Application of Cryo-EM Method to Study Covid-19 Proteins

By

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A thesis submitted to the School of Pharmacy in partial fulfillment of the requirements for the degree of Bachelor of Pharmacy (Hons.)

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Declaration

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Approval

The thesis titled “Application of Cryo-EM Method to Study Covid-19 Proteins” submitted by Farhana Akter, 18346032, of spring 2022 has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Bachelor of Pharmacy.

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Ethics Statement

This study does not involve any kind of animal and human trial.

Abstract

Researchers from all around the globe are working quickly to utilize cryo-electron microscopy (cryo-EM) to explain how the corona virus disease (COVID-19) infects human cells, in tandem to the global community's efforts to stop the spread of the illness and cure individuals who have been diagnosed. Through the use of cryo-EM, these researchers were able to determine the spike protein's structural characteristics as well as that of the virus' cellular receptor. Cryo-EM, which studies the structure of physiologically significant molecules like proteins and pathogens at almost atomic resolution to easily grasp 3D structure and activity, has emerged in recent years as a popular and successful approach for biological research. The creation of vaccinations and the advancement of COVID-19 therapies will be facilitated and accelerated by these discoveries. Public access to the research team's cryo-EM structures is now possible. About seven years ago, cryo-EM began to be used more widely as improvements in electron detectors, software, productivity, and other critical elements made it possible for scientists to resolve crucial biological material at better resolution. A lot of work has been achieved in understanding the structure and dynamics of the corona virus in far less than two months. In the past, it may have taken years to advance to this level. This article will be reviewing the applications of the Cryogenic electron microscopy for studying and understanding the characteristics of protein spikes of the SARS-CoV-2, which can originate COVID-19 in the human body.

Keywords: Cryo-EM, Covid-19, Corona viruses, Vaccination, SARS-CoV-2, Protein spikes

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Abbreviations

EM= Electron microscopy

SARS-CoV= Severe acute respiratory syndrome corona virus-2

MERS= Middle East respiratory syndrome

GI= Gastrointestinal

ACE2= Angiotensin-converting enzyme-2

NMR= Nuclear magnetic resonance

SPA= Single-particle analysis

Cryo-ET= Cryo-electron tomography

PDB= Protein Data Bank

kDa= kilo Dalton

GPCR= G-protein-coupled receptors

HSV-2= herpes simplex virus type 2

CCDs= charge coupled devices

RBD= receptor-binding domain

NTD= N-terminal domain

PHEIC = Public health emergency of international concern

RMSD= root mean square deviation

IC50= Half-maximal inhibitory concentration

RNA= Ribonucleic acid

Chapter 1

1.1. Introduction

The application of cryo-EM methods as a structural biology technique is well demonstrated by the quick progress made in using it to investigate SARS-CoV-2 proteins using cryo-EM techniques. Firstly, the composition of a soluble portion of the trimeric SARS-CoV-2 spike protein was discovered about one month after the gene sequence was made available to researchers. The spike protein has several structural forms, including natural spike structures visible on intact viral membranes, which have been quickly deciphered by almost a dozen research teams from across the world. These are significant accomplishments that also demonstrate the efficiency, effectiveness, and biological use of current cryo-EM techniques. Especially in terms of the conformational distribution of the region that activates the human ACE2 receptor, the structures offer significant new insights into the diversity of spike design. These results are vitally important for the development of COVID-19 treatments and vaccines because they combine newly available structural data on the hooking location of non-identical antibodies on the S-protein.

1.2. Rationale of the Study

Many biological research laboratories all around the world are focusing on looking into the COVID-19 virus due to the present worldwide health issue. The formulation of a vaccine can be aided by understanding these viral proteins through studies of their three-dimensional structure. The shape of the spike protein on the COVID-19 virus, often known as SARS-CoV-2, is very intriguing. Cryo-EM was used to study this, and researchers from The Texas State

University at Austin and the National Academy of Allergy and Contagious Diseases in the United States recently published their findings in Science. SARS-CoV-2 viral spike proteins are at approximately ten times more strongly attached to their host cell target than those of the SARS virus, the scientists find using high resolution cryo-EM (SARS-CoV). This explains why SARS-CoV-2 virus cannot attach to the spike protein and gives insight on how the development of a vaccine must be different from prior corona virus breakouts.

1.3. Aim of the Project

The widespread use of cryo-electron microscopy has grown greatly over the past few years (cryo-EM).82 of the 84 corona virus cryo-EM configurations that were deposited in the repository before to January 2020 were composed of trimeric spike peptides. Contritely, only around 50% of the thirty four entries of SARS-CoV-2, which spike proteins have indeed been deposited in 2020; the remainder is made up of additional entries. These advances are significant because complexes of this class are usually flexible and stereo chemically unexpected, creating it challenging by investigate them using X-ray crystallography. The potential possibility of shifting away from explaining proteins in context of static shapes and toward generating flexible landscapes, this may provide a much better understanding of their biological function is offered by the introduction of "single particle" technologies like cryo-EM. The goal of this study is to know the effectiveness of the application of current cryo-EM techniques used to determine the Covid-19 spike proteins which is important for the development of COVID-19 treatments and vaccines.

Chapter 2

Methodology

Initially, 46 papers were collected based on my topic from a various internet sources which include PubMed, Research Gate, Elsevier and Science direct by thorough research. From all of those papers, 29 were chosen based on relevancy and finally 24 papers which had most recent years of publications were selected with the intention of getting the most updated information. After that, all the information were mixed and matched and re-organized and an outline was created followed by a comprehensive review of all these papers. Lastly, all the selected information was re-written and references were added with the help of Mendeley library to specify the sources of those information. Thus, this review on 'Application of Cryo-EM Methods to Study Covid-19 Proteins' was carried out.

Chapter 3

3.1. What is COVID-19

SARS-COV2 is the major mediator of the potentially lethal disease known as COVID-19, which is an utmost issue for worldwide public health. It is hypothesized that this is probably the COVID-19 zoonosis due to a high figure of affected individuals who were into Wuhan City, China's wet animal market (Rothan, 2020). Patients who contracted COVID-19 from direct contact with another person had to be isolated and then received a range of therapies. To contain the present outbreak, several steps have been put in place to lessen COVID-19 transmission from person to person. (Rothan, 2020) (Subramaniam S, 2020)

In December 2019, the first instances were recorded. Acute respiratory distress syndrome hospitalized five patients from December 18 to December 29, and one of them tragically died.By January 2, 2020, 41 hospitalized individuals who had been admitted had been found to have COVID-19 infection, which had been verified by a laboratory. Below half of them suffered from underlying medical conditions such mellitus, high blood pressure, or heart disease. In that hospital, it was assumed that these individuals had contracted an illness, most likely from a nosocomial source. The COVID-19 virus was determined not to be a highly contagious one (distributed by one patient to many others), instead to spread most likely as a result of several patients becoming infected at various sites across the hospital through unidentified processes. (Rothan, 2020)

According to a study in Nature, Chinese health officials discovered that as of February 7, 2019, there had been 31,161 cases of the sickness in China, and more than 630 individuals had passed away as a result of the infection. At the time this article was published, the World Health

Organization (WHO) recorded 51,174 documented cases in China, including 15, 384 severe cases and 1666 deaths. As of the date of this publishing (February 16, 2020), there have been 51,857 verified cases worldwide across 25 different nations. (Rothan, 2020)

3.2. Cell biology of COVID-19

3.2.1. Pathogenesis of COVID-19

Particularly in the Chinese epidemic zone, the acute symptoms of COVID-19 are linked to an increase in the number and rate of mortality. On January 22, 2020, and January 25, 2020, the Chinese National Health Commission disclosed the details of the first 17 fatalities. There were 56 deaths recorded. As of January 25, 2020, it has 2684 affirm cases of COVID-19, of a mortality rate roughly 2.84% and a median age of 75 years (with a range of 48 to 89)(Figure 1). According to Rothan H & Byrareddy S, *(2020)* higher leukocyte counts, aberrant respiratory signs, and all COVID-infected individuals had higher plasma values of top player cytokines. In one of the case reports for COVID-19, a patient who had been experiencing a fever for five days appeared with a cough, rough breathing noises coming from both lungs, and a core temperature-39.0°C. RT-PCR findings from the patient's sputum were positive, confirming the COVID-19 infection. Laboratory tests revealed leucopenia with neutrophil levels of 70.0% of the total leukocyte count of 2.91x10^9 cells/L. Blood C-reactive protein was also measured and found to be 16.16 mg/L, which is higher than the usual range of 0 to 10 mg/L.High D-dimer levels and erythrocyte sedimentation rates were also noted. As a respiratory system-targeting virus, COVID-19 infection was mostly caused by severe

pneumonia, RNAemia, associated with a high prevalence of ground-glass opacities, and early cardiac damage. (Rothan, 2020)

3.2.2. Symptoms of COVID-19

From the time COVID-19 symptoms first appeared until death, it took an average of 14 days—a range of 6 to 41 days. The length of these symptoms depends on the human age and immune health. When compared to patients who were younger than 70, it was shorter for those who were older. Fever, coughing, and exhaustion are the most typical first signs of COVID-19 disease (Figure 2). (Rothan, 2020) (Larsen J, 2020)

In a study, the authors discovered data that lends credence to the idea that COVID-19 differs from other well-known respiratory disorders in having a most frequent order of observable symptoms. Fever in the three corona virus-based infections and congestion in influenza-virus are typical first symptoms. The very first two criteria of COVID-19 or MERS, or SARS are fever and cough. Contrary to MERS or SARS, COVID-19 appears to impact on the GI tract like nausea and vomiting, before the lower part of GI tract such as diarrhea. They've discovered that fever and cough always come first in illnesses. (Larsen J, 2020)

Authors also discovered that the beginning symptoms of the most probable route are similar to those seen in the outcome of the four signs when they observed the collection of 7 signs and symptoms. Additionally, GI tract symptoms come last in each of the both symptom fulfillments. The earlier signs and symptoms of patients upon admission were part of a different MERS data set, and they were listed in order of likelihood: fever, myalgia, cough, and diarrhea

(Table 1).This arrangement resembles the most obvious route that was identified. (Larsen J, 2020)

3.3. Phylogenetic analysis

Ortho corona virinae subfamily of the corona viruses includes COVID-19, which are its seventh member and the seventh corona virus to infect humans. The subgenus sarbecovirus has a clade that includes the COVID-19.COVID-19 can be regarded as a novel beta corona virus that infects people since it differs from SARS-CoV enough based on its genetic structural similarity and phylogenetic data. The significant amount of ACE2 transmitter homology observed in a wide range of species of animals, which suggests that animals may serve as potential intermediary hosts or experimental animals for COVID-19 infections, is another indicator that COVID-19 originated in bats. Furthermore, these viruses have a singular undamaged open framing of reading on chromosome 8, indicating that they represent CoVs of bat origin. Putative receptor-binding domain's amino acid sequence but it mimics the SARS-CoV amino acid sequence, suggesting that both viruses may share a receptor. (Rothan, 2020)

World Health Organization (WHO) showed that, COVID-19 is a category 2B CoV.COVID-19 genomic sequencing from ten different individuals showed 99.98% sequence similarity. In a different study, five patients had similarly 99.8–99.9% nucleotide identification, and these results are the sequence analysis indicated of having a novel beta-CoV strain. COVID-19 sequence revealed much than 80% similarity with the SARS-CoV and 50% identity with the MERS-CoV, both of which originated in bats. Accordingly, the results of the phylogenetic study show that COVID-19 is a belonging to the family beta corona virus. (Rothan, 2020)

3.4. Transmission of COVID-19

According to reports, the animal market in Wuhan City, whereby animal parts are frequently exchanged, which is the potential zoonotic origin of the COVID-19, a significant amount of affected individuals who were disclose to it. A latent host or intermediary carrier from whom the sickness could have spread to mankind is being sought. Initial reports described two snake species that might serve as a COVID-19 reservoir. However, other from mammals and birds, there hasn't been any reliable proof of other corona virus reservoirs to yet. The most likely source of COVID-19 in humans is mammals, according to a genomic sequence study, which revealed 88% similarity with 2 corona viruses that resemble acute-severe respiratory syndrome (SARS) and are produced from bats. (Rothan, 2020)

According to a number of investigations, transmission is probably how Covid-19 infections spread. Cases that occurred inside communities and among people, who did not visit the Wuhan wet livestock market, add validity to this. Person-to-person transmission often directs touch or by the droplets passed from an infected person's cough and sneeze. There was no proof of transfer from mother to kid in a short research done on women during their third trimester who'd been verified to have the corona virus. It is yet unknown, though, if transmission may happen during vaginal birth because all of the pregnant women underwent cesarean procedures. This is significant because severe pneumonia and infections caused by respiratory germs are more likely to affect pregnant women. (Chen H, 2020)

Based on the authors, the first step of infection involves the contact with a receptor generated by host cells, followed by merging with the cell membrane. The virus's main target is thought

to be the lung epithelial cells. Accordingly, it has been discovered that the ACE2 receptor is a cellular receptor that the domain of receptor-binding of the SARS-CoV virus spikes binds to in order to transmit from human to human. It's significant that the COVID-19 spikes' receptor-binding domain has a sequence with the SARS-CoV. This information clearly shows that the ACE2 receptor is the entrance point into the host cells. (Rothan, 2020)

Chapter 4

4.1. Introduction of Cryo-electron microscopy method

In recent years, Cryo-electron microscope (Cryo-EM) has supplanted X-ray crystallography as well as NMR to become a well-liked also a successful method for component identification. For the characterization of massive bimolecular assemblies, protein complexes, or samples that were already constrained, conformationally diverse, and resistant to crystallization, it has come to be regarded as essential. Additionally, it is the only technology available that can reveal in situ high-resolution structures of biological assemblies and macromolecules. High-resolution features of the biological material are preserved with the use of a cutting-edge electron microscope that can operate at cryo-temperature. (Assaiya A, 2021)

SPA, electron scattering, spiral modeling, or cryo-electron tomography can be used to determine the structures either in isolation or within the biological context (cryo-ET).The SPA, ED, and cryo-ET three streams have all made considerable strides recently. As a consequence, new records in the guise of the biomolecules and complexes whose topologies could be constructed and atomic aspects could be seen at precision never previously attained by cryo-EM have been shattered. (Assaiya A, 2021)

Additionally, the capacity to sort and analyze many conformational states from a single sample in combination with the gathering of bigger datasets is establishing the crucial connection between protein structures and activities. Overall, these advancements are advancing the fields of biology and cell biology by assisting researchers in understanding the molecular mechanisms underlying crucial cellular processes and resolving the formations of macromolecules that have previously been difficult to determine from a structural perspective.

Here, we provided an overview of recent developments and significant contributions made by the SPA, ED, and cryo-ET, cryo-electron microscopy streams. (Assaiya A, 2021)

4.2 Principle of Cryo-electron microscopy method

The principle behind cryo-EM is to project protein complexes in all directions by taking images of biological macromolecules that have been frozen and preserved in glassy ice. Following that, a desktop is utilized to operate or compute significant total of 2D-pictures and rebuild the higher realistic 3D shape of the bio-molecule. From 2D photos, adjustments are used to infer 3D structure. The core structure theorem, developed by Aaron Klug in 1982, serves as the theoretical underpinning for this assertion. According to it, the flourier transform predicted with a specific conducted is alike to the inter-section component of the function whose Fourier analysis is transverse to the prognosis orientation and passes through the onset. (Benjin X, 2020)

4.3. Cryo-electron microscopy over other structural biology methods

Currently, high-resolution structural biology research is based on three types of regularly utilized structure biological techniques: crystallography, NMR, and cryo-EM. X-ray crystallography has dominated instructional biology for the past 50 years. As from Oct 17, 2019, the PDB database's shapes make up around 89% of the whole, followed by NMR contents with 8.2% and EM contents with 2.5% (Figure 3).The majority of proteins with molecular weights between 10 and 150 kDa may be studied using X-ray crystallography. Over

than 140,000 3D structures have been resolved after decades of growth and development, and this has made protein structure analysis the primary method for understanding the composition of biological macromolecules. (Benjin X, 2020)

The minimal molecular weight restriction for sample fragments has been raised to 52 kDa due to the rapid growth of cryo-EM. Direct electron detector development is primarily responsible for the recent, massive improvement in resolution of cryo EM. Impact from switching from photographic term to electronic X-ray detectors on the EM field is comparable to that on macromolecular crystallography (Carroni M, 2016). The magnification has also been steadily increased, and the freezing technique has become more sophisticated. As a result, cryo-EM is now quicker and more effective, and in some cases, it may even compete with crystallography of X-ray. Moreover, Cryo-EM provides the following advantages over more established structural biology techniques like X-ray crystallography and NMR: (i) This does not necessitate the use of crystalline; (ii) it is suitable for elevated antigens and their derivatives; (iii) it minimizes harmful radiation and preserves and allows for flexibility of the samples, includes gene transcription; It is appropriate for the analysis of structures of protein structures such as GPCR and their complexes because and (iv) it can capture multiple different conformational states simultaneously. Again, EM has some drawbacks also: (i) It would only be good for large associated proteins, which typically require an at least 150 kDa or even more than 300 kDa; (ii) The present frequency of cryo-EM is around 3Ă, and it goes beyond 3Ă only in a few cases, which is below the demand of pharmaceutical R&D (2Å), (iii) The requirement of specimen uniformity is too big; If the polypeptide is pliable, getting satisfactory results will be difficult. (Benjin X, 2020)

4.4. Applications of Cryo-EM

4.4.1. Protein synthesis and degradation related complexes

The advancement of data classification techniques and the expansion of cryo-EM offer a potent tool for the analysis of internal translational machinery rearrangements and the assessment of mitochondrial ribosome structure. V. Ramesh of the MRC single - molecule biology laboratory employed cryo-EM to study a broad range of high-resolution configurations of mitochondria. They published the structures of the major subunits of the mitochondrial ribosome's from human and yeast in 2014 with 3.4Å and 3.2Å resolutions. We can reveal the shapes of the ribosome's from human and yeast with resolutions of 3.5Å and 3.3Å, respectively. (Benjin X, 2020)

These findings offer a crucial structural foundation for comprehending the mitochondrial protein production molecular process. The proteasome is a binding protein that is present in various prokaryotes as well as eukaryotes and archaea. The proteasome is extensively dispersed in the cytoplasm and the nucleus of eukaryotes. Its primary duties include controlling the levels of certain proteins and clearing cells of improperly folded proteins. It is the biggest protein degradation machine discovered to date and one of the vital bimolecular machinery inside the cell. In 2018, Dong et al. used cryo-EM and machine learning to jointly identify 2.8 Å -3.6 Å conformations of 7 median isoforms on the 26S proteasome of human as occurs during precursor breakdown (Dong, 2018). This research offers a crucial structural underpinning for the kinetic mechanism of the regulatory particle subcomplex's substrate transport channels and the triggering function of the proteasome core particulate gated switch. (Benjin X, 2020)

4.4.2. Viruses and related protein complexes

Pathogens called viruses are responsible for many human and animal illnesses. In addition to helping to clarify the process of viral self-assembly and infection, the identification of their three dimensional structures is crucial for medication. Since particles of the viruses are bigger from protein particles, cryo-EM is a better method for studying their three-dimensional structure. The atomic-scale function of nucleo-capsid of the HSV-2 of herpes virus was originally revealed by Yuan et al. in 2018. They presented the assembly method of the herpes virus nucleo-capsid and revealed the intricate structural details and complex interaction mode of the protein (Yuan, 2018). Song et al., published the cryo-EM composition of the spiked proteins of the SARS corona virus and its interface ACE2 combination the same year. The MERS-CoV along with SARS-CoV spike structure and function were discovered by Yuan et al., who also provided a crucial 3D structural map for the development of broad-spectrum antibodies and vaccines. (Benjin X, 2020)

4.4.3. Neurodegenerative disease-related proteins

Disorders known as neurodegenerative diseases, such as myotrophic motor neuron problem, Parkinson's, alzheimer's disease, and Huntington's disorder, among others, cause dysfunction

as a result of the slow mortality of the function of neuron. This type of sickness currently poses a major danger to people's health and way of life because its etiology is unclear and there is no known solution. The basic structure of cryo-EM γ-secretase linked a notch splinter was described by Yang et al. in 2018; this structure showed the structural underpinnings of splinter conceding and is influential for the ability to attract γ-secretase's. The human ATPase tetramer's cryo-EM composition was then determined by Gu et al. This provided a crucial structural foundation for the disease therapy related to Degenerative brain disorders and cell metabolism by explaining the structural design pattern and mammal ATPase biochemical mechanism. (Benjin X, 2020)

4.5. Recent advancement in Cryo-EM imaging

In the past, high resolution EM imaging depended either on photo-graphic film, which is very slow, bulky, and sensitive, or on more practical but secondary detection by charge coupled devices (CCDs).Despite having lower resolution than film, CCDs enable automated data collecting. The fundamental issue is that the received electrons must be transformed to visible region for the CCD sensor to detect them electronically, and this conversion results in significant loss of sensitivity and resolution. Conversely, the most recent direct detectors capture the emitted electrons in a tiny, sensitive layer, preventing the signal from being dispersed into adjacent pixels (Carroni M, 2016).

There are two key benefits to this movie mode. First and foremost, it is possible to correct specimen movement that is caused by the beam or by other factors, which enables the preservation of high-resolution features that were inadequately communicated in single photos

(Figure 4).Cryo EM investigations' resolution and effectiveness were previously severely constrained by specimen motion during data gathering. Direct detectors' quick readout and high DQE enable the correlation of characteristics between movie images, each of which contains a very tiny electron dosage (1 e-/pixel), allowing for the tracking and correction of specimen displacements (Carroni M, 2016).

The quick, flawless, autonomous, and simple to evaluate crystal structure screening technology is now in its mature state. What cryo-EM will develop into over the coming decades is difficult to foresee. Cryo-EM might perhaps eventually replace crystallography in the process of traditional drug development. However, it is more likely that the two methods will persist to be the primary methods of drug discovery for a very long time, complementing one another and growing together. The scientists are certain that as cryo-microscopy technology advances, their comprehension of various molecular pathways, including as those controlling translation or gene regulation, Nucleic acid synthesis, RNA scrambling, cytoskeletal transport, and channel gated mechanisms, will better. (Benjin X, 2020)

Chapter 5

5.1. Comprehending SARS-Cov-2 spike behavior by using Cryo-EM imaging

5.1.1. Behavior of SARS-CoV-2 spike protein

By applying Cryo-EM to highlight the spike of SARS-CoV-2 configurations, alignments, and distribution throughout the virion particles, (Ke, 2020) extensively analyze the spike of SARS-CoV-2 in situ behavior. The viral element, which is often a glycoprotein, is recognized by the receptors on the host cell as the basis of any viral infection. Understanding how viral glycoprotein acts is essential for developing a vaccination against viruses and comprehending the route of infection. The crucial viral component known as the spike glycoprotein is what allows human corona viruses to recognize, adhere to, and enter host cells. The transmembrane protein known as the trimeric spikes, which binds to the ACE2 protein on the host cell's surface, mediates subsequent membrane fusion and virion entrance. On February 26, 2020, the surge protein, firstly the SARS-CoV-2 molecule to be discovered clinically (6VSB), was disclosed in the Protein Database. Because of the massive structure of the spiked glycoprotein, microscopy was employed to get the 3D structures of the separate jurisdictions of the trimeric spike protein complex. (Ismail A, 2020)

5.1.2. Cryo-EM image analysis of SARS-CoV-2 spike protein

After infecting Calu-3 cells in lung and VeroE6 by SARS-CoV-2, Ke et al. used cryo-EM to image the preserved supernatant. Prefusion (97%) and postfusion (3%), the two primary

isoforms of the trimeric spike in both systems, are depicted in Figure 5. Perspectives of the receptor-binding region (RBD), *Ke et al*. divided the prefusion trimers into 3 classes: (i) closed conformation, (ii) open conformation, and (iii) spike trimers with mobility, but much closed conformation, which are designate by a lower density of electron. One or more of the spike monomer's, RBDs are exposed with ready for identification of ACE2 within open conformation. On the other hand, the spike of NTD covers all three RBDs between closed configurations of the prefusion. (Ismail A, 2020)

By analyzing the Cryo-EM image we can assume that the prefusion of the spike trimer initiates a compositional change to the isoform of postfusion after binding to ACE2 receptor, where a fusion peptide and domains will join to create a thread structure that is long. However, the postfusion specific niche observed on top of the unbound virions might be part of the SARS-CoV-2 survival tactic. As postfusions protect the prefusion trimers from antibody neutralization, trimers may be created post-transfusion to protect bacterium from the protective immune system. Therefore, it must be taken into account while developing vaccines. The most amazing feature is that the epithelium division serves as a rotating gate, permitting two spike triple helical isoforms to tilt beyond the bacterial surface with a tilt diffraction angle of 00-900. This perspective is also crucial for vaccine development because neutralizing antibodies can access the base of the head domain and the stalk area. (Ismail A, 2020)

5.2. COVID-19 spike protein

The new corona virus 2019-nCoV, which has just been given the disease name COVID-19, has recently becoming a causative agent in the Chinese metropolis of Wuhan in the Anhui province. It causes high fever or respiratory sickness. The World Health Organization (WHO) reports that as of 16 February 2020, there have been more than 51,000 valid cases worldwide, resulting in 1600 fatalities. This new discovered pathogen is identified as a beta corona virus genus, and severe-acute respiratory syndrome corona virus (SARS-CoV). In comparison to SARS-CoV, the 2019-nCoV looks to be easier to send from person to person, extending throughout several continents, and prompting the WHO to declare a public health emergency of international concern (PHEIC) on January 30, 2020. (Wrapp D, 2019)

5.2.1. Cryo-EM imaging of 2019-nCoV spike protein

By using a pure and complete glycosylated S protein, cryo-EM grids were created, and preparatory examination demonstrated a macro particle sizes on negligible conglomeration towards the borders of perforations. It expressed the 2019-nCoV S ectodomain sites 1 to 1208 combining extra sustaining proline alterations in the S2 fusion's C-terminus machineries adopting a prior stable method thus worked for another S proteins, in reference to the first known gene arrangement of 2019-nCoV.The domain structure of the expression construct is displayed in Figure 6A. Moreover, from the cryo-EM grid it is also attained a 3.5-resolution 3D representation of an polygonal trimer where a singular RBD is identified in the upper conformation after gathering and analyzing 3207 microscopy pictures.(Fig. 6B).The

asymmetry of this configuration was not immediately obvious due to the RBD's tiny size (21 kDa), hence 3D reconstruction and identification were conducted (Fig. 6B). (Wrapp D, 2019)

5.2.2. Comparison between the structure 2019-nCoV S and SARS-CoV S

2019-nCoV S shares several structural similarities with SARS-CoV S, having a RMSD of 3.8Å across the 959Cα atoms (Figure 7A).The location of RBDs in the two structures' respective down conformations is one of the bigger distinctions between them (though it is still only a small one). The descending conformation of such 2019-nCoV RBD is more angled to the trimer's body wall than the descending orientation of the SARS-CoV RBD, which firmly against with the NTD of that surrounding protomer (Figure 7B). Despite from the observed conformational difference between the NTDs or RBDs or the subdomains 1 and 2 and S2 subunits all provide RMSD character of 2.6, 3.0, 2.7, and 2.0, respectively, when the basic features of 2019-nCoV S are matched with those of SARS-CoV S. (Figure 7C). (Wrapp D, 2019)

To enter host cells, 2019-nCoV uses a spike (S) protein that has been heavily glycosylated. The S enzyme is a protein subunits class I fusion protein in a ground state prefusion and goes through significant systemic change to merge the membranes of the envelope and the host membrane. The S1 subunits indentured to a human cell receptor starts this process. The prefusion trimer is made more unstable by receptor contact, which causes the S1 subunit to shed and S2 to become a firm postfusion form. The RBD of S1 occupies a human cell receptor by hinge-like conformational motions that momentarily conceal and reveal the receptor binding determinants. The “down” conformation and the “up” conformation relate to these two

states, where down denotes the receptor- isolated state and up represents the more unstable receptor-convenient state. The S protein is a point for antibody-resolve neutralization due to its important function, and the identification of prefusion S form would offer atomic- volume knowledge for direct development of the vaccine. (Wrapp D, 2019) (Chan J, 2020)

5.3. N501Y SARS-CoV-2 spike protein

5.3.1. Cryo-EM structures of N501Y SARS-CoV-2 spike protein

Because of a number of modifications, notably the N501Y mutation, the variant of UK SARS-CoV-2 is expected to be extra contagious than previous strains. New SARS-CoV-2 strains mutations of various spike proteins were discovered in UK and South Africa in December 2020. Early epidemiologic studies results have suggested that these mutations exhibit greater population transmissibility. The alteration of remnant 501 on RBD from the Asn to Tyr is a shared characteristic of UK and South African versions despite their divergent evolutionary histories (N501Y). (Kashyap S, 2021)

N501 is a crucial remnant on spike protein at junction between the domain and ACE2 which is engaged to crucial interactions within multiple ACE2-residues, according to structural investigations using cryo-EM. Before the discovery of this novel UK variation, several researches using a mouse model showed that evolution of remnant 501 may also related to enhanced both infection and cell adhesion. Therefore, it is very important to understand how N501Y modulates viral entry, ACE2 adherence, and antibody suppression to effectively halt COVID-19 spreading. (Zhu X, 2021)

5.3.2. N501Y SARS-CoV-2 spike protein complex with ACE2

An in-depth view of receptor's general structure as well as the attaching between the domain and ACE2 is provided by the complex produced by the ectodomain of the N501Y spike and the ACE2 receptor (Figure 8).The "up" position of the RBD is where the ACE2 receptor is attached (Figure 8A).At a global resolution of 2.9Å, the complex's general structure was identified. Local RBD with ACE2 domain refinement raises regional design at the contact area around 3.3Å (Figure 8B), (Zhang X, 2010) allowing for the clear identification of the Y501 chain and nearby remaining (Figure 8C). Apart from the local reconfigurations that cause in the ring complex of Y501, fitted within a reservoir is in between ACE2 receptor of Y41 and K353, the entire shape at the attaching part is essentially same as unmediated form (Figure 8D) (Figure 8E). A perpendicular y-shaped "-" stacking contact is formed by Y501 on the spikes and Y41 into ACE2 receptor. (Zhu X, 2021)

We used biolayer interferometer to assess the binding characteristics involving enzyme and can either unmediated or N501Y ectodomain in order to determine if the N501Y mutation makes the Covid-19 spike more capable to bind with ACE2 (BLI). This showed that the dissolution rate constant was mostly responsible for the N501Y mutation's small increase in affinity for ACE2 (koff). Through preincubation before cell infection, it also evaluated the effectiveness of originating externally supplied mixed ACE2-mFc to eliminate undulated and N501Y pseudoviruses. N501Y mutant's lower IC50 for neutralization is evident from the study of neutralization profiles, which suggests that the total spikes with N501Y transformation have a stronger affinity for ACE2-mFc. These three findings, when combined with previous

publications, support the idea that the N501Y mutant's increased infectivity is caused by better ACE2 binding. It is likely that new mutations that improve viral fitness will develop as SARS-CoV-2 continues to spread. Thus, a crucial weapon might be added to the toolbox of attempts to prevent and cure COVID-19 by using cryo-EM techniques to quickly correlate the imprints of the antibodies created by current and next vaccine generations. (Zhu X, 2021)

5.4. SARS-CoV-2 omicron spike protein

5.4.1. SARS-CoV-2 omicron spike protein analysis

The newly found COVID-19 omicron strain poses additional risks to the world's economy and health. The omicron strain is more contagious and has milder symptoms than earlier strains. It also spreads more broadly and more readily infects persons who have received vaccinations. Most of the alterations with the membrane-anchored spike protein of SARS-CoV-2 protein occur as the virus progresses. The spike is the mechanism through which SARS-CoV-2 enters human cells, elicits human immune responses that are neutralizing, which is foundation of the existing mRNA vaccines of COVID. The three main receptor-bonded S1 subunits are placed over an S2 spike with a trimeric cell fusion in the clove-shaped homotrimer that makes up the mature virions' spike (also known as its prefusion structure) (Figure 9a). A human protease called furin also particularly cleaves a brief pattern at the S1/S2 border.S1 separates as a result of both furin cleavage and ACE2 binding. Then S2 experiences a significant structural shift that joins the host and viral membranes. (Ye G, 2022)

The RBD domains of the spike proteins of several CoV variants have been described by numerous structural investigations (Table 2). A mutation of D614G at S1/S2 barrier with several changes within subsequent delta-strain enabled additional molecule-opening spike against the first Wuhan strain, despite some discrepancies resulting from variations in experimental techniques. However it is unknown how these changes affect the protein's structure or immune evasion. Approximately 100% of the spiked particles for such omicron spike were in the extended conformation, including one Polymorphism in the standing position and the other RBDs in the laying position (Figure 9b). It was further shown that neither omicron was found in the closed-conformation by doing more concentrated 3D classification on all was in the open-conformation. In contrast, 49.7% of the spike particles in the prototypic spike were within the conformation rely on a 3.0 map which was closed, and other spike particles (50.3%) were located conformation which was open. These cryo-EM data were used to design and enhance the structural parts for the omicron active spike, the prototype exposed spike, and the prototypical sealed spike (Figure 9c; Figure 9d; Figure 9e). Therefore, many omicrons spiked fragments than the conventional spike are in the open configuration. (Ye G, 2022)

5.4.2. Comparisons between omicron open spike and prototypic open spike

Author’s examined that molecule's structure packing to comprehend the structural foundation for the omicron open spike. S1 adopts a Trans-packing approach, similar to other corona viruses of the β-genus, within the RBD packs conversely with NTD of that other isoforms. The standing-up RBD exhibits relatively modest densities because of its mobility. Therefore, we

determined the buried interface within the 3 S1 sections and its surrounding S2 isoforms as well as around the respective two RBDs that are lying and its neighboring NTD.

Prototypic open spike is therefore substantially loosely packed than the omicron spike. Either the NTD or SD1 of the omicron spike simultaneously moved in direction of the nearby S1 subunit as related to the prototypic spike, according to structural covering of these two spikes (Figure 10a). Due to these domain shifts, the lying-down RBDs in the omicron open spike each pack more closely with their adjoining NTD (Figure 10b). Additionally, a somewhat disordered loop in the prototypical open spike's S2 (loop 853, as detailed below) structurally orders in the spike of omicron, managing connections between two isoforms (Figure 10c). Overall, compared with the prototypic spike, the omicron open spike clusters more tightly. (Ye G, 2022)

5.5. Cryo-EM in vaccine development from inactivated SARS-CoV-2

The continuing worldwide pandemic of corona virus illness (COVID-19) on 2019 is the outcome of the SARS-CoV-2 epidemic. Numerous attempts are now underway to create COVID-19 vaccines and therapies as quickly as possible. Because current vaccine candidates employ inactivated SARS-CoV-2 viruses, understanding the structure of inactivated SARS-CoV-2 is critical. This was also possible by the Cryo-EM method. (Liu C, 2020)

One of the most prevalent vaccination techniques is the use of chemically inactivated microorganisms. However, the method may be not uniformly applicable or all the viruses and may be potentially devastating implications in the absence of a chemical and structural

knowledge of the antigen. Some studies showed that we must acknowledge the differences between mitigating and non-mitigating antibodies produced by vaccinations, as well as the challenge of eliminating antibody-dependent enhancement (Killi-kelly, 2016) (McLellan, 2013)

β-propiolactone is a agent that has been applied successfully in rabies or in other vaccinations. It showed that Covid-19 viruses can be treated with β-propiolactone have the majority of its spikes in the postfusion configuration. Although β-propiolactone may elicit this conformation shift, the influence of purification and concentration techniques cannot be ruled out. Because the S protein has predominant disclose protein on the upper side of the virus particle, most Covid vaccine candidates depend on its antigen. Twenty-one COVID-19 vaccine formulations were being tested in clinical trials before until July 7, 2020, and four of them used inactivated viruses *(WHO, 2020a*). Three of the four contenders employed β-propiolactone as an inactivation reagent (Chen H, 2020). PiCoVacc, one candidate was similarly recovered and attenuated in this experiment, and not surprisingly, it likewise showed significant postfusion spikes, despite the false assumption of a prefusion condition (Gao, 2020). As a result, structural investigations, for all these vaccine candidates, the findings about the viral spike in presence in whole virus particles are crucial, especially when the antigen's antigenicity is not indicative of the triggering of protective immunity. (Liu C, 2020)

Chapter 6

Future Aspects of Cryo-electron microscopy method

The arrangement of several previously intractable macromolecules is currently being resolved employing cryo-electron microscopy (cryo-EM).This is mostly due to the fact that it requires minimal amino-acid and can scan this as isolated particles that might build high-rezone particle theory. Indeed, arrangements have lately acquired besides the requirement for substantial cathartic from lysates, and the ability to visualize or identify molecules by imaging is a bonus. It will be feasible to acquire even better pictures and structures with improved specimen preparing procedures, next detectors, and up to date representation processing software. SP cryo-EM has been and also sustained to become a common approach, and for this time needed from gene to structure is sure to shrink in the next years. (Vinothkumar, 2021)

In situ structural characterization is a legitimate evolution of portraying using EM. Large molecules can currently be investigated at extremely high resolution in the living cell environment that is expected for substantially become more vigorous in the following time. Portraying limited big molecules or proteins assemblies within original context will be a future challenge (Vinothkumar, 2021). These proteins' compositions are frequently diverse. Integration of several methods, including as optical and E-microscopy, spectrometry, and genomics, might aid in achieving these aim.

According to (Wang H, 2015), Cryo-EM has recently become an essential method in bio-living structural research, thanks to considerable technological advances in both hardware equipment and software algorithms. The technological elements of cryo-EM determine its distinct benefits

and future development path. Cryo-EM has benefited from highly multidisciplinary research efforts as a fast expanding discipline.

Chapter 7

Conclusion

As cryo-EM method is being employed for obtaining information about SARS-CoV-2 at the nano scale, so it is important to exploring the method of E-microscopy in the analysis of these viruses. In future, one key objective of constitutional virology to fill the knowledge gap between resolution features of the viral proteins or the crucial molecular exchange that determine congregation in an entire, contagious virus together with RNA. These strains of integrated systemic investigations, together with natural assessments of viral replication procedure, will be critical for determining the Molecular pathways that SARS-CoV-2 operates by penetrating cells and causes the catastrophic consequences on living health. Recognizing the potential effect of this method, conduct to the establishment of several nationwide and regional institutes in various nations to support the development of the cryo-EM industry. As these capacities remain important, so we are witnessing the expansion in cryo-sector, which has being spurred by growing accessibility of sophisticated equipment in particular institutions. For this pattern to sustain, the expenditure of cryo-EM apparatus required for sophisticated-resolution structure identification must be greatly reduced. So, finally the determination of the shape of spike protein of the COVID-19 virus by Cryo-electron microscopy method is fascinating and should be investigated further.

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Table 1: Frequencies of Seven Symptoms from Various Clinical Datasets.

Datasets used for 7 symptom stochastic progression model					
	COVID-19 with N=55,924[1]	COVID-19 with N=1,099[2]	Influenza with N=2,470[3]	MERS with N=245[4]	SERS with N=357[4]
Fever	0.879	0.887	0.680	0.841	0.997
Cough	0.677	0.678	0.930	0.633	0.583
Sore throat	0.139	0.139	0.840	0.135	0.17
Headache	0.136	0.136	0.910	0.188	0.389
Myalgia*	0.148	0.149	0.940	0.400	0.591
Nausea/Vomiting	0.050	0.050	0.010**	0.151	0.154
Diarrhea	0.037	0.038	0.010**	0.204	0.174

*For the two COVID-19 datasets myalgia is reported as myalgia or arthralgia. We assume most of these cases have myalgia and use it as the frequency for myalgia.

**Although adult patients at times may experience vomiting and diarrhea when infected with influenza, these symptoms are rare. Therefore we approximate the frequency of these symptoms as 0.010 in this case.

Table 2: Summary of the RBD conformations of SARS-CoV-2 spikes.

Spike	Reference	Cleaved	Buffer	Closed	1-RBD up	2-RBD up	3-RBD up	Intermediate RBD
Omicron	This study	Yes	20mM Tris pH7.4, 200mM NaCl, 8mM CHAPSO		~100%			
Wuhan	1	Yes	20mM Tris pH8.0, 150mM NaCl, 0.1% OG	34%	27%			39%
		No		83%	17%			
Prototype (Wuhan + D614G)	This study	Yes	Same as omicron	49.7%	50.3%			
	2	No	10nm colloidal gold in PBS	31%	55%	14%		
	3	No	25mM NaHEPES pH7.4, 150mM NaCl	5%	36%	39%	20%	
	4	No	2mM Tris pH8.0, 200mM NaCl, 0.02% NaN3	56%	44%			
		Yes		69%	31%			
Gamma	5	No	2mM Tris pH8.0, 200mM NaCl, 0.02% NaN3, 0.5% glycerol	15%	85%			

Alpha	6	Yes	25mM Tris pH7.5, 150mM NaCl, 0.02%DDM	16%	81%	3%		
Beta				24%	76%			
Delta	7	Yes		31%	69%			
Kappa				54%	46%			
Gamma					100%			

Figure 1: The progression in China of COVID-19 infections and fatal cases. Infections with COVID-19 begin to surface in December 2019.As of the day this paper was created on February 16, 2020, 51,174 people had contracted the virus in China, and more than 1666 people had died as a result. (Rothan, 2020)

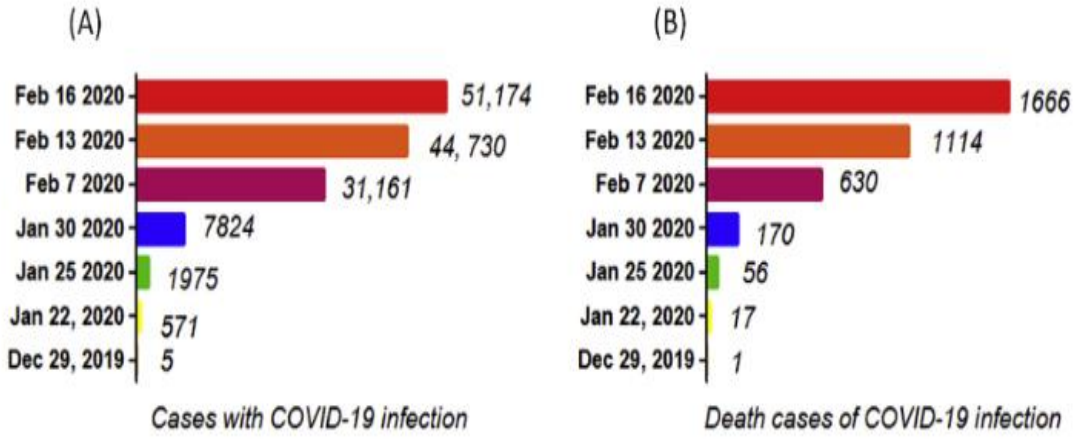

Figure 1: The progression in China of COVID-19 infections and fatal cases.

Figure 2: Symptoms of Covid-19

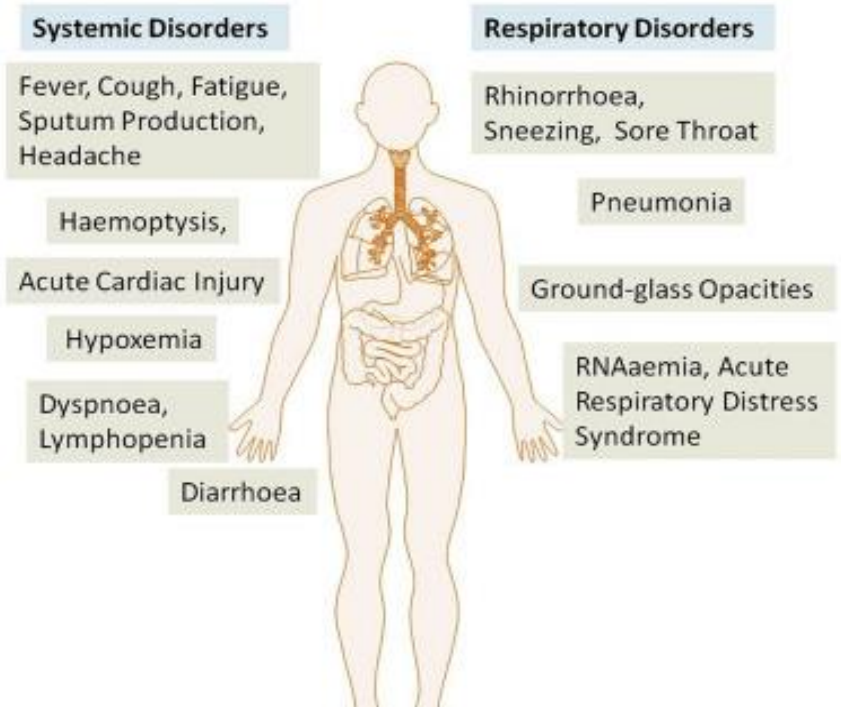

Figure 2: Symptoms of Covid-19

Figure 2 depicts the systemic and respiratory problems brought on by COVID-19 infection. It typically takes the COVID-19 infection 5.2 days to incubate. Early beta corona viruses and COVID-19 both have symptoms that are frequently comparable. But COVID-19 had certain unique clinical traits, such as the concentrating of the bottom airway, which was shown by upper respiratory tract symptoms as rhinitis, sneezing, and dry mouth. Additionally, those with COVID-19 had intestinal indications such dysentery; in contrasts, only a tiny percentage of people with MERS-CoV or SARS-CoV had diarrhea. (Rothan, 2020)

Figure 3: The three structural biology techniques have a similar structure percentage. Structures identified by X-ray crystallography are shown in blue, those identified by nuclear magnetic resonance are shown in dark orange, and those identified by electron microscopy are shown in gray. (Benjin X, 2020)

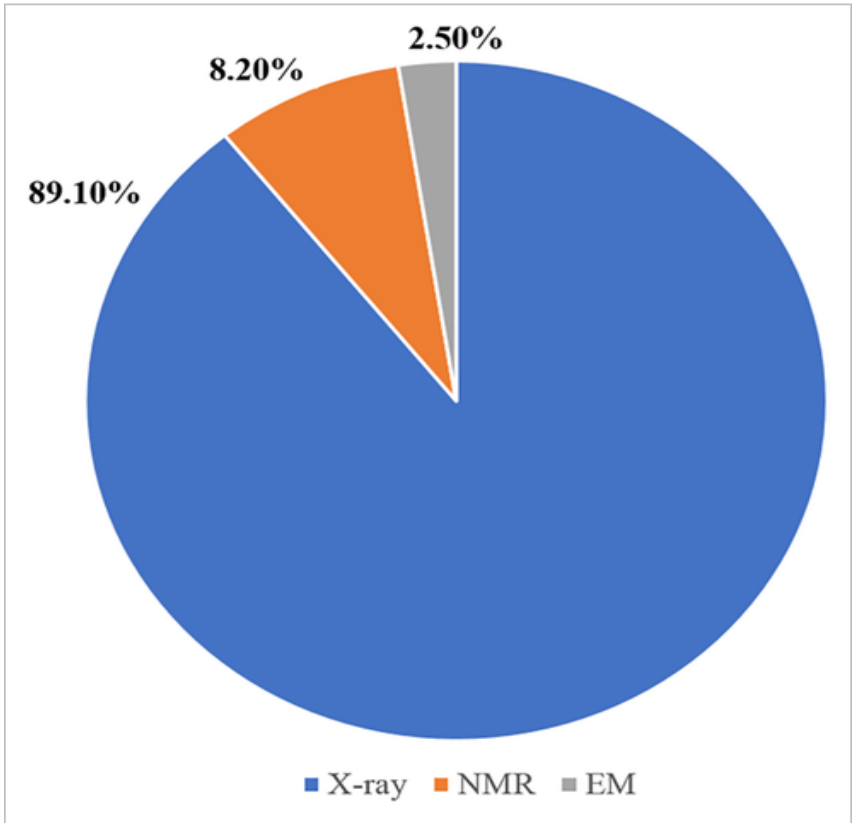

Figure 3: Three structural biology techniques have a similar structure percentage.

Figure 4: High-resolution information recovery and motion correction, Average of rotavirus particle frames before to translational alignment (A) and following it (B).Before alignment, features are blurred. (Carroni M, 2016)

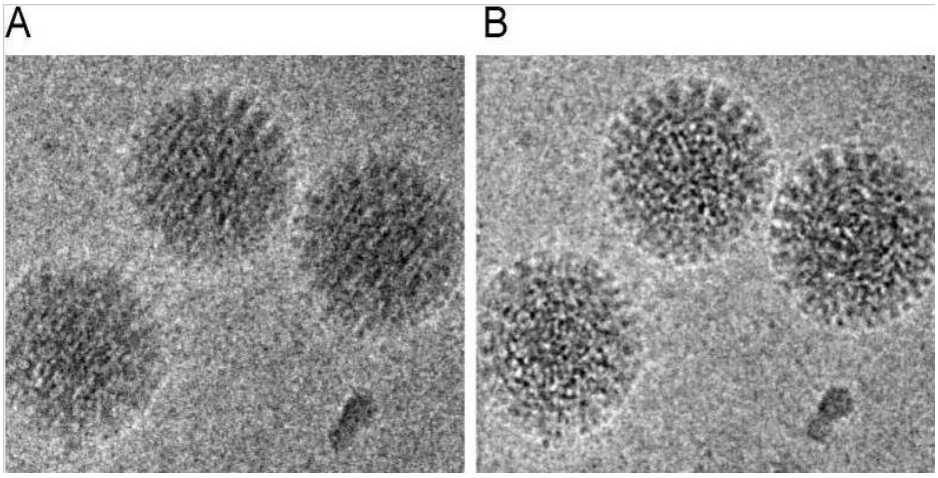

Figure 4: High-resolution information recovery and motion correction

Figure 5: The prefusion and postfusion trimeric spike structures for SARS-CoV-2. PyMOL software is used to depict the structures in the colored (by chained) surfaces.

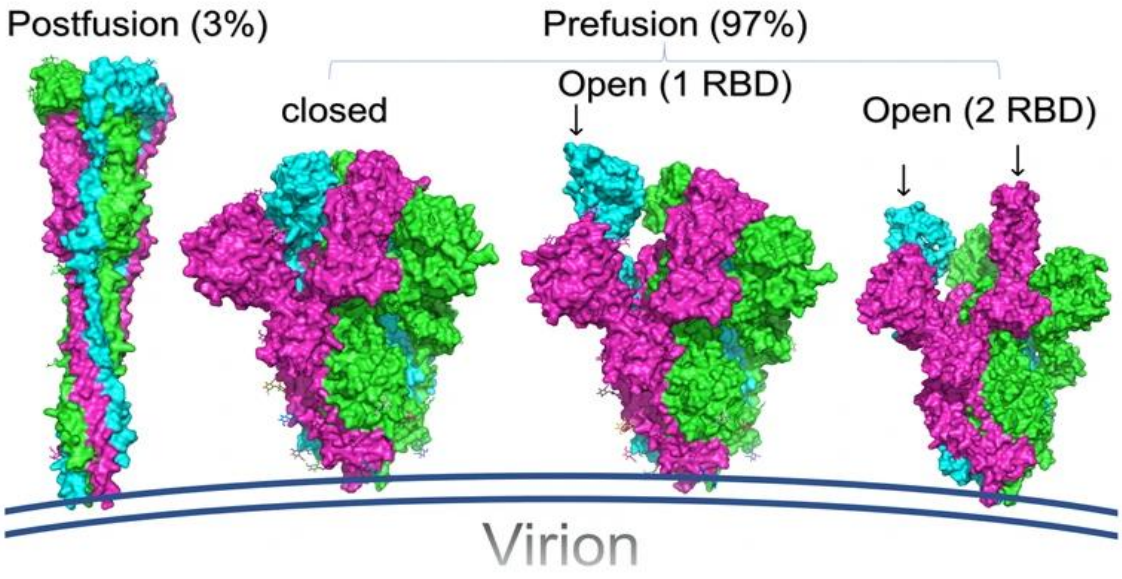

Figure 5: The prefusion and postfusion trimeric spike structures for SARS-CoV-2.

The open RBD in the prefusion phase is indicated by little arrows. Downloaded from the PDB with the following codes: 6M3W, 6VXX, 6VYB, and 6X2B, these are the configurations for the postfusion, closed prefusion, open 1 RBD prefusion, and open 2 RBD prefusion respectively. (Ismail A, 2020)

Figure 6: 2019-nCoV S's structure in the prefusion conformation is shown in Figure 6.

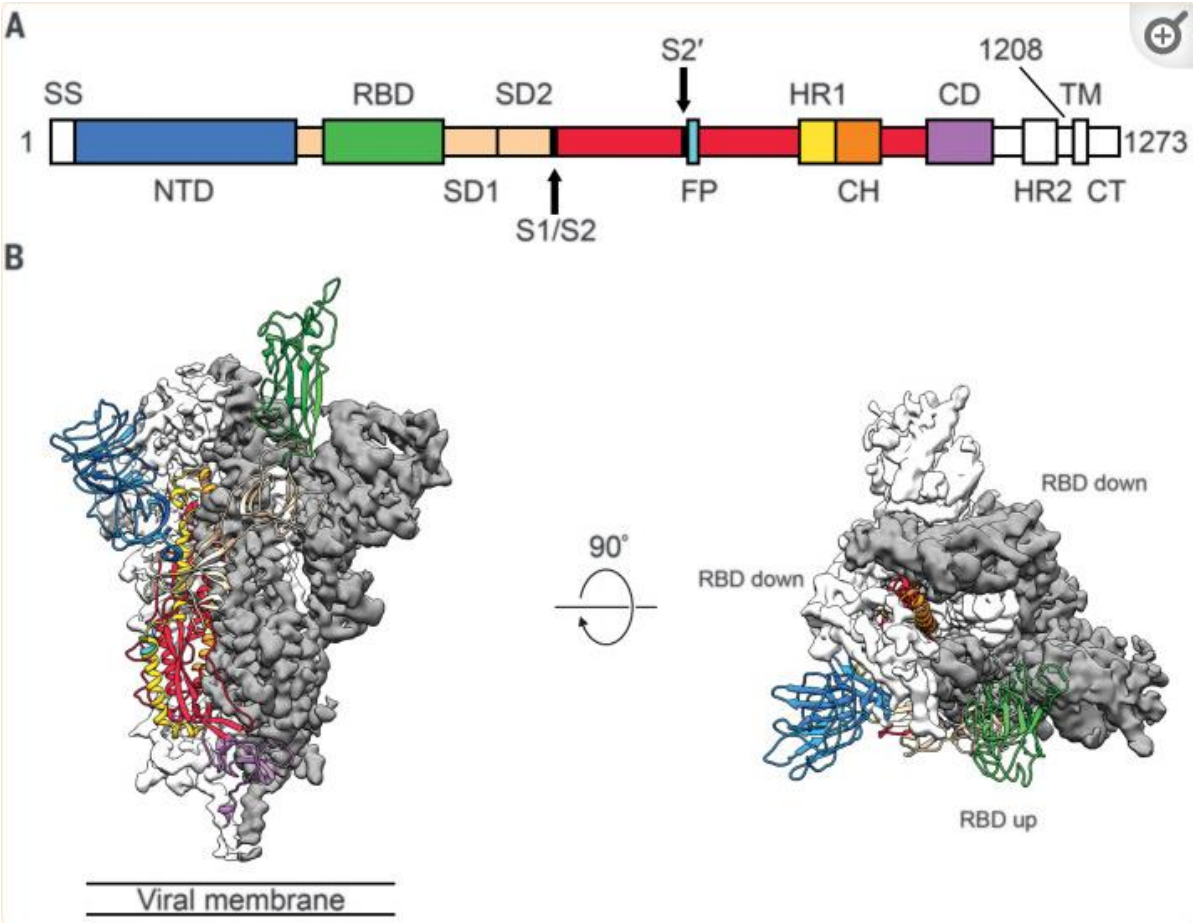

Figure 6: 2019-nCoV S's structure in the prefusion conformation.

2019-nCoVS primary structural schematic (A), colored by domain. White is used to indicate domains that were either left out of the ectodomain express construct or could not be seen on the final map. FP, fusion peptide; SS, signal sequence; S2′, S2′protease cleavage site; CH, central helix; CD, connector domain; HR2, heptad repeat 2; TM, transmembrane domain; CT, cytoplasm tail. Protein cleavage sites are shown by arrows. (B) Side and top views of the 2019-nCoVS protein with a single RBD in the up conformation's prefusion structure. The RBD up

protomer is depicted in ribbons colored in accordance with the design, and the two RBD down protomers are displayed as cryo-EM density in either white or gray (A). (Wrapp D, 2019)

Figure 7: Comparison between the structure of 2019-nCoV S and SARS-CoV S.

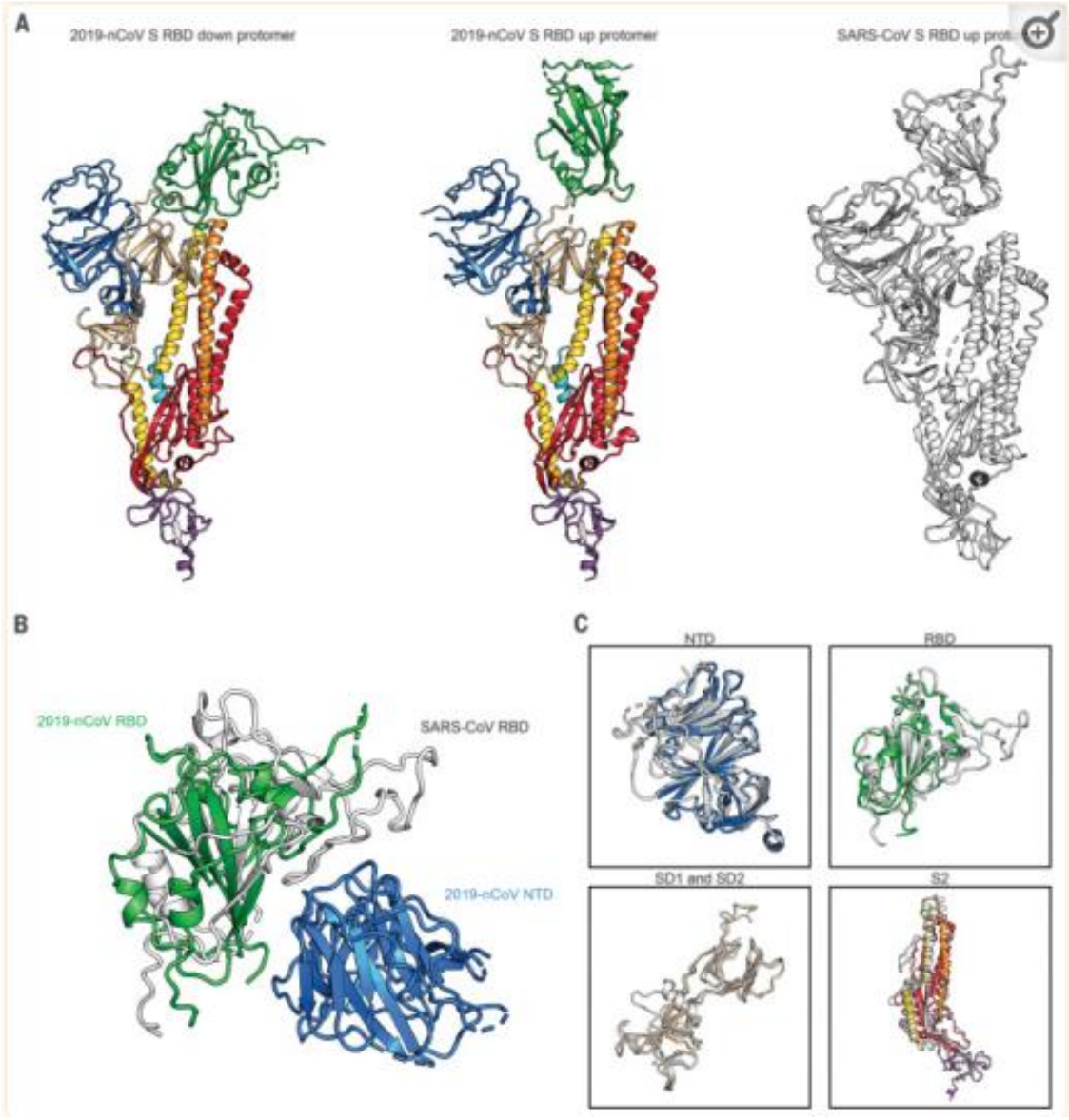

Figure 7: Comparison between the structure of 2019-nCoV S and SARS-CoV S.

(A) A single protomer of 2019-nCoV S is depicted in ribbons colored in accordance with Fig. 7 and has the RBD in the down conformation (left). 2019-nCoV protomer in RBD up

conformation (center) and SARS-CoV protomer in RBD up conformation (right), both depicted as ribbons and colored white (PDB ID: 6CRZ). (B) Based on the location of the adjoining NTD from the nearby protomer, the RBDs of the 2019-nCoV and SARS-CoV were aligned. The SARS-CoV RBD is white, while the 2019-nCoV RBD is green. The color of the 2019-nCoV NTD is blue. (C) The 2019-nCoV S structural domains have been matched with their SARS-CoV S equivalents as follows: NTD (top left), RBD (top right), SD1 and SD2 (bottom left), and S2 (bottom right) (bottom right). (Wrapp D, 2019)

Figure 8: Structure of the ACE2 ectodomain coupled to the SARS-CoV-2 N501Y mutant spike protein.

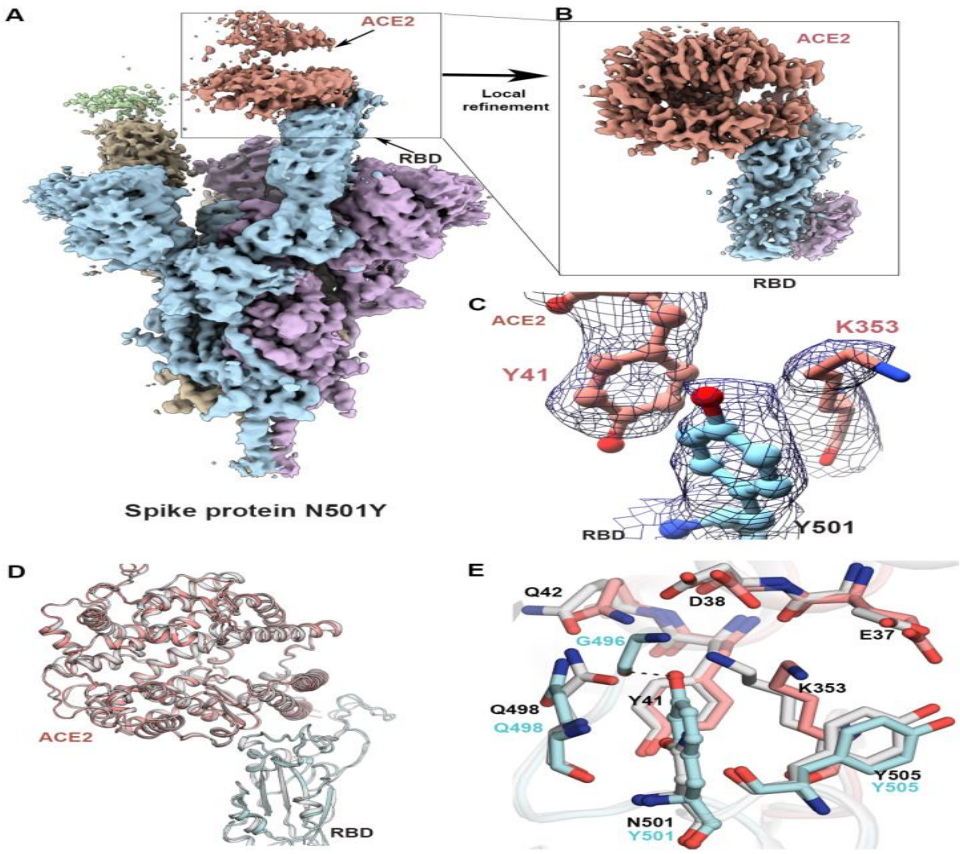

Figure 8: Structure of the ACE2 ectodomain coupled to the SARS-CoV-2 N501Y

(A) The complex's overall density map following the completion of universal structure refinement. The three spike protein protomers are colored cyan, purple, and yellow, and the densities of the ACE2 proteins that are highly and weakly bound, respectively, are shown in pale red and green.

The contact zone between the tightly bound ACE2 protein ectodomain and the receptor binding domain (RBD) is shown in (B) an improved density map. (C) Visualization of the density at the contact zone for residues Y41 and K353 in ACE2 and Y501 in the RBD.(D).Ribbon diagram showing the superposition of the N501Y RBD-ACE2 complex with the unaltered complex (PDB ID 7KMB).(E)Zoomed-in picture of the interface displaying a combination of the N501Y mutated spike protein and wild-type spike protein structures in conjunction with ACE2.Our structure's carbon atoms are labeled cyan and pale red for residues in the N501Y mutant and ACE2, respectively, while light gray for carbon atoms in the structure of the compound between unmutated spike protein and ACE2. (Zhu X, 2021)

Figure 9: Omicron and prototype spike protein general architectures.

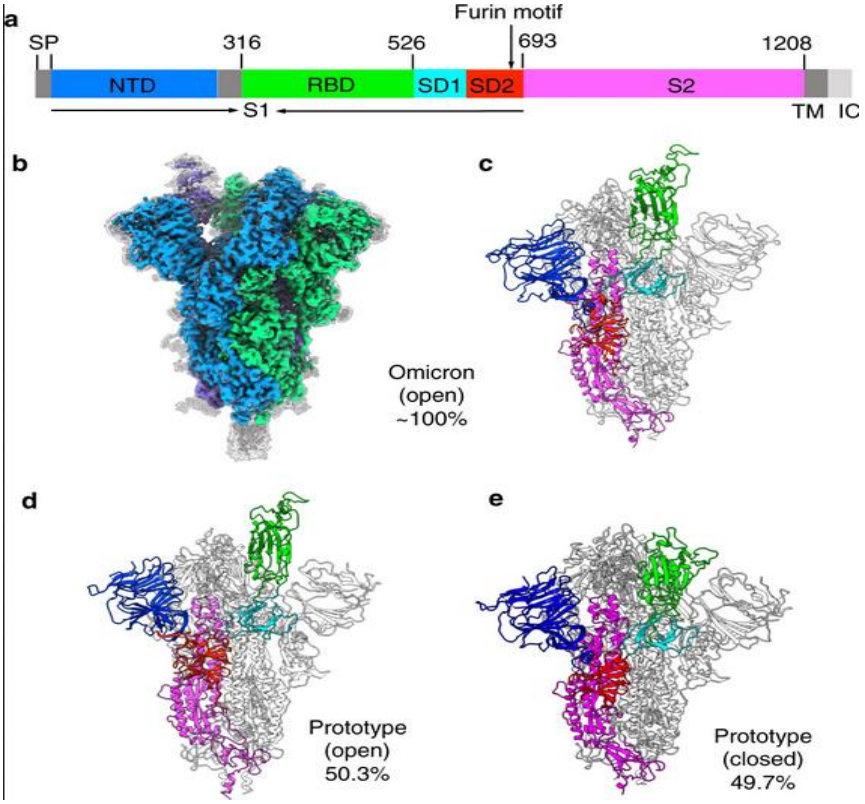

Figure 9: Omicron and prototype spike protein general architectures.

a. Schematic illustration of an omicron spike in its entirety. Signal peptide SP, transmembrane anchor TM, NTD N-terminal domain, RBD receptor-binding domain, SD1 subdomain 1, SD2 subdomain 2, and intracellular tail IC. The arrow points to the location of the Furin cleavage. **b**. Superimposition of sharpened and unsharpened cryo-EM maps for the omicron spike ectodomain.

The gray mesh displays the unsharpened map. Three distinct colors, one for each of the spike components, are displayed on the sharpened map. **c**. Omicron spike ectodomain structural model in the open conformation. **d.** Structural representation showing the open conformation of the prototypical spike ectodomain. **e.** Prototypical spike ectodomain structural model in closed shape.Two out of the three spike subunits are colored in gray for the structural models, while the third spike subunit is colored according to its domains: NTD is blue, RBD is green, SD1 is cyan, SD2 is red, and S2 is magenta. Based on the RBD conformation, population patterns for each spike ectodomain were shown as percentages. (Ye G, 2022)

Figure 10: Omicron open spike and prototypical open spike structural comparisons

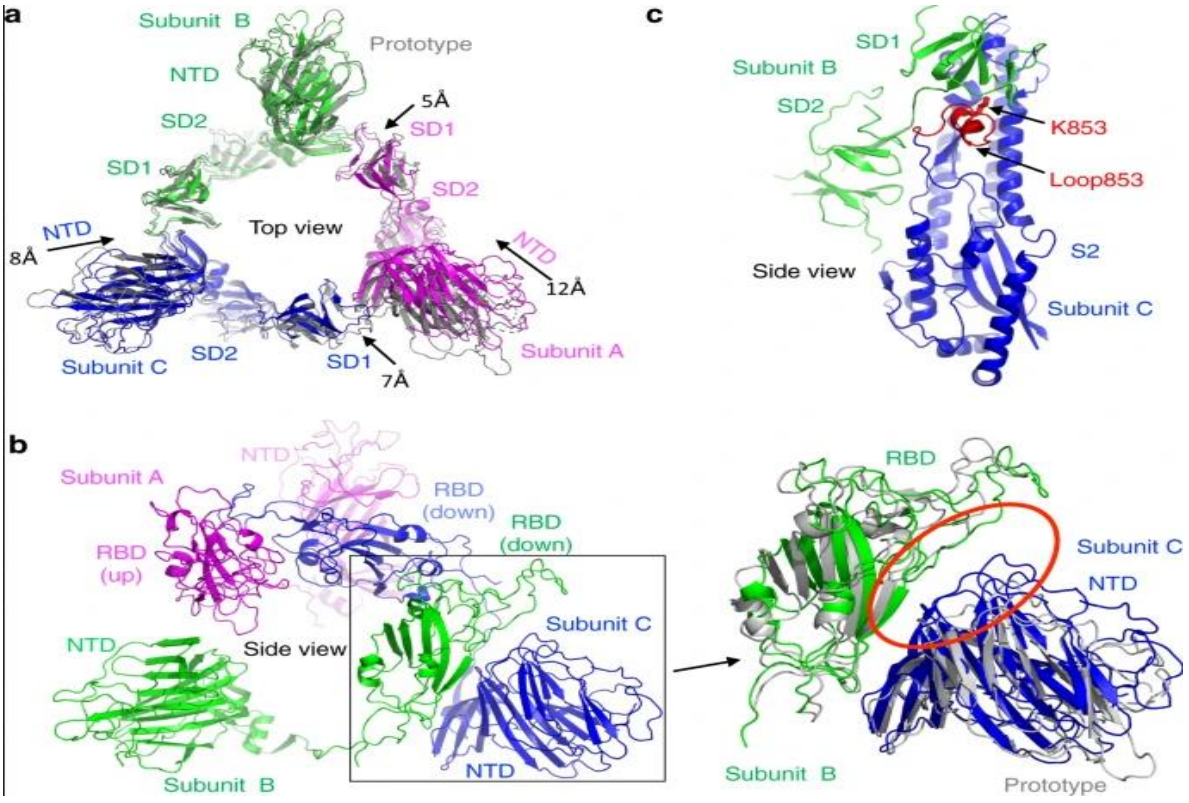

Figure 10: Omicron open spike and prototypical open spike structural comparisons

a. Top view morphological superimposition of the S1 subunits of the prototypical open spike and the omicron open spike (without the RBDs).The prototype spike is gray in hue. Different subunits contribute to the color of the omicron spike: subunit A includes the up-RBD and is colored magenta; subunits B and C contain the down-RBD and are colored, respectively, green and blue.

The domain changing of the omicron spike in relation to the prototypic spike is indicated by the arrows. **b.** Side view structural superimposition of the prototypical open spike and the omicron open spike's S1 subunit (without SD1 or SD2).The buildings have the same coloring as in panel.**(a).**The red circle indicates the location of the contact between the NTD from subunit C as well as the RBD from subunit B. The RBD/NTD interfaces are bigger in the omicron open spike compared to the prototypic open spike due to domain shifting, as illustrated in panel **(a). c.** The side view of the S1/S2 structural contact in the omicron open spike. The building is colored similarly to how it appears in panel **(a).**Lys853 (which varies from the equivalent residue in the prototypical spike) and Loop 853 (an S2 loop containing residues 831–854) are both highlighted in red. In the prototypic spike, loop 853 is somewhat disordered; however, in the omicron spike, it orders and facilitates S1/S2 connections. (Ye G, 2022)