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Reduction of SARS-CoV-2 Infectious Titers by Direct Contact with Cuprous-Oxide Impregnated Face Masks External Layers

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ABSTRACT

SARS-CoV-2 can remain viable on the protective face masks surface for several days. Mask touching, reuse and disposal occurs frequently, leading to increased risk of cross-contamination, infection and further transmission. Cuprous-oxide has potent virucidal properties. We determined the capacity of surgical face masks (type IIR) made with nonwoven fabric impregnated with cuprous-oxide microparticles (Test Fabric), to inactivate SARS-CoV-2 when in direct contact with the virus. The Test Fabric reduced the infectious titers of SARS-CoV-2 by 0.73, 3.02 and 4.19 log10 within 5, 30 and 60 minutes, respectively. In contrast, the infectious titers of the virus were reduced by Control Fabric by 0.24, 0.67 and 0.97 within 5, 30 and 60 minutes, respectively. The reductions were significantly higher in the Test Fabric than in the Control Fabric (0.49, 2.35 and 3.22 log difference, accordingly), reaching a statistically significant difference after 5 minutes ($p<0.01$). The mask filtration properties were not affected by the presence of the cuprous oxide microparticles. We conclude that the use of cuprous-oxide containing face masks in the external layers of respiratory face masks may significantly reduce the risk of SARS-CoV-2 cross-contamination, transmission and infection, due to masks handling and disposal, especially when used by the general population.

KEYWORDS: SARS-CoV-2; face masks; cuprous-oxide; transmission; cross-contamination

I. INTRODUCTION

The current ongoing pandemic caused by the highly pathogenic novel human coronavirus [1], named se-vere acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has infected more than 125 million individuals and caused more than 3.3 million deaths worldwide (https://covid19.who.int/, accessed May 05, 2021). The main mode of the virus transmission is through aerosol particles and droplets generated during coughing, sneezing or talking by symptomatic patients and from asymptomatic individuals, even before or without the onset of symptoms [2,3]. No significant differences were found in viral burden between symptomatic and asymptomatic individuals [4]. and thus asymptomatic individuals may unknowingly contribute significantly to the spread of the virus. One of the major effective measures taken worldwide to stop the transmission of the virus is the use of protective face masks by the general population [5].

Copper, and cuprous-oxide in particular, have potent virucidal properties [6], including against SARS-CoV-2 [7]. A platform technology that impregnates different textiles with cuprous-oxide microparticles was developed, endowing them with wide spectrum biocidal properties [8,9]. N95 respiratory masks in which the external layers were made with nonwoven fabric impregnated with the cuprous-oxide microparticles reduced by more than 99.9% the infectious titers of Human Influenza A (H1N1) and Avian Influenza Virus (H9N2) virions that remain on the mask [10]. The high capacity of the cuprous-oxide impregnated fibers and fabrics to neutralize 12 additional different pathogenic viruses was demonstrated in separate studies [11,12].

In the current study, we examined the capacity of the external layers of 3-ply respiratory masks, made of nonwoven cuprous-oxide impregnated fabric, to reduce the infectious titers of SARS-CoV-2 when in direct contact with the fabric.

II. MATERIAL AND METHODS

The test material was spunbond nonwoven polypropylene fabric containing ~3% cuprous-oxide microparticles, (MedCu Technologies, Ltd. Figure 1), hereafter referred as the Test Fabric.

The cuprous oxide particles were characterized by x-ray diffraction (XRD) using an x-ray diffractometer (Y-2000) with Cu Kα radiation (λ = 1.5418 Å). A scan efficiency of 0.1°·S-1 was applied to record the powder patterns in the range of $0° \leq 2\theta \leq 60°$. Three peaks at $2\theta = 29.61°$, 36.49° and 42.37° were indexed to (110), (111), and (200) planes of the cubic phase with lattice constant $\alpha = 0.4266$ nm, which is in accordance with the spectrum for Cu2O in JCPDS–International Centre for Diffraction Data (PDF, Powder Diffraction File, No. 05–0667, 1996).

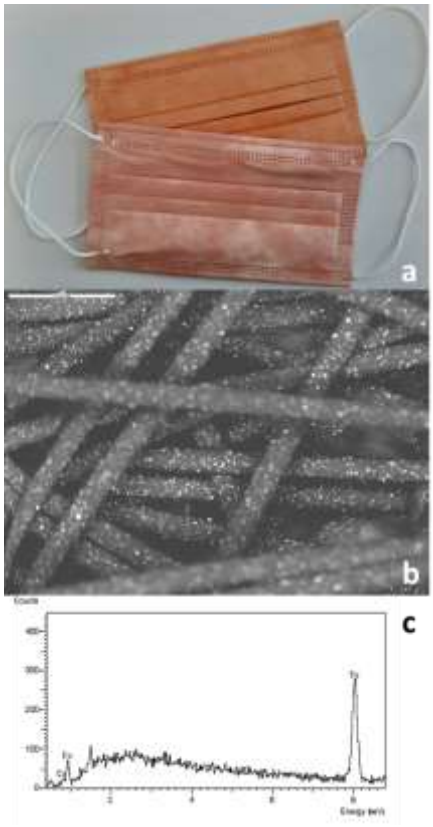

Figure 1. Surgical face masks. The external layers of the surgical masks (a) are made from polypropylene spunbond fabric impregnated with cuprous-oxide microparticles. (b) Scanning electron microscopy (SEM) imaging of the polypropylene fabric shows homogenous distribution of white dots on the surface of the poly-propylene fibers. (c) A representative Energy Dispersive X-Ray Analysis (EDX) of a white dot seen in (b) shows a peak at 8 keV, corresponding to copper.

Based on particle size distribution (PSD) analysis by intensity, the size of the cuprous oxide particles ranged from about 400 nm up to about 4000 nm (main peak at 1230 nm), with a minor peak (0.5%) above 5000 nm. Based on the PSD analysis by volume, the size of the cuprous oxide particles ranged from about 400 nm up to about 4000 nm (main peak at 1500 nm), with a minor peak (5.7%) above 5000 nm. SEM analysis shows large cubic particles in accordance with the PSD analysis (Figure 2a), however, enlarged magnification of the particles reveals that most of the dispersed cuprous oxide particles agglomerate into larger particles and that the minimal particle size detected is ~190 nm (Figure 2b).

Impregnation of cuprous oxide microparticles in the polypropylene fibers was achieved by adding the cuprous oxide microparticles to the polypropylene during the master batch preparation stage [8]. The concentration of the cuprous oxide microparticles in the fabric was based on previous studies that demonstrated that this con-centration endows the fabric with wide spectrum biocidal properties (e.g. ref [8,13]). As a negative test control material, the same spunbond polypropylene fabric without cuprous-oxide was used, hereafter referred as Control Fabric.

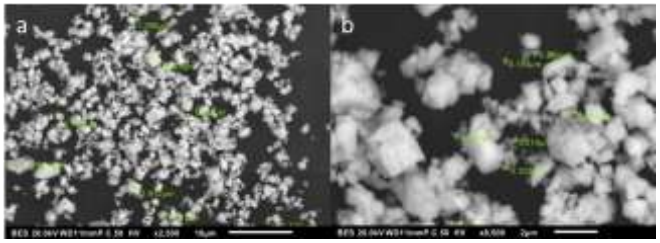

Figure 2. SEM analysis of the cuprous oxide microparticles dispersed in water at a) x2500 and b) x8500 magnifications.

After being UV sterilized for 1 hour, 20 mm by 20 mm pieces of Control and Test Fabrics were aseptically placed together in separate vial containers (final weight of 0.20 ± 0.005 g per tested sample), hereafter referred to as Control or Test Fabric specimens, respectively.

As the virus source, -80°C cryopreserved SARS-CoV-2 clinical isolate was used. The stock virus titer was determined by infecting Vero-E6 (CRL-1586™; American Type Culture Collection) plated cells grown in Gibco phosphate-free, high glucose DMEM media (catalog number: 11971025) containing 10% fetal bovine serum (FBS) and determining the plaque forming units (PFU)/ml.

The determination of the direct contact virus inactivation of the test items was performed as follows according to the ISO Standard 18184:2019 -Textiles — Determination of antiviral activity of textile products: 100 µl of the virus stock suspension were placed onto each test specimen, making sure all the liquid was thoroughly absorbed. The vial containers were then tightly closed and incubated at of 25°C for 5, 30, or 60 min (triplicate Control or Test Fabric specimens per time point). After the respective incubation time, 10 ml of Earle's Balanced Salt Solution (EBSS, Gibco) were added to each container; the containers were closed and agitated for 60 seconds by vortexing. The virus suspensions were collected. From each of the recovered eluent suspensions

after vortexing, 2-times sequential dilutions were prepared using phosphate free DMEM media.

For potential deactivation of the virus by possible eluting elements from the Control or Test Fabric specimens (i.e. not by direct contact inactivation), the following control was performed: 10 ml of EBSS were added to 3 Control Test specimens and to 3 Test Fabric specimens placed in the vial containers. The containers were then closed, vortexed for 60 s, and 5 ml of the solution from each vial container were transferred to new tubes. 50 µl of virus suspension were then added to each tube. The tubes were kept at 25°C in the CO2 incubator for 30 min.

The infectivity of the virus recovered from the Control or Test Fabric specimens were then determined by conducting a plaque assay using Vero-E6 cells plated in 96 well culture plates 12-24 hours in advance, and grown to 80-90% confluency. Briefly, the cells were washed once with PBS and then 100 µl of sequential dilutions the above-described suspensions were added to each well. The cells inoculated with the virus were incubated for 1 hour at 37°C in 5% CO2 incubator, with gentle shaking every 15 minutes. After 1 hour of ab-sorption, 100 µl of a semi-solid maintenance solution preparation (1% methylcellulose in DMEM) were added to each well. The plates were incubated for 3 days at 37°C in 5% CO2 incubator. The number of plaques formed was monitored every day. After 72 hours of incubation, when the formation of plaques was obvious, 50 µl of 2% paraformaldehyde were added per well in order to fix the cells. The supernatants were discarded after 30 minutes, and 30 µl of crystal violet were added per well. After 2 minutes, the cells were washed 5 times with water. The number of plaques was determined by using an inverted microscope.

The plaque forming units (PFU) per ml were calculated using the following formula:

PFU/ml = number of plaques counted/(dilution factors x inoculation volume).

Each PFU/ml was converted to log 10 and the average of the Initial viral load (Log10) and Output viral load (Log10) were then determined.

The log10 Reduction was calculated in the following manner:

Log10 Reduction = Average Initial viral load (Log10) – Average Output viral load (Log10).

The percent reduction of the infectious titers was calculated as follows:

Percent reduction from Initial titer = 100-[(Mean final titer / Mean initial titer)*100]

A t-test was performed to compare the means of log reductions from direct contact inactivation as well as the total reduction for statistical significance between control and test fabrics. A p-value of less than 0.05 was considered a statistically significant difference.

The amount of copper eluting from the mask into human dermatomed skin was determined in an independent laboratory (Dermal Technology Laboratory Ltd., Med IC4, Keele University Science and Business Park, Keele, Staffordshire, UK) according to the OECD Test No. 428: Skin Absorption: In Vitro Method. Regular surgical face masks without copper were used as negative controls. The surgical face mask materials (triplicate samples) were applied to the surface of the skin in static diffusion cells and the skin was otherwise left unoccluded for an exposure period of 24 hours. Various skin compartments of a typical OECD 428 study [14] were collected and analyzed for copper by Inductively Coupled Plasma - Optical Emission Spectrometry (ICP-OES).

The amount of copper that eluted from the masks into the air under simulated breathing conditions was determined by an independent lab (Nelson Laboratories, Inc. 6280 S. Redwood RD., Salt Lake City, UT, USA). To simulate human inhalation, air was pulled under vacuum of 1 CFM through a capsule HEPA filter and a Concha Therm III® unit, which can heat and humidify air. The temperature of the test was maintained at 37 ± 2°C and >90% relative humidity. In addition, a 4 ± 2% concentration of carbon dioxide was introduced into the air stream. The air was pulled through the test sample and releasable particles were drawn through a Pall® zefluor 3 µm PTFE membrane, with a particle collection efficiency of >99.996% of 0.3 µm diameter. After 5 hours of exposure to simulated inhalation, the test was stopped and the collection membranes were removed and the amount of copper was determined by ICP-OES. The test was done in triplicates. Regular surgical masks without copper were used as negative controls. As a positive control, 0.1 grams of cuprous oxide powder were added to 100 ml of USP water and 1 ml of the mixture was inoculated onto a PTFE particle collection membrane and the solution was allowed to dry.

The filtration properties of the masks were determined according to EN 14683 (European standard for face masks) by an independent lab (TUV SUD Products Testing Co., Ltd. B-3/4, No. 1999 Du Hui Road, Minhang District, Shanghai, China), which included determination of Bacterial Filtration Efficiency (BFE), Differential Pressure and Synthetic Blood Penetration tests.

III. RESULTS AND DISCUSSION

The capacity of copper to neutralize readily coronaviruses has been previously demonstrated [15]. More recently it has been found that the SARS-CoV-2 can remain viable on surfaces between hours and days, depending on the inoculum shed and environmental conditions [16]. While on plastic, stainless steel, and cardboard, the median half-life of survival for the SARS-CoV-2 is 6.81, 5.63 and 3.46 hours, respectively, on metallic copper it is 0.77 hours; less than in the aerosols (1.09) [16]. Copper ions released from the metallic copper and the generation of reactive oxygen species (ROS) was shown to be involved in the inactivation of the

viruses that come in contact with the copper surfaces [15]. We decided to use in our antiviral nonwoven fabrics' cuprous oxide as the oxidation state of copper is one very significant step closer to releasing the active copper ions that damage the viruses, and hence the faster inactivation of the virions, as opposed to the slower inactivation ob-served with pure copper [16].

Surgical masks, N95 and other respiratory masks, are now being used widely not only by healthcare and first responders, but by the wide public, in view of the current COVID-9 pandemic. Disturbingly, SARS-CoV-2 virus can be retrieved from the surface of regular face masks even after 7 days of exposure of the mask to the virus [17]. While it is recommended that respiratory face masks be used until they are soiled,[18] in reality the general population reuses their face masks even for several days, sometimes until the masks are disintegrated. It has already been demonstrated that face masks and respirators can become contaminated with viral pathogens following their prolonged use [19-21], and that mask and face touching is a frequent habit [22].

The presence of antiviral nonwoven fabrics, in the layer in contact with the face and in the external layer of the mask, may significantly reduce the risk of cross contamination during mask handling and disposal. The internal layer in contact with the face is especially relevant regarding asymptomatic individuals, who unknowingly contaminate their masks. Following the mask removal, they may contaminate their hands or gloves, and then unintentionally contaminate other high touch surfaces, such as door handles and elevator buttons. These sur-faces may be touched by unexposed individuals, who then can become infected. We thus developed 3-ply face masks in which cuprous oxide microparticles were embedded in both external nonwoven polypropylene layers, and we tested the capacity of the nonwoven fabric to reduce the infectious titers of SARS-CoV-2.

The virus infectious titers were not reduced following the exposure of the virus to the EBSS medium that was in contact with the Control and Test specimens (Table I). The obtained mean titers were somewhat higher than the mean value obtained from the virus suspensions that were not mixed with the tested fabrics. In order to account for the variability of the obtained initial titer, considering that Replicate 3 of the original virus suspension was an outlier, in the following calculations we referred to the initial viral titer as the average of all 8 wash-out measurements obtained, excluding the outlier, i.e. 232,000 PFU/ml, and the mean log 10 of all 7 wash-out measurements obtained is 5.35 log10.

Table I. Wash-Out Control Results

Virus Suspension	Replicate	Viral titre (PFU/ml)	Mean ± SD (PFU/ml)
Original	1	208,000	165,330.3 ± 73,900.1
	2	208,000	
	3	80,000	
Original + Washing Out Test Fabric	1	192,000	218,660.7 ± 46,180.8
	2	192,000	
	3	272,000	
Original+ Washing Out Control Fabric	1	272,000	234,660.7± 40,260.6
	2	272,000	
	3	240,000	

Following the direct contact of the virus suspension with the Test Fabric specimens, the infectious titers of SARS-CoV-2 were reduced by 0.73, 3.02 and 4.19 log10 within 5, 30 and 60 minutes, respectively (Table II).

Table II. Virus titers following direct contact with the Test Fabric

Contact Time (min)	Replicate	Calculated Titre (PFU/ml)	Mean Titre (PFU/ml ± SD)	Average Log10 Viral Load	Log10 Reduction From Initial Titer†
5	1	52,000	42,667.0± 8326	4.62	0.73
	2	36,000			
	3	40,000			
30	1	5,000	2,333.7±2516	2.33	3.02
	2	2,000			
	3	1‡			
60	1	3,000	1000.7±1731	1.16	4.19
	2	1			
	3	1			

†average of all data obtained in Table I, reflecting the titer of the initia inoculum (232,000 PFU/ml = 5.365 log10) ‡ Lower limit of detectio

The infectious titers of the virus that was in contact with the Control Fabric were also reduced in a time de-pendent manner, but to a significantly lower extent (Table III).

Table III. Virus titers following direct contact with the Control Fabric

Contact Time (min)	Replicate	Calculated Titre (PFU/ml)	Mean Titre (PFU/ml ± SD)	Average Log10 Viral Load	Log10 Reduction From Initial Titer†
5	1	192,000	149,330.3±36,950	5.11	0.24
	2	128,000			
	3	128,000			
30	1	80,000	53,330.0±24440	4.68	0.67
	2	48,000			
	3	32,000			
60	1	48,000	29,330.3±16650	4.38	0.97
	2	24,000			
	3	16,000			

†average of all data obtained in Table I, reflecting the titer of the initial viral inoculum (232000 PFU/ml = 5.365 log10)

As depicted in Figure 3, the reductions of the infectious titers were significantly higher in the Test Fabric than in the Control Fabric (0.49, 2.35 and 3.22 log difference after 5, 30 and 60 minutes, respectively), reaching a statistically significant difference already after 5 minutes ($p<0.01$).

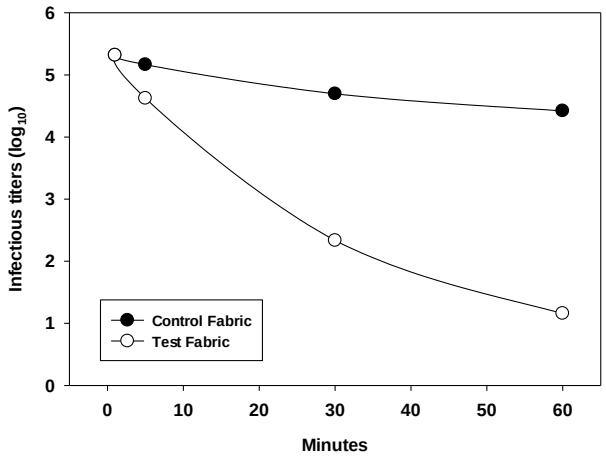

Figure 3. Viral infectious titers of SARS-CoV-2 after being in direct contact with the Control and Test Fabrics. The virions incubated with the fabrics were recovered after the respective incubation times. A plaque assay was then conducted to determine the infectious titers of the recovered virions. The infectious titers of the recovered virus from the Test Fabric were statistically lower than the titers of the virus re-covered from the Control Fabric after 5 minutes ($p<0.01$).

The cuprous oxide impregnated masks achieved ≥99.8% Bacterial Filtration Efficiency (n=5), 30.1 Pa/cm2 in the Differential Pressure test (n=5) and no penetration of synthetic blood through the masks (n=13). Thus, the masks passed successfully the EN 14683 tests, achieving the type IIR surgical mask parameters threshold, indicating that the presence of the cuprous oxide microparticles in the external layers of the masks did not affect the filtration properties of the mask, which depends on the internal filtration layer.

The cuprous-oxide impregnated nonwoven fabric has passed all the safety biocompatibility tests, such as skin sensitization or skin irritation, and is in use for years in adult diapers and antimicrobial wound dressings [23,24], which have been cleared by the FDA and other regulatory bodies for clinical use in acute and chronic wounds. Their safety in respiratory face masks was also demonstrated [10]. In the current study we further tested if there is elution of copper from the mask to the skin by using an ex-vivo human assay (OECD Test No. 428: Skin Absorption: In Vitro Method) and by measuring the amount of copper eluting to the air during simulating breathing conditions. No significant differences were found in the amount of copper detected in the unwashed, digested skin samples, and in the washed and tape stripped skin samples exposed to the cuprous oxide containing masks and the control masks without copper even when the fabric had been in continuous contact with the human skin for 24 hours. (1.62 ± 0.44 and 1.4 ± 0.8 µg copper, respectively; p= 0.64). The amount of copper that eluted from the masks during 5 hours of simulated breathing conditions was 0.09 pg/m3, which is ~ 100,000-fold lower than the respiratory copper permissible exposure limit (PEL) set by the USA Occupational Safety and Health Administration (''OSHA''). The lowest observed-adverse-effect levels (''LOAELs'') for chronic copper inhalation exposure was determined to be 0.64 mg/m3. The 0.09 pg/m3 of copper that eluted during the simulated breathing test is a tiny fraction of the copper LOAEL.

IV. CONCLUSIONS

Face masks are now widely used by the general population in view of the ongoing COVID-19 pandemic. Masks may become contaminated with the SARS-CoV-2 virus, including unknowingly by asymptomatic individuals. Since the virus may remain infectious on the masks for several days, and since masks are being reused by the general public for several days, viral cross-contamination and increased risk of viral infection due to the improper mask handling and disposal, may occur. Thus, the safe use of cuprous-oxide containing face masks in the external layers of the masks, which can significantly readily reduce the viral infectious titers, may contribute to reduction of viral cross-contamination, transmission and infection.

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