Highly Efficient and Rapid Inactivation of Coronavirus on Non-Metal Hydrophobic Laser-Induced Graphene in Mild Conditions

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The prevalence of COVID-19 has caused global dysfunction in terms of public health, sustainability, and socio-economy. While vaccination shows potential in containing the spread, the development of surfaces that effectively reduce virus transmission and infectivity is also imperative, especially amid the early stage of the pandemic. However, most virucidal surfaces are operated under harsh conditions, making them impractical or potentially unsafe for long-term use. Here, it is reported that laser-induced graphene (LIG) without any metal additives shows marvelous antiviral capacities for coronavirus. Under low solar irradiation, the virucidal efficacy of the hydrophobic LIG (HLIG) against HCoV-OC43 and HCoV-229E can achieve 97.5% and 95%, respectively. The photothermal effect and the hydrophobicity of the HLIG synergistically contribute to the superior inactivation capacity. The stable antiviral performance of HLIG enables its multiple uses, showing advantages in energy saving and environmental protection. This work discloses a potential method for antiviral applications and has implications for the future development of antiviral materials.

1. Introduction

The coronavirus 2019 (COVID-19) pandemic initiated by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has caused a myriad of infection cases and deaths (102 and 2.22 million, respectively, by February 2, 2021, updated)\[1\] across the globe and severely retarded the development of global economic.\[2-4\] Though numerous vaccines have been developed, challenges remain due to uncertainties in protection longevity, potential mutation of virus, and production capacity of vaccines.\[5-8\] For instance, the newly emerging variant has placed the world in a precarious situation.\[9\] The predominant mode of viral transmission is the spreading of respiratory droplets and physical contact.\[10-12\] Therefore, wearing face masks and
following hygiene practices have been widely recommended to contain the virus.[31–33] Previous reports showed that metals,[34–36] chemicals,[37–39] high temperature,[40,41] and UV irradiation[42,43] can inactivate the virus, but their cytotoxicity and damage to the human body have raised concerns.[36] Price pressure and availability of instruments further limit their widespread and daily applications, especially in underdeveloped countries. Therefore, the development of inexpensive materials that can effectively and rapidly inactivate the virus in mild conditions has broad interests amid the pandemic. Though previous studies showed that the graphene materials exhibited outstanding antibacterial activity under sunlight and proposed that the high temperature (e.g., 110 °C) resulted from photothermal effect might inactivate virus, there was no direct evidence on how effective the antiviral efficacy could be achieved.[27–29] In addition, such a high temperature could impractical in real scenario as the solar irradiance is often lower. Here we report that laser-induced graphene (LIG) and its composites[30–32] show excellent antiviral activity against HCoV-OC43 and HCoV-229E, two human coronaviruses serving as surrogates for SARS-CoV-2.[24,33] Surprisingly, hydrophobic LIG (HLIG), even without any metal additives, can inactivate HCoV-OC43 and HCoV-229E by >95% in 15 min at a low temperature of 46 °C. This temperature can be easily achieved from the broad-spectrum solar absorption of LIG or by Joule heating in winter. The silver nanoparticles/LIG composites (Ag NPs/LIG) also show similar performance, but it is highly cytotoxic.[34] The inactivation capacity of LIG and its composites is well preserved after several washing and drying, indicating their reusability. The porous LIG with excellent and stable antiviral activity under sunlight and proposed that the high temperature of 46 °C, surfaces such as doorknobs and ground could be endowed with antiviral effect.[35,36]

2. Results and Discussion

The LIG and HLIG can be directly converted from precursors such as wood, paper, cloth, and polymer[37] by a common CO₂ laser. It is a fast and green method that does not need harsh conditions, such as high temperature, high pressure, and massive chemicals consumption. Dropping a proper amount of AgNO₃ solution on LIG followed by a second lase can afford Ag NPs/LIG composite. Raman spectra show three characteristic peaks of graphene materials (Figure 1a), G peak at ~1350 cm⁻², D peak at ~1590 cm⁻², and 2D peak at ~2700 cm⁻², attributing to the graphite-derived structure, presence of defects, and the number of layers of the graphite structure.[38–41] The Ag NPs/LIG has a higher I_D/I_G ratio than LIG, indicating that the incorporation of Ag NPs from the second ablation slightly increases the defect or disorder of LIG. The X-ray diffraction (XRD) pattern of Ag NPs/LIG in Figure 1b shows the XRD peaks at 2θ of 38.2°, 44.3°, 64.72°, 77.55°, and 81.72° could be assigned as the (111), (200), (220), (311), and (331) crystallographic planes of the face-centered cubic silver crystals, respectively.[42] The broad peak at 2θ of 26° could be attributed to the (002) graphite crystal planes, giving an interlayer spacing of 3.4 Å.[43] When lased in air, the LIG shows a contact angle of ~20°. An obtuse angle of ~140° was obtained when lased under an inert atmosphere, demonstrating the hydrophobicity of HLIG (Figure 1c). The scanning electron microscopy (SEM) image in Figure 1d illustrates the high porosity of LIG with pore size of several hundreds of nanometers. The transmission electron microscopy (TEM) images in Figure 1e,f show the few-layered graphene structure and uniform distribution of spherical Ag NPs with a size of tens of nanometers.

The photothermal effect and Joule heating effect of LIG were explored. When exposed to 1 kW m⁻² sunlight, the temperature of LIG soared up to 55 °C within 10 s and then maintained at 62 °C (Figure 2a). For Joule heating, a DC voltage of 7.5 V could heat a 10 × 10 cm² LIG up to 50 °C. The temperature of LIG
increases with the increase of light intensity and power supply (Figure 2c,d). A temperature of 46 °C, which is sufficient for the virus inactivation as shown later, could be achieved by simply exposing LIG to ≈0.5 kW m⁻² sunlight or applying direct current power (≈20 mW cm⁻²). This temperature can be easily attained outdoor (Figure S4, Supporting Information) or powered LIG by 2–3 AAA batteries. A rough analysis of the production cost and energy cost is summarized in Table S1, showing the low-cost nature of LIG.

Two human coronaviruses, HCoV-OC43 and HCoV-229E, were used to evaluate the antiviral performance of three different types of LIG and melt-blown fabrics (MBF), the key filtering layer in commercial surgical masks. The viral fluid was first separately incubated with LIG, HLIG, Ag NPs/LIG, and MBF with or without exposure to sunlight irradiation for 5, 10, and 15 min. The viral fluid was then used to infect MRC-5 cells and the level of viral RNA extracted from the infected cells was measured by real-time polymerase chain reaction (RT-PCR). Cell only and cell + virus were the negative control and positive control, respectively. The results in Figure 3a,b show that cells infected with the sunlight-treated virus have much lower viral RNA copies compared with the MBF group. Without sunlight, the antiviral efficacy of LIG, HLIG, and Ag NPs/LIG was 2.4%, 4.7%, 18% against HCoV-OC43, and 19%, 64.3%, 51.4% against HCoV-229E, respectively. With 10-min sunlight irradiation, the level of HCoV-OC43 RNA decreased by 9%, 34.3%, and 77%, and the level of HCoV-229E decreased by 29%, 68.3%, and 28.3% for LIG, HLIG, and Ag NPs/LIG, respectively. Prolonged irradiation time to 15 min improved the inhibition rate of HCoV-OC43 mRNA level on LIG, HLIG, Ag NPs/LIG to 58.3%, 97.5%, and 85.7%, and that of HCoV-229E to 98%, 95%, and 75.67%, respectively. These results showed the extraordinary antiviral activity of HLIG. The viral RNA level in MRC-5 cells almost vanished after 15-min irradiation for both HCoV-OC43 and HCoV-229E. The surface temperature was ≈46 °C with sunlight irradiation for all the LIG samples and the viral fluid was kept wet throughout the test. In practical use, the viral inhibition performance of HLIG could be even stronger due to induced dryness upon absorption of sunlight. In addition, SARS-CoV-2 was classified as enveloped viruses due to its characteristic outer lipid membrane.[44] The hydrophobicity of HLIG might enhance the interaction between the graphene surface and fatty acid of viral membrane, which depleted the lipid membrane and weakened the virus under photothermal effect.[45]

We further evaluate the expression level of HCoV-OC43 in MRC-5 cells by immunofluorescence analysis (Figure 3c). Compared to the PC, almost no infected cells in LIG and HLIG could be seen. The average fluorescence of LIG and HLIG with sunlight is 13.416 ± 1.598 and 12.146 ± 0.577 per cell in the testing cohorts, respectively (Figure S5, Supporting Information). These counts were significantly lower than the PC and MBF group (52.475 ± 5.937 and 45.033 ± 2.760, respectively). The Ag NPs/LIG group result was not shown here because of its strong cytotoxicity for MRC-5 cells. The morphology of MRC-5 cells has a significant change when they are treated with Ag NPs/LIG; the cells became slightly round, and the vacuoles, granules, and intercellular space increased (Figures S6 and S7, Supporting Information). The detailed cytotoxicity was evaluated by Cell Counting Kit-8 (CCK-8) assay (Figure S8, Supporting Information). The cell viability rate of Ag NPs/LIG groups decreased to 52.42% after 24-h treatment,
while that of LIG and HLIG group kept ≥90%. Though the Ag NPs/LIG possessed moderate intrinsic antiviral capacity without sunlight irradiation when compared with LIG and HLG, the cytotoxicity and high cost made it difficult for widespread applications.

Median tissue culture infective dose (TCID$_{50}$) assay was conducted to detect the viral titers. The results in Figure 3d,e show that without sunlight, all the LIGs reduced the infectivity, but weak. Without sunlight, the virus titers of LIG, HLIG, and Ag NPs/LIG has a reduction of 41.38%, 41.38%, and 45.7% for HCoV-OC43 and 29.17%, 50%, and 36% for HCoV-229E, respectively. The virus titers of all sample groups have a considerable reduction with sunlight introduction. For example, significant reduction of HCoV-OC43 and HCoV-229E virus titers of ≈66% and ≈86% were achieved after the treatment on HLIG with sunlight. The performance is compatible to the literature,$^{[46,47]}$ and clearly showing that treatment on HLIG with sunlight can effectively reduce the establishment of infection and spread of coronaviruses.

We also tested the antiviral stability of LIG against HCoV-OC43 and HCoV-229E. The RT-PCR results showed that the HLIG surface can maintain strong antiviral activity even after multiple uses. As shown in Figure 4a the stability of HLIG against HCoV-OC43 was remarkable with an inhibition efficiency of ≈97% even after being reused three times. For HCoV-229E, the inhibition efficiency of LIG, HLIG, and Ag NPs/LIG decreased to 61.34%, 68.67%, and 60.3%, and the HLIG remained the best among all the sample groups. The results showed that the inactivating effects of HLIG to coronaviruses are stable and can be recycled for multiple uses.

Figure 3. Antiviral effect of LIG, HLIG, Ag NPs/LIG, and MBF. a) Anti-HCoV-OC43 and b) anti-HCoV-229E with different LIG material in MRC-5 cell line. Data were expressed as mean ± SE, n = 3, *p < 0.05. c) Immunofluorescence of MRC-5 after infected with HCoV-OC43, scale bars are 50 µm. TCID$_{50}$ assay of detecting the viral d) HCoV-OC43 and e) HCoV-229E titers. Data were expressed as mean ± SE, n = 8, *p < 0.05.
4. Experimental Section

Chemicals and Materials: All chemicals and reagents were used as received unless otherwise specified. Polyimide (PI) film was provided by Zeman Tape Material Technology, China. Minimum essential medium (MEM) was purchased from Gibco. Phosphate buffered saline (PBS), fetal bovine serum (FBS), penicillin, and streptomycin, were purchased from Millipore Corporation, United States. CCK-8 kit (Sigma) was used as the secondary antibody. Milli-Q water was supplied from Invitrogen. The antibody against Coronavirus Antibody, OC-43 and HCoV-229E were purchased from MedChemExpress. All other chemicals as well as solvents were all purchased from Aldrich.

Fabrication of Hydrophilic and Hydrophobic LIG: The PI film with thickness of 100 µm was irradiated by a 10.6 µm CO2 laser marking machine (Minsheng Laser #MSDB-FM60 CO2 Laser Marker, 60 W) in ambient atmosphere and nitrogen atmosphere to fabricate LIG and HLIG, respectively. The laser power, speed, pulses/dot, and line spacing were set as 1.8 W, 1000 mm s⁻¹, 5 and 0.03 mm, respectively. The laser mode was vector mode.

Fabrication of Ag NPs/LIG: First, one lase was applied to create a LIG film, the laser power, speed, pulses/dot, and line spacing were set as 1.8 W, 1000 mm s⁻¹, 5 and 0.03 mm, respectively. The laser mode was set as vector mode. 10 mg mL⁻¹ AgNO₃ solution was then loaded on the obtained LIG film drop by drop, with a loading amount of 1 mg cm⁻², then dried it in room temperature. Finally, a second lase with the same laser conditions as the first lase was applied, Ag NPs/LIG composite was obtained.

Materials Characterizations: SEM images and contact angle were performed using a QUATTRO S Scanning Electron Microscope from Thermo Fisher and Contact Angle Goniometer (ramé-hart Model 190) respectively. Raman spectrum was obtained by LabRAM HR800 Laser Confocal Micro-Raman Spectrometer with a laser wavelength of 514.5 nm. The infrared thermal images were taken by Flir C2 camera, and GLORIA-X500A from Zolix with a parallel light spot diameter of 4.6 cm was used to simulate sunlight. UV–vis–Near Infrared (UV–VIS–NIR) Spectrum was conducted by a UV–vis–NIR infra-red spectrophotometer (PE Lamda 750). XRD was tested by powder X-ray diffractometer (Bruker D2 PHASER with LYNXEYE XE-T detector). Fluorescent images were collected on Olympus IX71 inverted fluorescence microscope.

Cells and Virus: Human embryonic lung fibroblast cell line MRC-5 was provided by the China Center for Type Culture Collection. All cells were cultured in MEM medium supplemented with 10% heat-inactivated FBS (GibCO, USA), 100 units mL⁻¹ penicillin, and 100 µg mL⁻¹ streptomycin in a 5% CO₂ humidity incubator at 37 °C. One day before the infection experiment, the cells were seeded into a 24-well cell culture plate and infected with virus at ~70–80% confluence. Human coronavirus HCoV-229E (ATCC VR-740) and HCoV-OC43 (ATCC VR-1558) were purchased from the American Type Culture Collection, MD, USA. Cells were used to propagate viral stocks and to measure viral titers in plaque and 50% tissue culture infectious dose (TCID₅₀) assays. The MOI was calculated according to the manufacturer protocol. The integrity and purity of RNA were tested using 1.5% agarose gels, NANO DROP2000 (Thermo), and a UV–vis–NIR infra-red spectrophotometer (PE Lamda 750). Raman spectrum were obtained by LabRAM HR800 Gloria-X500A from Zolix with a parallel light spot diameter of 4.6 cm respectively. Raman spectrum were obtained by LabRAM HR800 Gloria-X500A from Zolix with a parallel light spot diameter of 4.6 cm respectively.

4. Conclusions

In this study, the antiviral effect of LIG, HLIG, Ag NPs/LIG, and MBF was comprehensively evaluated from the nucleic acid, protein expression and virus titers of two coronaviruses HCoV-OC43 and HCoV-229E. All sample groups exhibited weak virucidal capacity, but a sharp increase to 97.5% and 95% against HCoV-OC43 and HCoV-229E can be attained after 15 min exposure to sunlight for HLIG. And inactivation effect of LIG for HCoV-OC43 was very stable with a slight reduction of 0.5% after multiple uses. The low cost, scalable production, mild virucidal conditions, reusability, and sustainability make HLIG a promising and powerful tool for daily-use protection amid the pandemic. For example, the HLIG with tunable porosity could be used as a layer in surgical masks, water treatment, or air filtration systems, which serves for bacterial shielding and disinfection. Additionally, the formation of HLIG composites could further bring the scope of surface disinfection to a broader context.
Supporting Information. Each qRT-PCR reaction involved 0.25 μL SYBR green dye (Invitrogen), 12.5 μL Premix Taq (Promega), 0.5 μL of each primer (25 μmol L⁻¹), and 6.25 μL H₂O to a final volume of 25 μL. The amplification reaction was achieved through one cycle at 95°C for 10 min followed by 40 cycles at 95°C for 15 s and a final cycle at 60 °C for 60 s in the CFX96TM RT-PCR detection system (BIO-RAD). GAPDH was chosen as an endogenous control to normalize the expression levels of genes, and results were analyzed with the 2⁻ΔΔCt method. Each sample was subjected to three independent replicates. All assays were performed with three independent biological replicates. Data analyses were performed using Rotor-Gene 4.6.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

L.H. and M.G. contributed equally to this work. R.Y. designed the LIG synthesis and characterization with assistance from Z.Y. M.G., Y.Y., D.W., and C.S. carried out the virus studies. L.H., M.G., and R.Y. wrote the manuscript with comments from other authors.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords

antivirals, COVID-19, hydrophobic graphene, laser-induced graphene, mild conditions

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