SARS-CoV-2 Unrevealed: Ultrastructural and Nanomechanical Analysis

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ABSTRACT: The ongoing outbreak of the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) started in late 2019 and spread across the world, infecting millions of people, with over 3.3 million deaths worldwide. To fight back the virus, it is necessary to understand how the main structures work, especially those responsible for the virus infectivity pathogenicity. Here, using the most advanced atomic force microscopy techniques, SARS-CoV-2 viral particles were analyzed, with a special focus on their ultrastructure, adsorption conformation, and nanomechanical behavior. The results uncovered the aspects of the organization and the spatial distribution of the proteins on the surface of the viral particles. It also showed the compliant behavior of the membrane and ability to recover from mechanical injuries. At least three layers composing the membrane and their thickness were measured, protecting the virus from external stress. This study provides new insight into the ultrastructure of SARS-CoV-2 particles at the nanoscale, offering new prospects that could be employed for mapping viral surfaces. The understanding of the viruses’ capacity to survive mechanical disruptions at any level and their ability to recover from such injuries can shed light on the structure−function relationship and help us to find targets for drug action, especially for this virus that, to this day, has no course of treatment approved.

1. INTRODUCTION

Coronaviruses (CoVs) are enveloped viruses with a non-segmented single-stranded positive RNA (27–32 kb) genome belonging to the Coronaviridae family of the order Nidovirales. Their genome has 10 open read frames (ORFs).¹ Some of these ORFs are responsible for producing the structural proteins (sp): Spike (S), envelope (E), membrane (M), and nucleocapsid (N).² The nucleocapsid protein (N) interacts with the RNA producing a ribonucleoprotein (RNP) particle protecting the viral genome.³ Recently, many studies on the structural aspects of the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) have been done to understand the molecular and structural dynamics of the virus infection mechanisms.⁴−⁶ Structural crystallographic analysis provided essential information about the structural flexibility of S protein and its role in infection.⁷ Although many studies have evaluated the virus proteins, focusing on chemical composition, very few studies have been done on the virus particle conformation and nanomechanical properties.⁸−¹⁰ Nanomechanical studies provide essential insights into the unique mechanical properties of viruses. The viral capsid functions go beyond the protection of the genome being considered important to the viral life cycle, interaction with vectors, and pathogenicity. Thus, the understanding of its meta-stability, conformational plasticity, ultrastructural morphology, and mechanical properties (e.g., resiliency, brittleness/hardness, material fatigue, and resistance to osmotic stress) is essential to understand the virus behavior and allowing the development of drugs and vaccines based on this structural, mechanical, and physicochemical model.¹¹−¹³ Studies on physical virology have driven new approaches to developing antiviral strategies, viral-based drug delivery systems, and the design of proteins and peptides with therapeutic applications toward viruses.¹⁴−¹⁶ For instance, Yang and Lu¹⁵ have discussed that the knowledge produced by physical virology studies was employed to design new molecules-based drugs toward the Hepatitis B virus (HBV), which are now in clinical trials. Here, the knowledge produced about the SARS-CoV-2 nanomechanical properties could be
useful to design a new specific drug toward the SARS-CoV-2 to affect the aspects of physical properties of the virus.

In this study, the structure of the SARS-CoV-2 was investigated by AFM (atomic force microscopy), a powerful tool, which may be employed to reveal structural details with high reliability, bringing new insights into the ultrastructure, architecture, and nanomechanical properties of the SARS-CoV-2.

2. RESULTS AND DISCUSSION

2.1. Structure and Shape of SARS-CoV-2 Viral Particles. In the topographic maps (Figure 1), it is possible to observe the outer structure as the uppermost layer from the virus particle. We measured that the virus particle has a size of 92 nm with a standard deviation (SD) of 15.7 nm (Figure 1A). These data are corroborated by Laue et al. and Bar-On et al.
al., which stated that the viral particle size can be around 100 nm without considering the spike proteins.

On the top view of the viral particle, it is possible to observe in Figure 1B a complex structure with units having a measured diameter of 10.9 nm ± 1.9 nm SD. These findings corroborate the model proposed by Klein and collaborators for the diameter of the spike protein. The spacing from center to center of each of these units was measured to be 11.8 nm ± 2.5 nm SD. The cross section in Figure 1C highlights the particle height profile, with a height of 21.4 nm ± 4.4 nm SD after deposition in a glass substrate. The green arrows point to some of the protein units that have been measured, with an inset map showing the direction in which the particle was sectioned. S proteins tend to reorder their organization on the virion surface; in this sense, AFM images have an advantage: the ability to analyze the whole surface, suggesting a higher density of S proteins.

Neuman et al. have reported protein distribution across the SARS-CoV-1 membrane using Cryo-EM images and Fourier transform. Their findings suggest that the spikes interspacing is ∼15 nm and they reported other densities spaced 5 to 8 nm apart, connecting the RNP (ribonucleoprotein complex: composed by the N protein and the genome) to the membrane region, but could not confidently affirm if these densities were related to M or N proteins. For the SARS-CoV-2, Kiss et al. and Lyonnais et al. reported a “bald” viral particle showing a smooth surface, without clear smaller structures. Opposingly, our data show that the viral particle membrane is crowded with smaller structures, suggesting the structural description of the proteins on the membrane surface.

In Figure 2, it is shown the adhesion force maps resulting from the interaction between the AFM probe and the substrate containing the viral particles.

The bidimensional adhesion map shown in Figure 2B corresponds to the viral particles shown on the topographic map in Figure 2A. It is possible to identify the viral particle as being very distinguished from the substrate. The surrounding region of the viral particles has a scale of adhesion different from the center, with a measured thickness of ∼20 nm, compatible with the distance from the spike corona base on the membrane and its top portion, as reported in the literature.

In addition to this, it is possible to observe an interaction between the coronas of the particles. The region suggested as corona appears with a different scale around the particle because the peripheral spikes are interacting with the substrate and the spikes at the top of the virus do not interact with the substrate, only with the probe, resulting in a different distribution of adhesive forces.

Further analysis on the adhesion mode (Figure 2C) focusing on the center of the viral particle showed triangular shape structures, compatible with the spike proteins diameter. The distribution of the spike proteins on the surface of the viral particle is shown in Figure 2D. Different contrasts of adhesion forces on the top of the viral particle may be related to the width of the postfusion action of the S2 subunit to bundle. Postfusion is an essential process performed by the S2 subunit from the spike protein. During the SARS-CoV-2 infection and after the recognition of the ACE2 receptor by the S1 subunit, the spike protein changes to postfusion mode, and S2 drives the fusion between viral and cellular membranes that led to virus entry in the cell. In this direction, our findings suggest that the spike proteins are structures that can interact with a great range of molecular and cellular mechanisms, which corroborates the high virulence profile of the SARS-CoV-2 as stated by Kumar et al. It is important to address that the adhesion maps do not have the same physical nature as height maps. During peak force quantitative measurements, on AFM, the maps are obtained from the force curves composed from cycles of approximation and retraction of the tip relative to the sample. The topographic maps are derived from the approach curves while the adhesion maps are related to the retraction portion, and they both reveal different information about the sample structure.
In force spectroscopy measurements via AFM, the adhesion forces are a combination of electrostatic, van der Waals, capillary, and forces promoted by chemical bond breakage. In particular, in cases of nonfunctionalized probes (such as in this research), the adhesion forces are taken as nonspecific interactions and it is not possible to separate the contribution of each one of these forces. However, as the probes used here to analyze all samples are made of the same material and have the same specifications (geometry, tip radius, etc.), just as the experiments for all samples were performed under the same conditions (temperature and humidity of the air); thus, differences in the contrast of adhesion forces are mainly related to van der Waals and electrostatic interactions, representing different components of the particle surface.

Considering the influence of electrostatic forces, Butt and collaborators pointed out that, especially in measurements in air, contributions of electrostatic forces are expected, especially in nonconductive samples and in low air humidity, when charge dissipation is ineffective. Thus, changes arising from electrostatic forces result in a greater or lesser accumulation of charge on the surface, which is associated with the composition and/or the structure of the membrane. Liu and coworkers did show through surface plasmon resonance (SPR) the validation of the role of electrostatic force in the interaction between SARS-CoV-2 pseudoviruses and surfaces, providing evidence that negatively charged surfaces can inhibit the adsorption of SARS-CoV-2 pseudoviruses and suggesting new routes into the design of anticontamination personal protection equipment (PPE) and other technologies based on the viral surface charges. These results corroborate the ones presented here, in which the structures with higher adhesion force values are related to the substrate functionalized with poly-L-lysine, which has a positive charge, whereas the regions on the map with lower adhesion force values correspond to the triangular structures, associated with the spike protein.

The data presented in Figure 3 show the difference in adsorption patterns observed for the SARS-CoV-2 viral particle in a glass substrate (in air).

In Figure 3A–C, it is also possible to recognize the spike protein profile highlighted by the dashed line represented by triangular shapes, typical of the spike structure. Due to the charge interaction of the viral particle membrane with the substrate (glass), it is possible to observe internal particle structures. For instance, in Figure 3A–C, it is possible to observe a globular-like pattern and their different adsorption assemblies on the glass substrate. This result corroborates the findings of Lu and collaborators for the organization of the nucleocapsid. Figure 3D–F suggests a different internal assembly in different particles adsorption, with hexameric (Figure 3D) and tetrahedral assembly (Figure 3F) and in the
latter, with different pyramidal rotations, according to the model proposed by Yao and co-workers.\textsuperscript{24}

2.2. Indentation Analysis. In this study, we focused on measuring the resistance of each layer of the viral particle membrane. Thus, we performed several indentation cycles with up to 1 nN force with an indentation velocity of 50 nm/s. The reason for the chosen parameters is that viscoelastic materials under loading stress at low frequencies have a more soft behavior, opposing the more solid-like response at higher frequencies.\textsuperscript{19}

In this direction, we performed 70–100 cycles of force curves at the same region of the viral particles. The indentation results shown in Figure 4A represent the behavior of the viral particles upon different stages of indentation compared to the force curve performed on a glass slide substrate (a black dotted line). The separation between the curves gradually increases accordingly with the advance of the cycles performed, indicating the indentation of the probe on the viral particle. Each step observed in force curves (regions with constant force values—yellow circles in Figure 4A) is related to the rupture\textsuperscript{32} of a structural layer. In the 10th cycle, it is possible to observe the first step related to a constant force of \(~100\) pN, a second step around \(600\) pN, and the third step with a rupture plateau force around \(800\) pN, indicating the rupture of three layers (or individual membrane structures) that require forces of the order of \(100\), \(600\), and \(800\) pN, respectively, to be disrupted at an indentation speed of \(50\) nm/s. It is possible to see that a total layer width of \(\sim 6\) nm has been ruptured. These data confirmed the previous studies showing that SARS-CoV-2 may have a membrane with a width from 6 to 8 nm.\textsuperscript{19}

On the 20th cycle, it is possible to observe several rupture events (steps) between \(100\) and \(900\) pN of the rupture force interval. These multiple events, with smaller thickness plateaus, suggest that there is an attempt by the viral particle to reassemble the membrane proteins; however, the reassembly is not completely effective due to the continuous stress on the layer, even at low speeds.

Finally, on the 70th cycle, there is the common first step on \(~100\) pN and two smaller plateaus on a force scale of \(~400\) and \(~500\) pN, respectively. Correspondingly, there is a bigger displacement compared to the glass curve, indicating that the viral internal structure, the nucleocapsid, is continuously deformed by the indentation of the AFM probe, and because of that the disrupting of the layers requires smaller forces than the ones on the earlier cycles.

In the retraction curve shown in Figure 4B, three main penetration release events\textsuperscript{33} stand out from the SARS-CoV-2 membrane structure, evidenced by the yellow circles, confirming the presence of three main structures suggested in the result as shown in Figure 4A. The hysteresis observed between the approach and retraction cycles corresponds to the energy dissipation of the AFM probe over the surface of the viral particle, demonstrating its viscoelastic character, especially at low speeds of indentation. The first plateau common to all cycles shown in the graph in Figure 4A reveals something interesting: there is always a tendency to reassemble the viral membrane and an increasingly thicker layer is disrupted with smaller amounts of loading forces as the cycles advance.

Although the indentation analysis confirmed the rupture of the particle layers, further analysis on the AFM showed that local damage on the membrane layers can be fixed by reassembly of the membrane itself. In Figure 4C, the 40th cycle (magenta curve) shows a tendency to decrease indentation, when compared with previous cycles (20th cycle, blue curve). This effect may be explained by the reorganization of the membrane proteins, showing that a complete rupture of this organization is only possible using higher loading forces.

 Nonetheless, the viral particle reassembly effect could also be explained by the viscoelastic behavior of the membrane or the displacement and subsequent mobility of protein units. The protein mobility was explained by Alenghat and Golan,\textsuperscript{34} which have shown that membrane protein mobility may vary from simple random Brownian motion to both axial and orbital movement into the membrane itself. Finally, as shown in Figure 4D, it is possible to observe a conformation of proteins in a hexamer-like arrangement but with an apparently missing unit, and this could be related to the membrane protein mobility.

The nanomechanical analysis performed in this study corroborated the findings made by Yao et al.\textsuperscript{24} and Astuti and Ysrai,\textsuperscript{35} for the SARS-CoV-2 structure. The analysis showed that three layers may compose the virus structure, with them being mechanically resistant, suggesting that proteins and other membrane components may act as different layers that protect the virus from external stress and act as the infective structure. This protective behavior is important to better understand the fragility of the virus to the host immune system on the environmental conditions. These data are quite desirable to promote the evaluation of new products, especially for disinfection purposes. The nanoindentation analysis also corroborated the suggestion made by Lyonnais et al.\textsuperscript{25} confirming the deformable feature of the SARS-CoV-2. Finally, the most innovative result is that the SARS-CoV-2 virus may self-assemble after mechanical stress. These data are crucial to better understand the resistance of the virus and propose new drugs and techniques to fight back the disease.

Nanomechanical analyses are common to assess the mechanical properties of diverse biomaterials.\textsuperscript{36,37} AFM has proven to be a valuable approach to describe the detailed structural architectures and features of a wide variety of viruses.\textsuperscript{38–41} Regarding mechanical stiffness, nonenveloped capsids differ widely in their ability to withstand high mechanical loads without being physically disturbed or even irreversibly deformed.\textsuperscript{20} For example, it has been shown that procapsids of phage \(\Phi 29\)\textsuperscript{42} and MVM (minute virus of mice) particles\textsuperscript{43} can bear deformations, but that repeated deformations eventually lead to shell failure. The \(\Phi 29\) pro heads were able to withstand the loading force of \(2.8\) nN for \(~30\) cycles before the shell brakeage but the MVM collapsed after only seven deformation repetitions, with a maximum force of \(0.9\) nN. The CCMV (Cowpea chlorotic mottle virus)\textsuperscript{44} capsid had a plastic response to multiple indentations, at \(pH = 5\), with the capsids showing persistent deformation and slow partial restoration of the original height. In contrast, at \(pH = 6\), the capsid withstood the deformations and showed no signs of fracture. Thus, the fatigue of nonenveloped viruses is quite specific to the particular species of virus and may differ even for the same species at different environmental conditions, demonstrating that, even though they are structurally uniform in terms of capsids, they still present a wide range of materials properties.\textsuperscript{32,45}

For enveloped viruses, as is the case of SARS-CoV-2, notable differences were found in the investigation of their mechanical properties. Influenza viral particles are very deformable, mechanically behaving like its soft lipid envelope.\textsuperscript{46} In contrast, the immature virions of two retroviruses, murine leukemia-
(MLV)\textsuperscript{67} and human immunodeficiency (HIV-1)\textsuperscript{48} are mechanically very rigid, with Young’s modulus comparable to those of icosahedral capsids. The results of the mechanical properties for these three enveloped viruses (MLV, HIV-1, and Influenza) suggested that an effective fusion with the cell membranes may require a soft and flexible virion structure that allows extensive contact between the virus and the cell. In this direction, monitoring and changing the mechanical properties of the enveloped virus may promote a mechanical control of the virus entry into host cells.\textsuperscript{45}

3. CONCLUSIONS

In this study, the ultrastructure and nanomechanical properties of SARS-Cov-2 viral particles were evaluated by AFM. The high-resolution topographic revealed the structures of the membrane surface of the viral particle and its distribution, corroborating with theoretical models proposed in the literature for this virus. In addition, the adhesion force maps showing the interaction between the AFM tip and the viral particle surface revealed structures with size and shape compatible with spike proteins. For these structures, adhesion forces presented different values from the other membrane components, demonstrating a more negative nature of these structures. Another relevant finding was the observation of internal particle structures with a globular-like pattern and their different adsorption assemblies on a glass substrate. An adsorption study comparing between different substrates and calculation of the adsorption energy might be a useful further investigation. The membrane fatigue tests using successive indentations on the same region of the virus surface with curve acquisition speeds of 50 nm/s allowed us to see membrane rupture events, layer by layer, suggesting three main layers, with each one of them having their thickness measured individually and adding up to a total thickness of \(\sim 6\) nm. In addition, it was possible to observe the reorganization of membrane proteins, with force curves of late cycles showing a reduction in the amount of indentation on the particle membrane. The penetrative events were also confirmed by the retraction curves in advanced indentation cycles, showing peaks characteristic of penetrative release, with binding forces reaching up to 200 pN. As a characteristic of viscoelastic materials, at low indentation rates, the viral particle presented a quite evident hysteresis between the approach and retraction curves, characterizing the energy dissipation of the probe during indentation. The results presented here add new data in the characterization of the physical and structural properties of the SARS-Cov-2, reinforcing models presented in the literature.

4. METHODS

4.1. Origin of Virus. Nasopharyngeal (NP) swab samples were collected from symptomatic patients who had acquired COVID-19. These patients were treated in the same hospital and were the two first confirmed cases of COVID-19 in São Paulo city. The specimens were collected on days 2−4 postsymptom onset; placed in 1−2 mL of saline medium, and used for molecular diagnosis and virus isolation. The resulted strain was designated: SARS-CoV-2 isolate (EPI−ISL_413016).\textsuperscript{49}

4.2. Virus Isolation. Vero E6 cells (ATCC, no 1586) were cultured in Minimum Eagle’s medium (MEM; Gibco) supplemented with 10% FBS (Gibco) and plated at 100,000 cells/well in a 48-well plate at 37 °C with 5% CO\(_2\) atmosphere. The virus yield in culture supernatants was quantified by RT-qPCR (Real-Time quantitative Polymerase Chain Reaction) using specific primers and a probe against the RdRp gene according to RT-qPCR 2019-nCoV (Institute Pasteur protocol).\textsuperscript{50}

4.3. Inactivation. The inactivation followed the ATCC protocol, as described: The heat-inactivated preparation of 2019-nCoV/USA-WA1/2020 (ATCC VR-1986HK) was inactivated by heating it to 65 °C for 30 min, becoming, therefore, unable to replicate. The heat-treated material is tested for virus inactivation by culturing in Vero E6 cells (ATCC CRL-1586) for two passages of 14 days each to confirm that there is no evidence of viral replication.\textsuperscript{7} This protocol is corroborated by Betjat et al.\textsuperscript{52} It is important to notice that the inactivation process, used to manipulate the virus in the AFM, could affect the integrity of the surface of the sample. According to Lovey et al., the inactivation process used in the SARS-CoV-2 virus may affect the structural and genomic integrity of the virus.

4.4. Virus Stocks. Viable (titre 1.0 × 10\(^8\) PFUs in 500 μL) and inactivated from isolate SARS-CoV-2 (EPI_ISL_413016) were prepared and used in this study.

4.5. AFM Structure Analysis and Nanomechanical Experiments. For AFM measurements, 10 μL of MEM solution with viral particle suspensions were deposited on microscope slides (13 mm diameter), previously treated with poly-L-lysine 1% (Sigma, St. Louis, MO, USA). The slides were analyzed on MultiMode 8 (Bruker, Santa Barbara, CA, USA) and the probes used were SNL (Bruker) with 0.06 N/m nominal spring probes with 0.06 N/m nominal spring constant in the peak force quantitative nanomechanics (QNM) mode. The structural parameters of the viral particles were calculated using the Gwyddion 2.57 software,\textsuperscript{54} applying the boundary grain detection to the topographic images (5 μm\(^2\) scan area), masking the regions corresponding to the particles. From these regions, statistical information on the height and diameter of 415 particles was calculated. For the diameter and spacing of membrane proteins units’ measurements, five different viral particles (a scan area of 150 nm\(^2\)) were analyzed using the cross-section tool. Viral particle indentation experiments were performed on seven different virions, and each one has undergone 70 to 120 indentation cycles. Adhesion maps were analyzed on 13 viral particles (250 nm\(^2\) scan area).

For the indentation analysis, the measurements were done on the QNM Ramp Mode in fluid following the same protocol described by Kiss et al.\textsuperscript{22} Briefly, we also performed the measurements on the liquid mode, but in our case, the fluid used was culture medium solution (MEM). We used a force setpoint of 1 nN and a tip velocity of 50 nm/s, which, to our best knowledge, is lower than any other AFM study performed on the SARS-CoV-2. AFM data were analyzed and the maps were obtained with the aid of Mountains SPM\(_8\) software (Digital Surf France).

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