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ABSTRACT
Nanostructured surfaces enhance ion yields in matrix-assisted laser desorption–ionization mass spectrometry (MALDI-MS). The spike protein complex, S1, is one fingerprint signature of SARS-CoV-2 with a mass of 75 kDa. Here, we show that MALDI-MS yields of SARS-CoV-2 spike protein ions in the 100 kDa range are enhanced 50-fold when the matrix–analyte solution is placed on substrates that are coated with a dense forest of multi-walled carbon nanotubes, compared to yields from uncoated substrates. Nanostructured substrates can support the development of mass spectrometry techniques for sensitive pathogen detection and environmental monitoring.

The global SARS-CoV-2 pandemic since early 2020 has highlighted the need for low cost, sensitive, robust, and widely deployable techniques for detection of pathogens, such as the SARS-CoV-2 virus. Mass spectrometry techniques are very widely used in biomedical sciences. MALDI-MS (matrix-assisted laser desorption/ ionization mass spectrometry) is a relatively low cost and rapid technique.1–3 In response to the current crisis, MALDI-MS based detection of SARS-CoV-2 signatures has been reported from nasal swabs and from saliva samples.4–7 In MALDI-MS, the use of (nano)-structured substrates has been shown to enhance analyte ion yields and, in some cases, eliminate the need for a matrix altogether.7,12 However, these signal increases were limited to relatively low mass analyte ions, below 10 kDa. Here, we report on the detection of SARS-CoV-2 spike protein (S1) ions in the 100 kDa mass range. The spike protein complex is a signature for detection of SARS-CoV-2.4–7 With a mass of 75 kDa, direct detection in MALDI-MS is challenging as the desorption and ionization processes in the laser–matrix–analyte interaction can lead to fragmentation of the protein into a series of poly-peptide and small fragment ions. This makes unique identification of pathogens difficult in MALDI-MS experiments. Detection of robust signatures in mass spectra of SARS-CoV-2 samples is beneficial for the development of environmental sensors and for rapid, low-cost testing capabilities outside laboratory settings. We compare spike protein ion yields from carbon nanotube substrates to yields from standard flat substrates (made from single crystal silicon wafers) for a series of MALDI-MS conditions and find S1 analyte ion yields that are enhanced 50-fold when we used carbon nanotube substrates.

Recombinant SARS-CoV-2 spike protein, S1, was purchased from R&D Systems (Minneapolis, MN, USA). We prepared dilute solutions of S1 with sinapinic acid as a standard MALDI-MS matrix (purchased from ProteoChem, Hurricane, UT, USA). The matrix-analyte solution consisted of 10 mg of sinapinic acid in 1 ml of solution (50% acetonitrile and 50% water with 0.1% trifluoroacetic acid) and we prepared analyte–matrix samples with 0.05–0.2 µg of S1
protein per 1 \( \mu l \) of matrix solution. We then deposited drops of 2.5 \( \mu l \) of the matrix–analyte solution onto flat silicon single crystal samples and onto silicon samples which had been coated with a dense forest of multi-wall carbon nanotubes (CNTs).

Nanotube forests were grown on silicon wafers in a plasma-enhanced chemical vapor deposition (PECVD) process. The growth process results in a relatively uniform height of CNTs. A scanning electron microscope (SEM) image of a sample with a CNT forest is shown in Fig. 1(a). The average diameter of the CNTs is about 70 nm. In Fig. 1(b), we show a section of a dried drop of the matrix–analyte solution deposited on a CNT substrate. SEM images of mm-scale dried drops of the matrix–analyte solution on CNT and flat substrates are shown in Figs. 1(c) and 1(d), respectively. We observe the formation of much denser assemblies of matrix–analyte crystals on the CNT samples compared to flat substrates (silicon shown, also titanium, not shown). The images in Figs. 1(c) and 1(d) also show damage spots from the sample exposure to laser pulses during MALDI-MS experiments.

We conducted MALDI-MS experiments with the Sciex 4800 MALDI-TOF-TOF, and with a modified D850 time-of-flight (TOF) reflectron system from Jordan TOF Products, Inc. In the Sciex 4800, a Nd:YAG laser provides 3–7 ns long pulses at 355 nm with a

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**FIG. 1.** (a) SEM images of a CNT sample prior to deposition of the matrix–analyte solution. (b) and (c) SEM images of a CNT sample onto which a drop of the matrix–analyte solution had been deposited. The sample in (c) had been exposed to a series of laser pulses, which led to the removal of the matrix crystals and the CNT forest in the laser spot. (d) SEM image of a single crystal silicon substrate onto which a drop of the matrix–analyte solution had been deposited. The sample was then exposed to a series of laser pulses leading to local removal of the matrix–analyte crystals and ablation of the silicon substrate.
repetition rate of up to 200 Hz. The laser pulse energy was 15 μJ and the laser spots had a diameter of 75 μm, resulting in a laser intensity of 340 mJ/cm². We collected spectra with 900 shots in the mass range from 20 to 500 kDa in a linear mode and from 80 to 4000 Da in a reflectron mode. We measured samples several times in a series of laser spots and we measured samples with varying S1 concentrations. The instrument was calibrated using the mass peaks from a bovine serum albumin (BSA, Merck Life Science NV, Amsterdam, The Netherlands) sample (66.793 kDa).

In our modified Jordan TOF setup, we used a 532 nm laser with 23 ns pulse length [full width at half maximum (FWHM)] at a repetition rate of 10 Hz. Laser pulse fluences were tuned from less than 1 to over 500 mJ/cm² with laser pulse energies ranging from 50 to about 670 μJ/pulse and laser spot sizes from 0.1 to 0.3 mm². We used a micro-channel plate (MCP) detector as an ion detector in the linear mode. The pressure in the sample chamber was <10⁻⁵ Torr and we applied differential pumping to maintain pressures below 10⁻⁶ Torr in the mass spectrometer and near the MCP. The sample was held at a voltage of +1 to +4 kV for acceleration of ions into the linear mass spectrometer.

In Fig. 2, we show mass spectra in positive ion polarity from the Sciex 4800 from flat and CNT substrates (both prepared with a drop of 0.2 μg of S1 protein per 1 μl of matrix solution) in the mass range from 20 to 400 kDa. The S1 protein has a mass of 75 kDa. We observe strong mass peaks at 212, 106, 71, and 53 kDa. We interpret the peaks as derived from the spike protein S1 trimer at 212 kDa in the q = 1 to 4+ charge states, respectively. The observed S1 trimer peak in the 1+ charge state at 212 kDa indicates that a fragment with a mass of 13 kDa had been dissociated from the trimer, reducing the trimer mass from the expected 225 to 212 kDa. The peak of the doubly charged dimer is shifted by 6.5 kDa to the observed 106 kDa. The peak at 53 kDa, which we interpret as the trimer in the 4+ charge state is shifted by 3.25 kDa, consistent with the loss of a 13 kDa group in S1 trimer ion formation. The dominant peak at 106 kDa has a full width at half maximum (FWHM) of 10 kDa, broader than the reference peak from the BSA calibration sample, which had a FWHM of 2 kDa at 67 kDa [inset of Fig. 2, top]. We observe mass analyte ion intensities that are 50-fold higher from the CNT substrates compared to the flat substrates.

We now estimate the limit of detection for S1 in our experiments. A limit of detection for the spike protein of 10 pg has been reported in recent MALDI-MS studies. Our experiments were conducted using 2.5 μl droplets of 0.2 μg/μl S1 protein, thereby depositing 500 ng S1 protein per droplet. The droplets had a diameter of about 2 mm (Fig. 1). The laser spot for the data in Fig. 2 had a diameter of 75 μm. The sample was locally consumed during the analysis in the laser spot area, containing a proportional amount of the analyte, i.e., about 0.7 ng (700 pg). The signal-to-noise ratio in the trimer peak from the CNT sample in Fig. 2 is about 10:1. We can still detect the trimer with a signal-to-noise ratio of ~2:1, thus, from ~5 times fewer analyte molecules. We can, thus, estimate the sensitivity of our approach in the current implementation to be about 140 pg. Future studies for a series of S1 concentrations, CNT, laser, and mass spectrometer instrument parameters can be conducted to further improve the detection limit of detection using CNT substrates in MALDI-MS.

A concern when using CNTs as a substrate in MALDI-MS is potential mass interference from carbon clusters with analyte ions. In Fig. 3, we show mass spectra in the reflectron mode in the mass range from 100 to 800 Da for a CNT substrate with matrix solution (no S1 analyte, top) and with a drop from the matrix–analyte solution (bottom). These low mass range spectra were collected in the reflectron mode with the Sciex 4800 instrument. The inset in the top spectrum shows a mass spectrum from a CNT sample without the matrix or matrix–analyte solution, collected with the Jordan instrument in the
linear mode. The CNT sample without the matrix deposit shows a series of carbon cluster ions up to 240 Da. The spectrum from the matrix solution on CNTs shows two dominant peaks from the sinapinic acid matrix at 207 and 224 Da. We did not observe higher mass carbon cluster ions from the CNT samples. The observed low mass carbon cluster ions did not interfere with low mass S1 fragment ions.

In the standard MALDI-MS approach, the sinapinic acid matrix facilitates absorption of the laser photons, leading to desorption of intact and fragmented analyte molecules, a fraction of which is also ionized. The CNT forest can aid absorption of laser energy. This can lead to enhanced desorption and ionization of analyte ions. Laser pulse energy thresholds for efficient ablation and ionization of CNTs are higher than for desorption and ionization of matrix–analyte species, hence mass interferences from CNT based ions are not a concern, especially for the ion mass range above about 200 Da.

We conducted a series of experiments where we changed the laser pulse energy and detected ion yields in the low and high mass ranges for a series of laser pulses. Laser induced desorption processes lead to depletion of matrix–analyte crystals from the sample. We observe damage spots from laser exposures ex situ by SEM imaging (Fig. 1). In Fig. 4, we show relative ion intensities in a sequence of 300 laser shots applied to a fixed spot on a CNT sample with the matrix–analyte solution. Analyte ion intensities deplete to one-third of their peak intensity after about 60 laser shots. We observe a threshold laser fluence for ion desorption of about 20 mJ/cm² (a laser pulse energy of 50 μJ) in the Jordan instrument operated in the linear mode. While progressively more matrix crystals are desorbed and the high mass ion yields decreases, yields of low mass carbon ions, which can originate mostly from the CNT substrate, trend higher or remain about constant (inset in Fig. 4).

Spike protein ion yields are very strongly enhanced for CNT substrates compared to flat substrates. Figure 4 shows strongly enhanced high mass analyte ion yields for laser pulse energies of 270 and 670 μJ but not for 140 μJ. With a typical laser beam spot size of 0.1 mm² (Fig. 1), we can estimate a threshold laser intensity of about 200 mJ/cm² for the onset of the strong enhancement effect for high mass analyte ion formation. We do not have quantitative insight into absolute ion yields or on the ion to neutral fraction in MALDI-MS with CNT substrates or...
molecules were mixed with artificial saliva. We deposited drops of the increase in analyte ion intensities from CNT samples. Both effects contribute to the strong increase in analyte ion intensities from CNT samples.

We conducted a series of laser mass spectrometry experiments with CNT substrates but without matrix solutions. Here, spike protein molecules were mixed with artificial saliva. We deposited drops of the saliva-analyte solution on CNT substrates and found that the drops spread out over an area of about 1 cm². MALDI-MS experiments with the Sciex 4800 MALDI instrument did not detect analyte ions from dried drops of saliva-analyte solutions. This underscores the role of the matrix crystals to absorb laser excitations and the role of CNTs to form denser assemblies of matrix–analyte crystals. We report results from MALDI-MS studies of SARS-CoV-2 spike proteins with nano-structured substrates. We find that samples of the matrix–analyte solution prepared with carbon nanotube forests show 50-fold enhanced yields of high mass analyte ions compared to flat substrates. A combined effect of increased densities of matrix–analyte crystals and increased absorption of laser pulse energy with carbon nanotubes leads to strong analyte ion signal increases. Efficient formation and detection of characteristic fingerprint ions can enable detection of pathogens, such as SARS-CoV-2 with high sensitivity and specificity. Increased yields of high mass analyte ions from CNT substrates can also be useful for ion injection into MS/MS instruments. Carbon nanotube substrates and compact laser mass spectrometers can enable low cost testing and environmental monitoring.

In the future work, we suggest that it will be interesting to explore ion intensities from intact virus and pathogen samples deposited on CNT forest substrates. Droplets containing pathogens in matrix solutions could be collected on CNT surfaces, followed by MALDI-MS. Advances in laser technology make compact short pulse lasers available for integration into mass spectrometers. Developments in compact particle accelerators e.g., based on MEMS techniques can further support miniaturization of mass spectrometers. We suggest that CNT substrates that collect pathogens and administer efficient absorption of laser energy can increase yields for high mass analyte ions for rapid and reliable identification of pathogens with high sensitivity and in robust instruments that could be widely deployed at relatively low cost.

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AUTHOR DECLARATIONS
Conflict of Interest
The authors have no conflicts to disclose.

Author Contributions
Thomas Schenklen: Conceptualization (equal); Data curation (equal); Funding acquisition (equal); Investigation (equal); Supervision (equal); Writing – original draft (equal); Writing – review & editing (equal). Jeroen van Tilborg: Conceptualization (equal); Writing – review & editing (equal). Tobias M. Ostermayr: Conceptualization (equal); Writing – review & editing (equal). Sven Steinke: Conceptualization (equal); Writing – review & editing (equal). Eleanor A. Blakely: Conceptualization (equal); Writing – review & editing (equal). Qiang Ji: Conceptualization (equal); Methodology (equal); Writing – review & editing (equal). Rehan R. Kapadia: Methodology (equal); Writing – review & editing (equal). Ali Javey: Data curation (equal); Methodology (equal); Writing – review & editing (equal). Cameron G. R. Geddes: Supervision (equal); Writing – review & editing (equal). Eric Esarey: Supervision (equal); Writing – review & editing (equal). Antoine M. Snijders: Conceptualization (equal); Data curation (equal); Funding acquisition (equal); Investigation (equal); Methodology (equal); Writing – review & editing (equal). Peter A. Seidl: Data curation (equal); Investigation (equal); Methodology (equal); Software (equal); Validation (equal); Writing – review & editing (equal). Brian Matak: Data curation (equal); Software (equal); Writing – review & editing (equal). Lieselotte Obst-Huebl: Data curation (equal); Investigation (equal); Methodology (equal); Writing – review & editing (equal). Hugo Knobel: Data curation (equal); Methodology (equal); Visualization (equal); Writing – review & editing (equal). Ian Pong: Data curation (equal); Methodology (equal); Writing – review & editing (equal). Arun Persaud: Data curation (equal); Software (equal); Writing – review & editing (equal).

DATA AVAILABILITY
The data that support the findings of this study are available within the article.

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14 See http://www.rmjordan.com/ for information on the time of flight mass spectrometer.