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Major Article

Methylene blue applied to N95 respirators and medical masks for SARS-CoV-2 decontamination: What is the likelihood of inhaling methylene blue?



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Background: Global shortage of personal protective equipment (PPE), as consequence of the COVID-19 global pandemic, has unmasked significant resource inequities prompting efforts to develop methods for safe PPE decontamination for reuse. The World Health Organization (WHO) in their Rational Use of PPE bulletin cited the use of a photodynamic dye, methylene blue, and light exposure as a viable option for N95 respirator decontamination. Because WHO noted that methylene blue (MB) would be applied to surfaces through which health care workers breathe, we hypothesized that little to no MB will be detectable by spectroscopy when the PPE is subjected to MB at supraphysiologic airflow rates.

Methods: A panel of N95 respirators, medical masks, and cloth masks were sprayed with 5 cycles of 1,000 uM MB solution. Mask coupons were subjected to the equivalent of 120 L/min of 100% humidified air flow. Effluent gas was trapped in an aqueous solution and the resultant fluid was sampled for MB absorbance with a level of detection of 0.004 mg/m³.

Results: No detectable MB was identified for any mask using Ultraviolet-Visible spectroscopy.

Conclusions: At 500-fold the amount of MB applied to N95 respirators and medical masks as were used for the decontamination study cited in the WHO Rational Use of PPE bulletin, no detectable MB was observed, thus providing safety evidence for the use of methylene blue and light exposure for mask decontamination.

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BACKGROUND

By 2020, the coronavirus disease 2019 (COVID-19) pandemic created a global shortage of personal protective equipment (PPE) including N95 respirators and medical masks (MMs). What were once

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Article Summary Line: Off-gassing of methylene blue from N95 respirators and medical masks after application of high concentrations of methylene blue for PPE decontamination.

authorized for single use, the masks were now being recommended for reuse based on the World Health Organization (WHO) Rational Use of PPE guidelines and various national regulatory and health agencies including the Food and Drug Administration (FDA) in the United States.^{1,2} The WHO recommended ultraviolet light (UV), vaporized hydrogen peroxide (VHP), and dry heat decontamination methods.¹ In the December 23, 2020 Rational Use of PPE Bulletin, the WHO cited research pertaining to the use of a photodynamic dye methylene blue and light exposure (MBL) as another method for PPE decontamination.¹ Although there are a number of FDA-cleared or approved^{3,4} and off-label uses of methylene blue (MB) for its medicinal and antimicrobial capabilities,⁵⁻⁸ it was unknown whether MB applied to face masks could potentially be inhaled by health care workers (HCWs) and if so, at what concentration. We sought to establish whether MB that had been sprayed onto masks can be detected coming off of treated masks under high airflow rates using much higher concentrations of MB as was used during the original

Development of Methods for Masks and N95 Respirator Decontamination (DeMaND) study showing that MBL could decontaminate SARS-CoV-2 on those masks.⁹

Methylene blue is a dye developed in 1876 by Bandische Anilin und Soda Fabrik (BASF)'s Heinrich Caro and used for decades in the production of clothing textiles for its blue color.¹⁰ It is referred to as "Essential Blue." As newer dyes less resistant to fading were invented, the textile use waned. However, in 1940, the antimicrobial capability of MB was discovered specifically against a staphylococcus¹¹ and, thereafter, a number of other pathogens.^{8,12–14} The mechanism of action of MBL decontamination leverages photon energy from the visible light spectrum to create short-lived (nanoseconds to milliseconds) singlet oxygen which can travel up to a few millimeters in the air. The singlet oxygen destroys deoxyribo- and ribo-nucleic acids and viral envelopes rendering the virus inactive.¹⁴ This reaction has been witnessed for a number of light-activated dyes including riboflavin (Vitamin B2), turmeric, erythrosine (red food dye #3), Rose Bengal, and others.^{15,16} As the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) virus spread around the globe, investigators in China identified that MBL could be used to decontaminate convalescent serum from previously infected COVID-19 patients.¹⁷ MB is FDA approved for intravenous treatment of methemoglobinemia and as a topical wound dressing.^{3,4} MB is approved in the European Union (EU) to photodisinfect human donor plasma prior to transfusion¹⁸ and approved in Canada and the EU for intranasal application with light activation preoperatively to reduce methicillin-resistant *Staphylococcus aureas* (MRSA) colonization.^{19,20} In addition, an ingested MB formulation is approved in the EU as an aid for endoscopic colonic adenoma detection.²¹

To test the use of MBL on PPE, the DeMaND consortium successfully demonstrated that MBL could decontaminate PPE from 3 coronaviruses – SARS-CoV-2, a murine coronavirus, and porcine coronavirus – after PPE inoculation.⁹ Furthermore, the group demonstrated that pre-treated PPE could inactivate the coronaviruses suggesting that ongoing PPE decontamination was possible, making this uniquely suitable to protect HCWs about to don the PPE in addition to the postprocessing decontamination capabilities as seen by VHP and UV light. The DeMaND group also showed that even after 5 cycles of MBL decontamination, there was no degradation of mask performance as tested by the National Institute of Occupational Safety and Health (NIOSH). Various white and red-light conditions from bright (50,000 lux) to ambient (500 lux) light were tested and both showed at least a 3-log and 4-log reduction in viable virus suggesting that general purpose indoor medical setting light conditions would be adequate to stimulate the MB.

Even though the concentrations of MB that were shown through the DeMaND study to inactivate virus were far lower than the concentrations of MB used in the approved intranasal MRSA decolonization technology or even in the FDA-approved intravenous MB formulation for the treatment of methemoglobinemia and the wound dressing impregnated with MB,^{3,4} we sought to test the amount of MB that might come off the pre-treated masks that could be potentially inhaled by HCWs during the course of a work shift. The Materials Safety Data Sheet for MB denotes an acute toxicity estimate for

inhalation by humans of 4,794 parts per million or 20.5 gm/L.²² When testing for risk of chemical inhalation, Ultraviolet-Visible light (UV-Vis) spectroscopy has been utilized to detect chemicals passing through the air and getting trapped in a fluid that can then be analyzed for said chemical.²³ We established a model to detect MB as air-flow rates were passed through PPE materials that had been pre-treated with high concentrations of MB and hypothesized that no or very low levels of MB would be detected.

METHODS AND MATERIALS

Masks and N95 respirators tested

For this study, 6 different respirator and MMs (Halyard N95 46727, Kolmi FFP2 NR Type IIR, 3M VFlex 1,804, 3M 1,860, 3M 1,870 + N95 Respirator, and EN 14,683 Type II Medical Face Mask [generic]) and one cloth community mask (CM) were tested (see Fig 1). Each mask was sprayed using an off-the shelf spray bottle with 7–8 mL of 1000 μ M MB (Sigma-Aldrich) solution with tap water. A total of 3 replicates for each mask were tested except the EN Type II mask in which 5 replicates were tested. A replicate denotes a single mask and a full-thickness coupon was cut out of each mask from a randomly selected spot on the face of the mask for testing.

Mask and N95 respirator MB application

N95 respirators, MMs, and the CMs were placed on aluminum foil with overlying paper towel. Using the spray bottle with 1000 μ M (see Fig 2), 3 masks of each type were treated with MB using 6 sprays applied to the front (external side) of the respirator and 2 sprays applied to the back (internal side) in an overlapping spray pattern to ensure the entire mask was treated (see Fig 3). Each spray yielded approximately 1 mL of MB solution therefore depositing 7–8 mL onto each respirator or mask. All spray applications were performed in low ambient light conditions of approximately 100–150 lux with exposure time less than 10 minutes at room temperature. Masks were wrapped in aluminum foil to avoid photobleaching, then taken to a dark room to dry for 24 hours. The spraying and drying cycles were repeated a total of 5 times for each mask to simulate repetitive MB applications for decontamination.

Mask preparation for spectroscopy

For each mask type, samples were cut from 3 treated masks that underwent 5 spray cycles with 1,000 μ M MB solution, and one control sample was cut from an untreated control mask for each type. Each mask "coupon" was placed within a bolt that allowed airflow through the coupon for capture of any MB that may have been blown off the material (effluent gas) (see Fig 4).

Each sample had an area of 0.128 cm², which corresponded to 0.07% of the total area of a mask assuming a total mask surface area of 183 cm² (the surface area of a 3 M 1860S N95 Respirator). Flow rate was set at 85.33 mL/min, which corresponded to 120 L/min for the whole mask (43,200 liters in total). The minute volume of females



Fig 1. Panel of N95 respirators, Medical Masks (MM) and a community cloth mask tested. From left to right: Halyard Fluidshield Duckbill 46,727 N95 respirator, 3M 1,860 N95 respirator, Kolmi Duckbill Filtering Face Piece (FFP) 2 NR Type 2R, 3M VFlex 1804 N95 respirator, 3M 1,870 + N95 respirator, EN 14,683 Type 2 medical mask (generic), cloth community mask.

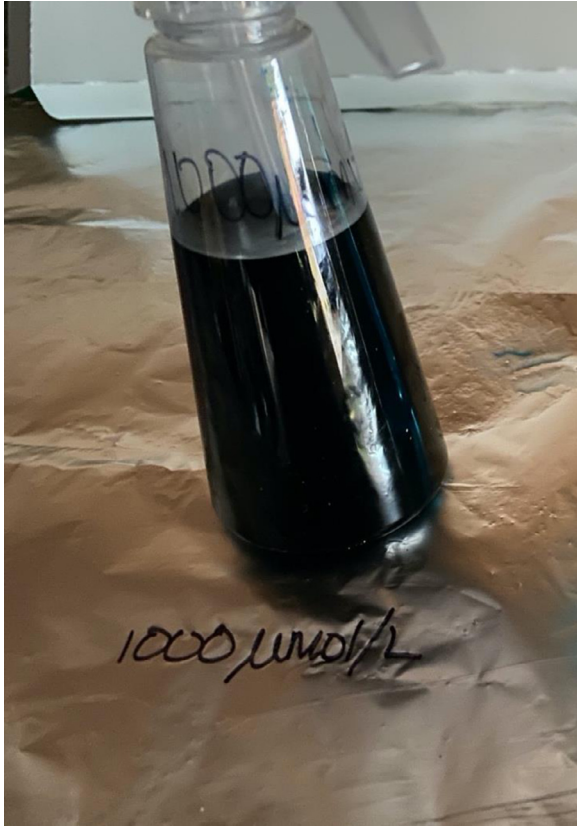


Fig 2. MB solution. Appearance of 1,000 μ M MB in spray bottle.

and males at 40% workload corresponding with light activity is 25–33 L/min, respectively.²⁴ If we are trying to see how much MB a female HCW would potentially be exposed to over a course of a 10 hour shift, we calculate the total volume of air to be (25 L/min)(60 min/hr)(10 hrs) = 15,000 liters of air. Thus, the amount of airflow past these coupons was almost 3-fold physiological respiratory flows.

Air with 100% relative humidity was used for the experiments, which was generated by flowing dry air through a bottle of water. In total, 30 independent experiments were conducted (7 mask types \times [3 MB-treated samples [except for the EN Type II mask which had 5 replicates] + 1 control sample for each mask]).

UV-Vis spectroscopy cannot be used to measure the concentration of MB in a gas sample because the concentration is so low that the

absorbance at 667 nm is below the limit of detection of any UV-Vis spectroscopy. In order to measure the concentration of MB in a gas sample, we used water to trap and concentrate the possible MB in the effluent gas that passed through the respirator and mask samples (see Fig 5). Specifically, we measured the concentration of MB in the liquid sample, the volume of the water that is used to trap MB, and the volume of gas sample that is swept into the water. The concentration of MB in the gas sample is then calculated by the following equation:

Concentration of MB in gas

$$= \frac{\text{Concentration of MB in water} \times \text{volume of water}}{\text{volume of gas}}$$

This equation holds because of the fact that MB is highly soluble in water. Therefore, all MB that is released into the effluent gas that passes through the mask samples will be trapped by the water. Such method of using a liquid absorbent to help determine the concentration of an analyte in gas sample has been used in a previous study by Xu et al.²³

For quantifying the concentration of released MB in the air passing through the samples, 51.2 L of air (10 hours for the flow rate of 85.33 mL/min) was swept into an absorber where MB was trapped by 3 mL of 0.5 M HCl aqueous solution. The absorber solution was then analyzed by a UV-Vis-Near Infrared (NIR) Spectroscopy (Agilent Cary 6000i). The integrated intensity of the absorption peak at 667 nm was used to quantify the concentration of MB.²³ The limit of detection, which is triple the intercept of the standard curve of this measurement method, is 0.1 mg/m³. To lower the limit of detection to see if we could detect any MB coming off the respirators or MMs, we extended the sample collection time from 2 hours to 2 days, which improved the limit of detection from 0.1 mg/m³ to 0.004 mg/m³ (or 4×10^{-6} mg/L).

Validation of our test method

To confirm that our results were not due to the failure of our test methods, we confirmed that 2 potential points of failure were not demonstrated:

- 1) Leakage in the gas flow system: The accuracy of this test method was validated using air samples with known concentrations of formaldehyde. This experiment confirmed that there is no leakage in the gas flow system.
- 2) Absorber failed to absorb released MB: We conducted an experiment with a vial of 10 mL 0.5 g/L MB solution replacing



Fig 3. Sprayed and dried N95 respirator. Example of mask immediately after MB spray application with 1,000 μ M MB (left) and after drying for 24 hours (right). MB is visible to the unaided eye on the respirator but with less intensity compared to immediately after application.

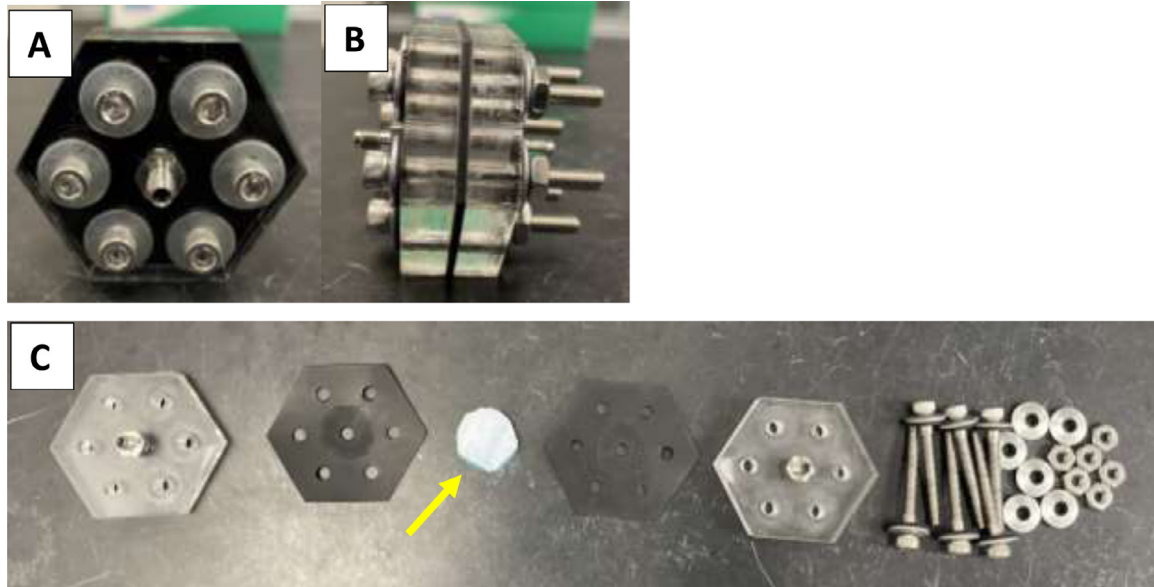


Fig 4. Mask coupon bolt. Experimental design of the mask coupons and the bolt through which air was passed. (A) Coupon bolt cross section; (B) Coupon bolt side view; (C) Components of bolt and a cut out mask coupon (yellow arrow) in the center of the image.

the masks sample and the experimental conditions of humid air at 120 L/min. After 24 hours, the concentration of MB in the absorber was still lower than the limit of detection (0.01 mg/m^3). This result confirmed the extremely low vapor pressure of MB.

RESULTS

According to the Safety Data Sheet (SDS) for MB,²¹ the acute inhalational toxicity threshold is 20.5 mg/L ($20,500 \text{ mg/m}^3$) of MB. In our model, we were able to obtain limits of detection at 0.004 mg/cm^3 . During the course of the study, all mask replicates yielded undetectable levels of MB absorbance by UV-Vis NIR spectroscopy. The control sample to demonstrate that the experimental design could detect MB using a 1 part per million stock of MB yielded an absorbance of 0.1735 (above the level of detection. For the controls and MB-treated samples tested for each respirator and medical mask there were no samples that yielded detectable MB absorbances.

DISCUSSION

We demonstrated that even at 500-fold amounts of MB applied to N95 respirators, a MM, and a cloth community mask then subjected to 3-fold airflow rates as would be expected for a HCW wearing MB-treated PPE there was not any detectable MB coming off the PPE. This establishes a level below any existing inhalational toxicity levels for MB vapor. The concentration of MB that demonstrated significant SARS-CoV-2 inactivation for the DeMaND study was $10 \mu\text{M}$ and so we created a study model to intentionally exceed the needed concentrations for decontamination.⁹

Other decontamination modalities such as gases (vaporized hydrogen peroxide, ethylene oxide), liquids (benzalkonium chloride, ethanol, hypochlorite), ultraviolet light, heat (moist or dry), microwave generators have their advantages and disadvantages.²⁵ These modalities are variously associated with significant space requirements, potential for toxicity to staff, added procedures related to transport of respirators off-site for disinfection, significant equipment costs, potential mask material and strap degradation resulting from the disinfection process, and inapplicability of these methods in resource constrained settings. Thus, alternative scalable and accessible decontamination methods are required to address existing and

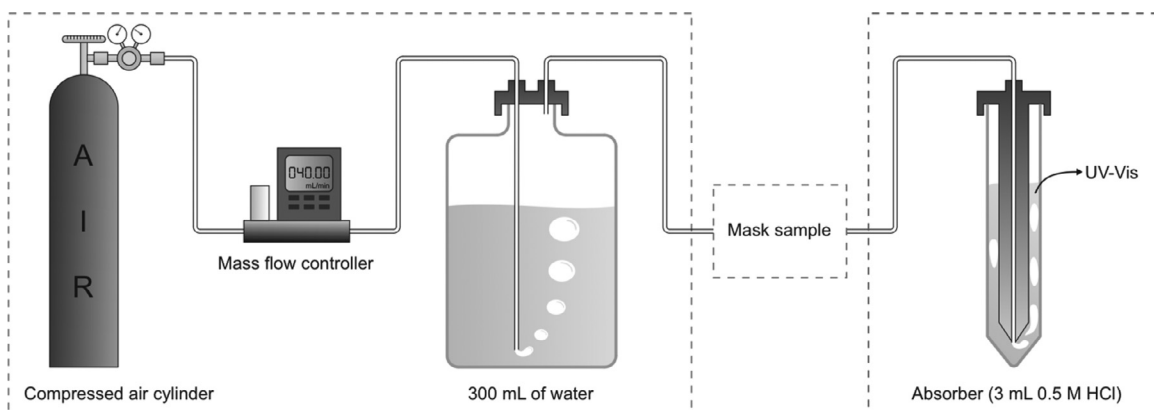


Fig 5. Testing apparatus. Diagram of how air was humidified and then passed through the various mask samples and trapped within a solution for subsequent spectroscopy.

future strains on PPE supplies. Furthermore, enhancing the performance of PPE through adding ongoing protection with MB and light could theoretically change the infection risks to HCWs when on the front lines exposed to COVID patients. Further human factors testing could assess the experience for the wearers. In the paper by Lendvay et al., HCW volunteers wore masks treated with 10 μ M MB during the course of a typical shift and reported no adverse reactions.⁹

The unique absorbance of MB at 667 nm makes it easy to measure the concentration of MB in a solution sample by UV–Vis spectroscopy. UV-Vis spectroscopy refers to absorption spectroscopy or reflectance spectroscopy in part of the ultraviolet and the full adjacent visible regions of the electromagnetic spectrum. UV-Vis spectroscopy is widely used to measure the concentration of organic compounds, especially those with a high degree of conjugation, because they absorb light in the UV or visible regions of the electromagnetic spectrum.²⁶ This makes our model ideal to assess whether MB comes off the respirators and masks.

Oral and nasally applied MB solutions undoubtedly lead to inhalation of MB and to date, no significant toxicities ascribable to inhalation have been noted. Since MB has shown efficacy as a facemask disinfectant, the need to demonstrate additional inhalational safety led to the present study using supraphysiologic inhalational conditions at 100% humidity over many consecutive hours at high applied MB concentrations. When used as a mask spray, no detectable off-gassing occurred. Of note is that MB was originally synthesized for use as a fade resistant textile dye, and exhibits a low atmospheric vapor pressure (17.535 mm Hg),²² properties consistent with experimentally demonstrated lack of off-gassing from the multiple types of facemasks used in this study.

When considering the toxicity of MB at higher concentrations, one must consider the known history. As it pertains to teratogenicity, Cragan in 1999 reported a consolidation of existing case series of children born with birth defects after intra-amniotic injection of MB during amniocenteses in some mothers who were being evaluated for the health of the placentas.²⁷ Cragan contended that the relationship between intestinal malformations in the newborns was more than just correlated, but was associated. One limitation of this study was that the amount of MB injected was not detailed. Furthermore, the MB was administered directly into the amniotic sac which would not parallel the inhalational entry point for any MB-treated PPE. A similar finding was made by Tiboni et al. in a mouse study²⁸ a few years after Cragan's report looking at fetal implant loss, neural tube defects and axial skeletal defects in mice. Doses of MB administered were 35–70 mg/kg. No statistically significantly higher implant loss or neural tube defects were identified at the 35 mg/kg dosing compared to the control of 0 mg/kg MB when MB was injected subcutaneously (implant loss) or intra-amniotically (neural tube defects). At the lowest dose given, 35 mg/kg, there was an increase in axial defects. To put this dosing into perspective, however, if a person wearing a MB-treated mask were to somehow inhale all the MB that was originally applied to the mask based on the original DeMaND study concentration of 10 μ M MB and 8 mL/mask, that would yield a potential dose of 0.026 mg. For a 50 kg person that would be an equivalent dose of 0.00052 mg/kg, a 67,000-fold higher concentration than the mouse study.

There are limitations to this study. Our study design involved using coupons of masks and translating the airflow rates and mask material sizes to what would be experienced were the tests to be performed on whole masks. Our methods have been validated²³ in the past as a means to reduce the level of detection to lower absorbances. Were a whole mask to be tested, more MB could come off the mask. Our method, however, detects the same concentration of MB that would be off-gassed from the mask whether a coupon or whole mask. Further, we did not test the variable of drying time to understand the minimum drying time before any MB might be detected.

All mask specimens were completely dried before they were cut into coupons. Another limitation is that since we only tested 7 mask types, we cannot generalize these data to all mask types. The cloth community mask used was a 3-ply mask and may not simulate all the other types of community masks possible.

The ability to provide a potential method for safe, scalable and affordable decontamination methods that may actually protect a HCW while they are actively wearing the PPE is a departure from existing decontamination technologies. There are less scalable and significantly more expensive ongoing mask protection technologies – earth metals such as copper, silver, and zinc – but these materials are unlikely to be applied to disposable PPE for issues of sustainability and cost.²⁹

CONCLUSION

Methylene blue applied to a facemask from a spray bottle represents an alternative, effective decontamination method that is convenient, fast, employable on demand, between patient encounters, after aerosol generating procedures, with the added protective benefit of continuous viral inactivation during use, and when doffing facemasks. Demonstration of safety is paramount and we have shown that the exposure risk to inhaling MB when applied to N95 respirators and facemasks may be exceedingly low and well within established safety thresholds.

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