Photochemistry and Photobiology, 20**, **: *–*

Rapid Communication

Photoinactivation of the Coronavirus Surrogate phi6 by Visible Light

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Received 3 September 2020, revised 7 October 2020, accepted 26 October 2020, DOI: 10.1111/php.13352

ABSTRACT

To stop the coronavirus spread, new inactivation approaches are being sought that can also be applied in the presence of humans or even on humans. Here, we investigate the effect of visible violet light with a wavelength of 405 nm on the coronavirus surrogate phi6 in two aqueous solutions that are free of photosensitizers. A dose of 1300 J cm^{-2} of 405 nm irradiation reduces the phi6 plaque-forming unit concentration by three log-levels. The next step should be similar visible light photoinactivation investigations on coronaviruses, which cannot be performed in our lab.

INTRODUCTION

The new severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which causes the severe lung infection Covid-19 and which was first diagnosed in December 2019 in Wuhan China, is still spreading worldwide, with almost 19 million confirmed infections and more than 700 000 deaths until August 2020 (1).

To reduce the risk of infection, potentially contaminated goods are successfully disinfected with chemical disinfectants as well as heat and ultraviolet radiation (2–7). However, these proven techniques have the disadvantage of damaging sensitive materials and human cells and can therefore not be applied directly on humans.

In contrast to ultraviolet radiation, visible blue and violet light is much less dangerous to humans but has shown an antimicrobial effect in experiments on bacteria and fungi (8,9). This is explained by endogenous photosensitizers such as porphyrins and flavins, which absorb visible light and subsequently produce reactive oxygen species like $^1O_2$, OH and H_2O_2 that destroy the cells from within (10–19).

For viruses, there are very few publications on this subject. Richardson and Porter observed inactivation of the ssRNA murine leukemia virus – apparently in a nutrient medium - by violet light of a white fluorescence lamp (20). In 2014 Tomb et al. (21) investigated the inactivation of phiC31, a nonenveloped, double-stranded DNA virus, by 405 nm irradiation. The authors observed a successful reduction of viruses in nutrient media, but almost no inactivation in phosphate-buffered saline (PBS). Further studies were performed on feline calicivirus, a nonenveloped RNA virus (22), with similar results: virus reduction was observed in irradiated organically rich media, which probably contained photosensitizers. In 1966, Cartwright et al. (23) reported a kind of accidental experiment in the summer month of June, in which medium, containing coronaviruses (Transmissible Gastroenteritis Virus), was unintentionally exposed to sunlight for one day. The authors observed a virus reduction by 2 log-levels, but it is unclear whether this effect was caused by photosensitizers in the medium and the intensity as well as spectral composition of the daylight is also unknown.

Besides this "accident", the inactivating effect of visible light on coronaviruses has not yet been specifically investigated. However, this would be of particular interest with regard to the coronavirus pandemic because visible light could not only be applied as a gentle disinfection technique, but could even be considered for therapeutic application in or on humans. Similar approaches have been suggested or even tried for bacterial infections (10,24–26).

Due to the security level of the available laboratory for this study, experiments on coronaviruses are not possible. Instead, visible light illumination experiments were carried out with the bacteriophage phi6, a member of the *Cystoviridae* (27). Phi6 is an enveloped dsRNA virus (28), with a RNA genome of 13.5 kbp and a size of 75 nm (29), which multiplies in *Pseudomonas syringae* strains. Phi6 has already been discussed as a useful surrogate for enveloped viruses, such as coronaviruses (approx. 30 kb ssRNA, size 100−150 nm). In studies on ultraviolet radiation inactivation (30,31), temperature and humidity properties (32), recovery from hands (33), and persistence in water, sewage or on surfaces (34–37) phi6 results were usually within the range of published results of different coronaviruses. Additionally, phi6 has also been suggested as surrogate for enveloped human viruses in visible light photodynamic inactivation (with additional photosensitizers) though without performing a direct comparison to coronaviruses (38) and in their review Costa et al. (39) conclude that the photodynamic inactivation mechanisms in mammalian viruses and bacteriophages are similar and therefore phages can be employed as useful surrogates in this kind of application.

It should be emphasized that the present virus irradiation experiments were not conducted in nutrient medium, but in phosphate-buffered saline (PBS) and saline magnesium gelatin buffer (SMG) to exclude a possible influence of external photosensitizers.

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MATERIALS AND METHODS

Bacteriophage phi6 (DSM 21518) and its host Pseudomonas syringae (DSM 21482) were purchased from Leibniz-Institute DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany). *P. syringae* was propagated in Tryptic Soy Broth (TSB, Sigma-Aldrich, St. Louis, USA). For preparation of host cells, 25 mL of TSB were inoculated with a single bacterial colony and the culture was grown overnight at 170 rpm and 25°C in order to obtain an optical density at 600 nm of 0.20–0.25. This was equal to 1–5 × 10^8 colony-forming units (CFU) mL^{-1}.

A high titer phage stock solution (10^9 plaque-forming units (PFU) mL^{-1}) was prepared by plate lysis and elution (40). Briefly, 10^5 PFU of phi6 in SMG buffer (50 mM Tris-HCl pH 7.5, 0.1 M NaCl, 8.1 mM $MgSO_4$, 0.01% (w/v) gelatin) were mixed with 100 µL of an overnight culture of the host bacterium *P. syringae*, incubated for 10 min at 25°C to allow the phages to attach to the cells, subsequently mixed with 3 mL of sterile soft agar (TSB with 0.6% agar, Agar Bacteriological, VWR, Darmstadt, Germany), which was kept melted in a water bath at 48°C, and poured onto the center of a TSB agar plate (20 mL TSB with 1.5% agar in a 90 mm Petri dish). After incubation for 18 h at 25°C and confluent lysis, 5 mL of SMG buffer were added onto the plate, and the plate was stored on a platform shaker (Duomax 1030, Heidolph Instruments, Schwabach, Germany) for 40 min with slow agitation. The lysate was transferred into a sterile tube, centrifuged at 4000 ***g*** (Multifuge 3S-R, Heraeus, Hanau, Germany) for 10 min at 4°C, and the supernatant was filtered through a cellulose acetate membrane with a 0.2 µm pore size (VWR, Darmstadt, Germany) to remove bacterial debris. The phage stock was stored at 4°C for further experiments.

The illumination setup is described in detail in (41). A high power 405 nm LED type LZ4-40UB00-00U8 (LED Engin, Inc., San Jose, USA) on top of a truncated reflective pyramid homogeneously irradiated the phi6 containing sample, 10^7 PFU mL^{-1} in 3 mL of PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4 pH 7.1) or SMG, inside a 5 mL beaker in a 20°C water bath with increasing doses. The irradiation intensity was 78.6 mW cm^{-2} and aliquots (100 µL) were taken after t = 0, 1.5, 3, and 4.5 h of incubation, corresponding to fluences of 0, 424, 848, and 1272 J cm^{-2}. Control samples were kept under normal laboratory lighting conditions in a water bath, whose temperature was continuously adjusted to that of the irradiated samples in order to exclude any temperature effects. Postexposure, the number of active phages was determined using the double agar overlay plaque assay (42): 100, and 200 µL of sequentially diluted phi6 samples in SMG, 100 µL of host bacteria *P. syringae*, and 3 mL of soft agar were mixed and plated over TSB agar plates. Plaques were counted after incubation for 24 h at 25°C and phage concentration was expressed in PFU mL^{-1}. At each sampling time point, three technical replicates were measured, and at least three independent experiments were performed for each condition.

RESULTS AND DISCUSSION

Example photographs of plaques formed in double agar overlay in a series of phi6 photoinactivation experiments are presented in Fig. 1. The subsequently calculated survival rate of phi6 is given as the ratio of the virus concentration in the irradiated sample at time t to the initial concentration at time zero, and is expressed as relative PFU mL^{-1} in Fig. 2. A three log-level reduction was obtained by a dose of approximately 1300 J cm^{-2}. With the assumption of a mono-exponential behavior, this means that the dose for one log-reduction is about 430 J cm^{-2}.

Phi6 is assumed to consist of RNA, proteins and phospholipids (43–45). These molecules are potential targets for viral photodynamic inactivation (46), but there is no direct hint of the existence of endogenous photosensitizers, and so we can only speculate about a possible inactivation mechanism that would fit to the so far obtained results:

It is known that bacteriophages, including phi6, can be inactivated by external photosensitizers during the illumination with visible light. Reactive oxygen species (ROS) are generated under

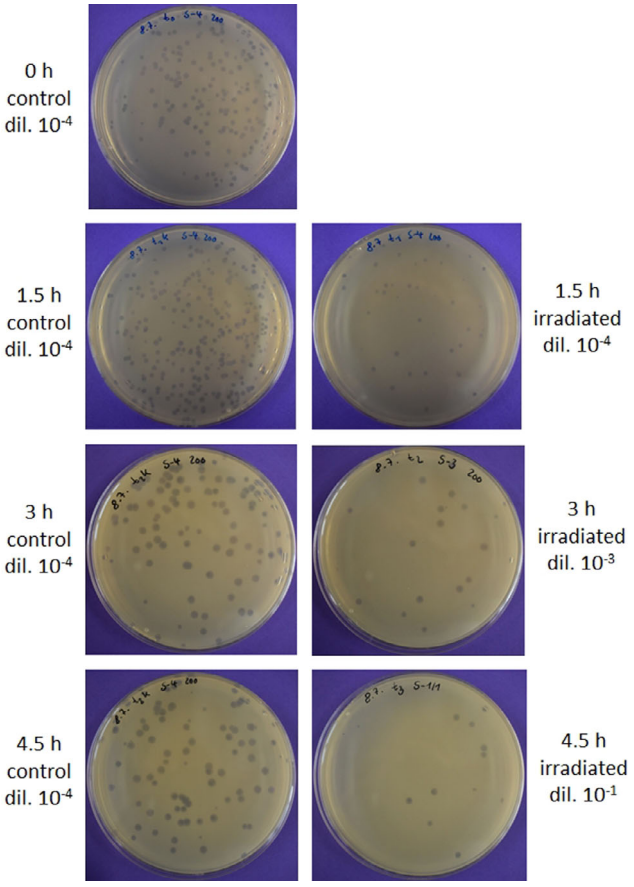

Figure 1. Plaques formed by phi6 in the double agar overlay after 0, 1.5, 3, 4.5 h of incubation without or with 405 nm irradiation. (Phage-suspension was sequentially diluted as indicated.)

irradiation and known to attack envelope, proteins, and nucleic acids (39,38). This is in good agreement with the results of Tomb et al. (22,21), who observed a stronger photoinactivation effect, when performing the irradiation in organically rich media or in media with increased photosensitizer (porphyrin) concentration.

In our experiments, we even employed two buffers (PBS and SMG) to be sure of the absence of external photosensitizer, so it cannot be exactly the same mechanism but maybe it is similar: Pseudomonads (phi6 host cells) are known to be sensitive to 405 nm irradiation, which is assumed to be caused by endogenous photosensitizers like porphyrins (47,14). These photosensitizers might have been carried along with the host´s membrane when building the phi6 envelope and now cause the ROS generation and the resulting photosensitivity.

Something similar could happen to coronaviruses. Human cells (coronavirus host cells) also contain photosensitizers like porphyrins, flavins, NADH and others (48–51), which might be incorporated in the coronavirus envelope and lead to a photosensitivity toward visible light.

The observed phi6 log-reduction dose of about 430 J cm^{-2} does not seem to be unreasonable. In their phiC31 photoinactivation study, Tomb et al. (21) observed a weak virus reduction of about 0.33 log-levels for 300 J cm^{-2} in PBS, which would correspond to 900 J cm^{-2} for a one log-reduction. Given the fact that phiC31 is a nonenveloped dsDNA virus and phi6 an

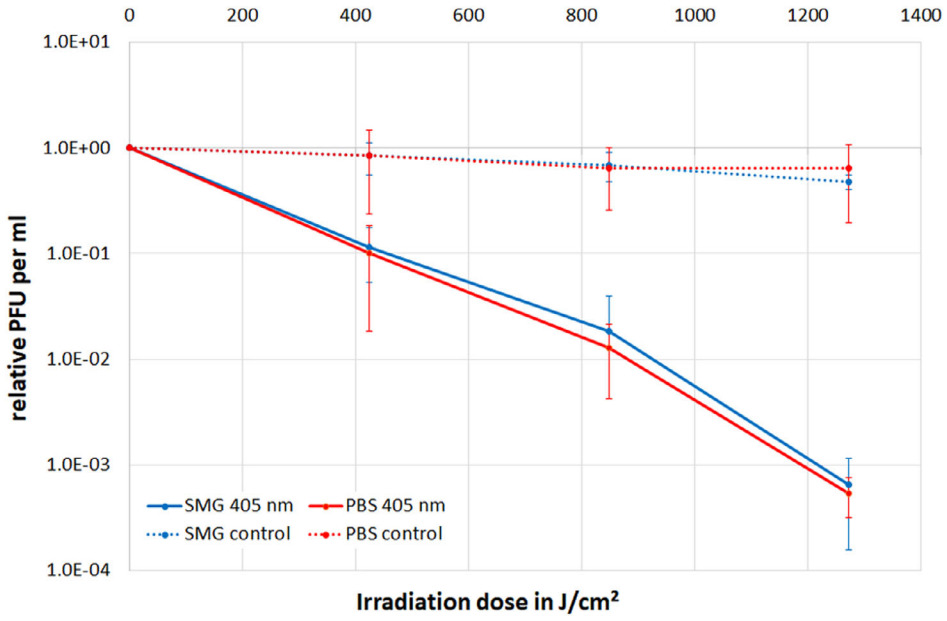

Figure 2. Survival rate of phi6 in relative plaque-forming units (PFU)/ml as a function of the 405 nm irradiation dose. Error bars represent one standard deviation of the mean of at least three trials.

enveloped RNA virus and enveloped viruses are usually more sensitive (39), the difference seems to be realistic. The much lower log-reduction doses for phiC31 and feline calcivirus in organically rich media (21,22) can be explained as the result of external photosensitizers in the medium.

Summarized, violet 405 nm irradiation is capable of inactivating the enveloped RNA virus phi6 without addition of photosensitizers. This gives reason to hope that coronaviruses can also be inactivated by light, but corresponding investigations must be performed in a laboratory with a higher safety clearance.

If this assumption is confirmed, visible light can not only be used to gently reduce pathogens in materials, surfaces or rooms, but one can also speculate whether there are also potential therapeutic approaches to reduce the viral load in the body. Human cells contain the above-mentioned endogenous photosensitizers such as porphyrins and flavins, which may reduce coronaviruses substantially at lower irradiation doses.

Study limitations: These results have been obtained for phi6, which has proven to be a useful coronavirus surrogate for many different applications but so far, photoinactivation with visible light (without additional photosensitizers) has not been among them. Therefore, photoinactivation results on coronaviruses might differ. Nevertheless, among the previously published virus photoinactivation studies, these results on phi6 – the first 405 nm photoinactivation experiments with an enveloped RNA virus - might provide the best available approximation to future coronavirus results.

Acknowledgements—Support by the Bundesministerium für Bildung und Forschung (Innosued, Grant number 03IHS024B) is gratefully acknowledged.

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