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## Towards Label-free detection of viral disease agents through their cell surface proteins: Rapid screening SARS-CoV-2 in biological specimens

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### ABSTRACT

Current methods for the screening of viral infections in clinical settings, such as reverse transcription polymerase chain reaction (RT-qPCR) and enzyme-linked immunosorbent assay (ELISA), are expensive, time-consuming, require trained personnel and sophisticated instruments. Therefore, novel sensors that can save time and cost are required specially in remote areas and developing countries that may lack the advanced scientific infrastructure for this task. In this work, we present a sensitive, and highly specific biosensing approach for the detection of harmful viruses that have cysteine residues within the structure of their cell surface proteins. We utilized new method for the rapid screening of SARS-CoV-2 virus in biological fluids through its S1 protein by surface enhanced Raman spectroscopy (SERS). The protein is captured from aqueous solutions and biological specimens using a target-specific extractor substrate. The structure of the purified protein is then modified to convert it into a bio-thiol by breaking the disulfide bonds and freeing up the sulfhydryl (SH) groups of the cysteine residues. The formed biothiol chemisorbs favourably onto a highly sensitive plasmonic sensor and probed by a handheld Raman device in few seconds. The new method was used to screen the S1 protein in aqueous medium, spiked human blood plasma, mucus, and saliva samples down to 150 fg/L. The label-free SERS biosensing method has strong potential for the fingerprint identification many viruses (e.g. the human immunodeficiency virus, the human polyomavirus, the human papilloma virus, the adeno associated viruses, the enteroviruses) through the cysteine residues of their capsid proteins. The new method can be applied at points of care (POC) in remote areas and developing countries lacking sophisticated scientific infrastructure.

### Introduction

The spread of the SARS-CoV-2 virus infections has caused millions of deaths and significant interruptions to human life with serious side effects to the global economy [1–6]. To manage the health challenges caused by SARS-CoV-2 and other viruses, it is necessary to detect their outbreak at an early stage [7]. Multiple analytical techniques have been demonstrated for the detection of SARS-CoV-2 virus in clinical specimens [8,9]. The gold standard for the detection of viral infections is RT-qPCR [10]. However, this method is time consuming, expensive and requires highly trained personnel to carry out the test. In addition, RT-qPCR methods might not detect the pathogen in asymptomatic individuals at the very early stages of infection [11]. Other

methods such as enzyme-linked immunosorbent assay (ELISA), RT-loop-mediated isothermal amplification (RT-LAMP), chemiluminescence immunoassays, lateral-flow immunochromatographic assays, amplicon-based metagenomic sequencing, and biosensors have been developed for the detection of SARS-CoV-2 in different biological specimens [12]. Nanotechnology-based methods have been also demonstrated for the point of care screening of the corona virus [12]. A colorimetric assay that uses gold nanoparticles (AuNPs) coated with antisense oligonucleotides was developed for the detection of SARS-CoV-2 RNA after its extraction from the pathogen cells. However, the method has low sensitivity (detection limit = 0.18 µg/ mL) [13]. The surface of the SARS-CoV-2 pathogen is covered with spike protein molecules that are responsible for membrane fusion [14,15]. The spike protein is a large protein that is made of two subunits, S1 and S2 [16]. The molecular structure of the S1, S2 proteins include several cysteine residues that are inter-

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connected through disulfide bonds [17–21]. New methods have been developed to detect of SARS-CoV-2 through its spike protein. For example, antibody-coated AuNPs were synthesised to bind the S1 protein from saliva samples. The protein was then detected by UV-Vis and electrochemical methods down to 250 ng/mL and 1 pg/mL, respectively [22]. Zhang et al demonstrated new dye-functionalised silver nanoparticles and antibody-coated gold film as smart nanomaterials to detect SARS-CoV-2 in spiked saliva down to 6.07 fg/mL by surface enhanced Raman spectroscopy (SERS) [23]. The method uses 4-mercaptobenzoic acid as a Raman label for the indirect detection of the spike protein and requires 4 h of processing/screening time per sample. Therefore, there is an ongoing need for a direct SERS method for the rapid detection of viral infections clinical settings. Herein, we present a label free SERS method for the fingerprint identification of the S1 protein in biological specimens within the hour. The specificity of the method is maintained by utilising a target-specific extractor substrate for the selective binding of the S1 protein of the pathogen from the biological specimen. The protein molecular structure is then modified to free up the SH groups of its cysteine residues. The modified protein is directly measured on a gold coated silicon nanopillar SERS substrate by a handheld Raman device down to 150fg/L without the need for a Raman reporter [24,25]. The new method has strong potential for the screening of many harmful viruses through the cysteine residues of their capsid proteins.

## Materials and methods

### Chemicals and reagents

Phosphate buffered saline (PBS), glycine, hydrochloric acid and tyrosine were purchased from Sigma Aldrich (USA). Tris(2-carboxyethyl) phosphine (TCEP) and 3-mercaptopropionic acid (MPA) were purchased from Thermo Scientific (USA). Recombinant spike S1 protein of SARS-CoV-2 (molecular weight = 78.3 KDa) and anti S1 monoclonal antibody were purchased from Sino Biological Inc. (China). Erythropoietin, troponin and insulin were purchased from Abcam (Australia). All preparations were made using deionized water (ultrapure Millipore, 18.2 MΩ cm, 25 °C). Gold coated silicon nanopillar substrate (Au@SNP) was outsourced from Silmeco ApS (Denmark), molecular sieve columns (illustra NAP-5) were outsourced from GE Healthcare Life Science (Australia).

### Preparation of unreduced and reduced spike protein standard solutions

A standard solution ( $1.9 \times 10^{-7}$  M) of unreduced S1 protein was prepared by diluting 75 μL of the S1 protein stock solution (2 mg/mL,  $25.5 \times 10^{-6}$  M) to 10 mL using 1 × PBS (pH = 7.4). To prepare  $1.9 \times 10^{-7}$  M stock solution of reduced S1 protein, 74.5 μL of the unreduced protein stock solution ( $25.5 \times 10^{-6}$  M), were combined with 74.5 μL of TCEP (40 mM) in a LoBind vial and the volume completed to 10 mL using 1 × PBS (pH = 7.4). Standard solutions of reduced S1 protein were prepared in range of  $1 \times 10^{-8}$  M to  $0.5 \times 10^{-18}$  M, by serial dilution of the reduced S1 protein stock using 1 × PBS (pH = 7.4).

### Raman measurements of unreduced and reduced S1 protein

20 μL of unreduced and reduced S1 protein solutions were loaded onto Au@SNP substrates and stood at room temperature for 15 min. The nanostructured chips were then washed with deionized water 3 times and dried using N<sub>2</sub> gas. The Raman measurements ( $n = 3$ ) were taken using the handheld Raman spectrometer (ocean optics, USA) in the wavelength range of 500–1700 cm<sup>-1</sup> (spectral resolution = 12 cm<sup>-1</sup>). A 785 nm laser beam was used to screen the samples, using the raster orbital scanning mode (ROS) for measurements. The laser power at the sample was 5 mW. Each measurement was carried out using 10 accumulations (each accumulation was for 100 ms, total acquisition time = 1s). The background noise and fluorescence were automatically corrected by the programmed software algorithm (OceanView Spectroscopy 1.5.07)

### Fabrication of target-specific extractor substrate

$1 \times 10^{-7}$  M anti S1 monoclonal antibody (50 μL) was combined with 40 mM TCEP (50 μL) in a LoBind vial and left to stand for 15 min to reduce the disulfide bonds in the hinge region of the antibody and form free sulfhydryl groups. An Au@SNP substrate was then inserted into the vial and left to stand for 4 h to chemisorb the antibody fragments through the development of Au-S bonds with the gold nanostructures within the Au@SNP substrate. To block the non-specific adsorption of foreign molecules, the remaining bare sites on the antibody-coated substrate was immersed in a LoBind vial that contains 50 μL of  $1 \times 10^{-8}$  M MPA aqueous solution for 1 h. The Au@SNP substrate was then washed 3 times using 1xPBS solution (pH = 7.4) and stored at 4 °C for future use.

### Negative and positive control measurements

A negative control sample was prepared by mixing amino acids and proteins (tyrosine, EPO, troponin and insulin) in 5 mL of deionized water to the final concentration of  $1 \times 10^{-8}$  M tyrosine,  $1 \times 10^{-8}$  M EPO,  $1 \times 10^{-7}$  M troponin and  $1 \times 10^{-9}$  M insulin, respectively. A positive control sample was prepared by taking 1 mL of the negative control sample and adding 0.11 mL of  $1 \times 10^{-11}$  M S1 protein standard solution. The concentration of the S1 analyte in the solution was  $9.9 \times 10^{-13}$  M. 15 μL of the negative and positive control samples were loaded onto antibody-coated extractor substrates and left to stand for 20 min to bind the S1 protein. The nanostructured chips were then washed 3 times with deionized water then immersed into LoBind vials that contain (100 μL) of acidic glycine (pH = 2.5) that was used as a buffer. The chip was left to sit for 10 min to recover the bound protein. The solution was then collected and loaded into a molecular sieve column to trap and separate the acidic glycine molecules from the captured protein. The purified protein was recovered from the column using 1xPBS (400 μL, pH = 7.4) as an eluant. For the Raman measurements, 10 μL of the eluted purified protein were combined with 10 μL of TCEP (40 mM), loaded onto Au@SNP substrates and stood at room temperature for 15 min. The nanostructured chips were washed 3 times with deionized water, dried using N<sub>2</sub> gas, reduced with TCEP then measured with the handheld Raman spectrometer ( $n = 3$ ) as described earlier.

### Selective extraction and detection of the S1 protein from spiked biological samples

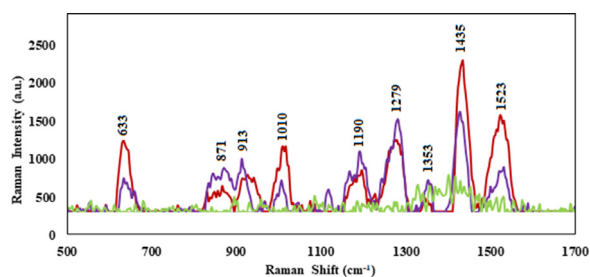
The S1 protein was spiked into human blood plasma, human mucus and saliva samples and diluted with PBS solution (pH = 7.4) to the final concentration of  $1 \times 10^{-11}$  M. 15 μL of each biological sample was loaded onto an antibody-coated extractor substrate and stood at room temperature for 20 min to readily attach the S1 protein to the surface. The substrate was then immersed into a LoBind vial that contains 100 μL of acidic glycine (pH = 2.5) for 10 min to recover the bound S1 protein. The solution was then collected and loaded into a molecular sieve column to trap and separate the acidic glycine molecules from the captured protein. The purified protein was recovered from the column using 1xPBS (400 μL, pH = 7.4) as an eluant. For the Raman spectroscopy, 10 μL of the eluted purified protein were combined with 10 μL of TCEP (40 mM), loaded onto Au@SNP substrates and stood at room temperature for 15 min. The nanostructured chips were washed 3 times with deionized water, dried using N<sub>2</sub> gas to avoid air oxidation of the analyte, reduced with TCEP, then measured with the handheld Raman spectrometer ( $n = 3$ ) as described earlier.

### Ethics approval

The study was approved by the ethics committee of Queensland University of Technology (approval numbers 1900000820, 2000001115,

**Table 1**  
Raman vibrational modes of the unreduced and reduced S1 protein.

Raman Shift (cm <sup>-1</sup> )	Unreduced	Reduced	Band assignment	Refs.
1523	x	x	Amide II vibrational modes.	[35,37]
1435	x	x	CH <sub>2</sub> bending, cysteine residues.	[32,39]
1353	x	x	Tryptophan residues, Alanine residues.	[32,38,39]
1279	x	x	Amide III vibration modes, histidine residues.	[32,39]
1190	x	x	C-N vibration modes, amide II vibrational modes.	[32,39]
1010	x	x	phenylalanine residues, histidine residues.	[37,39,40]
913	x	-	C-C stretching modes, glutamic acid residues.	[37,39]
871	x	x	Tyrosine residues.	[37,38,39]
636	x	x	C-S stretching mode, C-C twisting of tyrosine residues.	[32,38,39]



**Fig. 1.** SERS measurements of unreduced S1 protein ( $1.9 \times 10^{-7}$  M, purple spectrum) reduced S1 protein ( $1.9 \times 10^{-7}$  M, red spectrum). The green spectrum represents the SERS measurement of the blank substrate.

4652, 1900000820, 2000001115). Saliva and mucus samples were self-donated by the co-author Dr. Kristyan Guppy-Coles.

## Results and discussion

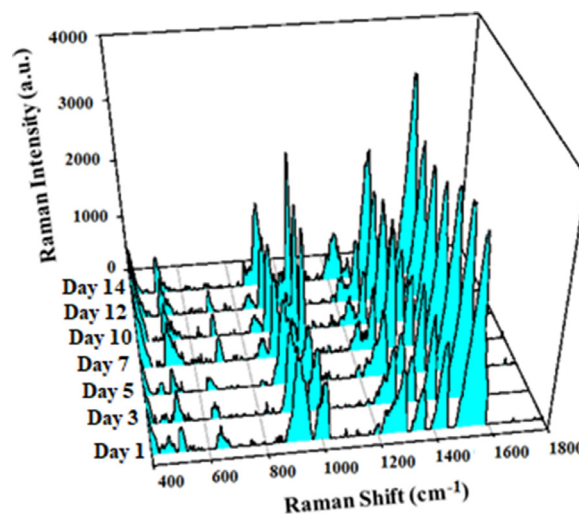
The detection of viral infections, such as SARS-CoV-2 infections, by antigen tests has been recommended by the world health organisation (WHO) as a cheap and rapid alternative for the RT-qPCR test [26]. The detection of the virus spike protein has been one of the useful approaches to develop new antigen tests for SARS-CoV-2 infections [10,23,27–31]. Many of the current antigen tests are designed to detect the spike protein through the change of the electric or optical properties of a sensor [Table S1]. However, none of these tests provide fingerprint identification of the spike protein. Herein we demonstrate label free Raman spectroscopy method for the direct determination of the S1 protein in aqueous solutions, spiked saliva, mucus and human blood plasma.

### SERS measurements of S1 protein

The fingerprint of the S1 protein structure was obtained by SERS (Fig. 1, purple spectrum). However, the random orientation of the physically adsorbed protein molecules impact on the reproducibility of the measurement which is critical for the quantitative analysis of the protein by SERS [32,33]. Therefore, we attempted to control the oriented immobilisation of the S1 protein molecules onto the SERS substrate through its cysteine residues [34,35]. The disulfide bond structure of the protein molecules was reduced to produce SH groups that chemisorb onto the SERS substrate and form stable Au-S bonds [16,36]. The SERS measurement of the reduced S1 protein is displayed by the red spectrum in Fig. 1. The Raman band assignments of reduced and unreduced S1 protein are given in Table 1.

### Reproducibility of the SERS measurements of reduced S1 protein

The reproducibility of the SERS measurement of reduced S1 protein was investigated at different time intervals over 14 days (Fig. 2). As shown by Fig. 2, the SERS spectrum of the reduced protein was highly



**Fig. 2.** Seven Raman measurements of reduced S1 protein ( $1 \times 10^{-11}$  M) on gold coated silicon nanopillar substrate within 14 days. RSD = 93.7%.

reproducible after two weeks from the first measurement. The reproducibility of the Raman spectra can be accredited to the oriented immobilisation of the reduced protein onto the substrate through the formation of Au-S bonds and the uniformity of hot spot distribution on the substrate surface [32,41,42].

### SERS quantification of reduced S1 protein

Due to the high reproducibility of its Raman spectrum, the reduced S1 protein was used for its quantification by SERS (Fig. 3a). The intensity of the Raman band at  $1433 \text{ cm}^{-1}$  was found to monotonically increase with the protein concentration. A linear relationship between the Raman signal intensity at  $1433 \text{ cm}^{-1}$  and the log concentration of the reduced S1 protein, was found in the range of  $10^{-8} \text{ M} - 10^{-18} \text{ M}$  and represented by the regression equation  $y = 99.65x + 2630.8$  ( $R^2 = 0.9679$ ) (Fig. 3b). The lowest limit of quantification (LOQ) of the protein was determined experimentally to be  $1 \times 10^{-18} \text{ M}$  ( $150 \text{ fg L}^{-1}$ ) [33–35].

### Synthesis of target specific extractor substrate

The direct SERS detection of proteins requires their purification from the complex biological matrix [43,44]. Therefore, we synthesised a target-specific extractor substrate to bind the S1 protein from biological specimens. Thiol-ended antibody fragments were produced by reducing the disulfide bonds within the hinge region of the S1 antibody [36,45]. The antibody fragments were chemisorbed onto gold coated silicon nanopillar substrate [32,46]. As indicated by Fig. S1, the attachment of the antibody fragments was evident by the emergence of Raman bands that are attributed to a glycoprotein structure [36,42,47]. To prevent the non-specific adsorption of interfering molecules from

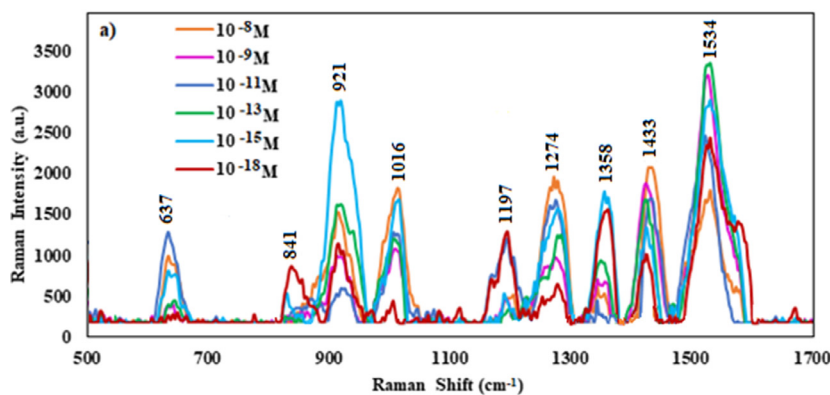


Fig. 3. (a) SERS measurements of different concentrations of reduced S1 protein on gold coated silicon nanopillar substrates, (b) relationship between the Raman signal intensity at  $1433\text{cm}^{-1}$  and the log concentration of reduced S1 protein.

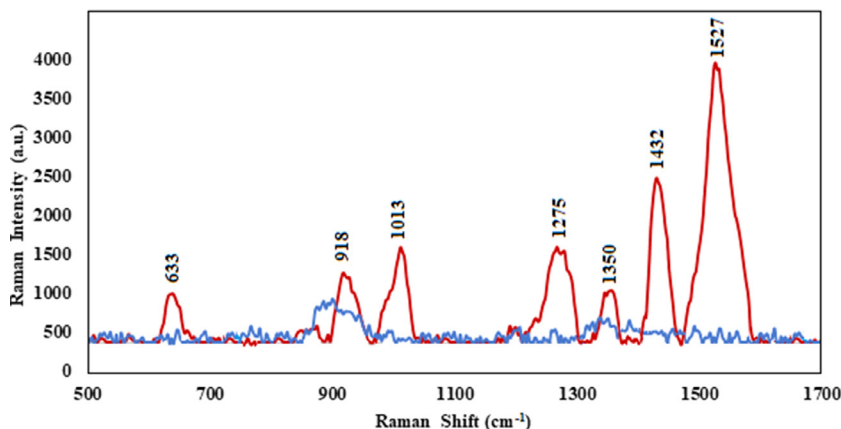
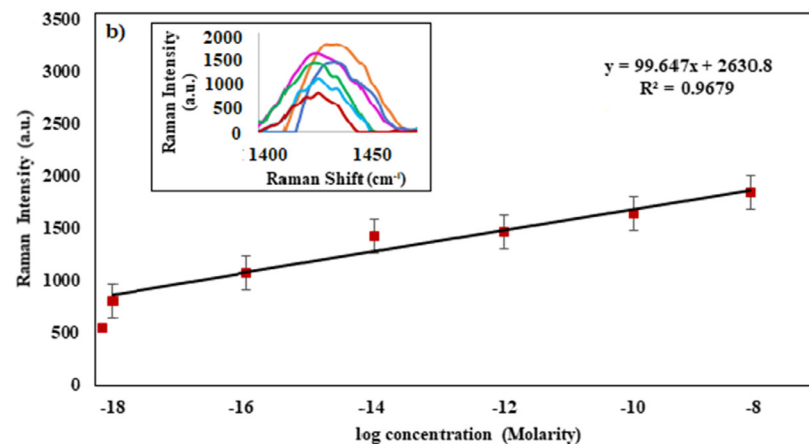


Fig. 4. SERS measurements of extracted and reduced proteins from negative and positive control samples.

the biological matrix, the unoccupied sites on the substrate surface were blocked with MPA [32,36,47]. In addition, the polarity of MPA increases the hydrophilicity of the surface and therefore facilitates the diffusion of the target molecules, from the aqueous phase towards the substrate surface to bind to the antibody fragments [47].

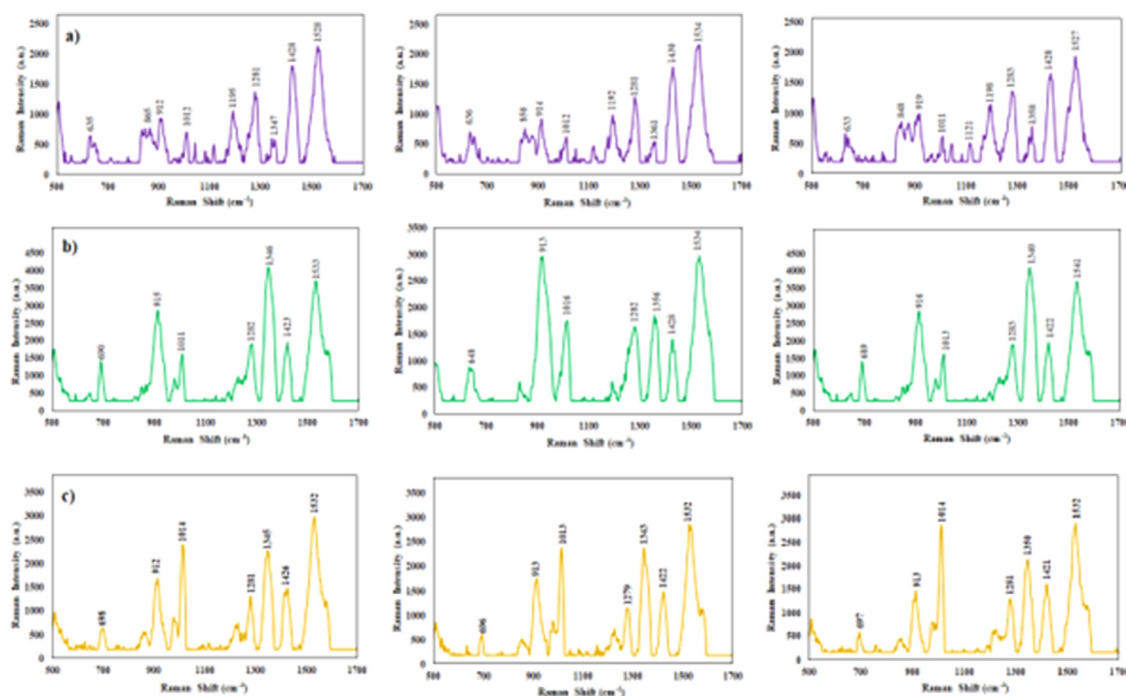
The fabricated substrate was utilised to bind the S1 protein from negative and positive control samples. The bound protein was recovered from the substrate, reduced and measured by the handheld Raman device [48]. As indicated by Fig. 4, the Raman measurement of the recovered protein from the negative control sample did not display any diagnostic Raman signals of a protein structure, thus confirming absence of the S1 protein and the failure of the extractor substrate to bind other proteins from the sample matrix. To the contrary, the SERS measurement of the protein that was recovered from the positive control sample showed Raman bands that are similar to those of the reduced S1 protein

(Fig. 1 red spectrum, Fig. 4 red spectrum). This result indicates the high specificity of the extractor substrate towards the target protein.

#### Determination of S1 protein in biological samples by label free SERS

The extractor Au@SNP substrate was used to bind the protein from the spiked human blood plasma, saliva and mucus samples at pH 7.4 which is suitable for the formation of an antibody-antigen complex between the antibody and the S1 protein [32,34–36]. The purified protein was reduced and measured on gold coated silicon nanopillar SERS substrate. As shown by Fig. 5, the SERS measurements showed Raman bands that are similar to those of the reduced S1 protein standard. As shown by Fig. 2 and Fig. 5a–c, the reproducibility in the spectra of the reduced S1 protein extends to its detection in the spiked human samples. The concentration of the S1 protein in the samples was found to





**Fig. 5.** Raman spectra of (a) Three spectra of S1 ( $1 \times 10^{-11}$ M) after extraction from spiked human plasma, (b) Three spectra of S1 protein ( $1 \times 10^{-11}$ M) after extraction from spiked human mucus, (c) Three spectra of S1 protein ( $1 \times 10^{-11}$ M) after extraction from spiked human saliva. The protein was reduced by TCEP after extraction, chemisorbed onto gold coated silicon nanopillar substrate and screened by handheld Raman spectrometer.

be  $1 \times 10^{-13}$ M. The qualitative differences in the spectra depicted in Fig. 5a–c may be attributed to slight differences in the arrangement of the different domains of the protein molecule onto the surfaces of the SERS substrate [49].

Nascimento et al, recently demonstrated a new electrochemical method for the detection of the S1 protein in saliva [26]. The method uses magnetic beads to bind the protein and after binding, ACE2-coated gold nanoparticles are used to sandwich bind the protein. The formed bioconjugate is then deposited an electrode and quantified down to  $0.35 \text{ fg L}^{-1}$  using differential pulse voltammetry (DPV). Despite the high sensitivity of the method, it lacks high specificity (RSD=93.7%), due to the residual current of the bare electrode in the absence of the S1 protein (as indicated by Fig. 3 in reference 26). Therefore, this method has the potential for false positive identification of S1 protein in the sample. In contrast, the new SERS method has shown high specificity towards the S1 protein as indicated by Fig. 5. In addition, it provides direct fingerprint identification of the protein through the cysteine residues in its molecular structure and without the use of a Raman reporter.

## Conclusion

In this work, we present a label free SERS method for the detection of viruses through the cysteine residues in their surface proteins. The new label free SERS detection was utilised for the screening of SARS-Cov-2 in biological specimens through its S1 protein. A target-specific extractor substrate was fabricated and used to bind and purify the protein from the sample matrix. The purified protein molecular structure was modified to free up the SH groups of its cysteine residues then chemisorbed in a unified orientation onto a SERS sensor and screened using the handheld device. The new method showed high sensitivity without the need for a Raman reporter and used for the detection of the protein down to  $150 \text{ fg L}^{-1}$  within the hour. The new direct SERS method has strong potential for the fingerprint identification of many viruses, through the cysteine residues in their capsid proteins, at points of care and in remote areas that lack scientific infrastructures for sophisticated techniques such as RT-qPCR. A kit that comprises of target specific extractor substrate and

Au@SNP SERS sensors, bond-breaker TCEP reagent and buffer solution can be pre-manufactured and commercialised to the health authorities in the remote areas that do not have advanced screening facilities. An advanced design of this kit can involve a 96 well plate that have 96 target-specific extractor chips to extract 96 different viruses from microliter aliquots of one biological sample. The extracts are then mixed with the reducing agent and loaded onto 96 SERS sensors then screened by the handheld Raman. Since gold and silicon materials are chemically inert towards air oxidation, the self-life of the plasmonic extractor substrate and SERS sensor is relatively long. To maintain the binding efficiency of the antibody coated extractor substrates, they should be stored in PBS buffer at pH 7.4. The target-specific extractor substrates can potentially be recycled for the repeated extraction of proteins. However, since the present method is proposed for the detection of viral infections, it is not recommended to recycle the extractor substrate and the SERS sensor to avoid contamination by the infectious disease agents (viruses) and the potential for false positive identification.

## Declaration of Competing Interest

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

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## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.slasd.2022.06.001](https://doi.org/10.1016/j.slasd.2022.06.001).

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