Cryo-EM in COVID-19 drug invention and discovery

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

School of Pharmacy
Brac University
March 2022

Declaration

It is hereby declared that

1. The thesis submitted is my own original work while completing degree at Brac University.
2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The thesis does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution.
4. I have acknowledged all main sources of help.

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Approval

The thesis/project titled “Cryo-EM in COVID-19 drug invention and discovery” submitted by Rabeya Rahman Trisha (17346017) of Summer, 2017 has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Bachelor of Pharmacy on March 12, 2022.

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Ethics Statement

No animal or human trials have been conducted in this study.

Abstract

Cryo-EM is a type of electron microscope that employs the cryomicroscopy technology, which involves cooling materials and examining them under a microscope to create 3D models of proteins. Throughout this COVID-19 pandemic, the development of medicines are critical, and the protein structures of the SARS-CoV-2 virus must be recognized in order to battle COVID-19. Cryo-EM may be used to find protein structural alignments, protein positioning, domain-motif interaction with known antiviral medications, and potential therapeutic routes for creating treatments and vaccines. Furthermore, because Cryo-EM allows scientists to examine dynamic models of proteins, it makes it simpler for them to analyze molecules of various shapes and dimensions, which may be a significant benefit over X-ray crystallography. The use of Cryo-EM in medication development for COVID-19 including how future drugs can be designed to counter COVID-19 are discussed in this review paper so that the pandemic can be brought to a close.

Keywords: Drug Discovery; Cryo-EM; SARS-CoV-2; COVID-19; Drug-Protein interactions.

Dedication

This article is dedicated to my family and my supervisor for always standing beside me.

Acknowledgement

To start off, I would like to thank Almighty Allah for giving me opportunity to be a part of Brac University and providing me such providence for doing this article. Secondly, I feel fortunate that I am supervised by Professor Dr. Hasina Yasmin (Deputy Chair, School of Pharmacy of Brac University) as her being so humble and consistent helping hand for finishing this article. Thirdly, I would like to thank Professor Dr. Eva Rahman Kabir (Dean, School of Pharmacy, Brac University) for giving me opportunity to work with Dr. Hasina Yasmin. Moreover, I would heartily like to thank all my faculty members for being part of my Brac University journey and teaching me some valuable life lessons that would really guide me in my future. I would also like to thank my batch mates, juniors and seniors always for being helpful in my academics. Finally, I would like to thank my family and well-wishers for always being supportive to me.

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List of Acronyms

SARS-CoV-2	Severe Acute Respiratory Syndrome Coronovirus 2
COVID-19	Coronavirus Disease of 2019
Cryo-EM	Cryogenic electron microscope
WHO	World Health Organization
Nsp	Non-structural proteins
RdRp	RNA-dependent RNA polymerase
NiRAN	Nidovirus RdRp associated nucleotidyl transferase domain
vRNA	Viral RNA

Chapter 1

Introduction

1.1 Cryo-EM

Cryogenic electron microscope (Cryo-EM) is a 20th century's 3D electron microscope which has been a unique tool in the field of structural biology that is applicable for achievable resolution of complex macromolecules and for the study of dynamic and challenging biological systems (Figure-1). It serves benefits over recent analytical methods for example X-ray crystallography, NMR by determining structural ensembles of dynamic macromolecules. Since crystallization is not necessary, little amount of samples are being used for analysis and images can be produced using computerized software, these criteria make Cryo-EM constitutively and constructively valuable over other analytical methods for structural biology investigation. Due to their richness in biological insight, Cryo-EM can be used to determine the dynamic and functional macromolecules complexes such as virus complex detection especially in this COVID-19 situation. For several years, Cryo-EM was confined to massive complexes or low resolution models for determining the structure of biological macromolecules. The resolution of the Cryo-EM is starting to compete with X-ray crystallography with the recent progress in electron detection and image processing. New image-processing techniques compensate for sample movement and categorize pictures according to distinct structural states, while the new technology for electron detection records images with remarkable quality (Fujiyoshi, 2013). These developments, when combined, produce density maps with enough precision to determine the atomic structure of variety of specimens. Simultaneously, these developments are reimaging a single particle analysis of Cryo-EM determining the structure. The Cryo-EM single particle is a strong alternative to widely apply over X-ray crystallography in both method and image software of recent technology advancements, the obtainable resolution, output and

capability of Cryo-EM to categorize configurations and conformational mixes have greatly increased.

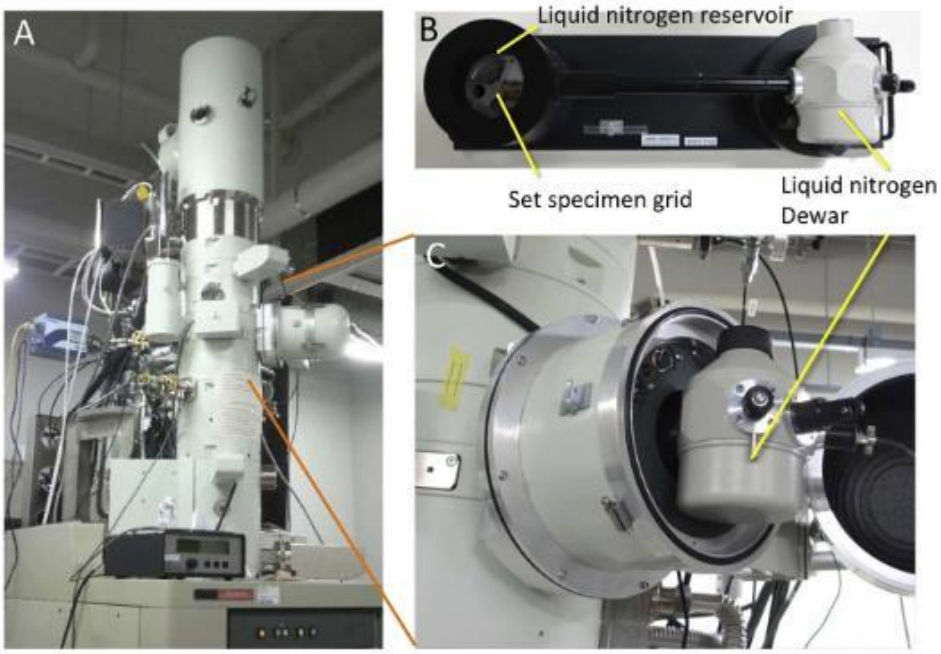


Figure 1. Representation of Cryo-EM equipment. (Murata & Wolf, 2018)

1.2 Use of Cryo-EM in Drug discovery

New era's Cryo-EM has been drastically enhanced the resolution performance by not only adding direct electron detectors and automated image collector software but also combined this technology with advanced image analysis system. Because of the high resolution performance of Cryo-EM, it has make it possible to access high resolution biological structures in which worth mentioning molecules larger than 500 kDa which was difficult to solve with some conventional methods namely X-ray crystallography, NMR. Additionally, Cryo-EM is also able to capture accurate pathways for cellular physiological process that would be essential in molecular biology. Thus, Cryo-EM also helps in gaining target proteins that might be useful in developing new therapeutics. Additionally, with the recent combination of Cryo-EM and image analysis, more information can be gathered about the macromolecule complexes which are conformationally variable (del Valle & Axel Innis, 2020). Due to the development of Cryo-EM, it was possible to achieve the configuration of several drug-protein interactions which help in determining therapeutic targets and develop therapeutic strategies. To mention, Cryo-EM revealed significant drug-protein interactions such as Emetine and *P.falciparum'*s ribosome (Figure-2), Glibenclamide and Potassium channel, Benzodiazepines and GABA receptor, Flecainide and Sodium channel respectively. Additionally, with the help of Cryo-EM, drug molecules such as Linezolid, Bictegravir also being resolved where the nature of drugs, their binding modes and sites are revealed that might help in designing future therapeutic strategies. Moreover, Cryo-EM plays a vital role in resolving protein structures by which drug binding sites, drug-protein interactions, nature of protein molecules can be determined. By using Cryo-EM, proteins such as TRP channels, AMPA receptor, Ryanodine receptor (Figure-3) and CFTR (The Cystic Fibrosis Transmembrane Conductance Regulator) has been revealed. These proteins have significant role in human body and Cryo-EM structure of these proteins

help to know more about their functions and nature which will aid in developing targeted therapies.

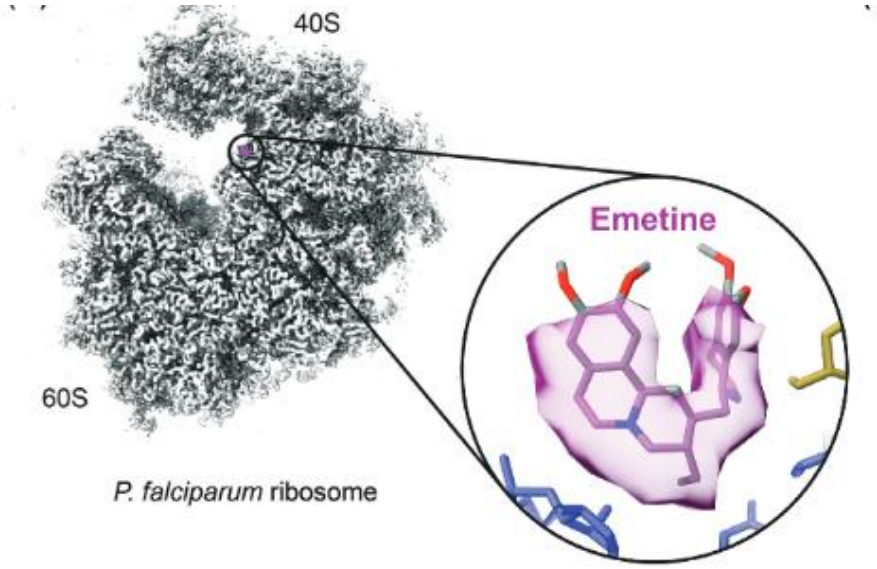


Figure 2. Interaction between drug Emetine and P.falciparum ribosome. (del Valle & Axel Innis, 2020)

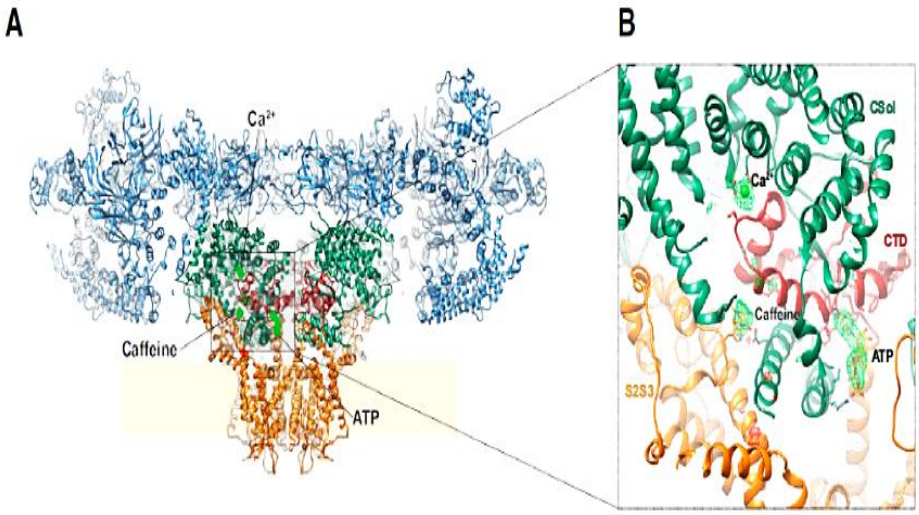


Figure 3. Representation of Cryo-EM structure showing binding modes of caffeine with Ryanodine receptors. (des Georges et al., 2016)

1.3 COVID-19

The WHO pronounced COVID-19 a pandemic in March 2020 due to SARS-CoV-2 virus, since it causes extremely contagious illness that has wreaked havoc on worldwide health care infrastructures (Faidon Brotzakis & Vendruscolo, 2021). As a RNA virus, coronaviruses are tend to go under frequent mutation and that's occur new variants. In recent S-proteins mutation, scientists gathered evidence about new Delta and omicron variant as well as their mechanisms (Haque & Pant, 2022). Omicron variant is more spreadable than any other previous variants, thus causing stress in healthcare system. These new Delta and omicron variants have great impact on SARS-CoV-2 virus's transmissibility as well as in host's immunity (Harvey et al., 2021; Lauring & Malani, 2021). Vaccines are now lowering the death rate however, according to researchers, with the introduction of super variant, these vaccines won't work upon virus and redesign of vaccines are needed (Haque & Pant, 2022). COVID-19 does not presently have any conventional treatment. However, by functions through several drugs, which would include antiviral agents such as Remdesivir, Favipiravir, and Ribavirin; interferons, protease inhibitors including Lopinavir/ritonavir; antibiotics like as Teicoplanin and other glycopeptides, anti-inflammatory agents, and monoclonal or polyclonal antibodies, have indeed been evaluated to ensure their efficacy in overcoming this pandemic (Vabret et al., 2020).

1.3.1 Treatment options of COVID-19

COVID-19 does not presently have any conventional treatment. However, by functions through several drugs, which would include antiviral agents such as Remdesivir, Favipiravir, and Ribavirin; interferons, protease inhibitors including Lopinavir/ritonavir; antibiotics like as Teicoplanin and other glycopeptides, anti-inflammatory agents, and monoclonal or polyclonal antibodies, have indeed been evaluated to ensure their efficacy in overcoming this pandemic (Vabret et al., 2020). Remdesivir targets RdRp of SARS-CoV-2 of COVID-19 which is one of the anti-hepatitis C virus drug same as ribavirin. It is a prodrug of 1′-cyano-substituted

adenosine nucleotide analog (Jiang et al., 2021). It works by competing with nucleotide counterpart's incorporation resulting in inhibition of viral RNA like other nucleotide analog inhibitors (Figure-4). In spite of having limitation in the efficiency of clinical treatment, it's being used in treatment of COVID-19 as RdRP inhibitor. Ribavirin belongs to guanosine analogue which has been shown antiviral activity against broad range of DNA and RNA viruses. Clinically, it has been used in mostly chronic and life threatening viral infections mentioning chronic hepatitis C infection, respiratory syncytial viral infection (Nyström et al., 2019). Despite of not showing satisfactory outcomes by this combination, these are recommended in Chinese guidelines as standard treatment option for COVID-19 (McCreary et al., 2020). Lopinavir is HIV-1 protease inhibitor which is used with combination of Ritonavir in a fixed dose, where ritonavir is a CYP3A4 inhibitor to boost the concentration of Lopinavir. This combination has shown activities in SARS and MERS, it's been recommended in COVID-19 treatment (Ratia et al., 2008). Additionally, according to article when used together, these agents give synergistic effect (Chu et al., 2004). Corticosteroids, which are potent immunomodulatory drugs used as a treatment for severe SARS-CoV-2 infections works by the mechanism of preventing or attenuating the hyper-inflammation effects caused by the virus. Anti-inflammatory effects are exerted by glucocorticoids as they stimulates the proteins which are responsible for the synthesis and release of anti-inflammatory and thus inhibit the pro-inflammatory proteins (Annane, 2021). Antimalarial agents such as Chloroquine and Hydroxychloroquine have also been suggested as possible SARS-CoV-2 inhibitors (Wang et al., 2020). Monoclonal antibodies are a type of therapy that is used for both diagnostic and therapeutic applications. Tocilizumab, Bevacizumab, in addition to Meplazumab are among the monoclonal antibodies being tested for treating SARS-CoV-2 infections (Pooladanda et al., 2020).

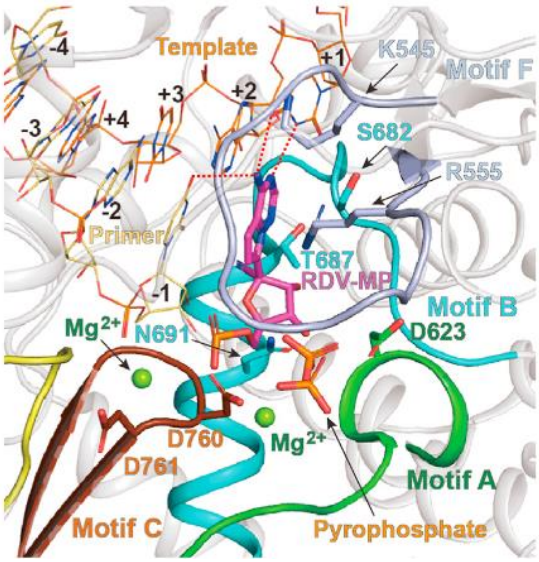


Figure 4. Representation of RdRp-RNA-Remdesivir complex by Cryo-EM. (Yin et al., 2020)

1.3.2 Challenges of COVID-19

With such advent of COVID-19 outbreak, experts all around the world are looking for possibly effective medicines to combat emerging coronaviruses. However, there are several challenges in the development of coronavirus medicines, limiting their development and applicability. To begin with, coronaviruses are RNA viruses with a wide range of configurations, which is why new varieties of coronaviruses with unique structures emerge often. The medications that have been used in the past may not have been effective or have very little impact against this new form of coronavirus. Second, many medications in the clinic have high EC50/C_{max} ratios and are prone to significant adverse effects. Furthermore, the coronavirus-caused severe epidemic exhibits features of precision. There is no clinical data to suggest that broad-spectrum antiviral medicines are effective against coronavirus because of the poor specificity of study and investigation. Vaccination remains the most efficient method of combating the infection. In comparison to the lengthy process of medicine development, the vaccination is reasonably

quick to create and has a powerful preventive effect. Monoclonal antibodies give excellent effects, higher specificity, and greater safety, but they take longer and cost more to produce (Han et al., 2020).

1.4 Rationale of the study

The rationale of this study is to focus on paving new ways for developing effective therapeutic strategies for COVID-19 as currently there is no potential therapeutics to combat COVID-19. Additionally, due to coronavirus's dynamic protein structures as well as frequent mutations its being an obstacle for the scientists to develop new drugs.

1.5 Objective of the study

In this review, the objective is to discover the role of Cryo-EM in observing high-resolution SARS-CoV-2 structural and non-structural protein, protein binding, drug-protein interactions to facilitate potential therapies such as vaccines, therapeutic antibodies in COVID-19 treatment.

Chapter 2

Methodology

This review paper has been conducted based on recent and relevant research papers and articles from peer reviewed journals. A comprehensive search has been performed through peer-reviewed journals, clinical trial reports and articles. To enrich the review paper, basic and additional information have been collected from different books. Following search engines have been used to collect data for this paper- ResearchGate, Science Direct, PubMed, Elsevier, etc. in which the major publications include- Nature, Journal of structural biology, Nature methods, bioRxiv: the preprint server for biology, science, Journal of virology, Signal Transduction and Targeted Therapy, Nature communications, eLife, Journal of Biological Chemistry, Cell Research etc. In depth screening of the journals followed by narrowing down to the most recent and relevant ones was done to create an ideal quality review on the potentialities of Cryo-EM in the context of COVID- 19.

Chapter 3

Cryo-EM Technique

3.1 Working Principle

Electron microscopes, instead of light microscopes, may dive approximately to 5,000 times closer into bio molecular frameworks. Cryo-EM, presents unerringly as the name suggests: the technique is carried out at extremely low temperatures, such as -200°C including using liquid ethane and propane. This remarkable approach has the capability to disclose a great deal more about matter's minute, hidden properties, advancing medicine, biotechnology, as well as fundamental knowledge of the cosmos (Zheng et al., 2017).

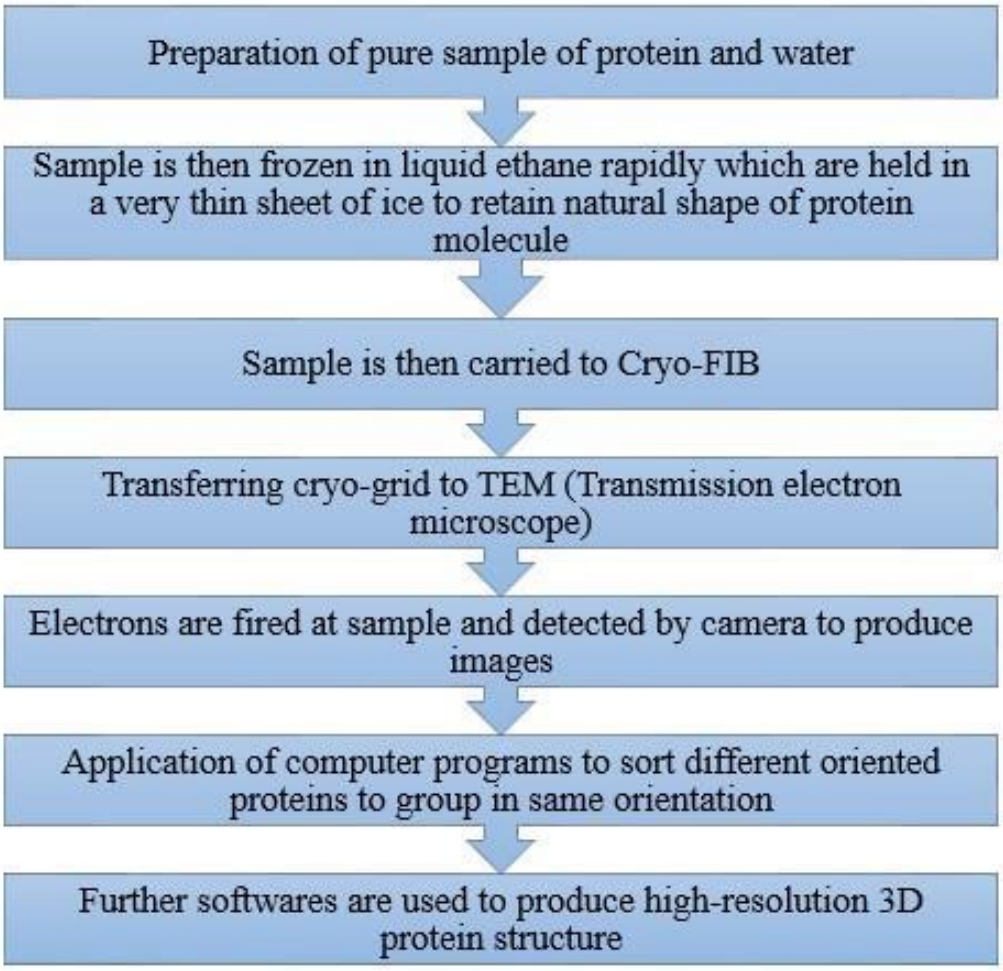


Figure 5. Working principle of Cryo-EM.

Step 1: Sample preparation by Vitrification

Standard culture protocols are used to grow cells on carbon-coated gold EM grid. Cells are flash-frozen instantly on the EM grid instead of colored to preserve the actual cellular environment. Cryogenic freezing freezes liquid water at such a fast pace that non-crystalline vitreous ice forms, avoiding the destruction done by crystal formation at delayed freezing rates. Vitification includes blotting, followed by plunging of samples where samples can be vitrified using two methods such as plunging method and Jetting method (Figure-6). This approach preserves the ultrastructure of the flash-frozen cell. Semi-automated freezing of samples is done with a Vitrobot System (Nogales & Scheres, 2015).

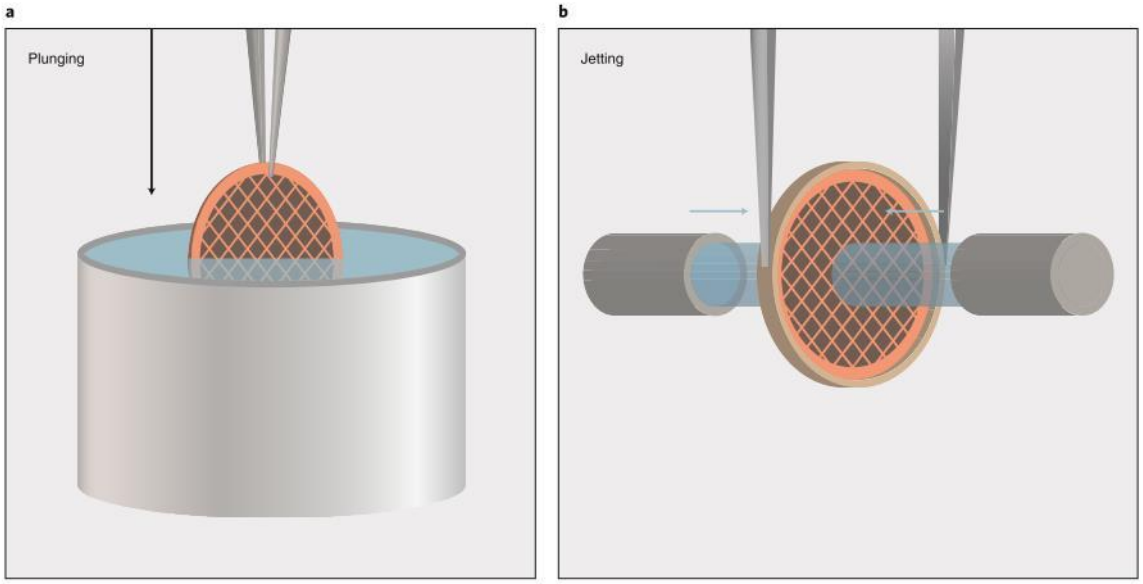


Figure 6. Vitrification method. (Weissenberger et al., 2021)

Step 2: Localization by fluorescence

Identifying the architecture of interest in the vast heterogeneity of the natural cellular environment can be difficult. Cryo-correlative microscopy is used to find the structures of

relevance in the Cryo-fluorescence light microscope, CLEM system. The sample is vitrified on a customized Cryo stage during Cryo-fluorescence imaging. The vitrified sample is carried to the Cryo-FIB (Cryo-focused ion beam electron microscope) along with the parameters of the target region for milling in a customized cartridge system that shields the sample from contamination. The Cryo-new FIB's optional iFLM Correlative System for Cryo-tomography combines light and electron microscopy into a single system, eliminating the need for extra sample transfer stages and enabling customers to design a streamlined Cryo-correlative solution for Cryo-tomography. By localizing fluorescent targets inside of the chamber, the iFLM (Integrated Fluorescence Light Microscope) Correlative System enables customers to examine the fluorescence signal inside milled lamellae and connect two imaging modalities simultaneously within one system (Lindert et al., 2012).

Step 3: Thinning by milling

After initial screening and targeting in the Cryo CLEM system, the sample is transported to the Cryo-FIB, a specialized Cryo-Dual Beam electron microscope, for thinning. A scanning electron beam (SEM) as well as a focused ion beam (FIB) are integrated inside the Cryo-FIB (FIB).The electron beam images the sample, whereas the ion beam ensures that material from vitrified cells is properly eliminated. Continuing correlative microscopy localization, material is removed across each target site using a concentrated ion beam, resulting in a thin, electron-transparent lamella. The Cryo-lamella, which may be carved as thin as 100–200 nanometers, contains the objects of interest. There seems to be no mechanical sectioning with the Cryo-FIB. Rather, the vitrified sample is flattened by sputtering, which involves scanning a focused stream of gallium ions over the frozen surface of the specimen, removing exterior atoms layer by layer (also referred to as ion beam milling). Sample thinning is crucial for tomography since

the electron beam in the TEM only can travel through materials thin enough to transmit 200–300 keV electrons. Cryo-FIB thinning is a straightforward and controlled process that avoids the intrinsic cutting distortions of mechanical sectioning at cryo-temperatures, as opposed to Cryo-ultramicrotomy (e.g. compression in the cutting direction) (Grassucci et al., 2007).

Step 4: Electron Cryo-Tomography

Following milling, thin Cryo-lamellas are transported to the Cryo-TEM (Cryo-transmission electron microscope), whereby tomographic images are captured. The images in the tomographic series are made by rotating the sample at specified intervals. In such a procedure termed as back projection, the 3D tomographic volume is formed by digitally merging separate projection photos (Lučić et al., 2013).

Step 5: Visualization and structural analysis

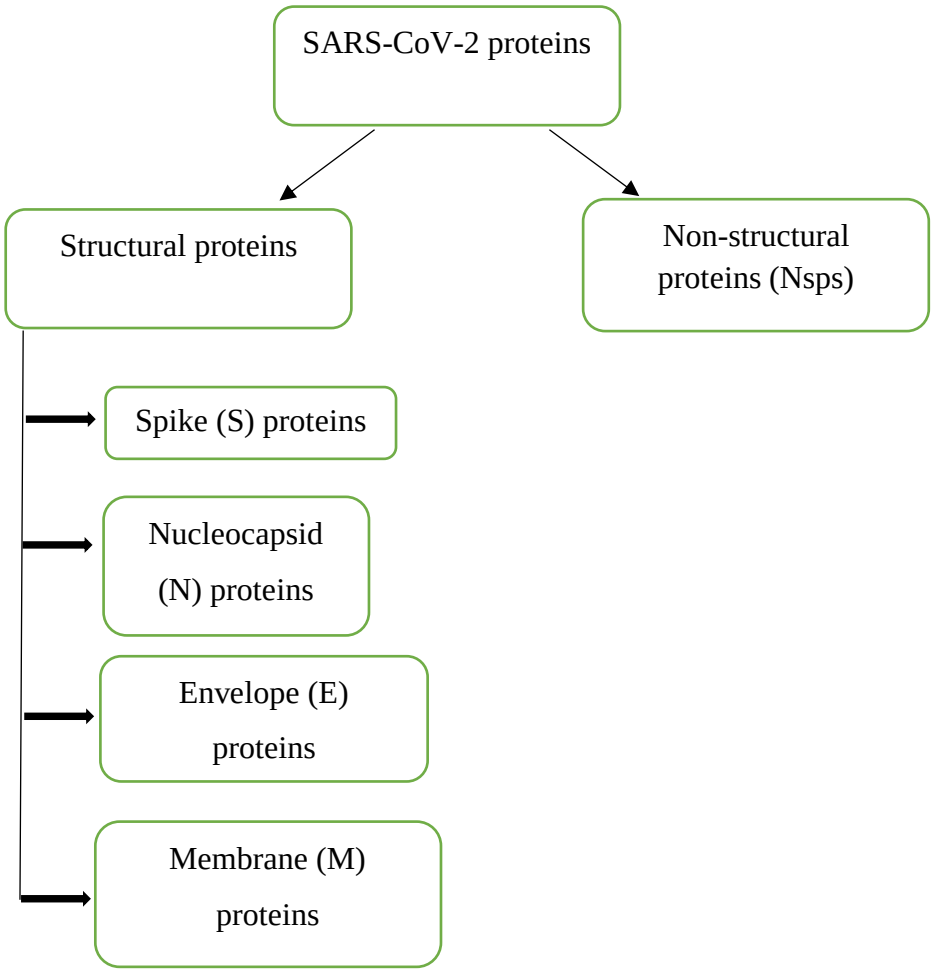
The 3D tomogram containing cellular structures may be split and tinted in a variety of ways to enhance its visualization and presentation utilizing Software, a worldwide 2D-5D platform for comprehensive visual analysis. Small amounts of data comprising the constructions of relevance can be extracted from the tomogram analytically and submitted to image-processing procedures. Image processing enables researchers to achieve nanometer-level resolution while preserving as much structural information as feasible (Bonomi et al., 2018).

Chapter 4

Cryo-EM in SARS-CoV-2 research

4.1 SARS-CoV-2 proteins

SARS-CoV-2 belongs to the coronaviridae family which has a large, enveloped, and positive (+) sense as well as single-stranded RNA (ssRNA) virus. SARS-CoV-2 proteins can be classified into two parts, one is structural proteins and another one is non-structural proteins (Nsps).



4.1.1 Structural proteins

Structural proteins consists of four parts include Spike (S) protein, Nucleocapsid (N) protein, Envelope (E) protein and Membrane (M) protein. SARS-CoV-2 genome is packed within a lipid bilayer aided by Nucleocapsid (N) proteins where a transmembrane envelop (E) protein as well as a corresponding membrane (M) protein are also incorporated. Spike (S) proteins are embedded in lipid bilayer which is unquestionably the most investigated component of SARS-CoV-2 (Fertig et al., 2022).

4.1.1.1 Spike (S) proteins

The trimer of S-protein are made with the association of the three of identical protomers that profoundly binds to human ACE2 receptor. 24 S-protein ectodomains on average irregularly decorate on each virions that gives them their typical "Crown-like" shape (Z et al., 2020). For each S-protein, they are 20nm long which are club-shaped along with comparatively wide head region that connect them with viral membrane via a thin stalk (Turoňová et al., 2020). Practically each protomer are composed with two regions include S1 and S2. S1 region contains N-terminal domain (NTD) along with the Receptor binding domain (RBD). On the other hand, the S2 region contains C-terminal domain (CTD) that shield the fusion protein (FP) which is accountable for the fusion with targeted cell membrane. Due to conformational changes occur in S-protein, they can easily invade in host cell by switching from pre-fusion to post-fusion state followed by the host cell proteases cleavage and S1 shedding. Along with the virions, SARS-Cov-2 is pleomorphic in structure which diameter may vary from 80-140nm (Liu et al., 2020). Due to the variations in shape and size of virions, it’s quite difficult to reconstruct the structure using a typical Cryo-EM image processing. However, digital image processing and reconstruction made it possible to reveal the near atomic structure of S-protein also with their fusion state. With Cryo-EM, scientists also highlighted on the immunogenicity of S-protein by revealing the existence of glycan shield which might be a major target for drug design (Walls

et al., 2020; Wrapp et al., 2020). These glycan shields not only help in hiding the epitopes of S-protein from host immune system detection but also take parts in folding and interactions with protease. Additionally, from these studies it also had been revealed that S-protein trimers can remain in two states. In one state, where all protomers remain in closed or incompletely closed conformation (addressed as "down" position of RBD) and thus hides the receptor-binding motifs (RBMs). On the other hand, where one to three RBDs remains open (addressed as "Up" RBD position) (Wrapp et al., 2020) (Figure-7). These structure helps to understand how SARS-CoV-2 binds with ACE2 receptors and thus paving the way for developing RBD-targeted therapeutics. In numerous studies, the co-express of TMPRSS2 and Furin with ACE2 has been revealed. These both proteases are needed for SARS-CoV-2's effective activation and pathogenicity (Hussain et al., 2020). Moreover, researchers are suggesting that conformational changes of S1and S2 could be mediated by Furin-dependent cleavage that results in exposure and binding of RBD and ACE2 as well as the disclosing of the cleavage site of TMPRSS2 (Hussain et al., 2020). However, molecular interaction details between Furin and S protein of SARS-CoV-2 are yet to be determined.

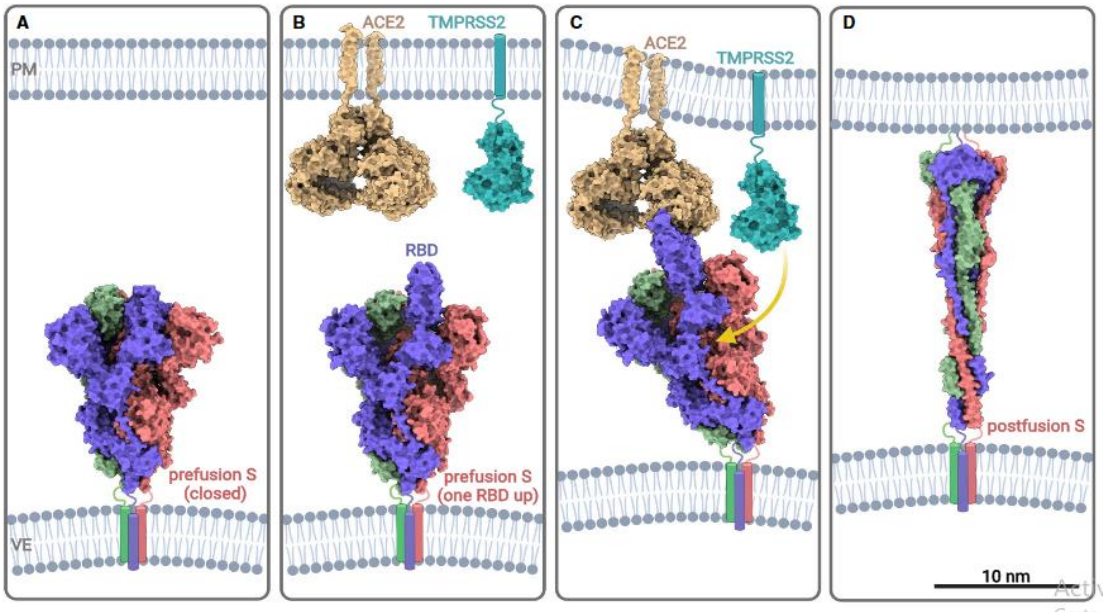


Figure 7. Cryo-EM structure showing S-proteins with their conformational changes. (Fertig et al., 2022)

4.1.1.2 Nucleocapsid (N) proteins, Envelope (E) proteins, Membrane (M) proteins

Even if there is success in retrieving the structure of S-proteins, it's more difficult to reconstruct the structure of N, M and E proteins using Cryo-EM. Reasons behind this could be portrait as such that studies about M proteins are held up due to the fact that they don't accumulate on visible matrix like other enveloped viruses for example influenza (Klein et al., 2020). On contrast, E-proteins are way too small such as ~8kDa while N-proteins are unorganized fundamentally (Mariano et al., 2020). However, coupling or infusing these proteins to molecular scaffolds might enlarge the field of Cryo-EM studies (Mariano et al., 2020).

4.1.2 Non-structural proteins (NSPs)

After infecting a cell, SARS-CoV-2 cascading intracellular functions occur including vRNA replication and structural proteins synthesis that will further contribute in assembling new virions. This whole process starts by encoding SARS-CoV-2 genome into two open reading frames (ORF1a and ORF1ab) that further encode a 16 non-structural proteins set (Nsps). Pp1a and Pp1ab are the initial translational products of ORF1a and ORF1ab which are later cleaved by viral and host proteases so that the discrete Nsps can be released for replication. Despite having numerous functions, these Nsps are less understood for example how these polyproteins perform proteolysis to form a replisome-like multi protein complex which takes part in vRNA's replication and transcription (Hartenian et al., 2020; V'kovski et al., 2020).

4.1.2.1 Nsp1

Functioning as a shutdown factor, Nsp1 binds with ribosome complexes mRNA entrance channel. A Cryo-EM construction of Nsp1 reveals that its two α-helices formed by C-terminal binds within 80s subunit's entrance channel (Figure-8). Such interactions allow Nsp1 to inhibit host mRNA translation (Schubert et al., 2020; Thoms et al., 2020; Yuan et al., 2020).

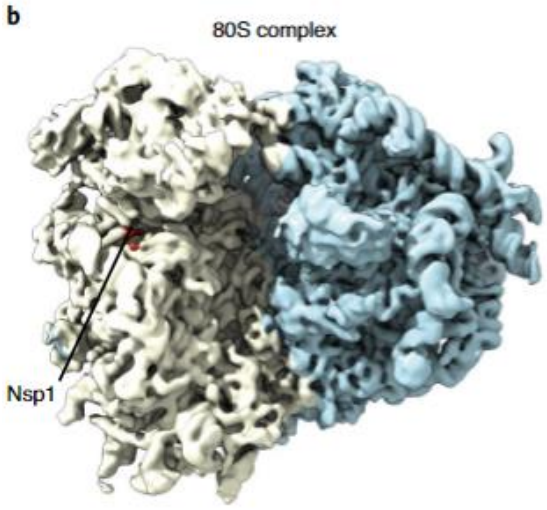


Figure 8. Cryo-EM structure of Nsp1 binding with ribosome (Schubert et al., 2020).

4.1.2.2 Nsp2

Nsp2 non-structural proteins are the less known among SARS-CoV-2 Nsps. The full structure is yet to be determined however, the Nsp2 NTD structure reveals that they contains three zinc finger domain which are ZnF1,ZnF2 as well as ZnF3. Still, the structure revelation of SARS-CoV-2 Nsp2 will help researchers to develop potential therapeutics for COVID-19 (Ma et al., 2021).

4.1.2.3 Papain-like protease (PL^{pro}) and Nsp3-Nsp4-Nsp6 complex

Nsp3 is considered as the largest protein in SARS-CoV-2. It composed of multiple domain among coronaviruses proposing its role as pleiotropic. Papain-like protease(PL^{pro}) domains present in Nsp3 can differ among coronavirus for example in cleavage site or target specificity (Lei et al., 2018). However similar to SARS-CoV and MERS-CoV, SARS-CoV-2 owns only one papain-like protease domain (PL^{pro}) which is accountable for cleavage activity (Rut et al., 2020). In addition to that, PL^{pro} is able to cleave poly-ubiquitin chains that restrains host response for inflammation and also weakens type I interferon response (Figure-9) (Clementz

et al., 2010). Particularly, Nsp3, 4, 6 forms complex that helps in adjusting the endoplasmic reticulum(ER) to incorporate within double membrane vesicles (DMVs). All these studies informs that Nsp3 CTD is abundant for DMVs formation and additionally Nsp4 is engaged with the formation of DMVs. However, accurate mechanism of Nsp3, 4, 6 complex is yet to determine. This complex take action for ER arrangements which further gather the RTC complex's remaining components (Cong et al., 2020). Since, there are still lot of domains remaining to be categorized, their relevance will help to understand the interactions better.

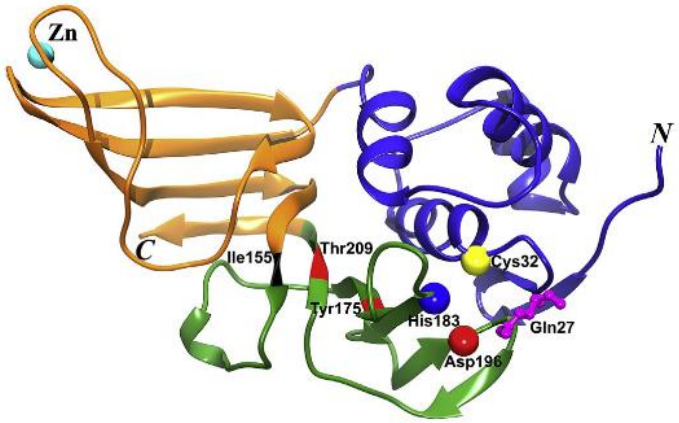


Figure 9. Cryo-EM structure revealing Nsp3 (PL^{pro}) (Hardenbrook & Zhang, 2022)

4.1.2.4 Nsp5 (Main protease, M^{pro})

One of the most fascinating drug target is M^{pro}. Main protease (M^{pro}) is Nsp5, along with Nsp3 (PL^{pro}) they're accountable for the cleavage of polypeptides that later release mature proteins. Mpro is a 3C-like protease that helps producing 16 Nsps by treating the M^{pro} specific sites of two SARS-CoV-2 polyproteins which are Pp1a and Pp1ab (Ullrich & Nitsche, 2020). Studying the crystal structure of M^{pro}, it was revealed that M^{pro} comprised of three domains (Figure-18)

includes a chymotrypsin-like domain I, Piconavirus 3C protease-like domain II and domain III (Zhang et al., 2020).

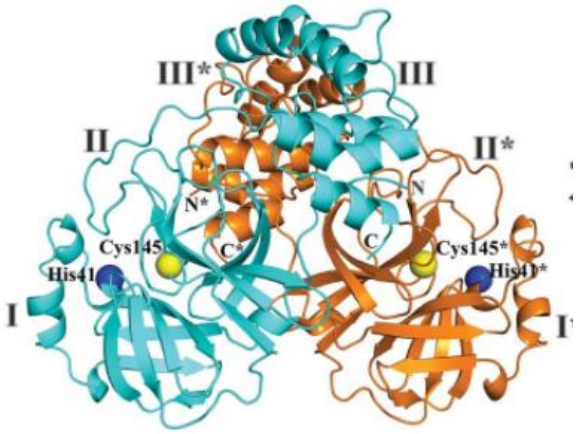


Figure 10. Cryo-EM structure showing M^{pro} with three domains (Zhang et al., 2020)

4.1.2.5 Replication-transcription complex (RTC) and RdRp

Replication-transcription complex (RTC) has a major role in causing infection that's why it became most prominent area for therapeutic development. The structure and functions of RTC are studied using Cryo-EM that reveals that RTC build up SARS-CoV-2's replication machinery using Nsp7,Nsp8,Nsp9,Nsp12 and Nsp13 (Figure-11) (Y. Gao et al., 2020; Hillen et al., 2020; Peng et al., 2020; Wang et al., 2020; Yin et al., 2020). Amongst them, Nsp12 is known as RNA dependent RNA polymerase (RdRp) got more priority as it is located at the very core of RTC that drives the synthesis of vRNA (Figure-12) (Y. Gao et al., 2020). Concisely, Nsp12 consists of an N-terminal extension domain (NiRAN) as well as the C-terminal RdRp domain. Nsp13, a helicase forms Nsp13-replication-transciption complex (Nsp13-RTC) by interacting with holo-RdRp:RNA complex which is necessary for replication and transcription (Y. Gao et al., 2020).

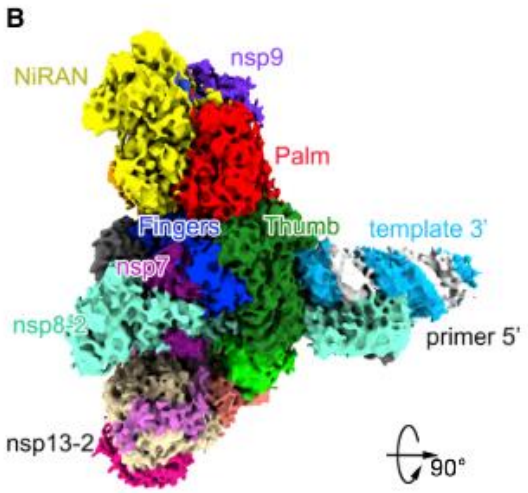


Figure 11. Representation of RTC by Cryo-EM (Hillen et al., 2020)

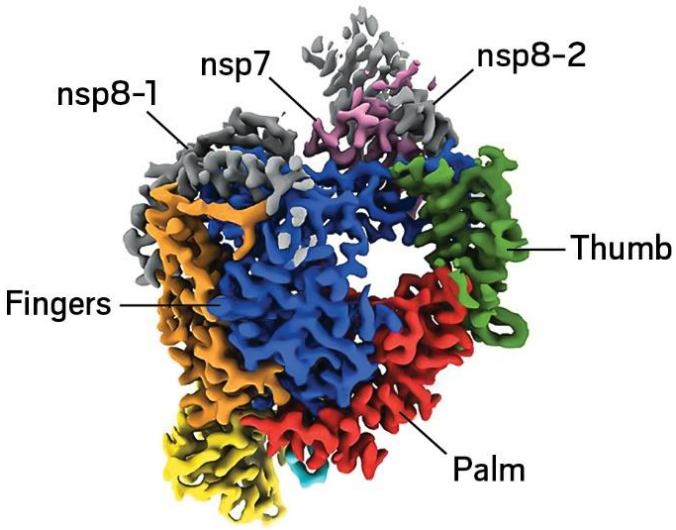


Figure 12. Cryo-EM structure showing RdRp of SARS-CoV-2 (Y. Gao et al.)

4.1.2.6 Nsp9, Nsp10, Nsp14

Dimerization of Nsp9 was seen in SARS-CoV that is essential for replication of the virus (Littler et al., 2020). However, Nsp9's role and protein interactions are yet to be determined. Acting as a cofactor, Nsp10 is needed for Nsp14 and Nsp16's stimulation (Rogstam et al., 2020). On the other side, Nsp14 acts as a bifunctional protein that composed of N-terminal exoribonuclease domain (ExoN) and a C-terminal domain guanine-NT-MTase associated with capping. Nsp9-Nsp12 facilitated the association of Nsp10 and 14 with Nsp13-RTC complex that forms cap(0)-RTC complex and thus reveals the proofreading activity of Nsp14 (Figure-13) (Yan et al., 2021).

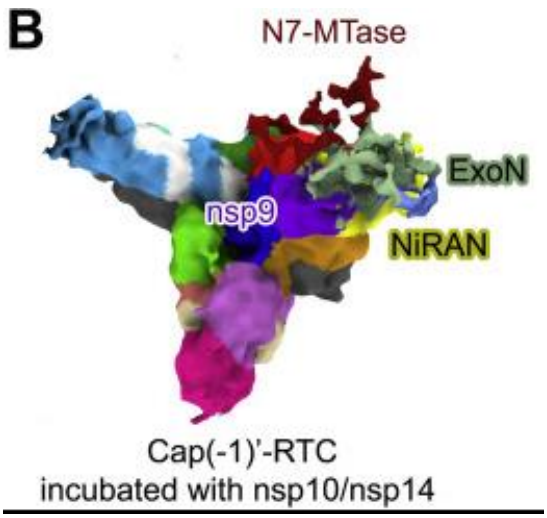


Figure 13. Cryo-EM structure revealing cap(0)-RTC complex of SARS-CoV-2 (Yan et al., 2021)

4.1.2.7 Nsp15

Nsp15 namely NendoU of SARS-Cov-2, with uridine specificity forms a hexameric endonuclease. Nsp15 comprises of three domains includes N-terminal oligomerization domain, middle domain as well as an endoU catalytic domain. Nsp15's Cryo-EM structure reveals that

endoU domain tends to be shaky without substrate and that results in poor local resolution (Figure-14) (Pillon et al., 2021).

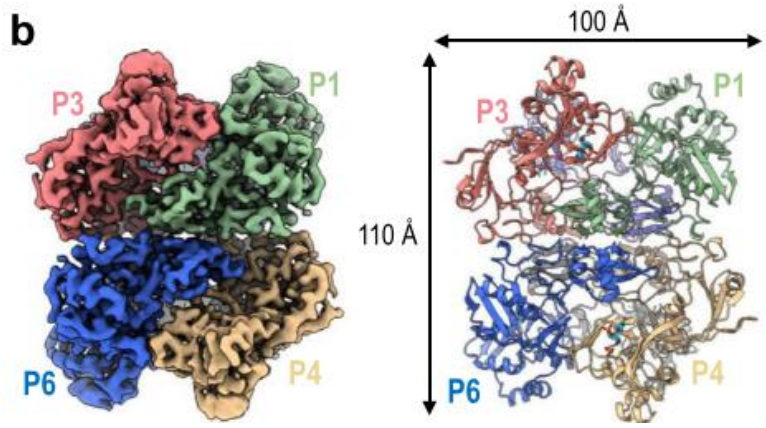


Figure 14. Structure of Nsp15 by Cryo-EM (Pillon et al., 2021)

4.1.2.8 Nsp16

Nsp16 of SARS-CoV-2 is S-adenosylmethionine-dependent methyl- transferase (SAM-MTase) which is necessary for the vRNA cap methylation. Nsp16 monomer remains on top of Nsp10 monomer that makes up the overall structure (Hardenbrook & Zhang, 2022). However, Cryo-EM construction is yet to be done.

4.1.2.9 Open reading frame accessory proteins

Accessory proteins of SARSCoV-2 are not that much well reconstructed and also most of their functions are not well understood yet. These proteins are highly variable even among close related coronaviruses. ORF3a of SARS-CoV-2 was resolved by Cryo-EM in the closed conformation of the virus and revealed the information that ORF3a adopts a novel fold that forms large and bifurcated channel to connect with cytoplasm (Figure-15) (Sorum et al., n.d.). Moreover, they form a dimeric or trimeric ion channel and take part in apoptosis. The structural information of ORF9a provides its mechanism that it interferes with host immune response type I interferon (X. Gao et al., 2021). However, some accessory proteins such as ORF3b, ORF7, ORF9a, and ORF10 are yet to be determined, whereas the mechanisms of ORF7a and ORF8 are yet to be known.

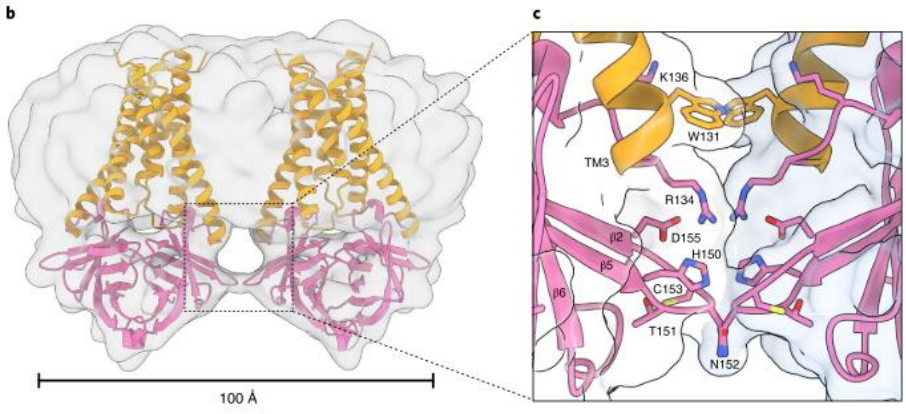


Figure 15. ORF3a ion channel resolved by Cryo-EM (X. Gao et al., 2021)

Chapter 5

Cryo-EM in COVID-19 drug discovery

5.1 COVID-19 drug discovery

SARS-CoV-2 as the novel coronavirus has been declared as global pandemic due to its effect on respiratory system causing acute respiratory distress syndrome (ADRS) (Jang et al., 2021). Still, there is no potential drug or vaccines that only target the SARS-CoV-2. Despite of having such condition, The FDA approved Remdesivir as a treatment option for COVID-19. But according to WHO and European Medical Association, Remdesivir alone isn't sufficient where there is also high mortality rate present using Remdesivir (Jang et al., 2021). So, to prepare for combating SARS-CoV-2 variants and other deadly pathogens, it's needed to be develop therapeutic drugs by founding strategies such as drug repurposing, molecular docking of drugs, structure based drug design. Here, some of the drugs are being reviewed such as Molnupiravir, Suramin, Paxlovid and Omipalisib which are discovered for using as a treatment of COVID-19.

5.1.1 Molnupiravir

Molnupiravir is orally existing antiviral medicine which is going under phase III trial (Kabinger et al., 2021). It is an RdRp inhibitor that interrupts the replication of SARS-CoV-2 in animals as well as humans. This drug causes mutagenesis of viral RdRp. The MOA of Molnupiravir is that the active form of Molnupiravir which is β-d-N4-hydroxycytidine (NHC) triphosphate, is used up as a substrate by RdRp instead of cytidine triphosphate or else uridine triphosphate. When this RNA is used by RdRp as template, that active form of Molnupiravir (NHC) incorporates G or A bases and that leads to the mutagenesis of viral RdRp (Figure-16) (Kabinger et al., 2021). Moreover, in structural investigation of mutagenesis containing RdRp-

RNA complexes, NHC forms constant bases pairs which results in proofreading escape and mutated RNA synthesis of this polymerase. Such mechanism mark Molnupiravir as a broad spectrum antiviral (Kabinger et al., 2021).

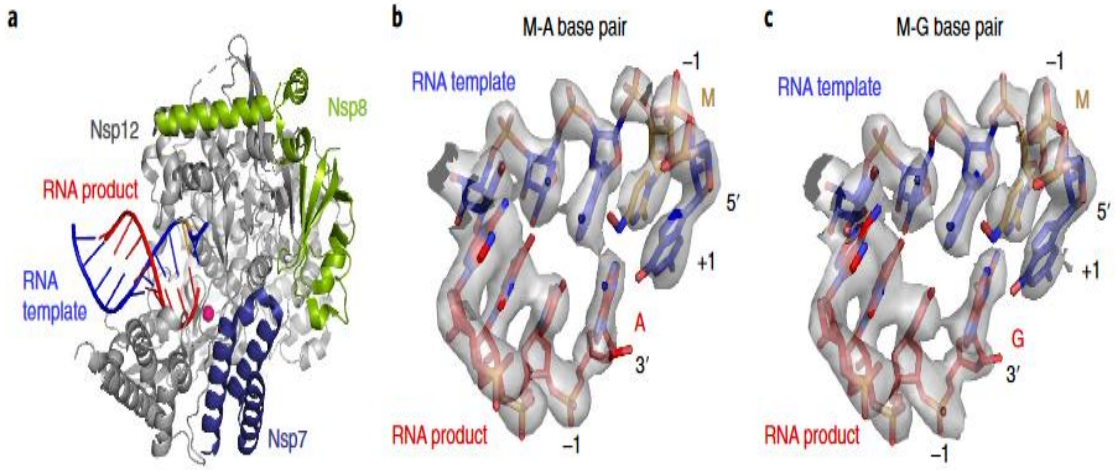


Figure 16. Cryo-EM structure showing NHC-induced mutagenesis in SARS-CoV-2 RdRp-RNA complex (Kabinger et al., 2021)

5.1.2 Suramin

Suramin, a non-nucleotide inhibitor form complex with RdRp of SARS-CoV-2 thus inhibit the polymerase activity (Yin et al., 2021). Two independent Suranim binding sites are harbors by the RdRp where one site binds together with RNA binding sites thus block binding of substrate. Another one binds with catalytic site and due to steric hindrance block RNA primer binding (Figure-17). Because of this binding pattern, potency of Suranim is at least 20-fold higher than Remdesivir's triphosphate form in the inhibition of polymerase activity in vitro (Wu et al., 2022).

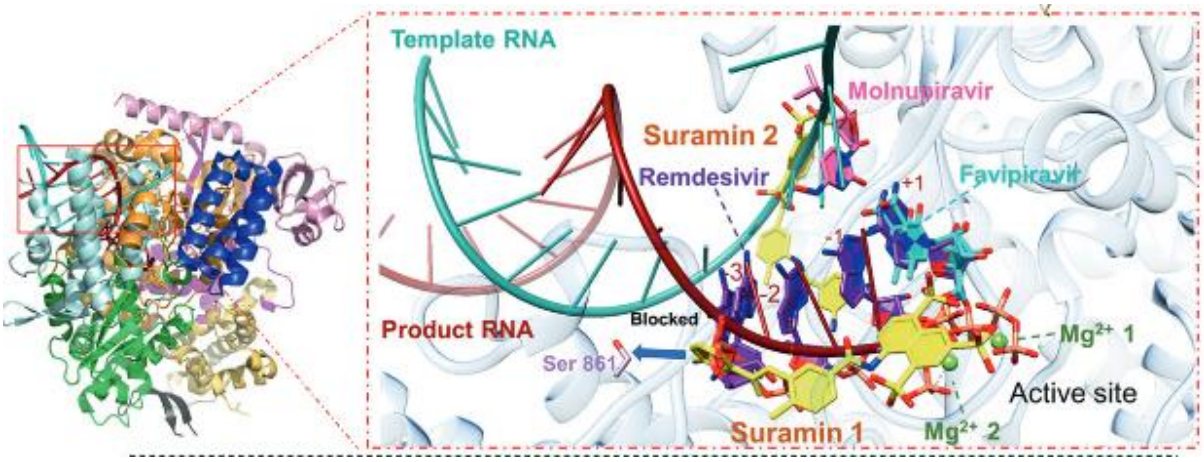


Figure 17. Cryo-EM structure revealing Suramin binding sites with SARS-CoV-2 RdRp (Wu et al., 2022)

5.1.3 Paxlovid

Pfizer has developed second generation SARS-CoV-2 M^{pro} inhibitor PF-07321332 which can be taken orally and this inhibitor was designed according to the structure of the complex of SARS-CoV-2 M^{pro} and inhibitor (Zhao et al., 2021). PF-07321332 when used with Ritonavir as a combination, showed positive result in phase III for COVID-19 treatment. This combination inhibit cytochrome enzymes thus maintaining PF-07321332's higher circulating concentrations. PF-07321332 also similar structurally to Boceprevir which is anti HCV drug (Fu et al., 2020). The physicochemical properties and affinity of PF-07321332 optimization from leads have been done speedily due to the structure based approach. Application was submitted to U.S FDA about PF-07321332 and Ritonavir (Paxlovid by brand name) combination for emergency COVID-19 treatment on November 16, 2021 (Zhao et al., 2021).

5.1.4 Omipalisib

Using computational screening, 38 compounds were found that endeavor antiviral activity along with cell cytotoxicity which target M^{pro} and RdRp. Among these, seven potential drugs has shown anti SARS-CoV-2 activities while testing on Vero cells as well as human lung

epithelial Calu 3-cells (Jang et al., 2021). One of the most potent inhibitor, Omipalisib also known as GSK2126458 has shown potential inhibition (Knight et al., 2010). Phase I clinical trial for Omipalisib has been done for solid tumors treatment as well as for idiopathic pulmonary fibrosis (IPF) (Lukey et al., 2019). As COVID-19 causes numerous respiratory disease including IPF, in that case Omipalisib can be beneficial in both antiviral and antifibrotic abilities of COVID-19 in patients (George et al., 2020). According to a study, Omipalisib exhibit different mechanism in humans for anti SARS-CoV-2 activity by inhibiting receptor signaling growth factor of host cell that remain activated as response of viral infection. Thus researchers suggesting that Omipalisib could be concurrently able to inhibit protein targets in both virus and host that leads Omipalisib having higher antiviral activity (Klann et al., 2020). The molecular docking of Omipalisib shows the interaction between M^{pro} and RdRp to understand the inhibition of viral proteins (Figure-18).

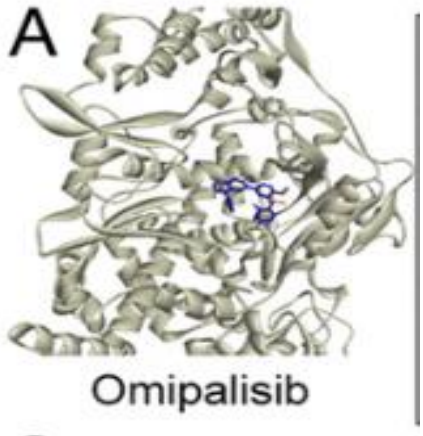


Figure 18. Molecular docking of Omipalisib showing interaction between M^{pro} and RdRp (Jang et al., 2021)

Chapter 6

Conclusion and Future prospect

The fast worldwide spread of covid-19, which caused the World Health Organization to declare a pandemic, underscores the critical need for coronavirus vaccines and treatments. Knowing the 2019-nCoV spike's atomic structure will enable for more protein engineering attempts to enhance antigenicity and protein expression for vaccine development. Cryo-EM structural data will also aid in the evaluation of SARS-CoV-2 spike mutations that will occur as the virus evolves, as well as determining if those residues have surface exposure and mapping of known antibody epitopes for other coronavirus spike proteins (Lyumkis, 2019; Nogales & Scheres, 2015; Walls et al., 2016). Productive anti–SARS-CoV-2 medicines are still unavailable, despite the fact that Remdesivir received full FDA approval in October 2020. (Schwartz et al., 2020). Using a variety of methodologies and tactics, the research establishment has made significant progress in discovering potential anti–COVID-19 drugs during the last year. Recent anti–SARS–CoV-2 drug discovery techniques generally focus on inhibiting viral replication by addressing M^{pro}, PL^{pro}, or RdRp, as well as disrupting S protein binding to ACE2 receptors. Researchers (Z.Wang et al., 2021) concentrated on all known Cryo-EM structures from the above targets in combination with newly discovered small-molecule inhibitors, comprising natural products, FDA-approved pharmaceuticals, and candidate medications, to enhance highly precise formulation and assessment of anti-SARS-CoV-2 therapies. On the other hand, structural analysis found that several non-covalent inhibitors (such as Favipiravir, Perampanel analogue 5) had no strong or long-lasting effects, indicating the need for more research into covalent inhibitors to decrease "off-target" dangers. In fact, combining Remdesivir with numerous small compounds have demonstrated strong additive/synergistic anti-SARS-CoV-2 effects in vitro, offering possible therapeutic compositions that could be efficient in managing

COVID-19 patients. This might be one of the reasons why inhibitors targeting SARS-CoV-2 have failed. Despite the fact that in vitro efficiency has been demonstrated and significant explanation of relevant processes and interactions has been provided, direct clinical proof of therapeutic efficacy for many of these medications is now absent. Addition to that, since SARS-CoV-2 virus is frequently mutating and their proteins are quite unstable so reconstructing structure by Cryo-EM seems hard as without proper optimization of samples, desired resolutions can't be obtained. This can be pointed out as one of the major drawback of Cryo-EM. Scientists are working on the progress of Cryo-EM, however it's pretty time consuming and costly as well. Despite this, it can be believed that Cryo-EM will aid in diminishing this pandemic by contributing revealing proteins structure. Finally, small-molecule inhibitors (including medication combinations) might be helpful in avoiding and controlling the COVID-19 pandemic.

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