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Evaluation of supercritical CO2 sterilization efficacy for sanitizing personal protective equipment from the coronavirus SARS-CoV-2

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HIGHLIGHTS

• Mask bioburden/crisis/airborne was the primary transmission pathway of coronavirus.
• ScCO2 could be a sanitization and reuse platform for surgical, cloth, and N95 masks.
• ScCO2 has advantages in safe and effective decontamination and reuse of the masks.
• Coronavirus is completely inactivated, and a possible mechanism is provided.
• Recommendations provided to improve decontamination and reuse of masks in pandemic.

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Abstract

The purpose of this research is to evaluate the supercritical carbon dioxide (scCO2) sterilization-based NovaClean process for decontamination and reprocessing of personal protective equipment (PPE) such as surgical masks, cloth masks, and N95 respirators. Preliminarily, Bacillus atrophaeus were inoculated into different environments (dry, hydrated, and saliva) to imitate coughing and sneezing and serve as a “worst-case” regarding challenged PPE. The inactivation of the microbes by scCO2 sterilization with NovaKill or H2O2 sterilant was investigated as a function of exposure times ranging from 5 to 90 min with a goal of elucidating possible mechanisms. Also, human coronavirus SARS-CoV-2 and HCoV-NL63 were inoculated on the respirator material, and viral activity was determined post-treatment. Moreover, we investigated the reprocessing ability of scCO2-based decontamination using wettability testing and surface mapping. Different inactivation mechanisms have been identified in scCO2 sanitization, such as membrane damage, germination defect, and dipicolinic acid leaks. Moreover, the viral sanitization results showed a complete inactivation of both coronavirus HCoV-NL63 and SARS-CoV-2. We did not observe changes in PPE morphology, topographical structure, or material integrity, and in accordance with the WHO recommendation, maintained wettability post-processing. These experiments establish a foundational understanding of critical elements for the decontamination and reuse of PPE in any setting and provide a direction for future research in the field.

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1. Introduction

The concern regarding the spread of airborne microbes, such as influenza and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), is amplified by the presence of saliva mucus, which acts as a protective physical barrier for the virus against environmental extremes (Tang, 2009). Pandemic transmission via aerosols has increased dramatically in recent years (Tellier, 2006), and deaths have risen significantly. The recent COVID-19 outbreak due to the new SARS-CoV-2 strain has caused illness in all countries. As of March 1, 2021, 113,820,168 cases of COVID-19 have been confirmed globally, including 2,527,891 deaths reported to WHO (WHO Coronavirus Disease (COVID-19) Dashboard Data last updated: 2021/3/1, https://covid19.who.int/). Every pandemic situation requires a tremendous number of respiratory PPEs such as surgical masks, cloth masks, and N95 respirators to protect healthcare workers and individuals from airborne transmission of the pathogens. For example, during the 2009 H1N1 pandemic, The Occupational Safety and Health Administration (OSHA) predicted that 360 million masks could be needed in the United States alone (OSHA, 2009). For that reason, the World Health Organization (WHO) and the United States Centers for Disease Control and Prevention (US CDC) recommended considering the reuse of respirators during an influenza pandemic (CDC, 2009), which it re-stated during the COVID-19 pandemic (CDC, 2020a,b; WHO, 2020). PPE bioburden has been identified as a potential source of infection with multiple factors influencing the level and tolerance of this bioburden. The bioburden found on the PPE increases with extended wearing time and reuse, resulting in a potential source of microbial contamination (Leung et al., 2020), and increasing the risk of infection (Zhiqing et al., 2018). So, safe and effective sterilization technologies are needed to eliminate the potential bioburden on the PPE. Three standard methods used for decontamination in biomedical settings include (1) radiation-based decontamination (gamma, X-ray), (2) gas-based decontamination (ethylene oxide), and (3) steam-based decontamination. All of these methods have disadvantages; e.g., irradiation can induce structural and property changes, gases often leave a toxic residue, and high temperatures can alter the structural properties. Some studies show no significant effects on N95 filtering after one to three decontamination cycles by ultraviolet germicidal irradiation (UVGI), moist heat incubation (MHI), or microwave-generated processes (Viscusi et al., 2011), however, found changes in mask fit, comfort, or donning difficulty (Dai et al., 2016; Viscusi et al., 2011). Existing available approaches are not meeting the demand for safe and effective decontamination and reuse of PPE because of the disadvantages outlined above (Dai et al., 2016). There is currently no process available in the healthcare setting to safely decontaminate PPE in different conditions (dry, hydrated), especially if biological fluids such as saliva are present, thus limiting the reuse of PPE.

Supercritical fluid technology presents an alternative sterilization method, which stands out as a potential platform to repeatedly decontaminate PPE, specifically with supercritical carbon dioxide (scCO2)-based processes (Dai et al., 2016). The scCO2 shares both liquid and gas properties, displaying liquid-like density and solvation capabilities, and gas-like viscosity, diffusivity, and is also lacking surface tension, which collectively allows scCO2 to penetrate complex, dense materials and act as a powerful solvent (Zhang et al., 2006). The scCO2 sterilization technique is considered a ‘green’ and sustainable technology because it does not leave a toxic residue. Decontamination by scCO2 and the use of dense phase CO2 to inactivate live bacteria date back to 1951 (Fraser, 1951). There are many advantages such as low cost of fluid, the potential reuse of CO2, lack of toxicity to humans and the environment, and easy-to-use operating systems. Sterilization using scCO2 under specific conditions (e.g., additive co-solvents, temperature, and pressure parameters) has been demonstrated to completely inactivate microorganisms. ScCO2 has excellent permeability into a wide range of materials to denature/inactivate microorganisms (Shieh et al., 2009). Herein, we evaluated the application of the scCO2-based NovaClean process for decontaminating and reprocessing PPE, with minor modifications, to achieve decontamination requirements in a safe and effective way. ScCO2 decontamination can be easily implemented across a diversity of healthcare settings (e.g., various outpatient care facilities). The NovaClean process parameters consist of pressure, temperature, mixing velocity, and additive concentrations (always constant). The NovaClean process is a low temperature (35 °C), minimally reactive, deep penetrating, bioburden reducing, and decontaminating technology. NovaSteril has previously shown that treatment with scCO2 and the peracetic acid-based NovaKill™ additive can achieve total inactivation of various microbes (Bernhardt et al., 2015; Qiu et al., 2009; Setlow et al., 2016). Here, the NovaClean process was applied to mask materials inoculated with human coronavirus strains to evaluate whether the NovaClean process inactivates human coronaviruses without damaging the morphological and topographical structure, scaffolds, and wettability of the mask. Bacillus atrophaeus (CDC recommended decontamination test spore, which is the most highly resistant to heat, desiccation, U.V., and γ-radiation, and a number of bactericidal chemicals because of the robust cell wall, thick cortex, and multi-layered structure (Setlow, 2006)) was tested as a proof-of-concept in the NovaGenesis500 instrument within a biosafety level 2 (BSL2) laboratory. This scCO2-based process was tested against standard commercial spore strips and spores in different artificial environments such as dry, hydrated, and in the presence of saliva. Next, two different strains of human coronavirus, HCoV-NL63, and SARS-CoV-2, were tested within a biosafety level 3 (BLS3) laboratory. Finally, we examined the reprocessing ability by wettability testing and surface mapping on a surgical mask, cloth mask, and 3M 1860 N95 respirator. Based on the data generated from this study, we demonstrated a method that allowed us to sanitize and potentially reuse the PPE.

2. Methods

2.1. Experimental design

Fig. 1 is a workflow diagram of the experiments performed in this study. Generally, the validation of a sanitization workflow includes preparing substrates, source, and treatment of microbes, instrumental analysis, validation, and interpretation. In this approach, commercial spore strips and spore samples were prepared under environmental conditions, including dry, hydrated, and saliva, followed by processing in the NovaGenesis500 instrument. The effectiveness was analyzed by various techniques as described below. In parallel, three different mask materials (surgical, cloth, and N95) were prepared and processed multiple times, and the reprocessing ability was investigated. Finally, HCoV-NL63 and SARS-CoV-2 strains were inoculated on the inside and outside of the mask materials, followed by processing and analysis of viral loads. The detailed procedures are provided below.

2.2. Test organisms and fluid preparation to mimic coughing and sneezing

We used B. atrophaeus spores (ATCC 9372; Crosstex, USA) as a surrogate for validation for coronavirus. Sterile techniques were used throughout. Spore strip, suspension handling and recovery procedures were carried out in a BSL-2 laboratory (Center for Applied Nanobioscience and Medicine, The University of Arizona). Human coronavirus, HCoV-NL63, and SARS-CoV-2 handling and recovery procedures were carried out in a BSL-3 laboratory (ZepToMetrix Corporation Buffalo, NY; in collaboration with iFlyer, LLC, Ithaca, NY). The artificial saliva was prepared using the ingredients listed in Table 1 (Woo et al., 2010). Saliva is an extracellular fluid in the mouth containing 98% water plus electrolytes, mucosal proteins, other proteins, and enzymes (Diaz-Arnold and Marek, 2002) which maintain osmotic balance (Dodd et al., 2005). Mucin from the porcine stomach (Sigma–Aldrich, St. Louis, MO) was used for mimicking mucus stimulant. All other artificial saliva components were analytical grade from Sigma–Aldrich.
2.3. Preparation of test substrate

2.3.1. Spore strips in the presence of different environments

Commercial biological indicator paper spore strips *B. atrophaeus* 1.4 × 10⁴ were placed into various environments (dry, no liquid; hydrated, phosphate-buffered saline (PBS); and artificial saliva, mucus). The strips were dipped into PBS/Saliva for 2 s, removed, and left to air-dry overnight in a fume cabinet (Lab Crafters Air Sentry model HBASC6, Lab Craffers Inc., NY USA). Three strips were used per test. Growth control (untreated) and death control (heat-treated at 120 °C for 20 min) were included in all experiments. All the strips were individually packaged in a sterilization pouch (Henry Schein Inc., NY, USA) and were heat sealed. All sealed pouches were designed to be positioned inside the vessel of a NovaGenesis500 (NovaSterilis) instrument.

2.3.2. Spore suspensions in the presence of different environments

Spore suspensions (Crosstex) were purified by centrifugation at 10,000 × g for 10 min, followed by resuspension in sterile distilled water (Tavares et al., 2013). A suspension of 1 × 10⁵ spores was then dispensed onto a piece of mask or nitrocellulose membrane and left to air dry. For dry environmental conditions, the dried spores were placed directly into the sterilization pouch. For hydrated environments, the dried spores were mixed with 1 mL of PBS and were placed into a sterilization pouch. The dried spores were spiked into saliva samples and placed into a sterilization pouch for mucus environments and were heat sealed. Again, all sealed pouches were designed to be positioned inside the vessel of the NovaGenesis500 instrument.

2.3.3. For human coronavirus in presence of mask

Human coronavirus strain HCoV-NL63 was tested in a Nova2200 instrument – a 20 L commercial sterilizer at NovaSterilis (Lansing, NY). HCoV-NL63 strain was inoculated on either the outside surface (simulating surface contamination) or the inside surface (emulating aerosol penetration). The coronavirus strain SARS-CoV-2 was inoculated on the exterior surface of the respirator material and tested using the NovaGenesis500 instrument. The inoculated swatches were left to dry, placed into a sterilization pouch, and subjected to a 90-min NovaClean process within a biosafety level 3 (BSL3) laboratory.

2.3.4. For mask reprocessing study

Three types of mask materials were utilized, 3M 1860 N95 respirator (A, respirator type), surgical mask (B, three-layer screens), and cloth mask (C, reusable cloth type with two filter screens). The same batch number (Lot #) for each mask type was utilized in the study to avoid variability. The masks were cut to 2 × 2 cm pieces using sterilized scissors to obtain 3 test pieces from each mask. These were then placed in
individual sterilization pouches and were heat sealed. The bags were then placed inside a NovaGenesis500 vessel and processed for up to 5 decontamination cycles (40 min each). After each decontamination cycle (5 cycles tested), the mask pieces were retrieved from the instrument, and the wettability, morphology, and topography of the mask materials were evaluated.

2.4. ScCO2-based NovaClean source and experimental sterilization of microbes

The low-temperature scCO2 source used in this study has previously been described in detail (Bernhardt et al., 2015; Qiu et al., 2009). Once all the samples were placed inside the vessel of the NovaGenesis500, 7.5 mL of 75% ethanol with and without 15% H2O2 or peracetic acid-based NovaKil™ were added to the vessel, which was sealed with 25-foot pounds of torque. The vessel was pressurized to 1500–2000 psi at 33–35 °C for periods of time ranging from 5 to 90 min. The instrument operated within the same processing parameters, including pressure, temperature, mixing velocity, and additive concentrations set for all experiments. After decontamination, all the strips/membranes were retrieved from the system and analyzed for inactivation/death.

Experiments. After decontamination, all the spore strips/membranes were re-operated within the same processing parameters, including pressure, temperature, mixing velocity, and additive concentrations set for all experiments. After decontamination, all the strips/membranes were retrieved from the system and analyzed for inactivation/death.

For viruses, the respirator material was retrieved from the instrument, treated with TcCl3 consisting of 1 M acetic acid (99.8%, Sigma-Aldrich) at pH 5.5. For evaluation of spore germination testing, live, heat-killed, and NovaClean-treated spores were used to determine release from the spore core into the extracellular environment. The DPA leaks were measured using a previously described fluorescence method (A. Hindle and A. H. Hall, 1999). Spore suspensions were purified by centrifugation at 10,000 × g for 10 min and resuspended in sterile distilled water (Tavares et al., 2013). A suspension of 1 × 10^8 spores was placed inside the vessel of the NovaGenesis500 instrument. Then, the samples were NovaClean processed immediately to evaluate the release of DPA from endospores. Treated spores were spun down at 10,000 × g for 10 min, and DPA was examined in the supernatant solution by fluorescence with terbium (III) chloride hexahydrate (TbCl3•6H2O, Sigma–Aldrich) in a 96-well plate. 100 μL of supernatant solution were added to 100 μL of 20 μmol/L TbCl3 consisting of 1 M acetic acid (99.8%, Sigma–Aldrich) at pH 5.5.

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2.5. Effectiveness of decontamination characterization

For evaluation of spore survival following scCO2 decontamination, all the strips/membranes were inoculated into test tubes containing 5 mL of nutrient broth (B.D. Tryptic Soy Broth, Soybean-Casein Digest Medium) (Crosstex International, Inc., NY, or Becton, Dickinson, and company, MD, USA). These tubes were incubated at 35 °C and 150 rpm for five days on an orbital shaker incubator (Amerex Instrument, Inc., CA, USA), then visually inspected for turbidity. A Samsung S7 edge camera was used to capture images. The orange pellicle is considered positive for the growth of the spore, while green is considered negative for growth. Additionally, the spore germination/turbidity was measured using a plate reader (Epoch: Bio-Tek Instruments, Inc., Winooski, VT, USA) to determine the optical density at 600 nm (OD600). Experiments were repeated in triplicate, and three sample replicates were measured each time.

For spore Live/Dead and membrane integrity assessment, the spores of live, heat-killed, and NovaClean-treated samples were stained using the Live/Dead BacLight bacterial viability kit (Molecular Probes, Eugene, OR) with minor modifications to the manufacturer protocol. Briefly, equal proportions of dye components A and B were mixed in each sample at a rate of 3 μL dye mix per 1000 μL, and the resulting solution was incubated at room temperature for 20 min in the dark before imaging.

For spore germination testing, live, heat-killed, and NovaClean-treated spore samples were incubated overnight in tryptic soy broth, and then stained with the BacLight reagent, as described above. To determine if the inactive spores were likely germinated (alive) or dead, we examined for live cells (green) and dead cells (red). After staining for 20 min with the BacLight fluorescent dye (SYTO® 9: Ex/Em = 485/498 nm, and B, propidium iodide (P.I.): Ex/Em = 535/617 nm), samples were examined under the microscope using a filter which detects EGFP and DsRed. The images were acquired with a Zeiss Axio Imager M2 epifluorescence microscope using a Zeiss AxioCam MRm camera, ZEN 4.5 (blue edition) software at the Biomedical Imaging Core Facility at the U.A. College of Medicine-Phoenix. Images were taken in 14-bit mode, using an objective plan-apochromatic 63x/1.40 Oil with a speed of 2.7 s. The detector master gain in each channel was adjusted to cover the full dynamic range, and saturation level was avoided. Imaging conditions for each experiment were held constant.

2.6. Measurement of spore component leaks in the extracellular environment

Dipicolinic acid (DPA), which is essential for spore survival, was measured to determine release from the spore core into the extracellular environment. The DPA leaks were measured using a previously described fluorescence method (A. Hindle and A. H. Hall, 1999). Spore suspensions were purified by centrifugation at 10,000 × g for 10 min and resuspended in sterile distilled water (Tavares et al., 2013). A suspension of 1 × 10^8 spores was placed inside the vessel of the NovaGenesis500 instrument. Then, the samples were NovaClean processed immediately to evaluate the release of DPA from endospores. Treated spores were spun down at 10,000 × g for 10 min, and DPA was examined in the supernatant solution by fluorescence with terbium (III) chloride hexahydrate (TbCl3•6H2O, Sigma–Aldrich) in a 96-well plate. 100 μL of supernatant solution were added to 100 μL of 20 μmol/L TbCl3 consisting of 1 M acetic acid (99.8%, Sigma–Aldrich) at pH 5.5. All the samples were analyzed with a Synergy Mx Multi-Mode Microplate Reader (BioTek Instruments, Inc., USA). Using the Gen5™ software, the samples were excited at 270 nm, and emission spectra were acquired at 546 nm. The autoclaved spores were used as a positive control (Kim et al., 2020), and untreated spores were used as a negative control. The following equation calculated the NovaClean-induced DPA release ratio: DPA (%) = [(E1 − E0) / (E2 − E0)] × 100, where E0 is the emission intensity of untreated spores, E1 is the emission intensity of NovaClean treated spores, and E2 is the emission intensity of autoclaved spores. To assess whether the scCO2-based NovaClean process yielded intra-cellular protein or pH leaks from the spore, we assayed the protein by bicinconinic acid (BCA) reagent and SDS–Page analysis and measured the extracellular pH. The procedures are given in the Section 1. 2.7. SEM-EDX analysis

The shape, surface morphology, and elemental characterization of the spores were analyzed using a high-resolution field emission scanning electron microscopy (SEM/FIB Focused Ion Beam - Nova 200 NanoLab (FEI) equipped with an EDX spectroscopy analyzer at an acceleration voltage of 20.0 kV). The samples were fixed with 2.5% glutaraldehyde in 1× PBS overnight at 4 °C. Then they were spun down at 10,000 × g for 10 min and dehydrated with increasing concentrations of ethanol, 200 proof 100% (50% > 70% > 85% > 95% > 99% (each 3 h at 4 °C), and > 100% overnight at 4 °C). The samples were then dried on a cover glass, which was attached to a metal stub (10 mm diameter, Ted Pella, Inc., CA) with double-sided adhesive tape. Samples were Au sputtered for 180 s under vacuum prior to examining the morphology by SEM at the Eyring Materials Center at Arizona State University. SEM analysis provided a visual inspection of the spores after the NovaClean process. EDX analysis was used to identify surface elements of the spore (elemental composition), which can elucidate the detailed response against sanitation.

2.8. Mask reprocessing characterization

2.8.1. Surface mapping

Morphological/topographical characteristics of the masks were obtained from images acquired from pre- and post-scCO2 exposure cycles using a tactile sensor pad with a GelSight, Inc., Benchtop Optical System, MA, USA. The deformable gel elastomeric pad (soft, T20180827-002, GelSight Inc., Waltham, MA) was pressed onto each mask. Six
photographs were acquired by a standard DSLR camera (Canon Rebel T3i) with 18 megapixels resolved with a 5× lens (Canon MP-E 65 mm 1–5× Macro Lens). Each image was taken from a different angle illuminated with LED lighting with a shutter 2 s, aperture F6.3, and live V ISO 800. The images represent a 4.5 mm × 3.0 mm area and are combined with GelSight software (GSCapture) to generate a textural map of the surface. The surface texture, uniformity, and fiber distribution of each mask were evaluated. The size and distribution were evaluated using Image J v1.8.0 software (NIH, Bethesda, MD). In all experiments, the images shown in individual panels were acquired using identical exposure times or scan settings, and they were adjusted identically for brightness and contrast.

2.8.2. Wettability properties analysis using contact angle

The water contact angle (WCA) of the surface of each mask was measured with a contact angle meter (Reme-Hart Model 100 Goniometer, Reme-Hart Inc., Mountain Lakes, NJ, USA) with 5 μL drops of distilled water at room temperature. The DROPimage Pro software was used to capture the image and contact angle of each sample. The average value was determined by measuring each sample at three different positions, and the hydrophobic nature of the mask was confirmed by contact angle measurements (°).

2.9. Statistical analysis

Data in the text and figures were presented as mean ± standard deviation (S.D.). All statistical analyses were performed in GraphPad Prism 9 (San Diego, CA, USA). The additive effects were compared with the scCO₂ treatment sample using ordinary one-way ANOVA with Tukey’s multiple comparisons test. The viral load was compared using an unpaired t-test. The mask contact angle was compared to each other using ordinary one-way ANOVA with Tukey’s multiple comparisons test. All tests were assessed for statistical significance among groups with *p < 0.05, **p < 0.01, and ***p < 0.0001.

3. Results

3.1. Proof-of-concept testing: effects on biological indicators (spore viability analysis)

To determine how the additive ingredient affects the scCO₂ NovaClean process, a B. atrophaeus spore strip decontamination test was carried out to confirm which co-solvent would provide optimum spore inactivation. Additives are necessary because scCO₂ alone does not effectively kill bacterial endospores (White et al., 2006). It has recently been demonstrated by (Peet et al., 2015) that microorganisms sustain metabolic activity under scCO₂. In a preliminary screening, the NovaClean process at two different pressures, 1500 psi and 2000 psi, was chosen to inactivate B. atrophaeus spore strips. Both 1500 psi and 2000 psi treatment gave similar results, as determined by a color change and OD, as depicted in Fig. S1A and B, respectively. As an endpoint measurement, the determination of the OD₆₀₀ confirms the decontamination efficiency. The results of commercial B. atrophaeus strips using different pressure conditions reveal that the low-pressure condition was enough to inactivate 100% of the spores in less than 40 min; thus, further experiments focused on the low-pressure process setting.

In the low-pressure experiments, both NovaKill (Fig. S2) and H₂O₂ (Fig. S1) based scCO₂ decontamination processes showed similar results. Fig. S2A and B showed the results of commercial B. atrophaeus strips in different conditions of (i) dry, (ii) hydrated, and (iii) artificial saliva following completion of the NovaClean process and assessment of viability determined by an OD, and color change, respectively. 100% viability was defined as growth in the untreated samples, with 0% as blanks (only media). A ‘positive’ growth (turbid) represented viable spores, and a non-sterile sample. A ‘negative’ (sterile) indicated that the B. atrophaeus was effectively decontaminated (i.e., 1.4 × 10⁴ reductions) as the broth remained clear. Three independent experiments were carried out to determine the 100% decontamination efficiency cycle that achieved the spore reduction by 1.4 × 10⁴ or complete inactivation. In less than 40 min, 100% of the spores were killed for all conditions studied, i.e., (i) dry, (ii) hydrated, and (iii) artificial saliva. These experiments were carried out using commercial sterilization test strips to determine the preliminary measurement of the killing rates. Further experiments were performed using purified spores spiked in different environments. Fig. S2C and D showed the results of B. atrophaeus spore at different conditions of (i) dry, (ii) hydrated, and (iii) artificial saliva subjected to the NovaClean process and assessed for viability, which was determined by OD and color change, respectively. We found that the NovaClean process completely inactivated both dry and hydrated spores within 30 min, and spores spiked into saliva within 40 min. Specifically, all of the treated samples were negative for growth, while no loss of viability was observed for untreated samples. Therefore, based on the results of both decontamination of commercial spore strips and spores spiked in different environments, a NovaClean process cycle time of 40 min was sufficient for complete decontamination in the NovaGenesis500. This correlates with 100% eradication of highly resistant spores as recommended by the CDC in minimum time.

3.2. Effect of NovaClean process on spore viability and cell recovery

The NovaClean-processed spores were then examined for viability. The live/dead assay helped to visually image the spores in different environments. The assay was carried out for endpoint measurements. Live/Dead spores in different environments and untreated control are depicted in Fig. 2A. The dead spores were visualized with a membrane-impermeable PI indicator, which can only enter cells with compromised membranes, bind to nucleic acid, and emit a red fluorescence signal and therefore act as an indicator of membrane integrity (inner membrane (I.M.) and outer membrane (O.M.) damage) (Mathys et al., 2007). Active spores were visualized with a membrane-permeable SYTO9 dye, which can enter all spores regardless of the level of membrane integrity, bind to nucleic acid, and emit a green fluorescence signal (Stocks, 2004). As expected, when staining with a membrane-permeable dye like SYTO9, no membrane damage was observed in the untreated spores, and they showed green fluorescence, proving the spores had an intact cortex. NovaClean-processed spores showed a complete red fluorescence of the membrane-impermeable dye, PI., at all environmental conditions tested, indicating that inner and outer membrane damage occurred. Compared with the untreated spores (growth control), the heat-treated spores (dead control, 160 °C for 20 min) exhibited a red fluorescence in all cells, indicating dead spores.

The results described above are insufficient to determine if a spore was dead or if just the outer membrane was damaged, leaving the opportunity for the spore to recover its native membrane permeability under special conditions (Domenici et al., 2017). In order to further evaluate this, an additional experiment was performed to ensure that the spores treated with the NovaClean process were dead and not just damaged. The recovered spores were incubated in the growth media and stained with BacLight reagent, as depicted in Fig. 2B. As expected, like the dormant spore results, the spore germination exhibited a complete red fluorescence in different environmental conditions, which indicates “no recovery” after the NovaClean process relative to the untreated control. The germination results suggested that the I.M. and O.M. of the spores had unrecoverable damage. The spore core proteins and enzymes, which are essential to hydrolyze the cortex and trigger germination, might be denatured or destroyed, which indicates a possible mechanism for spore damage. Usually, the germinant penetrates the outer-most coat and middle cortex and interacts with a receptor complex (Paidhungat and Setlow, 1999) located in the inner spore membrane (Hudson et al., 2001). Since there is no interaction with a
3.3. NovaClean process-induced damage to the spore membrane: morphology and leakage of intracellular components

To further examine the state of spores after the NovaClean process, spores were observed by SEM after drying on the sample holder. SEM images revealed a significant difference between the groups. The control spores found by SEM were ovoidal to cylindrical shape (Fig. 2C-i), and exosporium structure overlays on the coat were well visualized. In contrast, the NovaClean-processed spores exhibited morphology that differs from control spores and showed evidence of pitting and non-ovoidal shape (Fig. 2C-ii and high magnification image Fig. S3). These observed morphology changes may provide mechanistic insight into the inactivation mechanism. For example, the NovaClean process may generate large shear forces between the scCO2 and the sample, creating microdamage (Russell et al., 2015) and/or stress to the membrane resulting in an altered structure. Under scCO2 pressures, the excessive amount of CO2 might diffuse into the spore coat, accumulate and act as a powerful solvent to cause leakage and damage. The EDX data (Fig. 2D) showed that the abundance of elements, such as Na, Ca, and K, on the external side of the membrane is higher after treatment. This may suggest ion shifts across the membrane due to a high-pressure effect under CO2 as hypothesized by Hong and Pyun (2001). This ion shift across membranes could possibly inhibit germination initiation and disrupt spore metabolism (Lin et al., 1988). It has also been hypothesized that the energy from high-pressure treatment may disturb the membrane (Nguyen Thi Minh et al., 2010).

Additionally, we tested intracellular leakage by measuring DPA content. DPA is a major component (5–14% of spore dry weight) present in the inner core of bacterial spores (Tabor et al., 1976; Warth et al., 1963), which helps the spore resist inactivation (Slieman and Nicholson, 2001). Usually, a low DPA amount is released during germination, and if the spore is damaged, significant DPA leakage is observed (Sonenshein et al., 2001). Thus, DPA release from the inner core is another indicator of the damage to the I.M. and spore envelope. As shown in Fig. 2E, DPA release from the spores treated by scCO2 for 40 min was less than 25%, indicating little or low damage. The DPA

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**Fig. 2.** Extracellular factors analysis of *B. atrophaeus* spores upon NovaClean process. Spore inactivation by scCO2-based NovaClean (NovaKill) process. Fluorescence micrographs of spore (A), and germinated spore (B) stained with Live/Dead (BacLight staining reagent) for membrane integrity assessment. The spores were incubated for 20 min in the presence of mixtures of SYTO™ 9 green-fluorescent nucleic acid stain and the red-fluorescent nucleic acid stain, propidium iodide. Viable spores were stained green, and spores with compromised membranes were stained red. The scale bar is 5 μM for all images, and the micrographs in all pictures are at the same scale. (C) SEM pictures of untreated (i) and NovaClean processed (ii) spores. (D) EDX of respective *B. atrophaeus* spores before and after NovaClean process. EDX line profile was digitally magnified for clear visualization. (E) DPA release of spores treated by scCO2, NovaKill, and H2O2. (mean ± SD, n = 3). The additive effects were compared with scCO2 treatment sample using ordinary one-way ANOVA with Tukey’s multiple comparisons test in GraphPad Prism 9. *Significantly different from scCO2 treatment. * p < 0.05.
release from the spores after 45 min in the NovaClean process was far higher than scCO₂ alone, at more than 75%, indicating damage to the I.M. and spore envelope. In general, microbial inactivation is induced with increasing scCO₂ pressure, because it enhances CO₂ solubility, which facilitates both acidification and cellular contact (Iensenbed et al., 1995). When the additive (such as PAA and H₂O₂) (Wuytack et al., 1998) is added along with this pressure, it acts synergistically to inactivate various species of microbes (Setlow et al., 2016). The germination process is affected by different environmental factors, including intra- and extracellular pH (Bressuire-Isoard et al., 2018). The CO₂ enters into the spore, lowering core pH and deactivating enzymes (Spylimbergo et al., 2005), preventing the germination process, which was reported previously. To clarify whether the scCO₂-based NovaClean process generated protein leakage from the spore, we used bicinchoninic acid (BCA) reagent, and SDS-Page analysis to detect and quantify extracellular proteins. The results did not reveal any significant changes between protein levels in the supernatant of control and scCO₂-treated samples. To assess the change in core pH, we measured the pH of the inside and outside of control and scCO₂-treated spores. We noticed that the pH of the extracellular environment was lowered by NovaKill, which was diffused and dissolved in the extracellular environment, resulting in cell demise.

3.4. Coronavirus inactivation with NovaClean process

The tissue culture infectious dose (TCID₅₀) was determined using the Reed-Muench calculator, and Fig. 3 shows the logs of recovered viral load. Untreated controls were included to measure the magnitude of viral inactivation. As shown in Fig. 3, the NovaClean process caused complete inactivation of all coronaviruses. HCoV-NL63 viral load of 3.8 log₁₀ and 3.4 log₁₀ were recovered from the inside and outside of control mask pieces, respectively (Fig. 3), whereas NovaClean-processed samples showed no signs of infective virus whatsoever. Similarly, as shown in Fig. 3, SARS-CoV-2 controls showed a viral load of 5.5 log₁₀, whereas no infective virus was collected from NovaClean-treated samples with the standard processing time. Previous studies have demonstrated the NovaClean methodology to achieve sterility assurance levels (SAL) of 10⁻⁶, indicating that the technology is extremely effective against a wide variety of pathogens beyond the virus (Qiu et al., 2009). Altogether, the NovaClean process was efficient for SARS-CoV-2 inactivation.

3.5. Impact of reprocessing efficacy of PPE on N95, 3-layer surgical mask, and cloth mask

We compared the surface texture and wettability of three different mask materials, which were functionally characterized after reprocessing by their ability to block small water droplets, the vehicle for viral transmission (Asadi et al., 2020). We performed large-scale optical 3D topography and texture imaging using a GelSight tactile sensor (Johnson et al., 2011). Representative 3D topography and texture images of different face coverings are depicted in Fig. 4. In the pre-treatment panel, masks A (N95), B (surgical mask), and C (cloth) had a different topography and texture. Mask A and B possessed the highest-fiber density and smallest fiber size distribution. Mask C possessed the lowest fiber density and highest fiber size distribution. After reprocessing, the photometric results showed the preservation of the masks’ structural integrity throughout five cycles (maximum tested). As shown in Fig. 4, even after five cycles of reprocessing, the structure of the fibers remained sharp, with relatively distributed homogeneous structures, and no surface damage noted, suggesting that all the masks may maintain function.

In addition to the surface texture analysis, mask wettability was analyzed by contact angle measurement methodology as depicted in Fig. 5. The mask type differed significantly in contact angles for 5 μl droplet volumes tested (Fig. 5A). Specifically, cloth type masks differed highly from N95 and surgical masks. The magnitude of change from the pre- and post-NovaClean process (throughout the 5 cycles, maximum tested) to final contact angles was not significantly different for 5 μl droplet volumes tested (Fig. 5B). Since the mask materials are inherently hydrophobic, no evident damage or degradation of the surface hydratability were noticed upon several cycles of the NovaClean process (tested up to 5). Hydratability of the mask surface did not change upon the NovaClean process, so the NovaClean process was an effective decontamination method for respiratory PPE materials with respect to maintaining their hydrophobicity.

4. Discussion

ScCO₂ has distinctive properties that make it an attractive approach for sterilization. The NovaClean process is a low temperature (35 °C), minimally to non-reactive, deep penetrating, and biocidal technology. The characteristic features are innocuity, easily manageable parameters, penetration into dense materials (such as a virus and spore), and the ability to process multiple PPE items at the same time. Several studies have presented the use of scCO₂ for inactivating bacterial spores, fungi, and viruses (Qiu et al., 2009; Setlow et al., 2016), and this communication goes further to confirm that the scCO₂-based NovaClean process can inactivate coronaviruses, which may contaminate PPE. Additionally, our study demonstrates that the scCO₂-based NovaClean process can inactivate microorganisms in different environmental extremes without physically damaging the mask materials. In this manuscript, we suggest that the NovaClean process could inactivate endospores and enveloped coronaviruses through a similar kind of mechanism, including (i) outer envelope damage; (ii) breakdown of permeability barriers; (iii) inactivation of proteins and enzymes which are essential for cell function; (iv) strong reduction of the pH homeostasis of the cell and environment which is essential for cell functioning; (v) I.M. and O.M. damage, and (vi) excess of CO₂ and free radicals that can enter the cell resulting in deactivating enzymes and the receptor complex. Though we didn’t show that any free radicals are generated during the NovaClean process, previous work reported by (Lefévre et al., 1992) and (Gehr et al., 2003) suggest that the radical ions from the H₂O₂ (easily converts to free radicals) and peracetic acid (decomposes in water and generates free radicals) disrupt sulfhydryl (—SH) and disulfide (—S–S) bonds within enzymes in the cell membrane by oxidative disruption. So, the oxidative damage might play a major role in the NovaClean process.
These sanitization responses relate to the NovaClean process and primarily start from CO₂ affinity and permeation through the lipid bilayer of the cell membrane (Fig. 6, initial response), which has been reported in various cell types (García-González et al., 2007). This affinity and permeation between CO₂ and the lipid membranes have been theoretically confirmed (Spilimbergo et al., 2002), suggesting that the CO₂ can enter into the cells (Spilimbergo et al., 2009). This kind of response may lead to the loss of enzyme-substrate binding affinity. A similar response with inactivation have been previously reported (Ho-mu et al., 1993; Lin et al., 1992), which agree with the existing report.

Fig. 4. 3D surface mapping of N95 (A), 3-layer surgical mask (B), and cloth mask (C). A-1, B-1, and C-1, surface damage assessment for A, B, and C mask respectively by using gel-based photometric profilometry. Scale bars = 500 μm. A-2, B-2, and C-2, analysis of fiber distribution (provided with height and distance in micrometer) for A, B, and C mask respectively by using ImageJ. With the ImageJ software, a representative fiber image shows the longitudinal line drawn across from the mask surface non-flat region that defines the region of interest (ROI) for the pre-and-post NovaClean process. The gray pixel intensities in ROI were quantified for fiber distribution in the individual mask surface and illustrated histograms.

Fig. 5. Water contact angle measurement of three different masks (A) before reprocessing and (B) after reprocessing cycles. The mask contact angle was compared to each other using ordinary one-way ANOVA with Tukey’s multiple comparisons test. *Significantly different from others. **p < 0.001.
Moreover, during the NovaClean-based process, DPA leaked from the spore core, which is also considered a crucial step for inactivating the organism because the DPA release from the spores is another indicator of the damage to the I.M. and envelope of spores (Akasaka et al., 2017; Rao et al., 2016; Soares et al., 2019). These changes diminish cell function, and restoration of a favorable environment is not achievable, resulting in cell demise via deactivation of enzymes. Finally, 100% spore inactivation was achieved in 40-minute cycles in all experiments. Since the coronavirus sanitization process included a similar set of parameters to spore inactivation, the mechanism of coronavirus inactivation may be similar to endospore inactivation. A recent study shows that changes in pH altered the enveloped virus M1 protein scaffold, likely stretching the lipid membrane to the point of rupture, promoting functional inactivation (Batishchev et al., 2016) because of the continuous acidification action on proton channel M2, leading to deformation of the viral envelope (Ruigrok et al., 1984).

Finally, we discuss the impacts of reprocessing on the topography and wettability of the mask materials used to avoid airborne infections. As shown by the topography analysis (Fig. 4), surgical and N95 masks have a very similar texture, with densely packed fibers and no interwoven microfibers. However, the NovaClean-process did not alter the surface integrity of these 3 types of masks. After five cycles of reprocessing (max tested), the structure of the fibers maintained sharp edges and relatively homogeneous distribution in the mat, while no damages were visible on the surface, which means each type of tested masks should be able to physically block airborne particles and provide air permeability as originally designed. Moreover, in a recent study, the wettability of the surface of the N95 mask was characterized by contact angle and showed around 100° (John et al., 2020). The WHO recommends that PVC-coated PPE contact angle should be around 80°, while the low-cost polypropylene mask contact angle should be around 120° (Sarkar et al., 2020). Our results showing that the contact angles before processing are 137, 130 and 120° for surgical, N95 and cloth masks, respectively, are in the same range of hydrophilicity. Interestingly, after processing, the contact angle did not significantly change, suggesting that mask wettability stayed identical. To note, the masks are usually made up of a multi-layered structure, yet our analysis focused on changes of the superficial layer because it is considered a primary barrier. Although unlikely, further analysis would be required to assess that no damages are present in the internal layers, since some layers are much stronger than others in the same respirator and changes in some layers might be masked by other layers (Lindsley et al., 2015). In addition, although we tested the physical integrity of reprocessed PPE via topography and wettability testing, our testing did not consider the impact of repeated handling in a typical use scenario, which includes extended wear among other challenging variables. There was not a detectable odor of H2O2 from the NovaClean processed masks after any of the cycles, but the smell of residual acetic acid was noticeable (a by-product of PAA in NovaKill). Although reprocessing with the NovaClean process did not change topography or wettability for the mask types tested, each style of PPE is unique even if they are produced by the same manufacturer, so the NovaClean process suitability for decontamination will need to be evaluated on a product-by-product basis to ensure

![Fig. 6. The sequence of morphological and functional changes occurring at different stages of NovaClean process. Sanitization by scCO2 based NovaClean process induces CO2 and radical elements penetration in cells and disrupts cellular integrity. Continuous exposure to NovaClean process causes the germination and functional defect via intracellular component leaks, pH-dependent disintegration of the membrane protein and damage membrane integrity. After 40 min, all cells are completely inactivated. Scale bars, spore and virus fine arts representations = 200 nm.](image-url)
functionality as originally intended. However, our findings show no adverse effects on physical and structural integrity or wettability through five cycles on those mask types tested.

5. Conclusions

This study demonstrated that the NovaClean scCO₂-based decontamination process (1) inactivates B. atrophaeus endospores in a 40-minute cycle independently of the spore environmental properties (dry/hydrated, coated in saliva), (2) inactivates human coronavirus strains, including SARS-CoV-2, inoculated on respirator material and (3) maintain surface integrity and wettability of surgical, N95 and cloth masks. The investigation of the mechanisms of inactivation due to the scCO₂-based NovaClean process showed changes in spore morphology, DPA leakage and presence of Ca, Na and K elements at the membrane surface suggesting potential membrane damages, which is in line with previous reports. Further analyses are required to explore in detail this mechanism, in particular for coronaviruses such as SARS-CoV-2. The low operating temperature, efficient and deep penetrating capability of scCO₂ and its lethality when combined with a minimal amount of an oxidative additive make scCO₂-based decontamination an ideal solution for the healthcare setting. The availability of easy-to-use scCO₂ equipment platforms, which can be deployed in a distributed manner, makes this technology a desirable solution during periods of pandemic. Finally, the excessive use and consumption of single-use PPEs have become a severe threat to the entire environment due to resulting from increased medical waste (Caraka et al., 2020) and the edentate cycles on those mask types tested.

References

A. Hindle, A. A. H. Hall, E. 1999. Dipicolinic acid (DPA) assay revisited and appraised for spore detection. Analyst 124, 1599–1604.

Akasaka, K., Maeno, A., Yamazaki, A. 2017. Direct high-pressure NMR observation of dipicolinic acid leaking from bacterial spore: a crucial step for thermal inactivation. Biophys. Chem. 231, 10–14.

Araga, F.A. 2020. Surgical masks as a potential source for microplastic pollution in the COVID-19 scenario. Mar. Pollut. Bull. 159, 111517.

Asadi S, Bouvier N, Wexler AS, Ristenpart WD. The coronavirus pandemic and aerosols: does COVID-19 transmit via exhalatory particles? Aerosol Sci. Technol. 2020; 0: 1–4.

Batishchëv, O.V., Shilova, L.A., Kachala, M.V., Tashkin, Y.V., Sokolov, V.S., Fedorova, N.V., et al., 2016. pH-dependent formation and disintegration of the influenza A virus scaffold to provide tension for membrane fusion. J. Virol. 90, 575–585.

Bernhardt, A., Wehr, M., Paul, B., Hochmuth, T., Schmacher, M., Schütz, K., et al., 2015. Improved sterilization of sensitive biomaterials with supercritical carbon dioxide at low temperature. PLoS One 10, e0129295.

Bressure-Isoard, C., Broussolle, V., Carlin, F., 2018. Sporulation environment in Bacillus: evidence and insights on underlying molecular and physiological mechanisms. FEMS Microbiol. Rev. 42, 614–626.

Caraka, R.E.; Lee, Y.; Kurniawan, R.; Herliansyah, R.; Rahan, P.A.; Naution, B.J., et al., 2020. Impact of COVID-19 large scale restriction on environment and economy in Indonesia. Glob. Environ. Sci. Manag. 6, 65–84.

CDC. Questions & Answers Regarding Respiratory Protection for Infection Control Mea-

CRediT authorship contribution statement

Conceptualization, Formal analysis, Investigation, Resources, Methodology, Project administration, Supervision, Writing - review & editing; JB: Conceptualization, Formal analysis, Investigation, Methodology. Writing - original draft, Writing - review & editing; AH: imaging, visualization & review; JL: contributed to analyzing the data, & made important suggestions, review & editing; CB: visualization, editing & review; NB, AS & TE: performed the coronavirus related experiment, review & editing. All co-authors discussed the results and commented on the manuscript. All authors read and approved the final manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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