nature biomedical engineering

PEER-REVIEW INFORMATION

A lab-on-a-chip for the concurrent electrochemical detection of SARS-CoV-2 RNA and anti-SARS-CoV-2 antibodies in saliva and plasma

Corresponding author: Donald Ingber

Editorial note

This document includes relevant written communications between the manuscript's corresponding author and the editor and reviewers of the manuscript during peer review. It includes decision letters relaying any editorial points and peer-review reports, and the authors' replies to these (under 'Rebuttal' headings). The editorial decisions are signed by the manuscript's handling editor, yet the editorial team and ultimately the journal's Chief Editor share responsibility for all decisions.

Any relevant documents attached to the decision letters are referred to as **Appendix #**, and can be found appended to this document. Any information deemed confidential has been redacted or removed. Earlier versions of the manuscript are not published, yet the originally submitted version may be available as a preprint. Because of editorial edits and changes during peer review, the published title of the paper and the title mentioned in below correspondence may differ.

Correspondence

Fri 01 Oct 2021
Decision on Article nBME-21-1830

Dear Prof Ingber,

Thank you again for submitting to *Nature Biomedical Engineering* your manuscript, "Simultaneous detection of SARS-CoV-2 RNA and host antibodies enabled by a multiplexed electrochemical sensor platform". The manuscript has been seen by three experts, whose reports you will find at the end of this message.

You will see that the reviewers appreciate aspects of the work, and that they raise a few technical concerns. Most importantly, however, they are not convinced of the current degree of integration and utility of the assay.

For this work, our editorial assessment was based on the claims of advantageous performance and practical usability of the integrated multiplexed assay rather than on 'raw' technological novelty. However, all reviewers raise performance and usability concerns: the assay requires multiple steps, it may be too complex to operate, and it is not sufficiently integrated for eventual point-of-care use. Having considered the reviewers' advice, we have reached the conclusion that the work is unlikely to provide the scientific significance that we look for in manuscripts that we consider for further external peer review.

However, should further work allow you to optimize and better integrate the components of the assay, and provide evidence of advantageous point-of-care use as well as further validation (with samples from various relevant vaccinated, unvaccinated, previously infected, and asymptomatic populations), we would be willing to assess an appeal for reconsideration.

We hope that you will find the referee reports helpful when revising the work.

Best wishes,

Pep

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Pep Pàmies
Chief Editor, *Nature Biomedical Engineering*

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Reviewer #1 (Report for the authors (Required)):

Authors presented here an integrated multiplexed electrochemical approach to combine serological and nucleic acid diagnostics to improve the overall accuracy of SARS-CoV-2 diagnosis. This serological and NAT will potentially provide qualitative data on a individuals disease severity and progression. Authors claim that this is not possible right now at a point-of-care. While the combination approach of molecular and serological testing is a nice twist, the manuscript presents many fundamental flaws. This manuscript should not be accepted for publication in Nature Biomedical Engineering. Specific comments are given below.

1) The work lacks fundamental novelty. The CRISPR/Cas12a based platform has already been reported. Serological approach is also well established. This work is just integrating two well established techniques in one platform.

2) An RT-LAMP based amplification step is added prior to the CRISPR step. This reduces the enthusiasm. LAMP amplification step requires 65C to complete. This could present a major barrier for commercializing this process.

3) Because of the complexity of collecting both saliva and blood at a same time, it is highly unlikely that this approach will ever be able to receive CLIA approval.

4) I am not quite sure with the multiplexing terminology as the authors are performing the COVID-RNA test from saliva and the serological antigen test has been done by spiking human plasma into the saliva.

5) Even the RNA test depends on RNA purification, LAMP assay and then CRISPR. It also needs to be post treated with HRP conjugate, TMB. No direct assay has been proposed.

6) No linearity in the CRISPR based assay with Cp numbers (Figure S2, S8).

7) Figure S18 is too simplified. It is not giving the impression of multi-step addition of different chemicals for the antigen assay.

8) Number of clinical sample tested was remarkably low. The field has progressed quite a bit.

9) Overall, the work is not novel enough for Nature BME. Individually, both the RNA-based and antigen based electrochemical detection for COVID-19 has already been performed by other researchers. The authors are just combining two methods. Authors claimed a point of care test. Because of the complexity of combining two different biological fluids, this is not doable at POC.

Reviewer #2 (Report for the authors (Required)):

In this paper, the author discussed multiplexed electrochemical detection of host antibodies related to COVID-19 and of SARS-Cov-2 viral RNA. They used sandwich-based enzymatic assay for the detection. Three viral antigens were immobilized to the electrode surface. After antibody binding, a secondary HRP-conjugated antibody was used. Finally, TMB was used as enzymatic substrate and electrochemical read out. For viral RNA detection, a CRISPR-based technique and HRP-based assay was used to analyze 30 clinical samples. A serological assay was used to analyze 112 clinical plasma samples. The assay showed promising sensitivity and specificity for analysis of clinical samples.

However, it's not clear that the novelty of the approach is significant enough for this venue. Moreover, the complex assay format with multiple steps and needing of additional reagents and purification methods would not be practical for point-of care diagnosis.

Other comments:

1. In fig 1, the presence of the target leads to the decrease of the current signal. However, in figure S6, SARS-CoV-2 RNA produced a higher signal than the negative control. Please explain the inconsistence.

2. Line 168, SARS-CoV-2 negative should be orange and the positive should be blue.

3. S1-RBD, S1, and N proteins were picked as targets for multiplexed serology EC assay. The authors mentioned that “IgG antibodies targeting the S protein are more specific for SARS-CoV-2, while those targeting the N protein may be more sensitive, particularly in the early phase of infection. Therefore, to maximize our assay’s accuracy for both early and late infections, we fabricated a multiplexed serology assay capable of measuring antibodies against S1-RBD, S1, and N proteins.” The authors did not explain why it is necessary to detect both S1 and S1-RBD? Is S1-RBD part of the S1? What are the advantages to detect both targets?

5. What do orange dot and blue square represent in Figure S8?

6. In figure S9a, both P1 and P2 are SARS-Cov-2 positive samples. Why does P2 have a much lower signal?

7. In figure S14 c and d, why the high titer plasma generated lower signal than the low titer plasma when detecting S1 protein? Error bars are missed in Figure 14.

8. The authors claim that the platform can be used in POC applications. But the RNA detection method still requires multiple steps including the use of RNA extraction kit, centrifugation, heating steps at 65 °C and 37 °C etc. Is there any strategy to simplify the procedure to make it more user-friendly?

9. The authors claimed they have overcome the biofouling issue in current EC sensors. Their proposed method involves BSA GO scaffolds. This claim has been published in Ref 25 (Ad. Func. Mat) but there are no supporting data to back up this claim in this article. The need for plasma dilution (1:9) also suggests that this system might still face biofouling issues. To substantiate this claim, additional experiments and head-to-head studies with other systems are needed.

10. LOD is reported to be single molecule for CRISPR sensor. 0.8 ct/uL. What is the sample volume in this set up and what will be the final count of detected cells?

11. N protein is claimed to show higher sensitivity that S1 in early stage infections. This claims needs more careful attention to recent literature.

Reviewer #3 (Report for the authors (Required)):

The manuscript reports an antifouling electrochemical platform for multiplexed serological detection of SARS-CoV-2 antibodies based on immunosensing and viral RNA detection based on CRISPR/Cas detection. The platform reports good accuracy and sensitivity for viral RNA and SARS-CoV-2 antibodies and could potentially be applied for COVID-19 diagnosis. The serological electrochemical platform may also be relevant for seroprevalence study. However, the technology described in this manuscript does not have enough novelty to be published in Nature BME.

There have been numerous reports on low-cost, point-of-care electrochemical platforms for COVID-19 diagnosis. The use of CRISPR/Cas for electrochemical detection of RNA in samples (amplified and unamplified) have also been reported elsewhere (e.g., Zamani et al. ACS Cent. Sci. 2021, 10.1021/acscentsci.1c00186; Li et al. Biosens. Bioelectron. 2021, 179, 113073; Hajian et al. Nat. Biomed. Eng. 2018, 3, 427). It’s true that there is no single report on the detection of both nucleic acid and proteins at

the same time yet. It's also arguable whether the platform reported in this manuscript is doing nucleic acid and protein detection concurrently since there's an amplification step involved for the CRISPR-based assay. According to the methods, the serological assay is also conducted on a separate chip. As such, the novelty in this aspect is arguable. An interesting application aspect mentioned in the manuscript is the use towards seroprevalence and vaccine efficacy studies.
Unfortunately, the study of clinical samples is limited to the evaluation of positive/negative samples. It would be interesting if the authors could demonstrate the relevance of serological tests with different populations such as vaccinated individuals, asymptomatic infection and past infections since the levels of anti-spike and anti-nucleocapsid IgGs may vary. Since conventional laboratory methods and many other recent reports can also be applied for COVID-19 diagnosis; the distinct advantage of the platform is unclear here.

In addition, the method described is also not fully integrated to allow rapid on-site testing or fully optimized and evaluated to replace current laboratory techniques. The authors argue the EC sensor platform is better compared to traditional fluorescent diagnostics due to its simplicity, yet have not demonstrated this aspect in the manuscript. In fact, the authors' recent publication in Science Advances on fully integrated fluorescent CRISPR sensor for COVID-19 viral RNA diagnostics demonstrates that fluorescent diagnostic platform can be simple to operate with proper engineering as well.

Given the abovementioned reasons, this manuscript does not report sufficient technological novelty or engineering design for publication in Nature BME.

Sat 23 Apr 2022
Decision on Article nBME-21-1830A-Z

Dear Prof Ingber,

Apologies for the delay in providing you with the reviewer reports for your revised manuscript, "Lab-on-a-chip multiplexed electrochemical sensor enables simultaneous detection of SARS-CoV-2 RNA and host antibodies", which has been seen by the original reviewers. In their reports, which you will find at the end of this message, you will see that the reviewers acknowledge the improvements to the work and that Reviewer #3 raises a few additional technical criticisms that we hope you will be able to address. Also, in view of the comments of Reviewer #1, I suggest that the next version of the manuscript discusses further which technological and performance bottlenecks as well as use-case considerations may eventually need to be addressed for the multiplexed lab-on-a-chip device to be deployed in point-of-care settings.

As before, when you are ready to resubmit your manuscript, please upload the revised files, a point-by-point rebuttal to the comments of Reviewer #3, and the reporting summary.

We look forward to receive a further revised version of the work. Please do not hesitate to contact me should you have any questions.

Best wishes,

Pep

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Pep Pàmies
Chief Editor, *Nature Biomedical Engineering*

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Reviewer #1 (Report for the authors (Required)):

In this revised submission, the authors conducted more experiments, developed a prototype (with integrated heating) and added more clinical samples. Overall, with these changes, the quality of the manuscript has been improved w.r.t. the performance and usability concerns. The combination of serological and swab/saliva-based NA tests was also a concern for me. Authors now proposed a saliva-based antibody detection method.

Despite all these changes made, I'm still not convinced that this work merits a publication in Nature Biomedical Engineering. The reason for this is that none of the technologies presented here is novel. Overall, this is a good thorough work, however, not every meticulously performed work deserves a spot in Nature publications.

Both the RNA-based and antigen based electrochemical detection for COVID-19 has been shown to work by others. All the techniques and engineering approaches are well known and the work does not bring any fundamental novelty.

Authors claimed a point of care test. Because of the complexity of combining two different biological fluids, this is not doable at POC. Their claim is that this test will be performed at moderately complex lab. If so, then there are already available techniques.

The apparent enhancement in sensitivity can also be explained easily as the measurement is done electrochemically as oppose to fluorescence/absorbance based techniques in other CRISPR/LAMP works.

This is a good routine work and suitable for publication in a more specialized journal.

Reviewer #2 (Report for the authors (Required)):

The authors have addressed the concerns and requests of the reviewers and improved the manuscript significantly.

Reviewer #3 (Report for the authors (Required)):

This manuscript reports an integrated lab-on-a-chip device for samples processing and multiplexed detection of viral RNA and anti-SARS-CoV-2 antibodies for clinical POC settings. The authors proposed a simplified sample preparation protocol and an integrated prototype to demonstrate the feasibility of POC testing in clinical settings. Still, authors should address the following concerns regarding the advantages claimed.

The viral RNA sensor has a signal-off mechanism, low current correlates to positive samples. How can one differentiate between bad chips (no/ low polystrep-HRP loading due to poor preparation). It seems only qualitative information can be provided by this sensor.

Since the antibody sensors have a signal-on mechanism, is it possible to obtain quantitative information? Is there any significance in providing quantitative values? What are the LODs for these sensors? It seems the authors did argue that the sensors are capable of distinguishing samples with various antibody levels. If so, please provide cross-validation between the antibody sensors and ELISA. Figure S22 seems like ELISA results only. Also, figure S22 has the same caption as Figure S23.

Antibody loads seemed non-detectable in saliva samples due to heat inactivation. Were the serum samples also heat-inactivated? If so, does this mean that the antibody sensors are simply not sensitive enough for certain saliva samples, heat-inactivated ones in particular? Based on the literature, it seems “heating-activation does not decrease the diagnostic efficacy of SARS-CoV-2 IgM or IgG antibodies.” (10.1016/j.cca.2020.06.032). Spiking saliva samples with standards simply increase the intrinsic antibody loads beyond the detection threshold?

Thu 9 Jun 2022
Decision on Article nBME-21-1830B

Dear Prof Ingber,

Thank you for your revised manuscript, "Lab-on-a-chip multiplexed electrochemical sensor enables simultaneous detection of SARS-CoV-2 RNA and host antibodies". Having consulted with Reviewer #3, I am pleased to write that we shall be happy to publish the manuscript in *Nature Biomedical Engineering*.

We will be performing detailed checks on your paper and will send you a checklist detailing our editorial and formatting requirements in due course.

Please do not hesitate to contact me if you have any questions.

Best wishes,

Pep

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Pep Pàmies
Chief Editor, *Nature Biomedical Engineering*

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Reviewer #3 (Report for the authors (Required)):

The authors have fully addressed my previous comments. Now the paper is suitable for publication.

Rebuttal 1

RESPONSE TO EDITOR AND REVIEWERS
(De Puig et al., nNBE-21-1830; now Najjar et al.)

Editor:

1. ...all reviewers raise performance and usability concerns: the assay requires multiple steps, it may be too complex to operate, and it is not sufficiently integrated for eventual point-of-care use... should further work allow you to optimize and better integrate the components of the assay, and provide evidence of advantageous point-of-care use as well as further validation (with samples from various relevant vaccinated, unvaccinated, previously infected, and asymptomatic populations), we would be willing to assess an appeal for reconsideration.

In response to your concerns, we have conducted additional experiments focusing in four main areas:

· (1) Simplify sample preparation: We have developed a new and simpler enzymatic lysis method for sample preparation and demonstrate that it enables electrochemical detection of viral RNA in saliva.

· (2) Develop a prototype that enables sample-in/answer-out functionality: We have designed, built, tested, and validated the function of an integrated microfluidic platform that carries out all steps in the detection process, including viral lysis, sample preparation, nucleic acid amplification, and electrochemical output for both serological and viral RNA targets.

· (3) Use standardized methods to validate the prototype: As requested, we further improved the study by including additional clinical samples for testing. We now also use a National Institute for Biological Standards and Control (NIBSC) and World Health Organization (WHO)-approved calibrant to validate the combined serology and RNA detection assay in saliva.

Please note that due to the extensive additional studies requested and efforts required by different team members, we feel that it is most appropriate to reorder the authors, and thus, the first author is now D. Najjar.

Reviewer #1:

1. The work lacks fundamental novelty. The CRISPR/Cas12a based platform has already been reported. Serological approach is also well established. This work is just integrating two well established techniques in one platform.

The novelty of our assay is that it is the first sample-in/answer-out device that enables both serology and viral RNA detection in an integrated electrochemical readout platform, which includes the following features:

- Our CRISPR/Cas12a platform uses an electrochemical readout that is **2-5 orders of magnitude more sensitive than other published approaches that use electrochemical readouts and CRISPR-Cas enzymes**, including ones that also utilize LAMP and Cas12a to detect viruses (https://pubs.acs.org/doi/abs/10.1021/acscentsci.1c00186). Our ability to

achieve these low limits of detection is made possible by the use of a novel HRP-precipitating form of TMB that provides additional amplification of the signal (as well as novel multiplexing capabilities), together with the antifouling properties of the nanocomposite coating we apply to the electrodes.

- We are able to carry out sample-in/answer-out viral RNA electrochemical detection of SARS-CoV-2 because **we do not require a separate viral RNA purification step,** as used in all previously published nucleic acid detection tools with electrochemical readouts of viral RNA.

- **The flexible design of our platform enables both viral RNA and protein (antibody) detection.** This is very useful for diagnosis and clinical assessment of patients with COVID-19, but it also can be easily expanded for use with other diseases of concern.

- While the serological diagnosis of SARS-CoV-2 is well established, **the simultaneous detection of multiple relevant antigens in a low-cost and robust platform is novel.**

- Integrating approaches for viral RNA detection as well as protein detection is non-trivial because the surface chemistry requirements for the two assays are quite distinct. Our ability to combine protein and nucleic acid detection with high sensitivity in one electrochemical platform was only made possible by developing the novel assay we describe that integrates the precipitating HRP-TMB readout with the antifouling properties of our coated electrodes.

In response to the Reviewer's concern, we now more clearly describe the novelty of our work in the Introduction and Discussion, highlighting the above points.

2. An RT-LAMP based amplification step is added prior to the CRISPR step. This reduces the enthusiasm. LAMP amplification step requires 65C to complete. This could present a major barrier for commercializing this process.

The Reviewer brings up a valid point about the temperature requirements of the LAMP amplification step. To address this point, we have built an integrated device that controls the temperature during the LAMP amplification. In the current setup, the different temperatures required for the assay can be internally controlled using the same heat source. This makes the temperature requirements for our assay similar to other LAMP-based FDA-approved SARS-CoV-2 diagnostics, with the additional advantages that our prototype integrates sample preparation, RNA detection and electrochemical readout.

3. ***Because of the complexity of collecting both saliva and blood at a same time, it is highly unlikely that this approach will ever be able to receive CLIA approval.***

We agree with the Reviewer that it would not be possible to receive a CLIA waiver for a device that uses both saliva and blood. As an in vitro diagnostic, an assay involving blood would classify the device in the category of a moderate or high complexity device and require CLIA-certified labs to perform the reaction. Therefore, we have designed our multiplexed lab-on-a-chip device to detect both RNA and antibodies using only a saliva sample. We clarify this point in the revised text.

4. I am not quite sure with the multiplexing terminology as the authors are performing the COVID-RNA test from saliva and the serological antigen test has been done by spiking human plasma into the saliva.

The Reviewer raises a point regarding the use of spiked serum in saliva. Initially, we validated the antigens used for the serology assay using serum because we only had access to serum samples from previously infected patients at the time. But upon receiving saliva samples, we validated the RNA-based assay for detection of viral RNA in saliva using clinical saliva samples from infected patients. SARS-CoV-2 is an BSL-3 pathogen, and therefore, all clinical samples that contain SARS-CoV-2, including saliva, have to be heat-inactivated before use in our facilities. Unfortunately, this heating step also denatures antibodies present in the patient's saliva. Thus, in response to the Reviewer's comment, we now use a standardized calibrant approved by the National Institute for Biological Standards and Control (NIBSC) and World Health Organization that has been previously used for approval of serology-based assays to spike into saliva samples for testing the serology.

5. Even the RNA test depends on RNA purification, LAMP assay and then CRISPR. It also needs to be post treated with HRP conjugate, TMB. No direct assay has been proposed.

This is a valid concern. In response, we have carried out additional studies development work, and now describe a microfluidic device that automates all sample preparation, temperature control, and reagent addition steps to enable an easy to use sample-to-answer platform that is more appropriate for point-of-care applications.

6. No linearity in the CRISPR based assay with Cp numbers (Figure S2, S8).

The objective of our work is to use this platform at the point of care. Thus, we seek to obtain yes/no results with regards to whether the patient is infected with SARS-CoV-2. There is a clear difference between the current generated when the TMB precipitates and generates a signal at or above the LOD of the sensor as compared to when it does not. We rely on this strong difference to determine the status of a reaction. We now clarify this point in the Results section of the revised paper.

7. Figure S18 is too simplified. It is not giving the impression of multi-step addition of different chemicals for the antigen assay.

Given that we have now simplified and streamlined the entire detection process, we have removed **Fig. S18** and replaced it with detailed discussion of our LOC microfluidic device in Supplementary **Fig. S5**.

8. Number of clinical sample tested was remarkably low. The field has progressed quite a bit.

A total of 112 clinical samples were tested to validate assay components for the serology assay and 30 samples for the saliva assay. In addition, we now include results for the combined saliva and serology assays using 12 clinical saliva samples with commercially relevant calibrants for simulated antibodies (**Fig. 4b-e**). This number of samples is consistent with or higher than those used in other diagnostics applications.

9. Overall, the work is not novel enough for Nature BME. Individually, both the RNA-based and antigen based electrochemical detection for COVID-19 has already been performed by other researchers. The authors are just combining two methods. Authors claimed a point of care test. Because of the complexity of combining two different biological fluids, this is not doable at POC.

We believe that these concerns are addressed now that we have built an integrated microfluidic platform that enables sample-to answer processing using only saliva, which should facilitate the use of the device at the point of care.

Reviewer #2:

1. ...it's not clear that the novelty of the approach is significant enough for this venue. Moreover, the complex assay format with multiple steps and needing of additional reagents and purification methods would not be practical for point-of care diagnosis.

This is a valid concern. In response, we have carried out additional studies and development work, and now describe a microfluidic device that automates all sample preparation, temperature control, and reagent addition steps to enable an easy to use sample-to-answer platform that is more appropriate for point-of-care applications.

2. In fig 1, the presence of the target leads to the decrease of the current signal. However, in figure S6, SARS-CoV-2 RNA produced a higher signal than the negative control. Please explain the inconsistence.

Fig. S6 shows the SHERLOCK reaction using a fluorescent readout, while **Fig. 1** (now **Fig. 2**) shows the results on the electrochemical platform. We have edited the text in the Results section of the revised paper to clarify the difference between the results shown in these two figures.

3. Line 168, SARS-CoV-2 negative should be orange and the positive should be blue.

We have edited the figure accordingly.

4. S1-RBD, S1, and N proteins were picked as targets for multiplexed serology EC assay. The authors mentioned that "IgG antibodies targeting the S protein are more specific for SARS-CoV-2, while those targeting the N protein may be more sensitive, particularly in the early phase of infection. Therefore, to maximize our assay's accuracy for both early and late infections, we fabricated a multiplexed serology assay capable of measuring antibodies against S1-RBD, S1, and N proteins." The authors did not explain why it is necessary to detect both S1 and S1-RBD? Is S1-RBD part of the S1? What are the advantages to detect both targets?

S1-RBD is the binding site of the spike protein that attaches to the ACE2 receptor on the surface of host cells. S1-RBD is also highly immunogenic and the region of the spike protein that is most commonly mutated in the new variants of concern. The advantage of detecting both targets is that S1-RBD is expected to be more sensitive, but as it is potentially less specific, the

detection of the full S1 subunit of the spike protein ensures that novel variants are not missed. We now clarify these points in the Introduction and Results sections of the revised paper.

5. What do orange dot and blue square represent in Figure S8?

In **Fig. S8**, the orange dots represent the clinical saliva samples that were RT-qPCR negative and the blue squares represent the clinical saliva samples that were RT-qPCR positive with a range of Ct values. We now clarify this in the figure description.

6. In figure S9a, both P1 and P2 are SARS-Cov-2 positive samples. Why does P2 have a much lower signal?

In **Fig. S9a** (now **Fig. S13a**), we first tested the assay's reagents against two independent clinical samples at different stages post-infection (samples P1 and P2) to ensure that our assay would be sensitive enough to detect anti-SARS-CoV-2 antibodies in both high- and low-titer clinical samples. P2 is the sample from the patient with the lower antibody titers. We now clarify this in the Results section of the revised paper by changing the labels for these samples to P_{high} and P_{low}, respectively, as well as by providing the ELISA results for the IgG ranges for clinical samples used in **Fig. S22**.

7. In figure S14 c and d, why the high titer plasma generated lower signal than the low titer plasma when detecting S1 protein? Error bars are missed in Figure 14.

The graphs in **Fig. S14c,d** (now **Fig. S18c,d**) have different y-axes, and so the signal in the low-titer plasma was actually lower than that of the higher-titer plasma.

8. The authors claim that the platform can be used in POC applications. But the RNA detection method still requires multiple steps including the use of RNA extraction kit, centrifugation, heating steps at 65°C and 37°C etc. Is there any strategy to simplify the procedure to make it more user-friendly?

As described above, this is a valid concern. In response, we have carried out additional studies and development work, and now describe an integrated microfluidic device to automates sample preparation, temperature control, and reagent addition steps to enable an easy to use sample-to-answer platform that is more appropriate for point-of-care applications.

9. The authors claimed they have overcome the biofouling issue in current EC sensors. Their proposed method involves BSA GO scaffolds. This claim has been published in Ref 25 (Ad. Func. Mat) but there are no supporting data to back up this claim in this article. The need for plasma dilution (1:9) also suggests that this system might still face biofouling issues. To substantiate this claim, additional experiments and head-to-head studies with other systems are needed.

We have previously demonstrated that the use of the BSA/GO nanocomposite overcomes biofouling not only in plasma but in different biofluids including saliva, serum, urine, and even whole blood (*Advanced Healthcare Materials* (2021): 2102244, https://doi.org/10.1002/adhm.202102244; Accounts of Chemical Research 54 (18), 3529-3539; *Nature nanotechnology*, *14*(12), 1143-1149; Advanced Functional Materials 31 (16), 2170107).

In the most recent publication (*Advanced Healthcare Materials* (2021): 2102244, https://doi.org/10.1002/adhm.202102244), the nanocomposite demonstrated excellent anti-fouling properties even when incubated for up to 9 weeks in unprocessed human plasma. We did not dilute the plasma 1:9 in the present study to prevent the biofouling, but rather to prevent the hook effect (which can complicate some immunoassays) and have optimum sensitivity and specificity for all IgG, IgM, and IgA antibodies as shown in **Fig. S14**. Requirement of sample dilution to prevent the hook effect has been well reported in the literature by the scientific community (*ACS omega*, *5*(21), 12550-12556; *Bioanalysis* 13.1 (2021): 13-28). Moreover, as shown in **Fig. 3**, we also included BSA as negative control and it did not generate any background signal, which clearly demonstrates that biofouling is not a problem in this system.

10. LOD is reported to be single molecule for CRISPR sensor. 0.8 ct/uL. What is the sample volume in this set up and what will be the final count of detected cells?

The sample volume in the LAMP reaction is 5 ml in the integrated microfluidics platform, which means that our limit of detection is 4 copies of SARS-CoV-2.

11. N protein is claimed to show higher sensitivity that S1 in early stage infections. These claims need more careful attention to recent literature.

The literature indicated that while the N protein may appear earlier during infection, the S protein is more closely associated with viral neutralization. We now elaborate on the literature and rationale behind out antibody target selection in greater depth in the Introduction of the revised paper.

Reviewer #3:

1. There have been numerous reports on low-cost, point-of-care electrochemical platforms for COVID-19 diagnosis. The use of CRISPR/Cas for electrochemical detection of RNA in samples (amplified and unamplified) have also been reported elsewhere (e.g., Zamani et al. ACS Cent. Sci. 2021, 10.1021/acscentsci.1c00186; Li et al. Biosens. Bioelectron. 2021, 179, 113073; Hajian et al. Nat. Biomed. Eng. 2018, 3, 427).... It's true that there is no single report on the detection of both nucleic acid and proteins at the same time yet. It's also arguable whether the platform reported in this manuscript is doing nucleic acid and protein detection concurrently since there's an amplification step involved for the CRISPR-based assay. According to the methods, the serological assay is also conducted on a separate chip. As such, the novelty in this aspect is arguable...**the method described is also not fully integrated to allow rapid on-site testing or fully optimized and evaluated to replace current laboratory techniques.***

A key difference between our work and that of Zamani et al. and Li et al. is that we use HRP-TMB for readout, which enables further amplification of the electrochemical signal for both the serological sensors as well as the CRISPR-based RNA sensors. More importantly, we have carried out additional studies and now describe an integrated microfluidic device that automates sample preparation, temperature control, and reagent addition steps to enable an easy-to-use sample-to-answer platform that is more appropriate for point-of-care applications. There

has been work recently published by Wang. et al. (https://doi.org/10.1038/s41551-021-00833-7) that demonstrates an ultrasensitive graphene FET-based electrochemical biosensor targeting SARS-CoV-2. This technology was only validated for VTM-based nasopharyngeal swab samples, but more importantly, it does not have multiplexing capabilities. With our integrated device, we are able to perform both serological and RNA detection from a saliva sample, which does not require viral transport media or specialized collection equipment, and the results are reported concurrently. To our knowledge, our work is still the first to describe an electrochemical diagnostic device that is multiplexed, highly sensitive, and capable of processing raw biological samples such as saliva. We now cite these references and clarify these points in the revised text.

2. Unfortunately, the study of clinical samples is limited to the evaluation of positive/negative samples. It would be interesting if the authors could demonstrate the relevance of serological tests with different populations such as vaccinated individuals, asymptomatic infection and past infections since the levels of anti-spike and anti-nucleocapsid IgGs may vary. Since conventional laboratory methods and many other recent reports can also be applied for COVID-19 diagnosis; the distinct advantage of the platform is unclear here.

The key question raised here is whether the serological test we describe can discriminate between patients with different levels of anti-spike and anti-nucleocapsid IgGs, because those levels vary between different patient populations. However, these levels also vary over time in infected patients, and we took advantage of this fact in the present study. Specifically, while we tested only clinical samples from patients that had been exposed to SARS-CoV-2 as well as negative controls, the samples from immunized individuals displayed a wide range of IgG titers. We have now added **Fig. S22**, which shows the range of IgG values for different patient samples used this in this study and the excellent correlation between these levels and the measurements obtained with our assay.

3. The authors argue the EC sensor platform is better compared to traditional fluorescent diagnostics due to its simplicity, yet have not demonstrated this aspect in the manuscript. In fact, the authors' recent publication in Science Advances on fully integrated fluorescent CRISPR sensor for COVID-19 viral RNA diagnostics demonstrates that fluorescent diagnostic platform can be simple to operate with proper engineering as well."

We agree that fluorescent sensors can be fully integrated into diagnostic platforms and used for rapid diagnosis of SARS-CoV-2. However, electrochemical sensor platforms offer significant additional benefits including increased sensitivity, greater ability to multiplex, more readily integrated with digital healthcare platforms, and greatly increased robustness in the field which is critical for point-of-care applications. We now make these points more clearly in the revised text.

Rebuttal 2

RESPONSE TO REVIEWERS

Response to Editor:

1. ***"…I suggest that the next version of the manuscript discusses further which technological and performance bottlenecks as well as use-case considerations may eventually need to be addressed for the multiplexed lab-on-a-chip device to be deployed in point-of-care settings."***

As requested, we have modified the Discussion of the manuscript to clarify the technological and performance challenges as well as use-case considerations of our work, particularly for clinical and hospital POC settings. Given Reviewer 1's comments, we also more clearly describe the novelty and innovation of our work, which include:

- Engineering of a high specificity and sensitivity multiplexed electrochemical (EC) sensor chip that enables detection of both proteins and nucleic acids with clinically relevant samples of biological fluids perfused through a single microfluidic channel
- Development of a CRISPR-based detection assay that is optimized to function in a multiplexed electrochemical assay in parallel with antibody-based detection assays that is amenable for point-of-care (POC) applications
- Development of methods for automated microfluidic extraction and amplification of RNA from raw patient saliva samples
- Fluidic integration of sample preparation process with the EC sensor chip

Reviewer #3:

1. The viral RNA sensor has a signal-off mechanism, low current correlates to positive samples. How can one differentiate between bad chips (no/low polystrep-HRP loading due to poor preparation). It seems only qualitative information can be provided by this sensor.

The Reviewer raises an important point with regards to the "signal-off" mechanism. However, the same holds for a signal-on system, where a poorly prepared chip could relate to a lower signal. Therefore, the primary goal is to have a reliable and reproducible sensor irrespective of the assay being used on it. More importantly, this type of signal-off mechanism has been used previously in commercial products as well as peer-reviewed competitive assays that demonstrate successful real-world application of this type of assay:

- Meng et al. 2020 https://doi.org/10.1016/j.bios.2019.111861 (31)
- Malecka et al., 2016 https://doi.org/10.1016/j.snb.2015.10.044 (39)
- Chung et al. 2011 https://doi.org/10.1016/j.apsusc.2011.06.015 (49)
- Jampasa et al. 2014 https://doi.org/10.1016/j.bios.2013.11.023 (99)
- Xiong et al. 2015 https://doi.org/10.1016/j.aca.2014.10.015 (94)
- Chen et al. 2015 https://doi.org/10.1021/acs.analchem.5b01168 (123)

Additionally, the issue with HRP loading can be omitted by directly linking a redox-active marker (Fc, MB, enzyme) that removes the need for both the polystrep-HRP and TMB steps.

- Xiong et al. 2015 https://doi.org/10.1016/j.aca.2014.10.015 (94)
- Cui et al. 2018 https://doi.org/10.1016/j.bios.2017.11.025 (46)

It is true that the CRISPR sensor is only qualitative in nature, and it is precisely why detection of an on/off signal for yes/no detection is necessary for this type of application. We now clarify these points in the Discussion.

2. Since the antibody sensors have a signal-on mechanism, is it possible to obtain quantitative information? Is there any significance in providing quantitative values?

For a POC diagnostic platform designed to detect viral load in patients during a pandemic, it is important to detect clinically relevant levels of the biomarkers of interest in patient samples. Therefore, we added a pre-amplification step prior to CRISPR-based RNA detection, responsible for our highly sensitive LOD (0.8 ct/µl), which would not be detectable in an amplification-free CRISPR-based detection assay. Our proposed use case for this system is to determine the presence or absence of clinically relevant biomarkers. But quantification of this signal might allow clinicians to follow a patient over the course of a disease to determine changes in viral load and disease progression. This is beyond the scope of our current study, but we now explain this point as a possible future direction that could be explored in the Discussion.

3. What are the LODs for these sensors? It seems the authors did argue that the sensors are capable of distinguishing samples with various antibody levels. If so, please provide cross-validation between the antibody sensors and ELISA. Figure S22 seems like ELISA results only.

The LOD of our CRISPR-based sensor is 0.8 ct/µl, as shown in **Figure S7**. Because we used clinical samples for the serology assay, it was difficult to determine the LOD as every sample had a different unknown titer. However, we performed a calibration curve using Rabbit IgG and determined the LOD to be 0.7 ng/mL. We now clarify this point and added Fig. S23 as well as a description of the calibration method used to obtain these results to the Supplementary materials.

Also, when designing our EC sensors, our goal was to have a system that was highly selective for established samples as compared to standard ELISA assays. As seen in Fig. S22, clinical samples with a range of antibody levels were used to validate our platform. To cross validate our platform, we ran patient clinical samples on traditional ELISA assays as well as our EC platform. Importantly, we found that **our EC sensor platform was more selective and sensitive than the ELISA** (e.g., IgG data, Fig. S15 and Fig. 3 f-g).

4. Also, figure S22 has the same caption as Figure S23.

We apologize for the oversight. We have fixed the caption. It now reads: "*Figure S22: **IgG range for several SARS-CoV-2 antigens detected within 58 positive clinical plasma samples**. Levels of IgG directed against N (purple), S1 (grey) and RBD (yellow) detected by ELISA are plotted for each of the positive clinical plasma samples. The samples were collected were from convalescent patients at different stages post-disease and contained variable antibody titers, allowing us to validate that our multiplexed electrochemical sensors perform well across a range possible antibody levels."*

5. ***Antibody loads seemed non-detectable in saliva samples due to heat inactivation. Were the serum samples also heat-inactivated? If so, does this mean that the antibody***

sensors are simply not sensitive enough for certain saliva samples, heat-inactivated ones in particular? Based on the literature, it seems "heating-activation does not decrease the diagnostic efficacy of SARS-CoV-2 IgM or IgG antibodies." (10.1016/j.cca.2020.06.032). Spiking saliva samples with standards simply increases the intrinsic antibody loads beyond the detection threshold?

All SARS-CoV-2 patient samples were required to be inactivated before we could work with these clinical materials, as per our institution's biosafety protocol. The saliva samples we analyzed were subject to stricter sample preparation protocols than the serum samples because the saliva samples were taken from actively infectious patients whereas the serum samples were collected from post-infectious patients. Due to the risks inherent in the saliva samples that contained live virus, our institution's approved biosafety protocol required that these samples be exposed to high temperature (95°C for 10 min) prior to analysis. While the high heat inactivated the live virus, it also denatures the antibodies within the saliva which prevented us from being able to detect saliva-based antibodies from the clinical saliva samples in this study. In contrast, we were able to request a biosafety protocol to inactivate the post-infectious serum samples at a lower temperature for a longer period of time (65°C for 30 min) because these were unlikely to contain live virus. This lower inactivation temperature enabled us to retain antigenicity of the anti-viral antibodies within the serum samples and allow us to detect them.

The past publication you reference (10.1016/j.cca.2020.06.032) describes an inactivation protocol that exposes specimens to 56°C for 30 minutes, which is likely why the antigenicity of the antibodies were preserved in that study. As seen in Akazawa-Ogawa et al. (https://doi.org/10.1007/s12551-017-0361-8), while IgG antibodies are stable at temperatures of 56°C, even a few minutes at 90°C causes irreversible denaturation. Therefore, we spiked the saliva samples with diluted antibody standards to simulate a raw saliva sample due to the degradation of the antibodies within the patient samples. We have edited the Methods to clarify these points.

ADDENDUM: Final Responses to Reviewer #1:

1. I'm still not convinced that this work merits a publication in Nature Biomedical Engineering. The reason for this is that none of the technologies presented here is novel. Overall, this is a good thorough work, however, not every meticulously performed work deserves a spot in Nature publications. Both the RNA-based and antigen based electrochemical detection for COVID-19 has been shown to work by others. All the techniques and engineering approaches are well known and the work does not bring any fundamental novelty.

Our manuscript describes the design, fabrication, and experimental validation of a low complexity multiplexed electrochemical (EC) sensor chip that allows for *simultaneous* detection of nucleic acids and proteins with high sensitivity and specificity within a small volume of clinically relevant biological fluids perfused through a single microfluidic channel. While individual assay capabilities might have been described previously, it is the combination of these elements and their optimization to enable ***simultaneous and multiplexed detection*** in a single device with a simplified design that can enable its use for diagnosis at the point of care (POC) is where our work is clearly novel. To our knowledge, this is also the first EC sensor that enables simultaneously multiplexed detection for multiple target biomarkers, including antibodies, protein antigens, and nucleic acids associated with SARS-Cov-2. The ability to obtain information about multiple biomarkers for disease detection allows for a more robust and accurate estimation of a patient's disease stage and progression. Additionally, the RNA-based

CRISPR assay described in this paper uses a novel HRP/TMB-based reporting strategy that, together with the novel anti-fouling surface chemistry, allows for highly sensitive and selective EC detection. The use of EC sensors also provides an added level of simplicity and robustness in terms of readout that is of enormous value for engineering of POC diagnostic devices, relative to other types of sensors (e.g., optical).

In addition, we designed a microfluidic cartridge that allows for automated sample preparation, amplification, and detection of target RNA within raw patient saliva samples, which improves the usability of our system (sample-in-answer-out). Again, to our knowledge, this has not been demonstrated previously. The microfluidic cartridge is 3D printed, biocompatible, and easily modifiable for other assays. Similarly, the multiplexed EC chip design has highly specific surface chemistry, which can be simply drop casted on the chip providing a robust platform for linking biomolecules of interest (e.g., both PNA and proteins in the present study). The high selectivity and ultra-low fouling on our sensor surface allowed us to perform multiplexing of different targets in the single-channel without any signal cross-talk and the signal outputs it provides should be easily comprehensible by both clinicians and patients. We should note that all of the other Reviewers clearly appreciated this novelty, which comes from the unique combination, integration, and optimization of multiple existing technology and lead to highly valuable novel diagnostic capabilities.

2. Authors claimed a point of care test. Because of the complexity of combining two different biological fluids, this is not doable at POC. Their claim is that this test will be performed at a moderately complex lab. If so, then there are already available techniques.

We agree that using two different biological fluids as we suggested in our original manuscript submission would be difficult in a POC use case. However, we clarified this point in our last revision by explaining that all analysis could be done on a single saliva sample, and hence, this device would be extremely valuable for POC applications, both in hospitals (e.g., emergency room) and in the field. The automation of sample preparation on-chip and the ability to derive results on multiple biomarkers from a single patient saliva sample makes this assay particularly compelling in the hospital-based POC scenario. Moreover, the potentiostats and pumps used to provide the final readout could potentially be integrated into the same device in the future to make it useful for field and eventually home POC applications as well. We now clarify all these points in the Discussion.

3. The apparent enhancement in sensitivity can also be explained easily as the measurement is done electrochemically as oppose to fluorescence/absorbance based techniques in other CRISPR/LAMP works.

We agree that one of the advantages of our multiplexed electrochemical sensing system is the enhanced sensitivity relative to common fluorescent methods. We have detailed this in our Results and consider it to be an additional advancement in the field of CRISPR-based diagnostics that is worthy of note.