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Simple, low-cost and long-lasting film for virus inactivation using avian coronavirus model as challenge

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Abstract: COVID-19 infection, caused by SARS-CoV-2, is inequitably distributed and more lethal among populations with lower socioeconomic status. Direct contact with contaminated surfaces has been one of the virus sources, as it remains infective up to days. Several disinfectants have been shown to inactivate SARS-CoV-2 but they rapidly evaporate, are flammable or toxic and may be scarce or inexistent for the vulnerable populations. Therefore, we are proposing simple, easy to prepare, low-cost and efficient antiviral films, made with wide available dishwashing detergent, which can be spread in hands and inanimate surfaces and it should be expected to maintain virucidal activity for longer periods than the current sanitizers. Avian coronavirus (ACoV) was used as model of challenge to test the antivirus efficacy of proposed films. Polystyrene petri dishes were covered with a thin layer of detergent formula. After drying, the films were exposed to different virus doses for 10 minutes and virus infectivity were determined using embryonated chicken eggs and RNA virus quantification in allantoic fluids by RT-qPCR. The films showed to inactivate the ACoV (ranging from $10^{3.7}$ to $10^{6.7}$ EID_{50}), which is chemically and morphologically similar to SARS-CoV-2, and it may constitute an excellent alternative to minimize the spread of COVID-19.

Keywords: coronavirus; film; detergent; antiviral; virucide; inactivation; sanitization

1. Introduction

The World Health Organization (WHO) has declared the COVID-19 as a pandemic that is caused by the new human coronavirus (SARS-CoV-2) that already infected more than 20 million people and cause more than 700 thousands of deaths, worldwide by august 2020 [1]. Person-to-person and contact with contaminated surfaces has been identified as the most common transmission ways [2]. The length of time infectivity of SARS-CoV-2 was recently evaluated on several inanimate surfaces, and it ranges from hours to several days [3]. The efficiency of surface disinfectant has been investigated and it has been shown to inactivate SARS-CoV-2 in few minutes [3,4]. However, these virus inactivation agents rapidly evaporate, are flammable, become inefficient in short time after

application and consequently, the sanitized surfaces can be a new transmission source following novel contamination. These products are also hard to find or expensive in underdeveloped countries, which are the new hotspots for the disease. Prevention of the transmission by contact through washing hands with soaps and sanitization with alcohols have been implemented in several countries [5]. However, these procedures are efficient to eliminate contamination that happened only before the sanitization and the hands might be contaminated shortly after these sanitization procedures. Therefore, there is urgency for simple, low-cost and efficient antiviral procedure that is able to maintain virus inactivation efficiency up to several hours.

Our hypothesis was that the use of a long-lasting dry film, made with dishwashing detergent, has ability to reduce the viral infectivity by several orders of magnitude in few minutes, in inanimate surfaces. A similar film, applied on hands, could also reduce the risks of contamination when hand washing and/or sanitization are reduced, especially in locations where water is not easily available, which is a common scenario in developing countries where the majority of population has low socioeconomic status.

Detergents have high concentration of surfactants like sodium dodecyl sulfate (SDS) also known as sodium lauryl ether sulfate (SLES) or sodium lauryl sulfate (SLS), that is a well-known protein-denaturing agent [6] and linear alkylbenzene sulfonates (LAS). Therefore, surfactants could denature or induces small conformational changes in the supramolecular structure of S (spike protein) of SARS-CoV-2 that binds to the ACE-2 (angiotensin-converting enzyme 2) receptor of epithelial cell from the host [2], reducing the virus infectivity. Surfactant may also inactivate enveloped virus (like SARS-CoV-2), acting on the lipidic layer [7]. Scientific reviews by the independent Cosmetic Ingredient Review (CIR) initiative concluded that SDS is safe and it is not a cause for concern to the consumer use [8], the same conclusion was confirmed by other study [9]. In order to verify the potential inactivation efficiency of the detergent dry film on SARS-CoV-2, we have used the avian coronavirus (ACoV), also known as avian infectious bronchitis virus, as model of challenge, since chemical and structure are very similar between coronaviruses [10]. Both viruses are enveloped, single-strand, positive-sense RNA viruses. ACoV belongs to Gammacoronavirus, while SARS-CoV-2 is classified in the Betacoronavirus genera [11]. ACoV was the first coronavirus to be reported in 1937, from chickens with respiratory disease [12]. Due the huge economic impact caused by this virus, ACoV is one of the most studied coronaviruses over the last decades [13,14]. Main advantages of using ACoV model of challenge are non-zoonotic nature of this virus (the infectivity is restricted to chickens), which can be cultivated under lower biosafety levels. Additionally, there are numerous attenuated virus vaccines commercially available, allowing to reproduce the experiments world widely. Therefore, this study aimed to evaluate the chemical stability and ability to inactivate ACoV infectivity of two proposed detergent based films, one for applying in inanimate surfaces and the second one to be used on hands.

2. Materials and Methods

2.1. Protocols to prepare the antiviral films

Detergent Ype Clear (Quimica Amparo LTDA, Amparo, SP, Brazil) contains approximately 8% of surfactant (2% of SDS and 6% of linear alkylbene sulfonates) and others non-surfactants compounds. The soybean oil used was from Soya brand (Bunge, SP, Brazil). Two antiviral films, for application in inanimate surfaces and in hands were evaluated. The film for inanimate surfaces was prepared by diluting the detergent in distilled water (2:1 ratio). The film for hand application was prepared using detergent and soybean oil (20:1 ratio), and the mixture was completely homogenized until obtain a white emulsion (if stored the formula must be re-homogenized before use). The oil was added in this last formula as plasticizer, to improve flexibility [15] and reduces skin drying. Both formulas were immediately used after preparation. 200 µL of each formula was applied to sterile plastic petri dish (85 mm of diameter) and spread in its surface to form a thin film. After approximately 30 minutes the water evaporated and a thin, sticky and translucent dry film was obtained.

2.2. Chemical stability of the film.

The chemical stability of the films was analyzed by high resolution NMR spectroscopy. The ^{1}H spectra were acquired on a high-resolution 600 MHz (14.1 T) Avance III NMR spectrometer (Bruker, Karlsruhe, Germany) using a 5 mm broadband probe. The dry films were prepared with 200 µL of the detergent casted on petri dishes. The fresh film was analyzed 30 minutes after preparation and a second film evaluation was analyzed after 7 days in the laboratory at room temperature. The films components were extracted into 1 mL of deuterated water (D_2O) and transferred to 5 mm NMR tube. The spectra were acquired with and without water suppression sequences, using DSS as chemical shift standard. The experiment was performed with 30 ° pulses, 3.89 s acquisition time, 64 K data points, spectral width of 14 ppm, 32 scans and recycle delay of 5 s.

2.3. Avian coronavirus (ACoV) as model for challenge.

The commercial attenuated ACoV vaccine H120 strain/BRMSA 1775, from CMISEA (Collection of Microorganisms Important to Swine and Poultry), was used as challenge model for antiviral activity of tested films. Three bottles of lyophilized vaccine (1000 doses for each bottle) were diluted into 12 mL of Phosphate-buffered saline (PBS). Titration was carried out by inoculation of five 10-day-old specific pathogen-free (SPF) embryonated chicken eggs per dilution (from a ten-fold serial dilution of the virus) via the allantoic cavity route [16] and virus titre was expressed as 50 % embryo infectious doses (EID_{50}/mL) according to Reed and Muench (1938) [17]. The final titre was estimated as $10^{7.7}$ EID_{50}/mL.

2.4. Evaluation of film's antiviral activity

For each tested film, seven polystyrene petri dishes of 85 mm of diameter were used and distributed in A to G groups (Table 1). 200 µL of film was dispensed in the center of the petri dish from groups A, B and C. The film was uniformly distributed in whole petri dish surface using a sterile cell scraper. Plates were allowed to dry in room temperature (20 to 25 °C) during 30 to 40 minutes. The virus levels of $10^{6.7}$ EID_{50} (high challenge), $10^{4.7}$ EID_{50} (intermediate challenge) and $10^{3.7}$ EID_{50} (low challenge) were distributed in A/D, B/E and C/F groups, respectively. 200 µL of virus diluted in PBS pH 7.2 was dispensed per petri dish and incubated during 10 minutes at room temperature. The virus solution was recovered from each petri dish by washing with 1.8 mL of transport media containing antibiotics (10,000 IU/mL of penicillin G, 5 mg/mL of streptomycin and 0.65 mg/mL of kanamycin sulfate). For each group, 0.2 mL of recovered suspension was inoculated into the allantoic cavity of 11-day-old SPF embryonated chicken egg, four to six eggs were inoculated per group. Three additional groups were included, G group was inoculated with PBS recovered from the petri dish containing film but no virus, aiming to confirm absence of toxic effects in the chicken embryo, H group was inoculated with transport media and I group remained non-inoculated. The inoculated eggs were incubated at 37 °C and candled daily for 7 days, wherein mortality observed in the first 24 hours was considered as nonspecific. Typical embryonic changes, consisting of stunted and curled embryos with feather dystrophy (clubbing) were classified as positive for ACoV [16,18]. Allantoic fluid was individually harvested (on the day of embryo death or from all surviving embryos at seventh day post-inoculation) and stored at -80°C until be processed.

Table 1. Experimental groups used for evaluation of film's antiviral activity, including the number of replicates for hands formulation and inanimate surface film.

Group	Description	Replicates	Film	ACoV level
A – Film + High challenge	Efficacy of film	Hands: 5 Surface: 6	Yes	$10^{6.7}$ EID_{50}
B – Film + Intermediate challenge	Efficacy of film	Hands: 6 Surface: 6	Yes	$10^{4.7}$ EID_{50}
C – Film + Low challenge	Efficacy of film	Hands: 6 Surface: 6	Yes	$10^{3.7}$ EID_{50}

D – High challenge	Positive control	Hands: 6 Surface: 5	No	$10^{6.7}$ EID_{50}
E – Intermediate challenge	Positive control	Hands: 5 Surface: 5	No	$10^{4.7}$ EID_{50}
F – Low challenge	Positive control	Hands: 6 Surface: 5	No	$10^{3.7}$ EID_{50}
G – Film + Transport medium	Safety control	Hands: 0 Surface: 4	Yes	No
H – Transport medium	Negative control	Hands: 6 Surface: 6	No	No
I – Non- inoculated	Embryo control	Hands: 7 Surface: 7	No	No

2.5. Absolute quantification of avian coronavirus (ACoV) RNA copies by RT-qPCR

RNA extractions from 100µL of allantoic fluid or from virus suspension inoculated in eggs were performed using TRIzol Reagent (Invitrogen) followed by RNA purification using Rneasy Mini Kit (Qiagen®). All samples were tested for ACoV viral load by RT-qPCR (hydrolysis probe system) using AgPath-ID One step RT-PCR kit (Ambion®), primers and LNA-probe (5´FAM-3´BHQ1, IDT) for amplification of the 3´UTR genome region of ACoV, as described [19]. The standard curve was constructed using reverse transcribed RNA for estimation of absolute quantification of ACoV RNA copies. Briefly, the RNA extracted from ACov was submitted to conventional RT-PCR as described [19], targeting a fragment of 3' UTR of ACoV, with 276 bp of size. The PCR product was cloned into TOPO TA vector (Invitrogen), according to manufacturer instructions and the transformed plasmid was inserted in DH5alpha competent cells. The extracted plasmidial DNA was reverse transcribed using MEGAscript™ T7 Transcription Kit (Ambion) as recommended by the manufacturer, the transcribed RNA was quantified using Qubit™ RNA HS Assay Kit (Invitrogen) and stored at -70°C until be processed. The estimation of the number of RNA copies was calculated using the formula: $\{[(g/\ \mu L \text{ of RNA})/(\text{size of transcribed RNA} * 320)]/(6.022*10^{23})\}$. Cq (Cycle quantification) results were used to calculate the number of RNA copies (Log10) using the linear equation from the standard curve. Samples presenting Cq ≤ 36 were classified as positive for ACoV.

2.6. Ethical standards

All the chicken embryos used in this study were handled in accordance to the standards of animal welfare and ethics adopted by the Brazilian Board for Animal Experimentation (Colégio Brasileiro de Experimentação Animal, COBEA), and the experimental animal protocol was approved by the Embrapa Swine and Poultry Ethics Committee for Animal Experimentation (no. 21/2020).

2.7. Statistical analysis

The results of Log_{10} ACoVRNA copies were submitted to test of normality, and to Kruskal-Wallis comparison test between group previously treated with the film and respective positive control group. The qualitative results obtained by virus isolation and RT-qPCR were submitted to Fisher exact test. All analyses were conducted using SAS® (2002) version 8, and the probability level for significance was set at $p \leq 0.05$.

3. Results

3.1. Evaluation of film's chemical stability

The ^1H NMR spectra of the film extracts showed that the major components of the detergent were SDS and LAS. The spectra presented the two aromatic signals of LAS, between 7 and 8 ppm, some weak peaks between 2 and 4.5 ppm, related to the hydrogen bonded to carbon 2 of SDS and the other non-surfactant compounds present in the detergent. The strongest NMR signals were observed

between 0.5 to 1.5 p.p.m that are related to the CH_2 and CH_3 groups of SDS and LAS. The spectrum profile obtained from the fresh film was identical to the one obtained after 7 days, with absence of any new peak, indicating that during these 7 days, the detergent components remained stable. The long-term chemical stability of detergent/oil formula was not verified, since it is prepared to be used during following few hours.

3.2. Evaluation of film's antiviral activity

ACoV RNA copies in the virus suspensions (previously exposed or not to the films) were estimated by RT-qPCR before inoculation in the embryonated chicken eggs. The suspensions containing the highest dose of $10^{6.7}$ EID_{50} (groups A and D of Table 1) presented approximately $10^{3.2}$ ACoV RNA copies, the suspensions with intermediate dose ($10^{4.7}$ EID_{50}) (B and E) presented $10^{1.2}$ ACoV RNA copies while no ACoV RNA detection was observed in the suspensions containing the lowest virus dose or in the diluent of virus (H).

Infectivity in the virus suspensions treated or not with tested films was determined by virus isolation in embryonated chicken eggs and ACoV RNA quantification by RT-qPCR in the allantoic fluid collected from these eggs (Figure 1 and Table 2). The virus suspensions previously exposed to the dry detergent (inanimate surface) and detergent plus vegetable oil (hands film) films (A, B and C groups) presented no virus infectivity, as no typical ACoV embryo lesions or positive results by RT-qPCR in the respective allantoic fluids were observed in these groups. On the other hand, all the control virus suspensions (not previously exposed to the films) (D, E and F groups) presented virus infectivity, since typical ACoV lesions in the embryos were induced in all samples and also presented ACoV RNA positive results, except by one sample of E group (control group of intermediate challenge) which was negative for virus isolation, but was positive for RT-qPCR. The allantoic fluids from eggs inoculated with virus diluent also presented absence of virus activity, as no embryo lesions or positive ACoV results were observed. Significant differences ($P < 0.05$), were observed between groups previously exposed to film and respective control groups for Log_{10} ACoV RNA copies values and also for qualitative results obtained by virus isolation and RT-qPCR (Supplementary material 1).

Table 2. Qualitative results of embryonated chicken eggs from different experimental groups obtained from avian coronavirus (ACoV) isolation and RT-qPCR.

Experimental groups		Positive/ Total samples (% positive)			
		Virus Isolation[1]		RT-qPCR[2]	
		Hands formulation	Inanimate surface	Hands formulation	Inanimate surface
High Challenge	A – Film	0/5[3] (0 %)	0/6 (0 %)	0/5[3] (0 %)	0/6 (0 %)
	D – Control	6/6 (100 %)	5/5 (100 %)	6/6 (100 %)	5/5 (100 %)
Intermediate Challenge	B – Film	0/6 (0 %)	0/6 (0 %)	0/6 (0 %)	0/6 (0%)
	E – Control	5/5[3] (100 %)	4/5 (80 %)	5/5[3] (100 %)	5/5 (100 %)
Low Challenge	C – Film	0/6 (0 %)	0/6 (0 %)	0/6 (0 %)	0/6 (0%)
	F – Control	6/6 (100 %)	5/5 (100 %)	6/6 (100 %)	5/5 (100 %)
	G –Film control	0/6 (0 %)	0/4 (0 %)	0/6 (0 %)	0/4 (0 %)
	H - Negative control	0/6 (0 %)	0/6 (0 %)	0/6 (0 %)	0/6 (0 %)

[1] Embryos presenting stunting, curling and/or feather dystrophy (clubbing) were considered as positive for virus isolation.

[2] Samples presenting Cq values < 36 were considered as positive.

[3] There was unspecific mortality of one embryo at 24 hpi, therefore this embryo was not considered in the analysis.

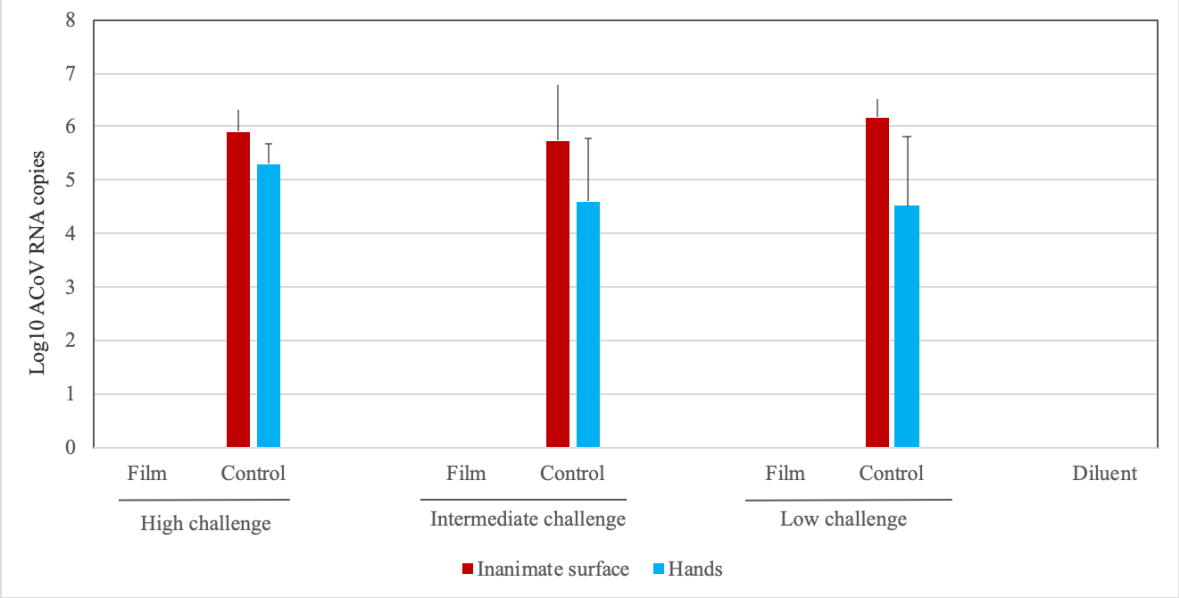

Figure 1. Absolute quantification of Log_{10} avian coronavirus (ACoV) RNA copies (only positive samples presenting Cq values < 36 were included) in the allantoic fluid from embryonated chicken eggs inoculated with virus suspension from different experimental groups previously treated or not with film and challenged with high, intermediate and low doses of ACoV.

4. Discussion

In view of current arising worldwide pandemic distribution of COVID-19, there is a realization that mortality of SARS-CoV-2 is inequitably distributed among vulnerable populations, especially related to lower socioeconomic status. Unlike the high-income countries of Europe, Northern Asia and North America, most countries of lower economic status face limited mitigation capacity, poor access to high quality public health and medical care, dense population and also have scarce access to commercial sanitizers and running water. Alternative formulations, as the here proposed film for virus inactivation, characterized by easy access, low cost and simple preparation may constitute a powerful and important tool for COVID-19 prophylaxis in these vulnerable populations.

In the present study, the antiviral activity of a simple and low-cost semi–permanent film was tested in plastic surface, using ACoV as model. A recent review about persistence of animal and human coronaviruses on different types of inanimate surfaces showed that coronaviruses remain infectious in plastic for the same or longer time than other surfaces (steel, aluminum, wood, paper, glass, silicon rubber, latex, disposable gown, ceramic and teflon) [20, 21]. The longest viral viability time (9 days) was found on a plastic surface [21]. Together, these results indicate that hard and non-porous plastic surface, as used in this study, is a good choice to screening the inactivation effects of disinfectants. Equally, despite this study has used the ACoV as virus model, the obtained findings may be extended to SARS-CoV-2, as similar chemical composition and structure were verified for the viruses of *Coronaviridae* family [10]. Similar environmental resistance was observed between these two viruses. At 56 °C, SARS-CoV-2 was viable after 10 minutes and inactivated after 30 minutes [3], while nine different ACoV strains were inactivated after 15 minutes at the same temperature [13]. Both, SARS-CoV-2 and ACoV were extremely stable in a wide range of pH values (3-10) at room temperature [13,3].

The presence of SARS-CoV-2 on surfaces is always a concern. In this study, film on plastic surface was able to inactivate the ACoV at all tested challenge doses. Firstly, the same virus RNA load was detected in the virus suspension previously exposed or not to the film, evidencing that same virus challenge was applied in film group and respective control. As result, no RNA detection (RT-qPCR) or virus activity in chicken embryos were observed in groups previously treated with film (Figure 1 and Table 2), while all control groups (challenged and not previously exposed to the film)

presented virus replication observed by macroscopic lesions and RNA virus detection in the embryos.

Antiviral activity of film can be attributed, in large part, to the biocidal action of the surfactants present in the detergent. Surfactant (surface active agents), are the single most important ingredients in laundry and household cleaning products [9,22], comprising from 1 % to 30 % in cleaning products formulation [9]. SLS surfactant could be a potent inhibitor of the infectivity of different types of pathogens without causing marked toxicity to skin and/or mucosae [23]. The mechanism by which SLS inactivates enveloped and non-enveloped viruses probably involves the denaturation of envelope or capsid proteins. These proteins may play different roles in the viral replicative cycle such as adhesion receptors, proteins involved in the encapsulation of viral genome [23]. Protein denaturation involves an initial rapid process where protein and SLS produce aggregates, followed by two slower processes, where the complexes first disaggregated into single protein structures situated asymmetrically on the SLS micelles, followed by isotropic redistribution of the protein [6].

Although the viral load of coronaviruses on inanimate surfaces is not clearly known to contribute as source of contamination, during an outbreak, it seems plausible that reduction of the viral load on surfaces by disinfection, especially those frequently touched by infected patients, may constitute an efficient tool for minimize the virus spread [20]. The prevalence of face-touching behavior in students was determined on average 23 times per h, where 10 times per hour for mucosae (eye, mouth and nose) touching [24]. Consequently, sanitization of both surfaces and hands are essential and inexpensive preventive methods for breaking the transmission cycle. Several biocidal agents such as 0.5 % hydrogen peroxide, 62-71 % ethanol or 0.1 % of sodium hypochlorite were able to inactivate coronavirus from inanimate surfaces within one minute [20]. However, these biocidal agents rapidly evaporate or become inefficient in a short period and consequently, these sanitized surfaces can be a new transmission source following novel contamination.

The results of the chemical stability evaluation of the films here tested showed that SDS and LAS compounds are highly stable in dry film casted in plastic surface for at least seven days. Chemical stability of here proposed films indicates that compounds properties, including antiviral activity, will be preserved for the same period, and may keep a residual protective effect. Our proposal is that the detergent film, applied in plastic, metal, glass, ceramic, laminated and other inanimate surfaces, in public areas, can be an efficient alternative to prevent the SARS-CoV-2 spread, especially in locations where scarce prophylaxis ways are reachable. Besides, diluted hand soap (1:49), which is well known to contain surfactants in the composition, was able to reduce 3.6 Log_{10} SARS-CoV-2 after 5 minutes and reached to undetected levels after 15 minutes [3], reinforcing the role of these substances in virus inactivation.

Therefore, the dry film might effectively maintain virus inactivation ability thorough hours to days, reducing the use of hazard chemical, and need of frequent sanitization procedures with the chemical compounds normally dissolved in water, generating large amount of toxic waste that may contaminates the ecosystems. New detergent film can be applied to the inanimate surfaces without removing the previously ones, that reduces the need of water and consequently reduces the environment contamination. The hand lotion prepared with detergent/vegetable oil could also reduce the risks of contamination especially in locations where water is not easily available for hand washing that is common scenario in populations in underdeveloped countries.

5. Conclusions

The antiviral film tested demonstrated excellent chemical stability and ability to inactivate ACoV infection at the three challenge doses tested. Further analysis will be performed regarding the shelf life of proposed films in different inanimate materials, hand application and with different virus models of challenge. Therefore, this study has demonstrated the efficacy of virus inactivation of a simple, low-cost and easy to prepare long-lasting detergent film, which may constitute an excellent alternative to mitigates the spread of SARS-CoV-2, particularly in population of low-income countries, as well as opening perspectives for studies with other enveloped viruses.

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