Shed the light on virus: virucidal effects of 405 nm visible light on SARS-CoV-2 and influenza A virus.

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Abstract

Germicidal potential of specific wavelengths within the electromagnetic spectrum is an area of growing interest. While ultra-violet (UV) based technologies have shown satisfactory virucidal potential, the photo-toxicity in humans coupled with UV associated polymer degradation limit its use in occupied spaces. Alternatively, longer wavelengths with less irradiation energy such as visible light (405 nm) have largely been explored in the context of bactericidal and fungicidal applications. Such studies indicated that 405 nm mediated inactivation is caused by the absorbance of porphyrins within the organism creating reactive oxygen species which result in free radical damage to its DNA and disruption of cellular functions. The virucidal potential of visible-light based technologies has been largely unexplored and speculated to be not effective given the lack of porphyrins in viruses. The current study demonstrated increased susceptibility of

lipid-enveloped respiratory pathogens of importance such as SARS-CoV-2 (causative agent of COVID-19) as well as the influenza A virus to 405nm, visible light in the absence of exogenous photosensitizers, indicating a potential porphyrin-independent alternative mechanism of visible light mediated viral inactivation. Given that visible light is generally safe to humans, our results support further exploration of the use of visible light technology for the application of continuous decontamination in areas within hospitals and/or infectious disease laboratories, specifically for the inactivation of respiratory pathogens such as SARS-CoV-2 and Influenza A.

Key words – Visible light, 405nm, Virucidal, SARS-CoV-2, Influenza, inactivation

Introduction

The severe-acute respiratory syndrome corona virus 2 (SARS-CoV-2), the causative agent of the COVID-19 pandemic, is a member of the beta-coronavirus family and it emerged at the end of 2019 in the Hubei province in Wuhan China[1]. By late February 2021, more than 112 million cases had been reported while accounting for approximately 2.5 million deaths, underscoring the rapid dissemination of the virus on a global scale[2]. As a complement to standard precautions such as handwashing, masking, surface disinfection, and social distancing, other enhancements to enclosed spaces such as improved ventilation and whole-room disinfection are being considered by segments beyond acute healthcare such as retail, dining, and transportation[3].

Initial guidance from health authorities such as the CDC and WHO on environmental transmission focused on contaminated surfaces as fomites[4]. Data pertaining to the survival of SARS-CoV-2 and other related coronaviruses to date has indicated that virions are able to persist on fomites composed of plastic[5], wood[6], paper[5], metal[7] and glass[8] potentially up to nine days. Recent studies have suggested that SARS-CoV-2 may also remain viable approximately at least three days in such surfaces and another two studies showed that at room temperature (20-25°C), a 14-day time-period was required to see a 4.5-5 Log_{10} of the virus[9, 10].

Since the start of the pandemic, transmission of the virus by respiratory droplets and aerosols has become an accepted method of transmission although the relative impact of each mode of transmission is the subject of much debate. Nevertheless, enclosed

spaces with groups of people exercising or singing have been associated with increased transmission. The half-life survival of SARS-CoV-2 in this type of environment has been estimated between 1-2 hours[6, 11, 12].

Taking this information into consideration, several methods have been evaluated to effectively inactivate SARS-CoV-2. Chemical methods, which focus on surface disinfection, utilize 70% alcohol and bleach and their benefits are well established. These methods are also episodic (or non-continuous) meaning that in-between applications, the environment is not being treated[13].

In addition to chemicals, one of the most utilized methods for whole-room disinfection is germicidal ultra-violet C (UVC; ~254 nm)[14]. This technology is well established[15] and has been shown to inactivate a range of pathogens including bacteria[16], fungi[17] and viruses[18]. The mechanism of action of UVC is photodimerization of genetic material such as RNA (relevant for SARS-CoV2 and IAV) and DNA (relevant for DNA viruses and bacterial pathogens, among others)[19]. Unfortunately, this effect has been associated with deleterious effects in exposed humans such as photokeratoconunctivitis in eyes and photodermatitis in skin[20]. For these reasons, UVC irradiation requires safety precautions and cannot be used to decontaminate fomites and high contact areas in the presence of humans[21].

Germicidal properties of violet-blue visible light (380-500 nm), especially within the range of 405 to 450 nm wavelengths have been appreciated as an alternative to UVC irradiation in whole-room disinfection scenarios where it has shown reduction of bacteria[22, 23] in occupied rooms and reductions in surgical site infections[24]. Although 405 nm or closely related wavelengths have been shown to be less germicidal than UVC, its

inactivation potential has been assessed in pathogenic bacteria such as *Listeria* spp and *Clostridium* spp[24, 25], and in fungal species such as *Saccharomyces* spp and *Candida* spp[26] . It is thought that the underlying mechanism of blue-light mediated inactivation is associated with absorption of light via photosensitizers such as porphyrins which results in the release of reactive oxygen species (ROS) [27, 28]. The emergence of ROS is associated with direct damage to biomolecules such as proteins, lipids and nucleic acids which are essential constituents of bacteria, fungi and viruses. Further studies have shown that ROS can also lead to the loss of cell membrane permeability mediated by lipid oxidation[29]. Given the lack of endogenous photosensitizers such as porphyrins in virions, efficient decontamination of viruses (both enveloped and non-enveloped) may require the addition of exogenous photosensitizers[23]. With the use of media suspensions containing both endogenous and/or exogenous photosensitizers, inactivation of viruses such as feline calcivirus (FCV)[30], viral hemorrhagic septicemia virus (VHSV)[31] and murine norovirus-1[32] has demonstrated the virucidal potency of 405 nm visible light. Of note, most studies virus inactivation studies have been performed in media containing porphyrins. In the current study, we show the impact of 405 nm irradiation on inactivation of SARS-CoV-2 and influenza A H1N1 viruses without the use of photosensitizers, supporting the possible use of 405 nm irradiation as a tool to confer continuous decontamination of respiratory pathogens such as SARS-CoV-2 and influenza A viruses. We further show the increased susceptibility of lipid-enveloped viruses for irradiation in comparison to non-enveloped viruses, further characterizing the virucidal effects of visible light.

Materials and methods.

405 nm Exposure System

The visible light disinfection product used in this study was Indigo-Clean from Kenall Manufacturing. The product form factor selected was a 6" downlight (M4DLIC6) to allow for use within a BSL-3 rated containment hood. Within the hood, the distance between the face of the fixture and the sample was 10"- much less than the normal 1.5m used in normal, whole-room disinfection applications. The output of the fixture was modified electronically during its manufacture to match this difference and ensure that the measurements would represent the performance of the device in actual use. For the range of output used in this study, multiple discrete levels were created using pulse width modulation within the LED driver itself. These levels were made to be individually selectable using a simple knob on the attached control module.

As expected, the amount of visible light within the 400nm-420nm bandwidth is a measurement of the "dose" delivered to the target organism, measured in $mWcm^{-2}$, is used to quantify this relationship similar to that used in UV disinfection applications.

To fully examine this effect, a range of irradiance values were used representing actual product deployment conditions in occupied rooms. The lowest value (0.035 $mWcm^{-2}$) represents a single-mode, lower wattage used in general lighting applications while the highest value (0.6 $mWcm^{-2}$) represents a dual-mode, higher wattage used in critical care applications such as an operating room.

The device was placed in a rig to ensure a consistent distance (10") between the fixture and the samples. The output of the fixture in the test rig was measured using a Stellar-RAD Radiometer from StellarNet configured to make wavelength and irradiance

137 measurements from 350nm-1100nm with < 1nm spectral bandwidth using a NIST
138 traceable calibration. To ensure that the regular white light portion of the illumination
139 (which is non-disinfecting) was not measured, the measurement was electronically
140 limited to a 1nm bandwidth over the 400nm-420nm range. The normalized spectral
141 profile is shown in Fig. 1 below. The absolute value of the measurement was
142 determined using a NIST traceable calibration as previously described.

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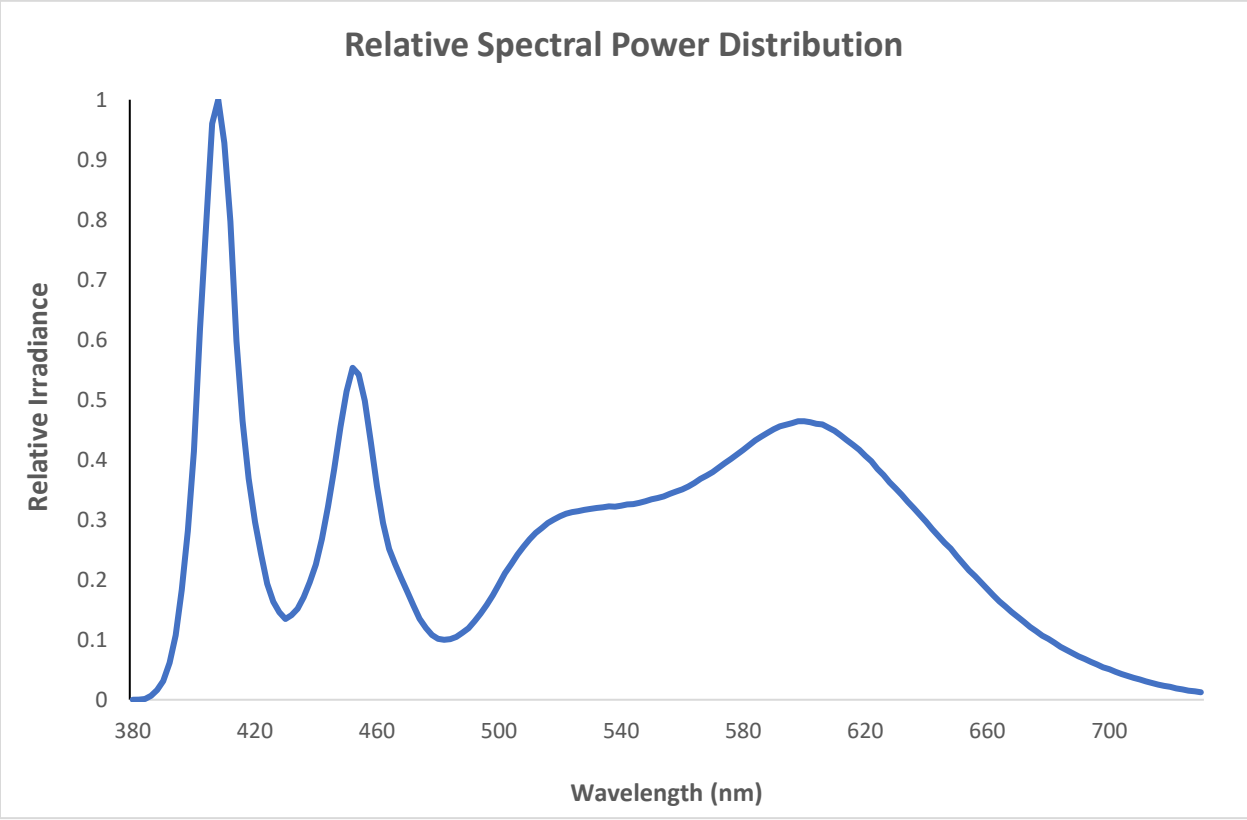

144

145 **Figure 1. Normalized spectral power distribution for Indigo-Clean M4DLIC6**
146 **showing peak irradiance at 405nm**.

147

Cells and viruses

Vero-E6 cells (ATCC® CRL-1586™, clone E6) were maintained in Dulbecco's Modified Eagle Medium (DMEM) complemented with 10% heat-inactivated Fetal Bovine Serum (HI-FBS; PEAK serum), penicillin-streptomycin (Gibco; 15140-122), HEPES buffer (Gibco; 15630-080) and MEM non-essential amino-acids (Gibco; 25025CL) at 37°C with 5% CO2. Vero-CCL81 (ATCC® CRL-81™) cells and MDCK cells (ATCC® CCL-34) were cultured in DMEM supplemented with 10% HI-FBS and penicillin/streptomycin at -37ºC with 5% CO2. All experiments involving SARS-CoV2 (USA-WA1/202, BEI resource – NR52281) were conducted within a biosafety-level 3 (BSL3) containment facility at Icahn school of medicine at Mount Sinai by trained workers upon authorization of protocols by a biosafety committee. Amplification of SARS-CoV-2 viral stocks was done in Vero-E6 cell confluent monolayers by using an infection medium composed of DMEM supplemented with 2% HI-FBS, Non-essential amino acids (NEAA), Hepes and penicillin-streptomycin at 37°C with 5% CO2 for 72 hours. Influenza A virus used here was generated using plasmid based reverse genetics system as previously described[33]. The backbone used in the study was A/Puerto Rico/8/34/Mount Sinai(H1N1) under the GenBank accession number AF389122. IAV-PR8 virus was grown and titrated in MDCK as previously described[33]. As a non-enveloped virus, the cell culture adapted murine Encephalomyocarditis virus (EMCV; ATCC® VR-12B) was propagated and titrated in Vero-CCL81 cells with DMEM and 2% HI-FBS and penicillin-streptomycin at 37°C with 5% CO2 for 48 hours[34].

405nm inactivation of viruses

The SARS-CoV-2 virus was exclusively handled at the Icahn school of Medicine BSL-3 and studies involving IAV and EMCV were handled in BSL-2 conditions. Indicated PFU amounts were mixed with sterile 1X PBS and were irradiated in 96 well format cell culture plates in triplicates. In these studies, A starting dose of $5x10^5$ PFU for SARS-CoV-2 and starting doses of $1x10^5$ PFU for IAV and EMCV were used. The final volumes for inactivation were 250 µl per replicate. The untreated samples were prepared the same way and were left inside the biosafety cabinet isolated from the inactivation device at room temperature. The plates were sealed with qPCR plate transparent seal and an approximate 10% reduction of the intensity was observed due to the sealing film. The distance from the lamp and the samples was measured to be 10". All samples were extracted at indicated times and were frozen at -80°C and were thawed together for titration via plaque assays.

Plaque assays

Confluent monolayers of Vero-E6 cells in 12-well plate format were infected with 10-fold serially diluted samples in 1X phosphate-buffered saline (PBS) supplemented with bovine serum albumin (BSA) and penicillin-streptomycin for an hour while gently shaking the plates every 15 minutes. Afterwards, the inoculum was removed, and the cells were incubated with an overlay composed of MEM with 2% FBS and 0.05% Oxoid agar for 72 hours at 37°C with 5% CO_2. The plates were subsequently fixed using 10% formaldehyde overnight and the formaldehyde was removed along with the overlay. Fixed monolayers were blocked with 5% milk in Tris-buffered saline with 0.1% tween-20 (TBS-T) for an hour. Afterwards, plates were immunostained using a monoclonal antibody against SARS-CoV2 nucleoprotein (Creative-Biolabs; NP1C7C7) at a dilution

of 1:1000 followed by 1:5000 anti-mouse IgG monoclonal antibody and was developed using KPL TrueBlue peroxidase substrate for 10 minutes (Seracare; 5510-0030). After washing the plates with distilled water, the number of a plaques were counted. Plaque assays for IAV and EMCV were done in a similar fashion. For IAV, confluent monolayers of MDCK cells supplemented with MEM-based overlay with TPCK-treated trypsin was used. For EMCV, Vero-CCL81 cells were used to do plaque assays in 6 well plate format. Plaques for IAV and EMCV were visualized using crystal violet. Data shown here is derived from three independent experimental setups.

Results.

Dose and time dependent inactivation of SARS-CoV-2 in the absence of photosensitizers.

The lowest irradiation dose of 0.035 $mWcm^{-2}$ was applied for SARS-CoV-2 and when compared to the initial input (T_0) of ~$5x10^5$ PFU, a reduction of 55.08% was seen as early as 4 hours and after 24 hours of irradiation, an inactivation of 90.17% (approximately 10 times reduction in infectivity) was observed for SARS-CoV-2 via plaque assays (Figure 2A). A slightly higher dose of 0.076$mWcm^{-2}$ resulted in a reduction of 98.22% (56 times) after 24 hours when compared to the original input at T_0 (Figure 2B). Subsequent increase of the irradiation dose to 0.150 $mWcm^{-2}$ resulted in a reduction of 63.64% after 4 hours which then reached 96.21% after 12 hours. Irradiation for 24 hours at 0.150 $mWcm^{-2}$ suggested a total reduction of 99.61% (256 times) for SARS-CoV-2 (Figure 2C). As a final experiment, a high irradiation dose of 0.6 $mWcm^{-2}$ was used to assess the inactivation potential within a much shorter time frame. Irradiation for one hour resulted in a reduction of 71.52% which reached 91.15% after

four hours and 99.74% (385 times) after 8 hours in comparison to the initial input (T_0) (Figure 2 D and E). All experimental conditions demonstrated the stability of untreated SARS-CoV-2 which was left at room temperature in PBS, as shown by the marginal reduction of viral titer over time.

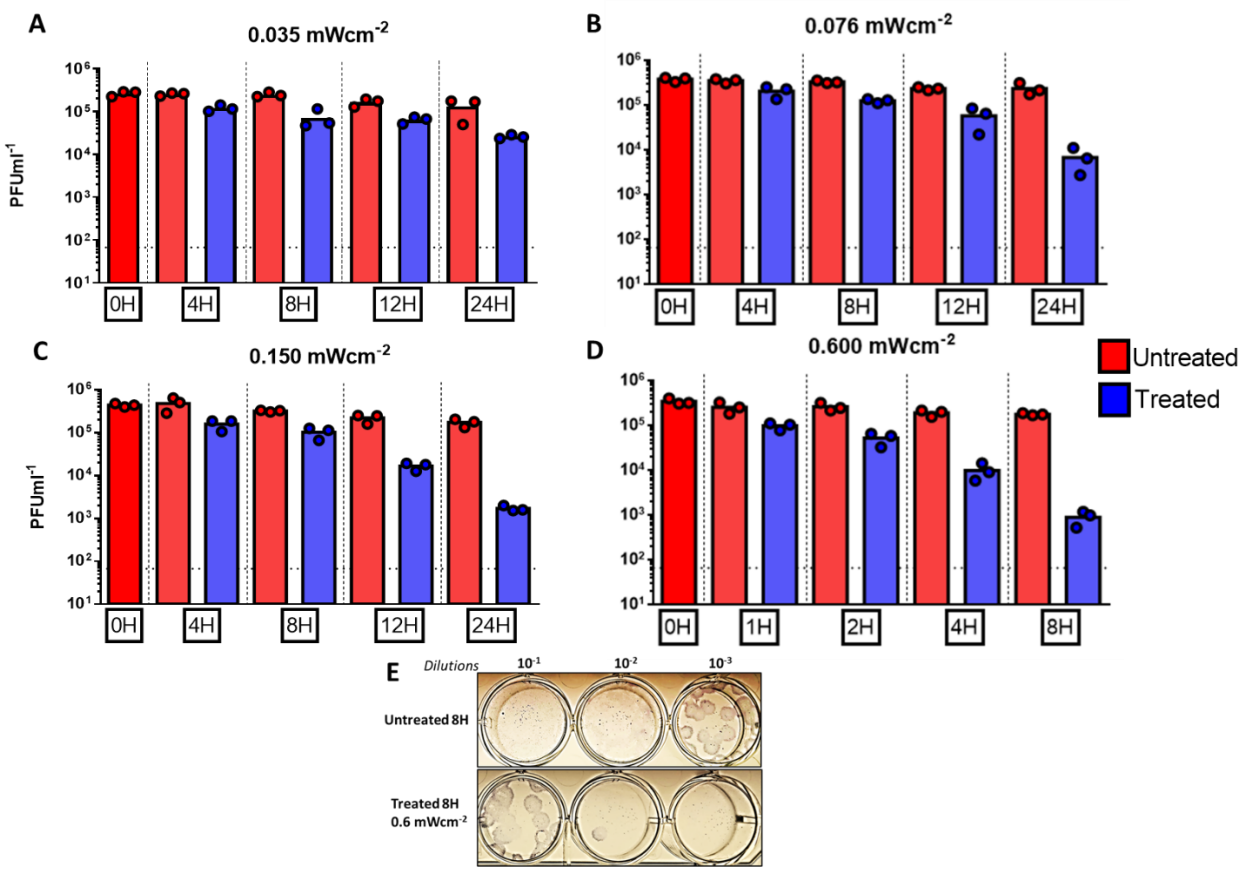

Figure 2. Dose and time dependent inactivation of SARS-CoV-2 virus in PBS by 405 nm irradiation. A. A dose of 0.035 $mWcm^{-2}$ or **B.** a dose of 0.076 $mWcm^{-2}$ or **C.** a dose of 0.150 $mWcm^{-2}$ or **D.** a dose of 0.6 $mWcm^{-2}$ was applied to irradiate samples at 405 nm over a course of 24 while sampling at 4, 8, 12 and 24 hours (for A, B and C) or over a course of 8 hours while sampling at 1, 2, 4 and 8 hours (D) was done in independent triplicates. Blue bars indicate treated samples and red bars correspond to the untreated equivalent that was left at the biosafety cabinet under the same conditions while not subjecting to irradiation. Data shown as $PFUml^{-1}$ in triplicate assessed by plaque assay. **E.** Plaque phenotype comparison from one independent experiment at an irradiation dose of 0.6 $mWcm^{-2}$. Fixed and blocked plaques were immunostained using anti-NP antibody before developing using TrueBlue reagent.

Influenza A virus is susceptible to 405 nm inactivation in the absence of photosensitizers.

Given the observations derived from SARS-CoV-2, a separate inactivation study using a different lipid-enveloped RNA virus was conducted by using influenza A Puerto Rico (A/H1N1/PR8-Mount Sinai) virus strain. Irradiation with a high dose of 0.6 $mWcm^{-2}$ suggested a time dependent reduction of infectivity of 31.11%, 63.33%, 81.56% and 98.49% (66 tiems) at 1, 2, 4 and 8 hours respectively (Figure 3A and 3B).

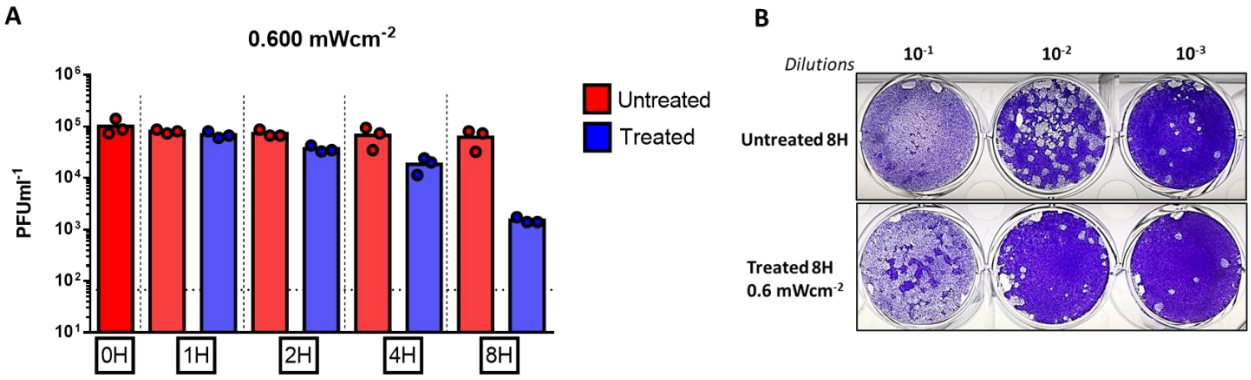

Figure 3 Inactivation of Influenza A virus in PBS by 405 nm irradiation. A. A dose of 0.6 $mWcm^{-2}$ was applied to irradiate samples at 405 nm over a course 8 hours while sampling at 1, 2, 4 and 8 hours (done in independent triplicates). Blue bars indicate treated samples and red bars correspond to the untreated equivalent that was left at the biosafety cabinet under the same conditions while not subjecting to irradiation. Data shown as $PFUml^{-1}$ in triplicate assessed by plaque assay. **B.** Plaque phenotype comparison from one independent experiment at an irradiation dose of 0.6 $mWcm^{-2}$. Fixed and blocked plaques were stained using crystal violet.

The stability of IAV virus at room temperature for a period of 8 hours was found to be the negligible in untreated IAV spiked PBS samples (Figure 3A).

Encephalomyocarditis virus (EMCV) as a model non-enveloped virus indicates reduced susceptibility to 405 nm inactivation in the absence of photosensitizers.

In order to better understand the effect of the lipid-envelope in viral inactivation by 405 nm irradiation, we used a non-lipid enveloped RNA virus derived from the *Picornaviridae* family. EMCV virus was irradiated at a high dose of 0.6 $mWcm^{-2}$ similar to SARS-CoV-2 and IAV.

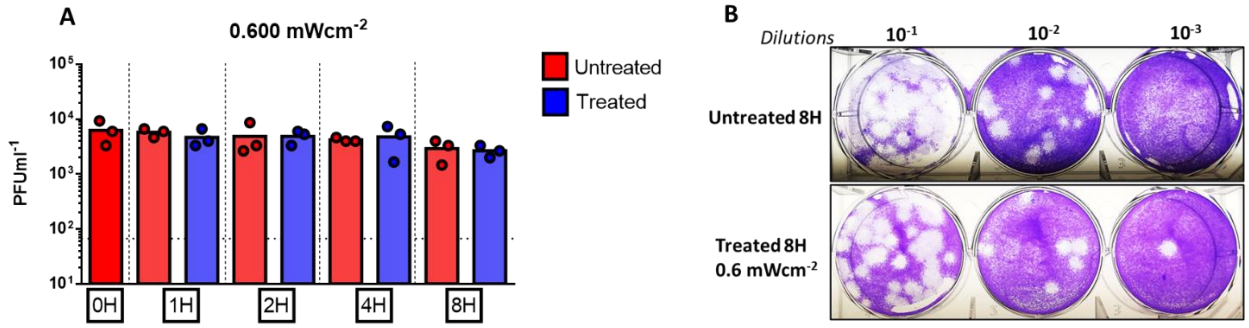

Figure 4. Encephalomyocarditis virus (EMCV) in PBS shows reduced susceptibility to 405 nm irradiation. A. A dose of 0.6 $mWcm^{-2}$ was applied to irradiate samples at 405 nm over a course 8 hours while sampling at 1, 2, 4 and 8 hours (done in independent triplicates). Blue bars indicate treated samples and red bars correspond to the untreated equivalent that was left at the biosafety cabinet under the same conditions while not subjecting to irradiation. Data shown as $PFUml^{-1}$ in triplicate assessed by plaque assay. **B.** Plaque phenotype comparison from one independent experiment at an irradiation dose of 0.6 $mWcm^{-2}$. Fixed and blocked plaques were stained using crystal violet.

In this case however, a total reduction of 9.1% (approximately 2 times) in comparison to the initial input (T_0) after 8 hours of irradiation was observed (Fig 4A and 4 B) indicating a lower rate of inactivation in contrast to the lipid-enveloped RNA viruses tested in this study. The plaque reduction at 8 hours did not indicate the same dramatic reduction as observed with the latter studies.

Discussion

The ongoing SARS-CoV-2 pandemic has affected the day-to-day functions in the entire world, raising concerns not only with regards to therapeutics but also in the context of

virus survivorship and decontamination[35]. Taking into consideration the rapid spread of SARS-CoV-2 from person to person by droplets, aerosols, and fomites, whole-room disinfection systems can be viewed as a supplement to best practices for interrupting transmission of the virus.

Given the ongoing COVID-19 pandemic, we wanted to explore the impact of 405 nm enriched visible light technology on inactivation of respiratory pathogens such as SARS-CoV-2 and influenza A virus.

Without the use of exogenous photosensitizers, we were able to show that irradiation with low intensity (0.035 $mWcm^{-2}$) visible light yielded a total of 55.08% inactivation after four hours and a total of 90.17% inactivation of SARS-CoV-2 after 24 hours. A slightly higher dose (0.076 $mWcm^{-2}$) resulted in 98.22% inactivation after 24 hours while an irradiation dose of 0.150 $mWcm^{-2}$ showed a reduction of 63.64% and 99.61% after four hours and 24 hours of irradiation, respectively. Finally, increasing the dose to 0.6 $mwcm^{-2}$ yielded 99.74% after eight hours, indicating a both time and dose dependent inactivation of infectious viruses. We selected conventional plaque assays as the read out to specifically estimate infectious virus titers upon disinfection. Methods based in the quantification of viral RNA via PCR based techniques might be misleading as they detect viral RNA from both infectious and noninfectious virions.

SARS-CoV-2 is a lipid-enveloped virus composed of a ssRNA genome and our data indicates its susceptibility to visible light mediated inactivation. To further confirm these observations, we used influenza A virus. which is another human respiratory virus with a lipid envelop and an RNA genome. Upon irradiating for 1 hour at 0.6 $mWcm^{-2}$, we observed a total reduction of 31.11% for the influenza A virus compared to the reduction

of 71.52% for SARS-CoV-2 under the same conditions. While both viruses have lipid envelopes, there is clearly a difference here that will require further study. One possible explanation is the difference in the virion size creating a physically smaller cross-section for absorption. (IAV ~120 nm and SARS-CoV-2 ~200 nm)[36, 37]. Nevertheless, both viruses were largely inactivated after eight hours- 98.49% for IAV and 99.74% for SARS-CoV-2. Intriguingly, it was observed that both RNA viruses were able to remain stable at room temperature for at least 24 hours, indicating minimal decay which is consistent with previous studies[35, 38]. We next irradiated a non-enveloped RNA virus, EMCV. Previous results for visible light against non-enveloped viruses demonstrated the need for external photosensitizers such as artificial saliva, blood, feces, etc[30, 35]. Without a porphyrin containing medium, we expected little to no inactivation when this virus was irradiated with visible light. For these measurements, we used the highest available irradiance of 0.6 $mWcm^{-2}$. As anticipated, we observed only a 9.1% inactivation after eight hours, however, this appears to be with the statistical precision of the measurement based on the results obtained from shorter irradiations (1, 2, and 4 hours). For comparison, a study involving the M13-bacteriophage virus (a non-enveloped virus) showed a 3-Log reduction using an irradiance of $50mWcm^{-2}$ (almost 100 times greater than the highest irradiance used in this study) for 10 hours at 425 nm further supporting the idea that non-enveloped viruses may require higher doses of visible light[39].

Our study was conducted using a neutral liquid media composed of PBS without any photosensitizers and we were able to show that visible light can indeed inactivate lipid-enveloped viruses, differing from the theory that states that photosensitizers are a

requirement for inactivation. Other studies which used visible light based irradiation have shown similar results in the absence of photosensitizers, indicating the possibility of an alternative inactivation mechanism[23, 25, 30]. Studies have proposed two theories for this observation. The first being that small amounts of 420-430 nm emitted from the source is contributing to the viral inactivation [40]. This theory most likely doesn't apply here as the spectrum of light used contains very little irradiance at these wavelengths. The other theory involves the presence of UV-A (390 nm) created as a byproduct. This wavelength is known to create oxidative stress upon viral capsids[41].

The results obtained suggest that the performance of visible light against SARS-CoV-2 is similar to organisms commonly found in the environment such as *S. aureus*. Previous studies have shown that the visible light irradiance levels used in this study (0.035 $mWcm^{-2}$ to 0.6 $mWcm^{-2}$) reduce bacteria levels in occupied rooms and improve outcomes for surgical procedures. It is therefore reasonable to conclude that visible light might be an effective disinfectant against SARS-CoV-2. More importantly, this disinfection can operate continuously as it is safe for humans based upon the exposure guidelines in IEC 62471[42]. This means that once it has been in use for a period of time, the environment will be cleaner and safer the next time it is occupied by humans.

One limitation of this study is that the inactivation assays were performed in static liquid media as opposed to aerosolized droplets. While the use of visible light in air disinfection has been briefly studied where it was shown that its effectiveness increased approximately 4-fold[43], further studies involving dynamic aerosolization are needed to better understand the true potential of visible light mediated viral inactivation.

In any case, our study shows the increased susceptibility of enveloped respiratory viral pathogens to 405 nm mediated inactivation in the absence of photosensitizers. The irradiances used in this study are very low and might be easily applied to safely and continuously disinfect occupied areas within hospitals, schools, restaurants, offices and other locations.

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Conflicts of interest

The García-Sastre Laboratory has received research support from Pfizer, Senhwa Biosciences, 7Hills Pharma, Avimex, Blade Therapeutics, Dynavax, ImmunityBio, Nanocomposix and Kenall Manufacturing. Adolfo García-Sastre has consulting agreements for the following companies involving cash and/or stock: Vivaldi Biosciences, Pagoda, Contrafect, 7Hills Pharma, Avimex, Vaxalto, Accurius, Pfizer and Esperovax. RR, CY and AGS have filed for a provisional patent based upon these results.

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