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Determination of the denaturation temperature of the Spike protein S1 of SARS-CoV-2 (2019 nCoV) by Raman spectroscopy

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Abstract

In the present work the temperature response of the constitutive S1 segment of the SARS-CoV-2 Spike Glycoprotein (GPS) has been studied. The intensity of the Raman bands remained almost constant before reaching a temperature of 133 °C. At this temperature a significant reduction of peak intensities was observed. Above 144 °C the spectra ceased to show any recognizable feature as that of the GPS S1, indicating that it had transformed after the denaturation process that it was subjected. The GPS S1 change is irreversible. Hence, Raman Spectroscopy (RS) provides a precision method to determine the denaturation temperature of proteins.

Keywords: Raman Spectroscopy; Spike Glycoprotein S1; Denaturation Temperature; SARS-CoV-2

Highlight:
- Raman Fingerprint of Spike Glycoprotein S1.
- Raman Bands assignment of Spike Glycoprotein S1.
- Contribution of specific AA-residues to the Raman Bands of Spike Glycoprotein S1.
- The Raman spectra before protein denaturation show characteristic Raman bands of Spike Glycoprotein S1, which disappear when the denaturation temperature onset is reached.
- The Raman resultant denaturation temperature of Spike Glycoprotein S1 are completely reproducible and easily accurate within 2 °C.
- Raman spectroscopy allowed us to obtain a precise determination of the denaturation onset temperature of the dry powder of Spike Glycoprotein S1 as 133 °C.

Graphical Abstract

Study and characterization Raman increasing the temperature of the SARS-CoV-2 Spike Glycoprotein S1 to determinate the Denaturation Temperature. Raman temperature dependence studies are applied to determine the denaturation temperature of proteins.
1. Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (2019-nCoV) is a newly emerging infectious disease caused by a new line of coronavirus, SARS-CoV-2 virus (SARS-CoV). A first specimen emerged in Guandong China in 2002 [1]. At the end of 2019 and during the following year 2020, a new recently emerged SARS-CoV-2 at Wuhan, China, produced a worldwide pandemic (COVID19) that has altered almost all aspects of human activities. The SARS-CoV-2 virus binds to the target cells via the ACE2 receptors protruding through the membrane cells (ACE2: angiotensin-converting enzyme 2) [2,3]. This bonding is mediated by the Spike Glycoprotein, henceforth referred as GPS, which is formed by homotrimers projected from the viral lipid bilayer. The GPS of SARS-CoV-2 is composed by two subunits, S1 and S2, as shown in Fig. 1. S1 subunit lies mainly at the head or flattened top of the spike, and conforms an important portion of this segment, and S2 on the stem. The GPS S1 subunit contains a receptor binding domain (RBD) that binds to the host cell ACE2 receptors. This process results in conformational changes in the S2 subunit, which allows the fusion peptide to insert into the host target cell membrane [4].

The three-dimensional structure of the spike glycoprotein was determined using cryo-electron microscopy [5]. This study confirmed that GPS couples to the ACE2 protein of human cells with a higher affinity than that of the SARS-CoV coronavirus [6]. Thence, it is now well established that the coronavirus GPS S1 subunit is essential in the viral binding to the host cell at the onset of the infection process [7].

![SARS-CoV-2 Spike Glycoprotein](image)

**Fig. 1.** Ribbon diagram of SARS-CoV-2 (2019-nCoV) Spike Glycoprotein GPS: a) S Trimmer, b) S Protomer, c) S1 Subunit, and d) S2 Subunit.

Given the crucial role that this GPS protein plays in the infection process has converted the study of its chemical and physical properties a highly relevant scientific and priority subject. The aim of the present work is that having determined and understood its physical, chemical, and biological functions as much as possible, these advances in scientific knowledge may lead to the development of successive generations of biotechnologically designed vaccines, as well as of medicaments, to inhibit the access of the SARS-CoV-2 virus to infect human cells [8,9].

A second particularly important reason to characterize all the physical properties of the GPS (and thence of subunits S1 and S2) is that it constitutes an abundant (protruding) surface protein on the SARS-CoV-2. Consequently, it becomes a primary target for neutralizing antibodies. The GPS undergo several structural rearrangements to fuse virus and host cell membranes, thus enabling the delivery of the virus genome into the target cells [10]. Thence, it is a primary target for virus-inactivating antibodies, and a natural target in vaccine development [10].

RS has successfully been applied to study several viruses, and in particular the SARS family of viruses [11]. In this study, conformational changes in glycoproteins were observed, and therefore RS was proposed as an effective and sensitive tool in the identification of these viruses. With respect to the virus SARS family, Zhang et al. [12] posted an assay using surface enhanced Raman scattering (SERS) coupled with multivariate analysis to diagnose SARS-CoV-2 on water. Recently, Sanket Desai et al. [13], described a test method to detect the RNA virus using RS, providing an essential framework in its application to virus detection in response to the needs of the COVID-19 Pandemic. Carlomagno et al. [14], reported on SERS application to detect the presence of SARS-CoV-2 in samples of human saliva. Additionally, Sánchez et al. [15] applied SERS to study the spectral signal of the GPS and N protein. This group showed the role of the GPS and N protein signals in the SERS Raman spectra of the complete virus.

As all proteins are sensitive to the temperature, which may cause structure and function changes, up to the protein unfolding, i.e. protein denaturation, it is necessary to study, among many other influential parameters (applied pressure, for instance), the temperature behavior of the GPS and separately that of the S1 subunit, which plays the primordial role in viral-human cell binding. A very comprehensive study on the temperature response of the GPS S1 subunit in contact with a wet environment such as that insides of an infected subject, has been performed by the molecular dynamics modeling by S. Rath and K. Kumar [16]. That study found that above 40 °C the RBD closes inhibiting the possibility of any binding to host cells, therefore, inactivating the SARS-CoV-2.

Thence, RS is evidently an analytical tool that helps to establish important properties of viruses. Within this context, we present in this work a study of the Raman spectrum response of the GPS S1 subunit from this virus, as a function of the temperature. In addition, the temperature response allowed us to reproducibly determine the denaturation onset temperature at 133 °C ± 2 °C and a final temperature of 144 ± 2 °C, where irreversible protein changes were clearly observed in the Raman spectra indicating that the denaturation was terminated. This result was contrasted with the application of RS to a study in the process of denaturation of
well-known proteins, and of the decomposing temperatures of AA, which allowed us to ascertain that RS provides reliable results in the determination of the $T_D$ when compared with those measured by differential scanning calorimetry (DSC) [17], the preferred technique in biochemistry studies to determine these temperatures [18,19], even in the case of proteins in which the Amide I band is silent, for example in the case of the GPS S1. The study of the temperature response of the main constitutive proteins of the SARS-COVID-2 virus, or of any similar virus is nowadays a priority subject. Mainly because its infectivity and total inactivation (destruction) largely depends on the temperature, along with other physical or chemical agents. The present study is providing new knowledge about the GPS S1 subunit response to temperature. This because the S1 subunit is the main element involved in the mechanism for the virus binding easing the infection of human cells.

2. Materials and methods

The of GPS S1 subunit (>95 % purity as determined by the manufacturer through size exclusion HPLC) refined from SARS-CoV-2 (2019-nCoV) viruses was obtained from Sino-Biological Incorporated in powder form. The protein consists of 670 AA with a molecular weight of 76.5 kDa. The GPS S1 samples were gently compressed to flat, thin, compact discs, before the Raman analysis.

2.1. Raman spectroscopy

The Raman spectra as a function of temperature for the GPS S1 were obtained using a HORIBA XploRa Plus Micro Raman, equipped with a solid-state infrared laser (785 nm wavelength) with an estimated power of 5 mW. The spectrometer grating conditions were 1800 grooves/mm, the laser wavelength and focal distance of 0.5 cm, the spectral resolution was 0.8 cm$^{-1}$ per pixel. Each spectrum had an acquisition time of 20 sec, and five consecutives were taken and averaged to provide the final spectrum. In order to acquire the temperature dependence measurements, we used an environmental cell with a transparent optical window and an electronic heating system. Moreover, to provide uniformly heat to the compact disc protein sample, was placed inside the chamber at a 6 mm depth. The temperature controller was set up to increase in 5 °C steps, remaining for 5 min at each temperature before recording the Raman spectra at the preset temperature. The whole temperature measurements were performed in three different occasions with several days between them, to check for reproducibility.

2.2. Spectral deconvolution

The deconvolution process was performed using the Fityk program (V 1.3.0) [20], which offers an assortment of functions to fit the Raman bands. The choice of fitting function does not alter the wavenumber position of a given band, but it may result in slightly different full-widths at half maximum (FWHM). Fitting the baseline of the Raman spectra was carefully established before data fitting. In order to do this, the fluorescence background was removed by fitting a polynomial curve to the measured spectrum where no Raman signal was expected. The treated spectra were used on data fitting. However, it is necessary to mention that the samples exhibited a very weak fluorescence, i.e., the subtraction performed is relatively small. Also, the background signal to noise ratio essentially appeared of the same magnitude for all spectra, similar to that shown and examined in Fig. 2, which is generally very good, better than 10$^2$.

2.3. Spike Glycoprotein modeling

The modeling process was performed using the UCSF chimera® program (V 1.15) which is feedbacked with the GPS data obtained from the Protein Data Bank, PDB: 7kdi [21].

2.4. DSC methodology

The denaturation profile of the proteins was determined by differential scanning calorimetry using a TA Instruments Calorimeter Model Discovery (New Castle, USA). To obtain the profile decomposition, 5 to 10 mg of the corresponding protein was sealed in T$_{zero}$ aluminum pans (TA Instruments, New Castle, USA). After 3 min at 25 °C the system was heated at a rate of 5 °C/min until achieving the full ending of the denaturation endotherm was achieved. The $T_D$ was determined at the maximum heat flow of the corresponding endotherm, using the first derivative of the heat flow and the equipment software (Trios V 3.3.0.4055; TA Instruments-Waters LLC, New Castle, DE).

3. Results and discussion

The interpretation of the Raman spectra of proteins has been a subject of study at least for the last two decades. In Table 1 the number and percentage contribution of the AA-residues present in the GPS S1, is listed [22]. The aim has been to understand the Raman bands observed in terms of the number of AA units (residues, when forming the peptide chains) present and the intensity of vibrational modes appearing at a given wavenumber where a Raman band is observed [23–27]. However, special care has to be taken in establishing definitive identifications, because it is well known that in complex folded structures, as that occurring in proteins, different peptide sections in close physical proximity to each other, would affect the emission strength of some vibrational modes [24–27]. Among these works, B. Sjöberg et al. [27] found that 8 AA linked in tripeptide units with Gly as initial and terminal residues (GlyAAGly) produced better agreements than the single AA-residue side-chains spectra present in some instance’s wavenumber displacements of almost 5–10 cm$^{-1}$ from those expressed in the protein either to higher or lower wavenumbers, but for high intensity
### Table 1

Quantity of amino acids constitutive of the GPS S1 subunit Protein [22].

| Full Name     | Abbreviation (3 Letters) | Abbreviation (1 Letter) | Quantity of AA | Percentage |
|---------------|---------------------------|-------------------------|----------------|------------|
| Threonine     | Thr                       | T                       | 57             | 8.51       |
| Asparagine    | Asn                       | N                       | 54             | 8.06       |
| Valine        | Val                       | V                       | 53             | 7.91       |
| Leucine       | Leu                       | L                       | 50             | 7.46       |
| Serine        | Ser                       | S                       | 50             | 7.46       |
| Phenylalanine | Phe                       | F                       | 47             | 7.01       |
| Glycine       | Gly                       | G                       | 45             | 6.72       |
| Proline       | Pro                       | P                       | 36             | 5.37       |
| Alanine       | Ala                       | A                       | 35             | 5.22       |
| Tyrosine      | Tyr                       | Y                       | 35             | 5.22       |
| Isoleucine    | Ile                       | I                       | 32             | 4.78       |
| Aspartic acid | Asp                       | D                       | 31             | 4.63       |
| Lysine        | Lys                       | K                       | 30             | 4.48       |
| Arginine      | Arg                       | R                       | 29             | 4.33       |
| Glutamine     | Gln                       | Q                       | 26             | 3.88       |
| Glutamic acid | Glu                       | E                       | 23             | 3.43       |
| Cysteine      | Cys                       | C                       | 19             | 2.84       |
| Histidine     | His                       | H                       | 9              | 1.34       |
| Tryptophan    | Trp                       | W                       | 7              | 1.04       |
| Methionine    | Met                       | M                       | 2              | 0.30       |
| **Totals**    |                           |                         | 670            | 100.00     |

### Table 2

AA side chains contributing to the Raman bands of the GPS S1 subunit of the SARS-CoV-2.

| Raman Band | Wavenumber/Relative intensity (cm⁻¹) | Identification/Relative intensity | Reference |
|------------|-------------------------------------|----------------------------------|-----------|
| B1         | 429.7/m                             | Glutamine/m                      | [23]      |
| B2         | 470.2/m                             | Glutamine/m                      | [23]      |
| B3         | 491.9/m                             | Asparagine/m                     | [23]      |
| B4         | 514.8/s                             | Asparagine/m                     | [23]      |
| B5         | 534.3/m                             | Isoleucine/s, Valine/s, Cysteine/m, Histidine/m, Alanine/m | [25] |
| B6         | 590.1/m                             |                                   |           |
| B7         | 636.2/m                             | Phenylalanine/m, Histidine/m, Lysine/m, Tyrosine/m | [25,27] |
| B8         | 704.3/m                             | Isoleucine/m (Tryptophan/w, Threonine/w) | [25,27] |
| B9         | 791.4/m                             | Cysteine/s (Histidine/m, Lysine/m, Tyrosine/m) | [25] |
| B10        | 836.7/m                             | Phenylalanine/s, Tyrosine/s, Leucine/w | [25,27] |
| B11        | 870.6/vs                            | Glutamic/vs, Threonine/vs, Aspartic acid/m, Arginine/m, Cysteine/m, Methionine/m, Tryptophan/m, Isoleucine/m | [25,26,27] |
| B12        | 882.4/s                             | Lysine/s, Histidine/s, Tyrosine/m | [25,27] |
| B13        | 910.1/m                             | Glutamic Acid/s, Histidine/m, Lysine/m, Phenylalanine/w, Valine/w | [25] |
| B14        | 932.4/m                             | Skeletal C-C, 3-helix, Aspartic Acid/s, Arginine/m, Histidine/m, Threonine/m, Alanine/w, Serine/w | [26] |
| B15        | 957.7/w                             | Valine/s (Cysteine/m, Histidine/m, Isoleucine/w, Methionine/w, Proline/w) | [25,27] |
| B16        | 986.9/w                             | Arginine/s, Glutamic Acid/m, Isoleucine/m, Lysine/m, Tyrosine/m, Aspartic Acid/w | [25,26,27] |
| B17        | 1013.0/m                            | Phenylalanine/vs, Serine/s, Tryptophan/s, Alanine/m, Isoleucine/m | [25,26,27] |
| B18        | 1033.1/m                            | Phenylalanine/s, Arginine/m, Isoleucine/m, Lysine/m, Threonine/w, Valine/w | [25,26,27] |
| B19        | 1048.3/s                            | Tyrosine/m (Glutamic Acid/w, Methionine/w, Tryptophan/w, Cysteine/vw, Proline/vw) | [25] |
| B20        | 1073.9/m                            | Arginine/s, Lysine/s, Aspartic Acid/m, Glutamic Acid/m, Valine/w | [25] |
| B21        | 1094.8/w                            | Histidine/s, Lysine/m, Proline/m, Cysteine/w, Isoleucine/w | [25] |
| B22        | 1114.9/s                            | Threonine/s, (Alanine/m, Histidine/m, Aspartic acid/m, Methionine/w) | [25] |
| B23        | 1131.7/m                            | Isoleucine/m, Serine/m, Valine/m, Cysteine/w, Leucine/w | [25,27] |
| B24        | 1142.7/s                            | Alanine/m, Lysine/m, Valine/m, Histidine/w | [25] |
| B25        | 1198.1/w                            | Amide III (Arginine/m, Isoleucine/m, Phenylalanine/m, Tyrosine/m, Valine/m, Cysteine/w) | [25,26,27] |
| B26        | 1224.8/m                            | Amide III (β sheet structure, Phenylalanine/s, Histidine/m, Serine/m, Tryptophan/m, Lysine/w) | [25,26,27] |
| B27        | 1251.8/s                            | Amide III, Histidine/m, Isoleucine/m, Aspartic Acid/w, Lysine/w, Methionine/w, Tryptophan/w, Tyrosine/w | [25,27] |
| B28        | 1304.4/m                            | Lysine/s, (Alanine/m, Arginine/m, Isoleucine/m, Phenylalanine/m, Serine/m, Cysteine/w) | [25] |
| B29        | 1321.2/m                            | Amide III (α-helix), Histidine/vs, Glycine/s, Isoleucine/s, Serine/s, Arginine/m, Glutamic Acid/m, Methionine/m, Valine/m | [25,26,27] |
| B30        | 1352.7/s                            | Alanine/s, Isoleucine/s, Methionine/m, Valine/m, Serine/m Histidine/m, Glutamic Acid/w, Aspartic acid/w | [25] |
| B31        | 1376.6/m                            | Cysteine/m, Glutamic acid/m, Alanine/m, Leucine/w, Proline/w | [25,27] |
| B32        | 1406.3/m                            | Histidine/vs, Alanine/m, Aspartic Acid/w, Cysteine/m, Glycine/m, Methionine/m, Phenylalanine/w, Leucine/w | [25,27] |
| B33        | 1453.7/s                            | Isoleucine/s, Alanine/s, Lysine/s, Glutamic Acid/m, Glycine/m, Leucine/m, Valine/m, Tryptophane/w | [25,27] |
bands in many instances the deviations of single AA-residue side-chains emissions were very modest. Those of tripeptides studied in [27] presented systematically modest deviations of ± 1 cm⁻¹, and seldom up to 5 cm⁻¹, when present in those three model proteins. In Table 2 we have included those AA-residues side-chains which are within an assumed region of ± 10 cm⁻¹ around a Raman band.

The full Raman spectrum (20 °C) for the compressed solid powder of the GPS S1 is shown in Fig. 2, near bottom axis, black thick line as it can be seen there are two spectral regions clearly separated, by a broad wavenumber expanse where no Raman signal is observed, from just above 1600 to 2800 cm⁻¹. Although the Raman signal was collected in the spectral interval from 100 cm⁻¹ and 3600 cm⁻¹, only the 400 to 1600 cm⁻¹ region is analyzed in this report. We also observe 1) a Raman band from 2800 to 3000 cm⁻¹, which in the literature has been identified as the signal from C-H bonds stretching modes, which have been studied by other authors to determine if the protein is either hydrophobic or hydrophilic in nature [28], and 2) the O-H broad band from 3100 to 3550 cm⁻¹, which similarly reflects information of the hydroxyl anions of the water molecules within the proteins. This region covers most of the Raman bands used to identify the fingerprints of the 20 constitutive AA-residues of all proteins [24–27].

The red line shows the data fitting using Lorentzian functions to deconvolute the 33 obtained Raman bands. In Table 2 we summarize the contributing vibrational bands resulting from the deconvolution analysis in comparison with published spectral positions of Raman bands from the side-chains of the 20 AA [23–25], mainly those of Zhu et al. [25], whose work lists a comprehensive library of the Raman Bands of 18 AA-residue side-chains. Table 2 lists both the AA-residues that produce high intensity Raman emissions at the observed position, and in parenthesis other AA-residues that might contribute in more modest magnitude to the resultant spectral band, as well as of some central AA-residue in some tripeptide sequences as studied by Sjöberg et. al. [27] and identified from the comparison with some proteins as mentioned above. In Fig. 3 the expected relative contributions of specific AA-residues to each Raman band of the Glycoprotein Spike S1 have been plotted.

In Fig. 2, the 870 cm⁻¹ band (B11) stands out notoriously. Two constitutive amino acids have strong Raman signals that are very close to this wavenumber, threonine (872 cm⁻¹) and glutamic acid (873 cm⁻¹) [23]. However, aspartic acid, arginine, cysteine, isoleucine, methionine, and tryptophan have medium intensity Raman bands at 873 cm⁻¹ [23]. Hence, it is expected that this strong band could be the collaborative signal of all these AA-residues, as illustrated in Fig. 3, with colored and/or hatched bars. These eight AA-residues comprise ≈ 29.85% of the total number of AA-residues constitutive of the GPS S1 subunit, (TNA).

The data deconvolution indicates that a second band at 882 cm⁻¹ (B12) is merged into this strong band, producing the asymmetric resultant shape. This band most likely corresponds to the fingerprints of lysine and histidine, both individually have strong intensity Raman dispersions at 881 and 882 cm⁻¹, and tyrosine, which has medium intensity emissions at 881 cm⁻¹. These constitute 12.09% of TNA. Since this is a relatively strong emission, this result suggested that the folded complex environment of the protein might be enhancing the emission intensity of these amino acids. There were another five bands of large intensity and standing out of the continuous spectral sections observed from the spectral deconvolution. These bands are at 515, 1115, 1252, 1321 and 1454 cm⁻¹, corresponding to B14, B22, B27, B29 and B33, respectively. The B14 band occurring at 515 cm⁻¹ has been identified by [25] as originating by vibrations of the asparagine amide terminal. The B22 band, occurring at 1115 cm⁻¹, coincides with the strong Raman signals of histidine (at 1113 cm⁻¹), and threonine (at 1116 cm⁻¹). Alanine, aspartic acid, and methionine present medium intensity bands at 1115, 1121, and 1122 cm⁻¹, hence, these three are also expected to contribute to this emission. All five AA-residues constitute 20.00% of TNA. At 1252 cm⁻¹ (B27), there exists a plethora of Raman emissions in the following 7 AA-residues and the amide region emissions of the peptide bonds: Amide III, histidine, isoleucine (medium intensity Raman intensity emissions); and the contribution with weak free AA-residues emissions of aspartic acid, lysine, methionine, threonine, and tryptophan. Once again, the complex folded protein structure might be enhancing the intensity of the histidine and isoleucine vibrations located at 1252 and 1257 cm⁻¹, respectively, in conjunction with the Amide III emissions, as well as of some of the weaker expected contributions of the other 5 AA-residues. The B29 band at 1321 cm⁻¹ corresponds to the amide III signal, present in all AA peptide bonds of proteins [25,26], as the 1321 cm⁻¹ signal from
the α-helix [24]. The Raman spectra for glycine, isoleucine and serine histidine (very strong signal), presented strong emissions at 1327, 1329, and 1327 cm⁻¹, respectively, very likely corresponding to their amide III regions [25,26]. Arginine, glutamic acid, methionine tyrosine and valine also exhibited medium intensity or weak emissions around 1328 cm⁻¹, for a TNA of 42.59%. A final-well resolved band, B33 at 1454 cm⁻¹, stood out at the higher wavenumber end of the spectrum. This band is most likely associated to alanine, isoleucine, leucine and lysine that produce strong strength signals in the isolated AA at 1461, 1450, 1457 and 1456 cm⁻¹, respectively. Additionally, the following AA-residues should also contribute, glutamic acid, glycine, leucine, and valine, all of which present medium intensity Raman scatterings as isolated AA, as well as tryptophan which has a weak emission, all of them around this wavenumber (TNA 41.49%) [23].

3.1. Temperature results

In Fig. 4, the Raman spectra recorded at varying increasing temperatures are shown. This figure shows that the spectra exhibit almost identical features from ambient temperature to 130 °C. In the spectra shown recorded at temperatures 135, 138, 140 and 144 °C, it is observed that the dominant spectral band at 870 cm⁻¹ rapidly diminishes in intensity, a situation that applies to the other spectral features. At 144 °C, hardly any recognizable GPS S1 subunit spectral features are observed, and at 148 and 150 °C, the GPS S1 spectra are totally deformed, i.e., all traces of the previous predominant transitions have disappeared. The spectra are now dominated by what seems to be interference bands.

In Fig. 5a a plot of the wavenumber positions dependence with temperature is presented for the 9 Raman bands of larger intensities (medium and large). It may be observed that B4, B11, B12, B19, B22, B24 and B27, display shifts toward lower wavenumbers (red-shift) with increasing temperatures, B30 displays a very moderate tendency for blue shifting, as well as B33, but this last band has almost null shifts. The wavenumber/temperature slopes (w/t) slopes are summarized below in Table 3.

According to the literature we found similar w/t slopes in a Raman study on the thermal response of two amino acids, L-alanine and L-threonine, as well as the related compound taurine [29]. Their magnitudes are very similar, although for some Raman band these slopes are larger.

With respect to changes in relative intensities to those at 20 °C for the same bands, it can be observed that for most of them their relative intensities suffer small decreasing variations with increasing temperatures. However, the Raman band B11 presents a noticeable diminution in the relative intensity above 80 °C. B11 is the strongest intensity Raman peak. This variation may correspond to the ongoing protein changes that antecede the final denaturized state, of several of the contributing AA to this collaborative signal. Nevertheless, for all these 9 bands at 132 °C, their relative intensities start to vary widely, with no discernible pattern for them a few degrees higher, followed by even more dramatic variations if the plot is extended to include temperatures above 144 °C, of the remaining spectral features at the wavenumbers at which these Raman bands appeared at 20 °C.

These dramatic changes of the GPS S1 subunit Raman spectra above 130 and 144 °C are clearly the fingerprint of the denaturation of this protein in a process that starts at 133 ± 2 °C, which is finished at 144 ± 2 °C.

The denaturation processes and Tp's are standardly measured using differential scanning calorimetry (DSC). Moreover, DSC is the preferred technique used in biochemistry and food studies for determining denaturation and decomposing temperatures of proteins and amino acids [18,19]. Due to our GPS S1 limited availability, and the amount of materials needed for DSC (milligrams to grams) we performed a subrogate experiment using the well-known proteins bovine serum albumin (BSA), lisozyme, ovalbumin, and of the amino acids L-glutamine, L-Cysteine, and L-Alanine.

In Fig. 6, we show as a comparative example our results for spectra of a standard protein in dry powder form, Bovine Serum Albumin (BSA), a) at room temperature and b) spectra after the protein BSA has been completely denaturized at 235 °C. The BSA spectrum at ambient temperature agrees well that reported for BSA under wet conditions [30]. However, the Tp's are different for wet and dry conditions, being once again higher than those of the wet forms [31]. In Table 4, we summarize the DSC results measured for these proteins and amino acids, in dry powder form, their denaturation or decomposing temperatures obtained, compared with the temperature response of the Raman bands of those three proteins and three amino acids, which disappeared at those temperatures, are presented. The almost total coincidence of the denaturation (proteins) or decomposing (amino acids) temperatures measured by DSC and Raman was very satisfactory. In fact, the linear regression of the temperatures measured by Raman on the temperatures measured by DSC, resulted in a regression coefficient of 0.986 (P < 0.01) with a slope of 1.04. The magnitude of this slope was not significantly different from 1.0, a value that we would expect if the denaturation or decomposing temperature measured by Raman and the ones measured by DSC were statistically the same. Further details of the complete study will be presented elsewhere [17]. These results support our contention that the dramatic changes observed in the Raman spectrum at a given onset temperature of these three proteins, as well as in the GPS

![Fig. 4. Raman spectrum of the GPS S1 observed at increasing temperatures.](Image)
S1 subunit corresponds to the actual denaturation onset temperature of this important protein.

In DSC the T_D is reported from the maximum of the endothermic peak measured during the denaturation process. This is usually located almost halfway between the onset and terminal temperatures of this endothermic process. Hence it may be adopted and reported as the T_D of powder of the GPS S1 of SARS-CoV-2, 

\[ T_D^{\text{GPS S1}} = 139 \pm 3 \text{ } ^\circ\text{C}, \]

It is important to remember that the T_D of all proteins depend on the medium and several chemical and physical aspects of the surrounding media in which they are immersed. The T_D of the dry solid protein is always substantially larger than when it is immersed in a given medium, in the GPS S1 case almost nominally a factor of 2, as expressed in centigrade degrees, i.e., from 70 \[ ^\circ\text{C} \] in wet conditions [16] to 140 \[ ^\circ\text{C} \] (a 20% increase in absolute Kelvin degrees). For instance, the surrounding medium PH, pressure, salinity, as well as the constitutive chemistry of the solvent or medium it resides affect, among many other factors, in an important magnitude the effective denaturation process and temperature of any protein. Hence, the actual T_D’s of the S1 subunit attached to the spike and ultimately the spike and the S1 protein.

**Table 3**

| Raman Band | Wavenumber position at 20 °C (\( \omega_0 \)) | Wavenumber position at 0 °C (Extrapolation) \( \omega_0^* \) | Wavenumber Temperature slope \( \frac{\text{cm}^{-1}}{\circ\text{C}} \) | Temperature slope \( \frac{\circ\text{C}}{\text{cm}^{-1}} \) |
|------------|------------------------------------------|-------------------------------------------------|---------------------|---------------------|
| B4         | 515                              | 516.6                             | 0.041               |                      |
| B11        | 871                              | 871.2                             | ---                 | 0.027               |
| B12        | 882                              | 883.6                             | 0.058               |                      |
| B19        | 1048                             | 1049.3                            | 0.029               |                      |
| B22        | 1113                             | 1114.9                            | 0.033               |                      |
| B24        | 1143                             | 1143.2                            | 0.032               |                      |
| B27        | 1252                             | 1252.5                            | 0.029               |                      |
| B30        | 1353                             | 1353.8                            | 0.009               |                      |
| B33        | 1454                             | 1454.5                            | 0.005               |                      |

**Table 4**

Comparison between RS and DSC to obtain the decomposing point in amino acids and denaturation in proteins in solid powder form [17].

| Sample   | Protein/ Amino acid | Decomposing or Denaturation Temperature \( ^\circ\text{C} \) | Decomposing or Denaturation Temperature \( ^\circ\text{C} \) |
|----------|---------------------|----------------------------------------------------------|----------------------------------------------------------|
| BSA      | P                   | 235 ± 3                                                  | 237 ± 2                                                  |
| Lisozyme | P                   | 230 ± 3                                                  | 232.5 ± 2                                                |
| Ovalbumin| P                   | 240 ± 3                                                  | 236 ± 2                                                  |
| L-Glutamine | A                | 185 ± 3                                                  | 187 ± 2                                                  |
| L-Cysteine| A                  | 245 ± 3                                                  | 245 [32]                                                 |
| L-Alanine | A                  | 285 ± 3                                                  | 297 [33]                                                 |

BSA: Bovine Serum Albumin.

Fig. 5. Plot of the Raman Behavior a) Raman shift as a function of temperature and b) relative intensities normalized to their intensity at 20 °C, of 9 Raman bands, as a function of temperature. The plots are displaced by arbitrary values in order to accommodate their intensity fluctuations above 133 °C. The shaded region indicates the range of temperatures at which the protein denaturation process is taking place.

Fig. 6. Raman spectra of a standard protein Bovine Serum Albumin (BSA) a) at room temperature and b) spectra of the denaturized protein at 235 °C.
mately to the encapsulating membrane of the SARS-CoV-2 depends on the environment it resides. Therefore, they are expected to substantially vary from the one determined for the protein in dry powder conditions. But for the fundamental understanding of GPS S1 subunit, it is important to determine as a reference magnitude the T_D of the solid protein. However, our RS experiment has the advantage to provide experimentally a clear visualization of what temperature the proteins spectra suffer their denaturation by a simple inspection, and without the need to resort to the analysis of the deconvoluted contributions of the Amide I and III bands, or even when one of them (Amide I) could be Raman silent as the case of the GPS S1 subunit.

Additionally, the full characterization of the denaturation of important constitutive proteins, or of the virus heat inactivation process, provide essential input parameters on the modeling of the permanence of live virus at different ambient and temperatures [34]. Also, it has been well established that protein denaturation strongly correlates with cell death and a similar expectation could be physically expected to be valid for the complete virus [35], although for the virus the denaturation of the envelope protein N for instance may play a larger role in the virus disintegration. Also, it has been well established that protein denaturation strongly correlates with cell death and a similar expectation could be physically expected to be valid for the complete virus [35], although for the virus the denaturation of the envelope protein N for instance may play a larger role in the virus disintegration. But for the fundamental understanding of GPS S1 subunit, it is important to determine as a reference magnitude the T_D of the solid protein. However, our RS experiment has the advantage to provide experimentally a clear visualization of what temperature the proteins spectra suffer their denaturation by a simple inspection, and without the need to resort to the analysis of the deconvoluted contributions of the Amide I and III bands, or even when one of them (Amide I) could be Raman silent as the case of the GPS S1 subunit.

4. Conclusions

In this work we report the Raman spectra response to controlled heating of the GPS S1 subunit of the SARS-CoV-2 (2019 nCoV). A careful, detailed and complete assignment of Raman bands in terms of the contribution of specific AA-residues on the Spike Glycoprotein S1 subunit have been performed. The study on the temperature response of this protein is necessary to understand its effect on the inactivation of the virus infectivity above 40–50 °C, as GPS S1 subunit contains the receptor binding domain that constitutes the entrance gate for the predominant mechanism for viral-human cells attachment in order for the SARS-CoV-2 to transfer its genetic code to the attacked cell. All recorded spectra showed similar spectral features and almost constant relative intensities to those at the spectrum recorded at 20 °C, but at 133 °C significant variations in these relative intensities, as well as decline in their intensities, are observed. Above temperatures of 144 °C, all distinctive spectral features disappear. These results are the fingerprint of the denaturation of the GPS S1 subunit in a process that starts at 133 ± 2 °C, which is finished at 144 ± 2 °C. The study results in a T_D of solid GPS S1 subunit of SARS-CoV-2 as T_D = 139 ± 3 °C. The result is supported by a parallel study by RS and DSC of three powder proteins and three amino acids, which indicates that Raman dependent spectroscopy provides reliable denaturation (proteins) or decomposing (amino acids) temperatures.

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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Credit author statement

All authors contributed to the study conception and design. Material preparation, Raman Spectroscopy, data curation and analysis were performed by Aída Hernández-Arteaga, Hiram Joaet Ojeda-Galván and M. C. Rodríguez-Aranda. The DSC data and analysis were performed by Jorge F. Toro-Vázquez.

Hugo R. Navarro-Contreras coordinated the integration of data; performed their final analysis together with Miguel José-Yacaman, who both wrote the manuscript, which was read and revised by all authors.

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