Water-Borne Nanocoating for Rapid Inactivation of SARS-CoV-2 and Other Viruses

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ABSTRACT: The rise in coronavirus variants has resulted in surges of the disease across the globe. The mutations in the spike protein on the surface of the virion membrane not only allow for greater transmission but also raise concerns about vaccine effectiveness. Preventing the spread of SARS-CoV-2, its variants, and other viruses from person to person via airborne or surface transmission requires effective inactivation of the virus. Here, we report a water-borne spray-on coating for the complete inactivation of viral particles and degradation of their RNA. Our nanoworms efficiently bind and, through subsequent large nanoscale conformational changes, rupture the viral membrane and subsequently bind and degrade its RNA. Our coating completely inactivated SARS-CoV-2 (VIC01) and an evolved SARS-CoV-2 variant of concern (B.1.1.7 (alpha)), influenza A, and a surrogate capsid pseudovirus expressing the influenza A virus attachment glycoprotein, hemagglutinin. The polygalactose functionality on the nanoworms targets the conserved S2 subunit on the SARS-CoV-2 virion surface spike glycoprotein for stronger binding, and the additional attachment of guanidine groups catalyze the degradation of its RNA genome. Coating surgical masks with our nanoworms resulted in complete inactivation of VIC01 and B.1.1.7, providing a powerful control measure for SARS-CoV-2 and its variants. Inactivation was further observed for the influenza A and an AAV-HA capsid pseudovirus, providing broad viral inactivation when using the nanoworm system. The technology described here represents an environmentally friendly coating with a proposed nanomechanical mechanism for inactivation of both enveloped and capsid viruses. The functional nanoworms can be easily modified to target viruses in future pandemics, and is compatible with large scale manufacturing processes.

KEYWORDS: SARS-CoV-2, virus inactivation, polymer nanostructures, responsive polymers, emulsion polymerization

INTRODUCTION

The COVID-19 pandemic caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has resulted in 185 million cases globally and over 4 million deaths as of March 2021. Transmission of SARS-CoV-2 occurs primarily through aerosols, liquid droplets and, consistent with other respiratory viruses such as influenza, rhinovirus, and norovirus, fomite transmission from surfaces. Variants of increased transmission and virulence have emerged with the evolution of SARS-CoV-2 during transmission either through immune pressure or natural evolution of the virus, despite global attempts at herd immunity via vaccination. Of particular concern are the alpha (B.1.1.7) and delta (B.1.617.2) variants, which originated in the United Kingdom and India, respectively. These variants contain mutations in the viral attachment spike protein that have led to a conformational change and a greater affinity for the ACE2 binding receptor on human cells for enhanced rate of infection. Therefore, there is even greater emphasis on eliminating both surface and airborne transmission to complement vaccination of the population to stop the current pandemic.

Face masks provide a physical barrier and are the first line of defense to prevent or reduce community transmission of SARS-CoV-2 via aerosols. Mask surfaces may accumulate the virus during use, and incorrect use or disposal provides a greater possibility of transmission. Antiviral coatings applied on surfaces can mitigate infection and provide long-lasting control measures to eliminate both surface and aerosolized transmission. There are now reports on the inactivation of...
SARS-CoV-2 using antiviral coatings, dealing mainly with copper-coated surfaces\textsuperscript{13,14} and silver nanoparticles\textsuperscript{15} impregnated into masks. Long-term exposure to such heavy metals may lead to deleterious health outcomes\textsuperscript{16} and microbe resistance.\textsuperscript{17} Moreover, these and other systems require the use of toxic organic solvents for coating applications. While masks have the potential for limiting aerosol transmission, fomite transmission from contaminated surfaces represents another significant infectious route that aids in SARS-CoV-2 spread.

Here, we custom designed a nanosystem that is an environmentally friendly water-based spray-on coating to inactivate infectious viruses on contaminated surfaces. The concept utilized thermo- and pH-responsive multifunctional nanoworms (Figure 1a) that were designed to change conformation on the nanoscale from collapsed (less than 100 nm) to elongated (\(\sim 1000\) nm) upon landing of the viral-loaded droplet on the surface, such as those that would be transmitted via saliva or mucosal secretions upon coughing and sneezing. We hypothesized that the conformational change of the nanoworm would enable rapid spreading of the droplet, increasing the area of potential contact between the nanoworm and virus for greater virus binding and capturing through the nanoworm-attached guanidine,\textsuperscript{18} polygalactose, and octane\textsuperscript{19–21} moieties. Inclusion of the basic groups on the nanoworms was designed to increase the pH, resulting in the collapse of the extended nanoworms achieved during the saliva or mucosal droplet exposure, and nanomechanical rupture of the viral membrane (Figure 1b). The nanoworms additionally have a covalently attached fluorescent probe for the continuous monitoring of the coating coverage on the surface, providing an indicator for coating reapplication (Figure 1c).

RESULTS AND DISCUSSION

The base nanoworms (Figure 2a) before functionalization with active components were synthesized using the temperature-directed morphology transformation (TDMT) method.\textsuperscript{24} TDMT is an emulsion polymerization technique that enables a wide range of multifunctional nanostructures to be produced directly in water and includes structures such as nanoworms\textsuperscript{24,25} tadpoles,\textsuperscript{26,27} vesicles,\textsuperscript{28} rods,\textsuperscript{24,25} toroids,\textsuperscript{29} multiarm spheres,\textsuperscript{30} and toroidal stacked nanorattles.\textsuperscript{29} Cylindrical polymer brushes have been alternatively synthesized in the literature directly in organic solvents,\textsuperscript{31,32} and anionic polymerization is required to first produce a polymer of a controlled length with functional side groups followed by

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\caption{Water-based and responsive nanocoating for disruption of SARS-CoV-2 and other viruses. (a) Decorated nanoworm in extended and collapsed conformation. (b) Illustration for the nanomechanical disruption and inactivation of viral particles; rapid droplet spreading leading to extension then collapse of nanoworms in response to change in pH. (c) Attachment of a fluorescence probe to the nanoworms for continuous monitoring of coated nanoworms on the surface at 356 nm.}
\end{figure}
either RAFT or ATRP to produce the brush polymers. Our nanoworms, which comprised the self-assembly of many block copolymers synthesized using the TDMT method (Figure 2a), utilized three functional and thermoresponsive MacroCTAs (macrochain transfer agents made by RAFT polymerization). The synthesis of all components, the MacroCTAs, and the emulsion polymerization are given in the Supporting Information. Mixing and then heating the MacroCTAs above their lower critical solution temperature (LCST) in water formed seed particles, which acted as the locus of polymerization of a styrene monomer to produce block copolymers. The resulting spherical nanostructures, when cooled in the presence of a small amount of plasticizer for polystyrene, transformed into nanoworms with a diameter of approximately 15 nm and a length distribution between 300 nm and 2 μm (Figure 2a). The R groups from the MacroCTAs that reside on the nanoworm surface were chosen to be an alkyne and a thiolactone group and can be added at any desired ratio (Figure 2a). While the thiolactone groups were not used to couple functional compounds in this study, it demonstrates the chemical versatility of our nanoworms to be further chemically modified, such as the addition of peptides to specifically target other microbial particles without changing the formulation.

The combination of three MacroCTAs enables rapid formation and subsequent stabilization of the nanoworms.
The random copolymer MacroCTA \( R_2-P(NIPAM_{49}-co-DMAEMA_{30}-S(C==S)SC_4H_9, \text{shown in Figure 2a}) \) consisted of \( N \)-isopropylacrylamide (NIPAM) and \( N,N\)-(dimethylamino)ethyl methacrylate (DMAEMA) and played a key role in constructing the four main types of nanoworms used here as antiviral coatings (Figure 2c). The incorporation of the tertiary amines from DMAEMA imparted cationic side groups and provided covalent bonding sites for further chemical functionalization of the nanoworm. The RAFT-mediated polymerization to make this MacroCTA showed that DMAEMA was initially incorporated at a significantly greater rate than NIPAM into the MacroCTA copolymer to form a gradient polymer (Figure 2b). On the basis of the determined reactivity ratios\(^{33} \) from this work \( (r_{\text{NIPAM}} = 0.37, r_{\text{DMAEMA}} = 7.58) \), the copolymer was initially rich in DMAEMA and then in NIPAM as the chain length increased. Upon formation of the nanoworms (Figure 2a), the water-soluble gradient copolymer resided as one component of the nanoworm corona, in which the DMAEMA-rich groups were located at the periphery of the corona, while the NIPAM-rich copolymer portion resided closer to the polystyrene (glassy) core. The advantage of such a gradient copolymer is nanoworm functionalization through the DMAEMA side groups with active components occurs at the periphery of the corona, thus providing the best opportunity to capture and intercalate with the viral particles, while the inner corona layer of PNIPAM responds to thermal and pH changes. The residual DMAEMA groups on the polymer further act to increase the pH upon spreading of the viral-loaded droplets and to drive the nanomechanical collapsed conformation response in combination with the thermo- and pH-responsive PNIPAM.

**Modeling Nanomechanical Action of Nanoworms.**

Receptor recognition and binding of coronaviruses to host cells relies on a complex interplay between the receptor-binding spike (S) glycoprotein S1 subunit and membrane-fusion S2 subunit on the 20 nm peplomers.\(^{34–36} \) The S1 subunit consists of three domains on the spike that act as the initial binding site for cell entry and can evolve for attachment to different cell-
surface receptors. SARS-CoV-2 is a β-coronavirus that is known to bind primarily to angiotensin-converting enzyme 2 (ACE2) on the mammalian host cell surface.37 The more conserved S2 subunit of the virion spike glycoprotein binds to sugars attached to proteins and fats on the cell surface.38 We designed our nanoworms to target this more conserved sugar-binding domain by incorporating a polygalactose (Figure 2) consisting of an oligomer of ∼10 sugar units. We hypothesized that the polygalactose attached to the nanoworm would have access to the S2 binding domain and increase binding affinity to the SARS-CoV-2 virion and its variants with similar glycoprotein binding domains regardless of mutations on the spike protein. We further reasoned that this binding affinity may be able to compete with and block binding to other biochemical structures presented by host cells, such as the sialic acids that are used as binding targets by another coronavirus surface glycoprotein known as homodimeric hemagglutinin-esterase (HE).38,39 The polygalactose presented by the nanoworm would represent a general linear oligomeric sugar that can be synthesized in a facile manner and can be scaled up. To facilitate binding to the negatively charged viral particles,19–21 positively charged nanoworms were synthesized by coupling with guanidine and through the quaternization of the DMAEMA groups (with a zeta potential of ca. +25 mV, see the Supporting Information). The second step for cell entry by the virus is fusion and intercalation between the virus and cell membranes.38 To mimic this step, we further incorporated hydrophobic octane19 groups into the nanoworm corona. Finally, the CuAAC “click” reaction between the coumarin azide and the nanoworm provides an in situ indicator to determine the amount of polymer on the surface. The fluorescence coumarin azide only becomes fluorescent upon formation of the triazol ring after the “click” reaction,39 making the nanoworms the only fluorescence component in the coating. As shown in Figure 1c (dark blue area = non-fluorescent surface, light blue area = fluorescent surface), the amount of nanoworms on the surface was accurately determined using the coumarin fluorescence probe attached to the nanoworms below 1500 mg/m². The nanoworm-coated surfaces used in this antiviral work consisted of approximately 1200 mg/m² (denoted with a red “X” in Figure 1c).

Therefore, for the purposes of this study, four functionalized nanoworms were constructed from the base-nanoworm (NWbase) (Figure 2c). One nanoworm was attached with octane (O), guanidine (G), and coumarin fluorescence probes (C), denoted as NWO,G,C and another nanoworm was coupled with an additional polygalactose (S) (NWs,O,G,C) via the copper(I)-catalyzed alkyne−azide cycloaddition (CuAAC) “click” reaction. Not all the DMAEMA units on the polymer were used in the coupling reaction, allowing the nanoworms to have a pH-responsive behavior. Hyperbranched nanoworms (HBNWs) were also constructed to significantly increase by ∼10-fold the amount of O, C, G, and/or S functional groups on the nanoworms, denoted as HBNWo,G,C and HBNws,O,G,C. A gradient polymer of NIPAM and DMAEMA, prepared by single-electron transfer living radical polymerization (SET-LRP),40–41 was “clicked” onto the base nanoworms (NWbase) to form the HBNWs (see Scheme S3) and then further coupled with O, C, G, and/or S to produce the resulting hyperbranched nanoworms (see Scheme S5 and S6). All nanoworms were extensively dialyzed, freeze-dried, and then rehydrated in water (without any change to the morphology of the nanoworms) prior to spraying on surfaces. To provide insight into role of each component, four additional nanoworms were also synthesized: NWs,O,C, NWs,G,C, and NWs,D,C (Figure 2d).

Mucosal droplets that carry viral particles generally have a low pH (∼6.5) and a wide range of salt components. Therefore, the nanoworms were tested to determine their conformational changes with temperature, pH and salt concentrations. The LCST of the PNIPAM MacroCTA (R1-P[NIPAM]16-S(C==S)SC,H2) in water was 29 °C, close to the LCST found in the literature. The LCST was found to be lower at 20 °C with the addition of PBS buffer (0.2 M) at pH 6.5, 7.4, and 8.5 (Figure 3a), being independent of pH at this salt concentration. When PNIPAM chains are tethered on a surface, the temperature-responsive nature of the polymer becomes less defined with an observed broader range of LCSTs, suggesting a density dependence for the chains tethered to the surface.42,43 Furthermore, incorporating either hydrophilic or hydrophobic comonomer into the PNIPAM chains leads to an increase and decrease in the LCST, respectively.44 We next tested the responsive nature of the functional HBNW and NW nanoworms with and without polygalactose (Figure 3b,c). It was found that the HBNWo,G,C- and HBNws,O,G,C- (i.e., without and with polygalactose) showed no change in size when the temperature was slowly raised from 3 to 70 °C in PBS at both pH of 6.5 and 7.4, suggesting that these hyperbranched nanoworms did not change conformation. In the case of the NWs without polygalactose (NWs,O,G,C), slow aggregation occurred upon heating from 3 to 40 °C at both pH values and with rapid precipitation above 40 °C. The incorporation of the polygalactose, in NWs,O,G,C, showed a similar trend to that without polygalactose (i.e., NWs,O,G,C), which had a lower precipitation temperature of 33 °C. These results support the broad change in nanoworm conformation below the bulk LCST (Figure 3a) resulting in aggregation, and at a certain temperature, depending upon chain functionality, we observe rapid precipitation.

The conformational behavior of individual fluorescent nanoworms mixed in a nonfluorescence nanoworm matrix was visualized through confocal microscopy. When a droplet of PBS buffer at pH 7.4 was added onto the surface, we observed many small aggregates of NWs,O,G,C and a few elongated nanostructures (Figure 3d). Decreasing the droplet pH to 6.5 (i.e., close to that of the mucosal droplet), the NWs,O,G,C were found to be elongated with very few aggregates (Figure 3e). These elongated nanostructures resulted from charge repulsion within the nanoworms due to protonation of the tertiary amines on the DMAEMA together with the positive charges from the guanidine groups. Confirmation of this was through the observation that a fully quarternized nanoworm showed nearly all NWs,O,G,C aligned perpendicular to the surface (Figure 3f). Taken together, these data showed little or no conformation change for the hyperbranched nanoworms (see extended confocal images in Figures S22 and S24) but quite large conformational changes for the nanoworms with and without polygalactose (NWs,O,G,C and NWs,D,G,C, respectively). The data supports the idea that only the nanoworms and not the hyperbranched nanoworms can undergo large nanoscale conformational changes.

A key design parameter for efficient capture of viral particles on surfaces is droplet spreading, which allows significantly greater interaction and virion attachment to the nanoworms.13 Droplets that cannot spread while maintaining a hemisphere geometry rely on Brownian motion of the viral particles to
interact with the polymer-coated surface and are less likely to be captured. We tested the spreading by adding 50 μL of Sorrentson’s Buffer (pH 6.5) to 1.5 cm² surfaces coated with each of the four nanoworms. It was found that after 5 min, the hyperbranched nanoworms (HBNW₅O,G,C and HBNW₅S,O,G,C) had only a small increase in spreading from 13% (uncoated surface) to ∼30%, whereas the NW₅O,G,C and NW₅S,O,G,C nanoworms revealed rapid and complete droplet spreading with 100% surface area coverage (Figure 3g).

Antiviral Activity of the Nanoworms. We next analyzed the potential antiviral properties of our functional nanoworms. Briefly, nanoworms with various combinations of octane (O), guanidine (G), and polygalactose (S) dispersed in water were spray-coated onto a hard plastic surface and then exposed to an adeno-associated virus (with a capsid protein shell) that expressed the influenza A virus attachment glycoprotein known as hemagglutinin (AAV-HA). To assess whether interaction of the AAV-HA after 30 min exposure to the nanoworm-coated surface resulted in reduced infectivity, the AAV-HA was eluted by washing, and then the eluate was added to HEK293 cells and incubated for 1 h. The cell monolayers were harvested, and the viral genome was extracted and then quantified via qRT-PCR. Our findings revealed that with the exception of the nanoworm with S alone (NW₅S,C), there was a significant reduction in genome copy number of AAV-HA compared to the untreated control (Figure 4a). The NW₅S,O,G,C coating showed the most virucidal potential but was not significantly different to samples coated with the blend of various NWs to produce the same number of functional S, O, and G groups to that of NW₅S,O,G,C. The NW₅S,O,G,C coating did cause a significantly reduced AAV-HA genome copy number compared to nanoworms with D,C and S,C alone (p < 0.05, one-way ANOVA and Dunnett’s multiple comparisons test). Taken together, these data suggest that co-locating S, O, and G on the same polymer chain provides a synergistic effect for greatest inactivation of the AAV-HA pseudovirus. We next tested the hyperbranched nanoworms (HBNW₅O,G,C and HBNW₅S,O,G,C) with significantly higher loadings of S, O, and G against AAV-HA (Figure 4b). The results for both the NWs and HBNWs caused a significant reduction in the AAV-HA genome copy number compared to the uncoated control. Of the 6 repeat samples analyzed per coating, no detectable AAV-HA genome copies were found for all eluates taken from the HBNW₅S,O,G,C. Only 1 sample had detectable copies after exposure to HBNW₅O,G,C, while 50% of the sample eluates taken from NW₅S,O,G,C did not contain detectable levels of AAV-HA.

Figure 4. (a and b) AAV-HA was exposed to each of the nanoworm-coated surfaces or uncoated control for 30 min and then washed. Eluate was then bound to HEK293 cells for 1 h to enable AAV-HA to infect, and then genome was extracted and quantified via RT-PCR. The inoculum control did not touch any surface prior to processing. ***, p < 0.01; ****, p < 0.001 compared to uncoated control using one-way ANOVA and Dunnett’s multiple comparisons test. (c and d) Nanoworm-coated plastic hard surfaces or uncoated surfaces were exposed to A/Puerto Rico/8/1930 (H1N1) influenza A virus resuspended in pH 6.5 buffer to contain ∼5 × 10⁴ infectious units (i.e., 10⁶ TCID₅₀/mL) for 30 min then washed and assayed for remaining infectious virus titer by (c) TCID₅₀ and intact virus NP gene segment by (d) qRT-PCR. ***, p < 0.001 compared to uncoated control, unpaired t test.
HA genome. Though it appeared that the hyperbranched nanoworms increased the virucidal activity against AAV-HA, the variance in sampling and detection of genome did not yield significantly different results between the HBNW and the NW coatings (p > 0.05 one-way ANOVA and Dunnett’s multiple comparisons test). To confirm the virucidal potential of our nanoworms containing S, O, G, and C against the actual enveloped influenza A virus respiratory pathogen, we next exposed A/Puerto Rico/3/1934 to surfaces coated in NW$_{S,O,G,C}$ for 30 min. After the incubation period, the surfaces were washed, and the eluate was collected. Samples were taken for the immediate quantitation of presence of infectious virus titer by one of two methods: performing a 50% tissue culture infectious dose (TCID$_{50}$) assay or extracting the viral genome followed by determining the amount of intact virus nucleoprotein (NP) gene segment present by quantitative RT-PCR. Results showed that the surfaces coated with NW$_{S,O,G,C}$ significantly reduced infectious virus titer by more than 2-log compared to the untreated surface (Figure 4c) and induced significant degradation of the virus genome as

Figure 5. Virucidal activity of nanoworm-coated surfaces against SARS-CoV-2. (a) Nanoworm-coated plastic hard surfaces or uncoated surfaces were exposed to SARS-CoV-2 isolate VIC01 for 30 min and then washed, and the eluate was assayed for infectious SARS-CoV-2 titer by TCID$_{50}$. (b) RNA extracted from eluate obtained from VIC01 was quantified via RT-PCR for SARS-CoV-2 E gene. (c) VIC17991 (B.1.1.7 variant) for 30 min, then washed and eluate assayed for infectious SARS-CoV-2 titer by TCID$_{50}$. (d) RNA extracted from eluate obtained from VIC01 then quantified via RT-PCR. (e) Nanoworm-coated surgical masks or uncoated masks were exposed to SARS-CoV-2 isolate VIC01 and VIC17991 (B.1.1.7 variant) for 30 min, then washed, and the eluate was assayed for infectious SARS-CoV-2 titer by TCID$_{50}$. (f) RNA extracted from eluate obtained from VIC01 and VIC17991 (B.1.1.7 variant) was quantified via RT-PCR for SARS-CoV-2 E gene. *, p < 0.05; **, p < 0.01; and ***, p < 0.001 compared to uncoated surface control using one-way ANOVA and Dunnett’s multiple comparisons test. Data were pooled from two independent experiments.
indicated by the reduction in the amount of viral RNA NP gene detected (Figure 4d).

Given the success of the nanoworm coatings against both a capsid pseudovirus presenting influenza A virus HA and infectious enveloped H1N1 influenza virus, we hypothesized that they would be effective against the enveloped SARS-CoV-2. Therefore, we next tested the virucidal activity of the nanoworms against SARS-CoV-2 with four coated surfaces (NW_{S,O,C}, NW_{S,O,G,C}, HBNW_{O,G,C} and HBNW_{S,O,G,C}). Using the original cultured human SARS-CoV-2 isolate (human/Victoria/01/2020; VIC01),\textsuperscript{45} virus inoculum (50 μL) prepared in Sorrenson’s buffer pH 6.5 was added dropwise onto the nanoworm-coated surface and incubated at room temperature for 30 min. Each surface was washed with 10-fold media, and the eluate was collected and assayed immediately for SARS-CoV-2 infectious titer via TCID\textsubscript{50}. For three of the four nanoworm-coated surfaces, the results showed significant reduction in the amount of infectious virus compared to the uncoated surface (Figure 5a). The hyperbranched nanoworm HBNW_{O,G,C} resulted in a mean TCID\textsubscript{50}/mL value of 10\textsuperscript{3.2} for the VIC01 isolate, which was 33-fold lower than that of the uncoated samples (average TCID\textsubscript{50}/mL = 10\textsuperscript{4.94}). The average infectious titer in the eluate taken from hyperbranched nanoworm with the attached polygalactose (HBNW_{S,O,G,C}) significantly decreased the infectious titer of VIC01 to 10\textsuperscript{5.9} TCID\textsubscript{50}/mL during the 30 min exposure period. In the case of NW_{O,G,C} a lower mean TCID\textsubscript{50}/mL value of 10\textsuperscript{4.8} was found compared to that of the hyperbranched nanoworm-coated surfaces. A significant result was found for NW_{S,O,G,C} (with attached polygalactose), whereby no virus-induced cytopathic effect was observed for the eluates assessed via TCID\textsubscript{50} assay, indicating no presence of infectious virus. To confirm lack of infectious virus titer in these samples, we repassaged them in Vero cells to amplify any potential low level of infectious virus particles within the sample cultures, but virus induced cytopathic effect remained undetectable. Supporting the virucidal activity of our nanoworms against SARS-CoV-2, all four polymers showed significant mRNA loss, and we propose that this may occur via mechanisms that result in either degradation or strong binding of mRNA to the surface compared to the uncoated control (Figure 5b). Furthermore, intact viral E gene was not detectable for NW_{S,O,G,C} indicating complete destruction of the viral RNA genome. In a separate set of experiments, we reduced the exposure period of SARS-CoV-2 VIC01 isolate to 5 and 15 min and found that significant virucidal activity could be detected at 15 min with a mean TCID\textsubscript{50}/mL value of 10\textsuperscript{2.1} and maintained our earlier findings that by 30 min no infectious SARS-CoV-2 was detectable (see Figure S33). Therefore, the results indicate that NW_{S,O,G,C} was completely virucidal against SARS-CoV-2.

With the emergence of a SARS-CoV-2 (alpha) variant of concern known as B.1.1.7, we repeated our surface exposure assay using the clinical human/Victoria/17991/2020 isolate which has the identical genome to this evolved alpha variant. Importantly, NW_{S,O,G,C} maintains its highly effective virucidal activity against the B.1.1.7 variant (Figure 5c), as after exposure we were unable to detect infectious virus via TCID\textsubscript{50} and subsequent repassaging in Vero cells. The effectiveness of the NW_{O,G,C} against the evolved B.1.1.7 variant was also significantly heightened, compared to tests conducted with VIC01 (p < 0.001, unpaired t test), and there was no detectable infectious B.1.1.7 variant virus present in the eluate. Quantitative RT-PCR of the E gene revealed significant loss of virus genome by all nanoworm coatings (Figure 5d), indicating all have the potential to inactivate SARS-CoV-2. Our results indicated that the evolutionary changes to the spike protein as this virus evolved during circulation can be neutralized by our functional nanoworm coatings. The increased effectiveness against the evolved variant also provided support for our hypothesis that polygalactose attached to the nanoworm would have access to S2 binding domain and increase binding affinity to the SARS-CoV-2 virion independent of mutations to the S2 domain amino acid sequence between the VIC01 isolate, which was the first detected clinical case in Australia at the beginning of the SARS-CoV-2 outbreak, and B.1.1.7 variant. In comparison to the AAV-HA pseudovirus, in which all four nanoworms showed near complete inactivation, it was found that NW_{S,O,G,C} gave complete inactivation (i.e., below detection limits) for both VIC01 and B.1.1.7, while only NW_{O,G,C} gave complete inactivation for B.1.1.7. The data strongly supports that binding alone was not enough for effective viral inactivation but required further nanomechanical disruption, which can only occur from the conformationally responsive NW_{O,G,C} and NW_{S,O,G,C} and cannot occur for the hyperbranched nanoworms. The data further supports that both S and G act synergistically for inactivation and RNA degradation.

Given that NW_{S,O,G,C} was found to be the most effective coating to exhibit antiviral activity, we decided to explore the potential for its use as an antiviral coating on surgical masks. Virus inoculum (50 μL) containing 10\textsuperscript{5} TCID\textsubscript{50} infectious units was prepared in Sorrenson’s buffer at pH 6.5 and added dropwise onto the nanoworm-coated masks, incubated at room temperature for 30 min, and then washed. The eluate was assayed for remaining infectious virus titer and intact genome. For the noncoated masks, complete recovery of the virus contained within the inoculum was detected, with average TCID\textsubscript{50} values of 10\textsuperscript{4.2} and 10\textsuperscript{4.6} for VIC01 and B.1.1.7, respectively, suggesting that masks: (i) only provide a physical barrier against transmission, (ii) do not preclude reaerosolized or fomite transmission viral particles during use, and (iii) require correct disposal after use. Coating the mask with NW_{S,O,G,C} showed good spreading of the droplet over the area and revealed that no infectious VIC01 or B.1.1.7 was recovered in the eluate after 30 min and significantly reduced amounts of intact virus genome detectable. This result strongly supports the complete virucidal activity of NW_{S,O,G,C} against SARS-CoV-2 and the potential for use on respiratory personal protective equipment (e.g., masks).

When examined more closely, the data suggests that S and G were key components for inactivation of the virus (Figure 5). However, when testing S by itself (i.e., NW_{S,C} with 25.9 S units per chain) or when combined with O (i.e., NW_{S,O,C}) the surface showed some inactivation (Figure 5a,c) and little or no mRNA loss (Figure 5b,d). Incorporating G alone (i.e., NW_{G,C} with 25.9 G units per chain) showed significant inactivation and loss of mRNA. This data suggests that S only acted to enhance binding to the SARS-CoV-2 viral particles, while G not only provided binding via hydrogen bonding and ion-pairing but also potentially catalyzes the hydrolysis of RNAs.\textsuperscript{23} Reducing the number of G units from 25.9 on NW_{G,C} to 7.9 on NW_{S,O,G,C} resulted in inactivation of the virus below detection limits with complete loss of RNA due to the synergistic effects of the S, G, and O. Collectively, the AAV-HA, influenza A virus, and SARS-CoV-2 data supported the efficient nanomechanical inactivation of a broad range of infectious
respiratory virial particles, including capsid and enveloped viral particles. The use of our environmentally friendly water-based, spray-on nanoworm coating in the public setting on high thoroughfare surfaces and on respiratory protective equipment may play an important role in the control and possible elimination of deadly viruses from the community.

CONCLUSION

In summary, all the nanoworms showed virucidal activity against the epidemiologically important respiratory pathogen SARS-CoV-2 and influenza A virus (H1N1). While our four different conventional and hyperbranched nanoworm compositions exhibited different levels of virucidal effectiveness, the physical nature of each polymer revealed the importance of the nanoworms to induce droplet spread on the surface and the ability of these nanostructures to undergo a conformational change to enable their virucidal effects. We found that attachment of the polygalactose (HBNW0,G,C) showed only a small increase in virucidal activity compared to the HBNNW0,G,C polymer. In the case of NW0,G,C the virucidal activity of NW0,G,C against SARS-CoV-2 isolate VIC01 was slightly better than that for the hyperbranched nanoworms, but it demonstrated highly significant inactivation of the B.1.1.7 variant. Notably, the virucidal activity of NW0,G,C was biologically significant against the enveloped viruses (VIC01, B.1.1.7, and H1N1 influenza A virus) with complete inactivation and degradation of their RNA genomes demonstrated. We propose that this virucidal action was due to the nanoworm’s (NW0,G,C and NW0,G,C) ability to induce droplet spreading through a rapid conformational response and thus increase the interaction between the viruses and nanoworms to bind through the polygalactose to the S2 domain and for the guanidine moieties to bind and potentially catalyze the hydrolysis of RNA. The data clearly demonstrate the important requirement of binding and capturing and a nanomechanical process to disrupt and inactivate enveloped viruses. In addition, the excellent virucidal activity of NW0,G,C on surgical masks for both VIC01 and B.1.1.7 reveals a potential expansion for use as a defense barrier to the aerosolized virus and may aid in eliminating any subsequent fomite virus transmission should the mask be touched. Furthermore, the inclusion of the fluorescence component in the functional nanoworm provides a means to assess reapplication of the coating for continued antiviral effectiveness of the surface. Therefore, our water-based coating offers an invaluable method to reduce the spread of infectious diseases, and due to the versatile chemistry, it may be readily redesigned to target newly emerging pathogens and aid in controlling future pandemics.

METHODS

Synthesis of Functional Nanoworms. Please see the Supporting Information for a complete description of the synthesis methodology and characterization of the nanoworms utilized by this study.

Materials. Reagents. Unless otherwise stated, all chemicals were used as received. The solvents used were either HPLC- or AR-grade; these included dichloromethane (DCM, Aldrich AR grade), DMSO (Aldrich, 99.9%), n-hexane (Emsure, ACS), chloroform (Emsure, ACS), acetone (ChemSupply, AR), acetonitrile (LiChrosolv, hypergrade for LC-MS), petroleum spirit (BR 40–60 °C, Univar, AR), methanol (Merck, Emsure, ACS), toluene (Merck, for analysis EMSURE ACS, ISO, Reag. Ph Eur), ethyl acetate (ChemSupply, AR), ethanol (ChemSupply, AR), N,N-dimethylformamide (DMF: Labscan, AR grade), 2-propanol (Aldrich, 99.5%), and N,N-dimethylacetamide (Aldrich, >99%). Activated basic alumina (Aldrich: Brockmann I, standard grade, ~150 mesh, 58 A), silica gel (Aldrich, 230–400 mesh, 60 Å), magnesium sulfate (anhydrous, Scharlau, extra pure), Milli-Q water (Biolab, 18.2 MΩ), sodium dodecyl sulfate (SDS, Aldrich, 99%), N,N′-dicyclohexylcarbodiimide (DCC, Aldrich, 99%), 4-(dimethylamino)pyridine (DMAP, Merck, 99%), 1-butanol-thiol (Aldrich, 99%), N-hydroxysuccinimide (NHS, Aldrich, 98%), dihomocysteine thiocacolate hydrochloride (Tla, Aldrich, > 99%), d- (+)galactose (Aldrich, ≥99%), propargyl alcohol (Aldrich, 99%), propargyl bromide solution (Aldrich, 80 wt % in toluene, contains 0.3% magnesium oxide as stabilizer), lithium chloride (Aldrich, 99%), triphenylphosphate (Aldrich, ≥98%), 3-chloropropylamine hydrochloride (Aldrich, 98%), triethylamine (Aldrich, ≥99.5%), acryloyl chloride (Merck, stabilized with phenothiazine), sodium hydrogen carbonate (Aldrich, 99.5%), sodium azide (Aldrich, ≥99.5%), hydrochloric acid (36%, Ajax, AR), copper(II) bromide (Cu(II)Br2: Aldrich, 99%), sodium borohydride (Aldrich, 99%), sulfuric acid (Aldrich, 98%), trifluoroacetic acid (Merck, >99%), carbon disulfide (Aldrich, >99.9%), 2-bromo-2-methoxypropanionic acid (Aldrich, 98%), methyl-2-bromopropionate (MP, Aldrich, 98%), ethyl α-bromoiso-butyrate (Aldrich, 98%), 2-ethyl-2-thiopropionourea hydrobromide (Aldrich, 98%), iodoaceton (Aldrich, 98%), copper(II) sulfate (Aldrich, 99%), copper(II) sulfate anhydrous powder (Aldrich, ≥99.99% trace metals basis), Cu(0) powder (Aldrich, ≤425 μm, 99.5% trace metals basis), and 1-ascorbic acid (Aldrich, 99%) were used as received. Phosphate buffers (0.2 M, pH 6.5 and 7.4) were prepared according to Sorensen’s protocol. Phosphate buffer (pH 8.5) was obtained by addition of 1.5 M NaOH to phosphate buffer (0.2 M, pH 7.8). Armrest surfaces were provided by Boeing Research and Technology center at The University of Queensland.

Monomers, Initiator, and Ligand. Styrene (STY, Aldrich, > 99%), N,N-dimethyl acrylamide (DMA, Aldrich), and N,N-(dimethylamino)ethyl methacrylate (DMAEMA, Aldrich, 98%) were passed through a basic alumina column to remove inhibitor. N-Isopropylacrylamide (NIPAM, Aldrich, 97%) was recrystallized from n-hexane/toluene (9/1, v/v), and azobis(isobutyronitrile) (AIBN, Riedel-de Haen) was recrystallized from methanol twice prior to use. Tris(2-(dimethylamino)ethyl)amine (Me3TREN)46 Cu(II)Br2/Me3TREN complex47 and 3-azido-7-hydroxycoumarin azide (coumarin azide)48 were synthesized according to the literature procedures.

RAFT Agents. Methyl 2-(butylthiocarbonothioylthio) propanoate (MCEBTTT) RAFT agent, γ-thiolactone, and alkyl RAFT agents were synthesized according to the literature procedures.49

Instruments. Nuclear Magnetic Resonance (NMR). All NMR spectra were recorded on either Bruker DRX 400 or 500 MHz spectrometers using an external lock (CDCl3, DMSO-d6, or D2O).

Size-Exclusion Chromatography (SEC) and Triple Detection—SEC. Analysis of the molecular weight distributions of the polymers were determined using a Polymer Laboratories GPC50 Plus equipped with differential refractive index detector. Absolute molecular weights of polymers were determined using a Polymer Laboratories GPC50 Plus equipped with dual-angle laser light scattering detector, viscometer, and differential refractive index detector. HPLC grade N,N-dimethylacetamide (DMAC, containing 0.03 wt % LiCl) was used as the eluent at a flow rate of 1.0 mL/min. Separations were achieved using two PLGel Mixed B (7.8 x 300 mm²) SEC columns connected in series and held at a constant temperature of 50 °C. InfinityLab Easivial polystyrene standards were used for SEC column calibration. Samples of known concentration were freshly prepared in DMAC + 0.03 wt % LiCl and passed through a 0.45 μm PTFE syringe filter prior to injection. The absolute molecular weights and dn/dc values were determined using Polymer Laboratories Multi Cirrus software based on the quantitative mass recovery technique.

Dynamic Light Scattering. The size and zeta potential of particles was measured by DLS which was performed with a Malvern Zetasizer Nano Series running DTS software and operating a 4 mW He–Ne laser at 633 nm. Analysis was performed at an angle of 173°.
and a constant temperature of 25 °C. The number-average and Z-average hydrodynamic particle sizes and PDI (PDI) are reported. The PDI (PDI) was used to describe the width of the particle size distribution, and calculated from a Cumulants analysis of the DLS measured intensity autocorrelation function and is related to the standard deviation of the hypothetical Gaussian distribution (i.e., PDI (PDI) = σ/σP, where σ is the standard deviation and σP is the Z-average mean size).

The LCST behavior of NWOGO, NW3GO, HBNWOGO, and HBNNWOGO in Milli-Q water, 0.2 M PBS pH 6.5, or 0.2 M PBS pH 7.4 was determined by DLS. Milli-Q water, 0.2 M PBS pH 6.5, and 0.2 M PBS pH 7.4 were filtered through a 0.1 μm filter (Millex-VV, Merck Millipore Ltd.) prior to sample preparation. The nanoworms were dispersed in cold Milli-Q water, 0.2 M PBS pH 6.5, or 0.2 M PBS pH 7.4 at ~ 4 °C at a 0.03 wt % dispersion. The nanoworm dispersion (1 mL) was transferred to a precooled cuvette and placed in the DLS instrument. The measurement was carried out from 5 to 70 °C at a ramp rate 2 °C/min.

The LCST of MacroCTA-A in Milli-Q water, 0.2 M PBS pH 6.5, 0.2 M PBS pH 7.4, or 0.2 M PBS pH 8.5 was determined by dissolving the polymer (40 mg at 2 wt %) in Milli-Q (2 mL) and placed in the fridge (at 4 °C) overnight. The polymer solution was warmed to ambient temperature and filtered through a 0.1 μm filter (Millex-VV, Merck Millipore Ltd.). A 1 wt % polymer solution was prepared as follows: A sample was diluted with Milli-Q water to dissolve the polymer (40 mg at 2 wt %) in Milli-Q (2 mL) and pressed directly onto the diamond internal reflection element of the ATR-FTIR.

Transmission Electron Microscopy (TEM). The nanostructure morphology and shape were determined using a JEOL JEM-7700 transmission electron microscope utilizing an accelerating voltage of 80 kV with a spot size of 1 at ambient temperature. A typical TEM grid was prepared as follows: A sample was diluted with Milli-Q water to approximately 0.02–0.05 wt % at room temperature. A Formvar precoated copper TEM grid was dipped into the solution, and the excess aliquot was blotted off and allowed to air-dry prior to imaging by TEM.

Attenuated Total Reflectance–Fourier Transform Spectroscopy (ATR-FTIR). ATR-FTIR spectra were obtained using a horizontal, single-bounce, diamond ATR accessory on a Nicolet Nexus 870 FT-IR. Spectra were recorded between 4000 and 4 cm⁻¹ with an OPD velocity of 0.6289 cm s⁻¹. Solids were pressed directly onto the diamond internal reflection element of the ATR without further sample preparation.

Laser Scanning Microscopy (LSM). LSM images were recorded on the Leica SP8 confocal laser scanning microscope (CLSM, Leica Microsystems). To prepare a sample, 1.5 wt % dispersion of nanoworms in Milli-Q water was sprayed onto a 26 mm × 76 mm microscopic slide (SuperFrost, cut edges, Assistent) and dried using an air dryer. Spraying–drying cycles were repeated 4 times more. After that, 10 μL of the Milli-Q water, 0.2 M PBS pH 6.5, or 0.2 M PBS pH 7.4 were spotted onto a coated microscopic slide and immediately covered with a square coverslip (Westlab, 22 mm × 22 mm, 0.13–0.17 mm thick). A Z-stack of a sample was performed at the excitation wavelength of 442 nm, 630X magnification, 4096 × 4096 pixels at a scan speed of 200 pixels/s, and 100 nm interval.

Virus Exposure to Coated Surface Assays. Surface Testing for Virucidal Activity against AAV-HA Recombinant Virus. The AAV-HA recombinant virus (Catalog No: AA02-CS9G303TAV01-01-400) was custom-designed and packaged from United Bioresearch, Australia. Briefly, the HA gene (Genbank Source Sequence Accession: CY181471.1) was inserted into the AAV vector under the CMV promoter. Stocks of virus were quantitated, aliquotted, and stored at −80 °C. For each test, AAV-HA was diluted in 0.2 M PBS (pH 6.5 or 7.4) to contain 10⁷ GC (genomic copy)/mL of AAV-HA (GC copy was determined by the manufacturer). A 10 μL droplet of AAV-HA containing 10⁵ AAV-HA viral particles was then added to the center of the surface (1 cm × 1 cm) and incubated for 30 min at room temperature. Each surface test included 3 repeats for coated and uncoated surfaces. The surface (in a 24-well plate) was washed with 250 μL of sterilized PBS (0.01 M, pH 7.4). To do this, the plate was vortexed for 2 s for a total of 5 times, and then the PBS solution was pipetted several times to wash the surface. The eluate was collected and transferred to a well containing a confluent monolayer of HEK293 cells. The eluate was gently mixed with the cells and incubated for 1 h at 37 °C in a 5% CO₂ incubator to enable remaining AAV-HA to bind to cells. The eluate was then removed, and 1 mL of DMEM complete medium was added and then incubated for another hour at 37 °C in a 5% CO₂ incubator to enable infection of cells. Following this, the cells were collected in the medium, and the rest of the cells were harvested with 200 μL of trypsin. All the cells were pelleted by centrifugation, and the genomic DNA was extracted following the Qiagen Kit protocol (Genetech Mammalian Genomic DNA Mini Prep Kit). DNA samples were finally eluted in 100 μL. The real-time PCR machine carried out in a 12 μL volume with 2 μL of the DNA sample obtained above. The program was 95 °C for 10 min, 58 °C for 15 min, and 72 °C for 20 min over 40 cycles on a Bio-Rad CFX real-time PCR machine. The primers were: Left: GGCCACAGGATTGCTTCTTCT; Right: TGGGACTTCTGTGTCCTTTC. After the PCR, the cycle number (Ct) was collected for each sample. ΔCt was calculated for each nanoworm-coated or uncoated surface using the mean Ct of the inoculum only that had not touched any of the test surfaces and was regarded as positive control. We then used the ΔCt value to calculate the genome AAV-HA copy number from the standard curve generated using titrated viral stock C values, which were performed in the same reaction. The final data of viral genome copy numbers were presented as mean ± SD of 3 repeats.

Surface Testing for Virucidal Activity against Influenza A Virus and SARS-CoV-2. All influenza A virus infection cultures were conducted within the physical containment level 2 (PC2) laboratory at the Doherty Institute. All SARS-CoV-2 infection cultures were conducted within the high containment facilities in a PC3 laboratory at the Doherty Institute. Influenza A virus isolate A/Puerto Rico/8/1934 was propagated in embryonated hen eggs and stocks stored at −80 °C as previously described. Stocks of SARS-CoV-2 isolates hCoV-19/Italy/37–1039 to hCoV-19/Italy/201101–1 were produced from infected Vero cell supernatants as previously described. The genomic sequence of each stock of SARS-CoV-2 isolate was confirmed to match the publicly available data of the original virus isolate (www.gisaid.org, accession numbers: EPI_ISL_406844 and EPI_ISR_779606, respectively).

Virus inoculum was created by adding an equal volume of virus stocks to filtered sterilized Sorenson's pH buffer at pH 6.5. Then, 50 μL of inoculum was added dropwise to the surface of each material and allowed to incubate at room temperature for the duration specified. The surface of each material was then washed 8 times by pipetting with 500 μL of infection media, with care taken not to scratch the surface. Eluate was then collected, and the infectious virus titer was quantified via a 50% tissue culture infectious dose (TCID₅₀) assay using the appropriate cell line (MDCK cells for influenza A virus and Vero cells for SARS-CoV-2) as previously described. Viral RNA was also extracted from the eluate using the QiaAmp Viral RNA extraction kit (Qiagen, Australia) as per the manufacturer’s instructions and stored at −80 °C. To evaluate the amount of virus genome present in each sample, we performed a quantitative reverse-transcription PCR (RT-PCR) for detection of the SARS-CoV-2 envelope (E) gene and influenza nucleoprotein (NP) gene segment. Using the SuperScriptIII OneStep RT-PCR System with Platinum Taq DNA Polymerase (Invitrogen, Carlsbad, CA, USA), the RT-PCR assay comprised of 5 μL of RNA, 12.5 μL of 2x Reaction Master Mix, 0.4 μL of 100x MgSO₄, 1 μL of SuperScript III/Taq Enzyme Mix, 0.4 μL forward and reverse primers, 0.2 μM primer probe (using previously published primer-probe sequences), 1 μL of 1 mg/mL Bovine Serum Albumin (BSA), and 2.6 μL of RNase-free H₂O. The RT-qPCR assay was performed on a Bio-Rad CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) using the following conditions: a denaturation step at 55 °C for 10 min and 95 °C for 3 min, followed by 45 cycles of amplification (94 °C for 15 s and 58 °C for 30 s). A known amount of influenza A
independent experiments were pooled and graphed unless otherwise deviation (SD) using GraphPad Prism (v8.4). Data from at least two with appropriate serum free media, before adding the samples. the day of testing, the medium was removed and monolayers washed 24 h before infection. Upon con

ASSOCIATED CONTENT

Supporting Information
The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsnano.1c05075.

Experimental section with synthesis of polymers and nanoworms, including NMRs, TEMs, SECs, FTIRs, and DLS data (PDF)

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Notes
The authors declare no competing financial interest.

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