts of microbial colonies were expressed as log CFU/mL (colony forming units per milliliter of sample) and converted to a survival ratio (N/N_0) where N_0 is the value of log CFU/mL at time 0 and N represents the same value at a different PCO reaction time.

Table 1 – Wavelengths, intensities, and dimensions of UV lamps used.

UV	λ (nm)	Intensity (mW/cm^2)	Length (mm)	Diameter (mm)	Lamp wattage (W)
UVA	352	8	580 ± 1.3	32.5 ± 1.5	20
UVB	306	9			
UVC	254	16			

2.4. *Gel electrophoresis for structural changes in DNA*

We used super-coiled plasmid DNA as an index of DNA damage caused by ROS on TiO_2 surfaces. Plasmid DNA pUC19, extracted using an Exprep® plasmid SV mini kit (Gene ALL, Seoul, Korea), was transferred to TiO_2-coated and clear quartz tubes (Sankyo Denki Co., Japan) and irradiated using different UV lamps (Table 1). Each sample was transferred to an e-tube for assay with an interval of 2 min for a total of 10 min of PCO exposure time. Linear standards were obtained by cutting the plasmid with BamH1, which is a restriction enzyme isolated from bacteria that has the capacity to recognize specific sequences in multi cloning sites and to cut DNA (Ogawa et al., 2012). Cleavage of pUC19 DNA was done using BamH1 in a mixture including BSA, 10× buffer, and dH_2O. Cleaved DNA was incubated at 37 °C for 24 h. Cleavage results in relaxation of the super-coiled DNA molecules. A linear form of plasmid DNA was used as a control and a size marker. Accordingly, a small aliquot was run on gel to check for digestion.

Genomic DNA was extracted after treating *E. coli* cells with TiO_2-UVC at 30 min intervals for a total of 120 min using the CTAB genomic DNA preparation method (Doyle and Doyle, 1987). Bacterial cells were harvested by spin down using a tabletop centrifuge at 1650× *g* for 5 min, and then washed in dH_2O. CTAB extraction buffer was then added and the mixture was incubated at 65 °C for 30 min. After adding 10 mL of chloroform, 7 mL of the aqueous phase was transferred to a tube containing 7 mL of isopropanol, and the pellet was obtained by centrifuging at 1650× *g* for 5 min. The genomic DNA pellet was washed in 70% ethanol and dissolved in 500 μL of TE buffer.

Agarose gel electrophoresis was used to separate and visualize DNA fragments based on their size topology. The gel was prepared by dissolving 1% agarose in 1× TAE buffer containing 5 μL/mL of ethidium bromide to stain the DNA, then heated until dissolved, followed by casting into a slab gel tray, and setting (Serpone et al., 2006). Samples were subjected to electrophoresis in agarose gel. Agarose forms a porous lattice in the buffer solution and the DNA must slip through the holes in the lattice in order to move toward the positive pole. The DNA migration pattern was recorded using WiseDoc® WGD-20 Gel Documentation. Plasmid DNA and genomic DNA strand-breaking activities were measured as a decrease in the ratio of linear to super-coiled plasmid DNA, and the concentration of the fragments.

2.5. *Scanning electron microscopy*

Cover slips for SEM were prepared using the critical-point drying technique. Cover slips were fixed in modified Karnovsky's fixative (2% paraformaldehyde, 2% glutaraldehyde, 0.5% $CaCl_2$ in 0.1 M phosphate buffer) overnight, washed with 0.1 M cacodylate buffer, and post fixed with 1% OsO_4 in 0.1 M

cacodylate buffer. Dehydration was achieved by passing the cover slips through 50, 60, 70, 80, 90, 95, and 100% ethyl alcohol for 5 min each, then finally 100% ethyl alcohol for 10 min. After being dried, the cover slips were mounted on SEM stubs, and the samples were coated with approximately 300 Å of gold and observed under a field emission scanning electron microscope (FE-SEM, S-800; Hitachi Ltd., Tokyo, Japan). The magnification (×20,000), resolution, and tilt angle were adjusted and photomicrographs were taken.

2.6. Confocal laser scanning microscopy

A rapid CLSM staining method using the LIVE/DEAD® BacLight™ Bacterial Viability Kit (Molecular Probes, Eugene, OR, USA) was used to reveal membrane damage in *S. typhimurium* after UVC-TiO_2-PCO treatment. According to the manufacturer, BacLight™ is composed of the two nucleic acid-binding stains SYTO 9™ and propidium iodide. SYTO 9™ penetrates all bacterial membranes and stains cells green, while propidium iodide only penetrates cells with damaged membranes. The combination of the two stains produces red fluorescing cells. The procedure follows a previously described method (Wu et al., 2010). The two BacLight™ stains were dissolved in DMSO, mixed together (300 μL + 300 μL), and diluted 1:10 in NaCl solution (0.85%) providing 6 mL of BacLight™ stock solution. The stock solution was stored at −20 °C in the absence of visible light. An amount of 30 μL of BacLight™ was added to 1 mL of sample when needed. Samples were incubated in the dark at room temperature for 20 min, followed by trapping between a slide and an 18 mm^2 coverslip. Samples were examined under a confocal microscope (LSM 510 Meta, Carl Zeiss, Jena, Germany). The excitation and emission wavelengths were set at 488 and 543 nm, respectively.

2.7. Statistical analysis

All bacterial inactivation treatments were repeated three times and data were recorded as mean and standard error. Morphological and DNA damage experiments were also repeated to ensure reproducibility of results. Statistical analysis was done using an analysis of variance (ANOVA) technique. Significance was defined at $p < 0.05$.

3. Results and discussions

3.1. Inactivation of pathogenic bacteria in liquid culture

The bactericidal effects of the UV-TiO_2-PCO (photocatalytic oxidation) reaction and UV irradiation alone on inactivation of pathogenic bacteria (*E. coli* O157:H7, *L. monocytogenes*, *S. typhimurium*) were evaluated and results are presented in Fig. 2. The initial populations of all three bacteria at the start of experiments were approximately 10^6 CFU/mL in liquid culture. The dark control (TiO_2 without UV light) produced no bactericidal effects. The bacterial inactivation efficiency depended on the type of UV light used. Regardless of the bacterial strains, UVC light showed a more significant disinfection effect than UVA and UVB lights, together with TiO_2-PCO or alone, resulting in an earlier bactericidal phase for all three bacterial types (within 60 s for UVC-TiO_2 and 90 s for UVC alone). For UVA, whether alone or with TiO_2-PCO, a 6 log reduction was not achieved, even after 180 min. UVA alone showed slightly quicker effects that UVA-TiO_2, particularly in case of *L. monocytogenes*, these were not consistent and

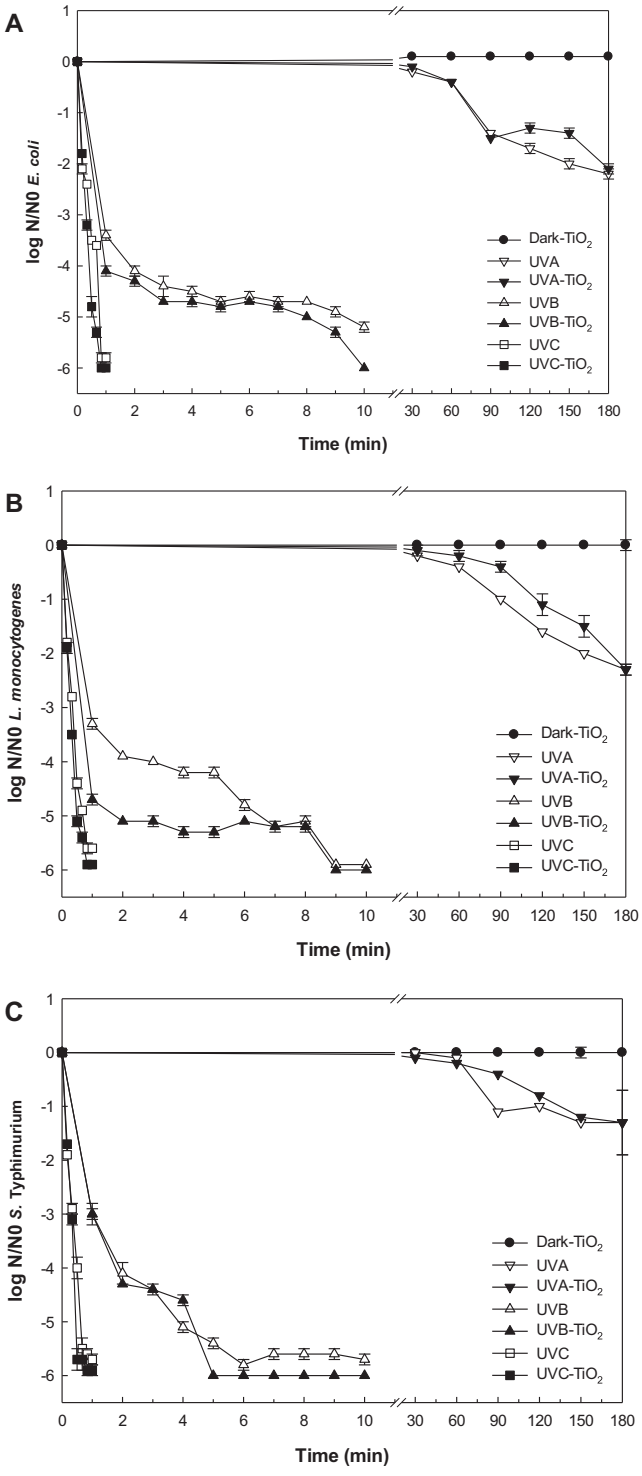

Fig. 2 – Bactericidal effects on *Escherichia coli* (A), *Listeria monocytogenes* (B) and *Salmonella typhimurium* (C) under different photocatalytic treatments. N/N_0 is the survival ratio along with the standard error of the means ($n = 3$).

probably a more prolonged UVA-TiO_2 treatment will cause more bacterial inactivation than induced UVA alone. UVB, on the other hand, resulted in a 6 log reduction in approximately 10 min. We used UVC with a wavelength of 254 nm in our experiments. Similar UVC light alone was used in a study by Cheigh et al. (2012) for observation of inactivation of *E. coli* and *L. monocytogenes*. They observed reductions of 5 and 4 log, respectively, after 20 min. The reason for this longer time might be because a layered bacterial suspension culture in a petri dish was used for irradiation experiments whereas we treated all bacteria in liquid culture, which resulted in significantly faster inactivation. TiO_2 also apparently increased the inactivation rates of bacteria in association with UVC (particularly for *E. coli* and *S. typhimurium*). These two bacteria are gram-negative and showed relatively lesser resistance than *L. monocytogenes*, which is gram-positive, to UVC-TiO_2. However, resistance to and UVB-TiO_2 treatment was different i.e. gram-negative bacteria being more resistant than gram-positive, especially during initial 4 min of treatment. More studies involving study of bacterial membrane structure in relation to their resistance to photocatalytic disinfection is required (Dunlop et al., 2010).

The use of UV light to inactivate cellular microorganisms is becoming a well-known disinfection method (Craik et al., 2001), however, use of UV light in association with TiO_2 is still in developing stage. Our results in Fig. 2 indicate that the UVC-TiO_2-PCO reaction is faster and more effective than using UVC alone or with other wavelengths of UV light, even in association with TiO_2. The antimicrobial effects of UV light are related to chemical modifications and cleavage of DNA, and other kinds of DNA lesions (Oguma et al., 2001). In a UV-TiO_2-PCO reaction, the interaction of UV light and semiconductor particles results in generation of highly reactive oxygen species (ROS) such as $OH^{\bullet}$, $O_2^{\cdot-}$, $HO_2^{\cdot}$, which are capable of destroying microorganisms (Benabbou et al., 2007; Dadjour et al., 2005). Other catalysts, such as ZnO, ZrO_2, CeO_2, Fe_2O_3, and WO_3 should also be tested; however, TiO_2 is preferable due to its important photoactivity, lack of toxicity, and high stability (Benabbou et al., 2007). Hence a combined UVC-TiO_2-PCO process is fast, non-thermal, and effective for inactivation of pathogenic bacteria such as *E. coli* and *S. typhimurium*, in water.

3.2. *In vitro plasmid (pUC19) and genomic DNA (E. coli TOP10) damage*

In order to verify that bacterial inactivation was a result of DNA damage, we carried out experiments to examine the effects (using gel electrophoresis) of the UV-TiO_2-PCO reaction and the effects of UV irradiation alone on plasmid DNA from *E. coli* for exposure periods of 0, 2, 4, 6, 8, and 10 min. The conversion of super-coiled plasmid DNA due to TiO_2 in dark (control) and UVA alone/UVA-TiO_2 and UVB alone/UVB-TiO_2 are shown in Fig. 3A and no DNA damaging effects by TiO_2 in dark, UVA and UVB were evident as expected due to our results regarding bacterial disinfection (Fig. 2). UVA-TiO_2 and UVB-TiO_2 showed some conversion of supercoiled DNA to its linear form (Fig. 3A). Fig. 3B illustrates the conversion of super-coiled plasmid DNA into its relaxed and linear forms while using UVC irradiation alone or in combination with TiO_2. After 6 min of treatment with UVC-TiO_2-PCO, most plasmid DNA was transformed to linear DNA. For UVC irradiation alone, some super-coiled DNA remained in the original form after 6 min, and super-coiled bands were clearly visible. The difference, in conversion of super-coiled plasmid DNA by the UVC-TiO_2-PCO system that of UVC alone, is prominent at 4 min and 6 min interval, where UVC-TiO_2 clearly show more effects (Fig. 3B). This difference of DNA damage by these two systems may become important in actual disinfection process. A similar, faster bacterial inactivation was achieved when TiO_2 was used, as discussed in the previous section. It should also be noted that, within 2 min after the start of this treatment, some of the plasmid DNA was already converted to the linear form. Therefore, cell inactivation was probably achieved within this period. Dunford et al. (1997) showed that sunlight illuminated TiO_2 catalyses DNA damage *in vitro* and demonstrated that super-coiled plasmid DNA with TiO_2 and under UV irradiation was converted first to the relaxed form and later to the linear form, indicating strand breakage. Nucleic acids are potential targets for PCO and damage is caused by ROS. Replication of DNA is prevented and many cellular functions are destroyed after nucleic acid damage, resulting in cell death (Yang and Wang, 2008). Therefore, we can correlate the bacterial inactivation achieved after 60 s of the UVC-TiO_2-PCO reaction with the start of the DNA or nucleic acid damage that disrupts cellular functions, as treated bacterial cells failed to grow and reproduce on nutrient agar. Complete conversion of such DNA *in vitro*, however, is significantly affected by the reaction time. Therefore, to study the effects of the UVC-TiO_2-PCO system and the time necessary to diminish the genomic DNA of *E. coli*, the exposure time was increased and damage was monitored at 30 min intervals for up to 2 h using gel electrophoresis (Fig. 3C). The effects of this system on genomic DNA became evident when the exposure time was increased up to 120 min. As treatment time progressed, the concentration of the genomic DNA band decreased due to structural damage caused by cross-linking of proteins and deformed genomic DNA. Our results demonstrate that genomic DNA damage takes much longer to become evident.

3.3. *Scanning electron and confocal microscopic observations*

SEM images were used to examine exposed membrane surfaces for visible changes or damage to the membrane caused by the UV-TiO_2-PCO treatment using different UV lamps. Based on the SEM photomicrographs in Fig. 4, all three bacteria types showed visible cell structural damage. However, cell damage due to UVA was not prominent, even after 60 min as evident in Fig. 4. Cell ruptures due to UVB-TiO_2-PCO and UVC-TiO_2-PCO were prominent after 30 and 60 min treatments, suggesting severe damage to the bacterial structure (Fig. 4). The UVC system caused more structural change in bacterial cells than the UVB system, as can be seen in SEM images after 3 min of treatment. This study has revealed destruction of the cytoplasmic membrane and rupture of the internal organization, leading to leakage of the cytoplasmic contents and cell death (Krishnamurthy et al., 2010). In another study (Cheigh et al., 2012) no damage to the membranes of *E. coli* and *L. monocytogenes* cells was observed when UVC was used alone for 10 min.

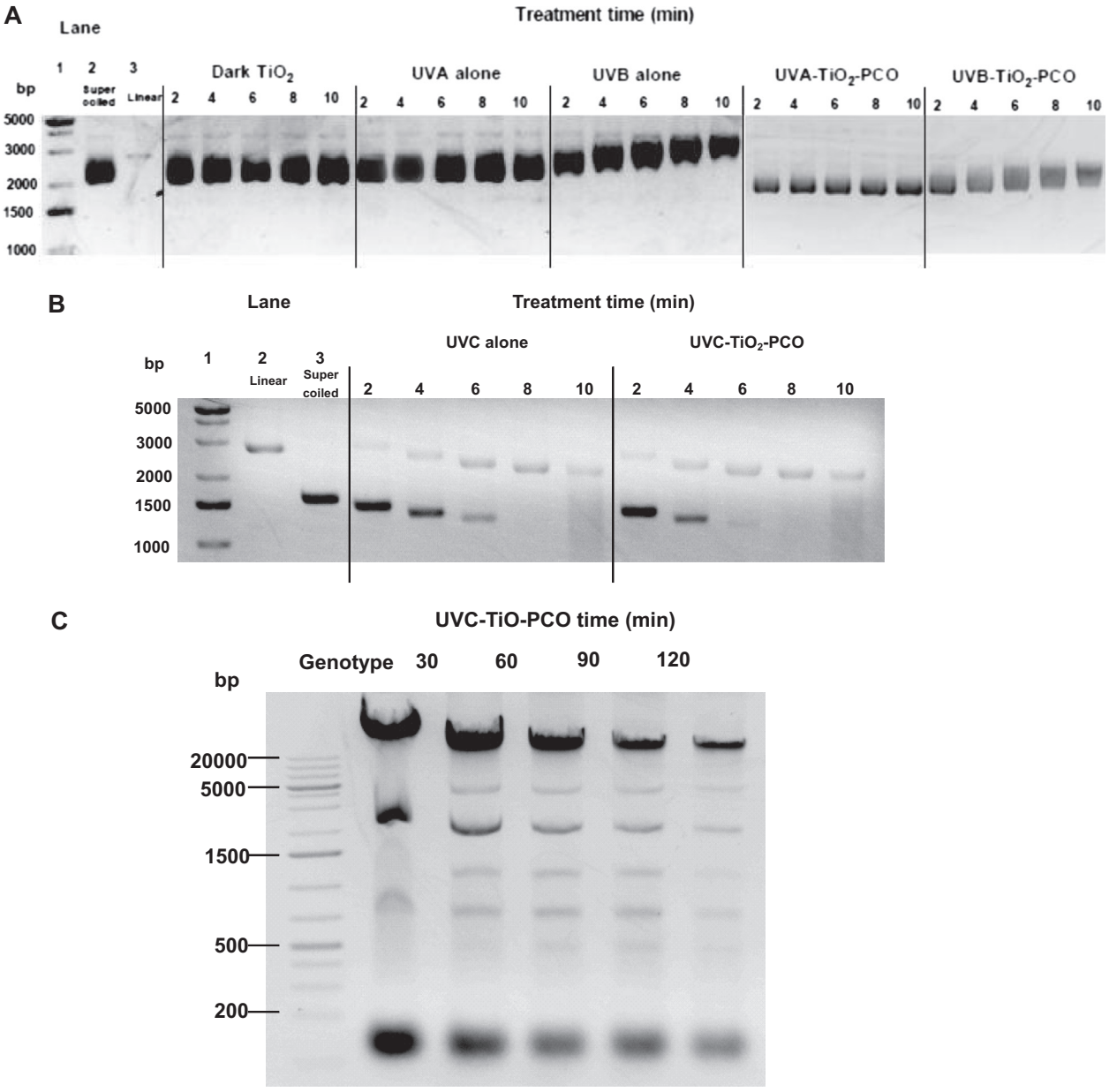

Fig. 3 – Changes in electrophoretic gel images of plasmid DNA with different treatment times (0–10 min) for dark TiO_2, UVA alone and UVA-TiO_2-PCO, UVB alone and UVB-TiO_2-PCO (A) UVC alone and UVC-TiO_2-PCO (B); lane 1 is a size marker; linear type of plasmid DNA (cut using BamH1), and a control sample (untreated pUC19 plasmid DNA) are also shown (A, B). Changes in electrophoretic gel images of genomic DNA of *E. coli* treated with UVC-TiO_2-PCO for reaction times of 30, 60, 90, and 120 min (C).

In order to further verify the time needed to cause sufficient bacterial membrane damage using a UVC-TiO_2-PCO system in liquid culture, confocal laser scanning microscopy (CLSM) was used. Fluorescent images of *S. typhimurium* cells prior to and after UVC-TiO_2-PCO treatment for 10 min are presented in Fig. 5A and B, respectively. All control cells prior to treatment were stained green by the nucleic acid-binding stain SYTO 9™, which penetrated through bacterial membranes and turned cells green. Propidium iodide only penetrated damaged cells, turning them red. All cells treated with UVC-TiO_2-PCO for 30 min were stained red under CLSM (Fig. 5C). The red color indicates that cell membranes were damaged during the UVC-TiO_2-PCO treatment (Wu et al., 2010). Hence, CLSM observation revealed that 30 min of treatment with UVC-TiO_2-PCO was necessary for complete damage of bacterial cell membranes. CLSM observations, more so than SEM, are strong confirmatory evidence of bacterial membrane damage due to UVC-TiO_2-PCO treatment. CLSM is also a good method for detecting cell viability and membrane integrity as it does not require cell culturing (Lopez-Amoros et al., 1997). Evidence that the UVC-TiO_2-PCO reaction is also capable of causing structural

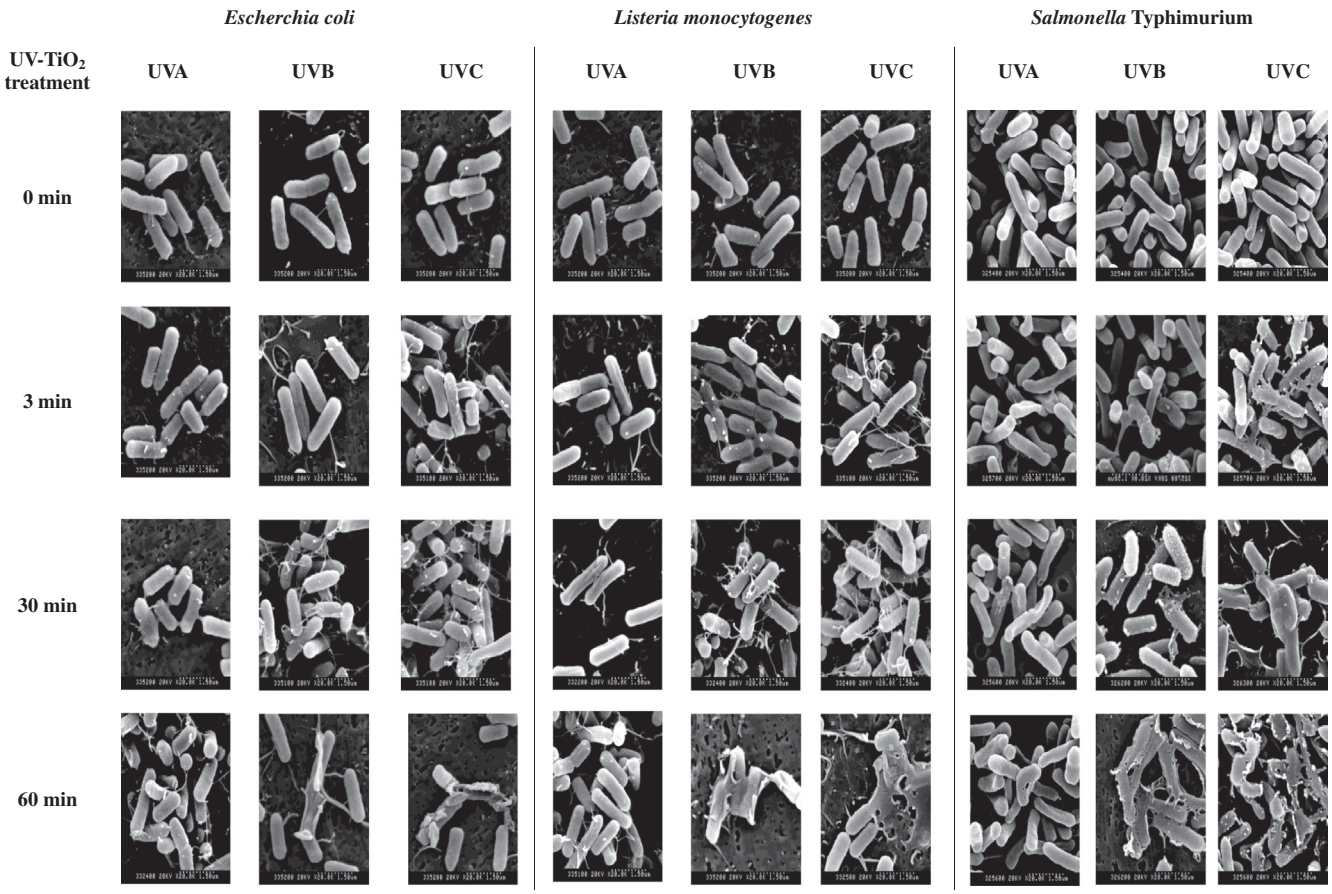

Fig. 4 – Scanning electron photomicrograph of *Escherichia coli* (A), *Listeria monocytogenes* (B) and *Salmonella typhimurium* (C) treated in a UV-assisted TiO_2-PCO reaction.

damage in pathogenic bacteria further supports the idea of using this system for bacterial pathogen inactivation. This is due to the fact that it can induce earlier cell inactivation, plasmid and genomic DNA damage, and structural disruption of living cells. The optimal time and other reaction conditions for ensuring full disinfection in waters containing different pollutants using TiO_2 based systems need to be established. We observed that treating pathogenic bacteria for 30 min in a UVC-TiO_2-PCO system caused irreversible changes in DNA and cell membranes, and that such a reaction is expected to cause complete disinfection in a water culture.

The UVC-TiO_2-PCO reaction induced generation of reactive oxygen species (ROS) in bacteria. ROS are inevitable by-products of biological redox reactions. ROS can inactivate enzymes and damage cellular components. The increased generation of ROS is considered to be a feature of acute stress conditions. UVC-TiO_2-PCO treatments significantly increased the release of ROS with treatment time. Such oxidative stress is detrimental to bacterial organisms. The biological targets for ROS generated from a UV-assisted TiO_2-PCO process are mainly DNA, RNA, proteins, and lipids in bacterial cells (Kim et al., 2005; Ashikaga et al., 2000). Matsunaga et al. (1988) proposed that direct photochemical oxidation of intracellular coenzyme A to its dimeric form is the main cause of a decrease in respiratory activity leading to cell death. Other authors (Saito et al., 1992) have suggested that the cell membrane and cell wall undergo disruption in the presence of irradiated TiO_2, as shown by leakage of intracellular K^+. It was also observed that TiO_2 photocatalysis promotes peroxidation of *E. coli* membrane phospholipids and induces major disorders in the cell membrane (Maness et al., 1999). The quantity of TiO_2 required in a photocatalytic process for bacterial inactivation is generally low and the type of UV light also plays an important role (Benabbou et al., 2007). These reports present different views on mechanisms that lead towards ROS induced inactivation of bacterial cells. In fact, ROS may either be produced directly from TiO_2 surface or more of OH radicals may result from indirect pathways from H_2O_2 and O_2^- through Fenton and Haber–Weiss reactions as discussed earlier (Dunlop et al., 2008). The ROS can cause damage to bacterial DNA inside the cell as results of which bacterial cells will cease to reproduce, as we observed in our study within 60–90 s of UVC-TiO_2 treatment and this effect is much earlier than that of cell membrane damage as observed in SEM and CLSM results which support the idea of indirect generation of ROS inside the cell which damaged the DNA much earlier than cell membrane. Sunnotel et al. (2010) also presented similar ideas of possible mechanism of UVA-TiO_2 induced inactivation of *C. parvum* and observed 73.7% reduction after 180 min in surface water as observed by dye exclusion assay.

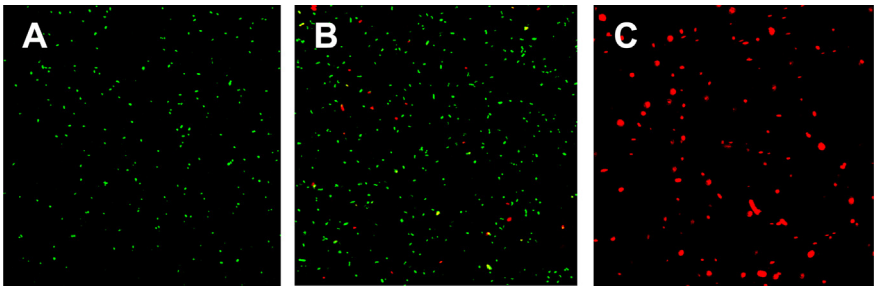

Fig. 5 – *Salmonella typhimurium* viability as detected under confocal laser scanning microscopy using SYTO 9™ and propidium iodide. Untreated (A), after UVC-TiO_2-PCO treatment for 10 min (B), and after UVC-TiO_2-PCO treatment for 30 min (C).

We observed UVC to be the most suitable type, whether alone or in combination with TiO_2. It has been reported that UVC effectively damages bacterial cells, most likely via a 3-step procedure, including impairment of the photosynthesis system, decomposition of cytoplasmic inclusions, and cell cytoclasis (Ou et al., 2011). Hence, we can infer that inactivation and structural damage in bacteria is achieved due to the combined effects of UVC and ROS generated from the TiO_2 surface due to UV light. Considering previous reports, we found some questionable systematic evidence in relation to the mechanism involved in PCO-assisted inactivation of bacteria. We observed that bacteria treated for 60 s in a UVC-TiO_2-PCO system ceased to reproduce and, when the treatment time was increased, all *E. coli* plasmid DNA became linear (6 min) and bacterial cell membranes were damaged (30 min). Therefore, a UVC-TiO2-PCO system is capable of inducing multiple bacterial cell inactivation processes and, hence, is recommended for ensuring the disinfection of water, provided more research is done to optimize this process in different culture environments.

4. Conclusions

- Photocatalysis involving use of TiO_2 and UV light in a lab-scale reactor can be effectively applied for disinfection of a water culture. A TiO_2 coated quartz tube with UVC light ($\lambda = 254$ nm, intensity $= 16$ mW/cm^2) was observed to cause a 10^6 CFU/mL reductions in the numbers of *E. coli*, *L. monocytogenes*, and *S. typhimurium* in about 60 s in liquid culture.
- Gel electrophoresis was used to study plasmid (pUC19) and genomic DNA (*E. coli* TOP10) after treatment in the UV-TiO_2 system. Photocatalytic systems involving use of UVA or UVB systems showed minimal or no damage in DNA after 10 min treatment. However, plasmid or super-coiled DNA started to become linear in 2 min of UVC-TiO_2 treatment and most of it was damaged in 6 min. Genomic DNA damage, however, required much longer times.
- Electron microscopic observations of bacterial pathogens treated for longer times in different UV-TiO_2 reactors revealed that membrane damage started to appear after 3 min and complete damage was evident after 30–60 min of treatment when using UVC in photocatalysis. Confocal laser microscopic observations, however, confirmed that membranes of treated (UVC-TiO_2) bacteria were completely damaged in 30 min, based on red stained cells under fluorescence. Hence for an effective photocatalytic-assisted disinfection use of UVC is recommendable along with TiO_2.
- Our study has revealed that the bactericidal effects of a UVC-TiO_2 system are 1) cessation of bacterial reproduction probably due to oxidative changes caused by indirect generation of OH radicals inside the bacterial cell and 2) onset of DNA and cell organelle damage. Complete DNA and cell membrane damage required a considerably longer time to become visible using existing detection methods, whereas disinfection of these bacteria required a significantly shorter time in liquid culture. Hence, the disinfection effect seems to be due to oxidative, chemical, and functional changes rather than due to apparent physical damage of cell organs in waterborne bacterial pathogens. The initiation of DNA damage due to possible generation OH radicals through indirect pathways, as discussed before, seem to have main bactericidal effect. However, induction of irreversible physical damage to microorganisms can be beneficial in a standard UVC-TiO_2 process for water disinfection.

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