Characterization of Rhamnolipids for the Inactivation of Enveloped Viruses

Ling Jin (✉ ling.jin@oregonstate.edu)

Department of Biomedical Sciences, Carlson College of Veterinary Medicine, Oregon State University, Corvallis, OR 97331

Wendy Black

Department of Biomedical Sciences, Carlson College of Veterinary Medicine, Oregon State University, Corvallis, OR 97331

Teresa Sawyer

Electron Microscopy Facility, 145 Linus Pauling Science Center, Oregon State University, Corvallis, OR, 97331

Research Article

Keywords: rhamnolipids, 222A, 222B, HSV-1, HSV-1-GFP, Coronavirus, Bovine Coronavirus

Posted Date: July 1st, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-38084/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.
[Read Full License](#)

Abstract

In the face of new emerging respiratory viruses, such as SARS-CoV2, vaccines, and drug therapies are not immediately available to curb the spread of the infection. Non-pharmaceutical interventions, such as mask-wearing and social distance, can slow the transmission. However, both mask and social distance are not 100% effective at preventing the spread of respiratory viruses, such as SARS-CoV2 and influenza viruses. There is an urgent need to develop an intervention that could reduce the spread of respiratory viruses. Rhamnolipids are environmentally friendly and biologically safe surfactants that can kill enveloped viruses. Two rhamnolipid products, 222A and 222B, were investigated in this study to determine their ability to inactivate two enveloped viruses, bovine coronavirus and herpes simplex virus 1. We found that 222B at 0.005%, which has no toxicity to cells, can inactivate 10^5 PFU/ml enveloped viruses in 3-5 min. Moreover, 50-100 μ l of 222B at 0.005% on 1 cm² mask fabrics can inactivate $\sim 10^3$ PFU /10 μ l in 3-5 min. These results suggest that 222B can be coated on masks to prevent or reduce the spread of enveloped viruses.

Introduction

Enveloped viruses, such as coronaviruses, influenza viruses, and herpesviruses, are all wrapped with a phospholipid bilayer that is called the envelope. Surfactants are amphiphilic organic compounds comprised of a hydrophobic non-polar hydrocarbon tail and a hydrophilic polar head. They possess an aqueous solubility (critical micelle or aggregation concentration) in the micro- or millimolar range above which they self-associate to micelles, bilayer vesicles or other aggregates (Heerklotz, 2008). The compatibility of the hydrophobic tails with phospholipid bilayer allows surfactants to penetrate through the membrane easily, consequently alter the conformation of phospholipid bilayer membrane or the envelope, which in turn dissociate the membrane lipid apart. Therefore, the interaction between the phospholipid bilayer and surfactant will disperse the envelope and strip off viral proteins on the surface of the virion (complete viral particle). Once the virion loses its surface envelope or viral proteins on the viral envelopes, it will lose its infectivity. Therefore, the surfactants can be used to kill or inactivate enveloped viruses.

Commercial bleach products, such as Clorox, Lysol, are commonly used to disinfect enveloped viruses in research labs and medical facilities. They contain synthetic chemicals and can cause skin irritation or harm the environment. Rhamnolipids are biosurfactants, which are of biological origin and have many advantages over synthetic counterparts, such as low toxicity and high biodegradability (Jahan et al., 2020; Kaskatepe and Yildiz, 2016; Madrid et al., 2020).

Rhamnolipids were first identified in 1949 and purified in 1968(Arima et al., 1968; Jarvis and Johnson, 1949). Since then, numerous microorganisms, including bacteria, fungi, and yeast, have been reported to produce rhamnolipids (Hoskova et al., 2015; Satpute et al., 2010; Twigg et al., 2018). The rhamnolipid structure is determined by the number of rhamnoses (one or two) and fatty acids (one or two) and the fatty acid composition. Therefore, rhamnolipids are commonly classified into two groups:

monorhamnolipids and dirhamnolipids. Rhamnolipids have been used as an emulsifier, stabilizer, solubilizer, wetting, foaming agent, bactericide and fungicide.

Similar to SARS-CoV2, bovine coronavirus (BCoV) is a member of the *Coronaviridae* family, which comprises enveloped positive-sense single-stranded RNA viruses. BCoV is a pneumoenteric virus that infects the upper and lower respiratory tract and intestine. Infection of BCoV can lead to calf diarrhea, winter dysentery, and respiratory infections in cattle of various ages (Saif, 2010). Human herpesvirus type 1 (HSV-1) is a member of the *Herpesviridae* family that include a large number of enveloped DNA viruses common in both animal and humans.

HSV-1 is the cause of cold sores or fever blisters in or around the mouth and encephalitis in newborns (Dhull et al., 2019; Patoulas et al., 2017). It is also the leading cause of corneal blindness in developing countries ((Jones, 2003; Moerdyk-Schauwecker et al., 2009). Both are enveloped viruses, and their surface proteins on the envelopes are essential for virus entry in infection. Rhamnolipids have been shown to be capable of inactivating both bacteria and fungi (Elshikh et al., 2017; Gaur et al., 2020; Radlinski et al., 2017). In this study, the ability of rhamnolipids to inactivate BCoV and HSV-1 was investigated.

Material And Methods

Rhamnolipids are provided by AGAE Technologies, LLC (Corvallis, Oregon, USA).

AGAE0222A is a 10% aqueous solution made with commercially available di-rhamnolipid predominant product R95Dd. AGAE0222B is a 10% aqueous solution made with commercially available mono-rhamnolipid predominant product R95Md. Both rhamnolipid solutions were adjusted to pH7.4 ± 0.1. In this study, rhamnolipids AGAE0222A and AGAE0222B are called 222A and 222B, respectively. Sodium dodecyl sulfate (SDS) (EM-7910) and Triton X- 100 (X-100) were purchased from OmniPur and Sigma-Aldrich, respectively.

Viruses and cells

HSV-1 McKrae (Watson et al., 2012) and HSV-1-GFP (Jin et al., 2005) were cultured in Vero cells in Eagle's Minimum Essential Medium (EMEM) supplemented with 5% fetal bovine serum (FBS) (GeminiBio, USA), penicillin (100 U/ml), and streptomycin (100 µg/ml) (Sigma-Aldrich, Inc, St. Louis, MO, USA). Vero cells were cultured in EMEM supplemented with 10% fetal bovine serum (FBS) (GeminiBio, USA), penicillin (100 U/ml), and streptomycin (100 µg/ml)

(Sigma-Aldrich, Inc, St. Louis, MO, USA) at 37°C with 5% CO₂ in a humidified incubator as we described previously (Eide et al., 2010; Moerdyk-Schauwecker et al., 2009).

Bovine coronavirus (BCoV) was the NADL-Nebraska Strain isolated by the National Veterinary Services Laboratories (NVSL) in 1981 (L'Haridon et al., 1981). BCoV was grown on human rectal tumor (Hrt-18G) cells (Jin et al., 2007; L'Haridon et al., 1981), which were maintained in EMEM supplemented with 10% FBS (GeminiBio, USA), penicillin (100 U/ml) and streptomycin (100 µg/ml) (Sigma-Aldrich, Inc.) at 37°C

with 5% CO₂ in a humidified incubator. The virus was propagated in EMEM supplemented with 2.5 µg/ml trypsin and 2.5 µg/ml pancreatin, 1× insulin–transferrin–selenium (Cat No. 51500-056, GIBCO) (Jin et al., 2007).

Rhamnolipid cytotoxicity assay *in vitro*

Ninety-six-well plates were seeded with ~2X10⁴ Vero cells per well and grown overnight at 37°C and 5% CO₂. The cells were washed once with phosphate-buffered saline (PBS) and treated in 100 µl 222A or 222B with indicated concentrations diluted in PBS at 37°C for 1h. The rhamnolipid-treated cells were then washed once with PBS and then replenished with EMEM containing 5% FBS (GeminiBio, USA) and antibiotics (as described above), and further incubated for 24 h. After incubation, cell morphology was examined under light microscope and cell viability was examined by using colorimetric cell viability kit III (XTT) (PromoKine).

Briefly, 50 µl of the XTT reaction solution was added to each well and the plate was incubated at 37°C incubator for 3-4h. Absorbance at a wavelength of 450-500 nm was read on a microplate fluoresce reader (BioTek) and recorded. Wells containing 222A or 222B at each concentration in media without cells served as a blank to ensure that rhamnolipids themselves were not registering fluorescence (Moerdyk-Schauwecker et al., 2009).

HSV-1 and BCoV infection *in vitro* in the presence or absence of rhamnolipids.

Twelve-well plates were prepared the day before infection by seeding each well with approximately 1´10⁵ Vero cells or Hrt18G cells. The cells were washed once with PBS and infected with 100µl of either ~2X10⁶ PFU of HSV-1-GFP or ~1X10⁶ PFU of BCoV in the presence or absence of rhamnolipids. Following 1-hr absorption, the cells were replenished with 1 ml culture medium containing 5% FBS and antibiotics (as described above) in the presence or absence of 222B at the indicated concentration. At 24 or 72 hr post-infection (hpi), the cytopathic effect (CPE) from virus infection was examined under a light microscope or fluorescent microscope.

Immunofluorescence staining

Hrt-18G cells infected with BCoV were washed with ice-cold PBS twice and fixed in 1 ml ice- cold methanol at -20°C for 20 min. Following fixation, the plates were washed again twice with cold PBS and blocked in 1% BSA in TBS at room temperature for 1 h, then stained with 1:40

diluted anti-BCoV monoclonal antibody conjugated with FITC at room temperature for 1 h on a rocker and washed twice with PBS before viewing under fluoresce microscope.

Electron microscopy of virions

Six 75-cm² flasks seeded with 90% confluent Vero cells were infected with 0.01M.O.I. HSV-1- McKrae. The infected flasks were harvested when 80-90% CPE developed at 3-4 days post- infection (dpi), and

subjected to two-freeze and thaw cycles between -80°C and room temperature. The supernatant was cleared of cells and cell debris by centrifugation at 9,000 X g for 30 min at 4°C. The virions were then centrifuged through a 60% sucrose cushion in a Beckman Model XL-70 ultracentrifuge at 25,000 rpm for 1h in a SW28 rotor at 4°C as we described previously (Jin et al., 2008). The virion pellets were suspended in 100 µl dH₂O first, and then 10 µl of virion was mixed with 90 µl of 0.01% SDS, 0.01% Triton X-100, and 0.01% 222B, respectively. The treated virions were stained as we described previously (Jin et al., 2008). Briefly, treated virion suspensions were adsorbed to formvar-coated carbon-stabilized copper grids by floating grids on ~ 20 µl drops of the sample spotted on parafilm. The grids were then blotted dry with Whatman filter paper and immediately floated on ~20 µl drops of 2% phosphotungstic acid (PTA) (pH 6.9) in water for 30 s. Excess PTA was removed by side- blotting and the grids were allowed to air dry (Jin et al., 2008). Images were obtained with a FEI Titan 80-200/ChemiSTEM Transmission Electron Microscope (TEM).

HSV-1 plaque reduction assay following rhamnolipid treatment

Vero cells were seeded in 12-well tissue culture plates at approximately 2 × 10⁵ cells/well on the day before infection. Three wells were infected with HSV-1 at each concentration in the presence or absence of rhamnolipids, 222B. Following a 1-h viral absorption, 3 ml of 2% methylcellulose overlay media was added to each well. Plates were incubated at 37°C with 5% CO₂ in a humidified incubator. The plates were fixed in 20% methanol, stained with 1% crystal violet, plaques were counted at 4-5 dpi (Moerdyk-Schauwecker et al., 2009).

Plastic surface coating with 222B and surface-contact assay

The 48-well Corning Costar Flat Bottom Cell Culture Plate with a growth area of 0.95 cm² was selected for 222B coating on the plastic surface. The bottom of the well was coated by adding 20 µl of 222B at 0.005% directly, which cover the entire surface of the well. The coated wells were air-dried overnight. Virus droplets in 10 µl containing 10³ or 10⁴ virions (PFU) HSV-1 were added to the bottom of the well for 1 min or 5 min. The contact between HSV-1 and 222B coated plastic surface was stopped by adding 1 ml of PBS. Viruses left on the plastic surface were eluted in 1 ml 1 X PBS for 1 hour at room temperature on a plate rocker. The control wells were coated with PBS only. The non-surface contact controls were wells that had 1 ml of PBS added

first, followed by adding 10 µl to the elution without contacting the coated surface. 100 µl of eluted viruses from each well were then titrated by standard plaque assay.

Fabric coating with 222B and surface-contact assay

Approximately 1 cm² square fabrics were cut from a commercial surgical mask, made of non- woven fabric (Tronex Healthcare, Mount Olive, US), and then soaked in 50-100µl of 0.005% 222B or PBS. The soaked fabrics were air-dried overnight after removing the excess liquid from the soaked fabrics. The dried fabrics were then placed in a well of 24-well plate (Corning). Virus droplets in 10 µl containing 10³

or 104 PFU HSV-1 were added to the coated fabrics for 3-5 min. The contact between viruses and 222B coated fabrics was stopped by adding 1 ml of PBS directly to the well. Viruses left on the fabric surface were eluted at room temperature for 1h on a plate rocker. The non-surface contact controls were 222B coated fabrics were eluted first with 1 ml of PBS, viral droplet was added to the elution media without contacting the fabrics. The mock controls were viral droplets added directly to 1 ml PBS in wells that were not coated. The eluted viruses were then titrated by standard plaque assay.

Statistics

All statistical analyses were performed using GraphPad Prism version 4 for Windows (GraphPad Software, San Diego, CA, USA). Plaque reduction results were analyzed by two-way ANOVA with Bonferroni post tests.

Results

Cytotoxicity assays of rhamnolipids on cultured cells

Given cell membranes are made of phospholipid bilayers, it is possible that surfactants may have negative effects on cell membranes as well. Therefore, the toxicity of rhamnolipids was examined in uninfected Vero cells by the light microscope and XTT viability assay. Fig. 1 shows the morphology of Vero cells treated with 222A over a concentration range from 0.001% to 0.1%. Cells treated with 0.1% or 0.05% 222A were mostly detached, and very few live cells were visible under the light microscope. Similar results were observed with cells treated with 222B (data not shown). However, cell monolayers looked similar to the uninfected cells in wells treated 222A or 222B at or below 0.01%. To determine whether rhamnolipid affects cell viability, treated cells were tested by colorimetric cell viability kit III (XTT). The assay is based on the ability of mitochondria enzyme from metabolic active cells to reduce the tetrazolium salt XTT to orange-colored formazan compounds. The dye formed is water-soluble, and its intensity is proportional to the number of metabolic active cells. The greater the number of active cells in the well, the greater the activity of mitochondria enzymes, the higher the concentration of the dye formed, the higher absorbance on the ELISA reader. As shown in Fig. 2, cells treated with rhamnolipids 222A or 222B at or above 0.05% were less than 10% viable compared to the mock-treated cells. Cells treated with rhamnolipids 222A at 0.01% and 0.009% were about 75% viable compared to the mock-treated control, respectively. Likewise, Cells treated with rhamnolipids 222B at 0.001% and 0.009% were about 50% and 70% viable compared to the mock-treated control, respectively. In contrast, cells treated with rhamnolipids 222A or 222B at or below 0.005% had similar viability as the mock-treated cells. Therefore, rhamnolipids at 0.005% were considered as biologically safe in this study.

The effect of rhamnolipid treatment on HSV-1 and Coronavirus (BCoV) replication *in vitro*

To determine whether rhamnolipids have anti-viral effects specific to enveloped viruses, about 2X10⁶ PFU HSV-1 was diluted in PBS containing 0.009% or 0.001% of 222B or 222A first, then inoculated to a 12-well plate seeded with Vero cells, and cultured in the presence or absence of rhamnolipids. Fig.3 shows the

cells infected with HSV-1 in the presence or absence of rhamnolipids. In HSV-1 infected wells without rhamnolipid, 90% cytopathic effect (CPE) was observed at 24 hour post-infection (hpi) (Fig. 3). In the presence of 0.001% 222A or 222B, 80- 90% CPE was observed, which was similar to those seen in HSV-1 control infection at 24 hpi.

However, no or less CPE was observed at 24 hpi in the presence of 0.009% 222B or 222A (HSV-1+222B or 222A). Since HSV-1 infection in the presence of 0.009% 222B did not produce any visible CPE at 24hpi, 222B is selected for the remaining study.

To prove the rhamnolipid's anti-viral activity is not HSV-1 specific, but specific to enveloped viruses, 222B was tested against bovine coronaviruses (BCoV). Hrt-18G cells in a 12-well plate were infected with BCoV at $\sim 1 \times 10^6$ PFU/well in the presence of 0.009% or 0.001% of 222B. At 72 hpi, BCoV CPEs, such as cell morphology change, presence of inclusion bodies, were visible in wells infected with BCoV in the presence of 0.001% 222B and BCoV only wells (Fig. 4A).

However, no CPE was visible in wells infected with BCoV in the presence of 222B at 0.009%. To further demonstrate the replication of BCoV, infected cells were stained with FITC labeled mAb specific to BCoV at 72h post-infection. As shown in Fig.4B, positive FITC staining was visible in BCoV only wells and wells infected with BCoV containing 0.001% 222B. In contrast, little FITC staining was visible in wells infected with BCoV containing 0.009% 222B. Noticeably, the BCoV only wells had significantly more positive BCoV staining than those infected in the presence of 0.001% 222B, suggesting that 222B even at 0.001% still had damaging effects on this virus.

Comparison of different surfactants against an enveloped virus, HSV-1

The above study demonstrated that rhamnolipids have antiviral activity toward the enveloped viruses that we tested. It suggests that its mechanism of action is specific to viral envelopes. To prove that viral

envelopes are common targets of surfactants, different surfactants were tested against HSV-1 infection. An ionic surfactant, sodium dodecyl sulfate (SDS), and a non-ionic surfactant, such as Triton X-100, were examined in this study. We used HSV-1 with GFP expression to visualize the replication of HSV-1 directly. Vero cells seeded in 12 well plates were infected with $\sim 1 \times 10^5$ PFU/well (i. e., 105 virions per well) in the presence of SDS, triton X-100, and 222B, respectively. The final concentration for each reagent was set at 0.009%. At 24h hpi, neither HSV-1 CPE, nor GFP expression was observed in the presence of SDS or Triton X-100, or 222B (Fig. 5). Cells infected with HSV-1 looked normal in the presence of 222B. However, 50% and 90% of Vero cells were detached in wells treated with SDS and Triton X-100, respectively.

HSV-1 CPE and green GFP expression were only observed in HSV-1 only infected wells. This proves all these reagents can inactivate the enveloped viruses.

Removal of HSV-1 envelopes by surfactants

To visualize the effect of surfactants on viral envelopes directly, HSV-1 virions were treated with different surfactants and examined under the transmission electron microscope (TEM). HSV-1 virions isolated from both tissue culture supernatant and cell lysates were suspended in H₂O containing 0.009% of 222B, SDS, and Triton X-100, respectively, before TEM negative staining. Negative staining is a unique technique of “negative contrast” staining, the contrast is not applied to the object but its environment, using heavy metal salts, such as phosphotungstic acid (PTA). The electron beam can cross biological material easier than the surrounding space. The heavy metal staining forms a dark contrast around viral particles (virions). The enveloped viruses or complete virions are enclosed by phospholipid bilayers (Fig. 6, white arrow) surrounding the capsid (Fig. 6, black arrow). In the untreated viral samples, enveloped virions were visible in each view (Fig. 6A, white arrows).

However, no enveloped virion was visible in samples treated with 0.009% of 222B (Fig. 6B). Likewise, fewer enveloped virions were seen in samples treated with 0.009% of SDS (Fig. 6C) or Triton X-100 (Fig. 6D). This suggests that envelopes were dispersed or removed in the presence of surfactants. Viral proteins on the envelope are essential for the initial infection step. If the viruses lose their envelopes, they will not be able to bind to the host receptors, consequently, lose their infectivity. To prove that viral envelopes or viral enveloped proteins were lost once in contact with rhamnolipids, 100 µl of HSV-1 viruses at ~1X10⁶ PFU/ml were mixed with either 0.9 ml of 0.01% of 222B or PBS alone briefly by vortexing. Afterward, 100 µl of the diluted HSV-1 were inoculated to a 12-well plate seeded with ~1X10⁵ Vero cells.

Following virus inoculation, the cells were cultured in media either without 222B or with 222B. Fig. 7 shows the cells infected with HSV-1 that was diluted with 222B but cultured with media without 222B (HSV-1=222B-pre) or infected with HSV-1 without 222B but cultured in 222B media (HSV-1+222B-post). No CPE was observed in cells infected with HSV-1 that was diluted with 0.009% 222B. On the other hand, 80-90 CPE was observed in cells infected with HSV-1 diluted only in PBS, but cultured with media containing 0.009% 222B. Likewise, GFP HSV-1 was observed in cells infected with HSV-1 diluted only in PBS, but cultured with media

containing 0.009% 222B (Fig. 7, HSV-1+222B-post). This further demonstrated that rhamnolipids 222B acted directly on the surface of viral envelopes.

Plaque reduction assay with 22B treatment before infection

To determine the potency of 222B against enveloped viruses, different concentrations of HSV-1 were treated with different concentrations of 222B before inoculation. The contact between HSV-1 envelopes and rhamnolipids took place when HSV-1 was diluted to different concentrations of 222B in PBS. Therefore, the interaction was within 5 min. The treated viruses were then titrated immediately by standard plaque assay. As shown in Fig. 8, 222B at 0.009% completely inactivated ~2X10⁶ PFU HSV-1 infections following brief treatment before viral inoculation. Similarly, 222B at 0.0045% reduced the input virus titer from ~2X10⁶ PFU to ~2X10² PFU, ~2X10⁵ PFU to 10-20 PFU, ~2X10⁴ PFU to a few PFU, respectively. 222 B at 0.001% produced about 10-fold reduction. 222B at a concentration below 0.001%

has little or no antiviral activity left (data not shown here). These results demonstrate that 222B at 0.0045% and 0.009% are capable of killing $\sim 1 \times 10^4$ and $\sim 1 \times 10^6$ PFU of enveloped viruses, respectively.

Contact inhibition against HSV-1 on 222B coated plastic surface

If a biologically safe antiviral product remains active on a coated surface, we could use it to create a physical barrier to prevent the spread of respiratory viruses, such as SARS-CoV2. To this end, 222B anti-HSV-1 activity was investigated on a 222B coated plastic surface. The well surface of a 48-well plate, $\sim 1 \text{ cm}^2$, was coated with 20 μl of 222B at 0.005%. Droplets in 10 μl containing $\sim 2 \times 10^4$ or $\sim 2 \times 10^3$ PFU of HSV-1 was directly applied to the surface. The contact between the coated surface and viruses was for 1 or 5 min, and then terminated by adding 1 ml of PBS. The post-contact viruses were eluted at room temperature for 1h on a plate rocker. Fig. 9 shows the number of HSV-1 PFU detected after the contact between HSV-1 and the 222B coated surface. When $\sim 2 \times 10^4$ PFU HSV-1 per droplet was applied to the 222B coated plastic surface, around average 200 PFU per ml was detected following 1-minute contact, and about 10 PFU/per ml on average was detected following 5-minute contact (Fig. 9A). Likewise, when 2×10^3 PFU per droplet was applied to the 222B coated plastic surface, about 30 PFU/ml on average were detected in the elution media following 1-min contact, no PFU was detected following 5-min contact (Fig. 9B). On the other hand, when $\sim 2 \times 10^4$ or $\sim 2 \times 10^3$ PFU HSV-1 in 10 μl was added to the PBS coated surface, similar numbers of PFU were recovered as the input viruses in the mock-treated control. Similarly, in the non-contact control, where 2×10^4 or 2×10^3 PFU HSV-1 (in 10 μl) was added directly to the elution buffer without contacting the 222B coated surface directly, only 10-fold reduction was observed compared to the mock-treated control and PBS control.

Contact inhibition against HSV-1 on 222B coated fabric surface

To determine whether 222B on the coated surgical mask can inactivate the enveloped viruses, about 1- cm^2 mask fabrics were coated with 50-100 μl of 0.005% 222B. The viral droplets in 10 μl were applied directly to the 222B coated mask fabric for 3-5 min. The interaction between viruses and 222B on the fabric surface was stopped by adding 1ml PBS, which diluted 222B to a concentration that has little or lacks antiviral activity. When 1.45×10^4 PFU per droplet were applied to the 222B coated mask fabric, less than 10 PFU/ml on average were detected in the elution media (Fig. 10 A).

Similarly, when 1.45×10^3 PFU per droplet were applied to the 222B mask fabric, no viral plaques were detected in the elution media (Fig. 10B). In contrast, when 1.45×10^4 PFU per droplet were applied to the elution buffer without contacting the 222B coated fabric directly, about 2.9×10^3 PFU were detected in the elution media (Fig. 10A). Likewise, when 1.45×10^3 PFU per droplet were applied to the elution buffer without contacting the 222B coated fabric directly, about 2.2×10^2 PFU were detected in the elution media (Fig. 10B). As seen in the 222B coated on the plastic surface, there was also a 10-fold reduction in the non-direct contact treatment compared to the mock-treated control and PBS control. To PBS coated fabric, about 2.2×10^3 PFU/ml and 2.2×10^2 PFU/ml were detected in the elution media with input viruses at 1.45×10^4 PFU and 1.45×10^3 PFU per droplet, respectively (Fig. 10). There was a little change in viral titer in the mock-treated control between input virus and post-contacting viral titer (Fig. 10). These results

demonstrated that 0.005% of 222B coated on the mask is capable of inactivating about 103PFU enveloped viruses per cm².

Discussion

The above study demonstrated that rhamnolipids could inactivate enveloped viruses by targeting the envelopes. Viral envelopes are derived from cell membranes. Given that cell membranes are made of the phospholipid bilayer, we sought to examine whether rhamnolipids are toxic to the cells by XTT viability assay. The assay is based on the ability of mitochondria enzyme from metabolically active cells to reduce the tetrazolium salt XTT to orange-colored formazan compounds. The higher the number of active cells is, the higher the color intensity is.

Unsurprisingly, rhamnolipids at or above 0.05% were toxic to the treated cells, and few cells were left following 1h exposure (Fig. 1). Cells treated with 0.1% or 0.05% rhamnolipids had less than 10% viable cells left following 1h exposure. When cells were exposed to 0.01% and 0.009%, around 50% and 70 % of cells were viable, respectively. Although, cells treated with 0.009% rhamnolipid looks similar to the untreated cells under the microscope (Fig. 1). However, when cells were treated with rhamnolipid at or below 0.005%, little or no viability loss was observed (Fig. 2). Therefore, rhamnolipids at or below 0.005% are completely biological safe within 1h exposure.

In this study, 222B, the predominant monorhamnolipids, were found to have better anti-HSV-1 activity than that of predominant dirhamnolipid, 222A (Fig. 3). Cells infected with HSV-1 in the presence of 0.009% 222B had less CPE than those in the presence of 0.009% 222A. In line with this observation, a higher viral titer was produced in the presence of 222A than that in the presence of 222B (data not shown). The difference between 222A and 222B could be the difference between the extra rhamnose sugar group (rha) in the 222A, which may interfere with the interaction between the lipid tail with the phospholipid bilayers of envelopes. Therefore, the monorhamnolipids are better anti-enveloped virus agents.

Surfactants have antiviral envelope properties as they causes envelopes to disintegrate when their hydrophobic tails come to contact with phospholipid bilayers. When enveloped viruses lose their membrane, they lose their ability to infect. Both SDS (an ionic surfactant) and Triton X-100 (a non-ionic surfactant) are surfactants commonly used for research. To prove envelopes are common targets of surfactants, the effect of SDS and Triton X-100 on viral envelopes was compared with rhamnolipids. As expected, virions treated with SDS, Triton X-100, and 222B, all had very few complete virions with envelopes left under TEM examination, while the untreated virions had complete virions with envelope in every view (Fig. 6). Although non-enveloped viruses were also visible in the untreated sample, this is because virions were isolated from both tissue culture supernatant and cell lysates. There are normally many incomplete virions inside of the infected cells, which lack envelopes. Only a small portion of virions are fully mature and enveloped. Here, to prove the principle of the action, the mixed virion preps were enough for us to tell the difference between treated and untreated virions. To support what we observed in TEM, we examined HSV-1 infection that was treated with 0.009% of 222B before infection. As

expected, HSV-1 exposed to 222B before inoculation failed to infect the cells. However, untreated HSV-1 was able to infect cells and produced 80-90% CPE at 24hpi, although the infection was cultured with media contains 0.009% 222B (Fig. 7). Both studies confirmed the mechanism action of rhamnolipids against enveloped viruses is specific to envelopes. The surfactant has little effect on virus replication inside of the infected cells.

A HSV-1 plaque reduction assay was used to evaluate the rhamnolipid anti-enveloped virus activity. This assay takes advantage of the observation that HSV-1 infections form large visible plaques at 4-5 days post-infection on cell monolayers (Eide et al., 2010). The number of plaques formed corresponds directly to the number of input infectious viral particles. Therefore, plaque-forming units (PFU) are used to quantify the infectious viral particles. In a 0.009% of 222B solution, $\sim 2 \times 10^6$ PFU (or virions) per 100 μl can be inactivated within 2-5 min. Similarly, 0.0045% of 222B can kill $\sim 2 \times 10^4$ PFU/100 μl . However, when 222B is at or below 0.001%, it can only reduce the infectivity to less than one log of the input viruses (Fig. 8). Since BCoV does not form visible plaques in the Hrt-18G monolayer, the plaque reduction assay was not performed in this study. Nevertheless, in a 0.009% of 222B solution, almost all 1×10^6 PFU of BCoV was inactivated as we saw in HSV-1, and no BCoV antigen was detected by direct antibody staining (Fig. 4). 222B at 0.001% was able to reduce the BCoV antigen production but did not completely block BCoV infection. Therefore, the rhamnolipid anti-enveloped virus activity is dose-dependent; the higher the concentration is, the higher the killing activity is.

Although 222B at 0.009% has some cytotoxicity, it is capable of killing $\sim 1 \times 10^6$ PFU/100 μl , or $\sim 1 \times 10^7$ PFU/ml enveloped viruses. Therefore, 222B at 0.009% was used in the initial characterization of rhamnolipids against enveloped viruses. Since 222B at 0.005% has no cytotoxicity, and it would be a safe concentration to use medically against enveloped respiratory viruses, such as SARS-CoV2, SARS, Influenza viruses, common cold viruses.

Considering the worldwide involvement with the 2019 Coronavirus disease (COVID-19) pandemic where there is neither an effective vaccine nor treatment available at this time, developing an antiviral mask or barrier could help to prevent the spread of not only this virus, but others. If rhamnolipids on coated surface can kill enveloped viruses, it will allow the production of antiviral masks or shields. Hence, 222B contact killing was investigated on a $\sim 1 \text{ cm}^2$ plastic or mask fabric surface in this study. To test the plastic surface, the 48-well plate with a growth area of 0.95 cm^2 ($\sim 1 \text{ cm}^2$) was coated with a 20 μl of 222B. To test the mask surface, the surgical mask was cut into $\sim 1 \text{ cm}^2$ and then coated with 50-100 μl of 222B. We used 10 μl of viruses in the contact assay. Because enveloped viruses tend to cluster together due to the nature of their lipid surface, volumes of less than 10 μl will produce variation between replicates. Another reason is 10 μl is close to droplet size from running nose or coughing. Since 222B at 0.005% is biologically safe, we chose to coat the surface with this concentration. To our surprise, 0.005% of 222B on the plastic surface is capable of killing at least 1×10^3 PFU HSV-1/ cm^2 within 5 min contact (Fig. 9). Similarly, 0.005% of 222B on mask fabric is also capable of killing $\sim 1 \times 10^3$ PFU HSV-1/ cm^2 within 3-5 min (Fig. 10). The contact killing is higher at 5 min than 1 min. In addition, we noticed that the coated fabric surface had higher killing capacity than the plastic surface. This could contribute to the fact

that mask fabrics had higher absorption and were coated with more 222B than the plastic surface. Given the viruses were applied to the surface, then eluted in 1 ml PBS, it is not surprising to see that not all the viruses were recovered in the elution media (Fig. 10, PBS). To the plastic-coated surface, the non-contact control had a 10-fold reduction compared to the mock-treated control (Fig. 9). It is possible that there were variations during virus titration. Regardless, $\sim 1\text{cm}^2$ surface coated with 222B can inactivate $\sim 1 \times 10^3$ PFU enveloped viruses.

Respiratory viruses are common in humans and are transmitted mostly via droplets or aerosols, or body secretions, such as tears, nasal or oral discharges. The amount of viruses shed in the nasal or oral secretion can range from 10^4 to 10^7 genome copies/ml depending on the severity of the infection and the types of viruses (Finberg et al., 2019; Liu et al., 2020a). For example, Middle East respiratory syndrome coronavirus (MERS-CoV) is a highly pathogenic coronavirus, can cause 34% mortality in infected humans (Ogimi et al., 2020). Around 5×10^6 genome copies/ml can be shed in sputum from patients with severe MERS (Corman et al., 2016). Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) emerged in Wuhan, China, in late 2019, is now causing a pandemic COVID-19. It was estimated that 10^4 - 10^7 genome copies/ml were present in nasal and pharyngeal swab specimens from COVID-19 patients (Liu et al., 2020a). In a similar study, 10^4 - 10^5 genome copies /ml SARS-CoV2 were detectable in sputum from severe infected COVID 19 patients (Huang et al., 2020; Liu et al., 2020b). In the case of Influenza A virus infection, 10^4 - 10^7 virions/ml can be detected from patients with uncomplicated acute influenza A (Finberg et al., 2019). If these respiratory viruses came out in a $10\mu\text{l}$ droplet size, there would be 10^2 - 10^5 virions per droplet from an infected patient. If an infected person wears a 222B coated mask, all those viruses would be killed inside the mask. A 222B mask would reduce the amount of emission of infected saliva or droplets shed into the environment, which will stop the spread of COVID 19.

Mask wearing by itself can reduce the transmission of respiratory viruses, such as flu and SARS- CoV-2. Community-wide mask-wearing has been shown to contribute to the control of COVID- 19 by reducing the amount of emission of infected saliva and respiratory droplets from individuals with subclinical or mild COVID-19 (Cheng et al., 2020). Countries like Japan and Slovenia, where people wear masks, were able to prevent the spread of COVID quicker than those non-mask wearing countries. In addition, recent reports on mask use found that the per- capita mortality tended to increase by 43% weekly in countries where people were not wearing masks, compared with a 2.8% increase in countries where people were wearing masks (Eikenberry et al., 2020; Lai et al., 2020). Due to the current pandemic, there is a shortage of both surgical masks and N95 masks available to the public. However, even home-made cloth masks can stop aerosol ($<5\mu\text{m}$) or droplets ($>5\mu\text{m}$) containing viruses (Clase et al., 2020; Ngonghala et al., 2020). The widespread use of 222B coated masks, could put an end of the COVID 19 pandemic much sooner. Therefore, there is an urgent need that we apply rhamnolipids to masks or make 222B coated masks available to the public to stop the pandemic of COVID19.

In summary, this study demonstrated that rhamnolipids have antiviral activity against enveloped viruses. Many respiratory viruses are enveloped viruses, such as SARS-CoV2, influenza viruses. Rhamnolipids not only can kill enveloped viruses in solution, but also can destroy enveloped viruses on coated surface.

Direct contact with rhamnolipids can destroy the envelopes within 5 min. Rhamnolipids, 222B at 0.005%, are completely biologically safe and can kill $\sim 1 \times 10^4$ PFU enveloped viruses in solution and $\sim 1 \times 10^3$ PFU enveloped viruses on the coated surface. The application of rhamnolipids 222B on masks will help us to end the pandemic of COVID19.

Declarations

Acknowledgments

We thank AGAE Technologies, LLC (Corvallis, Oregon, USA) for production of rhamnolipids used in this study. We also thank Dr. George Rohrmann (Oregon State University) for helping editing this manuscript.

Competing interests

The authors declare no competing interests.

References

- Arima, K., Kakinuma, A., Tamura, G., 1968. Surfactin, a crystalline peptidelipid surfactant produced by *Bacillus subtilis*: isolation, characterization and its inhibition of fibrin clot formation. *Biochemical and biophysical research communications* 31, 488-494.
- Cheng, V.C., Wong, S.C., Chuang, V.W., So, S.Y., Chen, J.H., Sridhar, S., To, K.K., Chan, J.F., Hung, I.F., Ho, P.L., Yuen, K.Y., 2020. The role of community-wide wearing of face mask for control of coronavirus disease 2019 (COVID-19) epidemic due to SARS-CoV-2. *J Infect.*
- Clase, C.M., Fu, E.L., Joseph, M., Beale, R.C.L., Dolovich, M.B., Jardine, M., Mann, J.F.E., Pecoits-Filho, R., Winkelmayer, W.C., Carrero, J.J., 2020. Cloth Masks May Prevent Transmission of COVID-19: An Evidence-Based, Risk-Based Approach. *Annals of internal medicine.*
- Corman, V.M., Albarrak, A.M., Omrani, A.S., Albarrak, M.M., Farah, M.E., Almasri, M., Muth, D., Sieberg, A., Meyer, B., Assiri, A.M., Binger, T., Steinhagen, K., Lattwein, E., Al-Tawfiq, J., Muller, M.A., Drosten, C., Memish, Z.A., 2016. Viral Shedding and Antibody Response in 37 Patients With Middle East Respiratory Syndrome Coronavirus Infection. *Clin Infect Dis* 62, 477-483.
- Dhull, D., Sharma, V., Sharma, Y., Kaushik, S., 2019. Applicability of molecular assays for detection and typing of herpes simplex viruses in encephalitis cases. *Virusdisease* 30, 504-510.
- Eide, K., Moerdyk-Schauwecker, M., Stein, D.A., Bildfell, R., Koelle, D.M., Jin, L., 2010. Reduction of herpes simplex virus type-2 replication in cell cultures and in rodent models with peptide-conjugated morpholino oligomers. *Antiviral therapy* 15, 1141-1149.

- Eikenberry, S.E., Mancuso, M., Iboi, E., Phan, T., Eikenberry, K., Kuang, Y., Kostelich, E., Gumel, A.B., 2020. To mask or not to mask: Modeling the potential for face mask use by the general public to curtail the COVID-19 pandemic. *Infectious Disease Modelling* 5, 293-308.
- Elshikh, M., Moya-Ramirez, I., Moens, H., Roelants, S., Soetaert, W., Marchant, R., Banat, I.M., 2017. Rhamnolipids and lactonic sophorolipids: natural antimicrobial surfactants for oral hygiene. *J Appl Microbiol* 123, 1111-1123.
- Finberg, R.W., Lanno, R., Anderson, D., Fleischhackl, R., van Duijnhoven, W., Kauffman, R.S., Kosoglou, T., Vingerhoets, J., Leopold, L., 2019. Phase 2b Study of Pimodivir (JNJ-63623872) as Monotherapy or in Combination With Oseltamivir for Treatment of Acute Uncomplicated Seasonal Influenza A: TOPAZ Trial. *The Journal of infectious diseases* 219, 1026-1034.
- Gaur, V.K., Tripathi, V., Gupta, P., Dhiman, N., Regar, R.K., Gautam, K., Srivastava, J.K., Patnaik, S., Patel, D.K., Manickam, N., 2020. Rhamnolipids from *Planococcus* spp. and their mechanism of action against pathogenic bacteria. *Bioresour Technol* 307, 123206.
- Heerklotz, H., 2008. Interactions of surfactants with lipid membranes. *Q Rev Biophys* 41, 205-264.
- Hoskova, M., Jezdik, R., Schreiberova, O., Chudoba, J., Sir, M., Cejkova, A., Masak, J., Jirku, V., Rezanka, T., 2015. Structural and physiochemical characterization of rhamnolipids produced by *Acinetobacter calcoaceticus*, *Enterobacter asburiae* and *Pseudomonas aeruginosa* in single strain and mixed cultures. *J Biotechnol* 193, 45- 51.
- Huang, J.T., Ran, R.X., Lv, Z.H., Feng, L.N., Ran, C.Y., Tong, Y.Q., Li, D., Su, H.W., Zhu, C.L., Qiu, S.L., Yang, J., Xiao, M.Y., Liu, M.J., Yang, Y.T., Liu, S.M., Li, Y., 2020. Chronological Changes of Viral Shedding in Adult Inpatients with COVID-19 in Wuhan, China. *Clin Infect Dis*.
- Jahan, R., Bodratti, A.M., Tsianou, M., Alexandridis, P., 2020. Biosurfactants, natural alternatives to synthetic surfactants: Physicochemical properties and applications. *Adv Colloid Interface Sci* 275, 102061.
- Jarvis, F.G., Johnson, M.J., 1949. A glyco-lipid produced by *Pseudomonas aeruginosa*. *J. Am. Chem. Soc* 71, 4124-4126.
- Jin, L., Cebra, C.K., Baker, R.J., Mattson, D.E., Cohen, S.A., Alvarado, D.E., Rohrmann, G.F., 2007. Analysis of the genome sequence of an alpaca coronavirus. *Virology* 365, 198-203.
- Jin, L., Lohr, C.V., Vanarsdall, A.L., Baker, R.J., Moerdyk-Schauwecker, M., Levine, C., Gerlach, R.F., Cohen, S.A., Alvarado, D.E., Rohrmann, G.F., 2008. Characterization of a novel alphaherpesvirus associated with fatal infections of domestic rabbits. *Virology* 378, 13-20.
- Jin, L., Perng, G.C., Mott, K.R., Osorio, N., Naito, J., Brick, D.J., Carpenter, D., Jones, C., Wechsler, S.L., 2005. A herpes simplex virus type 1 mutant expressing a baculovirus inhibitor of apoptosis gene in place of

latency-associated transcript has a wild-type reactivation phenotype in the mouse. *Journal of virology* 79, 12286-12295.

Jones, C., 2003. Herpes simplex virus type 1 and bovine herpesvirus 1 latency. *Clinical microbiology reviews* 16, 79-95.

Kaskatepe, B., Yildiz, S., 2016. Rhamnolipid Biosurfactants Produced by *Pseudomonas* Species
BRAZILIAN ARCHIVES OF BIOLOGY AND TECHNOLOGY 59.

L'Haridon, R., Scherrer, R., Vautherot, J.F., La Bonnardiere, C., Laporte, J., Cohen, J., 1981. [Adaptation of an isolate of bovine enteric coronavirus to serial growth in cell culture and characterization of the virus (author's transl)]. *Ann Rech Vet* 12, 243-251.

Lai, C.C., Wang, C.Y., Wang, Y.H., Hsueh, S.C., Ko, W.C., Hsueh, P.R., 2020. Global epidemiology of coronavirus disease 2019 (COVID-19): disease incidence, daily cumulative index, mortality, and their association with country healthcare resources and economic status. *International journal of antimicrobial agents* 55, 105946.

Liu, W.D., Chang, S.Y., Wang, J.T., Tsai, M.J., Hung, C.C., Hsu, C.L., Chang, S.C., 2020a. Prolonged virus shedding even after seroconversion in a patient with COVID-19. *J Infect*.

Liu, Y., Chen, X., Zou, X., Luo, H., 2020b. A severe-type COVID-19 case with prolonged virus shedding. *J Formos Med Assoc*.

Madrid, F., Rubio-Bellido, M., Morillo, E., 2020. Extraction of nonylphenol, pyrene and phenanthrene from sewage sludge and composted biosolids by cyclodextrins and rhamnolipids. *Sci Total Environ* 715, 136986.

Moerdyk-Schauwecker, M., Stein, D.A., Eide, K., Blouch, R.E., Bildfell, R., Iversen, P., Jin, L., 2009. Inhibition of HSV-1 ocular infection with morpholino oligomers targeting ICP0 and ICP27. *Antiviral research* 84, 131-141.

Ngonghala, C.N., Iboi, E., Eikenberry, S., Scotch, M., MacIntyre, C.R., Bonds, M.H., Gumel, A.B., 2020. Mathematical assessment of the impact of non-pharmaceutical interventions on curtailing the 2019 novel Coronavirus. *Math Biosci* 325, 108364.

Ogimi, C., Kim, Y.J., Martin, E.T., Huh, H.J., Chiu, C.H., Englund, J.A., 2020. What's New With the Old Coronaviruses? *J Pediatric Infect Dis Soc* 9, 210-217.

Patoulas, D., Gavriiloglou, G., Kontotasios, K., Tzakri, M., Keryttopoulos, P., Koutras, C., 2017. HSV-1 Encephalitis: High Index of Clinical Suspicion, Prompt Diagnosis, and Early Therapeutic Intervention Are the Triptych of Success-Report of Two Cases and Comprehensive Review of the Literature. *Case reports in medicine* 2017, 5320839.

Radlinski, L., Rowe, S.E., Kartchner, L.B., Maile, R., Cairns, B.A., Vitko, N.P., Gode, C.J., Lachiewicz, A.M., Wolfgang, M.C., Conlon, B.P., 2017. *Pseudomonas aeruginosa* exoproducts determine antibiotic efficacy against *Staphylococcus aureus*. *PLoS Biol* 15, e2003981.

Saif, L.J., 2010. Bovine respiratory coronavirus. *Vet Clin North Am Food Anim Pract* 26, 349-364.

Satpute, S.K., Bhuyan, S.S., Pardesi, K.R., Mujumdar, S.S., Dhakephalkar, P.K., Shete, A.M., Chopade, B.A., 2010. Molecular genetics of biosurfactant synthesis in microorganisms. *Adv Exp Med Biol* 672, 14-41.

Twigg, M.S., Tripathi, L., Zompra, A., Salek, K., Irorere, V.U., Gutierrez, T., Spyroulias, G.A., Marchant, R., Banat, I.M., 2018. Identification and characterisation of short chain rhamnolipid production in a previously uninvestigated, non-pathogenic marine pseudomonad. *Appl Microbiol Biotechnol* 102, 8537-8549.

Watson, G., Xu, W., Reed, A., Babra, B., Putman, T., Wick, E., Wechsler, S.L., Rohrmann, G.F., Jin, L., 2012. Sequence and comparative analysis of the genome of HSV-1 strain McKrae. *Virology* 433, 528-537.

Figures

Figure 1

Bright light images of Vero cells treated with rhamnolipids at different concentrations. The concentration of rhamnolipids is labeled above the image. The images were captured at 24h post-treatment. The bright light images were captured at 10X magnification.

Figure 2

Rhamnolipid cytotoxicity assay in vitro. Vero cells were incubated with the indicated concentration of rhamnolipids in PBS for 1 h. The treatment was then removed, and the cells were washed twice with PBS and further incubated for 24 h in DEMEM with 5% serum and antibiotics as described in materials and methods. Cell viability was evaluated with XTT cell viability kit III (PromoKine), which measures metabolic activity based on the extracellular reduction of XTT by NADH produced in the mitochondria, and expressed as a percentage of the mock-treated control (n = 3). A significant statistical difference from the mock-treated control is marked with **p < 0.001 (two-way ANOVA with Bonferroni post-test). The solid bar and open bar represents the percentage of cell viability following treatment of 222B and 222A, respectively.

Figure 3

HSV-1 infected Vero cells in the presence or absence of rhamnolipids. The concentration of rhamnolipids is indicated on the left of the images. HSV-1 infection with 222A or 222B is labeled above the image as HSV-1+222A or HSV-1+222B. Vero cells were infected with $\sim 2 \times 10^6$ FPU/well, and the images were taken at 24hpi at 10X magnification.

Figure 4

Bright light images and direct immunofluorescence staining of BCoV infected cells in the presence and absence of 222B. A: Hrt18G cells infected with ~10⁶ PFU of BCoV per well in a 12-well plate. The concentration of 222B is labeled above the images in BCoV infection. The bright light images were captured at 72hpi at 20X magnification. B: Hrt18G cells at 72hpi were stained with 1:40 diluted BCoV antibody conjugated with FITC and viewed under the fluorescence microscope. Fluorescent images were captured at 10X magnification.

Figure 5

Bright light (A) and fluorescence (B) images of Vero cells infected with GFP-HSV-1. GFP-HSV-1 was inoculated in the presence of SDS, Triton X-100, 222B, and HSV-1 only, respectively. All surfactants included in the HSV-1 infection media were made at 0.009%. A: Fluorescent images were captured at 20X magnification. B. The bright light image was captured at 10X magnification.

Figure 6

Electron micrograph images of HSV-1 virions treated with different surfactants. The images represent HSV-1 virions without treatment (A), virions treated with 0.009% of 222B (B), 0.009% of SDS (C), and 0.009% of Triton X-100 (D), respectively. The black arrow indicates the capsid of virions. The white arrow indicates the envelope of the virion. Scale bar=0.5 μ m

Figure 7

Bright light (A) and fluorescence (B) images of GFP-HSV-1 infected cells in the presence or absence of 222B. “HSV-1+222B-pre” represents cells infected with HSV-1 that were diluted with PBS containing 0.009% 222B, and cultured in a media without 222B. “HSV-1+222B-post” represents cells infected with HSV-1 that were diluted with PBS only and cultured in a media containing 0.009% 222B. Both bright light and fluorescent images were captured at 10X magnification.

Figure 8

Plaque reduction results from cells infected with 222B treated HSV-1 before infection. HSV-1 stock at $\sim 2 \times 10^7$ PFU/ml was diluted to $\sim 2 \times 10^6$ PFU/ml (solid bar), $\sim 2 \times 10^5$ PFU/ml (open bar), $\sim 2 \times 10^4$ PFU/ml (grey bar), $\sim 2 \times 10^3$ PFU/ml (stripped bar) in PBS containing 222B at 0.009%, or 0.005%, or 0.001% in the final concentration, respectively. The diluted viruses were then titrated in a 12-well plate seeded with Vero cells. Plaque formation was quantified at 3–4 dpi and expressed as average PFU post 222B treatment ($n=3$).

A:

B:

Figure 9

Contact inhibition against HSV-1 on 222B coated plastic surface. A: 222B contacts with input viruses at $\sim 2 \times 10^4$ PFU per well. B: 222B contacts with input viruses at $\sim 2 \times 10^3$ PFU per well. The X-axis stands for contacts between coated surface and HSV-1; the Y-axis represents the number of PFU post-contact with the coated surface. The checked bar stands for direct contact between 222B and HSV-1 for 1 min (222B-1mn); the solid bar stands for direct contact between 222B and HSV-1 for 5 min (222B-5min); the open bar stands for the no direct contact between HSV-1 and 222B coated surface (222B-NC); the striped bar stands for direct contact between PBS coated surface and HSV-1 for 5 min (PBS); and the gray bar stands for HSV-1 diluted in PBS directly (Mock-treated). Plaque formation was quantified at 3–4 dpi and expressed as average PFU post 222B treatment (n=3).

Figure 10

Contact inhibition against HSV-1 on a 222B coated fabric surface. A: 222B contacts with input viruses at $\sim 1.45 \times 10^4$ PFU per $\sim 1 \text{ cm}^2$ fabric. B: 222B contacts with input viruses at $\sim 1.45 \times 10^3$ PFU per $\sim 1 \text{ cm}^2$ fabric. The X-axis stands for contacts between coated surfaces and HSV-1; the Y-axis represents the number of PFU post-contact with the coated fabrics. The solid bar stands for direct contact between 222B and HSV-1 (222B); the open bar stands for the no direct contact between HSV-1 and 222B (222B-NC); striped bar stands for direct contact between PBS coated surface and HSV-1 (PBS), and the gray bar stands for HSV-1 diluted in PBS directly (Mock-treated). Plaque formation was quantified at 3–4 dpi and expressed as average PFU post 222B treatment (n=3).