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TMPyP functionalised chitosan membrane for efficient sunlight driven water disinfection

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Abstract

Sunlight-driven water disinfection system could help provide clean water to some of the world's poorest people. In this study, chitosan membranes were modified by pyromellitic dianhydride in order to introduce carboxyl groups and facilitate adsorption of the highly basic photosensitiser - 5, 10, 15, 20-tetrakis (1-methyl-4-pyridinio) porphyrin tetra *p*-toluene sulfonate (TMPyP). The physico-chemical properties of these modified membranes were investigated by microscopy, absorption spectroscopy and Midland surface blotting approaches. The chitosan membrane-TMPyP composite showed photodynamic inactivation of bacteriophage MS2 and *E. coli* BL21. For photodynamic inactivation under stationary conditions, complete inactivation of MS2 was observed after 90 min illumination at a light intensity of 32 mW cm^{-2} (which equates to around 3% of bright mid-day time sunlight under clear sky conditions in sub-Saharan Africa). For a flowing system, complete inactivation of MS2 was observed for sample flowing at 0.33 ml/min and passed over the surface of the modified membranes while being illuminated. Reduction of approximately 3 log PFU/ml were observed for samples passed just once over the membranes under the same conditions. With *E. coli*, under the same stationary conditions, a reduction of 3 log CFU/ml was observed. Each TMPyP functionalised chitosan membrane was used at least three times for the photodynamic inactivation of both MS2 and *E. coli* BL21 without any detectable loss of inactivation capacity. The re-usability of the membranes will reduce cost and increase the advantage of using an environmental-friendly technology for water disinfection.

1. Introduction

Conventional methods of waste water treatment and disinfection are costly and complex because of intensive use of chemicals, manpower and energy, as well as the centralised nature of their infrastructures and operations. As such, it is not affordable in most instances in rural areas of developing countries (Loeb et al., 2016). Water-borne pathogens such as *Cryptosporidium parvum*, cysts of *Entamoeba hystolytica*, *Giardia lamblia* and enteric viruses are resistant to chlorination which is presently the gold standard in water disinfection during wastewater treatment (Shannon et al., 2008, Silverman et al., 2013). Another major setback of using chlorine as disinfectant is that this can react with organic compounds and nitrite in wastewater, resulting in the formation of mutagenic and carcinogenic disinfection by products (DBPs) such as trihalomethanes, and haloacetic acids (Shannon et al., 2008).

Previous work has shown that photosensitiser such as 5, 10, 15, 20-tetrakis-

(1-methyl-4-pyridinio) porphyrin tetra *p*-toluene sulfonate (TMPyP) has been shown to result in rapid photodynamic inactivation (PDI) of the model virus, bacteriophage MS2 in solution(Casteel et al., 2004). This effect exploits a known phenomenon called the photodynamic effect which results from the interaction of three factors comprising a photosensitizer, light and oxygen. This interaction generates singlet oxygen and other reactive oxygen species (ROS) that can oxidise and cause irreversible damage to proteins, lipids, nucleic acid and other cellular components of microorganisms and ultimately inactivate these (Alves et al., 2013, Baumler and Maisch, 2012, Carvalho et al., 2007, Costa et al., 2012, Komagoe et al., 2011, Maisch et al., 2012b, Spannberger et al., 2012, Costa et al., 2010, Wainwright, 2004). Attaching TMPyP onto a solid support such as a chitosan membrane will make it suitable for use in water disinfection during wastewater treatment without releasing

TMPyP into the water after treatment. Also, TMPyP-functionalised chitosan membranes (CM-T) could be re-used, reducing the cost and increasing the advantage of using an environmental-friendly technology for water disinfection. Here, chitosan membranes were chosen as a solid support for attaching TMPyP for water disinfection because of their properties such as easy fabrication, presence of numerous reactive groups, rigid D-glucosamine structure, and lack of toxic reactions. Chitosan is also biodegradable, cheap and readily available (Crini and Badot, 2008, Amornchai et al., 2004, Krajewska et al., 1990, Martel et al., 2001, Meebungpraw et al., 2015).

The potential approach for water disinfection by CM-T could be by singlet oxygen/ROS generated in water by PDI driven by natural sunlight. ROS, including singlet oxygen undergoes rapid reactions with various classes of biomolecules but especially with proteins and unsaturated lipids. Singlet oxygen readily reacts with protein side chains with a bimolecular rate constant ranging from 10^5 to 10^9 M^{-1} S^{-1}. This is much higher than with other biomolecules including RNA, where the rate constant ranges from 10^4 to 10^6 M^{-1} S^{-1} (Cho et al., 2010, Davies, 2003). Side chains of some α-amino acids, principally the aromatic and sulphur containing amino acid residues, unsaturated lipids and nucleic acids are most likely the targets of PDI in microorganisms. These constituents are associated mainly with cellular and subcellular membranes such as plasma, mitochondrial, lysosomal and nuclear membranes in bacterial and eukaryotic cells, as well as the lipid envelopes, host attachment proteins, and other capsid proteins in viruses. However, little is known about the exact mechanism of PDI, especially in viruses because of diversity of the viral components. Gram positive bacteria are easily killed as compared to gram negative bacteria due to differences in the structure of their cell membrane (Alves et al., 2013, Bourre et al., 2010, Carvalho et al., 2007, Costa et al., 2012, Komagoe et al., 2011, Maisch et al., 2012a). Gram negative bacteria have an additional outer

membrane apart from the cytoplasmic (inner) membrane, giving them extra protection against antimicrobial agents including singlet oxygen and other ROS produced during photosensitisation. For viruses, although protein photo-oxidation by singlet oxygen has been extensively studied and the most sensitive viral components are protein in nature, it is an over-generalisation to assume a particular mechanism of protein photo-oxidation for viral PDI. This is because apart from diversity of PDI targets within viruses, the reaction of singlet oxygen with proteins can produce one or more of effects such as oxidation of side chains, peptide backbone fragmentation, dimerisation/aggregation, unfolding or conformal changes, enzymatic inactivation and alterations in cellular handling and turnover (Gracanin et al., 2009, Gracanin et al., 2007).

The objective of this work was to develop a sunlight-driven water disinfection system which is simple, cheap, re-usable, efficient and environmental friendly that could be used in rural areas of developing countries and or in developed countries in order to save energy. In this study, chitosan membranes were first modified by pyromellitic dianhydride in order to introduce carboxyl groups and facilitate electrostatic adsorption of the highly basic photosensitiser, TMPyP. The chitosan membrane-TMPyP composite produced was then used for photodynamic inactivation of MS2 and *E. coli* BL21. Both stationary and flowing water models were employed during PDI experiments in order to mimic what is obtainable in waste water treatment plants.

2. Materials and methods

2.1 Light source and conditions for PDI

The light source for PDI experiments was a Schott KL 2500 LCD (Schott Ltd., Stafford, UK) which provides a cool white light. Fluence rate of illumination during photoinactivation experiments were measured using a light meter (Clas Ohlson, UK).

Stationary and flow models were adopted for PDI using TMPyP functionalised chitosan membrane (CM-T). Visible light was used and fluence rates (radiant exposure) were 32 mW cm^{-2}. The buffer used for PDI was 1 x Phosphate buffer saline (PBS) (10 mM PO_4^{3-}, 137 mM NaCl, and 2.7 mM KCl) at pH 7.4, 20 - 22 ^oC under aerobic conditions.

2.2 Photosensitisers and other consumables

TMPyP, chitosan, pyromelitic dianhydride, succinic anhydride (Figure 1) and other consumables used were purchased without further purification from Sigma Aldrich unless otherwise stated. Reagents were the highest grade available.

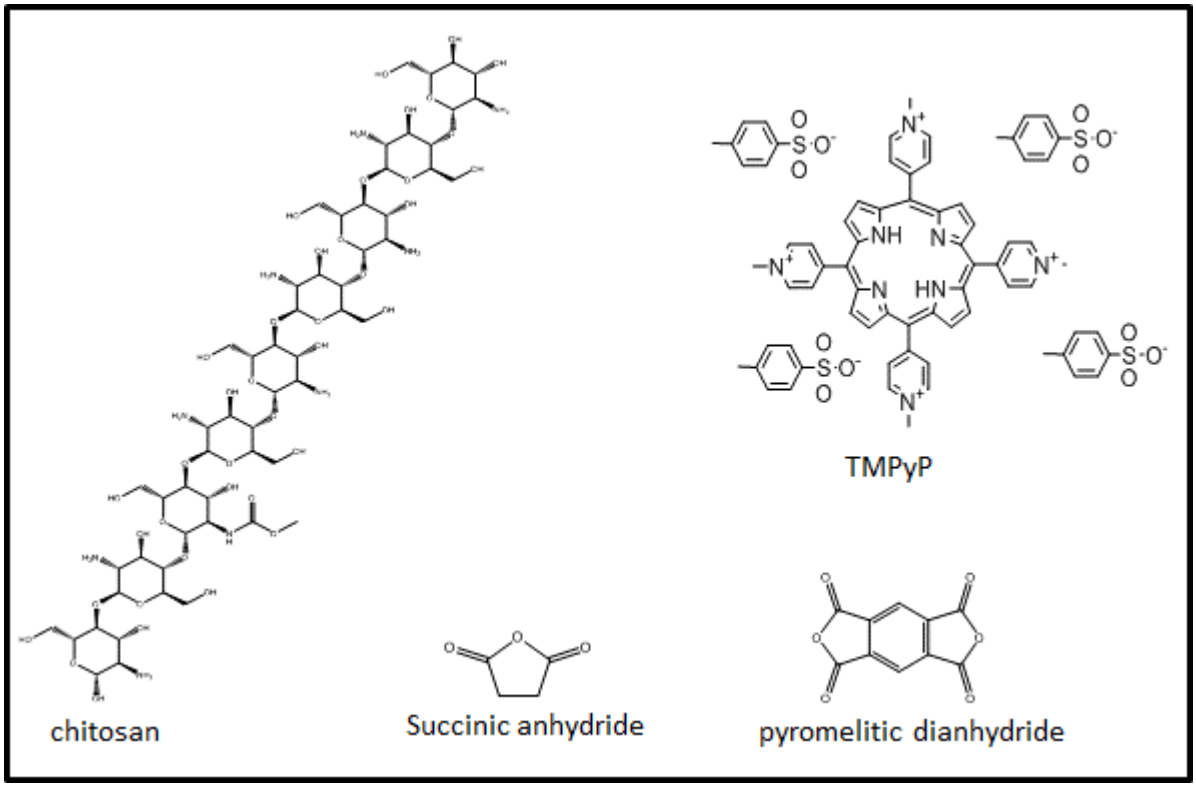

Figure 1: Chemical structures of TMPyP, chitosan, pyromelitic dianhydride and succinic anhydride. Prepared by using ChemDraw Pro 13.0.

2.3 Bacteriophage MS2 phage and E. coli strains

The MS2 phage ATCC 15597-B1 and *E.coli* host cell ATCC 15597 stocks were a kind gift from Prof. P.G Stockley, University of Leeds. BL21 *E. coli* were from standard laboratory stock.

2.4 Propagation and purification of bacteriophage MS2

One litre of exponential phase growth culture of *E. coli* (ATCC 15597) was infected with 300 µl of MS2 at 10^9 PFU/ml and incubated at 37 ^oC with shaking at 150 rpm for 48 h until *E. coli* cells were completely lysed. The lysate was then centrifuged at 4,000 x g rpm for 30 min to removed bacterial cell debris. The supernatant was precipitated using 50% (v/v) saturated ammonium sulphate overnight at 4 ^oC. After precipitation of the supernatant, the sample was centrifuged at 4,000 x g rpm for 30 min. Supernatant was discarded and the pellet re-suspended in 10 ml PBS. The re-suspended pellet was clarified again by centrifuging at 4,000

x g rpm for 30 min. Supernatant was taken and the pellet discarded. Phage particles in the supernatant were pelleted through 30% w/v sucrose cushion by ultracentrifugation at 32,000 rpm for 3 h using a Beckmann SW 32 Ti rotor. After ultracentrifugation, the supernatant was discarded and the pellet re-suspended in 800 µl PBS overnight. Re-suspended pellet was purified through 15% - 45% w/v sucrose gradient by ultracentrifugation at 50,000 rpm for 50 min using a Beckmann SW 55 Ti rotor. The gradient was then fractionated from top to bottom and fractions were analysed using SDS PAGE to identify the MS2 peak.

2.5 Infectivity and titre determination of bacteriophage MS2

The double layer agar plaque assay was used to determine the infectivity and titre of MS2 according to standard methods (Kropinski et al., 2009). Double layer agar plaque assays were undertaken (in duplicate) before and after PDI to determine titre after purification and the extent of PDI.

2.6 Production of chitosan membrane

Chitosan membranes were produced as described by Krajewska (1990, 1992). A solution of 1% (w/v) chitosan in 1% (v/v) acetic acid was made and then homogenised by shaking at 300 rpm and 60 ^{o}C for 30 min. The homogenised viscous chitosan solution was then cast into 4.5 cm diameter petri dishes, filling to depth of 3 mm. The petri dishes were placed in a hot air incubator set at 60 ^{o}C for 24 h to dry into a translucent chitosan membrane. The thickness of membranes after drying was about 50-100 µm. The chitosan membranes were then neutralised by treatment with 0.5% (w/w) of sodium triphosphate in 2M NaOH for 30 min. After neutralisation, the membranes were washed in distilled H_2O (dH_2O) until the solution was not alkaline and then air dried.

2.7 Modification of chitosan membrane using pyromelitic dianhydride

Each membrane was immersed in 1% (w/v) pyromellitic dianhydride (PMA) in DMSO contained in a closed bottle and then placed on a 3D rocking platform set at 30 rpm for 24 h at room temperature Control samples i.e. with PMA replaced with succinic anhydride were also used. The membranes were then washed in dH_2O several times and blow dried. The reaction scheme of PMA with chitosan amines is shown in Figure 2.

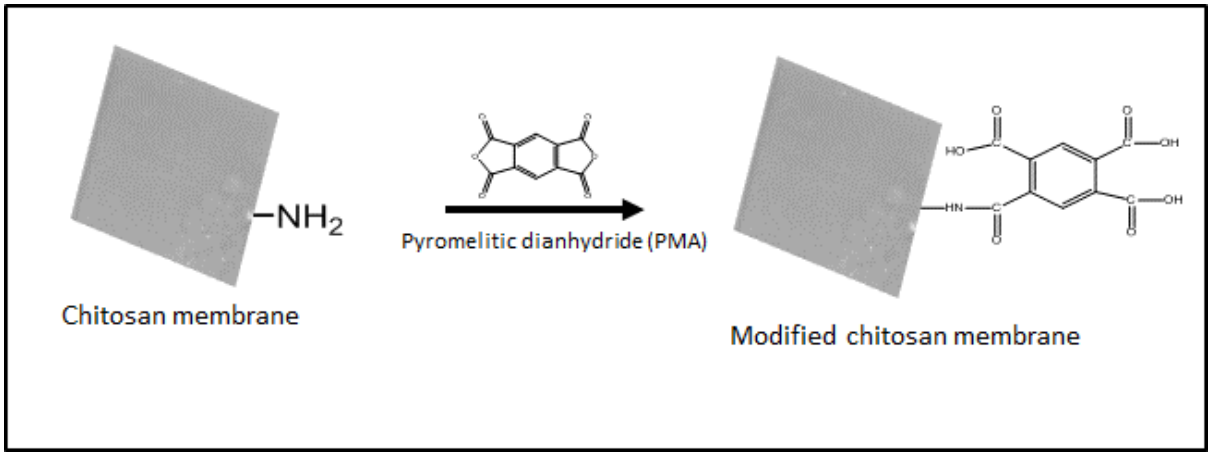

Figure 2: Reaction scheme for modification of chitosan membrane in order to introduce carboxyl groups and facilitate adsorption of the highly basic photosensitiser-TMPyP.

2.8 Midland blotting of PMA modified chitosan membrane

To detect free amine groups, PMA modified membranes and non-modified controls were incubated in the presence of NHS-biotin (4 mg/ml in PBS containing 20% (v/v) DMSO) for 30 min in order to attach biotin to the free amine groups. After three washes in dH_2O followed by drying in argon, the membranes were then incubated with HRP-streptavidin (1 µg/ml in PBS) for 30 minutes. Then, the membranes were incubated in the presence of an appropriate HRP-conjugated secondary antibody (1:1000 in PBS) for 1 hour to detect bound HRP-streptavidin. After the addition of the HRP-conjugated reagent, membranes were washed three times for 5 minutes each in PBS, once in PBS containing 0.1% (v/v) Tween-20 to aid

removal of non-specifically bound HRP-conjugated secondary antibody, with a final wash in PBS. The membranes were dried in argon in between incubations and after washing steps. Finally, ECL reagent was pipetted carefully onto the membranes and chemiluminescence detected after 1 minute using a G-BOX Gel Imaging System.

2.9 Functionalising PMA modified chitosan membrane with TMPyP

Modified membranes and non-modified membranes controls were stained in the dark with 200 µM of TMPyP placed on 3D rocking platform set at 30 rpm at room temperature. After staining, the membranes were thoroughly washed in dH_2O under mechanical agitation until zero absorbance from TMPyP was seen in the washed solution. Absorbance measurements at 421 nm (peak absorption of TMPyP) of reaction solution before and after immobilisation and wash solutions were determined. The amount bound of TMPyP per cm^2 of membrane will be the difference between the concentrations (µg/ml) of TMPyP before immobilisation and concentrations (µg/ml) of TMPyP contained in reaction solution and wash solutions after immobilisation.

2.10 PDI of bacteriophage MS2 using TMPyP functionalised chitosan membrane

PDI of MS2 was investigated using TMPyP-functionalised chitosan membrane (CM-T) illuminated at 32 mW cm^{-2}. Both stationary and flow water models were used. For the stationary model, CM-T was immersed in 10 ml 1x PBS containing MS2 at 10^9 PFU/ml while being illuminated. For the flowing water model, 10 ml 1x PBS containing MS2 at 10^8 PFU/ml was passed over the surface of CM-T using a peristaltic pump at a flow rate of 0.33 ml/min while being illuminated. Treatments were performed in the light with TMPyP loaded membranes (L), no TMPyP sensitiser membranes (NS) and with TMPyP membranes but in the

dark (D). All experiments were done in triplicate and a double layer agar plaque assay was used to evaluate MS2 viability after treatment.

2.11PDI of E.coli BL21 using TMPyP functionalised chitosan membrane

PDI of *E.coli* BL21 was investigated using CM-T illuminated for 90 min at 32 mW cm^{-2}. A stationary model was used for the PDI. Prior to PDI, 50 ml of 2 h log phase bacterial broth culture (about 10^8 CFU/ml) in TSB was washed three times by centrifugation at 1500 x g rpm for 10 min, supernatant removed and re-suspended in 50 ml PBS. From this, 10 ml each was used to set up 4 samples for PDI treatment using CM-T. Treatments were performed in the light with TMPyP loaded membranes (L), no TMPyP sensitiser membranes (NS) and with TMPyP membranes but in the dark (D). After PDI experiments, serial dilution of each sample was performed and dilutions plated for bacterial enumeration. Experiments were repeated in triplicate.

3. Results and discussion

3.1 PMA Modified and TMPyP functionalised chitosan membranes

Although the ultimate aim of this work was to get the TMPyP attached onto chitosan membrane before photodynamic inactivation of microbial pathogens in water, testing the photoinactivation capacity and efficiency of TMPyP in solution was the first step. To do this, we employed the bacteriophage MS2 as a model virus. Our data from preliminary data shows that TMPyP (at least 0.2 µM in solution) can achieve complete inactivation of MS2 within 60 sec when illuminated at 32 W cm^{-2} (Figure 3). At 10 seconds of illumination there were 1.5 log reductions in PFU/ml and at 30 seconds of illumination there were 4 log reductions in PFU/ml of MS2 (Figure 3). TMPyP alone in the dark (Dark experiment) or light alone (No sensitiser) do not cause any detectable reduction in log PFU/ml of MS2 (Figure 3). Previous work has shown that tetra-porphyrins like TMPyP can efficiently inactivate bacteriophages such as T4 in solution (Costa et al., 2008, Costa et al., 2010). It was reported that complete inactivation (>99.99% of inactivation, reduction of 7.2 log PFU/ml) of bacteriophage T4 from sewage was achieved only at highest TMPyP concentration of 5 µM and illuminated at 40 Wm^{-2} for 270 minutes. Complete inactivation within 1 minute was also reported but at higher concentrations of 1 mM and 0.01 mM of TMPyP illuminated at 2.2 mW cm^{-2} with a UV lamp (Casteel et al., 2004). In this work, we avoided using UV lamp as source of light and used cold visible light source instead, as UV light at certain wavelengths could inactivate microorganisms.

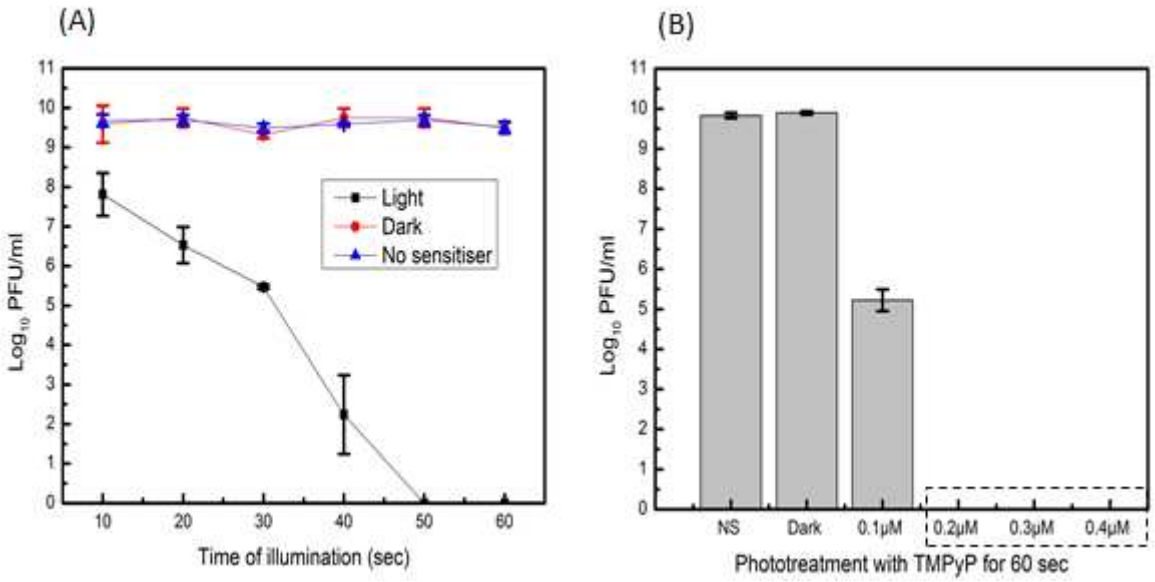

Figure 3: MS2 PDI in solution, illuminated at 32 mW cm^{-2}. (A), PDI using 0.5 µM TMPyP in solution from 10 to 60 sec. At 10 sec of illumination there were 1.5 log reductions in PFU/ml, at 30 sec of illumination there were 4 log reductions in PFU/ml and at 50 sec complete inactivation were observed. (B), PDI using different concentrations (0.1 µM to 0.4 µM) of TMPyP. Even 0.1 µM of TMPyP have caused 4 log reductions in PFU/ml after 60 seconds of illumination. Dark experiment was treated with 0.5 µM of TMPyP but was not exposed to light while NS (no sensitiser) was not treated with TMPyP but was exposed to light. Each value represents mean ± standard deviation of three independent experiments. Error bars show ± SD. ⬚; Complete inactivation.

It has been reported that photosensitiser-5,10,15,20-tetrakis(p-hydroxyphenyl) porphyrin (p-TAPP) could be immobilised onto polymeric chitosan membrane for possible water disinfection application (Bonnett et al., 2006). In that study, p-TAPP was attached onto chitosan membrane by adsorption without prior modification of the membrane and the p-TAPP-chitosan composite have shown photomicrobicidal activity against *E.coli* (Bonnett et al., 2006). However, in our case, direct adsorption of TMPyP onto polymeric chitosan membrane was not possible because the net charge of chitosan membrane is positive and is not optimal for adsorption of photosensitiser-TMPyP, which is a tetra cationic porphyrin (Figure 1). Therefore, the chitosan membranes were first modified by pyromellitic dianhydride (PMA) in order to introduce carboxyl groups and allow adsorption of the highly basic TMPyP (Figure 2).

PMA modifies primary amine groups on the membranes and generates three free carboxyl groups (Figure 2). The fourth carboxyl group becomes linked in a peptide bond to the amine (Figure 2). Scanning electron microscopy was undertaken to analyse the physical properties of PMA modified chitosan membranes as compared to unmodified chitosan membranes (Figure 4). The scanning electron microscopy images of dried modified chitosan membrane showed uniform contraction, compactness and folding of the membrane (Figure 4). The membranes modified with succinic anhydride became soluble in water while being washed in dH_2O (result not shown). Control experiments using succinic anhydride to modify the membrane amine group drastically affected its solubility and the whole membrane dissolved in water during washing steps in H_2O. In contrast, membranes modified with PMA remained intact while in water and became denser and more compact compared to unmodified membranes under both wet and dried condition as shown using SEM (Figure 4). It was expected that chitosan modification with acid anhydride derivatives might increase solubility in water as observed with succinic anhydride to modify the chitosan membrane. This was in agreement with a previous study (Tangpasuthadol et al., 2003) which reported that such modification with succinic anhydride could increase the hydrophilicity and hygroscopic property of the chitosan in film or powdered forms. However, unlike succinic anhydride PMA is a bis-anhydride with anhydride groups either side of an aromatic ring. Typically a PMA anhydride group will react with one amine group, with the other anhydride then being subjected to water attack. It is also likely that carboxyl-anhydride PMA molecules were able to form peptide bonds between two or more units of chitosan polymer, thereby crosslinking the chitosan units together. This could account for the compactness and insolubility in water of chitosan membranes modified with PMA (Figure 4). Chitosan membranes prepared as described by Krajeswka (1990, 1991) are brittle in nature(Bonnett et al., 2006). Some previous

studies have reinforced the chitosan membranes with nylon to overcome brittleness before functionalisation with photosensitisers (Bonnett et al., 2006). However, modification with PMA improved the mechanical strength of the membranes and therefore, there was no need for reinforcement of the membranes.

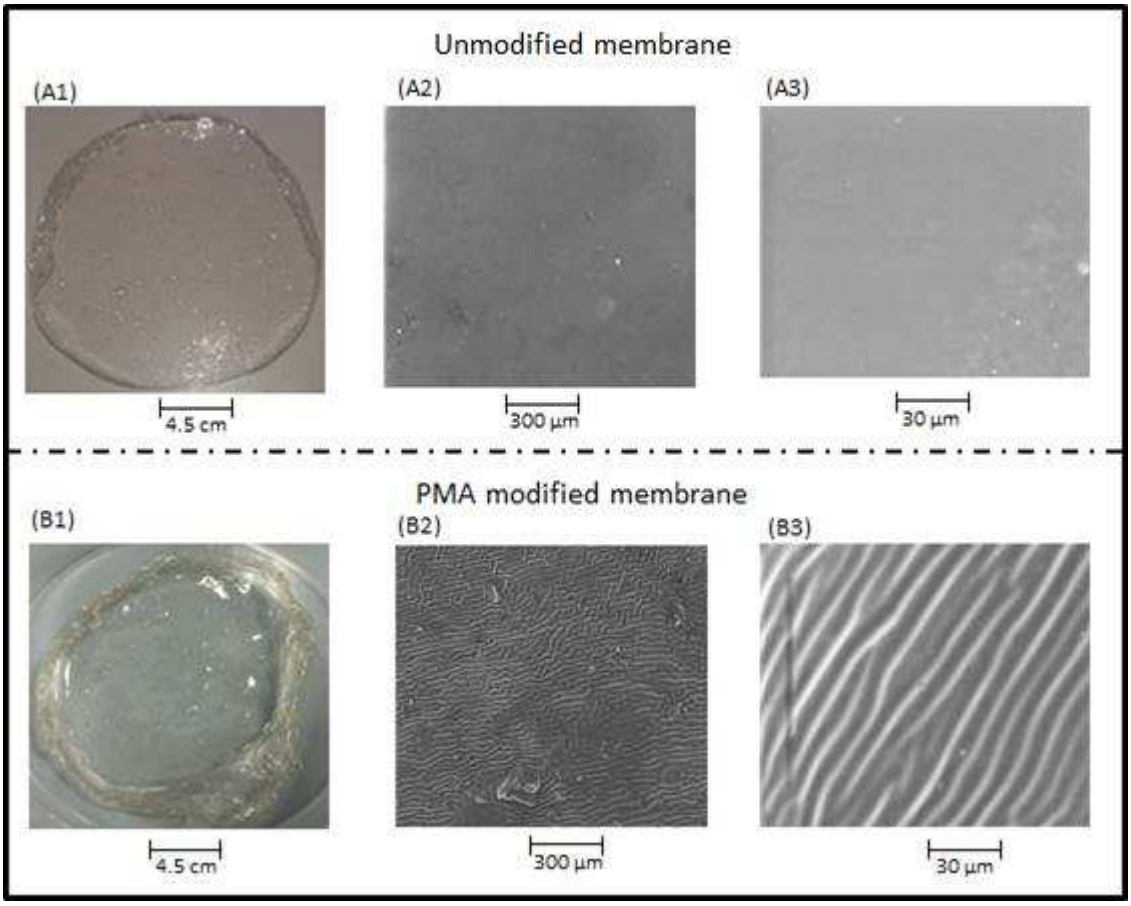

Figure 4: Images of PMA modified and unmodified chitosan membranes: (A1), unmodified chitosan membrane; (A2), (A3), SEM images of unmodified chitosan membrane; (B1), PMA modified chitosan membrane; (B2), (B3), SEM images of PMA modified chitosan membrane.

Midland blotting (Rushworth et al., 2014) was used to detect free amine (NH_2) groups in order to check complete modification of amine to carboxyl groups. The Midland blotting was

performed to analyse the chemical properties and success of PMA modified chitosan membranes as compared to unmodified chitosan membranes. Unmodified chitosan membranes have free amino groups at position 6 of the chitosan monomer while modified membranes have free carboxyl groups. Chemiluminescence was detected for unmodified membranes as they possess amino groups (and therefore became tagged by NHS-biotin, allowing subsequent labelling with streptavidin-peroxidase) while it was not detected for modified membrane as they have free carboxyl groups (Figure 5). The two controls; unmodified chitosan membrane but treated in DMSO and unmodified chitosan membrane showed chemiluminescence after Midland blotting thereby confirming the presence of free amino groups whilst PMA modified chitosan membrane did not show chemiluminescence after Midland blotting (Figure 5). The absence of chemiluminescence for the modified membranes shows that PMA modification was successful.

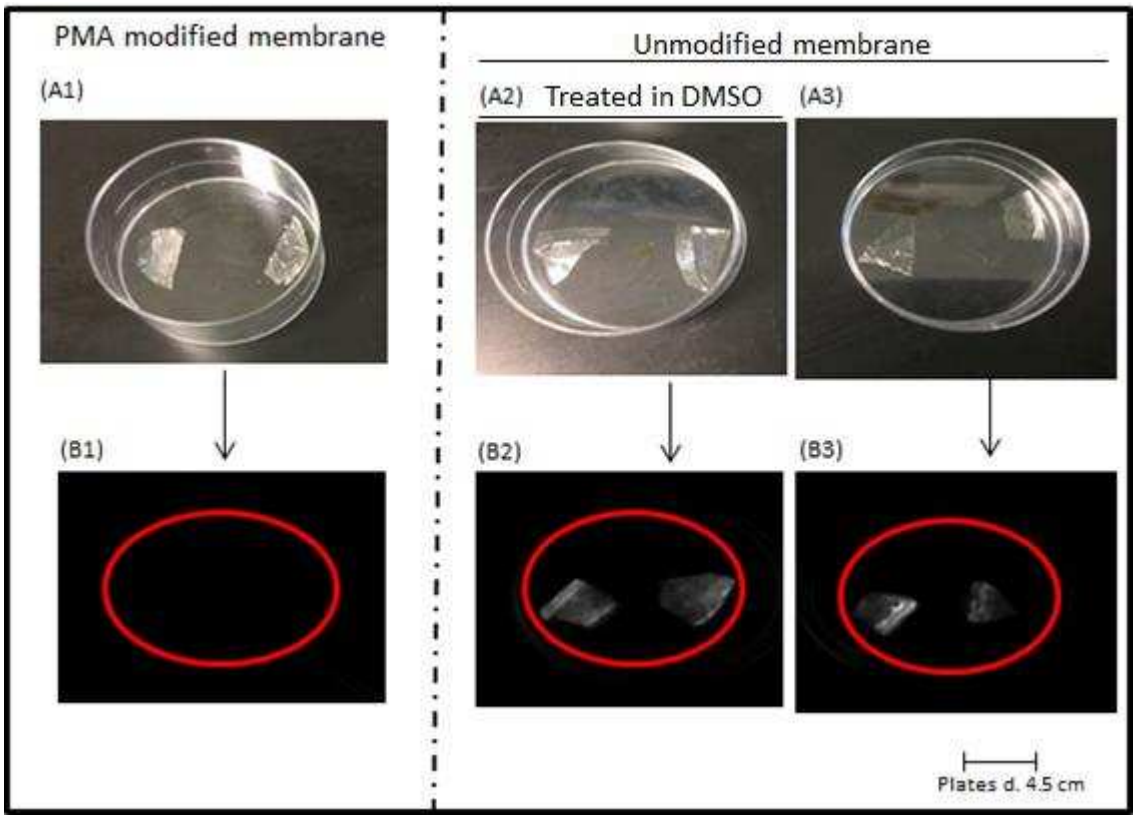

Figure 5: Images of chitosan membranes to confirm amine modification: (A1), PMA modified chitosan membrane; (A2), unmodified chitosan membrane but treated in DMSO; (A3), unmodified chitosan membrane; (B1), chemiluminescence not detected for PMA modified chitosan membrane; (B2), (B3), chemiluminescence detected for unmodified chitosan membranes.

PMA modified membrane and the 2 control samples (DMSO treated but unmodified membrane and unmodified membrane) were stained in TMPyP. However, only PMA modified membrane retained the TMPyP after vigorous washing in dH_2O (Figure 6). It is clear that most amino groups which gave net positive charge to the chitosan membranes had been modified allowing efficient adsorption of the cationic TMPyP. It is important to note that TMPyP attachment onto the PMA treated chitosan membranes is electrostatic and the dye could be released into PDI medium of very high ionic strength. We carried out all our investigations in PBS (10 mM PO_4^{3-}, 137 mM NaCl, and 2.7 mM KCl) and there was no release of TMPyP into

the solution as monitored by spectroscopy. In practice, most potential drinking water from the environment, freshwater streams or lakes will have low ionic strength. Within our study, monitoring the release of TMPyP from the chitosan membrane and washing to zero absorbance after staining of the membranes with TMPyP is very important as even 1 µM of TMPyP can achieve complete inactivation of MS2 phage in solution within few minutes. This is shown from preliminary PDI investigations using TMPyP in solution (Figure 3).

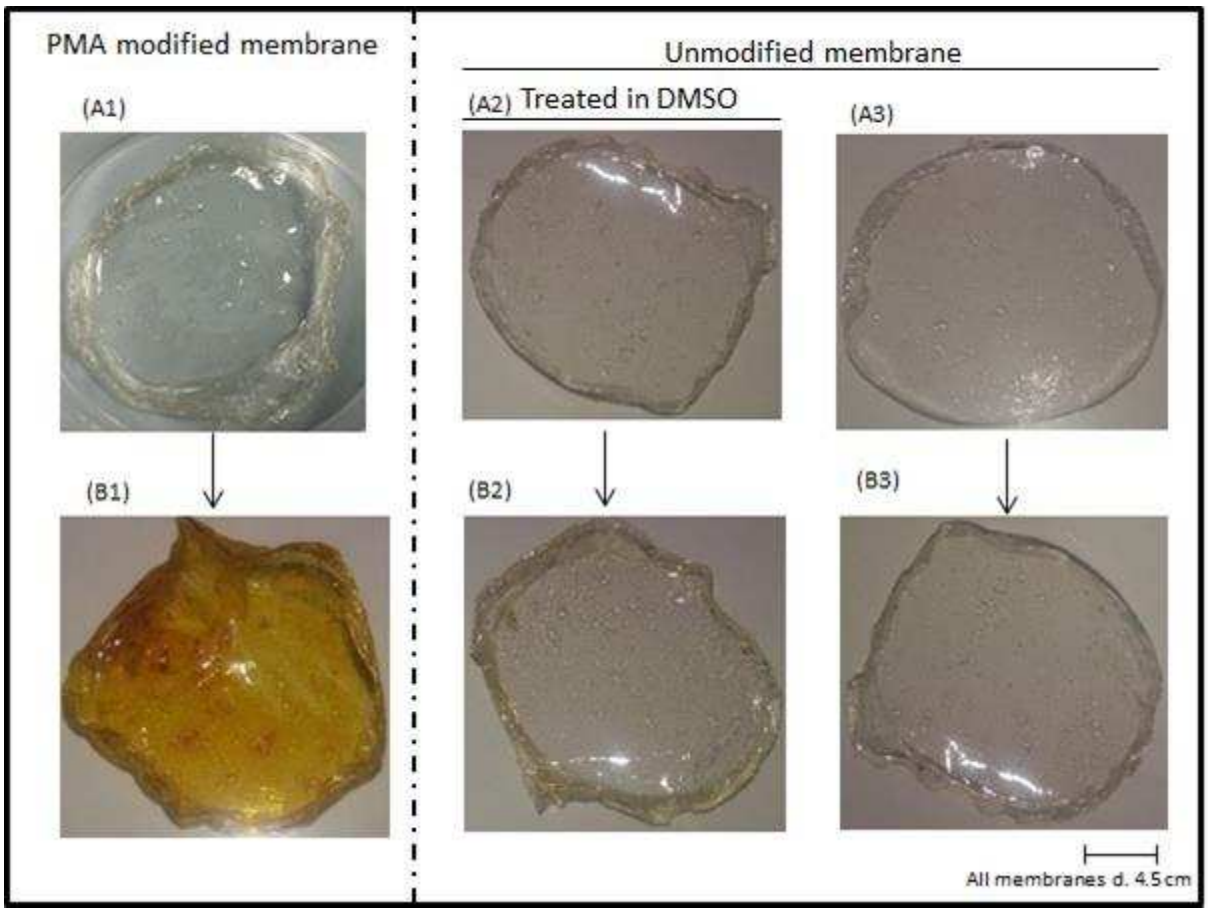

Figure 6: Chitosan membranes functionalised with TMPyP. (A1), PMA modified membrane; (A2), DMSO treated but unmodified membrane; (A3), unmodified membrane. The corresponding samples after staining with TMPyP and washing in H_2O are shown in B1-B3 respectively.

3.2 PDI of bacteriophage MS2 and E.coli BL21 using TMPyP functionalised chitosan membrane

TMPyP functionalised chitosan membranes (CM-T) were used to investigate microbial inactivation of MS2 and *E. coli* BL21 by PDI and for both, use of CM-T resulted in photodynamic inactivation (Figure 7 and Figure 8). For the MS2 PDI stationary model, complete inactivation (>99.99% of inactivation, reduction of 9.6 log PFU/ml) of MS2 was observed with CM-T after 90 min illumination with the light at 32 mW cm^{-2} (Figure 7). For the flowing water model, complete inactivation of MS2 (>99.9% of inactivation, reduction of 8.8 log PFU/ml) was observed for sample passed twice over the surface of CM-T at 0.33 ml/min while being illuminated. Reduction of approximately 3 log PFU/ml were observed for sample passed once under the same conditions (Figure 7). The same light intensity at 32 mW cm^{-2} was chosen and used as in our PDI studies in solution. This was to allow genuine comparison between rate and extent of PDI with TMPyP in solution and while attached onto chitosan membrane. This light intensity is low and it is just about 3% of bright midday sunlight under clear sky conditions in sub-Saharan Africa. And subsequent PDI investigation will be employ much higher light intensities. However, use of real world light intensities would just give complete inactivation and reveal little detail of the inactivation rates. It is also clear that the efficient PDI capacities of CM-T at low light intensity mean it could also be used in the UK and other northern European countries; in these nations the key advantage would be reduction in energy use for water treatment. The rate of PDI with TMPyP attached onto chitosan membrane is slower as compared to TMPyP in solution (Figures 3, 7). This was expected as proximity of unattached TMPyP in solution to the microorganism will be greater as compared with attached TMPyP. However, the ultimate aim was achieved as CM-T can cause PDI of MS2 phage under flow conditions and re-use with no detectable decline in its PDI capacity and efficiency.

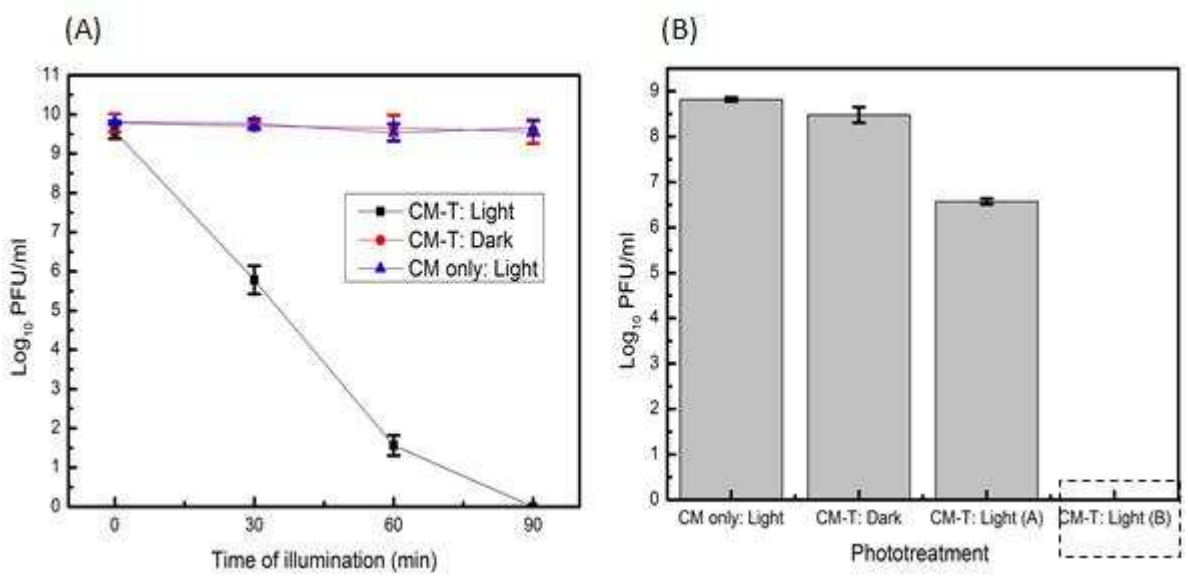

Figure 7: PDI of MS2 using TMPyP functionalised chitosan membrane, illuminated at 32 mW cm^{-2}. (A), stationary water model, It took 90 min for complete inactivation of MS2 (>99.9% of inactivation, reduction of 9.7 log PFU/ml); (B), flowing water model, CM-T: Light (A) was a sample passed once over the surface of CM-T at 0.33 ml/min while being illuminated, CM-T: Light (B) was a sample passed twice over the surface of CM-T at 0.33 ml/min while being illuminated. Complete inactivation of MS2 (>99.9% of inactivation, reduction of 8.8 log PFU/mL) was observed for CM-T: Light (B) while reduction of about 3 log PFU/ml were observed for CM-T: Light (A). CM-T: Dark was not exposed to light while CM only: Light (chitosan membrane not functionalised with TMPyP) was exposed to light. Each data point mean ± SD of three independent experiments. Error bars show ± SD.

For the photodynamic inactivation of *E.coli* using CM-T under the same stationary model used for MS2 phage, reduction of only 3 log CFU/ml of the bacteria was observed (Figure 8). Our data have also shown that washing of the cells lead to reduction of about 1 log CFU/ml of the bacteria and the actual photodynamic inactivation was about 2 log reductions under that conditions. It is important to state that more time of illumination and higher light intensity can increase the log reductions of the bacteria as these factors are proportional related to the extent of photodynamic inactivation. However, the use of MS2 photodynamic inactivation

(stationary model) conditions for the *E.coli* experiment allowed direct comparison. TMPyP in solution can bind to bacteria cell membranes, partition between lipid bilayer and even move into the cells to activate killing process in addition to ROS generated in solution. However the only possibility of PDI with TMPyP attached unto chitosan membrane will be direct cell damage by singlet oxygen (or other ROS) generated close to the support surface.

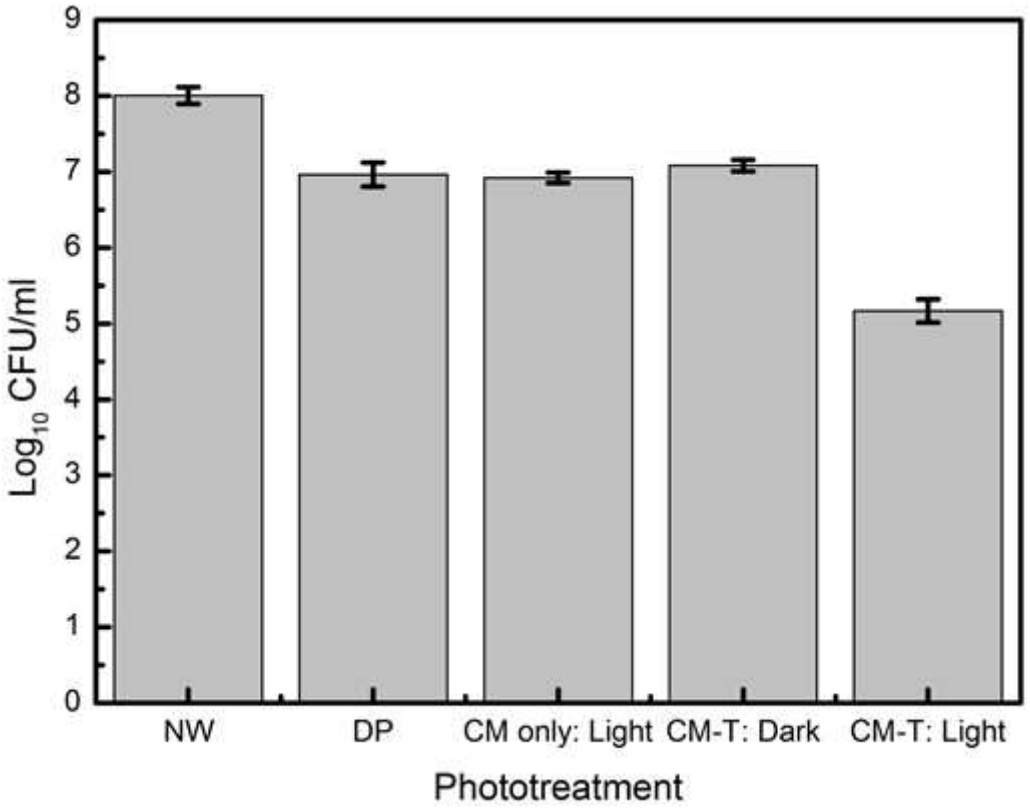

Figure 8: PDI of *E. coli* BL21 using TMPyP functionalised chitosan membrane, illuminated at 32 mW cm^{-2}. CM-T: Light, reduction of about 3 log CFU/ml was observed after 90 min of illumination. CM-T: Dark, was not exposed to light. CM only: Light (chitosan membrane not functionalised with TMPyP), was exposed to light. DP (direct plating) was washed but not treated either with CM-T or light and NW (cells not washed) and not treated either with CM-T or light. Each value represents mean ± standard deviation of three independent experiments. Error bars show ± SD.

4. Conclusions

No successful disinfection of water using the photodynamic effect of a photosensitiser during wastewater treatment has been reported in either developed or developing countries. However, there is ongoing worldwide research into the possibility of using photodynamic disinfection during wastewater treatment. Trending and emerging issues in this area have been finding the right solid supports, coupling chemistries and photosensitisers of appropriate properties and qualities suitable for sustainable usage in water disinfection. The driving force behind the research includes potential drastic cutting down of energy and chemical usage to the barest minimum as sunlight could be used as the source of light for the photodynamic effect and photosensitisers could be attached on to solid supports so that after phototreatment of water the supported photosensitiser is not released into the water. Photosensitiser-functionalised solid support could be re-used for water disinfection several times thereby making it cheap and environmentally friendlier compared to conventional methods of water disinfection during wastewater treatment.

This work has shown the possibility of making a simple, environmentally friendly and zero-man made energy input device for water disinfection. The CM-T has a number of merits:

It employed the use of chitosan membrane which is non-toxic, biodegradable, wettable, cheap and readily available, and of good mechanical strength to withstand the rigorous process of water disinfection without tear and wear. We described a method of modifying chitosan membrane without affecting its solubility in water using PMA. PMA modified chitosan membrane could also probably be used to adsorb positively charged dyes and heavy metals from contaminated water thereby remediating the water totally although this effect is not its primary function. CM-T was used and has shown that it has photodynamic inactivation capacity. CM-T has been re-used several times for the same purpose with no

detectable decline in its PDI capacity and efficiency. This will ultimately reduce cost. Furthermore, the flowing water model for PDI mimics something close to the process in water treatment plants.

The fact that CM-T was able to photodynamic inactivate MS2 and *E. coli* while using light intensity of 32 mW cm^{-2} which is low compared to daytime sun brightness, is an indication that this research can lead way to simple sunlight driven water disinfection system that could be used as a zero man-made energy input systems to produce clean and safe drinking water in both developed and developing countries.

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