Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

American Journal of Infection Control 50 (2022) 844–848

Contents lists available at ScienceDirect

American Journal of Infection Control

journal homepage: www.ajicjournal.org

Major Article

Inactivation strategies for SARS-CoV-2 on surgical masks using light-activated chemical dyes

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Key Words:
SARS-CoV-2 virus
Methylene blue
Riboflavin
Revolution-Zero
COVID-19
Photochemical inactivation

Background: Methylene blue (MB) and riboflavin (RB) are light-activated dyes with demonstrated antimicrobial activity. They require no specialized equipment, making them attractive for widespread use. Due to COVID-19-related worldwide shortages of surgical masks, simple, safe, and effective decontamination methods for reusing masks have become desirable in clinical and public settings.

Material and methods: We examined the decontamination of SARS-CoV-2 Beta variant on surgical masks and Revolution-Zero Environmentally Sustainable (RZES) reusable masks using these photoactivated dyes. We pre-treated surgical masks with 2 MB concentrations, 2 RB concentrations, and 2 combinations of MB and RB. We also tested 7 MB concentrations on RZES masks.

Results: Photoactivated MB consistently inactivated SARS-CoV-2 at >99.9% for concentrations of 2.6 μM or higher within 30 min on RZES masks and 5 μM or higher within 5 min on disposable surgical masks. RB alone showed a lower, yet still significant inactivation (~93-99%) in these conditions.

Discussion: MB represents a cost-effective, rapid, and widely deployable decontamination method for SARS-CoV-2. The simplicity of MB formulation makes it ideal for mask pre-treatment in low-resource settings.

Conclusions: The results demonstrate that MB effectively decontaminates SARS-CoV-2 at concentrations above 5 μM on surgical masks and above 10 μM on RZES masks.

The rapid spread of the coronavirus disease 2019 (COVID-19) pandemic caused by the severe acute respiratory coronavirus virus 2 (SARS-CoV-2) has occurred at such a swift pace that it has crippled worldwide supply chains and has most critically resulted in acute shortages of personal protective equipment (PPE) for healthcare personnel (HCP) and the general public. With these continuing PPE shortages, HCP have attempted to reuse PPE at a higher frequency than ever before. Although designed for single-use, potentially contaminated surgical masks and filtering facepiece respirators (FFRs) have been continually reused on an emergency basis.[1] Data from a web-based survey of physicians and nurses that was distributed worldwide in April 2020 indicated that up to 30% of the 2711 respondents reported reusing single-use PPE.[1] They commonly reported widespread shortages and frequent reuse of PPE.[1] The study concluded that access to appropriate PPE was the first of 8 sources of anxiety in HCP that were interviewed during the first week of the pandemic.[1] Furthermore, evidence has shown that SARS-CoV-2 can stay active on masks for hours, and even up to 7 days. Therefore, it has become desirable for simple, safe, and effective methods for the decontamination and reuse of face masks in both clinical and public settings.[2,3]

Several PPE decontamination methods have recently been examined in an attempt to safely prolong the use and enable reuse of surgical masks and FFRs.[4–6] The rapid development and deployment of both familiar and novel decontamination methods has led the World Health Organization (WHO) to issue guidance on the rational use of

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Funding/support: T. Lendvay: WHO grant funding, Singletto equity owner, CMO, P/T salary support; J. Chen: Singletto Inc. shareholder; P. Rolley: Singletto Inc. stock options, consulting fees, pending patents; T. Dawson: Revolution One Board Member, CEO, stockholder; C. Mores: WHO grant funding. Open access of this article is sponsored by the World Health Organization.

Conflict of interest: The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of The George Washington University.

https://doi.org/10.1016/j.ajic.2022.03.015

PPE.[7] These WHO guidelines include recommendations on proper decontamination methods for PPE prior to reuse. During 2020, the US Food and Drug Administration (FDA) issued emergency approval for vaporized hydrogen peroxide (VHP) treatment as a method of decontamination for N95 FFRs but stopped short of approval for surgical masks as well.[5,6] However, VHP decontamination requires specialized equipment that is often unavailable in health care settings in low resource settings. The critical worldwide demand for surgical masks and FFRs has necessitated the development and deployment of safe and effective strategies for viral decontamination of both mask types in order to safely provide protection from the rapidly changing viral dynamics that occur during pandemics. The development of such strategies will also ensure better preparedness for future pandemic-level threats, and protect against the ongoing pathogenetic threats faced by healthcare workers every day.

Recently, photoactivated methylene blue (MB) has been reported to decontaminate surgical masks with various coronaviruses, including SARS-CoV-2.[4] This light-activated dye is known to demonstrate antimicrobial activity.[8–14] Photoactivated MB generates singlet oxygen, which damages viral nucleic acids and/or viral envelopes.[15,16] It is used to sterilize donor plasma before transfusion and is approved by the FDA for the treatment of methemoglobinemia and in FDA-approved wound care dressings.[17] Its efficacy has been demonstrated against a wide range of viruses in donor plasma.[9,10] A growing body of evidence suggests that the novel method of surface application and infusion of photoactivated methylene blue (MB) into mask material can effectively decontaminate SARS-CoV-2 virus from surgical masks and FFRs.[4] It has been shown previously that surgical mask and FFR integrity and fit remain unaffected after 5 sequential applications of photoactivated MB, which could potentially enable the safe reuse of these types of masks.[4]

Here, we investigated methylene blue as a potentially effective pretreatment method for surgical masks, while also comparing the potential for using riboflavin (RB), another photosensitive chemical known to have antimicrobial properties.[18] We examined the ability of MB, RB, and a combination of both chemicals to inactivate the SARS-CoV-2 Beta variant on surgical masks. In addition, we investigated various concentrations of MB on Revolution-Zero Environmentally Sustainable (RZES) reusable face masks which represent a PPE category of growing demand among an increasingly environmentally conscious public and due to increasing concern that worldwide mask usage is leading to discarded mask pollution around the planet. The RZES face masks most commonly favor polyester material due to the ease of recyclability of this material.[19,20] These masks were designed and manufactured in accordance with European standards such as EN14683 (medical device standard for masks), EN149 (PPE standard for masks), and EN13795:1 (standard for surgical clothing).[19,20] EN14683 certification ensures that these face masks have met regulatory standards in Europe to be within healthcare settings in addition to the public setting.[21]

MATERIAL AND METHODS

Biosafety statement

All experiments with SARS-CoV-2 were performed at biosafety level (BSL) 3 facilities at the George Washington University Milken Institute School of Public Health (Washington, DC, USA). BSL-3 facilities are sufficient for experiments with SARS-CoV-2. Experiments involving recombinant viruses were performed in accordance with approved Institutional Biosafety Committee protocols.

Viruses and cells

The SARS-CoV-2 isolate was obtained from BEI Resources: SARS-CoV-2 isolate hCoV-19/South Africa/KRISP-EC-K005321/2020, lineage B.1.351 (Beta variant; BEI NR-54008). Viral titers were determined using plaque assays in Vero-E6 cells (American Type Culture Collection (ATCC)). Vero-E6 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% GlutaMAX at 37°C and 5% CO_2.

Photoactivated MB pretreatment and decontamination

The disposable surgical masks (ASTM Level 2) were treated with various concentrations of MB, RB, or in combination using the following methods. MB was obtained from Sigma-Aldrich and dissolved in ultrapure water to prepare 2 concentrations of MB (1000 μM and 5 μM). RB was obtained from Sigma-Aldrich and dissolved in ultrapure water to prepare 2 concentrations of RB (1000 μM and 50 μM). These solutions were applied to the surgical masks to create 6 different conditions using spray bottles that coated the masks with 160 μL per spray. These 6 conditions were the 1000 μM MB application (24 sprays of 1000 μM MB), 5 μM MB application (24 sprays of 5 μM MB), 1000 μM RB application (24 sprays of 1000 μM RB), 50 μM RB application (24 sprays of 50 μM RB), 500 μM MB+500 RB application (24 sprays of a 500 μM MB + 500 μM RB solution), and 2.5 μM MB +25 μM RB application (24 sprays of a 2.5 μM MB + 25 μM RB solution).

The RZES masks were treated with various concentrations of MB. The solutions were prepared by dissolving MB (Sigma-Aldrich) in ultrapure water to prepare 7 concentrations of MB. The concentrations prepared were 0.25 μM, 1.3 μM, 2.6 μM, 10 μM, 50 μM, 100 μM, and 500 μM. RZES masks were prepared for each condition by submerging and soaking them in each of their respective solutions.

The masks were dried and then cut into $\sim$1 cm^2 coupons, which were placed in empty tissue culture plates during testing. Control coupons were left untreated. For decontamination testing, the pre-treated coupons were inoculated with 10 μL virus stock and exposed to ambient fluorescent light for 5 min and 30 min. Virus inoculum was eluted in serum-free-media (DMEM supplemented with 1% GlutaMAX) and quantified by plaque assays. Control coupons were inoculated with 10 μL virus stock and eluted immediately. Input virus titer was $\sim 3.2 \times 10^4$ PFU per 10 μL virus stock before elution. Ambient light ($\sim$700 lux) was provided by the biosafety cabinet lights. Light conditions were quantified using a light meter (Cooke cal-LIGHT 400).

Treatment to control comparisons were made to determine the effect of various treatments upon the quantity of inoculated virus recovered. Statistical comparisons of duplicate coupons tested for each inoculated mask+treatment were performed in GraphPad Prism version 9.3.1 using an unpaired t test (where $P \leq .05$ for reporting significance).

RESULTS

Methylene Blue versus Riboflavin against SARS-CoV-2 on disposable surgical masks

One of the aims of this study was to investigate whether photoactivated MB and photoactivated RB demonstrate similar inactivation profiles against SARS-CoV-2 when applied as a pretreatment to surgical masks prior to exposure to the virus. In order to determine this, 2 concentrations of MB (1000 μM and 5 μM), 2 concentrations of RB (1000 μM and 50 μM), and 2 combination mixtures of both chemicals (500 μM MB + 500 μM RB and 2.5 μM MB + 25 μM RB) were applied to separate batches of surgical masks. After coupons from each pretreated mask type were exposed to 10 μL of SARS-CoV-2 Beta variant, they all were exposed to $\sim$700 lux of light for the indicated time

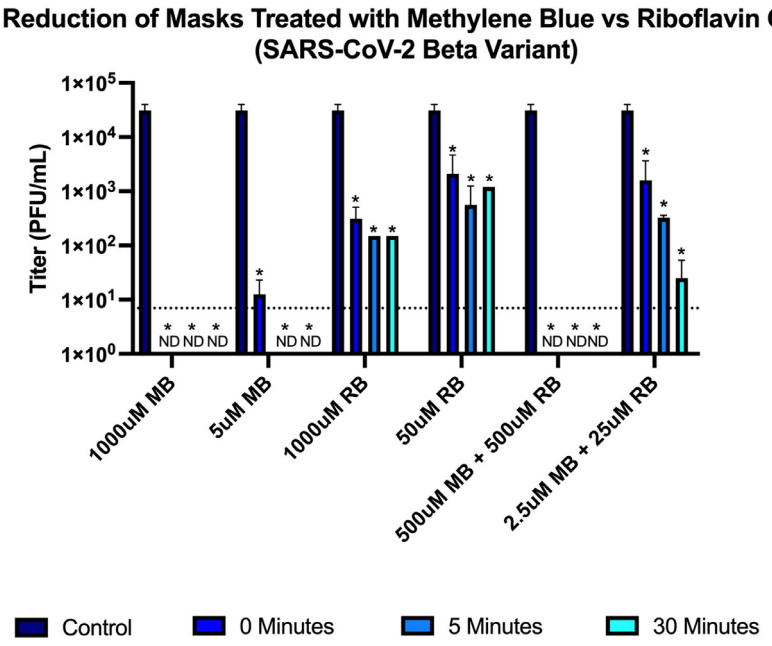

Fig 1. Methylene blue and riboflavin in light for inactivation of SARS-CoV-2 Beta variant on disposable surgical mask material. To compare the inactivation capabilities of photoactivated (~700 lux) methylene blue (MB) versus photoactivated riboflavin (RB) on surgical masks at various concentrations against the SARS-CoV-2 Beta variant, coupons of mask material with the indicated concentrations of these chemicals applied were inoculated with 10 μL virus and exposed to ambient fluorescent light (700 lux) for 5 min and 30 min. Control coupons of mask material were not exposed to any chemical dye, were inoculated with 10 μL virus and exposed to ambient fluorescent light (700 lux) for 5 min and 30 min. Titers of remaining infectious virus were determined by Plaque Assay. Values represent means and standard errors of duplicate samples with * denoting statistical significance ($P \leq .05$). Dotted line represents the limit of detection. ND, not detected. (Color version of figure is available online.)

periods (Fig 1). It was observed that masks that were pre-treated with MB alone and the mask containing the mixture of high concentration MB and RB produced complete inactivation of SARS-CoV-2 virus within 5 min while RB alone produced a slightly lower, yet significant inactivation of SARS-CoV-2 under these conditions (Fig 1 and Table 1). 1000 μM MB showed complete inactivation of SARS-CoV-2 (greater than 10,000-fold viral titer reduction) within less than 5 min. 5 μM MB showed complete inactivation of SARS-CoV-2 (greater than 10,000-fold viral titer reduction) by 5 min. 1000 μM RB showed significant inactivation of SARS-CoV-2 (~200-fold viral titer reduction) by 30 min. 50 μM RB showed significant inactivation of SARS-CoV-2 (~25-fold viral titer reduction) by 30 min. 500 μM MB +500 μM RB showed complete inactivation of SARS-CoV-2 (greater than 10,000-fold viral titer reduction) within less than 5 min. 2.5MB +25RB showed partial inactivation of SARS-CoV-2 (greater than 1,000-fold viral titer reduction) by 30 min.

Concentrations of methylene blue against SARS-CoV-2 on Revolution-Zero reusable masks

After establishing that MB is an effective pretreatment method of SARS-CoV-2 decontamination on disposable masks, we examined the ability of MB to inactivate SARS-CoV-2 Beta variant on the Revolution-Zero Environmentally Sustainable (RZES) reusable face mask at various concentrations of MB. In order to determine this, 7 different concentrations of MB were applied to separate batches of the RZES masks (Mask 1: 0.25 μM, Mask 2: 1.3 μM, Mask 3: 2.6 μM, Mask 4: 10 μM, Mask 5: 50 μM, Mask 6: 100 μM, and Mask 7: 500 μM). After coupons from each pre-treated mask type were exposed to 10 μL of SARS-CoV-2 Beta variant, they were exposed to ~700 lux of light for the indicated time periods (Fig 2). It was observed that masks that were pretreated with the 2 lowest concentrations had only partial inactivation of SARS-CoV-2 up through 30 min of light exposure time (Fig 2 and Table 2). Those pre-treated with 2.6 μM and 10 μM MB produced high inactivation of SARS-CoV-2 by 30 min and the masks with the 3 highest concentrations of MB produced complete inactivation of SARS-CoV-2 virus within 5 min. Mask 1 showed significant inactivation of SARS-CoV-2 (~200-fold viral titer reduction) by 5 min and complete inactivation (greater than 10,000-fold viral titer reduction) by 30 min. Mask 2 showed partial inactivation of SARS-CoV-2 (~300-fold viral titer reduction) by 30 min. Mask 3 showed a high level of SARS-CoV-2 inactivation (~1,200-fold viral titer reduction) by 30 min. Mask 4 showed a high level of SARS-CoV-2 inactivation (greater than 10,000-fold viral titer reduction) by 30 min. Mask 5, Mask 6, and Mask 7 all showed complete inactivation of SARS-CoV-2 (greater than 10,000-fold viral titer reduction) within 5 min or less.

DISCUSSION

The COVID-19 pandemic has demonstrated that rapidly emerging viruses capable of spreading worldwide in a matter of months have the potential to result in severe PPE shortages faster than previously anticipated. This underscores the importance of establishing effective methods of PPE decontamination for a wide variety of mask types that can enable rapid deployment of safe methods for the extended use or reuse of masks. In addition, the pandemic has highlighted the difficulties in deployment and inequity of complex methods for PPE decontamination. These are typically prohibitively expensive for resource poor settings and in some cases technically infeasible. The advantage of the MB pretreatment and decontamination method is that it can be made widely available in a short period of time and provides a simple, efficient, and cost-effective way to allow for mask reuse when warranted. This makes the MB method of pretreatment and decontamination a suitable method for both high- and low- resource settings.

Recently, photoactivated MB was shown to inactivate coronaviruses on respirator and medical mask material, forming the basis for its use as

Table 1
Methylene blue versus riboflavin activity in the reduction of SARS-CoV-2 on disposable surgical masks

Condition	Time	Average Titer	Titer Reduction	% Viral Reduction
Control	0 min	3.10×10^4	0.0	0.00%
1000 μM MB	0 min	ND	$\leq 3.10 \times 10^4$	≥99.98%
	5 min	ND	$\leq 3.10 \times 10^4$	≥99.98%
	30 min	ND	$\leq 3.10 \times 10^4$	≥99.98%
5 μM MB	0 min	1.25×10^1	3.09×10^4	99.68%
	5 min	ND	$\leq 3.10 \times 10^4$	99.98%
	30 min	ND	$\leq 3.10 \times 10^4$	≥99.98%
1000 μM RB	0 min	3.13×10^2	3.07×10^4	99.03%
	5 min	1.50×10^2	3.08×10^4	99.35%
	30 min	*1.50×10^2	3.08×10^4	99.35%
50 μM RB	0 min	2.10×10^3	2.89×10^4	93.22%
	5 min	5.60×10^2	3.04×10^4	98.06%
	30 min	*1.20×10^3	2.98×10^4	96.13%
500 μM MB + 500 μM RB	0 min	ND	$\leq 3.10 \times 10^4$	≥99.98%
	5 min	ND	$\leq 3.10 \times 10^4$	≥99.98%
	30 min	ND	$\leq 3.10 \times 10^4$	≥99.98%
2.5 μM MB + 25 μM RB	0 min	1.60×10^3	2.94×10^4	94.84%
	5 min	3.25×10^2	3.07×10^4	99.03%
	30 min	2.50×10^1	3.09×10^4	99.68%

*Titer and Log titer obtained from a single replicate. ND indicates Not Detectable.

a PPE decontamination method.[4] MB is approved by the US FDA and European Medicines Agency (EMA) to treat methemoglobinemia by intravenous injection.[13] It is commonly used to sterilize blood products before transfusion as well. MB is inexpensive, globally available, and can effectively inactivate viruses in combination with LED light of ~700 lux or greater, ambient light, or direct sunlight.[4] One of the main advantages of using photoactivated MB for virus inactivation is that MB activity is non-specific, and therefore the development of viral resistance is not expected. When combining MB with a light source, the energy is absorbed and transferred to molecular oxygen, resulting in the highly reactive singlet oxygen.[22] Singlet oxygen reacts with its cellular environment, leading to non-specific oxidative reactions. This results in damage to nucleic acids, proteins, and lipids.[15,16]

Although RB is an important vitamin commonly found in the diets of various cultures around the world, it did not show the same ability to inactivate SARS-CoV-2 as MB in the conditions of our study. RB has been generally known to have antimicrobial properties when exposed to ultra-violet A (UV-A) radiation.[18] Although it is clear that the levels of photoactivation that are sufficient for MB to effectively inactivate SARS-CoV-2 are insufficient for RB to inactivate this virus to the same degree, 1 limitation of our study was that no UV emissions were produced by our light source. Further investigation of high intensity light sources with various levels of ultra-violet light could indicate whether RB has enhanced ability to inactivate SARS-CoV-2. At least 1 study suggests that RB in the presence of UV light has the potential to inactivate SARS-CoV-2 in plasma and serum samples.[23] Determining whether this result can be translated to mask materials would require further investigation.

Our data demonstrates that photoactivated MB can inactivate SARS-CoV-2 Beta variant on both clinically and publicly available disposable surgical masks and reusable RZES masks. In this study, for practical and biosafety purposes, we tested ambient light of ~700 lux

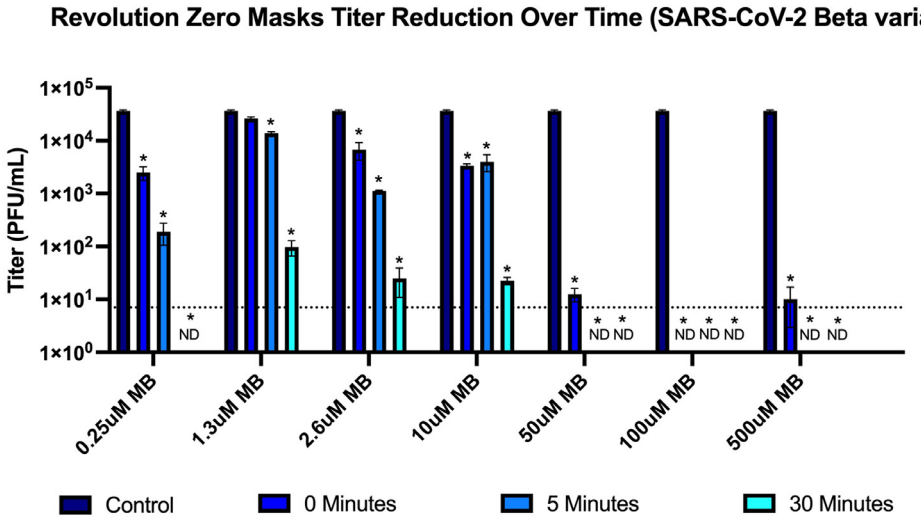

Fig 2. Methylene blue in light for inactivation of SARS-CoV-2 Beta variant on Revolution-Zero Environmentally Sustainable (RZES) reusable face masks. To determine the effectiveness of MB against SARS-CoV-2 Beta variant on reusable face masks used in the clinical and non-clinical setting, coupons of RZES mask material pretreated with the various concentrations of MB were inoculated with 10 μL virus and exposed to ambient fluorescent light (700 lux) for 5 min and 30 min. Control coupons of mask material were not exposed to any chemical dye, were inoculated with 10 μL virus and exposed to ambient fluorescent light (700 lux) for 5 min and 30 min. Titers of remaining infectious virus were determined by Plaque Assay. The masks were submerged with the following concentrations and methods: Mask 1 = 0.25 μM, Mask 2 = 1.3 μM, Mask 3 = 2.6 μM, Mask 4 = 10 μM, Mask 5 = 50 μM, Mask 6 = 100 μM, and Mask 7 = 500 μM. Values represent means and standard errors of duplicate samples with * denoting statistical significance ($P \leq .05$). Dotted line represents the limit of detection. ND, not detected. (Color version of figure is available online.)

Table 2
SARS-CoV-2 inactivation by various methylene blue concentrations on Revolution-Zero reusable masks

Condition	Time	Average Titer	Titer Reduction	% Viral Reduction
Control	0 min	3.10×10^4	0.0	0.00%
0.25 μM MB	0 min	2.50×10^3	2.85×10^4	91.94%
Mask 1	5 min	1.90×10^2	3.08×10^4	99.35%
	30 min	ND	$\leq 3.10 \times 10^4$	≥99.98%
1.3 μM MB	0 min	1.90×10^4	1.20×10^4	38.71%
Mask 2	5 min	2.10×10^4	1.00×10^4	32.26%
	30 min	9.75×10^1	3.09×10^4	99.68%
2.6 μM MB	0 min	6.75×10^3	2.43×10^4	78.39%
Mask 3	5 min	1.13×10^3	2.99×10^4	96.45%
	30 min	2.50×10^1	3.10×10^4	≥99.98%
10 μM MB	0 min	3.35×10^3	2.77×10^4	89.35%
Mask 4	5 min	4.00×10^3	2.70×10^4	87.10%
	30 min	2.25×10^1	$\leq 3.10 \times 10^4$	99.98%
50 μM MB	0 min	1.25×10^1	$\leq 3.10 \times 10^4$	99.98%
Mask 5	5 min	ND	$\leq 3.10 \times 10^4$	≥99.98%
	30 min	ND	$\leq 3.10 \times 10^4$	≥99.98%
100 μM MB	0 min	ND	$\leq 3.10 \times 10^4$	≥99.98%
Mask 6	5 min	ND	$\leq 3.10 \times 10^4$	≥99.98%
	30 min	ND	$\leq 3.10 \times 10^4$	≥99.98%
500 μM MB	0 min	1.00×10^1	$< 3.10 \times 10^4$	99.98%
Mask 7	5 min	ND	$\leq 3.10 \times 10^4$	≥99.98%
	30 min	ND	$\leq 3.10 \times 10^4$	≥99.98%

ND indicates Not Detectable.

generated by the lights of the biosafety cabinet. We demonstrate that 5–1000 μM MB combined with ~700 lux inactivates SARS-CoV-2 Beta variant within 5 min on surgical masks, while 50–500 μM MB combined with ~700 lux inactivates SARS-CoV-2 Beta variant within 5 min on RZES masks and 2.6–10 μM MB also showing high inactivation capability by 30 min on RZES masks. This indicates that both mask types are compatible with MB pre-treatment methods and result in successful inactivation of SARS-CoV-2 Beta variant, suggesting that there could be the potential for enhanced protection from this virus during MB use. In addition, our results also indicate the use of RB as a viable decontamination strategy under these conditions, but this may require additional testing. A clinical trial may provide additional support for the ongoing protection on pre-treated masks compared to non-treated masks.

Taken together, this study demonstrates that photoactivated MB pre-treatment is a viable method for inactivating SARS-CoV-2 Beta variant on at least 2 mask types used in both the clinical and non-clinical setting. This easily deployable low-cost decontamination method has the potential to mitigate PPE shortages and prolong safe PPE use within the healthcare setting and in a more general public setting.

Acknowledgments

We would like to thank Dr. Belinda Heyne for providing technical expertise for this project. We also thank Ms. Sarah Tritsch and Ms. Abigail Porzucek for critical assistance with data analysis and manuscript revision. We also acknowledge that funds for the project were provided by WHO based on a grant from the German Federal Ministry of Health (BMG).

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