An electrochemical SARS-CoV-2 biosensor inspired by glucose test strip manufacturing processes†

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Accurate and rapid diagnostic tests are critical to reducing the impact of SARS-CoV-2. This study presents early, but promising measurements of SARS-CoV-2 using the ACE2 enzyme as the recognition element to achieve clinically relevant detection. The test provides a scalable route to sensitive, specific, rapid and low cost mass testing.

SARS-CoV-2 came to the attention of health authorities during late 2019, shortly followed by declaration of a “public health emergency of international concern” on 30th of January 2020. SARS-CoV-2 quickly spread around the globe and was declared a pandemic by the World Health Organisation on 11th of March 2020. The virus, SARS-CoV-2, is the aetiological agent of coronavirus disease (Covid-19). Diagnostics have been a major priority and challenge thus far in the pandemic with assay quantity, reagent costs and time to result being of prime concern. SARS-CoV-2 has four major structural proteins,1 with the spike protein known to bind to the surface of cells expressing angiotensin converting enzyme 2 (ACE2) on their surface. The affinity between ACE2 and the spike protein has been shown to be in the low nanomolar range2 giving a similar level of affinity between ACE2 and the spike protein known to bind to the surface of cells expressing angiotensin converting enzyme 2 (ACE2) on their surface. The test provides a scalable route to sensitive, specific, rapid and low cost mass testing.

SARS-CoV-2 in adults or as a screening tool for identifying respiratory pathogen, either to allow definitive diagnosis of SARS-CoV-2 or as a screening tool for identifying positive cases, which then receive confirmatory lab testing. Finally, the ACE2 enzyme is a carboxypeptidase responsible for the hydrolytic cleavage of angiotensin II to angiotensin [1,7]-liberating phenylalanine in the process. The active site is positioned away from SARS-CoV-2 binding site meaning that enzyme activity and binding from other proteins or small molecules in human samples is likely to be insignificant when compared to binding of an entire SARS-CoV-2 viral particle (50–200 nm) and the resulting effect on electrochemical signal. A critical feature of the ACE2 enzyme is the hydrophobic region which normally facilitates insertion into cell membranes4 and allows ACE2 insertion into a synthetically made amphiphobic structures resembling cell membranes.

The fluorous effect is a well-known and well described tendency for fluorine atoms to avoid unfavourable interactions with other elements.5 Formation of fluororous self-assembled monolayers (SAMs) have been utilised in organic electronics to reduce biological fouling of surfaces.6 Here, a perfluorocarbon SAM formed of perfluorodecanethiol (PFDT) is deployed on a gold sensor surface to form a layer of amphiphobic character which facilitates straightforward insertion of ACE2 via its hydrophobic tail (ordinarily employed for membrane insertion in vivo). Use of a perfluorocarbon SAM with its unique properties provided a robust combination of anti-biofouling behaviour and enzyme insertion, allowing straightforward sensor preparation.

Electrochemical biosensors offer a very attractive route to sensitive and low cost detection of biological analytes. The most
notable example of this is the glucose biosensor, operating via
the glucose oxidase enzyme which is in widespread use each day
by diabetic patients for routine determination of blood glucose
levels. As a result of demand, facilities exist with very high
volume manufacturing capability (e.g. several million sensors
per day) and very well characterised chemical layer and enzyme
deposition protocols. Due to the volume of sales in the glucose
market (similar demand would exist for Covid-19 screening) unit
costs of 20 UK pence can be achieved using established manu-
facturing processes. Very few diagnostic technologies, including
lateral flow assays, can currently compete from a cost and volume
perspective (e.g. current lateral flow Covid-19 assays cost £5–20 per
test in the UK). In contrast, the sensor presented here requires a
simple two stage preparation procedure of (1) PFDT deposition on a
low cost PCB electrode and (2) ACE2 functionalisation through
physosorbtion into the PFDT (Scheme 1). These steps are compatible
with current manufacturing practices for glucose biosensors
and importantly provide opportunity to rapidly translate the
approach described here for SARS-CoV-2 detection into a glucose
test strip production environment. This unlocks potential to
achieve an extremely low cost Covid-19 assay, which utilises already
CE marked substrates and approved assay readers, thus mini-
mising the regulatory burden. Additionally, the assay is mutation
proof as it exploits the interaction between SARS-CoV-2 spike
protein and ACE2, has a degree of in-built surface orientation
through the ability of ACE2 to insert via its hydrophobic region and
offers the opportunity to develop similar tests for other respiratory
viruses entering cells via membrane bound surface proteins.

PFDT-SAM and ACE2 functionalisation experiments were
performed on a PCB sensor array bearing eight gold working
electrodes. Such PCB arrays are low cost and allow high
throughput assay development and represent an excellent test
bed for this assay. When functionalised with PFDT and ACE2, the
charge transfer resistance ($R_{CT}$) of the $[\text{Fe(CN)}_6]^{3–/4}$ redox
reaction, a signal parameter commonly used in impedimetric
biosensor measurements, increased as expected (Fig. S1, ESf†).
The PFDT SAM layer caused an increase in $R_{CT}$ and a further
increase was noted when ACE2 was physisorbed into the PFDT
layer, confirming successful insertion of the enzyme. This was in
contrast to experiments on facile ACE2 insertion with
thiolated-hydrocarbon layers, where ACE2 insertion behaviour
and sensor performance was not reproducible (Fig. S2, ESf†).

To characterise the sensitivity and specificity of the sensor,
experiments to assess dose response behaviour and determine
specificity of the ACE2 spike protein interaction were conducted
using recombinant spike protein, streptavidin and IL-6. IL-6, which is found at elevated levels in Covid-19 patients, and
streptavidin, widely used in bioassays, were employed as negative controls. A clear dose response effect was found for recombinant
spike-protein binding to ACE2 (Fig. 1). Fitting the dose response
curve gave a limit of detection for recombinant spike protein of
1.68 ng mL$^{-1}$. The binding between ACE2 and spike protein at
100 ng mL$^{-1}$ was shown to be significantly different to IL-6
(100 ng mL$^{-1}$, $P = 3.158E-7$) and streptavidin (200 μg mL$^{-1}$,
$P = 3.981E-4$) binding. This suggests it was possible to construct
a sensitive and selective sensor operating on the principle of
ACE2-spike glycoprotein interactions. Note – example Nyquist
plots, equivalent circuit and representative values are shown in
Fig. S3 (ESf†).

Next, assay performance in more complex samples was
explored. Due to biosafety restrictions, the sensor was tested
with inactivated virus samples at different concentrations
alongside a negative sample from a commercial kit. All samples
were provided in viral transport medium which contains pro-
teins and dead eukaryotic cells used to culture the virus and
was an appropriate proxy for a complex human tissue such as
saliva. Furthermore, these kits are used to validate diagnostic
tests in clinical biomedical laboratories because of their
close resemblance to clinical samples. When a viral dose response
curve was constructed, it was possible to detect the
inactivated virus with increasing concentration (Fig. 2). Curve
fitting and comparison to blank solution yielded an LoD of
38.6 copies mL$^{-1}$. At $10^4$ and $10^5$ copies mL$^{-1}$, the impedance
change was significantly different to the negative control
($P = 0.004$ and $P = 6.765E-4$ respectively). The background signal
increase observed in the negative control is most likely due to
residual non-specific surface interactions from the complex viral sample medium (proteins, cellular debris etc.). To provide context, RNA levels for SARS-CoV-2 in saliva samples have been estimated in the range of $10^5$–$10^{11}$ copies per mL$^{10,11}$ Therefore, these findings show it was possible to detect inactivated SARS-CoV-2 from a complex sample in 30 minutes at clinically relevant detection levels. Coupling this to the sensor being designed for manufacture in mass production environments, it raises the possibility of developing a Covid-19 assay using established sensors and instrumentation from the blood glucose industry.

To assess performance of the sensor more comprehensively, two human saliva samples were tested in bio secure conditions. One SARS-CoV-2 positive human saliva sample, confirmed through PCR based clinical diagnostics ($Ct = 26$ cycles, indicating high viral load), was transferred into inactivation solution called Viral Transport Medium (VTM), which require Biosafety Level 3 facilities. The ability to detect SARS-CoV-2 in VPSS using the biosensor reported here means that as well as for self testing, the assay is compatible with more centralised testing facilities where it is important to protect an operator running multiple samples such as hospital wards, GP surgeries, care homes, airports and workplaces. Adopting this revised approach, the $R_{CT}$ changes in the positive and negative samples were found to be significantly different (Fig. 3, $P = 1.2 \times 10^{-7}$). Changes to the assay approach with clinical samples resulted in an apparent drop in $R_{CT}$. This is attributed to a baseline artefact caused by Triton-X and sensor surface interactions in VPSS. Further investigation is being performed to fully understand and quantify these effects.

Numerous SARS-CoV-2 biosensor approaches have been developed and reported recently. Magnetic beads, carbon black electrodes and multiple antibodies were employed to achieve a detection limit of 6.5 plaque forming units mL$^{-1}$ in whole virus.$^{12}$ An ultrasensitive super sandwich-type assay has been presented to detect viral RNA from SARS-CoV-2 with an LoD of 1,13 and a FET biosensor capable of detecting spike protein with a LoD of 242 copies mL$^{-1}$ in transport medium has also been reported.$^{9}$ Plasmonic photothermal biosensors have been shown to detect down to 0.22 pM of viral RNA, which was estimated to be around $2.26 \times 10^4$ copies$^{14}$ Other electrochemical sensors have also shown good sensitivity, including molecular imprinted polymers (15 fM)$^{15}$ and graphene and gold nanostars ($1.68 \times 10^{-22}$ µg mL$^{-1}$).$^{16}$ The sensor developed here has comparable and clinically relevant sensitivity (LoD for inactivated virus of 38.6 copies mL$^{-1}$), with a clear route to manufacture. Importantly, the design of the sensor reported here is resistant to mutations in the spike protein. In terms of the chosen surface chemistry and the use of PFDT in a SAM layer, there are examples in the literature of other sensor systems with good anti-biofouling properties and these have been well reviewed.$^{17}$ Demonstrations of anti-fouling properties in ternary SAMs have recently been made$^{18,19}$ and there are advantages to these approaches for more complex sensors. However, in our experiments we could not reproduce the ACE2 insertion behaviour.
with standard hydrocarbon SAMs (Fig. S2, ESI†) and because of the emphasis on eventual sensor manufacture, PFDT was found to be well suited to the end application where monolayer coverage was sufficient to both retain the enzyme via amphiphobic interactions and provide anti-fouling properties.

Specificity of this sensor has been demonstrated against IL6, streptavidin, viral transport medium and one negative clinical saliva sample. This suggests that biofouling and non-specific binding of other commonly encountered biological proteins does not compromise assay sensitivity or specificity. Another consideration is other viral pathogens that bind to ACE2. Of the three viruses that are known to bind to ACE2, SARS-CoV-2 has the greatest affinity. SARS-CoV-1 is not present in the community and is therefore unlikely to impact the performance of this sensor. HCoV-NL63 primarily affects paediatric patient groups, which could limit the sensor to adult diagnostics if signal stratification is not possible. The current time to result based on the presented results is 30 min, which is competitive with existing SARS-CoV-2 assays but significant scope exists to reduce our assay time to faster sample turnaround times.

Further work will include sensor verification with a large set of clinical samples to determine, (1) whether low, medium and high viral load SARS-CoV-2 samples can be stratified alongside HCoV-NL63 samples or whether positive vs negative is the only feasible assay output, (2) fully understanding the measurement artefact effects of surfactants and detergents from VPSS and VTM, (3) testing shorter incubation times to reduce the overall assay time to result below the current 30 minutes and (4) testing simpler electrochemical measurements (e.g. amperometry) to determine if more straightforward measurement circuits (and therefore a lower cost reader can be employed) than those required for impedance.

These results present the basis of a novel, sensitive, selective and scalable biosensor assay which is based upon the principle of immobilising ACE2 into a layer with amphiphobic character. This sensor assay has sensitivity (1.68 ng mL⁻¹) with recombinant spike protein and specificity against two proteins (IL-6 and streptavidin). When evaluated with a SARS-CoV-2 analytical grade laboratory inactivated virus kit, an LoD of 38.6 copies mL⁻¹ was achieved. Finally, testing with high viral load clinical sample (Ct = 26) showed strong discrimination against a negative sample following inactivation in VPSS medium. By taking advantage of glucose biosensor production approaches (per sensor cost > £0.2) it is possible to demonstrate a clear path to translation through established manufacturing techniques, utilisation of existing sensor substrates and read out devices with prior CE marking. These findings together point towards a useful diagnostic tool, which can be rapidly deployed at low cost to screen for SARS-CoV-2 in a range of settings.

Conflicts of interest
There are no conflicts to declare.

Notes and references