Surface Activated Chemical Ionization – Cloud Ion Mobility Mass Spectrometry applied to SARS-CoV-2 spike proteins.

Conformational investigations under different chemical and physical conditions.

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Abstract

Surface Activated Chemical Ionization – Cloud Ion Mobility Mass Spectrometry (SACI-CIMS) technology was used to separate low and high molecular weight ionic clusters to investigate the feasibility of electromagnetically inducing the modulation of the spike protein conformation. SACI-CIMS delivers a high throughput and a strong reduction in the ion trap space charge effect. Solvent clouds containing medium-high molecular weight protein ions were in-source selected and focused to the mass analyzer. Thanks to soft SACI ionization conditions - due to the low surface ionization potential (+/-47 V) - it was also possible to preserve the protein molecular structure and to perform interatomic studies (e.g.: protein-protein, protein analyte interactions).

In this study, we investigated the spike glycoprotein and envelope protein of SARS-CoV-2 interaction with the mesenchymal cell membrane receptors under different chemical and physical conditions to better understand their impact on the protein expression. Thanks to the SACI-CIMS technology, we demonstrated electromagnetic frequency fields-induced conformational alterations of the spike protein, leading to protein denaturation. The magnitude of such effect was significantly augmented - up to 96% unfolding of the spike protein - when the protein was also exposed to N-acetylcysteine compounds.

Introduction

Ion mobility mass spectrometry (IM-MS)[1] combines ion-mobility and mass spectrometry, so that compounds of a pre-determined mass/charge (m/z) range in an analyte solution can be characterized together.[2–11]

A surface activated chemical-ionization (SACI) source employs a low-voltage ionization source, which increases spectral sensitivity by ionization of analyte solute molecules.[12,13] SACI is a ionization source based on polarization effects induced by a metallic surface layer inserted into an atmospheric pressure chemical ionization (**APCI**) chamber exposed to smaller potentials (40–300 V) than the ones employed by standard APCI (~3000–6000 V). Under SACI conditions, the solvent ion species are significantly reduced and the analyte ion population is increased, leading to better signal-to-noise ratios.[12,13]

SACI-Cloud Ion Mobility Mass Spectrometry (SACI-CIMS) is an innovative technology for separating wide molecular classes on the basis of their molecular weight.[14] The soft ionization conditions employed in SACI-CIMS are useful to investigate the interactions among molecules.[14]

The cloud ion mobility effect occurs under SACI conditions, by fixing the surface potential and allowing the mass spectrometry entrance capillary voltage to be tuned between -300V and -1500V. When the surface potential is at 47V, it is then possible to separate ions with *m/z* <300 from those with *m/z* >300 prior to their entrance into the mass analyzer to produce simplified spectra.

In this study, the SACI-CIMS technology was used to investigate the changes in the conformation (denaturation) of the spike protein interacting with mesenchymal cell ACE2 receptors, when stimulated with electromagnetic frequency fields. Prior in vivo studies did prove electromagnetic frequency-induced modifications of conformation and functionality of proteins and enzymes.[15,16] Frequencies ranging 7Hz to 80Hz were applied in this study to modify the conformation of the spike protein, thereby altering its functionality.[17]

In addition, a N-acetylcysteine chemical compound was combined to the electromagnetic irradiation to investigate the increased impact on the level of unfolding of the spike protein.[18]

SACI-CIMS was employed to analyze different spike protein water clusters and the protein conformation was evaluated on the basis of protein-water cluster ion distribution.

Materials and Methods

Chemicals

Spike proteins were purchased from Sigma-Aldrich (Milan, Italy). Methanol and doubly distilled water were purchased from VWR (Milan, Italy).

Sample preparation

Mesenchymal cells were provided by TebuBio (Milan, Italy). Mesenchymal cells growth was performed by using the Grow Medium M7765 TebuBio (Milan, Italy). Cells were incubated at 37 °C for 20 days and the number of cells was estimated by optical microscopy. Cell lines for negative control were washed with bi-distilled water, methanol (CH_3OH) and acetonitrile (CH_3CN); ultrasound was applied for 5 minutes after each washing step. Two cell lines were chemically treated by adding 3 L of N-acetylcystein solution at a concentration of 1 g/mL to the cell pellets containing at least 6 million cells. Such cell lines were then electromagnetically irradiated - using frequencies ranging from 7Hz to 80Hz - by means of a electromagnetic frequency fields generator.

Mass spectrometry

Acquisition parameters, unless stated otherwise, were as follows:

The direct infusion flow rate, provided by means of a syringe pump, was 5 L/min with additional solvent provided at a flow rate of 100 L/min by a HPLC pump placed in-line. A Bruker Daltonics HCTultra Ion-Trap mass spectrometer (Bruker Daltonics, Breme, Germany) was used. Each spectrum was obtained in the positive mode. An average of two microscans and a rolling average of four spectra were acquired. The nitrogen drying-gas temperature was 350°C and its flow rate was 1 L/min. The nitrogen nebulizing-gas flow rate was 12 L/min. The inner capillary voltage was between –100 V and –1500 V. The end-plate potential was –500 V. The N_2 curtain gas flow rate varied between 0.5 and 6 L/min. The ionization sources parameters were fixed as follows: ESI ionization voltage (3000 V) was applied via a home built external power supply to the spray needle; the APCI needle current was 3000 nA; the SACI surface potential was 47 V and was used to obtain the in-source ionization.

The inner capillary voltage gradient was used to produce the CIMS effect. HyStar software (Bruker Daltonics, Breme, Germany) was used for data acquisition and DataAnalysis (Bruker Daltonics) for data treatment. Different ion source pressures, similar to those described in literature for the IM effect,[15,16] were employed to investigate the CIMS effect. The pressure values were 0.76, 1, 1.5 and 2 bar. A dry gas line was utilized to vary the pressure of the ion source. The in-source pressure was monitored using a manometer.

Data Analysis

The SANIST – Conf platform (ISB, Milan, Italy) was used to extract and elaborate the SACI-CIMS spectra.

Results and discussion

Preliminary analysis were performed by directly injecting 1 µg/mL solution into the SACI-CIMS equipment to detect the SARS-CoV-2 spike protein $[M+mH2O+nH]^{n+}$ standard mass spectrum signal. Several inner capillary voltages were then tested to find optimal focalization conditions[14]. At -100 V the multicharged signals intensity of the protein was negligible (Figure 1a); the maximum intensity was reached at at -600 V (Figure 1b) and it disappeared at -1500 V (Figure 1c). At -600 V, the multicharged distribution values were detected between m/z 400 and m/z 800 (Figure 2).

The denaturation of the spike protein was first verified on protein water solutions (1 µg/mL) by adding 0.1% of formic acid in one case (Figure 3a) and by irradiating the protein with electromagnetic frequencies in the range of 7Hz to 80Hz in the second case (Figure 3b). The near doubling of the number of water molecules coordinated with the protein suggests a similar level of denaturation in both cases, therefore demonstrating the effectiveness of the electromagnetic irradiation.

The degree of denaturation of the spike protein attached to mesenchymal cells (minimum 6 million) was eventually tested and the following experiments were carried out:

a) Mesenchymal cells spiked with 5 µL of spike protein solution at a concentration of 1 µg/mL;

b) Mesenchymal cells spiked with 5 μL of spike protein solution at a concentration of 1 μg/mL and irradiated with electromagnetic frequencies (7Hz - 80Hz) for 30 minutes;

c) Mesenchymal cells spiked with 5 μL of spike protein solution at a concentration of 1 μg/mL and irradiated with electromagnetic frequencies (7Hz - 80Hz) for 90 minutes;

d) Mesenchymal cells spiked with 5 μL of spike protein solution at a concentration of 1 μg/mL treated with 3   L of N-acetylcystein solution at a concentration of 1   g/mL;

e) Mesenchymal cells spiked with 5 μL of spike protein solution at a concentration of 1 μg/mL treated with 3 μL of N-acetylcystein solution at a concentration of 1μg/mL and irradiated with electromagnetic frequencies (7Hz - 80Hz) for 90 minutes.

Figures 4a to 4e illustrate the resulting SACI-CIMS mass spectra.

While almost no denaturation occurs when the spike protein is simply attached to the mesenchymal cells (Figure 4a), the data clearly show a progressively increasing degree of protein denaturation from experiment b) to experiment e).

This can be observed by looking at both the increase in the molecular charge state and the increase in water and ion adducts species. Table 1 reports the ion species m/z ranges detected for each experiment and the corresponding degree of protein denaturation.

The highest degree, 96%, of protein denaturation (unfolding) was achieved by enhancing the effect of N-acetylcysteine[18] thanks to electromagnetic irradiation in the 7Hz to 80 Hz frequency range. It can be assumed that the combined effect of N-acetylcysteine together with the induced electromagnetic signals exhibit a synergic and cumulative effect on the spike protein folding/unfolding state.

Conclusions

Extensive and permanent conformational alteration (unfolding) of the SARS-CoV-2 spike protein attached to mesenchymal cells was observed when irradiated with 7Hz to 80Hz electromagnetic frequencies. The data suggest an alteration of the spike protein functionality and the magnitude of such effect is significantly augmented - up to 96% - when the protein is exposed to N-acetylcysteine compounds.

References

[1] R. T. Kelly, A. V. Tolmachev, J. S. Page, K. Tang, R. D. Smith. The ion funnel: theory, implementations, and applications. *Mass Spectrom. Rev*. **2010**, *29*, 294.

[2] C. Lapthorn, F. Pullen, B. Z. Chowdhry. Ion mobility spectrometry-mass spectrometry (IMS-MS) of small molecules: separating and assigning structures to ions. *Mass Spectrom. Rev.* **2013**, *32*, 43.

[3] Y. Zhong, S. J. Hyung, B. T. Ruotolo. Ion mobility-mass spectrometry for structural proteomics. *Expert Rev. Proteomics* **2012**, *9*, 47.

[4] A. Kiss, R. M. Heeren. Size, weight and position: ion mobility spectrometry and imaging MS combined. *Anal. Bioanal. Chem.* **2011**, *399*, 2623.

[5] E. Jurneczko, P. E. Barran. How useful is ion mobility mass spectrometry for structural biology? The relationship between protein crystal structures and their collision cross sections in the gas phase. *Analyst*. **2011**, *136*, 20.

[6] A.B. Kanu, P. Dwivedi, M. Tam, L. Matz, H.H. Hill. Ion mobility-mass spectrometry. *J. Mass Spectrom*. **2008**, 43, 1.

[7] S. R. Harvey, C. E. Macphee, P. E. Barran. Ion mobility mass spectrometry for peptide analysis. *Methods* **2011**, *54*, 454.

[8] C. Uetrecht, R.J. Rose, E. van Duijn, K. Lorenzen, A.J. Heck. Ion mobility mass spectrometry of proteins and protein assemblies. *Chem. Soc. Rev.* **2010**, *39*, 1633.

[9] F. Lanucara , S.W. Holman , C.J. Gray , C.E. Eyers. The power of ion mobility-mass spectrometry for structural characterization and the study of conformational dynamics. *Nat. Chem.* **2014**, *6*, 281.

[10] R. Guevremont. High-field asymmetric waveform ion mobility spectrometry: a new tool for mass spectrometry. *J. Chromatogr. A.* **2004**, *1058*, 3.

[11] A. A. Shvartsburg, A. G. Anderson, D. R. Smith. Pushing the Frontier of High-Definition Ion Mobility Spectrometry Using FAIMS. *Mass Spectrom. (Tokyo)*. **2013**, 2, S0011.

[12] S. Cristoni, L. R. Bernardi, I. Biunno, M. Tubaro, F. Guidugli. Surface-activated no-discharge atmospheric pressure chemical ionization. *Rapid Commun. Mass Spectrom.* **2003**, *17*, 1973.

[13] S. Cristoni, S. Rubini, L. R. Bernardi. Development and applications of surface-activated chemical ionization. *Mass Spectrom. Rev.* **2007**, *26*, 645.

[14] A. Arzoni, L. R. Bernardi and S. Cristoni. In-source cloud ion mobility mass spectrometry. *Rapid Commun Mass Spectrom.* **2015**, *29*, 690.

[15] Nishiyama H, Watanabe T, Inoue Y. Activation of immobilized enzymes by acoustic wave resonance oscillation. *Enzyme Microb Technol.* **2014** Dec; 67:27-31. doi: 10.1016/j.enzmictec.2014.08.005.

[16] Calabrò E, Magazù S. Resonant interaction between electromagnetic fields and proteins: A possible starting point for the treatment of cancer. *Electromagn Biol Med.* **2018**;37(3):155-168. doi: 10.1080/15368378.2018.1499031.

[17] Calabrò E. Competition between hydrogen bonding and protein aggregation in neuronal-like cells under exposure to 50 Hz magnetic field. *Int J Radiat Biol.* **2016** Jul;92(7):395-403. doi: 10.1080/09553002.2016.1175679.

[18] Akhter J, Quéromès G, Pillai K, Kepenekian V, Badar S, Mekkawy AH, Frobert E, Valle SJ, Morris DL. The Combination of Bromelain and Acetylcysteine (BromAc) Synergistically Inactivates SARS-CoV-2. *Viruses.* **2021** Mar 6;13(3):425. doi: 10.3390/v13030425.

Captions

Figure 1: SACI-CIMS spike protein $[M+mH2O+nH]^{n+}$ multicharged distribution monitored using a) -100 V, b) -600 V and c) -1500 V capillary voltage potential. The mass spectra where achieved by direct injection of 10 µL of spike protein solution at a concentration of 1 µg/mL.
The eluent composition was water and the eluent flow was 100 µL/min.

Figure 2: Magnification of spike protein multicharged distribution together with the water solvent cloud cluster ion. The mass spectra where achieved by direct injection of 10 µL of spike protein solution at a concentration of 1 µg/mL. The eluent composition was water and the eluent flow was 100 µL/min.

Figure 3: SACI-CIMS mass spectra of spike protein a) treated with 0.1% formic acit water solution; and b) irradiated with 7-80 Hz electromagnetic frequency signals. The mass spectra where achieved by direct injection of 10 µL of spike protein solution at a concentration of 1 µg/mL. The eluent composition was water and the eluent flow was 100 µL/min.

Figure 4: SACI-CIMS mass spectra obtained analyzing the extraction solutions of,
a) Mesenchymal cells spiked with 5 µL of spike protein solution at a concentration of 1 µg/mL; b) Mesenchymal cells spiked with 5 µL of spike protein solution at a concentration of 1 µg/mL and irradiated with electromagnetic frequencies ranging 7Hz to 80 Hz for 30 minutes; c) Mesenchymal cells spiked with 5 µL of spike protein solution at a concentration of 1 µg/mL and irradiated with electromagnetic frequencies ranging 7Hz to 80 Hz for 90 minutes; d) Mesenchymal cells spiked with 5 µL of spike protein solution at a concentration of 1 µg/mL treated with 3 µL of N-Acetylcystein solution at a concentration of 1µg/mL; e) Mesenchymal cells (6M cell) spiked with 5 µL of spike protein solution at a concentration of 1 µg/mL, treated with 3 µL of N-Acetylcystein solution at a concentration of 1µg/mL and irradiated with electromagnetic frequencies ranging 7Hz to 80 Hz for 90 minutes. All mass spectra where observed after direct injection of 10 µL of spike protein solution at a concentration of 1 µg/mL. The eluent composition was water and the eluent flow was 100 µL/min.

Table 1: Detection of the ion species and of the spike protein denaturation degree for each experiment.

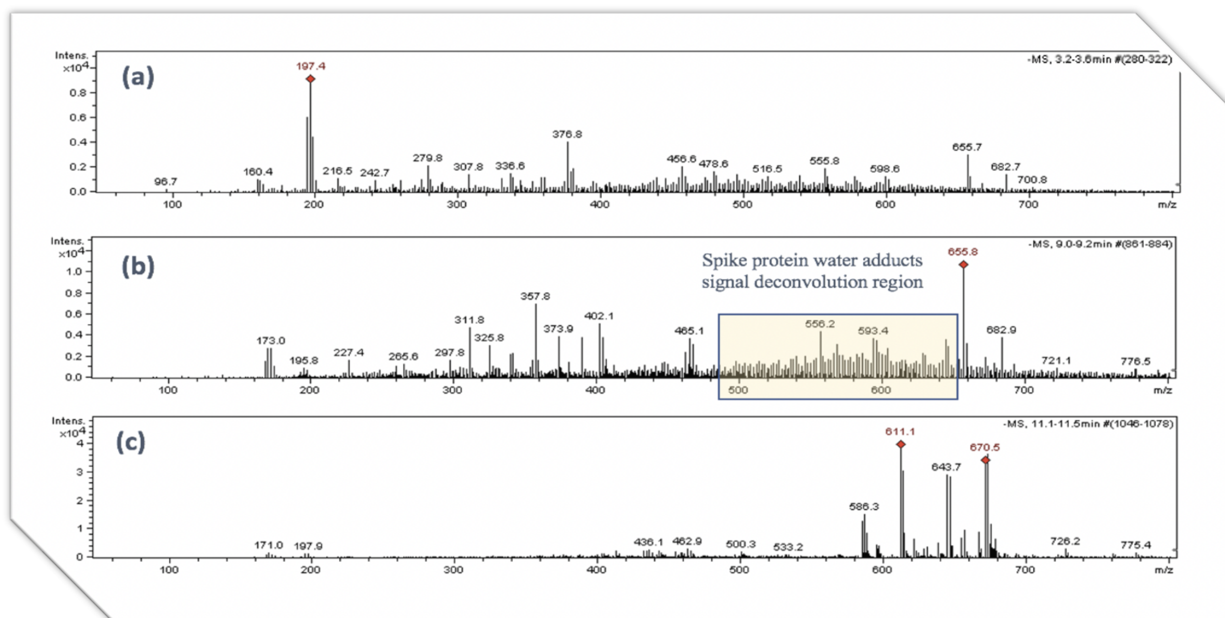

Figure 1

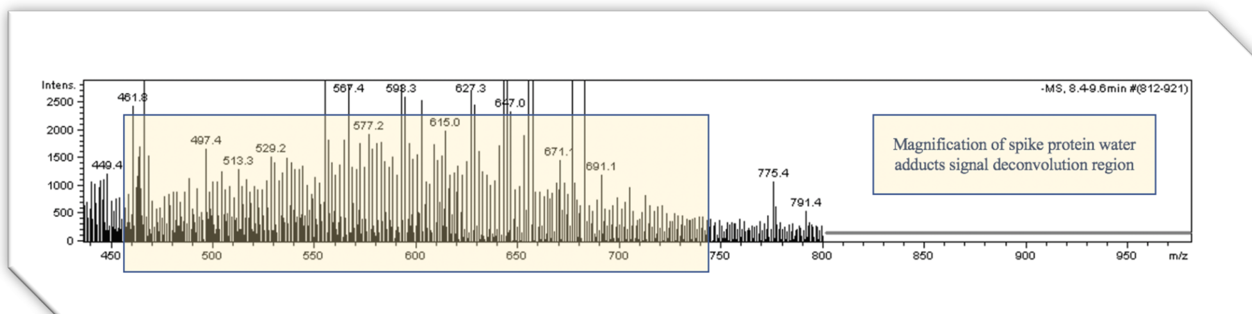

Figure 2

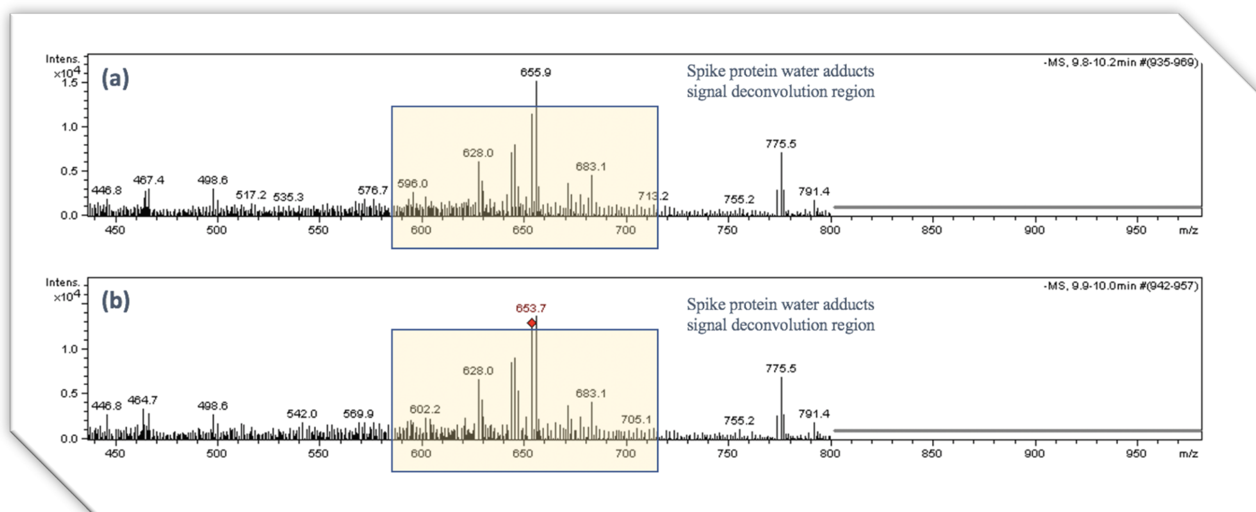

Figure 3

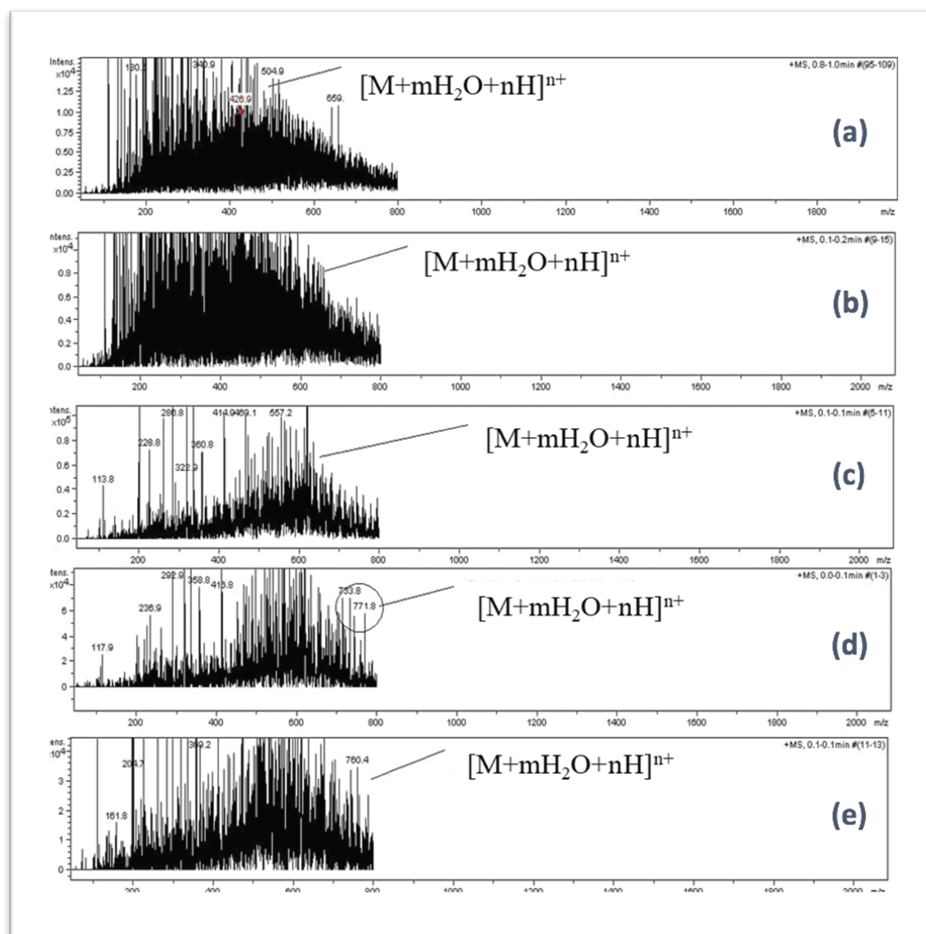

Figure 4

Experimental Settings	Detected Spike Ions	Denaturation Degree
a) Spike protein without stimulation	m/z 400 – 600	10%
b) Spike protein irradiated for 30 minutes	m/z 500 – 700	40 %
c) Spike protein irradiated for 90 minutes	m/z 500 – 700	66%
d) Spike protein with N-acetylcysteine (NAC)	m/z 500 – 700	64%
e) Spike protein with NAC and irradiation	m/z 400 - 700	96%

Table 1: *Detection of the ion species and of the spike protein denaturation degree for each experiment.*