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Electrical biosensing system utilizing ion-producing enzymes conjugated with aptamers for the sensing of severe acute respiratory syndrome coronavirus 2

Akira Nukazuka^{a,*}, Satomi Asai^b, Kei Hayakawa^a, Kazuhisa Nakagawa^a, Mana Kanazashi^a, Hidefumi Kakizoe^b, Kyoko Hayashi^c, Toshio Kawahara^c, Kazuaki Sawada^d, Hitoshi Kuno^a, Kazuhiko Kano^a

^a DENSO Corporation, 1-1 Showa-cho, Kariya, Aichi 448-8661, Japan

^b Department of Laboratory Medicine, Tokai University School of Medicine, 143 Shimogastuya, Isehara, Kanagawa 259-1193, Japan

^c College of Life and Health Sciences, Chubu University, 1200 Matsumotocho, Kasugai, Aichi 487-0027, Japan

^d Department of Electrical and Electronic Information Engineering, Toyohashi University of Technology, Hibarigaoka-1-1 Tenpakucho, Toyohashi, Aichi 441-8122, Japan

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ABSTRACT

Viral outbreaks, which include the ongoing coronavirus disease 2019 (COVID-19) pandemic provoked by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), are a major global crisis that enormously threaten human health and social activities worldwide. Consequently, the rapid and repeated treatment and isolation of these viruses to control their spread are crucial to address the COVID-19 pandemic and future epidemics of novel emerging viruses. The application of cost-efficient, rapid, and easy-to-operate detection devices with miniaturized footprints as a substitute for the conventional optic-based polymerase chain reaction (PCR) and immunoassay tests is critical. In this context, semiconductor-based electrical biosensors are attractive sensing platforms for signal readout. Therefore, this study aimed to examine the electrical sensing of patient-derived SARS-CoV-2 samples by harnessing the activity of DNA aptamers directed against spike proteins on viral surfaces. We obtained rapid and sensitive virus detection beyond the Debye length limitation by exploiting aptamers coupled with alkaline phosphatases, which catalytically generate free hydrogen ions which can readily be measured on pH meters or ion-sensitive field-effect transistors. Furthermore, we demonstrated the detection of the viruses of approximately 100 copies/ μ L in 10 min, surpassing the capability of typical immunochromatographic assays. Therefore, our newly developed technology has great potential for point-of-care testing not only for SARS-CoV-2, but also for other types of pathogens and biomolecules.

1. Introduction

The coronavirus disease 2019 (COVID-19) pandemic has remained a major global threat. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which is the causal agent [1,2], is highly contagious, posing a major public health challenge and socioeconomic burden in many countries [3,4].

SARS-CoV-2 is an enveloped virus with a positive-sense RNA genome, which encodes four major structural proteins: envelope (E), matrix (M), nucleocapsid (N), and spike (S) proteins [5]. The S protein is a large type-I transmembrane protein with a homotrimeric form, with each protomer containing two functional subunits, S1 and S2. The S1

subunit contains the receptor-binding domain (RBD), which engages angiotensin-converting enzyme 2 (ACE2) as the cognate receptor on host cells [6–12], while S2 plays a role in membrane fusion on invasion into the host [13,14]. Therefore, the S protein is pivotal in determining the infectivity of the virus to the host.

Many individuals with SARS-CoV-2 infection experience respiratory and gastrointestinal complications with symptoms such as fever, cough, vomiting, and diarrhea [15–19]. However, other infected individuals are asymptomatic or minimally symptomatic [20,21]. One major challenge in managing this pandemic is that asymptomatic individuals are at risk of becoming silent spreaders, which increases the number of COVID-19 cases [22,23]. Therefore, it is important to conduct accurate and

* Corresponding author.

E-mail address: akira.nukazuka.j8y@jp.denso.com (A. Nukazuka).

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frequent testing of individuals, irrespective of symptoms, to maintain the viral transmission under surveillance and apply appropriate preventive measures [24,25].

Currently, real-time quantitative polymerase chain reaction (RT-qPCR) and enzyme-linked immunosorbent assay (ELISA)-based antigen tests are the standard techniques for detecting SARS-CoV-2 and other pathogens in clinical diagnostics [26]. These traditional methods depend on measurement of optic signals, which requires large and costly devices for signal readout, thereby rendering them inadequate for repeated diagnosis. Notably, in terms of realizing these methods in point-of-care diagnostics, semiconductor-based electrical biosensor platforms, which include ion-sensitive field-effect transistors (ISFETs), have gained much attention because of their small size and low cost [27–31]. Despite their great potential, many attempts to employ ISFETs to detect analytes have shown poor sensitivity and reproducibility, mainly because of the inherent limitations associated with the Debye–Hückel theory [32]. For a solution-gate ISFET to effectively change the carrier density in response to the analytes, the balance of positive and negative charge densities near the planar surface of the ISFET gate insulator, which is the effective sensing distance, needs to be disturbed by the potential variations caused by molecular adsorption/desorption events on the surface. According to this theory, the distance, which is designated as the Debye length, decays exponentially with the distance from the ISFET surface and is <1 nm in typical physiological fluids [33,34], considerably smaller than that of most biological analytes, including proteins and nucleic acids. Furthermore, some analytes with few or no net charges cannot disturb the ISFET transconductance within the Debye length [35], which makes this platform impractical for their measurement.

We hypothesized that the biosensing capability of an ISFET could be extended beyond these obstacles by using freely dispersed charged ions as intermediate reagents that convert the target analyte information into electrical signals sensed by an ISFET. Our technique employed a DNA aptamer as the analyte recognition moiety. The aptamer is directed against the S protein of SARS-CoV-2 [36]. We engineered an aptamer to be tethered to alkaline phosphatase (ALP), which catalyzes the enzymatic hydrolysis of its substrates to concomitantly produce hydrogen ion (H^+) byproducts. Instead of immobilizing the aptamers onto the surface of the ISFET gate, enzyme-conjugated aptamers were suspended and mixed with SARS-CoV-2 in suspension to increase their binding and enhance the resultant output intensity [37]. Enzymatic catalysis was performed after the bound-free separation of the aptamers, and the H^+ ions produced were readily measured using ISFET and portable pH meters. In this study, we were able to detect SARS-CoV-2 in clinical specimens of patients hospitalized with COVID-19. In this proof-of-concept experiment, we obtained a limit of detection of approximately 100 copies/ μ L in 10 min, which surpassed the capability of standard immunochromatographic assays. Our technology could be employed to detect other types of pathogens and biomolecules by replacing the S protein-binding aptamers with their equivalent binding counterparts while using the same enzymes and platforms. Additionally, using enzyme-derived diffusing ions as signals provides the advantage of signal amplification over the time course, generating a very low detection limit and good reliability. Furthermore, it is suitable for the

industrial mass production of sensing devices that are reliable and cost-effective since the devices are free from complicated downstream processes, such as chemical functionalization with aptamers or other organic compounds.

2. Material and methods

2.1. Reagents and materials

Previously reported DNA aptamers targeting the RBD, S1 region, or trimer of the SARS-CoV-2 S glycoprotein were employed in this study [36,38–41]. Table 1 shows the aptamer names and their sequences. DNA aptamer oligomers with 5'-biotin modifications were synthesized and high-performance liquid chromatography (HPLC)-purified using Eurofins Genomics K.K. (Tokyo, Japan). Bovine serum albumin (BSA), streptavidin, sodium bicarbonate ($NaHCO_3$), sodium carbonate (Na_2CO_3), magnesium chloride ($MgCl_2$), phosphate-buffered saline (PBS), phosphate-buffered saline with 0.1% (v/v) tween-20 (PBS/T), ammonium molybdate, L(+)-ascorbic acid, sulfuric acid, and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) were purchased from Fujifilm Wako Pure Chemical Corporation (Osaka, Japan). p-Nitrophenyl phosphate (pNPP) and 4-methylumbelliferyl phosphate (4-MUP) were purchased from the Tokyo Chemical Industry (Tokyo, Japan). Additionally, CDP-Star® chemiluminescent substrate and 0.65 μ m Durapore® polyvinylidene fluoride (PVDF) membranes were purchased from Merck Millipore (Darmstadt, Germany). N-cyclohexyl-3-aminopropanesulfonic acid (CAPS) was purchased from Dojindo Laboratories (Kumamoto, Japan). Streptavidin-coupled ALP and avidin agarose were purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA). The S ectodomain proteins of SARS-CoV-2 were purchased from Sino Biological (Beijing, China). Alpha-amylase was purchased from Lee Biosolutions (Maryland Heights, MO, USA). Furthermore, biotinylated anti-SARS-CoV-2 RBD monoclonal antibodies were purchased from AcroBiosystems (Newark, DE, USA). Ultraviolet (UV)-inactivated SARS-CoV-2 was procured from ZeptoMetrix (Buffalo, NY, USA), and Nanosep® centrifugal devices (10 and 300 kDa molecular weight cutoffs) were purchased from Pall Corporation (Port Washington, NY, USA). The ELISA plates and the viral RNA extraction and RT-qPCR reagent kits were purchased from Sumitomo Bakelite (Tokyo, Japan) and Qiagen (Venlo, Netherlands), respectively.

2.2. Preparation of the DNA aptamer-ALP conjugates

5'-biotinylated DNA aptamer oligomers at a concentration of 20 μ M in PBS/T + Mg suspension (137 mM NaCl, 8.1 mM Na_2HPO_4 , 2.68 mM KCl, 1.47 mM KH_2PO_4 , 0.1% [v/v] Tween-20, and 3 mM $MgCl_2$ [pH 7.4]) were heated at 95 °C for 5 min, followed by gradual cooling to 25 °C for 60 min to form their distinct secondary structures. Next, 1 μ M of streptavidin-coupled ALPs was mixed with a 10-fold stoichiometric molar excess of DNA aptamer oligomers in PBS/T + Mg, and the streptavidin-biotin biochemical process was allowed to proceed with gentle agitation at 25 °C for 60 min. Subsequently, the unreacted oligomers were removed from the reaction by applying the mixture to an avidin agarose resin and discarding the precipitated resin. Finally, the

Table 1
Aptamer names, the sequences, and their citations used in this study.

Aptamer name	Sequence (5' ... 3')	Length (nt)	Reference
RBD-1C	CAGCACCAGCCTTGTGCTTTGGGAGTGCTGGTCCAAGGGCGTTAATGGACA	51	[36]
RBD-4C	ATCCAGAGTGACGCAGCATTTTCATCGGGTCCAAAAGGGGCTGCTCGGGATTGCGGATATGGACACGT	67	[36]
SNAP1.66	TTTTTCGCTTCTTCGCGGTGCTTGTGCATCCTGACTGACCCTAAGGTGCGAACATCGCCCGCTAAGTCCG	71	[38]
SP6.51	TTTTTGATATCAACCCATGGTAGGTATTGCTTGGTAGGGATAGTGGGCTTGATGTT	56	[39]
S1-A1	TTTTTAGCAGCACAGAGGTGAGATGCCGACAGGACGCTGACATTAGTCTCTATCCGTGACGGTATGCCTATGCGTGCTACCGTGAA	85	[41]
MSA1-T1	TTTTTGTGCTACTCCCACTTTCCGGTTAATTTATGCTCTACCCGTCCACCTACCGGAAGCATCTC	65	[40]
MSA5-T3	TTTTTCTCCACGGGTTTGGCGTGGGCGCTGGCGGGGGGATAGTGCCTGGAG	52	[40]

Abbreviation: nt, nucleotides.

concentration of the resultant conjugates was measured by absorbance at 260 nm and was adjusted to 2 μ M in PBS/T + Mg resuspension for downstream applications. Conjugation of the biotinylated anti-S protein antibody with ALPs was performed using the same procedure.

2.3. Characterization of the DNA aptamer- or antibody-ALP conjugates for binding to their target antigens

First, the SARS-CoV-2 S trimeric proteins, the alpha-amylase proteins, or the UV-inactivated SARS-CoV-2 viruses were directly immobilized onto the polystyrene surface of the ELISA titer plate by passive adsorption in 0.1 M Na₂CO₃/NaHCO₃ buffer (pH 9.6) at 4 °C overnight. After three consecutive washes with PBS/T, the wells were blocked with 3% (w/v) BSA in PBS/T. Next, the antigens were probed with 10 nM of the DNA aptamer- or antibody-ALP conjugates in PBS/T + Mg at 25 °C for 1 h, followed by three washes with PBS/T + Mg. Subsequently, enzymatic activity was measured by hydrolyzing 0.25 mM CDP-Star® in a substrate solution containing 0.1 M Na₂CO₃/NaHCO₃ buffer and 1 mM MgCl₂ (pH 9.6), using a chemiluminescent assay. Chemiluminescent signals were recorded using Cytation™ 3 (Biotek). Additionally, negative control experiments without antigen immobilization were performed in a similar manner. Data were obtained from three or four independent experiments, and the differences in signal intensities were evaluated using paired *t*-tests.

2.4. Determination of kinetic parameters of ALP

The kinetic parameters, including affinity of the substrate to the active site of the enzyme (K_m), maximum rate of reaction (V_{max}), and rate constant for the catalytic conversion of substrate into product (k_{cat}), of ALPs conjugated with the RBD-1C aptamer [36] were determined for four types of substrates: pNPP, BCIP, 4-MUP, and CDP-Star®. Briefly, 1 nM of the RBD-1C aptamer-ALP conjugates were incubated at 37 °C for 15 min in a solution containing 0.1 M Na₂CO₃/NaHCO₃ buffer (pH 9.6) and 1 mM MgCl₂, with the final concentration of each substrate ranging from 0.23 mM to 2.50 mM. After the enzymatic reaction was terminated, the concentration of inorganic phosphate ions liberated from each solution was quantified using the conventional colorimetric molybdenum blue method [42]. Finally, the approximate K_m and V_{max} values were determined using KaleidaGraph 4.0 (Synergy Software, Reading, PA, USA).

2.5. Preparation of SARS-CoV-2

Nasopharyngeal specimens were collected from outpatients and inpatients with COVID-19 at the Tokai University Hospital. First, the swab was transferred to 2 mL of PBS and vortexed to retrieve the SARS-CoV-2 specimen. Next, an aliquot of the viral specimen was used to determine its viral copy number using the RT-qPCR. After determining the viral load by RT-qPCR, another aliquot of the residual specimen was used to perform a 10-fold serial dilution of SARS-CoV-2 in PBS/T + Mg, which contained 0.5% (w/v) BSA, from 10¹ to 10⁶ viral copies/ μ L. The prepared viral series were filtered through 0.65- μ m pore size membranes to remove cells and cellular debris. All investigations, which involve infectious SARS-CoV-2 specimens, were undertaken at the Tokai University Medical School Biosafety Level 2 (BSL2) laboratory. This study was approved by the Review Board of Tokai University (approval number: 20R-052).

2.6. Real-time quantitative PCR

RT-qPCR of the SARS-CoV-2 genome was conducted following the protocols of the Japanese National Institute of Infectious Diseases [43]. Briefly, 140 μ L of the nasopharyngeal specimen from the 2-mL PBS suspension was used to extract the viral genome RNA with the QIAamp Viral RNA Mini Kit (Qiagen), which was then collected in 60 μ L of the

elution buffer. Next, 5 μ L of the eluted RNA sample was evaluated for viral load using the QuantiTect® Probe RT-PCR Kit (Qiagen) on a LightCycler® 480 System II (Roche Diagnostics). The nucleocapsid gene loci were amplified using the N set no.1 and 2 primer sets. Thermal cycling was performed at 55 °C for 10 min for reverse transcription, followed by 95 °C for 3 min, and then 45 cycles of 95 °C for 15 s and 58 °C for 30 s. Quantification was performed according to the standards.

2.7. pH meter-based SARS-CoV-2 assay

First, aliquots of 25 μ L of each viral preparation were added to an equal volume of the 200 nM DNA aptamer-ALP conjugates for the binding reaction with gentle shaking at 25 °C for 10 min. Second, unbound aptamer-ALP conjugates were removed through ultrafiltration with a 300 kDa cutoff filter out of the fraction containing SARS-CoV-2 with the conjugates attached to them. Third, the cutoff filter was washed thrice with PBS/T to further remove the unbound conjugates. Fourth, 500 μ L of a prewarmed substrate solution, containing 10 mM pNPP, 1 mM CAPS, and 1 mM MgCl₂ (pH 10.5), was added to the viral fraction and incubated at 37 °C for 10 min. Finally, the ALP enzymatic reaction was terminated by separating the virus-loaded conjugates through a 10 kDa cutoff filter. The resultant pH of the retrieved solution was measured using a LAQUAtwin pH-33 pH meter (Horiba, Kyoto, Japan). Data were obtained from at least five independent experiments, and the differences in pH changes were evaluated using paired *t*-tests.

3. Results and discussion

3.1. Selection of the enzyme-conjugated aptamers with the ability to bind to the spike glycoproteins of SARS-CoV-2

Immunoassays using a combination of selective aptamers and signaling enzymes typically require a two-step reaction. They usually comprise probing the targeted molecules with end-modified aptamers and conjugating the aptamers with the enzymes, which are followed by visible detection through enzymatic activity. In this study, conjugation between aptamers and enzymes was performed before the target-binding procedure to realize a simpler and faster detection of targets without requiring multiple labeling steps. The following previously reported DNA aptamers that have been shown to have an affinity to the S glycoproteins of SARS-CoV-2 (S proteins), all of which were 5'-biotinylated, were examined: RBD-1C, RBD-4C [36], SNAP1.66 [38], SP6.51 [39], S1-A1 [41], MSA1-T1, and MSA5-T3 [40]. Commercially available calf intestinal ALPs (EC 3.1.3.1), which are coupled with streptavidin, were conjugated with biotinylated aptamers. Following the immobilization of recombinant S proteins onto the titer-well surface, the resultant conjugates bound to the S proteins were measured, using a chemiluminescent assay that detected intensity on hydrolysis of the CDP-Star® ALP substrate. The conjugates of anti-S protein antibodies with ALPs were prepared and assayed similarly. The conjugation of single aptamers or antibodies alone did not affect the catalytic activity of ALPs (Supplementary Fig. S1).

Among the tested conjugates, ALP-conjugated aptamers, which include RBD-1C, RBD-4C, and MSA5-T3, and the conjugated antibodies, were shown to bind to the S proteins (Fig. 1a). Unexpectedly, the other aptamer-ALP conjugates did not exhibit obvious binding to the S proteins (Fig. 1a). These non-binding aptamers may be susceptible to steric hindrance upon conjugation with streptavidin and/or ALPs, which may attenuate their affinity to the S proteins. Alternatively, the immobilization of S proteins onto titer wells may mask their epitope regions, which would otherwise be targeted by these aptamers. Streptavidin-coupled ALPs alone did not bind to the proteins. Additionally, none of the tested conjugates showed an affinity for alpha-amylase proteins when used as negative controls. Because the RBD-1C aptamer-ALP conjugates showed the strongest binding to the S proteins, we used them in the downstream experiments. The excess amount of unmodified free

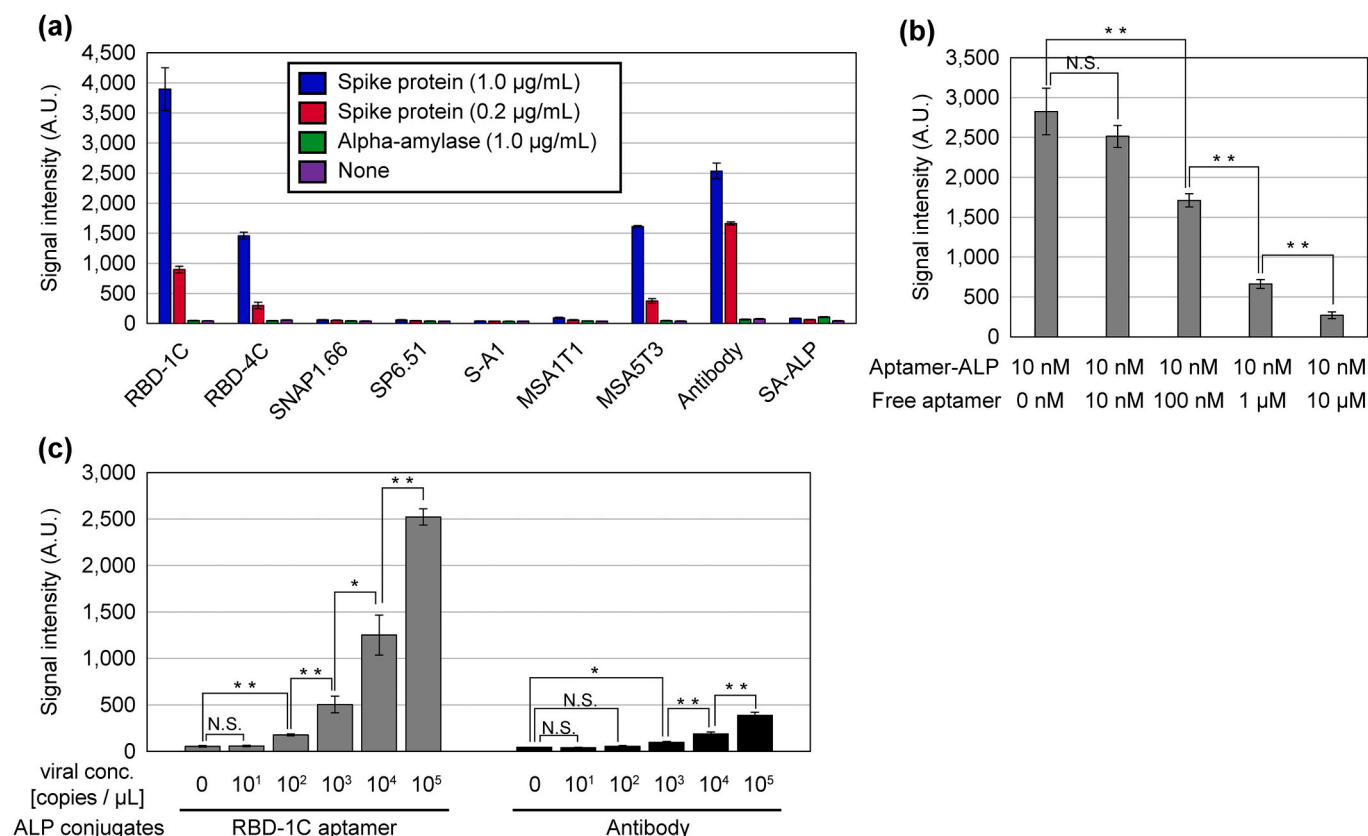


Fig. 1. Binding of the receptor-binding domain 1C aptamer conjugated with alkaline phosphatases to the spike proteins of SARS-CoV-2.

(a) Binding assay of different kinds of the aptamer- and the antibody-ALP conjugates to the indicated proteins immobilized onto the surface of titer wells. The graphs show the means $\pm$ SD of three independent experiments.

(b) Competitive binding assay of the RBD-1C aptamer-ALP conjugates with excess free aptamers. The conjugates and free aptamers were mixed to the indicated concentrations, which were then subjected to binding to the immobilized spike proteins. The graphs show the means $\pm$ SD of five independent experiments.

(c) Binding assay of the RBD-1C aptamer- and the antibody-ALP conjugates to the inactivated SARS-CoV-2. The concentration of the viruses varied from 10^1 to 10^5 copies/ μ L. The graphs show the means $\pm$ SD of four independent experiments.

The enzymatic activity was measured for (a)–(c). The P-values were calculated using paired *t*-tests. * $P < 0.005$; ** $P < 0.001$; N.S., no significant difference ($P > 0.05$).

Abbreviations: ALP, alkaline phosphatase; RBD, receptor-binding domain; SA-ALP, streptavidin-coupled alkaline phosphatase; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; SD, standard deviation

RBD-1C aptamers combined with the RBD-1C aptamer-ALP conjugates competitively interfered with the catalytic activity of ALPs (Fig. 1b), which confirms that the aptamers play a role in target recognition.

Next, we conducted a binding experiment of ALP conjugates with RBD-1C aptamers or antibodies against UV-inactivated SARS-CoV-2. Interestingly, we observed a more noticeable binding to inactivated viruses with the aptamer conjugates than with the antibody counterpart (Fig. 1c). The limit of detection was 100 copies/ μ L when the RBD-1C aptamer-ALP conjugates were used, which was 10 times lower than that when the antibody-ALP conjugates were examined. Due to the tetrameric nature of streptavidin and the smaller size of the aptamers compared with antibodies, the conjugated aptamers with streptavidin-coupled ALPs may confer architectural multivalency to the aptamers, which potentially increases their avidity and elevates binding to the densely positioned S proteins on the surface of SARS-CoV-2 [44]. Therefore, aptamers may be more advantageous than conventional antibodies for detecting the S proteins of intact SARS-CoV-2.

3.2. pH response by the catalytic activity of alkaline phosphatases

ALPs act on a wide variety of phosphate compounds to catalyze the release of inorganic phosphate, which then ionizes in the solution to produce H^+ ions. We compared the commonly used ALP substrates, which include pNPP, BCIP, 4-MUP, and CDP-Star®, for their ability to

generate inorganic phosphates to understand an appropriate substrate compound for electrical sensing of the targets and thereby estimate H^+ ion production. To determine the kinetic parameters for each compound, the RBD-1C aptamer-ALP conjugates were mixed with varying concentrations of the compound; the released inorganic phosphates were quantified using the colorimetric molybdenum blue method (Supplementary Fig. S2). The calculated kinetic values are given in Table 2. The examined substrates yielded comparable amounts of inorganic phosphate, as is evident from the k_{cat} values. Therefore, considering its commercial availability, pNPP was selected as the substrate material for our system.

Next, we conducted a proof-of-concept investigation to assess whether the RBD-1C aptamer-conjugated ALPs reduced the pH through the hydrolysis of pNPP. The reaction was performed in a minimally buffered solution containing 1 mM CAPS to minimize quenching of the released H^+ ions through the buffering effect. After adding the conjugates at final concentrations of 1, 10, and 100 pM, the pH of the solution was measured every 10 min. The reduction in pH in the presence of the conjugates was concentration-dependent (Fig. 2), which is consistent with the assumption that ALPs produce H^+ ions. The lower limit of the conjugate that yielded a pH change beyond the measurable performance of the meter was 1 pM. Aptamer-conjugated streptavidin alone without ALP coupling had little effect on the solution pH. The slight reduction in pH in the absence of ALP may be attributable to dissolved atmospheric

Table 2

Kinetic parameters of the receptor-binding domain 1C aptamer-alkaline phosphatase conjugates for several alkaline phosphatase substrates.

Parameter	Substrate			
	pNPP	BCIP	4-MUP	CDP-Star®
K_m (mM)	3.14 ± 0.26	1.53 ± 0.07	1.39 ± 0.14	3.61 ± 0.16
V_{max} ($\mu\text{M min}^{-1} \text{ nM}^{-1}$)	224 ± 12	143 ± 4	143 ± 8	252 ± 8
k_{cat} ($\text{sec}^{-1} \times 10^3$)	3.73	2.39	2.39	4.20
k_{cat}/K_m ($\text{sec}^{-1} \text{ M}^{-1} \times 10^6$)	1.19	1.56	1.72	1.16

The approximate K_m and V_{max} values were estimated by quantifying the concentration of the free inorganic phosphates, as described in Supplementary Fig. S2.

Abbreviations: 4-MUP, 4-methylumbelliferyl phosphate; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; k_{cat} , rate constant for the catalytic conversion of substrate into product; K_m , affinity of the substrate to the active site of the enzyme; pNPP, *p*-Nitrophenyl phosphate; V_{max} , maximum rate of reaction.

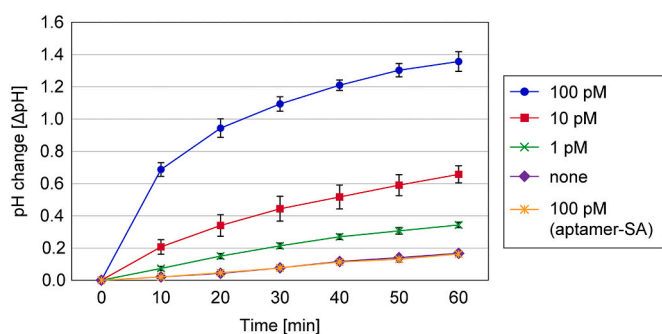


Fig. 2. Time course of pH changes generated by the receptor-binding domain 1C aptamer-alkaline phosphatase conjugates.

The RBD-1C aptamer-ALP conjugates at final concentrations of 1, 10, or 100 pM were mixed with pNPP substrate, and the resultant pH changes were recorded every 10 min. The graphs represent the mean $\pm$ SD of three independent experiments.

Abbreviations: ALP, alkaline phosphatase; pNPP, *p*-Nitrophenyl phosphate; RBD, receptor-binding domain; SD, standard deviation

carbon dioxide during measurement. Therefore, we confirmed that ALPs function as H^+ ion-producing agents.

3.3. SARS-CoV-2 assay

Having established the roles for the RBD-1C aptamers as S protein-binding moieties and ALPs as H^+ ion-producing machinery, we proceeded to demonstrate the applicability of the conjugates to detect SARS-CoV-2 electrically. Nasopharyngeal clinical specimens from patients who are positive for COVID-19 were provided for SARS-CoV-2 retrieval. Next, the viral copy number was determined using RT-qPCR for diagnosis before this study. After that, the residual specimen was provided and serially diluted to obtain the viral isolates at concentrations of 10^1 to 10^6 viral copies/ μL .

Fig. 3a schematically depicts the operational flow of the SARS-CoV-2 biosensor. An aliquot from each viral series was added to an excess aptamer-ALP conjugate (to a final concentration of $1 \mu\text{M}$) for the binding reaction with gentle shaking for 10 min. Next, unbound aptamer-ALP conjugates were washed and removed through ultrafiltration from the fraction containing SARS-CoV-2 bound to the conjugates. Subsequently, the prewarmed substrate solution, which contains pNPP with minimal buffering capacity, was added to the viral fraction and incubated for 10 min at 37°C . Finally, the reaction was terminated by removing the virus, and the resulting pH of the solution (hereafter, virus-reacted solution) was measured.

We used a portable pH meter to measure the pH change (LAQUAtwin pH-33, Horiba). First, the entire electrode was calibrated before measurement by immersion in a substrate solution without aptamer-ALP conjugates. After the 5-min calibration, the substrate solution was removed from the electrode surface, and the virus-reacted solution was dropped to record its pH. Notably, the pH of the solution decreased after the drop of the virus-reacted solution (Fig. 3b). The pH decreased as the viral copy number increased. In this experiment, the detection limit was 100 viral copies/ μL (Fig. 3b), which corresponds with the current detection limit for diagnosis in clinical settings. As the enzymatic response increased with a longer incubation time (Fig. 2), it is likely that the viral detection limit could be enhanced by incubating the virus-loaded aptamer-ALP conjugates for a longer time. We also found that the pH change was almost saturated at a viral concentration of 10,000 viral copies/ μL or above (Fig. 3b). This is attributable to the attenuated ALP activity at pH levels below its optimum. As the enzymatic reaction velocity as a function of time increases according to the viral load, installing a real-time pH monitoring setup, rather than our current endpoint measurement, would facilitate detection with a broader dynamic range.

In summary, this study was a proof-of-concept series of experiments to demonstrate the availability of amplifying H^+ ions as electrical signaling media. Remarkably, H^+ ion-producing ALPs were conjugated with aptamers against SARS-CoV-2 for their binding in suspension. This trend significantly contrasts with findings of recent reports on label-free SARS-CoV-2 detection that commonly utilizes aptamers, wherein both binding and signaling events occur on the two-dimensional surface of sensor electrodes [45–48]. Although our procedure still requires binding and washing steps before the electrical readout, we believe this is practically valuable for repeatable, recyclable, reliable measurement as an alternative to label-free systems, which require chemical modification of the sensor surfaces. Furthermore, freely dispersed charged ions, which are intermediate signals, enable sensitive and rapid detection of targets beyond the Debye length constraint. The results were consistent with those of the proton-ELISA immunoassay platform, which is highly sensitive at detecting antigen using H^+ -producing glucose oxidases conjugated to antibodies [49]. In this study, the S proteins on the surface of SARS-CoV-2, which are responsible for viral infection, were the targets. According to a recent report on a proposed aptamer sensor [50], detecting the S proteins on intact viruses has the potential to discriminate between infectious and noninfectious viruses, which is difficult using conventional PCR or N-antigen tests. Therefore, such technological advancement could significantly facilitate precise medical care diagnosis and isolation measures in the future.

4. Conclusions

In this proof-of-concept study, the proposed detection technology was developed using a novel combination of aptamer recognition moieties, ion-producing enzymes, and electrical sensing platforms. Compared to antibodies, small aptamers are highly avid when conjugated with scaffolds such as multimer-forming streptavidin or enzymes, rendering them particularly applicable to detecting densely assembled targets, including membrane-bound targets and cellular or viral capsid proteins [44]. An important limitation of this method is that it requires the use of costly pH meters, which are not suitable for extensive clinical trials. However, the platform can be extended to a semiconductor-based ISFET. Due to the scalability and cost-effectiveness of semiconductor manufacturing technology, ISFET-based devices could easily be reproducibly mass-produced as compact units at low cost. Additionally, aptamers can be rapidly and flexibly replaced or improved over iterative molecular evolution cycles; therefore, our system has the potential to rapidly adjust to other pathogens and biomolecules and is not limited to the detection of newly emerging viral species.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.sbsr.2023.100549>.

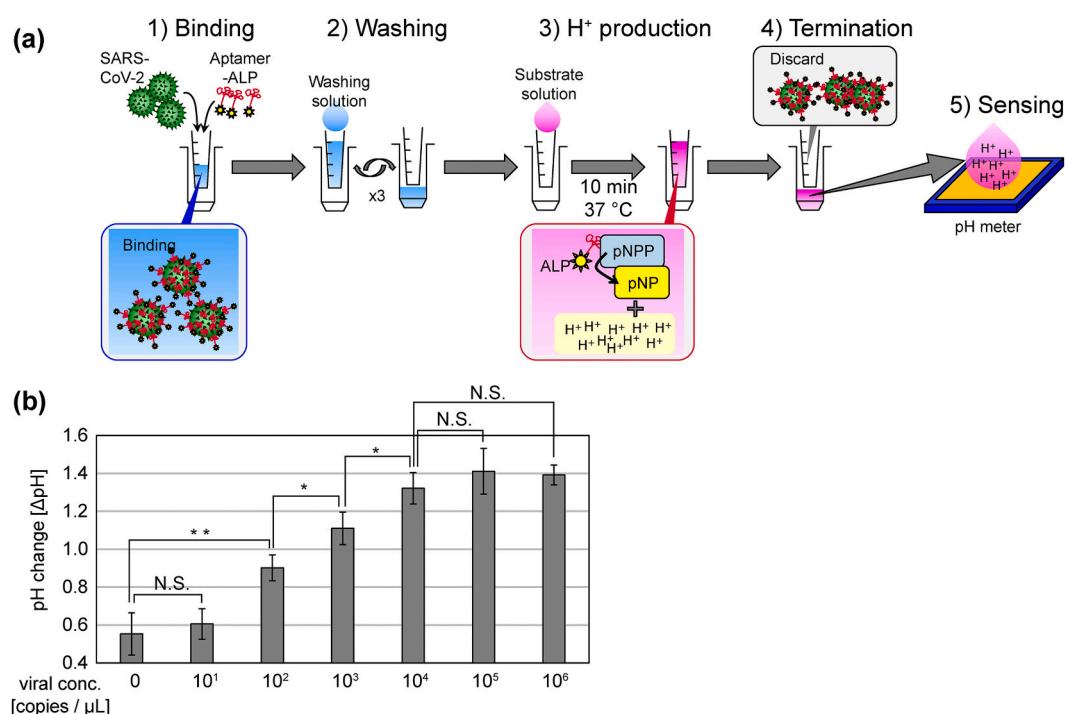


Fig. 3. SARS-CoV-2 assay.

(a) Scheme depicting the operational flow of SARS-CoV-2 sensing.

(b) pH changes after the binding and enzymatic reactions. The concentration of the viruses varied from 10^1 to 10^6 copies/ μL . The graphs present the means $\pm$ SD of at least five independent experiments. The P-values were calculated using paired *t*-tests. * $P < 0.005$; ** $P < 0.001$; N.S., no significant difference ($P > 0.05$).

Abbreviations: SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; SD, standard deviation

Patient consent

Consent to publish this case report was not obtained. This report does not contain any personal information that could lead to the identification of the patient.

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Authorship

All authors attest that they meet the current ICMJE criteria for Authorship.

CRediT authorship contribution statement

Akira Nukazuka: Conceptualization, Methodology, Investigation, Writing – original draft. **Satomi Asai:** Conceptualization, Resources, Writing – review & editing, Funding acquisition. **Kei Hayakawa:** Methodology, Investigation, Validation. **Kazuhisa Nakagawa:** Methodology, Investigation. **Mana Kanazashi:** Methodology, Resources. **Hidefumi Kakizoe:** Investigation, Resources. **Kyoko Hayashi:** Writing – review & editing, Supervision. **Toshio Kawahara:** Supervision, Funding acquisition. **Kazuaki Sawada:** Supervision, Funding acquisition. **Hitoshi Kuno:** Supervision, Funding acquisition. **Kazuhiko Kano:** Supervision, Project administration.

Declaration of Competing Interest

The authors declare no competing financial interest.

Data availability

Data will be made available on request.

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References

- [1] F. Wu, S. Zhao, B. Yu, Y.M. Chen, W. Wang, Z.G. Song, Y. Hu, Z.W. Tao, J.H. Tian, Y.Y. Pei, M.L. Yuan, Y.L. Zhang, F.H. Dai, Y. Liu, Q.M. Wang, J.J. Zheng, L. Xu, E. C. Holmes, Y.Z. Zhang, A new coronavirus associated with human respiratory disease in China, *Nature*. 579 (2020) 265–269, <https://doi.org/10.1038/s41586-020-2008-3>.
- [2] A. Gorbelenya, S. Baker, R. Baric, R. de Groot, C. Drosten, A. Gulyaeva, B. Haagmans, C. Lauber, A. Leontovich, B. Neuman, D. Penzar, S. Perlman, L. Poon, D. Samborskiy, I. Sidorov, I. Sola, J. Ziebuhr, The species severe acute respiratory syndrome-related coronavirus: classifying 2019-nCoV and naming it SARS-CoV-2, *Nat. Microbiol.* 5 (2020) 536–544, <https://doi.org/10.1038/s41564-020-0695-z>.
- [3] R. Keni, A. Alexander, P.G. Nayak, J. Mudgal, K. Nandakumar, COVID-19: emergence, spread, possible treatments, and global burden, *Front. Public Health* 8 (2020) 216, <https://doi.org/10.3389/fpubh.2020.00216>.
- [4] C. Sohrabi, Z. Alsafi, N. O'Neill, M. Khan, A. Kerwan, A. Al-Jabir, C. Iosifidis, R. Agha, World Health Organization declares global emergency: a review of the 2019 novel coronavirus (COVID-19), *Int. J. Surg.* 76 (2020) 71–76, <https://doi.org/10.1016/j.ijsu.2020.02.034>.
- [5] J.F. Chan, K.H. Kok, Z. Zhu, H. Chu, K.K. To, S. Yuan, K.Y. Yuen, Genomic characterization of the 2019 novel human-pathogenic coronavirus isolated from a patient with atypical pneumonia after visiting Wuhan, *Emerg. Microbes Infect.* 9 (2020) 221–236, <https://doi.org/10.1080/22221751.2020.1719902>.
- [6] T.M. Clausen, D.R. Sandoval, C.B. Spliid, J. Pihl, H.R. Perrett, C.D. Painter, A. Narayanan, S.A. Majowicz, E.M. Kwong, R.N. McVicar, B.E. Thacker, C.A. Glass, Z. Yang, J.L. Torres, G.J. Golden, P.L. Bartels, R.N. Porell, A.F. Garretson,

- L. Laubach, J. Feldman, X. Yin, Y. Pu, B.M. Hauser, T.M. Caradonna, B.P. Kellman, C. Martino, P.L.S.M. Gordts, S.K. Chanda, A.G. Schmidt, K. Godula, S.L. Leibel, J. Jose, K.D. Corbett, A.B. Ward, A.F. Carlin, J.D. Esko, SARS-CoV-2 infection depends on cellular heparan sulfate and ACE2, *Cell*. 183 (2020) 1043–1057.e15, <https://doi.org/10.1016/j.cell.2020.09.033>.
- [7] J. Lan, J. Ge, J. Yu, S. Shan, H. Zhou, S. Fan, Q. Zhang, X. Shi, Q. Wang, L. Zhang, X. Wang, Structure of the SARS-CoV-2 spike receptor-binding domain bound to the ACE2 receptor, *Nature*. 581 (2020) 215–220, <https://doi.org/10.1038/s41586-020-2180-5>.
- [8] J. Shang, G. Ye, K. Shi, Y. Wan, C. Luo, H. Aihara, Q. Geng, A. Auerbach, F. Li, Structural basis of receptor recognition by SARS-CoV-2, *Nature*. 581 (2020) 221–224, <https://doi.org/10.1038/s41586-020-2179-y>.
- [9] A.C. Walls, Y.J. Park, M.A. Tortorici, A. Wall, A.T. McGuire, D. Velesler, Structure, function, and antigenicity of the SARS-CoV-2 spike glycoprotein, *Cell*. 181 (2020) 281–292, e286, <https://doi.org/10.1016/j.cell.2020.02.058>.
- [10] D. Wrapp, N. Wang, K.S. Corbett, J.A. Goldsmith, C.L. Hsieh, O. Abiona, B. S. Graham, J.S. McLellan, Cryo-EM structure of the 2019-nCoV spike in the prefusion conformation, *Science*. 367 (2020) 1260–1263, <https://doi.org/10.1126/science.abb2507>.
- [11] R. Yan, Y. Zhang, Y. Li, L. Xia, Y. Guo, Q. Zhou, Structural basis for the recognition of SARS-CoV-2 by full-length human ACE2, *Science*. 367 (2020) 1444–1448, <https://doi.org/10.1126/science.abb2762>.
- [12] B. Jawad, P. Adhikari, R. Podgornik, W.Y. Ching, Key interacting residues between RBD of SARS-CoV-2 and ACE2 receptor: combination of molecular dynamics simulation and density functional calculation, *J. Chem. Inf. Model.* 61 (2021) 4425–4441, <https://doi.org/10.1021/acs.jcim.1c00560>.
- [13] M. Hoffmann, H. Kleine-Weber, S. Schroeder, N. Krüger, T. Herrler, S. Erichsen, T. S. Schiögen, G. Herrler, N.H. Wu, A. Nitsche, M.A. Müller, C. Drosten, S. Pöhlmann, SARS-CoV-2 cell entry depends on ACE2 and TMPRSS2 and is blocked by a clinically proven protease inhibitor, *Cell*. 181 (2020) 271–280.e8, <https://doi.org/10.1016/j.cell.2020.02.052>.
- [14] S. Xia, Y. Zhu, M. Liu, Q. Lan, W. Xu, Y. Wu, T. Ying, S. Liu, Z. Shi, S. Jiang, L. Lu, Fusion mechanism of 2019-nCoV and fusion inhibitors targeting HR1 domain in spike protein, *Cell. Mol. Immunol.* 17 (2020) 765–767, <https://doi.org/10.1038/s41423-020-0374-2>.
- [15] N. Chen, M. Zhou, X. Dong, J. Qu, F. Gong, Y. Han, Y. Qiu, J. Wang, Y. Liu, Y. Wei, J. Xia, T. Yu, X. Zhang, L. Zhang, Epidemiological and clinical characteristics of 99 cases of 2019 novel coronavirus pneumonia in Wuhan, China: a descriptive study, *Lancet*. 395 (2020) 507–513, [https://doi.org/10.1016/S0140-6736\(20\)30211-7](https://doi.org/10.1016/S0140-6736(20)30211-7).
- [16] R. Chen, Y.L. Yu, W. Li, Y. Liu, J.X. Lu, F. Chen, Q. Zhou, Z.Y. Xia, L. Gao, Q. T. Meng, D. Ma, Gastrointestinal symptoms associated with unfavorable prognosis of COVID-19 patients: a retrospective study, *Front. Med. (Lausanne)* 7 (2020), 608259, <https://doi.org/10.3389/fmed.2020.608259>.
- [17] C. Huang, Y. Wang, X. Li, L. Ren, J. Zhao, Y. Hu, L. Zhang, G. Fan, J. Xu, X. Gu, Z. Cheng, T. Yu, J. Xia, Y. Wei, W. Wu, X. Xie, W. Yin, H. Li, M. Liu, Y. Xiao, H. Gao, L. Guo, J. Xie, G. Wang, R. Jiang, Z. Gao, Q. Jin, J. Wang, B. Cao, Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China, *Lancet* 395 (2020) 497–506, [https://doi.org/10.1016/S0140-6736\(20\)30183-5](https://doi.org/10.1016/S0140-6736(20)30183-5).
- [18] A. Perisetti, H. Goyal, M. Gajendran, U. Boregowda, R. Mann, N. Sharma, Prevalence, mechanisms, and implications of gastrointestinal symptoms in COVID-19, *Front. Med. (Lausanne)* 7 (2020), 588711, <https://doi.org/10.3389/fmed.2020.588711>.
- [19] D. Wang, B. Hu, C. Hu, F. Zhu, X. Liu, J. Zhang, B. Wang, H. Xiang, Z. Cheng, Y. Xiong, Y. Zhao, Y. Li, X. Wang, Z. Peng, Clinical characteristics of 138 hospitalized patients with 2019 novel coronavirus-infected pneumonia in Wuhan, China, *JAMA*. 323 (2020) 1061–1069, <https://doi.org/10.1001/jama.2020.1585>.
- [20] Z. Hu, C. Song, C. Xu, G. Jin, Y. Chen, X. Xu, H. Ma, W. Chen, Y. Lin, Y. Zheng, J. Wang, Y. Yi, H. Shen, Clinical characteristics of 24 asymptomatic infections with COVID-19 screened among close contacts in Nanjing, China, *Sci. China Life Sci.* 63 (2020) 706–711.
- [21] Q.X. Long, X.J. Tang, Q.L. Shi, Q. Li, H.J. Deng, J. Yuan, J.L. Hu, W. Xu, Y. Zhang, F.J. Lv, K. Su, F. Zhang, J. Gong, B. Wu, X.M. Liu, J.J. Li, J.F. Qiu, J. Chen, A. L. Huang, Clinical and immunological assessment of asymptomatic SARS-CoV-2 infections, *Nat. Med.* 26 (2020) 1200–1204, <https://doi.org/10.1038/s41591-020-0965-6>.
- [22] G. Qian, N. Yang, A.H.Y. Ma, L. Wang, G. Li, X. Chen, X. Chen, COVID-19 transmission within a family cluster by presymptomatic carriers in China, *Clin. Infect. Dis.* 71 (2020) 861–862, <https://doi.org/10.1093/cid/ciaa316>.
- [23] C. Rothe, M. Schunk, P. Sothmann, G. Bretzel, G. Froeschl, C. Wallrauch, T. Zimmer, V. Thiel, C. Janke, W. Guggemos, M. Seilmaier, C. Drosten, P. Vollmar, K. Zwirgmaier, S. Zange, R. Wölfel, M. Hoelscher, Transmission of 2019-nCoV infection from an asymptomatic contact in Germany, *N. Engl. J. Med.* 382 (2020) 970–971, <https://doi.org/10.1056/NEJMc2001468>.
- [24] D.B. Larremore, B. Wilder, E. Lester, S. Shehata, J.M. Burke, J.A. Hay, M. Tambe, M.J. Mina, R. Parker, Test sensitivity is secondary to frequency and turnaround time for COVID-19 screening, *Sci. Adv.* 7 (2021) eabd5393, <https://doi.org/10.1126/sciadv.abd5393>.
- [25] M.J. Mina, K.G. Andersen, COVID-19 testing: one size does not fit all, *Science*. 371 (2021) 126–127, <https://doi.org/10.1126/science.abe9187>.
- [26] T. Ji, Z. Liu, G. Wang, X. Guo, S. Akbar Khan, C. Lai, H. Chen, S. Huang, S. Xia, B. Chen, H. Jia, Y. Chen, Q. Zhou, Detection of COVID-19: a review of the current literature and future perspectives, *Biosens. Bioelectron.* 166 (2020), 112455, <https://doi.org/10.1016/j.bios.2020.112455>.
- [27] R. Ahmad, T. Mahmoudi, M.S. Ahn, Y.B. Hahn, Recent advances in nanowires-based field-effect transistors for biological sensor applications, *Biosens. Bioelectron.* 100 (2018) 312–325, <https://doi.org/10.1016/j.bios.2017.09.024>.
- [28] T. Goda, Y. Miyahara, Label-free and reagent-less protein biosensing using aptamer-modified extended-gate field-effect transistors, *Biosens. Bioelectron.* 45 (2013) 89–94, <https://doi.org/10.1016/j.bios.2013.01.053>.
- [29] Y. Li, Z. Peng, N.J. Holl, M.R. Hassan, J.M. Pappas, C. Wei, O.H. Izadi, Y. Wang, X. Dong, C. Wang, Y.W. Huang, D. Kim, C. Wu, MXene-graphene field-effect transistor sensing of influenza virus and SARS-CoV-2, *ACS Omega*. 6 (2021) 6643–6653, <https://doi.org/10.1021/acsomega.0c05421>.
- [30] Y. Ohno, K. Maehashi, K. Matsumoto, Label-free biosensors based on aptamer-modified graphene field-effect transistors, *J. Am. Chem. Soc.* 132 (2010) 18012–18013, <https://doi.org/10.1021/ja108127r>.
- [31] N. Nakatsuka, K.A. Yang, J.M. Abendroth, K.M. Cheung, X. Xu, H. Yang, C. Zhao, B. Zhu, Y.S. Rim, Y. Yang, P.S. Weiss, M.N. Stojanović, A.M. Andrews, Aptamer-field-effect transistors overcome Debye length limitations for small-molecule sensing, *Science*. 362 (2018) 319–324, <https://doi.org/10.1126/science.aao6750>.
- [32] J.T. Davies, E.K. Rideal, *Interfacial Phenomena*, second ed, Academic Press, New York, 1961, <https://doi.org/10.1016/B978-0-12-206056-4.X5001-2>.
- [33] E. Stern, R. Wagner, F.J. Sigworth, R. Breaker, T.M. Fahmy, M.A. Reed, Importance of the Debye screening length on nanowire field effect transistor sensors, *Nano Lett.* 7 (2007) 3405–3409, <https://doi.org/10.1021/nl071792z>.
- [34] A. Vacic, J.M. Criscione, N.K. Rajan, E. Stern, T.M. Fahmy, M.A. Reed, Determination of molecular configuration by Debye length modulation, *J. Am. Chem. Soc.* 133 (2011) 13886–13889, <https://doi.org/10.1021/ja205684a>.
- [35] P.A. Neff, B.K. Wunderlich, R.V. Klitzing, A.R. Bausch, Formation and dielectric properties of polyelectrolyte multilayers studied by a silicon-on-insulator based thin film resistor, *Langmuir*. 23 (2007) 4048–4052, <https://doi.org/10.1021/la063632t>.
- [36] Y. Song, J. Song, X. Wei, M. Huang, M. Sun, L. Zhu, B. Lin, H. Shen, Z. Zhu, C. Yang, Discovery of aptamers targeting the receptor-binding domain of the SARS-CoV-2 spike glycoprotein, *Anal. Chem.* 92 (2020) 9895–9900, <https://doi.org/10.1021/acs.analchem.0c01394>.
- [37] M. Stenberg, H. Nygren, Kinetics of antigen-antibody reactions at solid-liquid interfaces, *J. Immunol. Methods* 113 (1988) 3–15, [https://doi.org/10.1016/0022-1759\(88\)90376-6](https://doi.org/10.1016/0022-1759(88)90376-6).
- [38] N. Kacharovskiy, L.F. Yang, H.V. Dang, E.L. Cheng, I.I. Cardle, A.C. Walls, M. McCallum, D.L. Sellers, F. DiMaio, S.J. Salipante, D. Corti, D. Velesler, S.H. Pun, Discovery and characterization of spike N-terminal domain-binding aptamers for rapid SARS-CoV-2 detection, *Angew. Chem. Int. Ed. Eng.* 60 (2021) 21211–21215, <https://doi.org/10.1002/anie.202107730>.
- [39] A. Schmitz, A. Weber, M. Bayin, S. Breuers, V. Fieberg, M. Famulok, G. Mayer, A SARS-CoV-2 spike binding DNA aptamer that inhibits Pseudovirus infection by an RBD-independent mechanism*, *Angew. Chem. Int. Ed. Eng.* 60 (2021) 10279–10285, <https://doi.org/10.1002/anie.202100316>.
- [40] J. Li, Z. Zhang, J. Gu, H.D. Stacey, J.C. Ang, A. Capretta, C.D.M. Filipe, K. L. Mossman, C. Balion, B.J. Salena, D. Yamamura, L. Soleymani, M.S. Miller, J. D. Brennan, Y. Li, Diverse high-affinity DNA aptamers for wild-type and B.1.1.7 SARS-CoV-2 spike proteins from a pre-structured DNA library, *Nucleic Acids Res.* 49 (2021) 7267–7279, <https://doi.org/10.1093/nar/gkab574>.
- [41] G. Yang, Z. Li, I. Mohammed, L. Zhao, W. Wei, H. Xiao, W. Guo, Y. Zhao, F. Qu, Y. Huang, Identification of SARS-CoV-2-against aptamer with high neutralization activity by blocking the RBD domain of spike protein 1, *Signal. Transduct. Target. Ther.* 6 (2021) 227, <https://doi.org/10.1038/s41392-021-00649-6>.
- [42] P.C. Bartels, A.F.M. Roijers, A kinetic study on the influence of the parameters in the determination of inorganic phosphate by the molybdenum blue reaction, *Clin. Chim. Acta* 61 (1975) 135–144, [https://doi.org/10.1016/0009-8981\(75\)90307-1](https://doi.org/10.1016/0009-8981(75)90307-1).
- [43] K. Shirato, N. Nao, H. Katano, I. Takayama, S. Saito, F. Kato, H. Katoh, M. Sakata, Y. Nakatsu, Y. Mori, T. Kageyama, S. Matsuyama, M. Takeda, Development of genetic diagnostic methods for detection for novel coronavirus 2019(nCoV-2019) in Japan, *Jpn. J. Infect. Dis.* 73 (2020) 304–307, <https://doi.org/10.7883/yoken.JJID.2020.061>.
- [44] Z. Wang, X. Yang, N.Z. Lee, X. Cao, Multivalent aptamer approach: designs, strategies, and applications, *Micromachines (Basel)* 13 (2022) 436, <https://doi.org/10.3390/mi13030436>.
- [45] J.C. Abrego-Martinez, M. Jafari, S. Chergui, C. Pavel, D. Che, M. Siaz, Aptamer-based electrochemical biosensor for rapid detection of SARS-CoV-2: nanoscale electrode-aptamer-SARS-CoV-2 imaging by photo-induced force microscopy, *Biosens. Bioelectron.* 195 (2022), 113595, <https://doi.org/10.1016/j.bios.2021.113595>.
- [46] N. Liu, R. Liu, J. Zhang, CRISPR-Cas12a-mediated label-free electrochemical aptamer-based sensor for SARS-CoV-2 antigen detection, *Bioelectrochemistry*. 146 (2022), 108105, <https://doi.org/10.1016/j.bioelect.2022.108105>.
- [47] Z. Rahmati, M. Roushani, H. Hosseini, H. Choobin, Label-free electrochemical aptasensor for rapid detection of SARS-CoV-2 spike glycoprotein based on the composite of Cu(OH)₂ nanorods arrays as a high-performance surface substrate, *Bioelectrochemistry*. 146 (2022), 108106, <https://doi.org/10.1016/j.bioelect.2022.108106>.
- [48] J. Xue, Y. Li, J. Liu, Z. Zhang, R. Yu, Y. Huang, C. Li, A. Chen, J. Qiu, Highly sensitive electrochemical aptasensor for SARS-CoV-2 antigen detection based on aptamer-binding induced multiple hairpin assembly signal amplification, *Talanta*. 248 (2022), 123605, <https://doi.org/10.1016/j.talanta.2022.123605>.
- [49] D.S. Juang, C.H. Lin, Y.R. Huo, C.Y. Tang, C.R. Cheng, H.S. Wu, S.F. Huang, A. Kalnitsky, C.C. Lin, Proton-ELISA: electrochemical immunoassay on a dual-gated ISFET array, *Biosens. Bioelectron.* 117 (2018) 175–182, <https://doi.org/10.1016/j.bios.2018.06.012>.
- [50] A.S. Peinetti, R.J. Lake, W. Cong, L. Cooper, Y. Wu, Y. Ma, G.T. Pawel, M.E. Toimil-Molares, C. Trautmann, L. Rong, B. Marinas, O. Azzaroni, Y. Lu, Direct detection of human adenovirus or SARS-CoV-2 with ability to inform infectivity using DNA

aptamer-nanopore sensors, Sci. Adv. 7 (2021) eabh2848, <https://doi.org/10.1126/sciadv.abh2848>.