

ACE2 Receptor-modified Algae-based Microrobot for Removal of SARS-CoV-2 in Wastewater

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ABSTRACT:

The coronavirus SARS-CoV-2 can survive in wastewater for several days with a potential risk of waterborne human transmission, hence posing challenges in detaining the virus and reducing its spread. Herein, we report on an active biohybrid microrobot system that offers highly efficient capture and removal of target virus from various aquatic media. The algae-based microrobot is fabricated by using click chemistry to functionalize microalgae with angiotensin-converting enzyme 2 (ACE2) receptor against the SARS-CoV-2 spike protein. The resulting ACE2-algae-robot displays fast ($>100\text{ }\mu\text{m/s}$) and long-lasting ($>24\text{ h}$) self-propulsion in diverse aquatic media including drinking water and river water, obviating the need for external fuels. Such movement of the ACE2-algae-robot offers effective ‘on-the-fly’ removal of SARS-CoV-2 spike proteins and SARS-CoV-2 pseudovirus. Specifically, the active biohybrid microrobot results in 95% removal of viral spike protein and 89% removal of pseudovirus, significantly exceeding the control groups such as static ACE2-algae and bare algae. These results suggest considerable promise of biologically functionalized algae towards the removal of viruses and other environmental threats from wastewater.

As an emerging coronavirus associated with formidable infectiousness and lethality, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) can spread through multiple transmission routes, including direct airborne transmission from respiratory droplets or aerosols and indirect fomite transmission upon contacting contaminated subjects or surfaces.¹ While SARS-CoV-2 is known to infect the respiratory tract, it can also infect the gastrointestinal tract with a prolonged residence in fecal samples.² In addition to its presence in stool samples, SARS-CoV-2 has also been detected in domestic wastewater in sewage and downstream rivers.³⁻⁵ These reports have raised concerns that wastewater could be a potential route of SARS-CoV-2 infection via fecal-oral transmission.⁶⁻⁷ To minimize the risk of secondary transmission to humans, there remains an urgent need to develop wastewater treatment strategies for the effective removal of SARS-CoV-2. Several physical, chemical, and biological processes, including sedimentation, filtration, disinfection with UV or oxidants, and enzymatic degradation, have been proposed to tackle SARS-CoV-2 decontamination in wastewater.⁸ While these conventional wastewater purification approaches are effective in general, it is still necessary to explore new techniques that are easy, fast, and effective in resolving the wastewater contamination issue of SARS-CoV-2.

We present here a biohybrid microrobot for the efficient removal of SARS-CoV-2 from contaminated aquatic media. Owing to their robust self-propulsion ability and facile surface functionalization, microrobots offer a dynamic and powerful strategy for rapid decontamination of water matrices from a wide range of environmental pollutants, including dyes,⁹⁻¹⁰ heavy metals,¹¹⁻¹² oil,¹³ pathogenic organisms,¹⁴⁻¹⁵ nitroaromatic explosives,¹⁶ and chemical and biological warfare agents.¹⁷ Such movement of functional microrobots provides an enhanced collision/contact and adsorption of the target contaminants along with localized self-mixing as compared to their static counterparts, and thus enabling efficient and rapid decomposition and accelerated ‘on-the-fly’ removal of pollutants.¹⁸⁻¹⁹ However, widespread environmental and defense applications of current synthetic microrobotic platforms have been hampered by their short life span, need for toxic fuels, or complex external actuation equipment, and restricted operating media. To address these challenges faced by synthetic microrobots,

biohybrid microrobots - combining self-propelled microorganisms with functional biomaterials - have recently demonstrated significant promise for large-scale environmental remediation.²⁰⁻

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The new microrobotic strategy for SARS-CoV-2 removal relies on angiotensin-converting enzyme 2 (ACE2) receptor functionalized algae microrobot (denoted ‘ACE2-algae-robot’). The ACE2 receptor is responsible for the recognition of the target virus with a high binding affinity to the S1 subunit of the viral spike protein and has been reported as an effective cellular receptor for SARS-CoV-2 towards diverse virus-related sensing,²³ therapeutic,²⁴ and neutralization²⁵ applications. Algae have been used for wastewater treatment,²⁶ but not in connection to active microrobots or towards the management of SARS-CoV-2 contaminated water. Here we select *Chlamydomonas reinhardtii* as a model algae because of their attractive properties, including easy large-scale production, fast motion in diverse aqueous environments, long life span, and facile surface functionalization.²⁷⁻²⁸ As illustrated in **Figure 1a**, the ACE2-algae-robot is fabricated using a click chemistry reaction for anchoring the ACE2 receptor onto the algae surface. The resulting ACE2-algae-robot displays fast movement ($>100\text{ }\mu\text{m/s}$) in various media, without compromising the intrinsic mobility of unmodified algae. Using SARS-CoV-2 spike protein (S protein) and pseudovirus as model contaminants, the moving ACE2 receptor on the algae surface leads to remarkable binding to the targets, enabling about 95% removal of the S protein and about 89% removal of SARS-CoV-2 pseudovirus from various testing wastewater (**Figure 1b**). The pseudovirus bears the same spike protein as live SARS-CoV-2 virus and has been shown extremely useful for developing SARS-CoV-2 detection and neutralization technologies.²⁹ These results clearly illustrate the feasibility of using the biohybrid microrobot for large-scale ‘on-the-fly’ decontamination of coronavirus and possibly other diverse environmental threats in wastewater.

Figure 1 schematically displays the fabrication process of the ACE2 modified algae (**a**) and the targeting and removal of S protein and SARS-CoV-2 pseudovirus from wastewater (**b**). In the study, the ACE2 receptors were conjugated to the algae surface using a click chemistry

approach. Specifically, the azide and dibenzocyclooctyne (DBCO) groups were conjugated to the ACE2 receptors and the algae, respectively, via the N-hydroxysuccinimide (NHS) ester reaction. The conjugated N_3 on the ACE2 receptors will then react effectively with the DBCO on the algae, resulting in the formation of ACE2-algae-robot. Next, immunostaining was performed to visualize the ACE2 receptors attached on the algae surface. Here, Fluor488-conjugated anti-ACE2 antibody was used to label the ACE2 receptors on the algae. As illustrated in **Figure 1c**, the signals from immunostaining of fluorescent anti-ACE2 antibody in the GFP channel co-localized with those of the autofluorescence of algae in the Cy5 channel. The enlarged image in **Figure 1d** further indicates the coverage of ACE2 receptors on the algae surface. In addition, the unmodified algae incubated with fluorescent anti-ACE2 antibody exhibited a negligible change of GFP fluorescence intensity as compared to the bare algae alone (**Figures 1e** and **Figure S1**). In contrast, the ACE2-algae-robot incubated with fluorescent anti-ACE2 antibody showed a significant increase in fluorescent intensity as compared to the two control groups, reflecting the effective conjugation of ACE2 receptors onto the algae via click chemistry. The pseudocolored scanning electron microscopy (SEM) images further illustrate the ACE2-algae-robot before (**Figure 1f** and **Figure S2i**) and after (**Figure 1g** and **Figure S2ii**) contact with SARS-CoV-2 pseudovirus. The bound virus particles can be clearly observed on the ACE2-algae-robot from the inset of SEM images in **Figure 1g**.

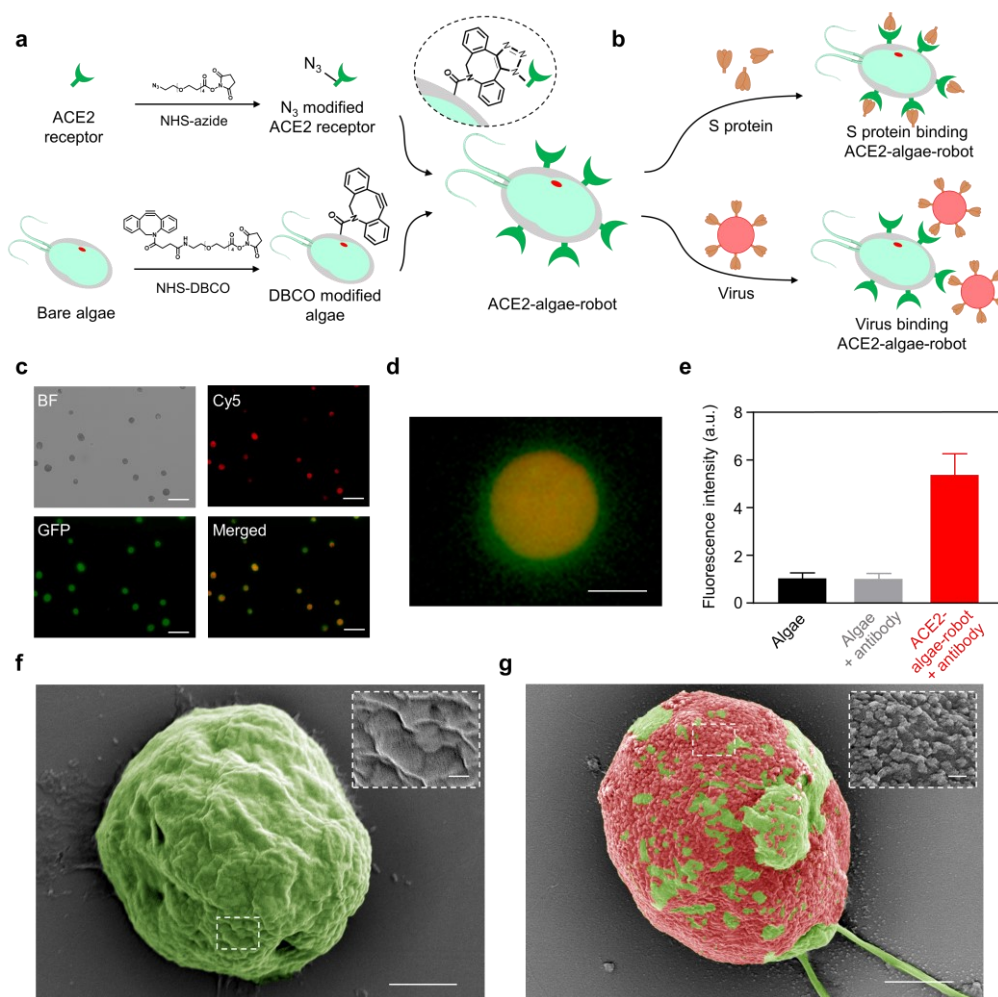


Figure 1. Fabrication and characterization of ACE2-functionalized algae microrobot (denoted ‘ACE2-algae-robot’). **a.** Schematic of the functionalization of microalgae with ACE2 receptor. **b.** Schematic depicting the use of ACE2-algae-robot for the binding and removal of spike protein and SARS-CoV-2 virus. **c.** Brightfield, fluorescent, and merged images of ACE2-algae-robot after immunostaining. Autofluorescence of natural algae chloroplast in Cy5 channel; immunostaining of Fluor488-conjugated anti-ACE2 antibody in GFP channel. Scale bar: 50 μ m. **d.** Enlarged fluorescence image from (c) clearly shows the full coverage of the ACE2 receptor onto the algae surface. Red core: chloroplast of the algae; Green: Fluor488-conjugated anti-ACE2 antibody. Scale bar: 5 μ m. **e.** GFP fluorescence intensity of bare algae, bare algae with Fluor488-conjugated anti-ACE2 antibody, and ACE2-algae-robot with Fluor488-conjugated anti-ACE2 antibody. **f, g.** Pseudocolored scanning electron microscopy (SEM) images of ACE2-algae-robot before (f) and after (g) contact with the virus. Green: ACE2-algae-robot; red: SARS-CoV-2 pseudovirus. Scale bar: 2 μ m. Zoom-in images show the surface morphology of ACE2-algae-robot before (f) and after (g) contact with the virus. Scale bar: 200 nm.

We next investigated the motion behavior of the biohybrid microrobot. After confirming the DBCO-NHS ester conjugation (**Figure S3**), the ACE2-algae-robot was fabricated by mixing DBCO modified algae with the azide-ACE2 receptor in DI water for 2 h. The speed of

the DBCO modified algae and the ACE2-algae-robot were measured to be 112 $\mu\text{m/s}$ and 108 $\mu\text{m/s}$ (~ 11 body length/s), respectively, compared to 115 $\mu\text{m/s}$ of the bare algae, indicating that the functionalization process has a negligible effect on the motion of the algae (**Figure 2a** and **Video S1**). Once prepared in DI water, the ACE2-algae-robot was transferred into various aqueous media to test their mobility. As illustrated in **Figure 2b** and **Video S2**, the ACE2-algae-robot displayed efficient motion (>100 $\mu\text{m/s}$) in TAP medium, 0.1 $\times$ PBS, drinking water, and river water without a need of any external fuel to propel the robot. In addition, ACE2-algae-robot demonstrated long-lasting motion in both drinking and river water matrices (**Figure 2c, 2d**, and **Video S3**), indicating its capability for extended operation towards the removal of SARS-CoV-2 S protein and pseudovirus. The images in **Figures 2e** and **2f** (along with **Video S4** and **S5**) illustrate representative tracking trajectories of individual ACE2-algae-robot, over a 2.5 s interval, at different times [0 h (i) and 24 h (ii)] in drinking and river water, respectively, reflecting the highly stable algae motion in these media.

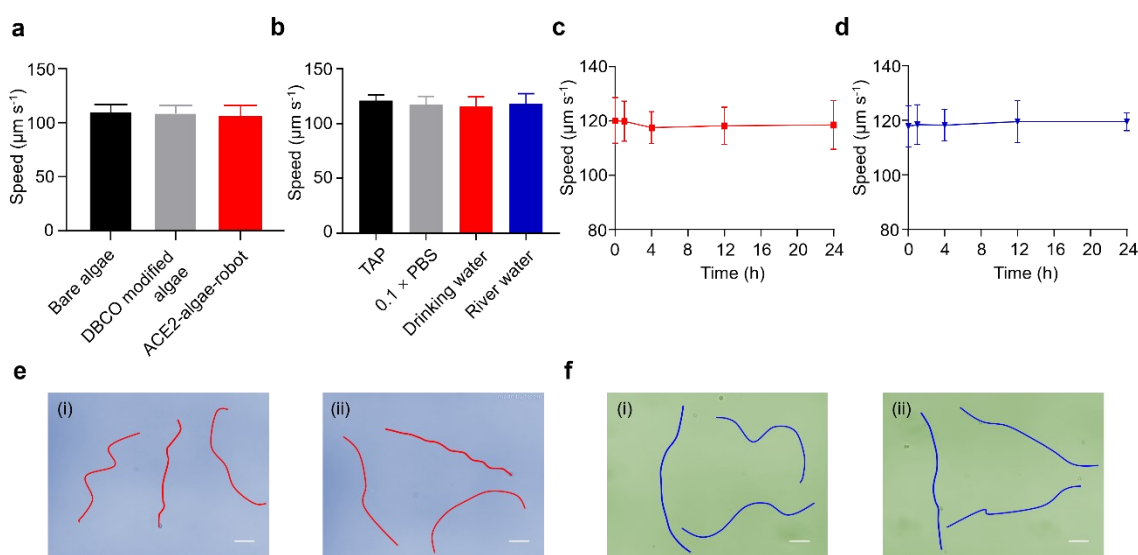


Figure 2. Motion behavior of ACE2-algae-robot. **a.** Effect of the algae functionalization upon its swimming behavior: Speeds of bare algae, DBCO modified algae, and ACE2-algae-robot ($n = 6$; mean $\pm$ s.d.). **b.** Movement of the ACE2-algae-robot in different media: tris-acetate-phosphate medium (TAP), 0.1 $\times$ phosphate-buffered saline (PBS; diluted from 1 $\times$ PBS with DI water), drinking water, and river water ($n = 6$; mean $\pm$ s.d.). **c,d.** Speed of ACE2-algae-robot at different time points (0 h, 1 h, 4 h, 12 h, and 24 h) in drinking water (c) and river water (d) ($n = 6$; mean $\pm$ s.d.). **e,f.** Representative optical trajectories of the movement of ACE2-algae-robot over 2.5 s motion in drinking water (e) and river water (f) obtained at 0 h (i) and 24 h (ii). Scale bar: 40 μm .

Figure 3a illustrates that the presence of viral S protein in the drinking water (red) and river water (blue) did not affect the mobility or the lifespan of the ACE2-algae-robot, which showed highly stable motion in both media for over 24-h operation. This was supported by the tracking trajectories of about 200 individual ACE2-algae-robots (during 0.6 s) after moving for 24 h in drinking water and river water, respectively (**Figure 3b** and **3c**). The corresponding speed distribution indicates that 80% of algae moved faster than 100 $\mu\text{m/s}$ (**Figure S4**). Such fast and continuous motion of the ACE2-algae-robot could accelerate its collision with viral S protein and thus improve the specific binding and removal of the target protein. **Figure 3d** examines the effect of ACE2-algae-robot density on the kinetic removal efficiency of S protein from drinking water. As expected, the speed and efficiency of the removal process increased upon increasing the density of the microrobots, with $5 \times 10^7 \text{ ml}^{-1}$ ACE2-algae-robot removing 95% of 2.88 ng/ml S protein from the water sample within 6 h. To compare the S protein removal capability between different algae groups, the same number of algae ($5 \times 10^7 \text{ ml}^{-1}$) from different control groups, including ACE2-algae-robot, static ACE2-algae (de-flagellated algae with the ACE2 receptor modification), bare algae, and cell wall deficient algae, was added to 500 μl drinking water containing 2.88 ng/ml S protein. The ACE2-algae-robot shows highly efficient binding and 95% removal efficiency after 6 h continuous motion (**Figure 3e**, i). In comparison, the active bare algae lacking ACE2 (**Figure 3e**, ii) and static ACE2-algae (**Figure 3e**, iii) displayed only 46% and 23% removal efficiency after 16 h operation, respectively, indicating the critical role of the ACE2 receptor modification and algae motion on the speed and efficiency of the S protein removal. The S protein removal by the bare algae is likely attributed to non-specific binding associated with the presence of diverse functional groups (e.g. carboxyl or amino groups) on the algae surface.³⁰ The images shown in **Figure S5** represent a homogenous mixture of ACE2-algae-robot and S protein after 6 h incubation compared to clear sediment of static ACE2-algae after 6 h incubation. These results explain further the fast and efficient removal using ACE2-algae-robot in **Figure 3e**. The ACE2-algae-robot also exhibits effective S protein removal in various media, including DI water, drinking

water, and river water, as indicated by the similar kinetic profiles in **Figure 3f**. These results reveal that ACE2-algae-robot, with long-lasting motion and ACE2 receptor for S protein recognition, represents an attractive system to enhance environmental remediation in complex aqueous surroundings.

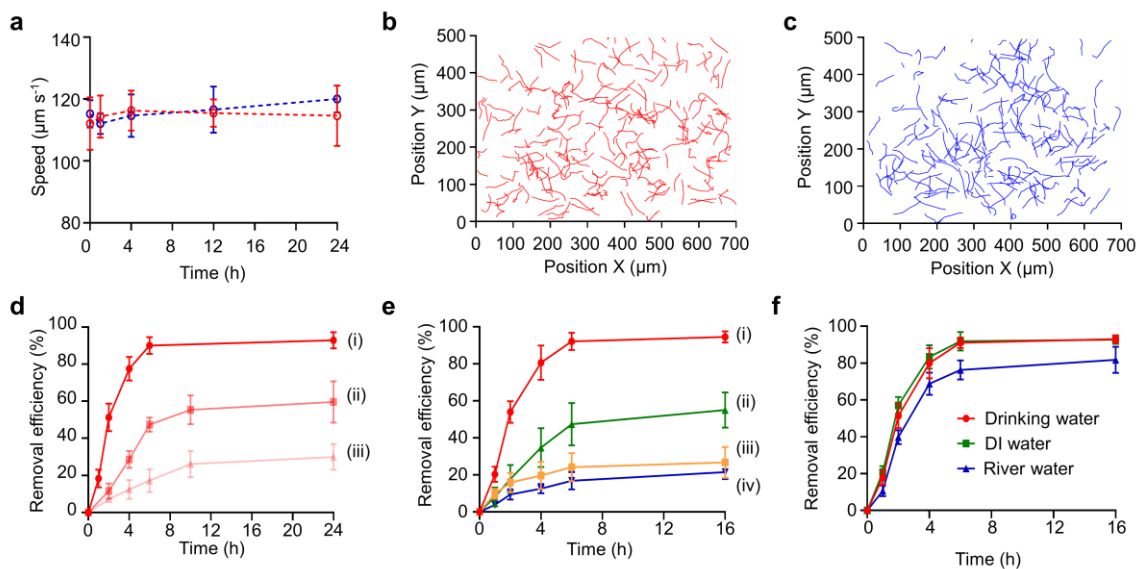


Figure 3. Use of ACE2-algae-robot for the removal of SARS-CoV-2 spike protein. **a.** Speed comparison of ACE2-algae-robot in drinking water (red) and river water (blue) containing spike protein. ($n = 6$; mean $\pm$ s.d.) **b,c.** Motion trajectory of ACE2-algae-robot in drinking water (b) and river water (c) samples containing spike protein. **d.** Effect of ACE2-algae-robot density on the spike protein removal kinetic profile in drinking water. Algae input density: (i) $5 \times 10^7 \text{ mL}^{-1}$ (ii) $2 \times 10^7 \text{ mL}^{-1}$ (iii) $5 \times 10^6 \text{ mL}^{-1}$. ($n = 3$; mean $\pm$ s.d.) **e.** Kinetic profile of the spike protein removal efficiency from drinking water with the treatment of (i) ACE2-algae-robot, (ii) bare algae, (iii) static ACE2-algae, or (iv) cell wall deficient algae. **f.** Kinetic profile of the spike protein removal efficiency by employing ACE2-algae-robot in different media, including drinking water, DI water, and river water ($n = 3$; mean $\pm$ s.d.).

Following the effective removal of viral S protein, the next set of experiments examined the ability of the ACE2-algae-robot to remove SARS-CoV-2 pseudoviruses, which represents an effective alternative of live human SARS-Cov-2 virus for research to evaluate new anti-viral technologies.²⁹ **Figure 4a** schematically illustrates the efficient removal of the SARS-CoV-2 pseudovirus from water samples using ACE2-algae-robot. After ACE2-algae-robot treatment,

the pseudoviruses are largely captured and removed from the solution, validated and visualized by an NL-20 cell-based assay. The NL-20 cells remained nonfluorescent following 24 h incubation in pseudovirus contaminated water samples upon ACE2-algae-robot treatment, confirming the highly effective pseudovirus removal by the robot. In comparison, without the ACE2-algae-robot treatment, the cells displayed bright fluorescence, reflecting the pseudovirus entry and significant expression of fluorescent protein in the host cells. **Figure 4b** and **S6** show the motion behavior (speed and lifespan) of ACE2-algae-robot in various water matrices containing the SARS-CoV-2 pseudovirus. The biohybrid microrobot displays similar speed in these aqueous media, which is similar to that observed in **Figure 2b** without the pseudovirus, indicating that the presence of the pseudovirus does not hamper the movement of ACE2-algae-robot.

Next, we investigated the effect of ACE2-algae-robot density on the removal efficiency of SARS-CoV-2 pseudovirus from drinking water. As shown in **Figure 4c**, the results demonstrated a gradual increase of the removal efficiency, from 19% to 90%, upon increasing ACE2-algae-robot concentrations from $5 \times 10^4 \text{ ml}^{-1}$ to $5 \times 10^7 \text{ ml}^{-1}$. The pseudovirus removal efficiency was also visualized by fluorescent imaging. As expected, the highest robot concentration resulted in a minimal fluorescent virus signal (**Figure 4d** and **S7**). The ACE2-algae-robot also offers significant improvement (89%) in the removal of SARS-CoV-2 pseudovirus compared with control groups, including static ACE2-algae (31%), bare algae (63%), and cell-wall deficient algae (21%) (**Figure 4e**). These experiments were performed by immersing 2.5×10^5 of algae in a 50 μL drinking water sample containing $2 \times 10^9 \text{ VG ml}^{-1}$ (VG: viral genomes) pseudovirus for 16 h. In the control experiments, the viral binding capability of the bare algae is possibly attributed to both biosorption³⁰ and physical trapping of the virus into the porous structure of algae cell wall.³¹ The fluorescence images in **Figure 4f** and **S8** illustrate the minimal fluorescent virus signal with the ACE2-algae-robot treatment, corresponding to the virus removal efficiency data of **Figure 4e**. Furthermore, we evaluated and compared the virus removal kinetic profiles in drinking water treated with the ACE2-algae-robot, bare algae, or static ACE2-algae (**Figure 4g**). These data illustrate that the ACE2-algae-

robot can effectively remove 85% of pseudovirus after 4 h treatment, compared to 60% and 18% for bare algae and static ACE2-algae, respectively. Therefore, each ACE2-algae-robot can bind to ~340 VG of virus. Such results indicate the significant contributions of both the ACE2 receptor for virus targeting and the fast microrobot motion for contacting the virus towards highly efficient viral binding and removal. The representative fluorescence images also show the progression of the viral infection over time with samples treated with ACE2-algae-robot (**Figure 4h** and **S9**). Furthermore, the ACE2-algae-robot can be reused to reach 90% viral removal efficiency after five repeated cycles (**Figure S10**). A post-treatment of the ACE2-algae-robot by flocculant was performed to clean the robot in the water sample (**Figure S11**). The flocculant can isolate the ACE2-algae-robot from the water matrices without affecting the virus removal efficiency, holding considerable potential for practical future applications.

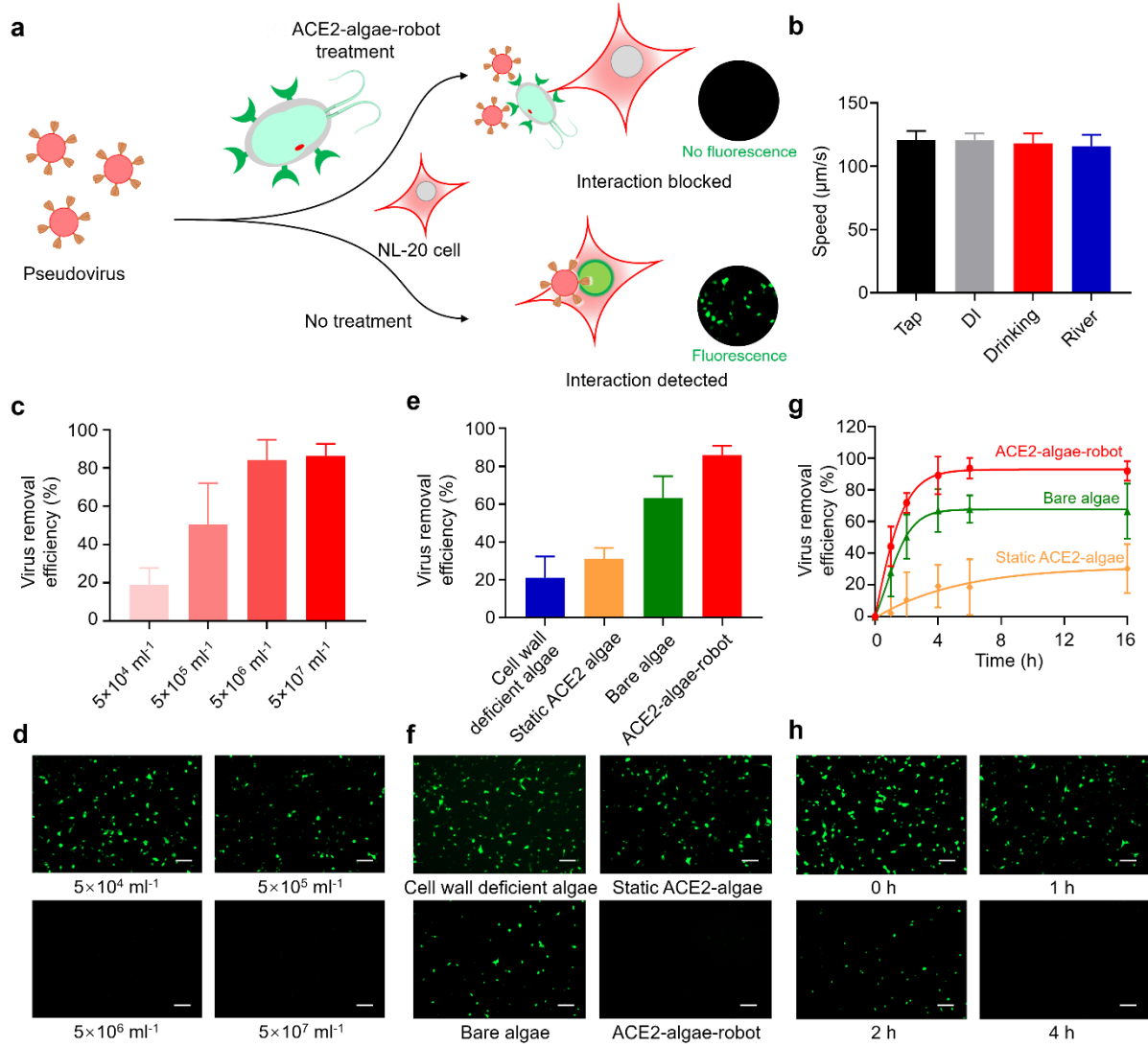


Figure 4. Use of ACE2-algae-robot for the removal of SARS-CoV-2 pseudovirus. **a.** Schematic illustrating the viral removal by ACE2-algae-robot, which blocks the virus from entering cells. Inset: Viral infection was indicated by expression of green fluorescent protein, whereas the virus treated by ACE2-algae-robot shows low viral infection. **b.** Speed comparison of ACE2-algae-robot in TAP, DI water, drinking water, and river water containing 1×10^8 VG of the virus, ($n = 3$; mean $\pm$ s.d.) **c.** Effect of ACE2-algae-robot density on the efficiency of virus removal in drinking water. **d.** Representative fluorescent images of NL-20 cells infected with virus treated by different densities of ACE2-algae-robot. Scale bar: 100 μ m. **e.** Efficiency of the virus removal in drinking water with the treatment of ACE2-algae-robot, bare algae, static ACE2-algae, or cell wall deficient algae. **f.** Representative fluorescent images of NL-20 cells infected with virus treated by ACE2-algae-robot, bare algae, static ACE2-algae, and cell wall deficient algae. Scale bar: 100 μ m. **g.** Kinetic profile of the virus removal efficiency in drinking water with the treatment of ACE2-algae-robot, bare algae, and static ACE2-algae. ($n = 3$; mean $\pm$ s.d.) **h.** Representative fluorescent images of NL-20 cells infected with the virus, which is treated by ACE2-algae-robot for different times (0 h, 1 h, 2 h, and 4 h). Scale bar: 100 μ m.

In summary, we have developed an effective biohybrid microrobotic method to actively remove the SARS-CoV-2 spike protein and pseudovirus from various water matrices using ACE2 receptor-modified microalgae. The ACE2-algae-robot can be readily fabricated by an efficient click chemistry approach, without compromising the motion behavior of algae and the function of the ACE2 receptor. The resulting ACE2-algae-robot displays excellent motion ability in various water matrices and offers considerable potential to clean contaminated water samples. The binding and removal capability of the ACE2-algae-robot were demonstrated and characterized using both SARS-CoV-2 spike protein and SARS-CoV-2 pseudovirus, resulting in 95% and 89% removal efficiency, respectively, at the experimental conditions. Given the high concentration of viral load (2×10^9 VG ml⁻¹) in our experiment, the removal efficiency of 89% and binding capability of ~ 340 VG algae⁻¹ represent a high efficacy compared to the traditional wastewater viral treatments.³² The enhanced removal of the viral spike protein and pseudovirus is attributed to the continuous self-propulsion of the ACE2-algae-robot and corresponding mixing and collision with the target protein and virus. The reusability and post-treatment of the microrobot were also studied, demonstrating considerable promise for removing waterborne pathogenic viruses from contaminated water. Although current platform can reach 89% viral removal, it is difficult to directly convert viral concentration in wastewater to disease prevalence in hosts.³³ However, any reduction of viral load would have a positive correlation with the control of viral infectivity and the illness of patients³⁴. The algae microrobots are expected to have greater mobility and longer lifetime in an open wastewater reservoir when compared to a confined test tube, and thus hold considerable potential for future scaling-up applications. Given that new emerging SARS-CoV-2 variants show a similar binding mechanism and higher binding affinity with the ACE2 receptor,³⁵⁻³⁶ the functionalized algae microrobotic platform is expected to efficiently remove different virus variants from wastewater. Overall, by relying on click chemistry to attach protein receptors onto natural algae surface, such functionalized algae-based microrobot offers an attractive strategy for a variety of environmental remediation applications.

ASSOCIATED CONTENT

Supporting Information

Experimental section and additional figures are available free of charge on the ACS Publications website.

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F. Z. and Z. L. contributed equally to this work. All authors supervised all of the experiments, discussed the results, commented on the manuscript, and revised the final manuscript.

Notes

The authors declare no competing financial interest.

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Table of Conetents (TOC) Graphic

