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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 reused 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...
PPE. 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. However, VHP decontamination requires specialized equipment that is often unavailable in healthcare 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. This light-activated dye is known to demonstrate antimicrobial activity. Photoactivated MB generates singlet oxygen, which damages viral nucleic acids and/or viral envelopes. 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. Its efficacy has been demonstrated against a wide range of viruses in donor plasma. 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. 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.

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. 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. 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). EN14683 certification ensures that these face masks have met regulatory standards in Europe to be within healthcare settings in addition to the public setting.

**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/SouthAfrica/KRISP-EC-K0005321/2020, 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% CO2.

**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. These were dissolved 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 ~1 cm² coupons, which were placed in empty tissue culture plates during testing. Control coupons were left untreated. For decontamination testing, the pre-treated coupons from each pretreatment method were performed in GraphPad Prism version 9.3.1. Statistical comparisons of duplicate coupons tested for each inoculation were performed using an unpaired t test (where α = 0.05 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 pre-treated mask type were exposed to 10 μL of SARS-CoV-2 Beta variant, they all were exposed to ~700 lux of light for the indicated time.
Control coupons of mask material were not exposed to any chemical dye, were inoculated with 10^30 min. Titers of remaining infectious virus were determined by Plaque Assay. Values represent means and standard errors of duplicate samples with * denoting statistical significance.

SARS-CoV-2 Beta variant, they were 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 < .05). Dotted line represents the limit of detection. ND, not detected. (Color version of figure is available online.)

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.

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

After establishing that MB is an effective pretreatment method for both high- and low-resource settings.

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...
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 x 10^4  | 0.0             | 0.00%             |
| 1000 µM MB| 0 min  | ND            | ≤3.10 x 10^4    | ≥99.98%           |
|           | 5 min  | ND            | ≤3.10 x 10^4    | ≥99.98%           |
|           | 30 min | ND            | ≤3.10 x 10^4    | ≥99.98%           |
| 5 µM MB   | 0 min  | 1.25 x 10^1   | 3.09 x 10^4     | 99.68%           |
|           | 5 min  | ND            | ≤3.10 x 10^4    | ≥99.98%           |
|           | 30 min | ND            | ≤3.10 x 10^4    | ≥99.98%           |
| 1000 µM RB| 0 min  | 3.13 x 10^2   | 3.07 x 10^4     | 99.03%           |
|           | 5 min  | 1.50 x 10^2   | 3.08 x 10^4     | 99.35%           |
| 50 µM RB  | 0 min  | 2.10 x 10^5   | 2.89 x 10^4     | 93.22%           |
|           | 5 min  | 5.60 x 10^2   | 3.04 x 10^4     | 98.06%           |
|           | 30 min | 1.20 x 10^3   | 2.98 x 10^4     | 96.13%           |
| 500 µM MB + 500 µM RB | 0 min | ND            | ≤3.10 x 10^4    | ≥99.98%           |
|           | 5 min  | ND            | ≤3.10 x 10^4    | ≥99.98%           |
|           | 30 min | ND            | ≤3.10 x 10^4    | ≥99.98%           |
| 2.5 µM MB + 25 µM RB | 0 min | 1.60 x 10^3   | 2.94 x 10^4     | 94.84%           |
|           | 5 min  | 3.25 x 10^2   | 3.07 x 10^4     | 99.03%           |
|           | 30 min | 2.50 x 10^1   | 3.09 x 10^4     | 99.68%           |

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

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. Titors 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 ≤ .05). Dotted line represents the limit of detection. ND, not detected. (Color version of figure is available online.)
generated by the lights of the biosafety cabinet. We demonstrate that 5–1000 μg MB combined with ~700 lux inactivates SARS-CoV-2 Beta variant within 5 min on surgical masks, while 50–500 μg MB combined with ~700 lux inactivates SARS-CoV-2 Beta variant within 5 min on RZES masks and 2.6–10 μg 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 photocatalyzed 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.

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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 x 10^4   | 0               | 0.00%             |
| 0.25 μg MB    | 0 min| 2.50 x 10^3   | 2.85 x 10^4     | 91.94%            |
| Mask 1        | 5 min| 1.90 x 10^4   | 3.08 x 10^4     | 99.35%            |
|               | 30 min| ND           | ≤3.10 x 10^4    | ≥99.98%           |
| 1.3 μg MB     | 0 min| 1.90 x 10^4   | 1.20 x 10^4     | 38.71%            |
| Mask 2        | 5 min| 2.10 x 10^4   | 1.00 x 10^4     | 32.26%            |
|               | 30 min| 9.75 x 10^3   | 3.09 x 10^4     | 99.68%            |
| 2.6 μg MB     | 0 min| 6.75 x 10^3   | 2.43 x 10^4     | 78.39%            |
| Mask 3        | 5 min| 1.13 x 10^3   | 2.99 x 10^4     | 96.45%            |
|               | 30 min| 2.50 x 10^3   | 3.10 x 10^4     | ≥99.98%           |
| 10 μg MB      | 0 min| 3.35 x 10^3   | 2.77 x 10^4     | 89.35%            |
| Mask 4        | 5 min| 4.00 x 10^3   | 2.70 x 10^4     | 87.10%            |
|               | 30 min| 2.25 x 10^3   | ≤3.10 x 10^4    | 99.98%            |
| 50 μg MB      | 0 min| 1.25 x 10^3   | ≤3.10 x 10^4    | 99.98%            |
| Mask 5        | 5 min| ND            | ≤3.10 x 10^4    | ≥99.98%           |
|               | 30 min| ND            | ≤3.10 x 10^4    | ≥99.98%           |
| 100 μg MB     | 0 min| ND            | ≤3.10 x 10^4    | ≥99.98%           |
| Mask 6        | 5 min| ND            | ≤3.10 x 10^4    | ≥99.98%           |
|               | 30 min| ND            | ≤3.10 x 10^4    | ≥99.98%           |
| 500 μg MB     | 0 min| ND            | ≤3.10 x 10^4    | 99.98%            |
| Mask 7        | 5 min| ND            | ≤3.10 x 10^4    | ≥99.98%           |
|               | 30 min| ND            | ≤3.10 x 10^4    | ≥99.98%           |

ND indicates Not Detectable.

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