Rapid Communication

Photoinactivation of the Coronavirus Surrogate phi6 by Visible Light

Petra Vatter, Katharina Hoenes and Martin Hessling*

Ulm University of Applied Sciences, Ulm, Germany

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 $^1$O$_2$, OH and H$_2$O$_2$ 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.
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 was 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 × 10^14 colony-forming units (CFU) mL⁻¹.

A high titer phage stock solution (10⁹ plaque-forming units (PFU) mL⁻¹) was prepared by plate lysis and elution (40). Briefly, 10⁶ PFU of phi6 in SMG buffer (50 mM Tris-HCl pH 7.5, 0.1 M NaCl, 8.1 mM MgSO₄, 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 x g for 40 min with slow agitation. The lysate was transferred into a sterile tube, centrifuged at 4000 x g for 40 min with slow agitation. 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. 10⁷ PFU mL⁻¹ in 3 mL of PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄ 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 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). The subsequently calculated survival rate of phi6 is given in Fig. 1. The number of actively infective phages was calculated using the equation \( K = \frac{t}{t_0} \), where \( t_0 \) is the time of the initial concentration and \( t \) is the time of the final concentration. The survival rate was determined using the equation \( S = \frac{t}{t_0} \).

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 mon-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 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 enveloped virus, this is reasonable.
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 calicivirus 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.

REFERENCES

1. Coronavirus Resource Center (2020) COVID-19 Dashboard. (Global Map). Available at: https://coronavirus.jhu.edu/map.html. Accessed on 6.8.2020.

2. Cadnum, J. L., D. F. Li, S. N. Redmond, A. R. John, B. Pearlman, and C. J. Donskoey (2020) Effectiveness of ultraviolet-C light and a high-level disinfection cabinet for decontamination of N95 respirators. Pathog. Immun. 5, 52–67.

3. Kampf, G., D. Todt, S. Pfander and E. Steinmann (2020) Persistence of coronaviruses on inanimate surfaces and their inactivation with biocidal agents. J. Hosp. Infect. 104, 246–251.

4. Kampf, G., A. Voss and S. Scheithauer (2020) Inactivation of coronaviruses by heat. J. Hosp. Infect. 105, 348–349.

5. Kratzel, A., D. Todt, P. V’kovski, S. Steiner, M. Gultom, T. T. N. Thao, N. Ebert, M. Holwerda, J. Steinmann, D. Niemeyer, R. Dijkman, G. Kampf, C. Drosten, E. Steinmann, V. Thièl and S. Pfander (2020) Inactivation of severe acute respiratory syndrome coronavirus 2 by WHO-recommended hand rub formulations and alcohols. Emerg. Infect. Dis. 26, 1592–1595.

6. Hessling, M., K. Hoenes and C. Lingenfelder (2020) Selection of parameters for thermal coronavirus inactivation – a data-based recommendation. GMS Hygiene Inf. Control. 15, 1–7. https://dx.doi.org/10.3205/dgkh000351

7. Hessling, M., K. Hönes, P. Vatter and C. Lingenfelder (2020) Ultraviolet irradiation doses for coronavirus inactivation - review and analysis of coronavirus photoinactivation studies. GMS Hygiene Infect. Control 15, Doc08.

8. Hessling, M., B. Spellerberg and K. Hoenes (2016) Photoactivation of bacteria by endogenous photosensitizers and exposure to visible light of different wavelengths - A review on existing data. FEMS Microbiol. Lett. 364, fnw270.

9. Tomb, R. M., T. A. White, J. E. Coia, J. G. Anderson, S. J. MacGregor and M. Maclean (2018) Review of the comparative susceptibility of microbial species to photoinactivation using 380–480 nm violet-blue light. Photochem. Photobiol. 94, 445–458.

10. Ashkenazi, H., Z. Malik, Y. Harth and Y. Nitzan (2003) Eradication of Propionibacterium acnei by its endogenic porphyrins after illumination with high intensity blue light. FEMS Immunol. Med. Microbiol. 35, 17–24.

11. Guffey, J. S. and J. Wilborn (2006) In vitro bactericidal effects of 405-nm and 470-nm blue light. Photomed. Laser Surg. 24, 684–688.

12. Maclean, M., S. J. MacGregor, J. G. Anderson and G. Woolsey (2008) High-intensity narrow-spectrum light inactivation and wavelength sensitivity of Staphylococcus aureus. FEMS Microbiol. Lett. 285, 227–232.

13. Feuerstein, O., I. Ginsburg, E. Dayan, D. Veler and E. I. Weiss (2005) Mechanism of visible light phototoxicity on Porphyromonas gingivalis and Fusobacterium nucleatum. Photochem. Photobiol. 81, 1186–1189.
14. Amin, R. M., B. Bhayana, M. R. Hamblin and T. Dai (2016) Antimicrobial blue light inactivation of *Pseudomonas aeruginosa* by photo-excitation of endogenous porphyrins: In vitro and in vivo studies. *Lasers Surg. Med.* 48, 562–568.

15. Dai, T. (2017) The antimicrobial effect of blue light: What are they behind? *Virulence* 8, 649–652.

16. Dai, T. and M. R. Hamblin (2017) Visible blue light is capable of inactivating *Candida albicans* and other fungal species. *Photomed. Laser Surg.* 35, 345–346.

17. Wang, Y., Y. Wang, Y. Wang, C. K. Murray, M. R. Hamblin, D. C. Cooper and T. Dai (2017) Antimicrobial blue light inactivation of pathogenic microbes: State of the art. *Drug Resist. Updates* 33–35, 1–22.

18. Plavskii, V. Y., A. V. Mitulich, A. I. Tret’yakova, I. A. Leusenkа, L. G. Plavskaya, O. A. Kazyuchits, I. I. Dobysh and T. P. Krasenkovа (2018) Porphyrins and flavins as endogenous acceptors of optical radiation of blue spectral region determining photoinactivation of microbial cells. *J. Photochem. Photobiol. B, Biol.* 183, 172–183.

19. Cieplik, F., A. Spath, C. Leibl, A. Gollmer, J. Regensburger, L. Tabenski, K.-A. Hiller, T. Maisch and G. Schmalz (2018) Survival of the Enveloped Virus Phi6 in Droplets as a Function of Relative Humidity, Absolute Humidity, and Temperature. *Applied and Environmental Microbiology* 84, 1–10. http://dx.doi.org/10.1128/aem.00551-18

20. Casanova, L. M. and S. R. Weaver (2015) Evaluation of eluents for the recovery of an enveloped virus from hands by whole-hand sampling. *J. Appl. Microbiol.* 118, 1210–1216.

21. Silverman, A. I. and A. B. Boehm (2020) Systematic review and meta-analysis of the persistence and disinfection of human coronaviruses and their viral surrogates in water and wastewater. *Environ. Sci. Technol. Lett.* 7, 544–553.

22. Aquino de Carvalho, N. E. N., N. Stachler, N. Cimabue and K. Bibby (2017) Evaluation of *Ph×* persistence and suitability as an enveloped virus surrogate. *Environ. Sci. Technol.* 51, 8692–8700.

23. Ye, Y., E. M. Ellenberg, K. E. Graham and K. R. Wigginton (2016) Survivability, partitioning, and recovery of enveloped viruses in untreated municipal wastewater. *Environ. Sci. Technol.* 50, 5077–5085.

24. Whitworth, C., Y. Mu, H. Houston, M. Martinez-Smith, J. Noble-Wang, A. Coulliott-Salmond and L. Rose (2020) Persistence of Bacteriophage Phi 6 on Porous and Nonporous Surfaces and the Potential for Its Use as an Ebola Virus or Coronavirus Surrogate. *Applied and Environmental Microbiology* 86, 1–11. http://dx.doi.org/10.1128/aem.01128/aem.01482-20

25. Lytle, C. D., A. P. Budacz, E. Keville, S. A. Miller and K. N. Poudouz (1991) Differential inactivation of surrogate viruses with merocyanine 540. *Photochim. Photobiol.* 54, 489–493.

26. Costa, L., M. A. F. Faustino, M. G. P. M. S. Neves, A. Cunha and A. Almeida (2012) Photodynamic inactivation of mammalian viruses and bacteriophages. *Viruses* 4, 1034–1074.

27. Sambrook, J. and D. W. Russell (2001) *Molecular Cloning. A Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

28. Hoernes, K. U., W. Wenzel, B. Spellerberg and M. Hessling (2020) Photoinactivation sensitivity of *Staphylococcus carnosus* to visible-light irradiation as a function of wavelength. *Photochim. Photobiol.* 96, 156–169.

29. Kropinski, A. M., A. Mazzocco, T. E. Waddell, E. Lingroh and R. P. Johnson (2009) Enumeration of bacteriophages by double agar overlay plaque assay. In *Bacteriophages*. Methods in Molecular Biology, Vol. 501 (Edited by J. M. Walker, M. R. J. Clokie and A. M. Kropinski), Humana Press, Totowa, New Jersey: pp 69–76.

30. Sands, J. A. (1973) The phospholipid composition of bacteriophage *φ×*6. *Biochem. Biophys. Res. Comm.* 55, 111–116.

31. Sinclair, J. F., A. Tzagoloff, D. Levine and L. Mindich (1975) Proteins of bacteriophage *φ×*6. *Virol.* 16, 685–695.

32. Laurinavicius, S., R. Käkelä, D. H. Bamford and P. Somerharju (2004) The origin of phospholipids of the enveloped bacteriophage *φ×*6. *Virology* 326, 182–190.

33. Wiehe, A., J. M. O’Brien and M. O. Sengé (2019) Trends and targets in antiviral phototherapy. *Photochim. Photobiol. Sci.* 18, 2565–2612.

34. Fila, G., M. Kychowiak, M. Rychlowski, K. P. Bielawski and M. Grinholc (2018) Antimicrobial blue light photoinactivation of *Pseudomonas aeruginosa*. Quorum sensing signaling molecules, biofilm formation and pathogenicity. *J. Biophoton.* 11, e201800079.

35. Wondrak, G. T., M. K. Jacobson and E. L. Jacobson (2006) Endogenous UVA-photosensitizers: Mediators of skin photodamage and novel targets for skin photoprotection. *Photochem. Photobiol. Sci.* 5, 215–237.

36. Lavi, R., R. Ankri, M. Sinyakov, M. Eichler, H. Friedmann, A. Shainberg, H. Breitbart and R. Lubat (2012) The plasma membrane is involved in the visible-light-tissue interaction. *Photomed. Laser Surg.* 30, 14–19.

37. Zan-Bar, T., B. Bartoov, R. Segal, R. Yehuda, R. Lavi, R. Lubat and R. R. Avtalion (2005) Influence of visible light and ultraviolet irradiation on motility and fertility of mammalian and fish sperm. *Photomed. Laser Surg.* 23, 549–555.

38. Baier, J., T. Maisch, M. Maier, M. Landthaler and W. Bäumler (2007) Direct detection of singlet oxygen generated by UVA irradiation in human cells and skin. *J. Invest. Dermatol.* 127, 1498–1506.