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Detection of SARS-CoV-2 in clinical and environmental samples using highly sensitive reduced graphene oxide (rGO)-based biosensor

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\textbf{ABSTRACT}

Quantitative RT-PCR (qRT-PCR) is the most commonly used diagnostic tool for SARS-CoV-2 detection during the COVID-19 pandemic. Despite its sensitivity and accuracy, qRT-PCR is a time-consuming method that requires expensive laboratories with highly trained personnel. In this work, on-site detection of SARS-CoV-2 in municipal wastewater was investigated for the first time. The wastewater was unprocessed and did not require any pre-filtration, prior spiking with virus, or viral concentration in order to be suitable for use with the biosensor. The prototype reported here is a reduced graphene oxide (rGO)-based biosensor for rapid, sensitive and selective detection of SARS-CoV-2. The biosensor achieved a limit of detection (LOD) of 0.5 fg/mL in phosphate-buffered saline (PBS) and exhibited specificity when exposed to various analytes. The response time was measured to be around 240 ms. To further explore the capabilities of the biosensor in real clinical and municipal wastewater samples, three different tests were performed to determine the presence or absence of the virus: (i) qRT-PCR, (ii) a rapid antigen-based commercially available test (COVID-19 Test Strips), and (iii) the biosensor constructed and reported here. Taken together, our results demonstrate that a biosensor that can detect SARS-CoV-2 in clinical samples as well as unfiltered and unprocessed municipal wastewater is feasible.

1. Introduction

Human coronaviruses have always played a role in human disease and have been associated with the common cold. However, in 2003 and 2012, Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV) and Middle East Respiratory Syndrome Coronavirus (MERS-CoV) gained the attention of researchers due to their virulence and ability to cause fatalities in seemingly healthy young adults [1–3]. While both of these viruses disappeared from the population, SARS-CoV-2 has been marked with unprecedented infectivity and spreading rate [4]. Indeed, on March 11, 2020, the outbreak of the 2019 coronavirus disease (COVID-19) was declared a pandemic by the World Health Organization. Since then, the world has witnessed substantial morbidity and mortality rates.

Up to the current COVID-19 pandemic, the main emerging infectious diseases that have caused concern in the world include the 2009 swine flu pandemic, the 2013–2016 Ebola virus disease epidemic, and the 2015 Zika virus disease epidemic [5]. In all these cases; challenges existed due to the absence of rapid and accurate molecular diagnostic tests [6]. Similarly, the COVID-19 pandemic has resulted in a substantial demand for diagnostic tests and this was a major bottleneck early in the pandemic. Quantitative RT-PCR (qRT-PCR) is often used for pathogenic identification, and remains the gold standard for the diagnosis of SARS-CoV-2 infection, due to its high accuracy and sensitivity. Other convenient diagnostic methods include enzyme-linked immunosorbent assay (ELISA), and lateral flow immunoassay tests [4]. However, certain limitations drive the need for the development of new and alternative diagnostic methods. For example, qRT-PCR requires well-equipped laboratories and highly trained personnel, with a turnaround time of...
4–6 h [6]. As such, novel diagnostic methods that are fast, accurate, sensitive and do not require capital expenditures and highly trained personnel would represent welcome tools in the quest to control the COVID-19 pandemic.

The development of biosensors for biological diagnostics is an emerging and growing area in modern science [7,8]. Generally, biosensors are defined as chemical sensors that use biochemical reactions to detect targets. Over time, electrochemical, optical, thermal, and fluorescent approaches have been used to detect various targets. For instance, Chen et al. developed a sensing methodology which combined an optical sensing technology, namely surface plasmon resonance (SPR) with the gene scissors’ clustered regularly interspaced short palindromic repeat (CRISPR). Besides achieving high sensitivity towards SARS-CoV-2, the platform also exhibited selectivity towards viral variants [9]. Similarly, Broughton et al. (2020) utilized CRISPR for the detection of SARS-CoV-2 in clinical samples, achieving a 95 % positive predictive capability, with the potential to be miniaturized in order to provide portability [12–16]. Indeed, many electrochemical biosensors have been successfully developed for the detection of numerous viruses and bacteria, and electrochemical immunosensors, in particular, have become the captivating option due to their low-cost and high sensitivities. Zhao et al. (2021) reported an electrochemical biosensor for the detection of SARS-CoV-2’s RNA with a limit of detection (LOD) of 200 copies/mL [17]. Other electrochemical biosensors focus on targeting the SARS-CoV-2’s spike (S) protein [18–20]. The spike protein is primarily responsible for the ability of the virus to bind the ACE-2 receptor on human cells, and is therefore considered important for the development of sensors [4].

Wastewater-based epidemiology (WBE) of SARS-CoV-2 is recognized as a powerful and reliable method of tracking the prevalence of the virus in the population and serves as an early-warning tool for predicting outbreaks and new waves of infections. Consequently, several countries have used qRT-PCR for the detection of SARS-CoV-2 in wastewater [21–24]. Overall, there is a strong correlation between WBE data and clinically reported COVID-19 cases in a population that produces the sampled municipal wastewater [24]. However, due to the limitations imposed by the use of qRT-PCR discussed above, the development of a biosensor that can detect SARS-CoV-2 in wastewater would bolster the efforts of WBE programs around the world. The complexity of municipal wastewater in terms of solutes and suspended solids that can interfere with the functioning of a biosensor is considered one of the challenges of developing such a biosensor. Previous studies that have attempted to develop such biosensors have been limited to synthetic wastewater spiked with their virus of interest or proteins from their virus of interest [25–27].

Recently, most research on biosensors have utilized 2D materials such as graphene, black phosphorus, transition metal dichalcogenides, ternary chalcogenides, and MXene materials [28,29]. Graphene and its derivatives, in particular, are identified as ideal platforms for surface functionalization which is often required for targetted sensing mechanisms [30]. In fact, the aforementioned electrochemical immunosensors [31] and optical biochemical sensors which are immobilized on sensor platforms through appropriate functionalization [20,31,32]. Such applications is typically be challenged by the biocompatibility of 2D materials [33]. In this study, we report the development of a reduced graphene oxide (rGO)-based biosensor for detecting SARS-CoV-2 in real municipal wastewater. The material developed in this study was designed for the selective detection of SARS-CoV-2 in clinical and raw wastewater samples. The municipal wastewater was unprocessed and did not require any prefiltration, prior spiking with virus or viral concentration in order to be suitable for use with the biosensor. The biosensor presented here is based on an electrochemical assay through a four-point Kelvin sensing system.

2. Materials and methods

2.1. Synthesis of graphene oxide (GO)

Graphite flakes were used for the synthesis of graphene oxide (GO) by the simplified Hummer method. First, a total of 3 g of graphite flakes and 9 g of KMnO4 were slowly added to H2SO4:H2PO4 solution in a 1:9 ratio. The solution was placed in an ice bath for 20 to 30 min. The mixture was then stirred for 3 days at room temperature (25 °C) to complete the oxidation reaction, which was indicated by the solution’s color change from dark purple to dark green. 35 % H2O2 was added to stop the reaction, during which a color change to bright yellow was observed. The obtained solution was washed several times with 1 M HCl followed by de-ionized water (DI) until a pH of between 5 and 6 was observed. The thick, dark-brown GO solution was stored at room temperature (25 °C). The GO concentration was determined by measuring the mass of GO after drying a specific volume of GO solution.

2.2. rGO/PBASE/antibody biosensor development

The fabricated biosensor was based on uniformly coated rGO layers. First, the Si wafer with 300 nm thermally deposited SiO2 (Ted Pella, Redding, CA) was cut into equal substrates of 20 × 20 mm². The substrate was sequentially cleaned with acetone, isopropyl alcohol (IPA), and DI water by sonication for two minutes each. Plasma treatment (PE-75 Plasma Asher, Plasma Etch, Nevada, USA) was performed for two minutes to ensure that the surface properties were tuned to become hydrophilic, increase wettability, and avoid aggregation of the solution [34]. 2 mg/mL GO solution was then spin-coated on the substrate to form uniform layers using a resist coat and develop a platform (RCD8, SUSS MicroTec, Garching, Germany). The program entered comprised three steps that run at 500, 800, and 1600 rpm for 40, 30, and 30 s respectively [35]. These increased speeds were previously proven to result in continuous and uniform GO layers. Seven layers were deposited on the substrate, with each layer followed by heating at 100 °C using a hotplate to enhance the adhesion of GO to the substrate. GO was then thermally reduced in a tube furnace (OTF-1200X, MTI Corporation, Richmond, CA) at 450 °C to form conductive rGO. Previously reported thermogravimetric analysis (TGA) data confirms that at this temperature, a stable weight loss is correlated with the loss of oxygen groups [36]. The program was first set to step the heat from room temperature to 450 °C in 60 min, then remain at 450 °C for 90 min. Specific SARS-CoV-2 S1 antibodies were immobilized on the rGO surface through 1-pyrenebutyric acid N-hydroxysuccinimide ester (PBASE, C24H17NO4; Ms:385.41 g/mol; purity ≥ 95 %, Sigma-Aldrich, Germany) linker molecules. First, the rGO coated substrate was soaked in 2 mM PBASE in methanol for one hour, rinsed with methanol, and de-ionized (DI) water. The device was then exposed to 250 μg/mL of S1 antibodies (Thermoﬁsher Cat# MA5-35940) in phosphate-buffered saline (PBS) and left overnight to allow maximum immobilization. Rinsing with PBS and DI water followed. The immunosensor was then immersed in 20 mg/mL of glycine in DI water to prevent possible nonspecific binding of the protein to unreacted PBASE molecules. Without glycine, non-speciﬁc binding is possible because PBASE has an N-hydroxysuccinimide group that can react with amino groups in protein molecules. After capping the immunosensor with glycine, it was rinsed with DI water. The prepared immunosensors were stored at 5 °C.

2.3. Detection of SARS-CoV-2 S1 protein in synthetic analyte

The SARS-CoV-2 S1 antibody recognizes the S1 protein, which contains the receptor-binding domain (RBD) of the virus. The S1 protein...
was diluted to 0.1 mg/mL in PBS, after which several concentrations were prepared using serial dilutions. A well made from 1,6-Hexanediol diacrylate (HDDA), with a size of $7 \times 7$ mm$^2$, was 3D printed using a micro-stereolithography 3D printing system (BMF S130). During testing, the well was fixed on top of the functionalized rGO/PBASE/antibody biosensor surface so that the synthetic analyte is injected into the well. Approximately 10 µL of S1 protein at varying concentrations were added to the well consecutively to determine the LOD of the biosensor. Furthermore, the prepared rGO/PBASE/antibody biosensor was also characterized in terms of selectivity toward the bovine serum albumin (BSA) protein, commercial vegan protein, glucosidase enzyme, and the SARS-CoV-2 nucleopcapsid (N) protein.

### 2.4. Detection of SARS-CoV-2 in clinical samples

To validate the effectiveness of our developed biosensor in more complex media, SARS-CoV-2 containing clinical samples were tested. Nasopharyngeal swab samples were collected from COVID-19 patients and non-infected individuals and stored in clinical transport mediums. The samples were then stored at $-20 \degree$C prior to testing. The presence of SARS-CoV-2 in the samples was determined using qRT-PCR as well as the CT value of each sample.

### 2.5. Detection of SARS-CoV-2 in wastewater samples

Testing for pre-known concentrations of SARS-CoV-2 in wastewater was possible due to the alliance of this work with a surveillance study conducted on SARS-CoV-2 in the United Arab Emirates (UAE) [24]. Municipal wastewater samples were collected from various locations across the UAE. After collection, the samples were preserved in ice and transported to the Water Intelligence Testing Center (WITC) at Khalifa University of Science and Technology (Abu Dhabi, UAE).

The wastewater samples were then tested for SARS-CoV-2 using a qRT-PCR based assay described before [24], SGTi-flex COVID-19 Ag rapid test strips, and the rGO/PBASE/antibody biosensor constructed in this study (Fig. 1). Samples were stored at 5 $\degree$C before directly being applied to the biosensor or rapid antigen testing strip. As for the qRT-PCR measurements, thermal virus deactivation was carried out at $60 \degree$C for 90 min using a water bath. Filtration was followed using 0.22 µm Millipore filters in order to remove bacteria and large sediments and particles commonly present in wastewater. 50 mL of the 0.22 µm filtrates were then concentrated using the 30 kDa molecular weight cutoff (MWCO) Ultra-30 Amicon polypropylene (PP) concentration/spin column. Through concentration, a final volume between 250 and 400 µL is obtained. After concentration, viral RNA was extracted and analyzed by qRT-PCR as described before [24].

### 2.6. rGO/PBASE/antibody biosensor material characterization

X-ray diffraction (XRD) analysis was conducted on GO and rGO samples to confirm reduction by investigating the crystal phase as well as the interlayer spacing in GO and rGO. An empyrean diffractometer (PANalytical, Germany) was used to obtain XRD patterns in the $2\theta$ ranges $5\degree$ to $50\degree$. Measurement conditions were set to scan the samples at $0.02\degree$/s, at 30 kV and 10 mA. The Raman spectra presented and discussed in this work were recorded using the Alpha 300 RA (WITec, Ulm, Germany) equipped with Confocal Fluorescence microscopy, a UHTS spectrometer (Ultra High Through Spectrometer) and a CCD camera operating in the default mode. The 532 nm excitation laser line was focused on the sample through a lens of 50x magnification. Line scans

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**Fig. 1.** Schematic diagram for the detection of SARS-CoV-2 in wastewater samples using qRT-PCR, rGO/PBASE/antibody biosensor, and rapid antigen test.
were performed on each sample using an integration time of 1 s, 50 points, and 5 accumulations. Additionally, Atomic Force Microscopy (AFM) (MFP-3D Origin instrument, Asylum Research, Santa Barbara, CA) was used to obtain surface roughness data. The topography of the rGO/PBASE/antibody biosensor was analyzed using AC-air topography mode and the average roughness was taken using a line scan. Keithley 2400 source meter and KickStart software (Keithley Instruments, Cleveland, OH) were used to conduct all electrical measurements. A silver conductive paste was used to contact the rGO layer via the four-point probe measurement configuration. The electrical stability of the sensor was verified by sweeping the voltage from –1 to 1 V in 101 steps and obtaining a constant resistance. Kickstart software was also used to obtain real-time measurements, where the current was recorded as a function of time.

3. Results

3.1. Development and characterization of the rGO/PBASE/antibody biosensor

Successful biosensor immobilization with SARS-CoV-2 S1 antibodies was confirmed via analytical and electrical characterization. Graphene oxide (GO) deposition, thermal reduction, and surface functionalization were characterized using X-ray Diffraction (XRD), Raman spectroscopy, and Atomic Force Microscopy (AFM). The XRD patterns of GO and rGO obtained are shown in Fig. 2A. The XRD pattern of GO exhibits a sharp peak at 2θ = 11.52°, while after the reduction, the peak is shifted to 2θ = 22.1°. In GO, the peak is sharp, while a broader peak is observed for rGO, which implies that the crystal phase (0 0 2) is randomly arranged in rGO compared to a high crystallization structure in GO [37]. Moreover, the observed shift is a result of a reduction in the interlayer distance within GO layers, indicating the elimination of oxygen-containing groups [38]. Fig. 2B shows the Raman spectra of GO, rGO, and rGO/1-pyrenebutyric acid N-hydroxysuccinimide ester (PBASE). The spectrum of all samples displayed two prominent peaks detected at ~1345 and ~1597 cm⁻¹ corresponding to the D and G bands, respectively. These two bands correspond to two fundamental vibrations with the D band symbolizing the disorder, while the G band correlates to the in-plane stretching of the C–C bond [37]. In addition to XRD, the reduction of GO was verified via Raman spectra with a clear shift in the G-band from 1598 to 1686 cm⁻¹, which is in line with previous reports [37]. Raman spectra also showed an increase in defects after reduction and PBASE functionalization. PBASE is a non-covalent linker commonly used in graphene-based materials. The PBASE molecule exhibits dual functionality with pyrene and succinimidyl ester groups. When the rGO film is exposed to PBASE, strong binding occurs due to π-stacking and van der Waals forces [31]. It is important to note that the chances of desorption are significantly hindered by such tight binding. The increase in defects upon PBASE functionalization is characterized by the peak ratio between the intensity of the D peak and the G peak (I_D/I_G), which is

![Fig. 2. Analytical and electrical characterization of the rGO/PBASE/antibody biosensor. (A) XRD of GO and rGO films. (B) Raman spectra of GO, rGO, and rGO/PBASE. (C) Current-voltage characteristics of the biosensor throughout functionalization. (D) AFM images and surface roughness profiles of rGO and rGO/PBASE over a 2 μm line scan.](image-url)
used as a common index to determine the density of defects. The reduction in GO to rGO increases the number of disordered phases translated by a larger $I_D/I_G$ for rGO [39]. Moreover, rGO functionalization results in an increase in defects as with chemical vapor deposition (CVD) graphene [39]. This is clearly demonstrated by the results obtained with $I_D/I_G$ increasing from 0.488 to 0.559 when moving from GO to rGO/PBASE, as shown in Fig. 2B, where a pyrene group of PBASE is bonded to the surface of rGO, resulting in some additional disorder. Furthermore, Fig. 2D reveals that the surface roughness of rGO, extracted from the AFM topography roughness surface analysis, increased significantly from 1.370 to 4.129 nm with PBASE functionalization. Finally, to confirm the presence of S1 antibody on the surface, electrical measurements were carried out. Fig. 2C shows the current–voltage (I-V) curves of the biosensor in a range from −1 to +1 V before and after attachment of S1 antibody. An increase in resistance, associated with a decrease in the slope of (dI/dV), was observed after each step. This increase in resistance confirms the successful introduction of the S1 antibody.

### 3.2. Detection of SARS-CoV-2 S1 protein

As a proof of concept, the biosensor was tested with commercial SARS-CoV-2 S1 proteins first. The biosensor was designed with a binding affinity to the SARS-CoV-2 S1 protein through functionalization. The electrochemical reaction that occurs in the presence of the S1 protein is converted into a detectable electrical signal. To evaluate the performance of the rGO/PBASE/antibody biosensor in the detection of SARS-CoV-2 S1 protein, the real-time dynamic response of the biosensor toward the introduction of increasing concentrations of S1 proteins (Fig. 3A) was investigated. 10 ul of serially diluted S1 protein solutions (0.5, 1, 1000, 10,000, and 100,000 fg/mL) were injected consecutively into the well. Since the receptor-binding domain (RBD) in the S1 protein is positively charged [40], a constant voltage of −0.8 mV was applied during the testing. The initial current value ($I_o$) and the real-time current ($I$) were used to calculate the normalized current using Eq. (1):

$$\Delta I/I_o = (I - I_o)/I_o$$ (1)

No leakage from the well was observed during the analysis. Fig. 3B shows the changes in normalized current with time upon the addition of different concentrations of S1 protein. A sharp increase in the current values suggests binding of the S1 protein to the immobilized S1 antibody, after which the value stabilized. The S1 protein was detected at concentrations as low as 0.5 fg/mL of the SARS-CoV-2 S1 protein in phosphate-buffered saline (PBS). It was observed that as the concentration of the S1 protein increases, the sharp peak current value increases. Real-time current measurements were also carried out using S1 protein concentrations below the LOD, but no changes in the current values were observed. These results indicate that our fabricated rGO/PBASE/antibody biosensor is highly sensitive with a lower LOD than those previously reported in the literature (Table 1). In addition, the biosensor’s specificity was explored by using non-specific interfering proteins. 20 µL of 0.3 mg/mL of bovine serum albumin (BSA) protein, commercial vegan protein, SARS-CoV-2 S1 protein, glucosidase enzyme, and SARS-CoV-2 N protein were injected consecutively into the test pool while measuring real-time current. As expected, the rGO/PBASE/antibody biosensor did not generate any response (i.e., the current remained

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**Fig. 3.** Biosensor response towards S1 protein. (A) Detection scheme of biosensor towards SARS-CoV-2 S1 protein. (B) Real-time response of the biosensor to different concentrations of SARS-CoV-2 S1 protein in PBS. (C) Response toward BSA, vegan, glucosidase, and SARS-CoV-2 N protein.
Data from Tables S1 and S2 were used to generate this table. Sensitivity and selectivity were found by calculating the positive and negative agreements, respectively, between the biosensor and the reference qPCR result.

| Method                                | Biomarker | Target                        | Type of Sample | Detection Limit | Detection Time | Reference |
|---------------------------------------|-----------|-------------------------------|----------------|-----------------|----------------|-----------|
| Graphene FET                          | SARS-CoV-2 spike antibody | SARS-CoV-2 spike protein | Synthetic       | 1 fg/mL          | ND             | [20]      |
| Carbon nanotube FET                   | Probe     | RdRP                          | Synthetic       | 10 fm           | ND             | [41]      |
| Surface enhanced Raman scattering biosensor | SARS-CoV-2 spike antibody | SARS-CoV-2 spike protein | Synthetic       | 0.77 fg/mL      | ND             | [42]      |
| Electrochemical impedance spectroscopy | Angiotensin-converting enzyme-2 | SARS-CoV-2 spike protein | saliva         | 6.07 fg/mL      | 4 min          | [19]      |
| Colorimetric/SERS/fluorescence triple-mode biosensor | RdRP and E gene | Target RNA | Synthetic       | 160 fim          | 40 min         | [43]      |
| Electrochemical                       | RT-LAMP reaction | SARS-CoV-2 N and ORF1ab genes | Concentrated wastewater | 2.5 x 10^6 ng/μl. | 2 h           | [44]      |
| Electrochemical                       | MB-DNA adsorption | SARS-CoV-2 N gene | Spiked wastewater | 1.7 fm | ND             | [45]      |
| Colorimetric                          | Metallopeptidase angiotensin-convertin enzyme 2 | Spike protein receptor binding domain | Spiked surface water | 5 x 10^5 copies/mL/ | <30 min      | [26]      |
| Colorimetric                          | RT-RAMP reaction | SARS-CoV-2 RNA | Spiked wastewater | 100 genome equivalent/mL | 1 h           | [27]      |
| Graphene oxide Kelvin immunosensor    | SARS-CoV-2 spike antibody | SARS-CoV-2 spike protein | Synthetic       | 0.5 fg/mL        | 240 ms         | This work |

ND: Not detected.

unchanged) after it was exposed to these non-specific proteins, with only a sharp current peak observed upon the introduction of S1 protein, its target analyte. These results confirmed the specificity of the biosensor (Fig. 3C). The response time, measured as the interval between the initial stable current and the peak current value was around 240 ms.

3.3. Validation of rGO-based biosensor for the detection of SARS-CoV-2 in real samples

3.3.1. Biosensor detection of SARS-CoV-2 in clinical samples

Validation of the developed biosensor was investigated using clinical samples. Upon collection of the clinical samples, they were maintained at a temperature of –20 °C to preserve the virus molecules. Before testing the samples using the prepared biosensor, they were brought to room temperature. The results of 19 clinical samples were collected and reported in Table S1. In Table 2, the sensitivity of the biosensor is found by calculating the positive agreement with the qRT-PCR results taken as a reference. Similarly, the selectivity is found by calculating the negative agreement with the qRT-PCR results taken as a reference. As shown in Tables S1 and 2, the biosensor detected positive clinical samples as accurately and selectively as qRT-PCR. The results obtained using the biosensor showed full agreement with those of qRT-PCR, indicating the high sensitivity and selectivity of the biosensor in a more complex medium than that investigated in Section 3.2. Given that clinical samples present a wide range of pathogens along with the target, the biosensor did generate noise signals. However, the biosensor could clearly distinguish between the negative and positive samples. When testing clinical samples using the commercially available rapid tests, only 1 out of the 3 positive samples indicated a positive result with a double line, lines on C and T (Fig. S2). As displayed in Table S3, only the clinical sample with the lowest CT value gave a positive result with the commercial rapid test. The same 3 samples were tested with the developed biosensor and all generated clear detection peaks, which were in line with the obtained qRT-PCR results.

3.3.2. Biosensor detection of SARS-CoV-2 in municipal wastewater samples

The performance of the rGO/PBASE/antibody biosensor was finally investigated in real municipal wastewater samples (Fig. 4A), which presents the most complex medium tested in this study. Autosamplers were used to collect 24 h composite wastewater samples from various locations.
locations in the UAE. Upon arrival to Khalifa University laboratories, the samples were tested for SARS-CoV-2 using qRT-PCR, rapid antigen test, and the biosensor developed in this study as described previously and summarized in Fig. 1. Given that qRT-PCR remains the gold standard in SARS-CoV-2 detection, it was used as a standard against which the rapid antigen test and the rGO/PBASE/antibody biosensor results were compared to. Initially, SARS-CoV-2 concentrations in 24 h composite municipal wastewater samples collected from 23 different locations in the UAE were determined by qRT-PCR. The same samples were tested without any prior treatment using the biosensor developed in this study. The obtained results of 90 wastewater samples are summarized in Table S2. As shown in Table 2, the biosensor correctly identified 65 positive samples out of 70 actual positive samples, as determined by qRT-PCR. Thus, a positive agreement of 92.9 % is obtained indicating the sensitivity. Similarly, the biosensor could distinguish the absence of SARS-CoV-2 in 16 negative samples out of 20 actual negative samples, as determined by qRT-PCR.

Subsequently, the samples with the highest concentrations (wastewater samples 86 through 90) were also tested using the rapid antigen based lateral flow tests. The rapid antigen tests failed to detect the virus in all the wastewater samples tested, including the highest concentration (104.79 copies/mL) (Table 4). It is important to acknowledge that municipal wastewater samples are complex, with many solutes that could interfere with such assays. The rapid antigen tests failed to detect SARS-CoV-2 in all the municipal wastewater samples that we tested. In comparison, rapid commercial antigen based tests failed to detect SARS-CoV-2 in all the municipal wastewater samples that we tested.

Several analytical features were used to compare the performance of the reported rGO-based biosensor to similar methods previously reported in literature (Table 1). Those include the biosensor type, biomarker used, analyte targetted, samples tested, and the method’s sensitivity. In comparison, our biosensor demonstrated the highest sensitivity with a very rapid response. Moreover, the robustness of our biosensor was investigated against several randomly chosen wastewater samples. All the acquired results were compared to those obtained by qRT-PCR, ensuring the reliability of our biosensor. Our prototype is cost-effective and can be used at room temperature, with minimal equipment and reagents. Importantly, our results demonstrate that a biosensor that can detect SARS-CoV-2 in unfiltered and unprocessed municipal wastewater is feasible. The biosensor described here is highly scalable and its fabrication could be automated for mass production. Not only could the biosensor be used at the point-of-care or in the field, but its design could also be modified for integration into wastewater pipelines. This would allow appropriate control strategies to be taken in time to prevent further outbreaks of the disease. Furthermore, the fabrication procedure described is highly modular and could be used to rapidly diagnose future pandemic outbreaks. The biosensor could also be designed to allow for multipurpose detection of emerging harmful pathogens in wastewater pipelines, thereby addressing the urgent need of mass testing entire populations.

4. Conclusion

In this study, we report the development of a highly sensitive, selective and rapid biosensor for the detection of SARS-CoV-2 in clinical and raw wastewater samples. Our proposed prototype does not require prior filtration or concentration of wastewater for the detection of SARS-CoV-2. Self-prepared GO was spin coated and thermally reduced to form conductive rGO films that serve as sensing platforms for the immuno-sensor. The device is based on a simple four terminal Kelvin sensor that allows real time data to be obtained and analyzed. The biosensor responded to as low as 0.5 fg/mL of S1 protein in PBS with a response time of an average of 240 ms. The biosensor also exhibited specificity when exposed to various analytes including BSA, commercial vegan protein, glucosidase enzyme, and SARS-CoV-2 nucleocapsid protein. It is also worth noting that our biosensor showed high sensitivity towards untreated wastewater samples, making it an ideal candidate for the development of instruments that can be used in the field. qRT-PCR was used to quantify the SARS-CoV-2 concentration in wastewater, and is considered the gold standard in diagnostics and detection of SARS-CoV-2. The biosensor prototype outperformed the rapid antigen test in identifying wastewater samples that were confirmed to contain SARS-CoV-2 through qRT-PCR. The prototype was able to identify positive samples with a sensitivity and selectivity of 92.9 % and 80 %, respectively. In comparison, rapid commercial antigen based tests failed to detect SARS-CoV-2 in all the municipal wastewater samples that we tested.

Table 4
Comparison of results of wastewater samples tested using qRT-PCR, the reported biosensor, and a commercial rapid test

| Sample | Concentration (copies/mL) | qRT-PCR | rGO/PBASE/anti | Commercial rapid test |
|--------|---------------------------|---------|----------------|----------------------|
| WWS86  | 18.95                     | +       | +              | −                    |
| WWS87  | 19.38                     | +       | −              | −                    |
| WWS88  | 34.03                     | +       | +              | −                    |
| WWS89  | 39.17                     | +       | +              | −                    |
| WWS90  | 104.79                    | +       | −              | −                    |

Fig. 4. Biosensor response toward real municipal wastewater samples. (A) Schematic diagram of municipal wastewater sample analysis using qPCR, rapid antigen tests or the biosensor described in this report. (B) Real-time response of biosensor to wastewater sample 46 with a viral concentration of 2.91 copies/mL. (C) Real-time response of the biosensor to wastewater samples 12 (negative) and 89 (positive). The rapid antigen tests tested negative in both cases (picture above the curve).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

All data generated or analysed during this study are included in this
Acknowledgment

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cel.2022.139750.

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