Antiviral Nanostructures That Reduce the Viability of Coronaviruses SARS-CoV-2 and HCoV-NL63

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Abstract

Background: The rapid emergence and global spread of the COVID-19 causing Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2) and its subsequent mutated strains, has caused unprecedented health, economic and social devastation. Respiratory viruses can be transmitted through both direct and indirect channels, including aerosol respiratory droplets and contamination of inanimate surfaces. Current methods of virus inactivation on surfaces include chemicals and biocides and while effective, continuous, and repetitive cleaning of all surfaces is not always viable. Recent work in the field of biomaterials engineering has established the antibacterial effects of hydrothermally synthesised TiO_2 nanostructured surfaces against both Gram-negative and positive bacteria. This study investigates the effectiveness of TiO_2 nanostructured surfaces against two human coronaviruses: SARS-CoV-2 and HCoV-NL63 for surface-based inactivation.

Results: Results show that structured surfaces reduced live infectious viral loads of SARS-CoV-2 and HCoV-NL63 by 5 log and 3 log, respectively after 5 hours compared to non-structured surfaces. Interestingly, infectious virus remained present on the control plastic surface after 7 hours exposure.

Conclusions: These encouraging results establish the potential use of nanostructured surfaces to reduce the transmission and spread of coronaviruses, by reducing the virus' infectious period on a surface. The dual antiviral and antibacterial properties of these surfaces give them potential application in high-risk environments such as hospitals and healthcare settings.

Background

Coronaviruses are positive-stranded RNA viruses, with a genome consisting of major structural proteins in the 5' to 3' order, including spike (S), envelope (E), membrane (M) and nucleocapsid (N) proteins (1, 2). Seasonal coronaviruses, HCoV-NL63, HKU1, HCoV-229E and OC43 are a common cause of paediatric respiratory infections (2–4). In the last two decades, various pandemic coronaviruses have emerged, including SARS-CoV in 2002 (10% mortality rate) and Middle East respiratory syndrome coronavirus (MERS-CoV) in 2012 (37% mortality rate), and have caused wide-spread infection and death, amongst both children and adults (5, 6).

The emergence and rapid spread of SARS-CoV-2, which causes the disease known as COVID-19, has caused global social, economic, tourism and healthcare devastation since its detection in late 2019 (7). To contain the spread, governments have imposed city and nation-wide lockdowns, closed both domestic and international borders, implemented mask-wearing mandates and imposed quarantine measures on international and domestic arrivals. While multiple vaccines have been developed to minimize the risk of severe infection and hospitalisation of COVID-19, there are currently no specific antiviral preventative or curative treatments for coronavirus infections (6, 8). Therefore, engineering surface and aerosol transmission controls remain an essential part of controlling infection spread. In addition, beyond the

current crisis, it can be expected that new viruses will emerge with pandemic potential that will require rapid surface inactivation to control transmission.

Studies show that SARS-CoV-2 is transmitted through a number of avenues (9), with surface transmission responsible for approximately 25% of deaths in lockdown (10). Surface transmission is caused when infected respiratory droplets (between 5 – 10 μm) and aerosols, generated when an infected person coughs or sneezes (11–13), land on and contaminate a surface or inanimate object (14). Subsequent users then contact that surface, transferring the virus to their hands, where infection can occur after touching their eyes, nose or mouth. Studies suggest that transmission of SARS-CoV-2 and seasonal coronaviruses, such as HCoV-NL63, is due to their ability to survive and remain infectious on different surfaces for long periods of time at room temperature (2, 15, 16). For example, SARS-CoV-2 can remain infectious on stainless steel for 3 – 4 days (17) and on smooth surfaces for 7 days (18). SARS-CoV-2 RNA has been detected in hospital rooms, with the most contaminated surfaces found to be the floor, electrical switches, chairs, and toilet seats and flush buttons (19). Recommended disinfection methods for surfaces include ethanol, bleach and peroxide, however these methods do not provide on-going protection (11), and given the rapid evaporation of alcohol-based solutions, surfaces can become reinfected within minutes. In addition, constant cleaning and disinfection can be expensive and time-consuming.

Recent advances in nanotechnology, namely nanomaterials and nanoparticles offer potential solutions for surface transmission. Some nanomaterials can be used to capture and inactivate or inhibit the replication or entry of the virus into human cells thus preventing infection (5). Some antiviral agents such as copper, silver nanoparticles, nanocarbons, zinc and polyethyleneimine are used in personal protective equipment (PPE) such as face masks, immunodiagnostic assays, drug administration and vaccines (5, 8, 20–24).

This work investigates the antiviral properties of previously established antibacterial TiO_2 nanostructured surfaces (25–29) against human coronaviruses SARS-CoV-2 and HCoV-NL63, as a method of viral inactivation. The aim of this study is to develop inherently antiviral surfaces which deactivate coronavirus particles without the need for chemical disinfectants. This research is a step towards installation and implementation of these surfaces in high traffic or highly touched areas to reduce the transmission and infection of coronaviruses such as SARS-CoV-2 through communities.

Results

Figure 1 shows SEM images of TiO_2 nanostructures formed via hydrothermal synthesis. General morphology of the surfaces shows that structures are random in nature, with no consistent orientation angle or uniform pattern. Average structure diameter was measured to be approximately 20 nm in diameter and 300 nm in height, using in-built JEOL software. Previously reported characterisation of hydrothermally synthesised TiO_2 is shown in Table 1, which shows surfaces are hydrophilic in nature. In

addition, it was found that structures remained mechanically stable, with no significant change in mechanical properties after 6 months (27).

Table 1
Characterisation of hydrothermally nanostructured TiO2

Property	Value	Reference
Contact Angle	14.3° (hydrophilic)	(25)
Elastic Modulus	12.2 GPa	(26)
Hardness	14.7 MPa	(26)

The results of the $TCID_{50}$ assays (Figure 2) show significant antiviral properties of nanostructured TiO_2 against both SARS-CoV-2 and HCoV-NL63. Nanostructured TiO_2 surfaces reduced live infectious SARS-CoV-2 by 3 log from the initial viral titre (1 $x10^6$ $TCID_{50}$/mL) after 2 hours and 5 log after 5 and 7 hours (Figure 2a). In contrast, polished Ti-4Al-6V control surfaces showed a 1 and 2 log reduction after 2 and 5 hours respectively, with no significant reduction after 7 hours. Similarly, virus exposed to tissue culture plastic showed 1 and 2 log reductions after 2 and 5 hours of exposure respectively, with no further significant reduction at 7 hours. While viable virus was still detected in small amounts after 7 hours exposure to nanostructured surfaces, this amount was significantly lower than both tissue culture plastic and polished Ti-6AL-4V controls.

HCoV-NL63 $TCID_{50}$ assay results (Figure 2b) show similar trends to SARS-CoV-2 $TCID_{50}$ test results (Figure 2a). Infectious HCoV-NL63 was significantly reduced by exposure to nanostructured TiO_2 for 2, 5 and 7 hours (2, 3 and 3 log reductions, respectively), whereas, both tissue culture plastic and polished Ti-6Al-4V produced a maximum of 1.6 log reduction over 7 hours. Interestingly, the virucidal activity of TiO_2 was greater against SARS-CoV-2 than HCoV-NL63 when compared to smooth Ti-6Al-4V surfaces, resulting in a larger reduction in live infectious virus at 7 hours post-exposure. While viral infectious dose decreased on all surfaces after 7 hours, nanostructured TiO_2 significantly accelerated this activity at all timepoints.

Discussion

This study tests the antiviral properties of nanostructured TiO_2 surfaces against human coronaviruses and reports encouraging results regarding a significant reduction in the recovery of live infectious coronaviruses SARS-CoV-2 and HCoV-NL63. We have previously reported that several viruses, including SARS-CoV-2, human rhinovirus (HRV) and respiratory syncytial virus (RSV) demonstrate reduced infectivity following exposure to aluminium surfaces treated with wet-etching (30, 31). This study is a novel finding for nanostructured TiO_2 surfaces and adds critical evidence that etched nanostructures can

be applied to different metals to produce antiviral surfaces that may reduce the risk of transmission. Regarding self-cleaning antiviral surfaces to date, most effort has been invested in studying silver, copper and polymer-based nanomaterials (5, 8, 20–24). However, these materials are not always cheap or suitable for application that require durability. Therefore, a more generic method for generating antiviral surfaces on a range of metals may be more suitable.

The TiO_2 surfaces produced in this study are mechanically stable and have proved effective against Gram-positive *Staphylococcus aureus* and Gram-negative *Pseudomonas aeruginosa* bacteria, reducing bacterial viability within 3 hours (25–27). The exact mechanism by which nanostructures reduce infectivity of viruses is not known. However, a significant amount of research exists investigating mechanisms of action against bacteria (32). One proposed mechanism of bacterial killing is via a biophysical model of interaction, in which the bacterial cell walls are stretched and disfigured in the regions suspended between two nanopillars, causing tearing and subsequent cell death (33). However, recently this hypothesis has been challenged, with computational modelling finding that nanopillar tips create critical sites at the pillar apex, causing strains that lead to local rupture and penetration of the cells (34).

Coronaviruses are enveloped viruses and so it may be postulated that the viral envelope, which is a lipid bilayer derived from the host cell membrane, is disturbed, torn or ruptured by the surface roughness of nanostructured surfaces. However, while similarities between bacterial and viral deactivation may exist, direct parallels cannot be drawn due to size and structural differences between bacterial cell and viral particle structure. In addition to having a lipid envelope with glycosylated surface attachment protein, and no peptidoglycan cell wall like bacteria, coronavirus particles are 118 – 140 nm in size, much smaller than bacterial cells (35). We are currently investigating the nature of this viral deactivation mechanism via nanostructured surfaces at a sub-cell level through coarse-grained molecular dynamic modelling methods.

Another possible explanation for the reduced infectious dose of SARS-CoV-2 and HCoV-NL63 is that the highly rough and hydrophilic nature of the TiO_2 nanostructured surface caused viral particles to become trapped through irreversible adsorption onto the surface. This phenomenon would result in less virus being recovered from the surface and therefore reduced infectivity of the recovered viral suspension (11) as seen in this study. This theory suggests the idea that nanostructured surfaces may entrap virus particles instead of deactivating them through physical deformation.

Some studies suggest that nanostructure size and surface charge may interact with the spike protein to block the initiation of viral infection (5) and disrupt infectivity (8), thereby producing a reduction in infectious dose as found in this study. Some studies also suggest that hydrophilic surfaces, such as the nanostructured TiO_2 surface tested here (with contact angle of 14.3°), may facilitate the inactivation of viruses due the increased contact area between the surface and the infected droplet (11, 36, 37). The interpretation of the data that suggest nanostructures affected the infectivity of SARS-CoV-2 more than HCoV-NL63 needs to be carefully considered, as the $TCID_{50}$ assays for these two viruses were performed

using different cell lines, which may influence viral attachment and entry, and thus the sensitivity of the assay.

Although the exact mechanism of action of nanostructures against viruses is yet to be elucidated, this study and others demonstrate the efficacy of this approach to the manufacture of antiviral surfaces. Most importantly, these nanostructures are effective against several coronaviruses and other viruses, both enveloped and non-enveloped, such as HRV. Therefore, nanostructures surfaces are an ideal strategy for inclusion in the arsenal required to protect humans from future emerging viruses for which vaccines and antiviral treatments may not be immediately available or may not be completely protective. Despite excellent progress in the development of vaccines against SARS-CoV-2, it is not apparent that public health measures and engineered transmission controls are still required to reduce the risk of exposure to the virus (38). Thus, it is essential to continue studies such as this to develop effective antiviral surface treatments.

Conclusions

We have shown that exposure to previously established antibacterial nanostructured TiO_2 surfaces significantly reduce the infectivity of human coronaviruses SARS-CoV-2 and HCoV-NL63 within 2 hours. These results provide evidence that nanostructured surfaces could reduce the transmission and spread of respiratory coronaviruses. In addition, the proven antibacterial properties of these surfaces position nanostructured materials as a potential solution to wider pathogen transmission control. Adoption of these surfaces in various high-risk settings such as hospital and healthcare environments, transportation hubs and community centres could reduce the spread of both viruses and bacteria through the community. Further investigation into the antiviral behaviour and deactivation mechanism caused by the nanostructured surfaces will allow optimisation and enhancement of this effect and will be fundamental to designing targeted antiviral surfaces for reducing the spread of viral infections.

Methods

The aim of this study is to investigate the antiviral properties of hydrothermally synthesised TiO_2 nanostructured surfaces against two human coronaviruses, SARS-CoV-2 and HCoV-NL63. Viral infectivity was tested using the $TCID_{50}$ assay, with both polished Ti-6Al-4V and tissue culture plastic control surfaces.

Nanostructure Fabrication

Ti-6Al-4V (medical grade 5) was polished to a 0.04 μm surface roughness (mirror shine), sonicated in acetone for 10 minutes and rinsed thoroughly with 18.2 MΩ H_2O. Samples were then placed in a custom-made PTFE holder with 60 mL 1M NaOH in a 125 mL Parr acid digestion vessel at 180°C for 2 hours. After cooling to room temperature, samples were rinsed with 18.2 MΩ H_2O and dried using N_2 gas.

Samples were then annealed in a furnace for 1 hour at 300°C and once cool, submerged in 0.6 M HCl for 30 minutes. After rinsing with 18.2 MΩ H_2O, samples were lastly calcined for 2 hours at 600°C.

Surface Characterisation

Surfaces were characterised using JEOL 7001F scanning electron microscopy (SEM) to visualise nanostructure morphology at various magnifications. The SEM was operated using 15 eV and 8 mA probe current. Surfaces have previously been characterised using nanoindentation, X-ray diffraction and contact angle (25–27).

Cell Culture

VERO and Rhesus Monkey Kidney Epithelial (LLC-MK2) cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) and Reduced-Serum Medium (Opti-MEM) respectively, with 2% Fetal Calf Serum and 1% Antibiotic/Antimycotic and incubated at 37°C under 5% CO_2.

Virus Testing

SARS-CoV-2 stock (strain QLD02/2020 GISAID accession number EPI ISL 407896, kindly provided by Alyssa Pyke, Queensland Health) was propagated in VERO cells (ATCC®, C1008, CRL-1586™, Manassas, USA) as previously described (39) in DMEM, supplemented with 1 µg/mL tosyl phenylalanyl chloromethyl ketone (TPCK)–treated trypsin (Worthington Biochemical). HCoV-NL63 (Amsterdam-1 strain, kindly provided by Lia van den Hoek, University of Amsterdam) was propagated in LLC-MK2 (ATCC®, CCL-7) cells as previously described (40).

Triplicate nanostructured TiO_2, polished Ti-6Al-4V and tissue culture plastic surfaces were exposed to 10 µL of $1x10^6$ $TCID_{50}$/mL virus suspension and incubated at room temperature for 2, 5 or 7 hours. At each time point, exposed surfaces were gently washed by pipetting 60 µL DMEM (SARS-CoV-2) or Opti-MEM (HCoV-NL63) 10 times to retrieve live virus. Viral titre was calculated using the Spearman-Karber algorithm for $TCID_{50}$/mL. Three independent exposure experiments were performed.

Statistical Analysis

Statistical analysis for $TCID_{50}$ assay results were completed using two-way ANOVA Tukey's multiple comparison test. Significant results are indicated in figures, where *p<0.1, **p<0.01, ***p<0.001 and ****p<0.0001.

Declarations

Ethics approval

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The data generated or analysed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

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Figures

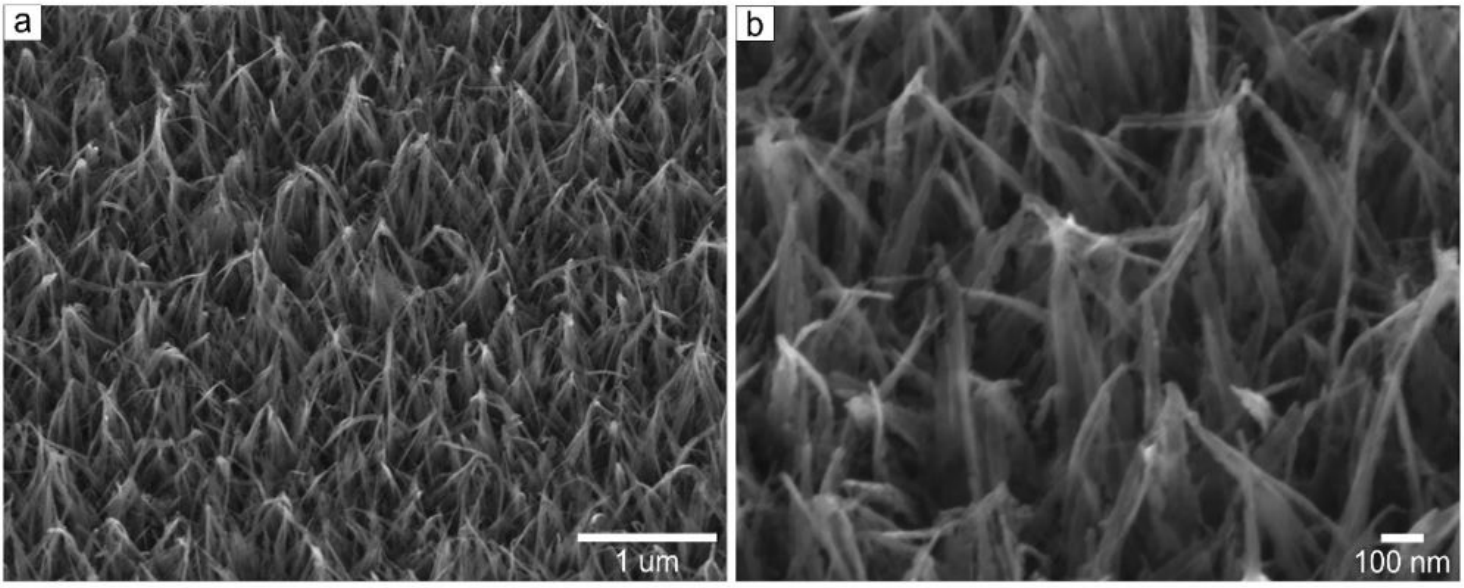

Figure 1

SEM images of hydrothermally synthesised nanostructured TiO2 surface at various magnifications.

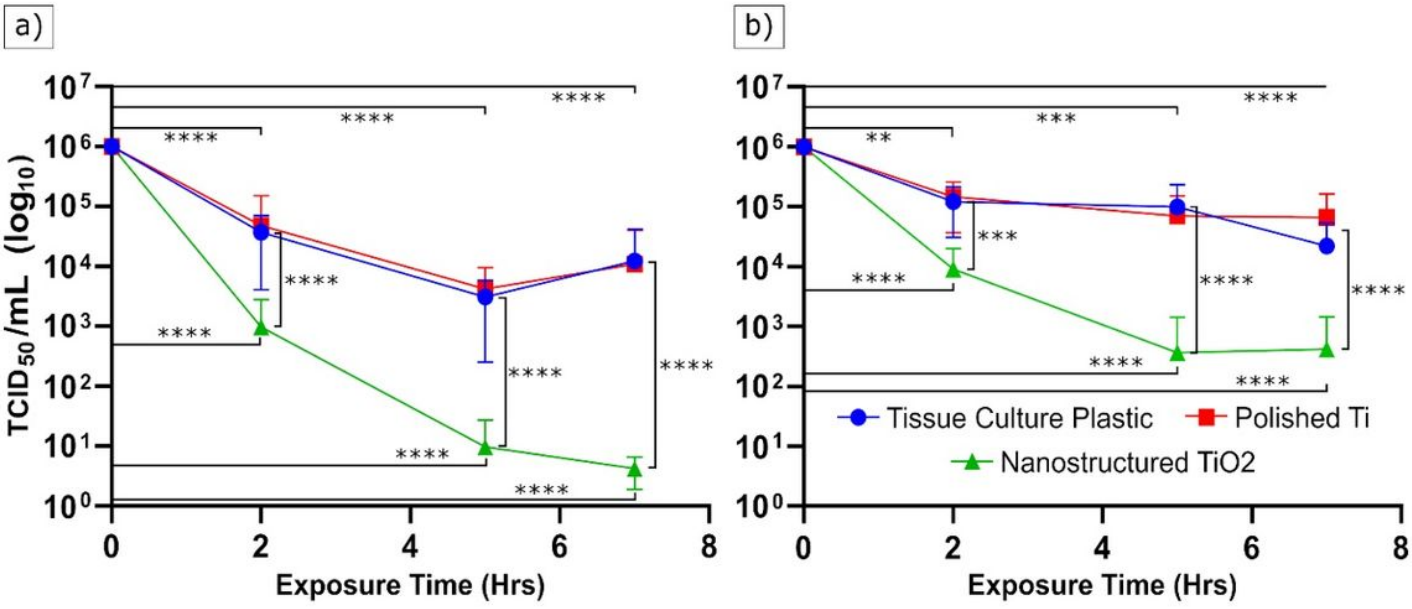

Figure 2

TCID50/mL of a) SARS-CoV-2 in VERO E6 cells and b) HCoV-NL63 in LLC-MK2 exposed to tissue culture plastic, polished Ti and nanostructured TiO2. Significant results are shown where *p<0.1, **p<0.01, ***p<0.001 and ****p<0.0001.

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