



## Photo-Antiviral Activity of Cationic and Anionic Dyes

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### ABSTRACT

Photo-antiviral activity of organic dyes, porphyrins, phthalocyanines, aromatic hydrocarbons and quinones has been shown to be an efficient alternative antiviral agent in the control of resistant and emerging viruses. These high coloured compounds are photosensitisers and in the presence of light and oxygen in solution generates singlet oxygen and other reactive oxygen species which can ultimately inactivate viruses and other microorganisms. Although many photosensitisers can have good yield of singlet oxygen, it has been shown that the type of photosensitiser and the number of their charge in aqueous solution affects the rate and extent of photodynamic inactivation of microorganisms. In this work, we used three (3) different photosensitisers which are TMPyP (tetra-cationic porphyrin), methylene blue (mono-cationic phenothiazinium dye) and Rose Bengal (di-anionic xanthene dye) to carryout photodynamic inactivation of a bacteria virus MS2. The results observed in this study showed that the type and concentration of the photosensitisers, time of illumination and light intensity all affect the rate and extent of PDI of MS2. The photosensitiser TMPyP is the most efficient in facilitating PDI of MS2 compared to the other photosensitisers tested, Rose Bengal and methylene blue. The order of rate and extent of PDI of MS2 by these photosensitisers in solution was TMPyP >>> methylene blue > Rose Bengal. This is likely to be related to the net charge on these photosensitisers as it has been established that cationic photosensitisers are generally more efficient in the PDI of microorganisms including viruses.

**Keywords:** MS2, singlet oxygen, TMPyP, Methylene blue, Rose Bengal, photodynamic inactivation

### INTRODUCTION

Organic dyes (Rose Bengal, eosin, methylene blue etc.), aromatic hydrocarbons (naphthalenes, anthracenes, biphenyls etc.), porphyrins, phthalocyanines, quinones and anthraquinone derivatives are classes of photosensitisers (Figure 1) that have been shown to have good quantum yield of singlet oxygen in solution. Photosensitisers, when irradiated with visible light and in the presence of oxygen can generate singlet oxygen and other reactive oxygen species (ROS) in aqueous solution which may efficiently inactivate microorganisms including viruses (Figure 2). The ability and effectiveness of singlet oxygen and other ROS to oxidise biomolecules has been studied and exploited with some success in the treatment of cancer, dental and dermatological diseases, as well as for synthetic chemistry and environmental remediation (DeRosa and Crutchley, 2002, Costa et al., 2013, Dolmans et al., 2003,

Dwivedi and Pande, 2012, Lucena et al., 2015, Usuda et al., 2006).

Photodynamic inactivation (PDI) of viruses has been shown to be an efficient alternative to antiviral agents in the control of resistant and emerging viruses (Casteel et al., 2004, Costa et al., 2008, Costa et al., 2010, Costa et al., 2009, Wainwright, 2004). Singlet oxygen and other ROS can react rapidly and cause irreversible damage to biomolecules thereby leading to the inactivation of viruses and other microorganisms (Costa et al., 2013). Singlet oxygen is the most likely ROS involved in the viral PDI (Costa et al., 2013, Silverman et al., 2013). All ROS have a short-life and high reactivity, thereby causing damage only to the surrounding molecules close to the point of

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ROS generation (Costa et al., 2013). Capsid proteins including host recognition proteins are immediate targets of singlet oxygen mediated oxidation in non-enveloped viruses, while envelope glycoproteins, including host-recognition proteins are potential targets of

singlet oxygen oxidation in enveloped viruses. Prolonged exposure to singlet oxygen may also result in oxidative damage to viral nucleic acid (Davies, 2003, Gracanin et al., 2009, Hotze et al., 2009, Cho et al., 2010).

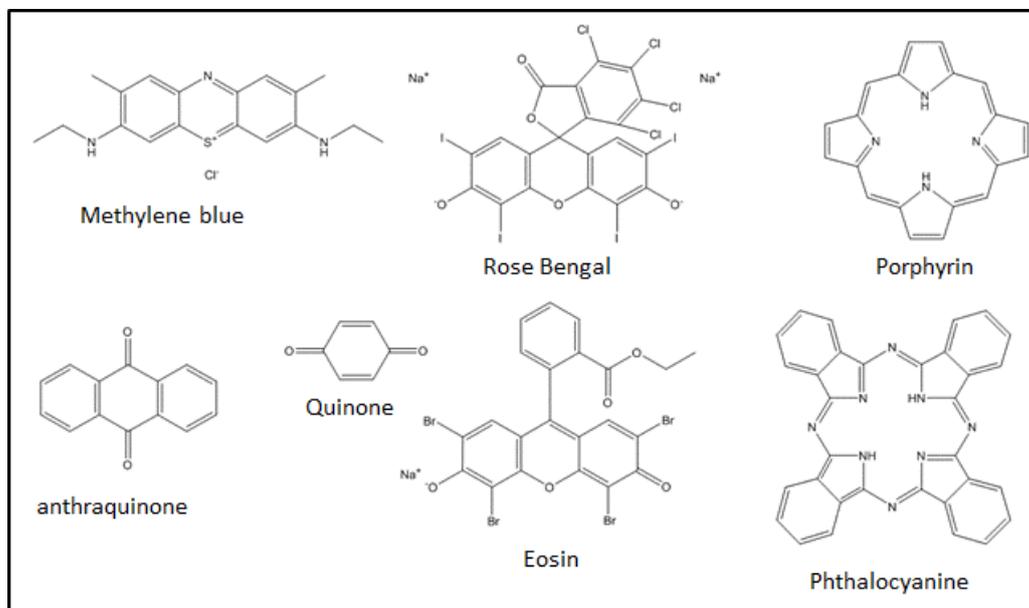


Figure 1: Chemical structures of some groups of photosensitisers. Prepared by using ChemDraw Pro 13.0.

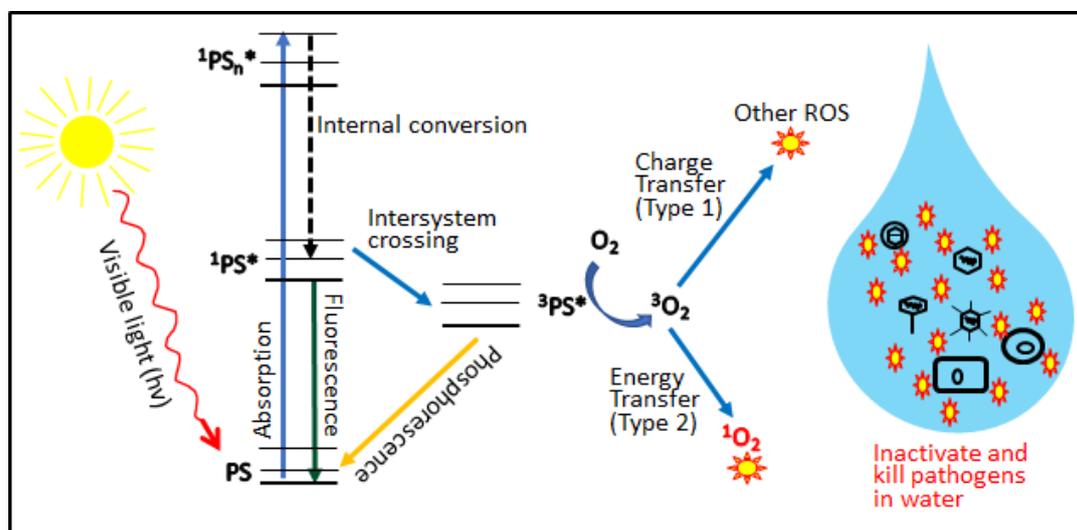


Figure 2: Jablonski diagram showing photosensitisation. PS, photosensitiser at ground state;  $^1PS^*$ , photosensitiser at singlet excited state;  $^3PS^*$ , photosensitiser at triplet excited state;  $O_2$ , molecular oxygen in aqueous solution;  $^3O_2$ , triple oxygen;  $^1O_2^*$ , singlet oxygen; other ROS:  $O_2^{\cdot-}$ , superoxide anion;  $O_2^{\cdot-2}$ , peroxide;  $\cdot OH$ , hydroxyl radical;  $OH^-$ , hydroxyl ion. (Costa et al., 2013, DeRosa and Crutchley, 2002).

Photodynamic inactivation (PDI) of viruses has been shown to be an efficient alternative to

antiviral agents in the control of resistant and emerging viruses (Casteel et al., 2004, Costa et al., 2008, Costa et al., 2010, Costa et al., 2009, Wainwright, 2004). Singlet oxygen and other

Although many photosensitisers can have good yield of singlet oxygen, it has been shown that the type of photosensitiser and the number of their charge in aqueous solution affects the rate and extent of photodynamic inactivation of microorganisms. Some experts have shown and argued that since most microorganisms have net negative charge, cationic photosensitisers should be more efficient in PDI of bacteria and viruses. This is due to positive charge favouring binding of photosensitiser molecule at critical cellular sites, that once damaged by exposure to light, cause loss of cell viability. Also, because the negatively charged microorganisms can bind to the positively charged photosensitiser, this increases the proximity microorganisms to the singlet oxygen generated. Thus, rate and extent of PDI is faster and higher as compared to anionic and neutral photosensitisers (Alves et al., 2009, Eichner et al., 2012). Some few studies to determine the effect of the photosensitiser type and number of charge on the inactivation of microorganisms were done using bacteria models (Alves et al., 2009, Eichner et al., 2012). However, there is only one study to the best of our knowledge that showed the effect of photosensitiser's charge on the inactivation of viruses (Costa et al., 2008). In that study anionic photosensitiser was not used and only di-, tri- and tetra-cationic porphyrins were used.

In this work we used three (3) different photosensitisers which are TMPyP (tetra-cationic porphyrin), methylene blue (mono-cationic phenothiazinium dye) and Rose Bengal (di-anionic xanthene dye) to carryout photodynamic inactivation of a bacteria virus MS2.

## METHODOLOGY

### Light source, photosensitisers and virus strain and host bacteria cells

A Schott KL 2500 LCD (Schott Ltd., Stafford, UK), which provides a cool white light in the visible region of the spectrum was applied as the light source for the PDI experiments. The spectral properties of the light source was determined using a QE Pro, high sensitivity spectrometer, (Ocean Optics, USA). Fluence rates of illumination during photoinactivation experiments were measured using a light meter (Clas Ohlson, UK). Photosensitisers-TMPyP, methylene blue (MB) and Rose Bengal (RB) were purchased without further purification

from Sigma Aldrich. Absorption spectra of the photosensitisers in phosphate buffered saline (PBS) (10 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.8 mM KH<sub>2</sub>PO<sub>4</sub>, 137 mM NaCl, and 2.7 mM KCl) were determined using a NanoDrop 2000<sub>C</sub> micro-spectrometer (Thermo Scientific) at 20-22 °C under aerobic conditions and a light intensity of 32 mWcm<sup>-2</sup>. Stock of bacteriophage MS2 the *E. coli* host cells were a gift from Prof. P. Stockley University of Leeds, UK..

### Detection of singlet oxygen generated by photosensitisers

Singlet oxygen generated by photo-irradiation in the presence of photosensitiser was measured by spectrophotometry. The method is based on the measurement of a decrease in the absorbance (318 nm) of 2-amino-3-hydroxypyridine when it reacts with singlet oxygen (Komagoe et al., 2011). An assay solution of 2-amino-3-hydroxypyridine (200 µM) and photosensitiser (1 µM) dissolved in PBS was put in a conventional quartz cell with a light path length of 1 cm and illuminated at 76.63 Wm<sup>-2</sup> for 1 - 5 minutes and changes in the A<sub>318</sub> before and after photo-irradiation were measured.

### Propagation and purification of bacteriophage MS2

The bacteriophage MS2 was propagated in *E. coli* and purified as previously described (Majiya et al., 2018).

### Photodynamic inactivation of MS2

A range of concentrations of the photosensitisers 0.5, 1 and 5 µM were applied at light intensities of 32 and 466 mW.cm<sup>-2</sup> for durations of illumination 1 and 5minutes. As controls, dark experiments (D) were carried out in the presence of photosensitizer but without illumination, while "no sensitiser" (NS) experiments were exposed to light but in the absence of photosensitiser. All experiments were repeated in triplicate.

The double layer agar plaque assay (Kropinski et al., 2009) was used to determine the infectivity and titre of bacteriophages MS2 after purification and before and after PDI to know the rate and extent of PDI.

### Statistical and graphical software

Data from different experiments were imported to Microsoft Excel. All graphical outputs were plotted in Origin Pro 8. The

schematic was drawn in Microsoft Powerpoint. Chemical structures were drawn using ChemDraw Pro v13.0

## RESULTS AND DISCUSSION

### Spectral properties of PDI light source and absorbance spectra of photosensitisers

Spectral properties of the PDI light source (Schott KL 2500 LCD, Schott Ltd., UK) and the absorbance spectra of photosensitisers-TMPyP, MB and RB were determined. The PDI light source spectra (400 nm – 786 nm) (Figure 3 A) showed that it mainly emitted visible light including some near infrared. However, the emission was within the visible light regions. The PDI light spectra and its peak did not seem to be affected drastically by the different light intensities and colour temperatures/ filters available (Figure 3 A). The spectra absorption peaks for 32 mW.cm<sup>-2</sup> (2650 K, A), 200 mW.cm<sup>-2</sup> (2950 K, A), 450 mW cm<sup>-2</sup> (3000 K, B) and 950 mW cm<sup>-2</sup> (3000 K, C) were at 661 nm, 642 nm, 643 nm and 645 nm respectively (Figure 3 A). The absorption spectra of the photosensitisers in PBS showed that their peak absorption was within visible light regions (Figure 3 B). The absorption peaks for TMPyP, MB and RB were at 422 nm, 550 nm and 666 nm respectively (Figure 3 B).

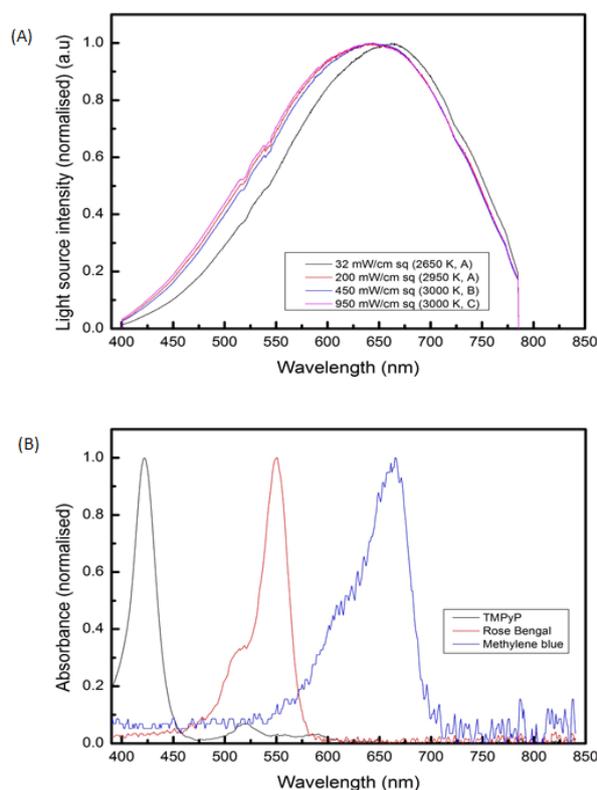


Figure 3: Spectral properties of PDI light source and absorption spectra of photosensitisers. (A), light spectra of our PDI light source (Schott KL 2500 LCD, Schott Ltd., UK). The PDI light source spectra (400 nm – 786 nm) showed that it is mainly emitted visible light including some near infrared. The spectra peak is between 641 nm - 661 nm; (B), absorption spectra of the photosensitisers used in PDI investigations. The absorption peaks for TMPyP, Rose Bengal and methylene blue were at 422 nm, 550 nm and 666 nm respectively.

### Detection of singlet oxygen generated by photosensitisers

Spectrophotometric measurement of the decrease in  $A_{318}$  of 2-amino-3-hydroxypyridine (AHP) when reacted with singlet oxygen was used to detect the singlet oxygen generated by TMPyP, RB and MB. An assay solution of 2-amino-3-hydroxypyridine (200  $\mu$ M) and a photosensitiser (1  $\mu$ M) dissolved in PBS was put in a conventional quartz cell with a light path length of 1 cm, and photo-irradiated with light of fluence rate of 466.45 mW.cm<sup>-2</sup> for 1 to 5 minutes. Changes in  $A_{318}$  before and after photo-irradiation were measured (Figure 4).

There are several methods of detecting singlet oxygen generated by photosensitisers in solution (Komagoe et al., 2011, Kohn and Nelson, 2007, Kraljic and Mohsni, 1978). We measured singlet oxygen produced by the photosensitisers. (Komagoe et al., 2011). However, it is an indirect and semi-quantitative method. (Komagoe et al., 2011). Our data showed that singlet oxygen was generated by photosensitisers in solution as shown by the decrease in  $A_{318}$  (Figure 3.14). The decrease in  $A_{318}$  was proportionally related illumination time (Figure 3.14), and the implication of this is that photodynamic inactivation of waterborne pathogens using these photosensitisers (TMPyP, RB and MB) will also be dependent on the illumination time amongst other factors.

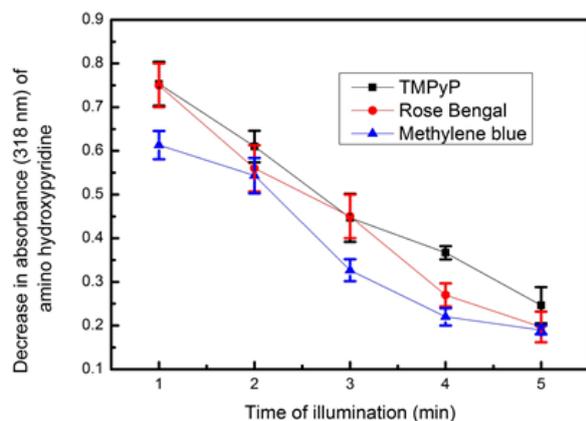


Figure 4: Decrease in absorbance (318 nm) of 2-amino-3-hydroxypyridine due to singlet oxygen generated by the photosensitisers in solution after illumination at different time. Data are mean  $\pm$  standard deviation ( $n = 3$ ).

#### Photodynamic inactivation (PDI) of MS2

The PDI conditions of  $0.5 \mu\text{M}$  photosensitisers each,  $32 \text{ mW}\cdot\text{cm}^{-2}$  and 1 minute of illumination were first used to investigate the PDI of MS2. These PDI conditions caused complete inactivation of MS2 with TMPyP in solution (Figure 5 A) while no inactivation was observed with MB and RB in solution (Figure 5 A). Also, other PDI conditions ( $1 \mu\text{M}$  and  $5 \mu\text{M}$  of RB and MB,  $32 \text{ mW}\cdot\text{cm}^{-2}$  and  $466 \text{ mW}\cdot\text{cm}^{-2}$  and 5 min of illumination) were used to investigate PDI of MS2 in solution. Our data showed that even at higher concentrations of  $1 \mu\text{M}$  and  $5 \mu\text{M}$  each of RB and MB, no inactivation of MS2 was observed for samples illuminated at  $32 \text{ mW}\cdot\text{cm}^{-2}$  for 5 min (Figure 5 B). However, when illuminated at  $466 \text{ mW}\cdot\text{cm}^{-2}$  for 5 min, reductions of 3 log PFU/ml of MS2 and complete inactivation were observed for samples treated with  $1 \mu\text{M}$  and  $5 \mu\text{M}$  of MB respectively (Figure 5 B). At this light intensity and 5 min illumination, only reduction of 1 log PFU/ml MS2 was observed for sample treated with  $5 \mu\text{M}$  RB and no inactivation was observed for sample treated with  $1 \mu\text{M}$  RB (Figure 5 B).

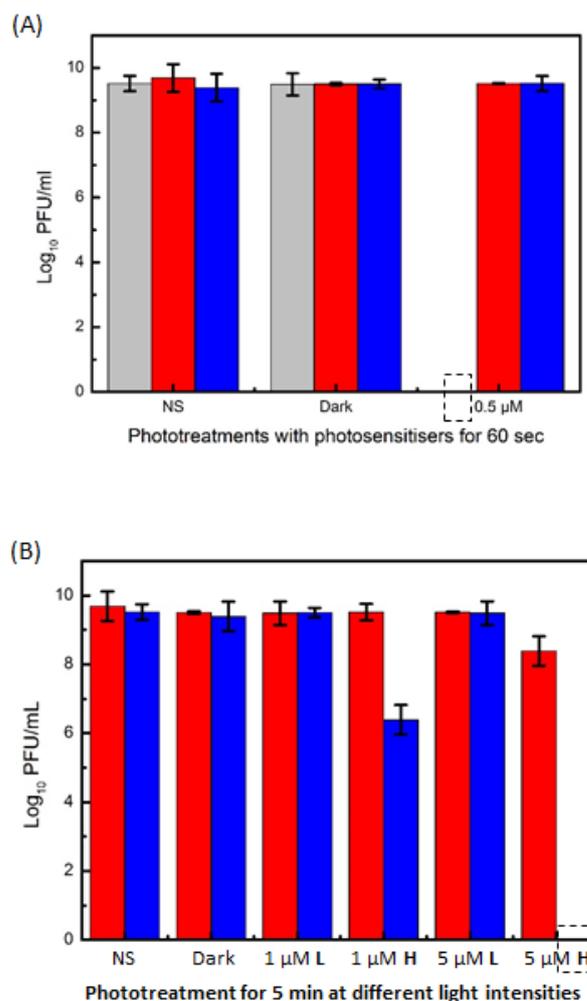


Figure 5: MS2 phage PDI in solution using TMPyP, Rose Bengal and methylene blue. (A), PDI using  $0.5 \mu\text{M}$  of photosensitisers each and illuminated at  $32 \text{ mW}\cdot\text{cm}^{-2}$ ; (B), PDI using different light intensities. Samples with  $1 \mu\text{M}$  and  $5 \mu\text{M}$  photosensitisers were illuminated at  $32 \text{ mW}\cdot\text{cm}^{-2}$  which is a low (L) light intensity. Also, samples with same concentrations of the photosensitisers were illuminated at  $466 \text{ mW}\cdot\text{cm}^{-2}$  which is a high (H) light intensity. The dark controls were treated with the concentration of photosensitiser shown but not illuminated whilst no photosensitiser controls (NS) were illuminated without photosensitiser present. Data are mean  $\pm$  standard deviation ( $n = 3$ ).

(■), TMPyP; (●), Rose Bengal;  
(▲), Methylene blue; (□), Complete inactivation

## DISCUSSION

We used 3 photosensitisers (TMPyP, Rose Bengal and methylene blue) for the PDI of our model virus – bacteriophage MS2 in solution and we found out that TMPyP was the most efficient. TMPyP is a porphyrin, Rose Bengal is a xanthene dye and methylene blue is a phenothiazinium dye (DeRosa and Crutchley, 2002). These photosensitisers have the following required properties of a good photosensitiser;

- i. High absorption coefficient in the visible spectra; the emission spectra of light source we used in this work was 400 nm – 786 nm and the absorption peaks for TMPyP, Rose Bengal and methylene blue were at 422 nm, 550 nm and 666 nm respectively. Also, sunlight could as well be used to excite these photosensitisers. A typical solar spectrum (solar AM1.5) is 300- 1400 nm (Loeb et al., 2016) with a peak at 500 – 600 nm (Jemli et al., 2002).
- ii. A triple state of appropriate energy ( $ET \geq 95 \text{ kJ mol}^{-1}$ ); this allows for efficient energy transfer to ground state oxygen in the water which can then be converted to singlet oxygen. The ET of methylene blue and Rose Bengal are  $133.9 \text{ kJ mol}^{-1}$  and  $175 \text{ kJ mol}^{-1}$  respectively (Loeb et al., 2016, DeRosa and Crutchley, 2002).
- iii. High quantum yield of the triple state ( $\Phi_T > 0.4$ ) and long triplet state lifetimes ( $TT > 1 \mu\text{s}$ ); the efficiency of the photosensitiser is dependent on the photophysical properties of its lowest excited triple state. The  $\Phi\Delta$  (quantum yield) of methylene blue and Rose Bengal in aqueous solution are 0.52 and 0.76 respectively (DeRosa and Crutchley, 2002). The singlet oxygen  $\Phi\Delta$  (which is directly proportional to triple state energy and quantum yield of the triple state of the photosensitiser) of TMPYP is 0.74 in PBS (Lei et al., 2010).

The photosensitisers TMPyP, RB and MB are all soluble in water and have their absorption peaks within visible light range (Figure 3 B). This means that our source of light for PDI which generates only visible light (Figure 3 A) can excite these photosensitisers to generate singlet oxygen and other ROS in solution and ultimately inactivate MS2 and other

microorganisms. We have also confirmed that the photosensitisers do generate singlet oxygen (Figure 4). Many studies have been reported that TMPyP, RB and MB can cause PDI of viruses in solution (Casteel et al., 2004, Wainwright, 2004, Cho et al., 2010). However, our data (Figure 5) indicated that TMPyP is the most efficient in the PDI of MS2. This may be due to differences in the net charges of the photosensitisers used. In solution, TMPyP will have net charge of +4, methylene blue +1 and Rose Bengal -2. Attraction between MS2 (negatively charged) and TMPyP will be greater and thus there will be better proximity of MS2 to singlet oxygen generated by TMPyP. This is in agreement with many reports that tetra and tri cationic photosensitisers causes rapid and high rates of inactivation of microorganisms during PDI (Costa et al., 2008, Alves et al., 2009).

## Conclusion

The results observed in this study showed that the type and concentration of the photosensitisers, time of illumination and light intensity all affect the rate and extent of PDI of MS2. The photosensitiser TMPyP is the most efficient in facilitating PDI of MS2 compared to the other photosensitisers tested, Rose Bengal and methylene blue. The order of rate and extent of PDI of MS2 by these photosensitisers in solution was TMPyP >>> methylene blue > Rose Bengal. This is likely to be related to the net charge on these photosensitisers as it has been established that cationic photosensitisers are generally more efficient in the PDI of microorganisms including viruses. The net charge for TMPyP is +4, +1 for methylene blue and -2 for Rose Bengal.

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