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Majiya, H, Adeyemi, O orcid.org/0000-0002-0848-5917, Stonehouse, NJ
orcid.org/0000-0003-1146-5519 et al. (1 more author) (2018) Photodynamic inactivation of bacteriophage MS2: the A- protein is the target of virus inactivation. *Journal of Photochemistry and Photobiology B: Biology*, 178. pp. 404-411. ISSN 1011-1344

<https://doi.org/10.1016/j.jphotobiol.2017.11.032>

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Photodynamic inactivation of bacteriophage MS2: the A- protein is the target of virus inactivation

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Abstract

Singlet oxygen mediated oxidation has been shown to be responsible for photodynamic inactivation (PDI) of viruses in solution with photosensitisers such as 5, 10, 15, 20-tetrakis (1-methyl-4-pyridinio) porphyrin tetra *p*-toluenesulfonate (TMPyP). The capsids of non-enveloped viruses, such as bacteriophage MS2, are possible targets for viral inactivation by singlet oxygen oxidation. Within the capsid (predominantly composed of coat protein), the A-protein acts as the host recognition and attachment protein. The A-protein has two domains; an α -helix domain and a β -sheet domain. The α -helix domain is attached to the viral RNA genome inside the capsid while the β -sheet domain, which is on the surface of the capsid, is believed to be the site for attachment to the host bacteria pilus during infection. In this study, 4 sequence-specific antibodies were raised against 4 sites on the A-protein. Changes induced by the oxidation of singlet oxygen were compared to the rate of PDI of the virus. Using these antibodies, our results suggest that the rate of PDI is relative to loss of antigenicity of two sites on the A-protein. Our data further showed that PDI caused aggregation of MS2 particles and crosslinking of MS2 coat protein. However, these inter- and intra-capsid changes did not correlate to the rate of PDI we observed in MS2. Possible modes of action are discussed as a means to gaining insight to the targets and mechanisms of PDI of viruses.

Highlights

- TMPyP in the presence of light and oxygen generates singlet oxygen ($^1\text{O}_2$).
- The $^1\text{O}_2$ mediated oxidation resulted to photodynamic inactivation (PDI) of MS2.
- MS2 capsid (i.e. coat protein and A-protein) was the primary target of PDI.
- PDI caused cross-linkage of the coat protein and aggregation of the MS2 particles.
- The rate of PDI correlates to antigenic loss at 2 antigenic sites on the A-protein.

Key Words

A-protein; MS2; singlet oxygen; TMPyP; photodynamic inactivation

1. Introduction

Photodynamic inactivation (PDI) of viruses has been shown to be an efficient alternative to antiviral agents in the control of resistant and emerging viruses [1-10]. When irradiated with visible light and in the presence of molecular oxygen in aqueous solution, photosensitisers such as 5,10,15,20-tetrakis (1-methyl-4-pyridinio) porphyrin tetra *p*-toluenesulfonate (TMPyP) can generate singlet oxygen by a type 2 reaction (and other reactive oxygen species (ROS) by type 1 reaction) [11-13]. Singlet oxygen and other ROS can react rapidly and cause irreversible damage to biomolecules thereby leading to the inactivation of viruses and other microorganisms [11, 14-19]. Singlet oxygen is the most likely ROS to be implicated in viral PDI [11, 20]. All ROS have a short-life and high reactivity, thereby causing damage only to surrounding molecules close to the point of generation [11]. Capsid proteins (including host recognition proteins) are immediate targets of singlet oxygen 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 by PDI [21-24].

Although virus capsids serve to protect the genome, they may contain small pores. Furthermore, under physiological conditions, the capsids of non-enveloped viruses can undergo a constant movement that suggests a dynamic state otherwise referred to as “capsid breathing” e.g. picornaviruses [25-27], nodaviruses [28], tombusviruses [29] sobemoviruses [30]. By means of a thermofluor assay that incorporates a pair of dyes to bind the nucleic acid and hydrophobic capsid residues, respectively, capsid permeability of picornaviruses has been shown to increase with temperature [31-33]. Although the permeability of viral capsids to singlet oxygen molecules has not been shown, the presence of small pores in the capsid and

access to the viral genome could result to oxidation-induced damages to the viral capsid and genome. Reaction of proteins with singlet oxygen occurs within a range of bimolecular rate constants of 10^5 to 10^9 $M^{-1} S^{-1}$. This range is much higher than for other biomolecules including RNA, which has a rate constant range of 10^4 to 10^6 $M^{-1} S^{-1}$ [24]. Amongst amino acids, the bimolecular rate constants and oxidation effects vary [21, 22, 34-36], with tryptophan, histidine, methionine, cysteine and tyrosine being the most susceptible to singlet oxygen oxidation under physiological pH conditions [21, 22].

Changes to viral proteins as result of PDI have been measured by SDS-PAGE and infrared (IR) spectroscopy [12]. However, these methods are not specific to PDI-mediated effects. The use of SDS-PAGE is limited to overall effects on viral proteins but cannot give detail of the domains affected. In addition, the reaction of singlet oxygen with proteins can result in multiple effects including oxidation of side chains, backbone fragmentation, dimerisation and/or aggregation, unfolding or conformational changes. These effects can result in enzymatic inactivation and alterations in cellular handling and turnover [21, 22]. Owing to the presence of reducing agents in SDS-PAGE as well as heat-treatment of proteins, there is significant reduction in assay sensitivity. Similarly, IR spectroscopy cannot detect changes induced by PDI on specific protein residues but can only provide an overall effect on proteins. Matrix assisted laser desorption ionization (MALDI) and electrospray ionisation (ESI) mass spectrometry have been applied to the evaluation of site-specific protein damage due to singlet oxygen oxidation [37]. In most studies aimed at photoinactivation and chemical disinfection of human viruses, bacteriophage MS2 has been used as a model organism owing to its similarity in size and morphology to some human viruses [38] as well as its non-pathogenicity to humans and ease of propagation [38, 39]. It has been recently shown that A-protein of MS2 is the main target

of chemical oxidants such as chlorine dioxide (ClO₂). The ClO₂ inactivates MS2 by modifying structural proteins especially the A-protein, thereby hindering the ability of MS2 to attach and infect host bacteria cells [39]. MS2 is a bacteriophage of the genus *Levivirus* within the family *Leviviridae*. It has a single stranded positive sense (3,569 nt) RNA genome which is enclosed within a ≈27 nm icosahedral capsid that is composed of 178 copies of coat protein (≈13.7 KDa) and one copy of maturation protein also called the A-protein (≈44 KDa). The A-protein is attached to viral RNA from inside the inner surface of the capsid shell, with a portion embedded within the capsid such that its external surface which blends with the outside of the capsid (Figure 1), recognises and binds to the host bacteria pilus in the course of infection [40]. The assembled MS2 capsid has 32 pores, each of which is about 1.8 nm in diameter, and may be permeable to small molecules [41, 42].

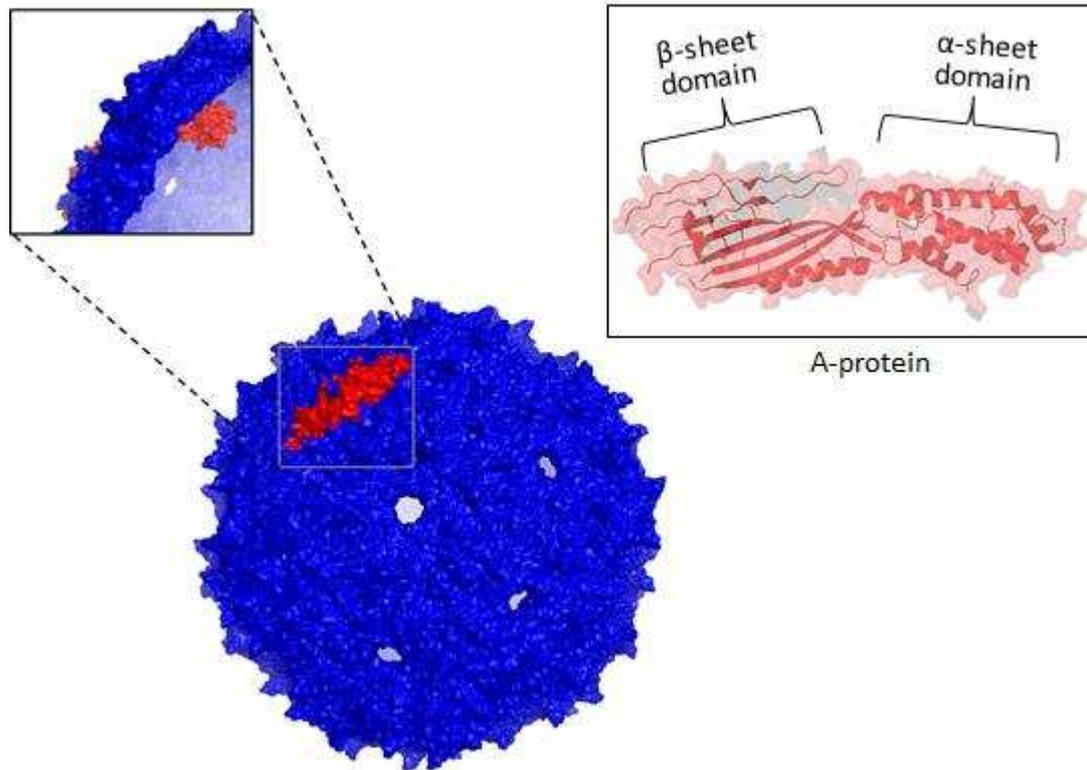


Figure 1: MS2 capsid showing: the coat protein 13.7 KDa (178 copies) labelled blue and the A-protein 44 KDa (one copy) labelled red. The A- protein is shown slightly tilted from the surface of the coat protein and (as inset) projecting into the capsid lumen. The α -helix domain of the A-protein is attached to the RNA inside the capsid, while the β -sheet domain is surface-exposed and is believed to recognise and bind to the host bacteria pilus during infection. The model was created by docking the MS2 A-protein [PDB-5tc1] onto the MS2 capsid [PDB-2MS2] using PyMOL version 1.7rc1.

Polyclonal sequence-specific antibodies are collections of immunoglobulin molecules that react against a specific epitope of an antigen (e.g. A-protein of MS2). Sequence-specific antibodies are sensitive enough to detect antigens via western blot. Changes to antigenic epitopes caused by oxidation could result to a switch in antigenicity from a native and detectable state to a non-native state and may correlate with the rate of PDI. Antigenic switches vary across epitopes even under similar treatment conditions. In order to detect such antigenic changes, standard polyclonal antibodies are not ideal owing to a binding to a range of epitopes in the target protein. In this study, sequence-specific antibodies against the

MS2 antigenic sites on the A-protein were generated. This proposes a novel method to evaluate the effect of singlet oxygen induced oxidation, caused by PDI on different regions of viral target proteins.

2. Materials and methods

2.1. Light source and conditions for PDI

The light source used 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). Visible light was used and the fluence rate (radiant exposure) was 32 mW cm^{-2} . This light intensity corresponds to approximately 3% of bright mid-day time sunlight under clear sky conditions in the Sub-Saharan Africa and about 10% of mid-day time sunlight during summer in the Northern Europe. The buffer used for PDI was 1x phosphate buffer saline (PBS) (10 mM Na_2HPO_4 , 1.8 mM KH_2PO_4 , 137 mM NaCl, and 2.7 mM KCl) at 20-22 °C under aerobic conditions.

2.2. Photosensitisers and other consumables

TMPyP (Figure 2) and other consumables used were purchased without further purification from Sigma Aldrich unless otherwise stated. MS2 coat protein antibodies were produced by Genscript, USA. Reagents were the highest grade available.

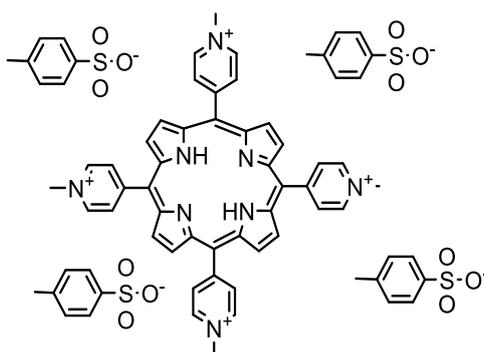
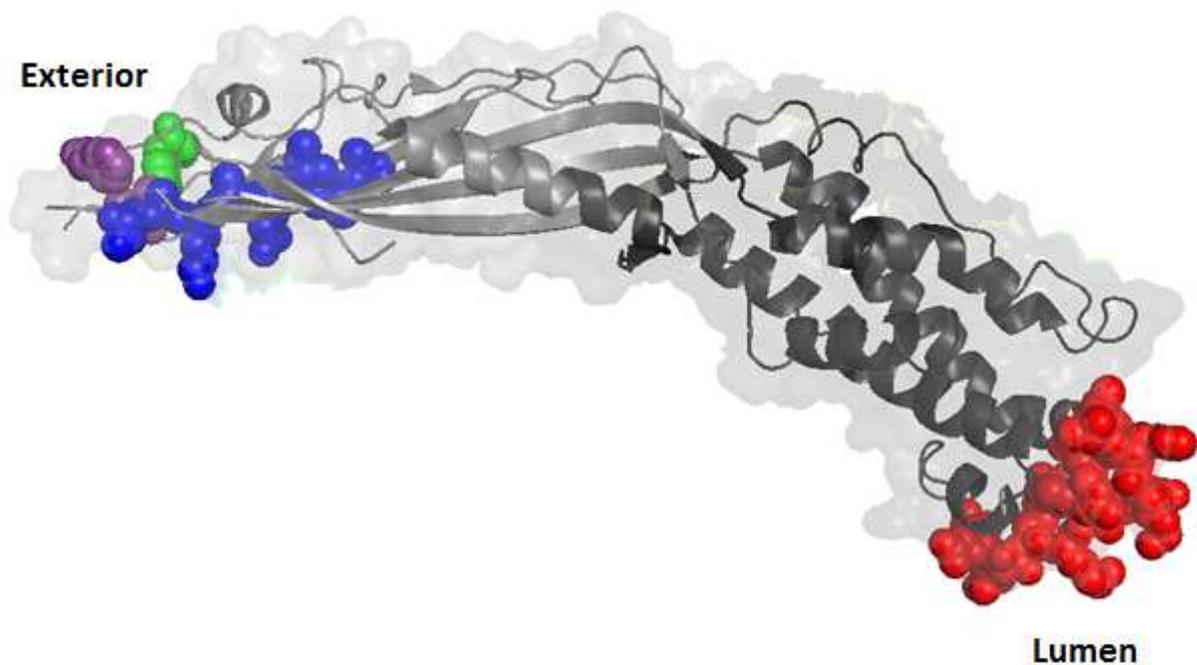


Figure 2: Structure of TMPyP (toluene sulfonate salt) used for MS2 photodynamic inactivation. The TMPyP structure was from ChemACX.com, ChemDraw Pro 13.0.

2.3. Sequence-specific antibodies

Four synthetic peptides that mimic the amino acid sequences of predicted antigenic sites of MS2 A-protein (Figure 3) were commercially synthesised by Genescript, USA. Antibodies against these peptides were raised in rabbits and purified by peptide affinity chromatography by Genescript, USA.



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MRAFSTLDRENETFVPSVRVYADGETEDNSFSLKYRSNWTPGRFNSTGAKTKQWHYPSY  
SRGALSVT SIDQGAYKRSGSSWGRPYEEKAGFGFSLDARSCYSLFPVSQNLTYIEVPQNV  
ANRASTEVLQKVTQGNFNLGVALAEARSTASQLATQTIALVKAYTAARRGNWRQALRYLA  
LNEDRKFRSKHVAGRWLELQFGWLPLMSDIQGAYEMLTKVHLQEF LPMRAVRQVGTNIKL  
DGRLSYPAANFQTTCNISRRIVIWFIINDARLAWLSSLGILNPLGIVWEKVPFSFVVDWL  
LPVGNMLEGLTAPVGCYMSGTVTDVITGESII SVDAPYGWTVERQGTAKAQI SAMHRGV  
QSVWPTTGAYVKSPFSMVHTLDALALIRQLSR
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Figure 3: MS2 A-Protein, showing positions of the four predicted antigenic sites as a ribbon diagram with the Van der Waals surface overlaid. The RNA-binding domain of protein A, located within the capsid lumen, is shaded in darker grey, while the surface exposed domain is shaded in lighter grey. Antigenic sites 1, 2, 3 and 4 are shown in green, red, blue and purple underlined bold characters and spheres, respectively on the amino acid sequence and the model. The model was created from the MS2 A-protein [PDB-5tc1] using PyMOL version 1.7rc1. Predictions of antigenic binding sites were performed by Genescript using proprietary software.

2.4. MS2 and *E. coli* strains

The bacteriophage MS2 ATCC 15597-B1 and *E. coli* host cell ATCC 15597 stocks were kindly donated by Prof. Peter Stockley (School of Molecular and Cellular Biology, University of Leeds, UK).

2.5. Propagation and purification of MS2

The bacteriophage MS2 was propagated and purified according to the previously described method [43].

2.6 Photodynamic inactivation (PDI) of MS2

PDI of MS2 was investigated using a range of concentrations of TMPyP from 0.1 μM to 1 μM in solution. The PDI light experiments (L) were illuminated at light intensity of 32 mW cm^{-2} for a duration of 10 sec to 60 min. As controls, a dark experiment (D) was carried out in the presence of photosensitizer but without illumination, while “No sensitiser” (NS) experiments were exposed to light at intensity of 32 mW cm^{-2} but in the absence of photosensitiser. All experiments were repeated in triplicate.

2.7. Infectivity and titre determination of MS2

The double layer agar plaque assay was used to determine the infectivity titres of bacteriophage MS2 using standard methods [44].

2.8. SDS-PAGE and western blots

Samples of pre-PDI and post-PDI treated of MS2 were analysed by SDS-PAGE and immunoblotted against anti-MS2 capsid protein polyclonal and/ or sequence-specific anti MS2 A-protein antibodies (anti-peptides 1, 2, 3 and 4) according to standard methods [45,46].

2.9. Native agarose gel electrophoresis

Native agarose gel electrophoresis was carried out on pre- and post-PDI treatment samples of MS2 according to standard methods [47].

2.10. Transmission electron microscopy and dynamic light scattering

Samples of pre-PDI and post-PDI treated MS2 were dialysed into buffer ((10 mM Hepes, 100 mM NaCl, 1.274 mM EDTA)). Sample dialysate were negatively stained with 2% (w/v) uranium acetate and analysed by transmission electron microscopy (TEM) according to standard methods [48,49]. Dynamic light scattering (DLS) measurements of the samples were also carried out according to standard methods [50]. DLS is widely used to measure the average size (hydrodynamic radius) of particles in solution.

3. Results

3.1. Photodynamic inactivation (PDI) of MS2

PDI of MS2 was investigated with different concentrations of photosensitisers in solution and at different times of illumination. Our data [43] suggested that at a minimum concentration of 0.2 μM in solution, TMPyP can inactivate MS2 (9.5 log reductions PFU/ml) within 60 sec when illuminated at 32 mW cm^{-2} . At 10 sec of illumination there were 1.5 log reductions in PFU/ml and at 30 sec of illumination there were 4 log reductions in PFU/ml of MS2. Photosensitiser alone in the dark (D) or light alone (NS) did not cause any detectable reduction in MS2 titre.

3.2. SDS-PAGE and western blotting using MS2 coat protein antibodies

The MS2 capsid consists of two structural proteins; 178 copies of coat protein and 1 copy of A-protein. The MS2 coat protein is ≈ 13.7 KDa, while A-protein is ≈ 44 KDa. Owing to the relative abundance of the coat protein over A-protein, we were not able to detect A-protein on SDS-PAGE (Figure 4Ai). This was also the case for western blots, although antibodies that were raised against MS2 capsid were used (Figure 4Aii). However, bands corresponding to 13.7 KDa MS2 coat proteins were detected for all PDI treated samples, including the dark experiment samples (Figure 4B). Furthermore, samples exposed to light as shown in lanes L3-L8 of figure 4B had additional bands. These bands were ≈ 27 KDa that are likely to correspond to dimers of MS2 coat protein.

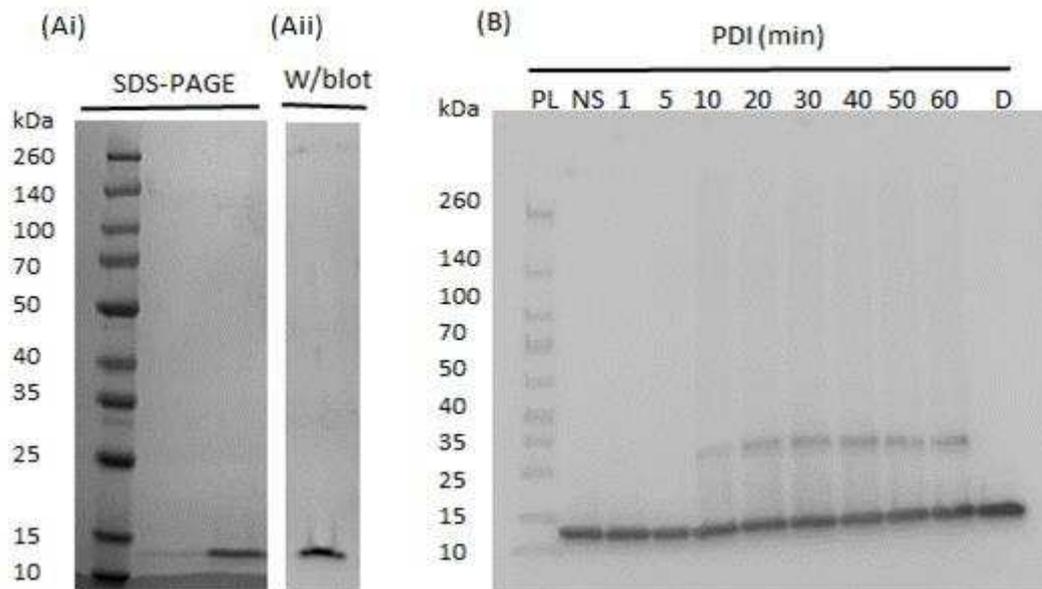


Figure 4: SDS PAGE and western blots of MS2 before and after PDI using 1 μM TMPyP in solution, illuminated at 32 mW cm^{-2} . (Ai), SDS PAGE of purified MS2 showing only coat protein 13.7 KDa; (Aii), western blot of purified MS2 showing only coat protein 13.7 KDa; (B) western blot of PDI treated MS2 samples, NS = no sensitiser; D = dark experiment.

3.3. Native gel electrophoresis of PDI treated MS2

In order to investigate the effect on the capsid induced of singlet oxygen, native gel electrophoresis of PDI-treated MS2 samples was carried out. This allowed us to visualise the PDI treated MS2 samples in their native form without reducing agents and heat-treatment. Clear bands were observed around the wells of PDI-treated samples using 50 μM of TMPyP even at 1 min of PDI (Figure 5A). These are suggested to be aggregates of MS2 particles that were retained within the wells. Complete retention within the well was observed for 60 min PDI -treated MS2 (Figure 5A).

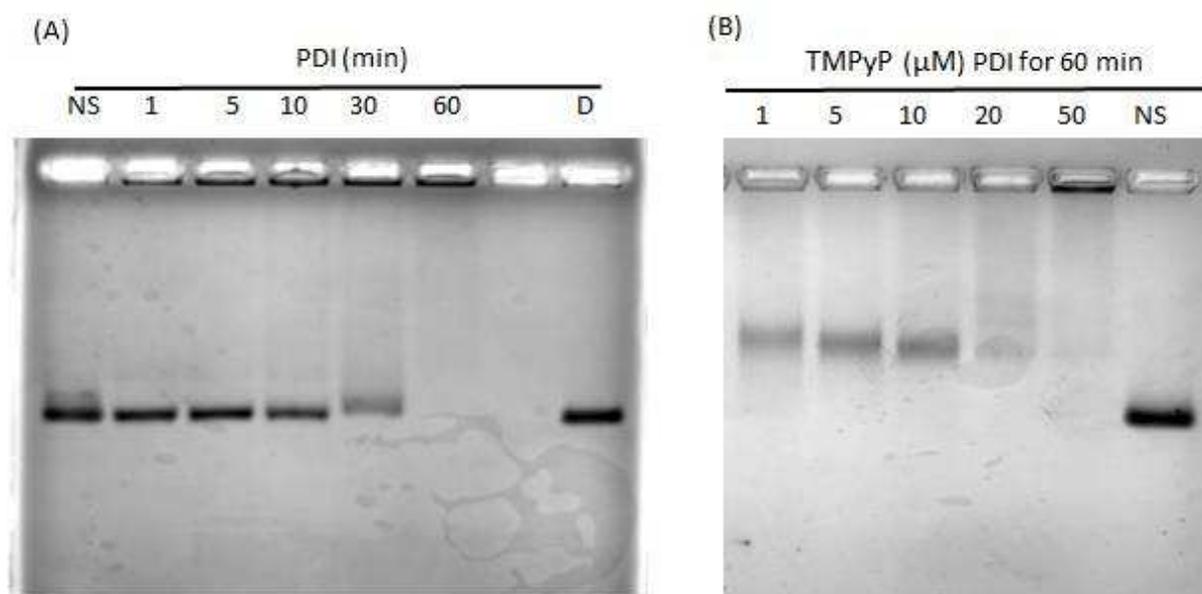


Figure 5: Native agarose gel electrophoresis of PDI treated MS2 illuminated at 32 mW cm^{-2} ; (A), Gel of MS2 PDI sample with $50 \mu\text{M}$ TMPyP in solution. NS = No sensitiser; D = dark experiment. (B), Gel of PDI treated MS2 with TMPyP in solution for 60 min. NS= No sensitizer.

The formation of aggregates appeared to be proportional to the time of PDI. Lower concentrations of TMPyP seemed to result in the formation of smaller aggregates of MS2 that could not be retained by the well but seemed to be restricted by the gel matrix (Figure 5B). It is possible that the cause of this is aggregation and/or a change in charge as a result of PDI (Figure 5B). The formation of aggregates were further confirmed by TEM and DLS (Figure 6). The TEM of samples treated for 30 and 60 min showed larger MS2 aggregates (Figure 6, A). DLS measures Brownian motion of particles in solution and correlates this to hydrodynamic radius (D_H). The larger the particle, the slower the Brownian motion will be [50]. The peak shifts (Figure 6, B) correspond to increase of average MS2 particle size (d. nm) as the time of PDI increases (Figure 6, C).

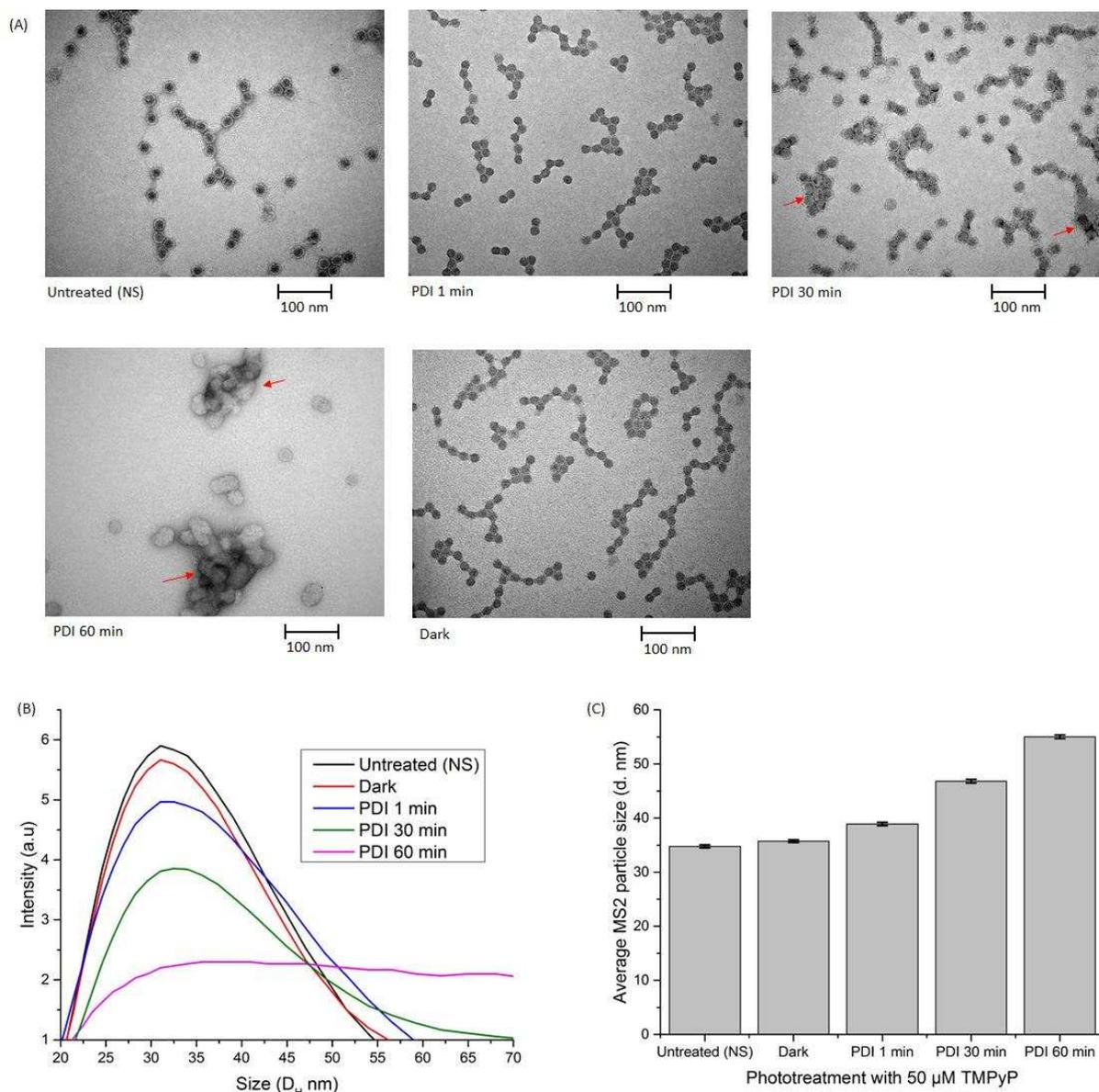


Figure 6: TEM and DLS analysis of PDT treated MS2. (A) TEM images of PDI treated MS2, illuminated at 32 mW cm^{-2} in $50 \mu\text{M}$ TMPyP solution. Untreated, PDI treated for 1 to 60 min and dark treated MS2 in $50 \mu\text{M}$ TMPyP solution are shown. The red arrow indicates large MS2 aggregates. (B) DLS measurements of PDI treated MS2 in $50 \mu\text{M}$ TMPyP solution, illuminated at 32 mW cm^{-2} . (C) Average MS2 particle sizes of PDI treated MS2. Dark, treated with TMPyP but illuminated. Data are means \pm standard deviation ($n = 3$).

3.4. Detecting changes to A-protein of MS2 induced by singlet oxygen using sequence-specific antibodies

Changes to the defined antigenic regions (see Fig 3) caused by oxidation could result in loss of antigenicity and may correspond to the rate of PDI. In order to detect such antigenic changes, sequence-specific antibodies Ab1, Ab2, Ab3 and Ab4 raised against 4 predicted epitopes of A-protein were used. Each of the sequence-specific antibodies was able to detect A-protein at ≈ 44 kDa in the untreated or dark-treated MS2 samples (Figure 7). We proposed that if oxidative damage affect specific targets on the protein, antigenicity is altered, then the ability for the antibodies to detect A-protein would be lost. Pre-treatment of these sequence-specific antibodies with their cognate peptides blocked antibody-binding thereby making them unable to detect A-protein by western blot (Figure 7E) as predicted. The PDI of MS2 phage was performed with $1 \mu\text{M}$ TMPyP for all samples but with different time of illumination; 1 - 60 min (Figure 7). Ab1 and Ab3 failed to detect A-protein for MS2 phage samples after 10 min of PDI (Figure 7A and 7C). However, both were able to detect A-protein for MS2 phage after 1 - 10 min of PDI (Figure 7A and 7C). Ab2 and Ab 4 failed to detect MS2 A-protein after even 1 min of PDI (Figure 7B and 7D). Photosensitiser alone in the dark (D) or light alone (NS) did not cause any loss of antigenicity as all the 4 sequence-specific antibodies were able to A-protein in these samples (Figure 7).

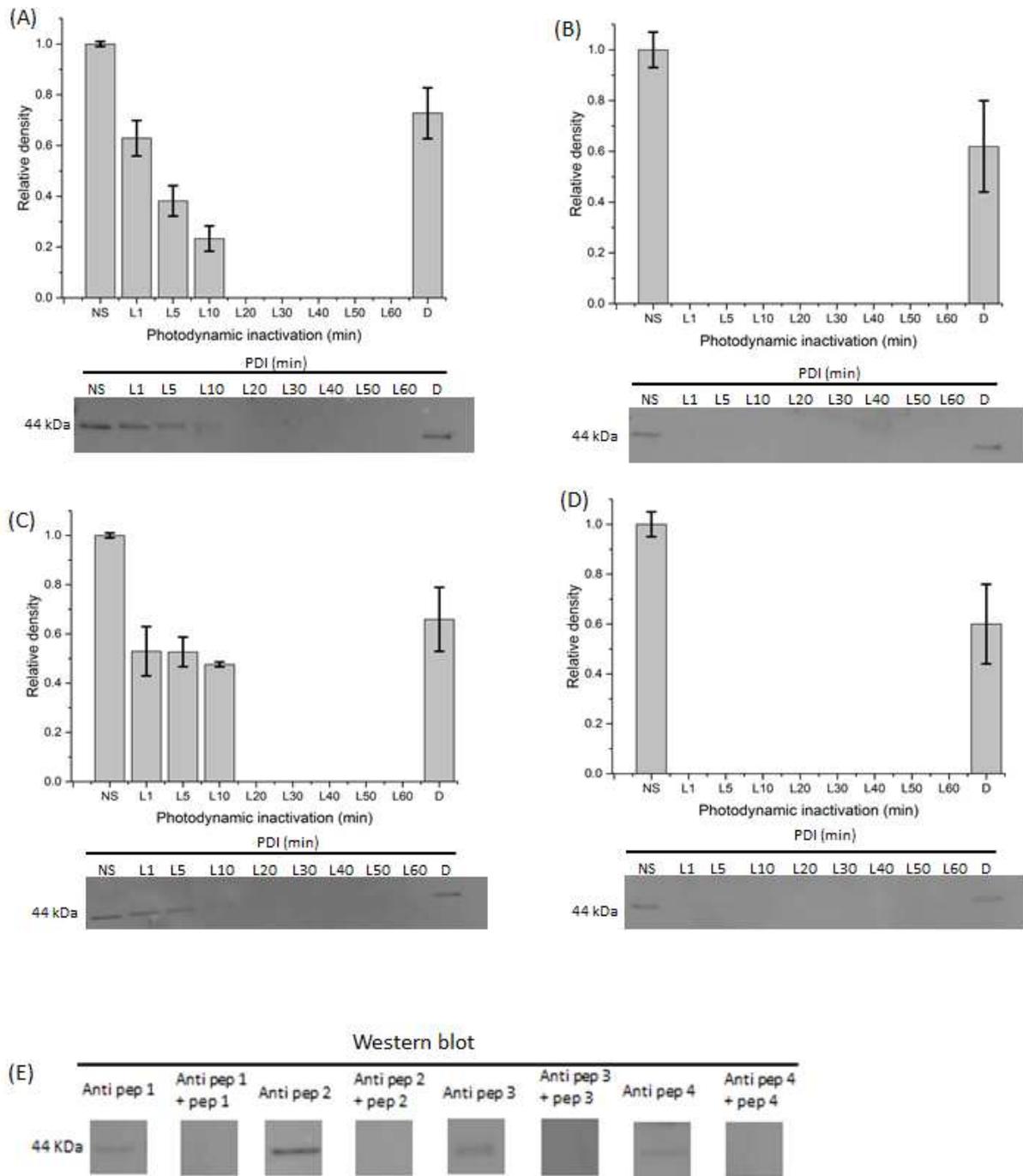


Figure 7: Western blot of PDI MS2 samples using sequence-specific antibodies to detect A-protein. 1 μ M of TMPyP and illumination at 32 mW cm^{-2} were used for PDI of MS2 and the following antibodies were used for detection after PDI: (A), Ab1; (B), Ab2; (C), Ab3; (D), Ab4. NS; no sensitiser; D, dark experiment and L1 to L60 denotes 1 min to 60 min of illumination. (E), shows blocking of the antigenic recognition by incubation of each antibody with its cognate peptide. Data are means \pm standard deviation ($n = 3$).

4. Discussion

PDI of viruses in solution by photosensitisers has been reported previously [1-6, 20, 38, 51, 52]. However, the molecular effect of PDI on virus particles has not been fully described. In this study, we have used MS2 as a model virus. In our previous work [43] we showed that, at a minimum concentration of 0.2 μM TMPyP in solution, MS2 was inactivated within 1 min under illumination at 32 mW cm^{-2} . The primary steps of viral infections involve recognition and attachment of the virion to the host cell receptor. Changes to host receptor recognition sites on the virus capsid could inhibit host receptor attachment thereby resulting in its inability to infect the host cell. The MS2 capsid comprises 178 copies of a 13 KDa coat protein and 1 copy of a 44 KDa host recognition and attachment protein known as the A-protein [40, 53]. We hypothesised these two proteins to be potential direct oxidation targets of singlet oxygen generated by TMPyP in solution because of the short life and high reactivity of singlet oxygen. However, PDI of MS2 phage is very fast and as suggested by other reports, this points to the fact that inactivation of the A-protein (and not effects on the coat protein) might be responsible for inactivation [12, 23]. As shown in figures 4 - 6, our data suggests that 1 min PDI-treatment of MS2 phage with 1 μM of TMPyP does not result in major changes to the viral capsid protein. Prolonged PDI treatment for 10 to 60 min caused aggregation of MS2 (Figures 4-6) and from western blot data (using MS2 capsid antibodies) monomers and dimers of MS2 coat protein were observed for PDI treated MS2 after 10 – 60 min illumination (Figure 4).

Aggregation of the MS2 particles was seen and was proportional to the time of PDI and concentration of TMPyP; this was demonstrated by agarose native gel electrophoresis, TEM and DLS (Figures 5 and 6). Physical, chemical and biological consequences of singlet oxygen mediated oxidation of proteins is likely to result from multiple effects such as enzyme

inactivation, protein peroxide formation, side change product formation, backbone fragmentation, formation of cross links and aggregates, and effect on protein properties and turnover [21]. In contrast to very few reports of backbone fragmentation, there is substantial evidence for formation of high-molecular-weight aggregates (dimers and higher species) of proteins that were oxidised by singlet oxygen [21, 54, 55]. There are conflicting reports on the exact causes of cross-linking and aggregate formation during oxidation of proteins. Some of the aggregates may be formed by radical-radical termination reactions of tyrosine-derived phenoxyl radicals to give di- (or bi-) tyrosine [21, 55-57]. However, other reports stated that di-tyrosine is not generated and not implicated in the formation of cross links during singlet oxygen modification of proteins [21, 56]. It has also been reported that aggregates and crosslinks may arise from secondary or dark reactions independent of the continuing formation of singlet oxygen [21]. Histidine has been often implicated in the formation crosslinks and aggregates of oxidised proteins. It has been proposed that the products of histidine oxidation by singlet oxygen can couple to lysine, cysteine or other histidine residues. Some studies suggested histidine may be very important in aggregate formation as proteins that lack histidine residues do not form cross links [21, 57]. This does not agree with our data, as we observed formation of dimers of MS2 coat protein for PDI samples of 10 – 60 min (Figure 4) and MS2 coat protein does not contain histidine residues. Therefore, dimerisation of coat protein is most likely as a result of di-tyrosine formation and or secondary or dark reaction. Similarly, aggregation of PDI treated MS2 samples (Figures 5 and 6) may also be due to di-tyrosine formation and or secondary or dark reaction. However, the presence of histidine residues in A-protein (Figures 1 and 3) which is part of capsid of MS2 may contribute to formation of viral particle aggregates during PDI.

After analysis of the coat protein as a target of PDI, we turned our attention to A-protein (Figure 1). The A-protein of MS2 has two domains; an α -helix domain (amino acids 140-225, 269-313, and 375-393) with a bundle of six α -helices and a β -sheet domain (amino acids 1-139, 226-268 and 314-374) with six anti-parallel β -strands sandwiched between an N-terminal loop and helix-loop-helix motif [40]. It is believed that interactions that occurred between helix-loop-helix motifs of one side of the β -sheet domain may be responsible for the attachment of MS2 via the A-protein to the bacterial F-pilus during infection [40]. We used sequence-specific antibodies made against four selected antigenic regions of the A-protein. The antibodies were specific as pre-treatment with their respective peptides blocked their binding to A protein. We hypothesised that at least three of the four selected antigenic determinant sites would be accessible to singlet oxygen during PDI of MS2. Oxidation could cause damage to these sites leading to loss of their antigenicity. However, we believed that the rate of damage caused and loss of antigenicity may vary according to their amino acid composition. Our data show that sequence-specific antibodies against antigenic sites 1 and 3 failed to detect the A-protein after 10 min PDI (Figure 7) whilst sequence-specific antibodies against antigenic sites 2 and 4 did not detect A-protein even after 1 min PDI (Figure 7). This rate of loss of antigenicity of site 2 and site 4 corresponds to the rate of PDI we observed in previous work. Although antigenic site 2 is within α -helix domain of A-protein which is attached to the viral RNA genome inside the capsid, it is possible that singlet oxygen generated during PDI can reach the site, thereby oxidising this leading to loss of antigenicity. We believed that antigenic site 4 which is within β -sheet domain of A-protein might be one of specific regions of the A-protein responsible for MS2 attachment to the bacterial pilus and especially the delivery of its genome inside the host. Antigenic sites 1 and 3 are also within β -sheet domain of A-protein. Antigenic site 1 has one tyrosine as the last amino acid of its

sequence, site 2 has one histidine whilst site 4 has a tryptophan and two tyrosines. The amino acids tyrosine, histidine and tryptophan in addition to methionine and cysteine are the most sensitive to oxidation by singlet oxygen. At physiological pH, (the pH condition of our experiments), bimolecular rate constants of these amino acids with singlet oxygen are around $10^7 \text{ k (M}^{-1} \text{ S}^{-1})$ [21, 58]. We suggest that the positions of histidine and tryptophan, almost at the middle of antigenic sites 2 and 4 respectively, contribute to the fast rate of loss of antigenicity. Tyrosine (which is the last amino acid of the sequence in site 1) is less likely to be important here. The two tyrosines in site 4 might also be a contributory factor to the fast rate of loss of antigenicity. The impact of change to the chemical structure of sites 2 and 4 caused by histidine and tryptophan oxidation will be more than that of tyrosine of antigenic determinant site 1. This might be especially so as singlet oxygen oxidation of histidine and tryptophan lead to ring opening, while leading to ring closure in tyrosine [21].

5. Conclusion

The results obtained from our previous study [43] suggested that a minimum concentration of 0.2 μM of TMPyP inactivates bacteriophage MS2 under 1 min illumination at 32 mW cm^{-2} . In this study, complete MS2 particle aggregation was observed after 60 min of PDI. Formation of dimers of MS2 coat protein was observed after 10 min PDI. The cause of aggregation of MS2 particles might be due to either and or cumulative effects of formation of di-tyrosine from tyrosine residues that are present in the coat protein and the A-protein, histidine residues present in A-protein and secondary/dark reaction. However, the formation of dimers of MS2 coat protein could be caused by formation of di-tyrosine or secondary/dark reactions and/or cumulative effects of these two. Sequence-specific antibodies for antigenic site 2 and 4 on the A-protein did not detect these after 1 min of PDI. This rate of loss of antigenicity corresponds to the rate of PDI of MS2 we observed previously. We believed that histidine and tryptophan being most sensitive amino acids to singlet oxygen and their position almost in the middle of the sequence of these antigenic sites on the A-protein might be responsible for the fast rate of loss of antigenicity. We proposed that site 4 may be one of specific regions of the A-protein responsible for MS2 attachment to the bacterial pilus and delivery of its genome into the host. This work could lead way to understanding the targets and mechanisms of photodynamic inactivation of viruses from a biological perspective.

Acknowledgements

This work was supported by Petroleum Technology Development Fund (PTDF), Nigeria. The authors would like to thank members of the Millner and Stonehouse labs at the University of Leeds, UK.

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