

INSTITUTE OF PHYSICS, POLISH ACADEMY OF SCIENCES



DOCTORAL DISSERTATION

SARS-CoV-2: Antibodies and effect of non-structural proteins on protein synthesis in human ribosomes

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in the Division of Theoretical Physics*

Warsaw, September 2024

Declaration

I, Hung Van Nguyen, declare that this thesis titled “SARS-CoV-2: Antibodies and effect of non-structural proteins on protein synthesis in human ribosomes” and the work presented within it are my own. I would like to confirm that:

- This work was carried out entirely during the period of pursuing a research degree at the Institute of Physics, Polish Academy of Sciences, Poland.
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- Where I have consulted the published work of others, I always consistently provide clear attributions.
- Where I have quoted from the work of others, the source is always given or cited. Aside from these quotations, this thesis solely represents my own work.
- I have acknowledged all primary sources of help.
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Warsaw, September 2024

A handwritten signature in black ink, appearing to read 'Van Huu', with a stylized flourish extending from the end.

Hung Van Nguyen

Abstract

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the causal agent responsible for the formidable global COVID-19 pandemic, officially declared by the World Health Organization in March 2020. Notably, the relentless pursuit of scientific endeavors has led to the development of numerous vaccines, pharmaceuticals, and immunotherapies, which have undoubtedly played an instrumental role in saving countless human lives. However, the fight against the COVID-19 pathogen continues, marked by the emergence of immune-evading variants of concern, such as the Delta and Omicron strains.

In an effort to develop more effective treatments and to understand the intricacies of adverse effects caused by vaccines and therapeutic agents, it is imperative to gain a deep understanding of the molecular interactions of SARS-CoV-2 with these interventions and the cellular constituents of the human body. Computational methods play a crucial role in improving the design of antiviral drugs, vaccines, and antibodies/nanobodies (Abs/Nbs) for the treatment of COVID-19. They are also essential for understanding complex processes such as membrane fusion, RNA splicing, messenger RNA (mRNA) translation, and protein trafficking, especially when SARS-CoV-2 non-structural proteins (NSPs) are involved in the ribosome. Computational approaches span the spectrum from all-atom to coarse-grained models, enabling a deeper understanding of these complex phenomena.

In this dissertation, three computational studies focus on SARS-CoV-2, pursuing two main objectives. First, we delve into the interactions of Abs and Nbs with the SARS-CoV-2 spike (S) protein. Second, we shed light on the impact of SARS-CoV-2 non-structural protein 1 (NSP1) on the protein synthesis process in the human ribosome. The dissertation consists of five chapters detailing these research endeavors.

Chapter 1 provides an introductory section covering key aspects, including the COVID-19 pandemic, the structure of SARS-CoV-2 and its variants, the life cycle of SARS-CoV-2, an overview of the interaction of Abs and Nbs with the S protein, and the interaction of NSP1 with the human ribosome.

Chapter 2 provides an overview of the computational methodologies used in the research presented in this dissertation. It includes a brief summary of the molecular dynamics simulations used to estimate the binding affinity of various SARS-CoV-2 biomolecular complexes, such as steered molecular dynamics (SMD), umbrella sampling (US), and alchemical simulations.

Chapter 3 is dedicated to the interaction of Abs and Nbs with the receptor binding domain (RBD) of the SARS-CoV-2 S protein, where their binding affinity is assessed through all-atom SMD and coarse-grained US simulations. This chapter encompasses two separate research publications: In the first publication, the study revolves around the binding of REGN10933 Ab, REGN10987 Ab, and their combination to RBD. It is observed that REGN10933 exhibits a stronger binding affinity to RBD than REGN10987. Interestingly, the combination of REGN10933 and REGN10987 displays even stronger binding to RBD. The stability of REGN10933-RBD and REGN10933+REGN10987-RBD complexes is primarily governed by electrostatic interactions, whereas the stability of REGN10987-RBD depends on

van der Waals (vdW) interactions. In particular, REGN10933 and REGN10933+REGN10987 show similar potency against the Delta variant and the wild type. However, they are less effective against the Omicron variant, confirming recent experimental results. The second publication examines the concurrent binding of H11-H4 Nb and CR3022 Ab to RBD, revealing a markedly increased binding affinity compared to their individual associations with RBD. The combination of H11-H4 and CR3022 increases the ability to neutralize SARS-CoV-2. The stability of the H11-H4-RBD complex is mainly driven by vdW interactions, while electrostatic interactions play a more significant role in the stability of CR3022-RBD and H11-H4+CR3022-RBD complexes. CR3022 is a promising candidate for the treatment of COVID-19, especially against the wild type strain. In addition, it is noteworthy that H11-H4 exhibits strong neutralizing capabilities against Alpha, Kappa, and the highly concerning Delta variants, consistent with recent experimental data.

Chapter 4 focuses on the interaction between mRNA and the 40S ribosome in the presence and absence of NSP1. Using all-atom SMD and coarse-grained alchemical simulations, our analysis revealed that mRNA exhibits significantly stronger binding affinity for the 40S-NSP1 complex compared to the 40S ribosome alone. These results are in close agreement with experimental observations. Furthermore, our studies showed that the electrostatic interaction between mRNA and the 40S ribosome plays a key role in driving the mRNA translation process. Upon entry into host cells, NSP1 can bind to the 40S ribosome, thereby interfering with the translation process.

Finally, Chapter 5 provides a summary of the findings presented in this thesis and outlines potential directions for future research.

Streszczenie

Ciężki ostry zespół oddechowy koronawirus 2 (SARS-CoV-2) odpowiedzialny jest za wybuch pandemii COVID-19, ogłoszonej przez Światową Organizację Zdrowia w marcu 2020 roku. W celu przeciwdziałania skutkom koronawirusa prowadzone są liczne badania naukowe. Doprowadziły one do opracowania wielu szczepionek, leków i terapii immunologicznych, które odegrały kluczową rolę w ratowaniu niezliczonych ludzkich istnień. Jednak przeciwdziałanie COVID-19 ciągle trwa, co jest szczególnie istotne ze względu na pojawienie się nowych wariantów koronawirusa, takich jak Delta i Omikron, które potrafią unikać odpowiedzi immunologicznej organizmu.

W dążeniu do opracowania bardziej skutecznych terapii i zrozumienia złożoności skutków ubocznych wywołanych przez szczepionki i środki terapeutyczne, konieczne jest dogłębne zrozumienie ich oddziaływań z molekułami koronawirusa oraz składnikami komórkowymi ludzkiego ciała. Metody obliczeniowe odgrywają kluczową rolę w udoskonalaniu metod projektowania leków przeciwwirusowych, szczepionek oraz przeciwciał/nanocząsteczek (Abs/Nbs) do leczenia COVID-19. Są one również niezbędne do zrozumienia złożonych procesów, takich jak fuzja błon, składanie RNA, translacja mRNA i transportu białek. Jest to szczególnie istotne, ponieważ niestrukturalne białka koronawirusa (NSP) biorą udział w funkcjonowaniu rybosomu. Podejścia obliczeniowe obejmują szerokie spektrum od modeli na pełnoatomowych do modeli zgrubnych (gruboziarnistych), umożliwiając pełne zrozumienie tych skomplikowanych zjawisk.

W niniejszej dysertacji przedstawione są trzy badania obliczeniowe, które koncentrują się na SARS-CoV-2, realizując dwa główne cele badawcze. Po pierwsze, zbadałem oddziaływania Abs i Nbs z białkiem kolca (S) SARS-CoV-2. Po drugie, dostarczam wskazówek molekularnych dotyczących wpływu niestrukturalnego białka 1 (NSP1) SARS-CoV-2 na proces syntezy białek w ludzkim rybosomie. Dysertacja składa się z pięciu rozdziałów opisujących te badania.

Rozdział 1 zawiera wstępną sekcję, obejmującą kluczowe aspekty, w tym pandemię COVID-19, strukturę SARS-CoV-2 i jego wariantów, cykl życia koronawirusa, opis oddziaływania Abs i Nbs z białkiem S oraz NSP1 z ludzkim rybosomem.

Rozdział 2 przedstawia przegląd metod obliczeniowych użytych w badaniach przedstawionych w tej dysertacji. Obejmuje on także krótki opis symulacji dynamiki molekularnej użytych do oszacowania siły wiązania różnych kompleksów biomolekularnych SARS-CoV-2, takich jak sterowane dynamiki molekularne (SMD), próbkowanie typu „parasola” (US) i symulacje alchemiczne.

Rozdział 3 poświęcony jest oddziaływaniu Abs i Nbs z domeną wiążącą receptor (RBD) białka S SARS-CoV-2, gdzie oceniana jest ich siła wiązania przy użyciu symulacji SMD na poziomie pełnoatomowym i gruboziarnistym. Rozdział ten obejmuje dwie oddzielne publikacje badawcze: W pierwszej publikacji badania koncentrują się na wiązaniu przeciwciała REGN10933, REGN10987 i ich kombinacji z RBD. Obserwuje się, że REGN10933 wykazuje większą siłę wiązania z RBD niż REGN10987. Co ciekawe, kombinacja REGN10933 i REGN10987 wykazuje jeszcze silniejsze wiązanie z RBD, a

stabilność kompleksów REGN10933-RBD i REGN10933+REGN10987-RBD jest głównie determinowana przez oddziaływania elektrostatyczne, podczas gdy stabilność kompleksu REGN10987-RBD zależy głównie od oddziaływań van der Waalsa (vdW). W szczególności, REGN10933 i REGN10933+REGN10987 wykazują podobną skuteczność przeciwko wariantowi Delta i dzikiemu typowi, są jednak mniej skuteczne przeciwko wariantowi Omikron, co potwierdza niedawne wyniki eksperymentalne. Druga publikacja bada jednocześnie wiązanie nanocząsteczki H11-H4 i przeciwciała CR3022 z RBD, wykazując znacznie większą siłę wiązania w porównaniu do ich indywidualnych oddziaływań z RBD, a kombinacja H11-H4 i CR3022 zwiększa zdolność do neutralizacji SARS-CoV-2. Stabilność kompleksu H11-H4-RBD jest głównie wynikiem oddziaływań vdW, podczas gdy oddziaływania elektrostatyczne odgrywają większą rolę w stabilności kompleksów CR3022-RBD i H11-H4+CR3022-RBD. CR3022 wydaje się być obiecującym kandydatem do leczenia COVID-19, zwłaszcza przeciwko standardowemu typowi. Ponadto warto zauważyć, że H11-H4 wykazuje silne zdolności neutralizujące przeciwko wariantom Alpha, Kappa i wariantowi Delta, co jest zgodne z najnowszymi danymi eksperymentalnymi.

Rozdział 4 koncentruje się na oddziaływaniach między mRNA a rybosomem 40S w obecności i nieobecności NSP1. Korzystając z pełnoatomowych symulacji SMD i gruboziarnistych symulacji alchemicznych wykazałem, że mRNA charakteryzuje się znacznie większą siłą wiązania z kompleksem 40S-NSP1 w porównaniu do samego rybosomu 40S – wyniki te są zgodne z obserwacjami eksperymentalnymi. Ponadto nasze badania wykazały, że oddziaływania elektrostatyczne między mRNA a rybosomem 40S odgrywają kluczową rolę w napędzaniu procesu translacji mRNA. Po wnikięciu do komórek gospodarza, NSP1 może wiązać się z rybosomem 40S, zakłócając w ten sposób proces translacji.

Rozdział 5 zawiera podsumowanie wyników przedstawionych w tej dysertacji oraz przedstawia potencjalne kierunki przyszłych badań.

Publications

➤ Publications presented in this thesis:

1. **Nguyen, H.**; Pham, D. L.; Nissley, D. A.; O'Brien, E. P.; Li, M. S. Cocktail of REGN antibodies binds more strongly to SARS-CoV-2 than its components, but the Omicron variant reduces its neutralizing ability. *J. Phys. Chem. B.* 2022, 126(15), 2812-2823.
2. **Nguyen, H.**; Li, M. S. Antibody-nanobody combination increases their neutralizing activity against SARS-CoV-2 and nanobody H11-H4 is effective against Alpha, Kappa and Delta variants. *Sci. Rep.* 2022, 12 (9701).
3. **Nguyen, H.**; Nguyen, H. L.; Li, M. S. Binding of SARS-CoV-2 non-structural protein 1 to 40S ribosome inhibits mRNA translation. *J. Phys. Chem. B.* 2024, 128(29), 7033-7042.

➤ Other publications during my doctoral program:

1. **Nguyen, H.**; Pham, D. L.; Nissley, D. A.; O'Brien, E. P.; Li, M. S. Electrostatic interactions explain the higher binding affinity of the CR3022 antibody for SARS-CoV-2 than the 4A8 antibody. *J. Phys. Chem. B.* 2021, 125(27), 7368-7379.
2. **Nguyen, H.**; Nguyen, H. L.; Lan, P. D.; Thai, N. Q.; Mateusz, S.; Li, M. S. Interaction of SARS-CoV-2 with host cells and antibodies: Experiment and simulation. *Chem. Soc. Rev.* 2023, 52(18), 6497-6553.

➤ Other publications:

1. **Nguyen, H.**; Le, L.; Ho, T. B. Computational study on ice growth inhibition of Antarctic bacterium antifreeze protein using coarse grained simulation. *J. Chem. Phys.* 2014, 140(22), 225101.
2. **Nguyen, H.**; Le, L. Steered molecular dynamics approach for promising drugs for influenza A virus targeting M2 channel protein. *Eur. Biophys. J.* 2015, 44(6), 447-455.
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6. **Nguyen, H.**; Van, T. D.; Tran, N.; Le, L. Exploring the effects of subfreezing temperature and salt concentration on ice growth inhibition of Antarctic Gram-negative bacterium *Marinomonas Primoryensis* using coarse grained simulation. *Appl. Biochem. Biotechnol.* 2016, 178(8), 1534-1545.

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10. **Nguyen, H.**; Nguyen, H. L.; Linh, H. Q.; Nguyen, M. T. Binding affinity of the L-742,001 inhibitor on endonuclease domain of A/H1N1/PA influenza virus variants: Molecular simulation approaches. *Chem. Phys.* 2018, 500, 26-36.
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12. Pham, T.; Nguyen, H. L.; Phan-Toai, T.; **Nguyen, H.** Investigation of binding affinity between potential antiviral agents and PB2 protein of influenza A: Non-equilibrium molecular dynamics simulations approach. *Int. J. Med. Sci.* 2020, 17(13), 2031-2039.

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In this thesis, I have included my three first-author articles: Two published in The Journal of Physical Chemistry B and one in Scientific Reports. I have meticulously listed the permissions from the respective journals below.

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Figure 3.1: REGN10933 and REGN10987 in complex with RBD of A) wild type, B) Delta variant, and C) Omicron variant. Notably, mutations in the Omicron variant RBD are located in the binding regions for both components of REGN-COV2 cocktail. Mutations in the Delta variant RBD specifically affect REGN10933 binding site. Residues that carry a charge in the wild type RBD are shown in blue, while those that gain a charge after mutation are highlighted in red.

Figure 3.2: 3D structures of A) H11-H4 Nb and CR3022 Ab bound to RBD, and B) RBD mutations of variants Alpha, Beta, Gamma, Kappa, Delta, Lambda and Mu. They have contact with H11-H4, but not with CR3022.

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Table 2.1: The η values determined for the stability of protein domains and interfaces, as well as for interactions between Abs and RBD in C_α coarse-grained simulations.

Abbreviation

COVID-19	C ORONA V IRUS D isease of 2019
SARS-CoV-2	Severe A cute R espiratory Syndrome C ORONA V IRUS 2
SARS-CoV	Severe A cute R espiratory Syndrome C ORONA V IRUS
MERS-CoV	M iddle E ast R espiratory Syndrome C ORONA V IRUS
WHO	W orld H ealth O rganization
ORF	O pen R eading F rame
S	S pike
RBD	R eceptor B inding D omain
RBM	R eceptor B inding M otif
NTD	N - T erminal D omain
FP	F usion P eptide
HR1	H eptad R epeat 1
HR2	H eptad R epeat 2
TMH1-TMH3	T rans M embrane H elices (1-3)
ACE2	R eceptor A ngiotensin C onverting E nzyme 2
Mpro	M ain p rotease
3CLpro	3C - L ike p rotease
RdRp	R NA- d ependent R NA p olymerase
SAM	S - A denosyl M ethionine
VOCs	V ariants O f C oncerns
ER	E ndoplasmic R eticulum
FDA	F ood and D rug A ministration
Ab	A ntibody
mAb	m onoclonal A ntibody
Nb	N anobody
mRNA	m essenger R NA
rRNA	r ibosomal R NA
rprotein	r ibosomal p rotein
NSP	N on- S tructural P rotein
MD	M olecular D ynamics

SMD	S teered M olecular D ynamics
TI	T hermodynamics I ntegration
FEP	F ree E nergy P erturbation
LJ	L ennard- J ones
US	U mbrella S ampling
vdW	v an d er W alls
AFM	A tomc F orce M icroscopy
SPR	S urface P lasmon R esonance
BLI	B io L ayer I nterferometry
DFS	D ynamic F orce S pectroscopy
FFC	F luorescence F low C ytometry
1D-PMF	O ne- D imensional P otential of M ean F orce
CoM	C enter o f M ass
SRP	S ignal R ecognition P article

Chapter 1: Introduction

1.1 COVID-19 pandemic

The first case of coronavirus disease 2019 (COVID-19) was detected in Wuhan, China, at the end of 2019¹⁻². The disease rapidly spread throughout the world, prompting the World Health Organization (WHO) to declare it a pandemic in March 2020 due to a substantial surge in both the number of cases and fatalities. The total cumulative number of confirmed cases has far surpassed those observed during the severe acute respiratory syndrome coronavirus (SARS-CoV) period during 2003³. Following the emergence of SARS-CoV in 2003 and the Middle East respiratory syndrome coronavirus (MERS-CoV) in 2012, the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) constitutes the third zoonotic human coronavirus of the twenty-first century⁴.

The number of cases and the death toll from COVID-19 classify it as one of the most catastrophic infectious diseases in human history. Although several years have passed since the onset of the pandemic, the origin of SARS-CoV-2 remains enigmatic. One hypothesis posits a zoonotic origin for SARS-CoV-2, wherein the virus is transmitted to humans from wildlife, such as bats^{3, 5-6}. A meta-analysis indicates that the probability of a natural disaster that causes millions of deaths, similar to COVID-19, is extremely small. In contrast, the probability of a virus leaking from a laboratory accident is higher⁷, thereby supporting the hypothesis that the virus may be the product of genetic research. Another plausible scenario is the emergence of the virus through an evolutionary and selective process, involving a human-transmitted variant of the coronavirus that circulates within the population, culminating in a pandemic⁸.

Since the onset of the pandemic, the research community has exerted tremendous effort to identify effective treatments for COVID-19. Despite the notable success of current vaccines, particularly messenger RNA (mRNA) vaccines⁹, antibodies (Abs)¹⁰⁻¹¹, and pharmaceuticals such as Remdesivir, Molnupiravir¹²⁻¹⁴, and Paxlovid¹⁵, the battle against SARS-CoV-2 continues, with the emergence of an increasing number of variants of concerns, such as Delta and Omicron. The pandemic has progressed through multiple waves¹⁶⁻¹⁷, including the initial, second, and third waves, attributed to the ancestral Wuhan strain, Beta and Delta variants¹⁸, respectively. Subsequently, the fourth and fifth waves have been ignited by the Omicron variant and its sub-lineages^{17, 19}, which are competing among themselves to establish dominance within the viral lineage²⁰. Even individuals who have received the original mRNA vaccine and a bivalent BA.5 booster have displayed limited neutralization efficacy against Omicron subvariants, including BA.2.75.2, BQ.1.1, XBB.1, *etc.*²¹, posing fresh challenges to public health²²⁻²³. Such observations underscore the imperative for researchers to gain a deeper understanding of the interaction between novel variants and human cells. This understanding must encompass immune evasion and intricate details of viral replication mechanisms to facilitate the development of more potent Abs and vaccines²⁴. These circumstances emphasize the enduring commitment to COVID-19 research within the scientific community.

1.2 SARS-CoV-2 structure

SARS-CoV-2 belongs to the beta-coronavirus within the Coronaviridae family, which comprises four genera: Alpha, Beta, Gamma, and Delta coronaviruses. It is spherical in shape and has a size of about 100 nm. SARS-CoV-2 genome is a single-strand positive-sense RNA, which distinguishes it as larger than most other RNA viruses²⁵. This molecule possesses a 5'-cap and a 3'-poly(A) tail, enabling it to function as functional mRNA for the translational synthesis of replicase polyproteins²⁶. Approximately two-thirds of the viral genomic region is occupied by the replicase gene, referred to as open reading frames (ORFs), which have the potential to encode non-structural proteins (NSPs) known as pp1a and pp1ab polyproteins, respectively (Figure 1.1).

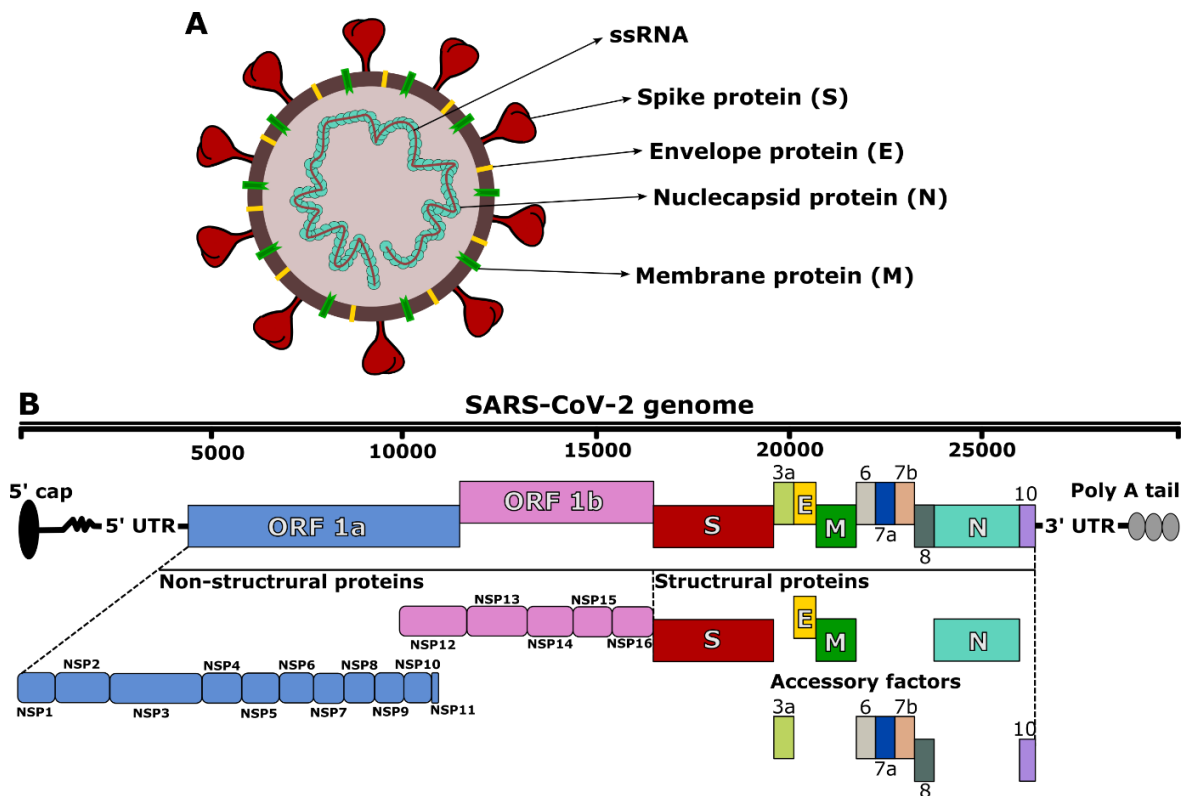


Figure 1.1: Illustration shows A) SARS-CoV-2 structure and B) a schematic of SARS-CoV-2 components.

The pp1a encompasses NSP1 to NSP11, while the pp1ab consists of NSP12 to NSP16. The remaining region preceding the 3'-end encodes various structural proteins, including spike (S), envelope (E), membrane (M), and nucleocapsid (N) proteins²⁷ (Figure 1.1). Here, structural genes also encode accessory proteins, such as ORF3a, ORF6, ORF7a, ORF7b, ORF8, ORF10, and others²⁸. Moreover, the genomic region immediately preceding the 5'-end contains two distinct domains: The leader sequence and the untranslated region. These domains are capable of forming a multitude of stem-loop structures that are essential for the replication and transcription of the viral genome²⁶.

1.2.1 Structural proteins

Structural proteins are essential components that living organisms employ to maintain their shape and structural integrity. These proteins are composed of amino acids, which serve as their fundamental building blocks or monomers. Amino acids are analogous to beads on a pearl necklace, binding together to form proteins. Each amino acid consists of a carbon alpha (C_α) linked to an amino group (NH_2), a carboxyl group ($COOH$), hydrogen (H), and a variable side chain denoted as (R), which confers different chemical properties on the amino acid. The complement of amino acids comprises twenty canonical types, each possessing unique chemical characteristics. These canonical amino acids are further categorized into groups based on the chemical properties of their side chains, which include positively charged amino acids, negatively charged amino acids, uncharged polar amino acids, and nonpolar amino acids. Proteins exhibit four hierarchical levels of structural organization. The primary structure refers to the linear sequence of amino acids in a polypeptide chain that extends from the N-terminus (NH_2) to the C-terminus ($COOH$). The secondary structure in proteins is defined by the localized spatial arrangement of the polypeptide chain, which is stabilized through hydrogen bonds within the peptide backbone. Common secondary structures include the α -helix and β -strand. The three-dimensional conformation of a single polypeptide chain, shaped by interactions between its side chains, is referred to as the tertiary structure. When a protein is composed of multiple polypeptide chains, its overall structure is referred to as the quaternary structure.

The SARS-CoV-2 genome encodes four structural proteins - S, E, N, and M - using genes located near the 3' end. The S protein plays a crucial role in binding to and neutralizing the host cell membrane. The M protein, the most abundant structural protein in the viral structure, plays a key role in protecting the virus. The M protein aids in the formation of the virus and reinforces the curvature of the viral membrane by interacting with the N protein. Within the virus, the E protein plays a role by harboring a small quantity of a transient membrane protein, often referred to as the coat protein. The E protein is instrumental in the processes of assembly, release, and pathogenicity of the virus. The N protein is another crucial viral component that tightly binds to the RNA genome and forms a symmetrical helical nucleocapsid structure. The N protein comprises two domains that facilitate their attachment to the RNA genome through various mechanisms²⁹. Detailed information on the structural proteins of SARS-CoV-2 is provided in the following.

a) S protein: The entry of SARS-CoV-2 into host cells is facilitated by the S glycoprotein³⁰⁻³². This transmembrane S glycoprotein assembles into homotrimers (Figure 1.2A) that protrude from the viral surface (Figure 1.1). Due to its crucial role in facilitating the entry of coronaviruses into host cells, the S protein is an attractive target for antiviral intervention. The S protein consists of two functional subunits: S1 and S2. The S1 subunit binds primarily to host cell receptors, whereas the S2 subunit facilitates the fusion of viral and host cell membranes. At the junction between the S1 and S2 subunits, there is a cleavage site known as the S1/S2 protease cleavage site (Figure 1.2A). Host proteases are responsible for cleaving the S protein at the S2 cleavage site in all coronaviruses. This cleavage event activates the

protein and is essential for triggering irreversible conformational changes necessary for the fusion of viral and host cell membranes³³⁻³⁴.

In the prefusion conformation, the S1 and S2 subunits remain associated via non-covalent bonds. Various coronaviruses employ specific domains within the S1 subunit to interact with distinct entry receptors. To enter into host cells, SARS-CoV-2 relies on the recognition of the receptor angiotensin-converting enzyme 2 (ACE2) on the surface of host cells, a process primarily mediated by the receptor binding domain (RBD) within the S1 subunit (Figure 1.2A). The S protein can adopt two distinct structural forms: The "closed" and "open" states. In the "closed" state, the three receptor recognition motifs do not extend beyond the interface formed by the three S protein protomers. The "open" state exhibits an upward orientation of RBD, which is essential for the fusion of SARS-CoV-2 with the host cell membranes, thereby enabling the virus to enter host cells efficiently³³.

The formation of the six-helical bundle is based on the interaction between heptad repeat 1 (HR1) and heptad repeat 2 (HR2) (Figure 1.2A), and this structural motif plays a crucial role in membrane fusion, mediated primarily by the S protein. Consequently, HR1 and HR2 have become attractive targets for drug development³⁵⁻³⁶. Three HR1 domains converge to create a spiral coil trimer oriented in a parallel configuration. At the same time, three HR2 domains intertwined around the center antiparallelly, primarily driven by hydrophobic forces. Hydrophobic residues located on the HR2 domain form interactions with the hydrophobic grooves formed by every pair of neighboring HR1 helices³⁷.

Because RBD specifically binds to the ACE2 receptor, it is a crucial target for antiviral drugs and Abs³⁸. RBD consists of two structural domains: The core and the external subdomains. The core subdomain exhibits a high degree of conservation and is composed of five β strands arranged in an antiparallel fashion, featuring a disulfide bond bridging two of these β strands. Conversely, the external subdomain is predominantly defined by a loop region that is stabilized through another disulfide bond³⁹. The core of RBD consists of five antiparallel β sheets connected by loops and short helices. Situated between the antiparallel β 4 and β 7 strands is the receptor binding motif (RBM), characterized by loops, α helices, and short β 5 and β 6 strands. The RBM contains the majority of the binding sites for both SARS-CoV-2 and ACE2. Within RBD, eight out of the nine Cysteine residues engage in the formation of four pairs of disulfide bonds. Three of these disulfide bonds are located within RBD's core, enhancing the stability of the β sheet structure. The remaining disulfide bond's role is to facilitate connections between the loops within the RBM. The N-terminal peptidase domain of ACE2 encompasses the binding site, formed by two lobes involving the RBM and ACE2. Specifically, the RBM binds to the smaller lobe of ACE2 on its lower side. The surface of the RBM exhibits a slight inward curvature to accommodate the presence of ACE2³³. RBD undergoes conformational changes reminiscent of a hinge, leading to the exposure or concealment of S protein elements that engage with host cell receptors. These conformational shifts manifest as two distinct states: the "up" and "down" conformations. Here, only the "up" conformation of SARS-CoV-2 is capable of binding to ACE2; the "down" conformation of SARS-CoV-2 cannot recognize ACE2 on host cells.

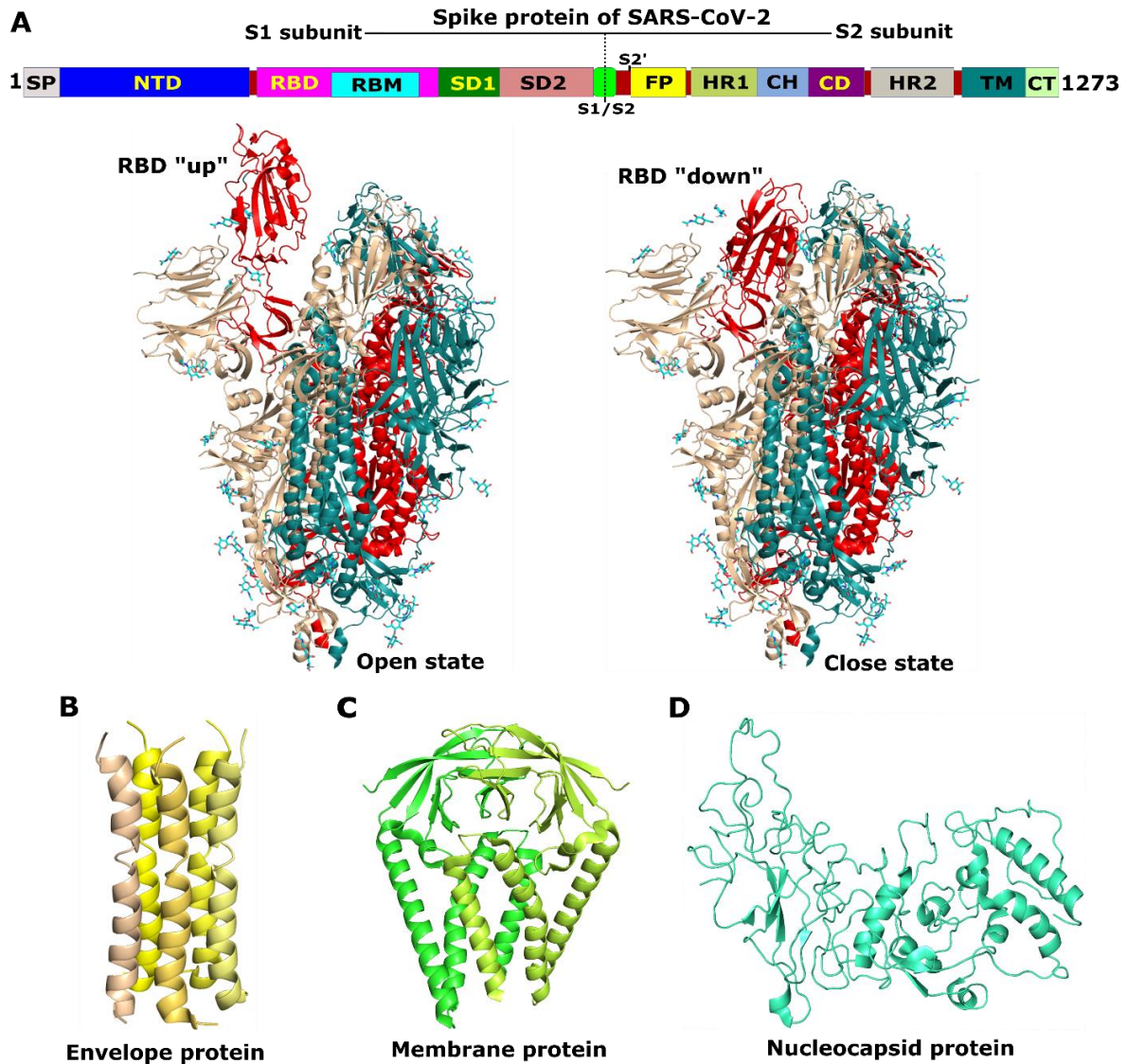


Figure 1.2: SARS-CoV-2 structural proteins include A) S protein structures in open (PDB ID: 6VYB) and closed (6VXX) states, B) E protein transmembrane domain (7K3G), C) M protein (8CTK), and D) N monomer protein (8FD5).

b) E protein: The E protein in SARS-CoV-2 is the smallest among all the structural proteins but plays a significant role in pathogenesis, virus assembly, and release ⁴⁰. The E protein is characterized by a five-helix bundle surrounding a narrow, dehydrated pore that contains a bipartite channel (Figure 1.2B). Although the amino acid compositions of the E protein vary considerably, their structural features remain conserved across different genera of β coronaviruses. Typically, this protein displays a short hydrophilic N-terminus, a sizable hydrophobic region, and a hydrophilic C-terminal tail ⁴¹.

c) M protein: The M protein is the most abundant structural protein and features three distinct transmembrane domains ⁴². These domains include an ectodomain at the N-terminus, three transmembrane helices (TMH1-TMH3), and an endo-domain at the C-terminus ⁴³ (Figure 1.2C). Specifically, the TMH1-TMH2 intersegment is located in the interior, while the

TMH2-TMH3 intersegment is on the exterior. The C-terminal region is predicted to contain at least two casein kinase II phosphorylation sites, which are relevant to interactions with S, E, and N proteins ⁴⁴. These interactions play a vital role in inducing membrane bending and act as a checkpoint for new virion formation ⁴⁵. The M protein is associated with other viral structural proteins, including the N protein, thereby facilitating the molecular assembly of virus particles and potentially contributing to pathogenesis ⁴⁶. Although the amino acid composition of the M protein varies, its structural features are conserved in different genera ⁴⁷. In particular, O-linked glycosylation is observed in β and δ coronaviruses, while other coronavirus M proteins undergo N-linked glycosylation ⁴⁸⁻⁴⁹. This glycosylation plays a crucial role in organ tropism and interferon signaling ⁵⁰.

d) N protein: The N protein plays a vital role exclusively in structural organization. It is characterized by three highly conserved domains: an N-terminal domain, an RNA-binding domain (or linker region), and a C-terminal domain ⁵¹ (Figure 1.2D). These domains are believed to collectively regulate RNA binding ⁵², with the phosphorylation status of the N protein being a critical factor that induces structural changes, thereby enhancing its affinity for viral RNA over non-viral RNA ⁵³. The N protein is actively involved in the RNA packaging, adopting a “beads-on-a-string” conformation. Beyond its role in organizing the viral genome, the N protein also contributes to virion assembly and improves virus transcription efficiency, among other functions ⁵².

1.2.2 Non-structural proteins

A non-structural protein (NSP) is a protein encoded by a virus but not part of the viral particle and arises during viral replication. SARS-CoV-2 contains sixteen NSPs, designated as NSP1 to NSP16 (Figure 1.3). Below is a description of the NSPs of SARS-CoV-2.

a) NSP1: NSP1 is composed of three domains: an N-terminal domain, a linker domain, and a C-terminal domain. The C-terminal domain can bind to the 40S subunit of the human ribosome, resulting in inhibition of mRNA translation ⁵⁴. The N-terminal and linker domains of NSP1 do not bind directly to the 40S mRNA entry channel; instead, they play a role in stabilizing its association with the ribosome and mRNA ⁵⁵.

b) NSP2: NSP2 consists of the N-terminal and C-terminal domains. Although NSP2 is involved in viral processes, its precise functions and structural basis remain unknown ²⁹. Notably, a highly conserved cysteine residue that coordinates a zinc ion within a zinc ribbon-like motif exhibits significant structural similarity to RNA-binding proteins. This motif plays a crucial role in NSP2's interactions with nucleic acids ⁵⁶.

c) NSP3: NSP3 is the largest membrane-bound protein, encompassing several domains ⁵⁷. NSP3 functions as a membrane-anchored scaffold that associates with host proteins and other NSPs to form the viral replication-transcription complex ⁵⁸.

d) **NSP4**: NSP4 consists of four transmembrane domains: N-terminal, luminal, TM3 and C-terminal⁵⁹. Transmembrane domains 1 to 3 (the N-terminal, luminal, TM3), along with a specifically charged residue, play a crucial role in facilitating productive virus infection, while the C-terminal domain is exposed on the cytoplasmic side of the membrane⁶⁰. NSP4 plays a role in anchoring the viral replication-transcription complex, in conjunction with other integral viral membrane proteins such as NSP3 and NSP6⁶¹. The co-expression of NSP4 and NSP3 leads to the induction of concentrated foci in the perinuclear region and the redistribution of proteins from the endoplasmic reticulum (ER) to the foci⁵⁸.

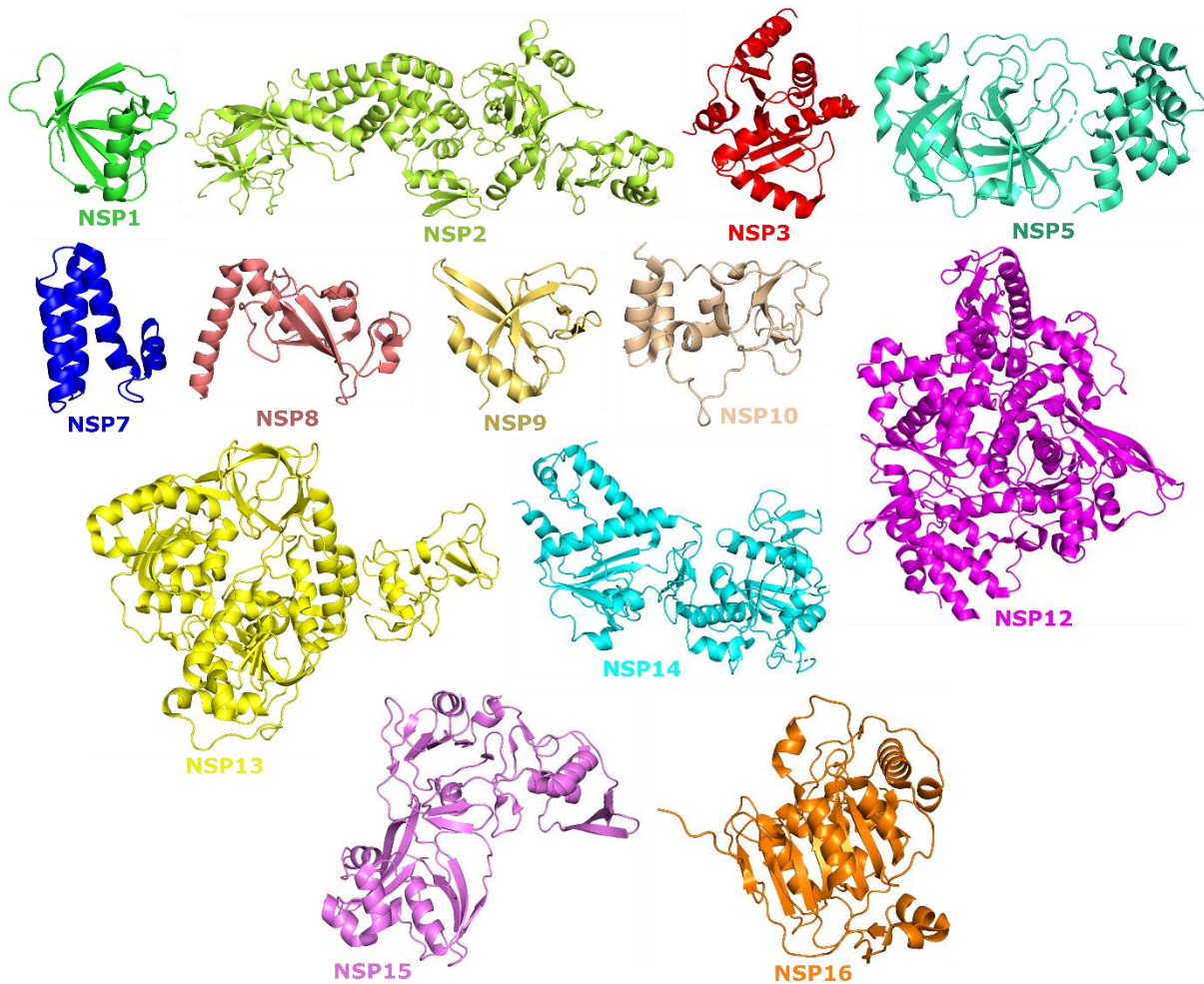


Figure 1.3: Illustration is NSPs of SARS-CoV-2, including NSP1 (7K7P), NSP2 (7MSW), NSP3 (6YWL), NSP5 (7QBB), NSP7 and NSP8 (7DCD), NSP9 (7BWQ), NSP10 (7ORR), NSP12 (6NUR), NSP13 (6ZSL), NSP14 (7R2V), NSP15 (7KOR) and NSP16 (6WVN).

e) **NSP5**: NSP5, known as the main protease (Mpro) or 3C-like protease (3CLpro), is comprised of monomers with N-terminal domains (domain I and domain-II) and a C-terminal domain (domain-III)⁶². Its primary function is to catalyze the processing of viral polyproteins, making it a promising target for antiviral therapy with SARS-CoV-2⁶³.

f) *NSP6*: NSP6 is a multi-spanning transmembrane protein consisting of six transmembrane domains ⁶⁴, which are located in the ER ⁶⁵. It plays a role in facilitating the generation of autophagosomes, which are responsible for releasing viral components to lysosomes for degradation ⁶⁶.

g) *NSP7, NSP8, and NSP12*: NSP7 consists of an α -helical structure with three helical bundle folds, while NSP8 is comprised of two subdomains: an N-terminal “shaft” domain and a C-terminal “head” domain ⁶⁷. The crystal structure of the NSP7-NSP8 complex forms a hollow cylindrical hexadecameric structure with a dimer conformation. This complex has a negatively charged outer surface and a positively charged inner core ⁶⁸, facilitating the passage of the nucleic acid phosphate backbone through the cylindrical channel without electrostatic repulsion. The cylindrical NSP7-NSP8 complex is stabilized by a salt bridge ⁶⁹.

NSP12 functions as a multi-subunit RNA-dependent RNA polymerase (RdRp) ⁷⁰, including two main functional domains: an N-terminal domain and a polymerase domain ⁷¹. The polymerase domain is at the C-terminus, and it adopts a “right hand” cupped-shaped conformation, comprising finger, palm, and thumb subdomains ⁷². The N-terminus contains a nidovirus RdRp-associated nucleotidyl transferase (NiRAN) domain linked to the C-terminus of the RdRp, along with an extended N-terminal β -hairpin domain ⁷¹. The NiRAN domain is followed by an interface domain, and the β -hairpin domain inserts into a groove formed by the palm domain and NiRAN domain ⁷³. The active site of RdRp is located at the interface between the finger and thumb subdomains, which is the center of the substrate domain where RNA synthesis occurs ⁷⁴. RdRp is known to be an important target for drug development to combat coronavirus infections, including COVID-19 ⁷⁵⁻⁷⁶.

NSP12 forms a complex with NSP7 and NSP8, connected by two salt bridges to NSP7 and NSP8. The binding of the NSP7-NSP8 heterodimer to the finger loop stabilizes the polymerase domain, enhancing its affinity for the template RNA. The second subunit of NSP8 is believed to play a crucial role in polymerase activity, possibly by binding to template RNA and providing an expanded interaction surface, helping anchor the RNA strand. In the presence of both NSP7 and NSP8, NSP12's binding affinity to template primer RNA is significantly enhanced, leading to increased polymerase activity ⁷³.

h) *NSP9*: NSP9 is composed of a central core featuring a six-stranded barrel, which is flanked by a C-terminal helix and an N-terminal extension. NSP9 serves as a single-stranded RNA binding protein, facilitated primarily by its β -barrel loop structure. Dimerization and interactions with other proteins are likely facilitated by the C-terminal β -hairpin and helix, and these structural elements are conserved across various coronaviruses ⁷⁷. NSP9 has a role in the synthesis of viral RNA. It exists in a dimeric form, and forms a unique structure, providing a nucleic acid binding site crucial for efficient virus replication ⁷⁸.

i) *NSP10, NSP14, and NSP16*: NSP10 is a single-domain protein that binds two zinc ions. It plays a pivotal role in SARS-CoV-2 viral transcription by stimulating both the 3'-5'-exoribonuclease activity of NSP14 and the 2'-O-methyltransferase activity of NSP16.

Therefore, NSP10 is essential for the methylation of the viral mRNA cap. It can bind to single- and double-stranded RNA and DNA and has an allosteric effect on the activity of NSP14 3'-5'-exoribonuclease, allowing the formation of the substrate binding pocket⁷⁹⁻⁸⁰. Similarly, the allosteric interaction of NSP10 with NSP16 improves mRNA binding to the 2'-O-methyltransferase activity of NSP16⁸¹.

NSP14 is a multidomain protein that acts as an enzyme with two distinct activities: an exoribonuclease activity that acts on single- and double-stranded RNA and an N7-guanine methyltransferase activity⁸². The exoribonuclease domain of NSP14 features a DEEDh motif similar to the DEDD motif found in other exoribonuclease enzymes. This domain, the zinc-binding sites, is necessary for exoribonuclease activity^{79, 83}. NSP10 interacts with NSP14, leading to an allosteric effect on its exoribonuclease activity^{80, 84}. The Guanine-N7-methyltransferase domain of NSP14 lacks the Rossmann fold typically observed in methyltransferase enzymes. This Guanine-N7-methyltransferase domain is responsible for the initial 5' methylation of the GpppA cap, enabling efficient RNA translation and protection from the host's innate immune system⁸⁵.

NSP16 is a ribose 2'-O-methyltransferase that forms a heterodimer with its allosteric activator, NSP10⁸⁶⁻⁸⁷. Its role in the virus's life cycle is to perform the final step of RNA cap synthesis. Capping the 5'-end of the mRNA stabilizes it, preventing degradation by the host cell and reducing the innate immune response⁸⁸⁻⁸⁹. The NSP10-NSP16 complex modifies the cap-0 structure of mRNA, previously methylated by NSP14, another S-adenosyl methionine (SAM)-dependent methyltransferase. NSP16 converts cap-0 (m7GpppN-RNA) to a cap-1 structure (m7GpppNm-RNA) by adding a methyl group at the ribose 2'-O position of the first nucleotide, using SAM as a methyl donor. Here, NSP10 acts as a cofactor for NSP16, stabilizing the SAM-binding pocket and significantly improving the enzymatic activity^{86, 90}.

j) NSP11: NSP11 is a short peptide formed through the cleavage of the pp1a polyprotein by the 3CLpro/Mpro proteinase at the NSP10/NSP11 junction. NSP11 is encoded in the genomic RNA region where the translational reading frame shift takes place, transitioning from ORF1a to ORF1b. This frameshift leads to the generation of NSP12 to NSP16 proteins from the pp1ab polyprotein⁹¹.

k) NSP13: NSP13 plays a pivotal role in viral replication and exhibits the highest degree of sequence conservation among its counterparts⁹². Its primary function is to safeguard the virus from degradation. NSP13 performs the conversion of double-stranded DNA into two single-stranded RNAs, making them suitable for replication⁹³⁻⁹⁴. The terminal portion of NSP13 is predicted to form a cluster of zinc, which provides resistance against coronaviruses and nidoviruses⁹⁵. NSP13 has NTPase activity, using the energy derived from ATP hydrolysis to facilitate the unwinding of base pairs. This activity is believed to be crucial for RNA-related processes, including transcription and translation⁹⁶.

l) NSP15: NSP15 is a nidoviral RNA uridylylate-specific endoribonuclease, its C-terminal catalytic domain belongs to the EndoU family, involving various critical biological functions

associated with RNA processing. It produces 2'-3' cyclic phosphodiester and 5'-hydroxy termini following RNA endonuclease activity on single- and double-stranded RNA, specifically targeting uridine. The precise functional significance of NSP15 remains elusive. Coronaviruses lacking NSP15 are viable and capable of replication. Nevertheless, conflicting research findings exist regarding NSP15's impact on impeding the innate immune response. Some suggest that NSP15 may degrade viral RNA as a strategy to conceal it from host defenses⁹⁷⁻⁹⁸.

1.2.3 Accessory proteins

Accessory proteins are essential virulence factors involved in various pathogenesis pathways during SARS-CoV-2 infection. Most of these proteins are believed to contribute to immune evasion strategies. Below is information about several types of SARS-CoV-2 accessory proteins.

a) *ORF3a*: ORF3a, situated between the S and E proteins, is the largest accessory protein in SARS-CoV-2. The ORF3a is O-linked glycosylated and features three transmembrane domains. ORF3a forms dimers and its six transmembrane helices collectively create an ion channel in the host cell membrane that exhibits higher permeability to $\text{Ca}^{2+}/\text{K}^{+}$ cations than Na^{+} ions. Additionally, the ORF3a is involved in virus release, apoptosis, and pathogenesis⁹⁹⁻¹⁰⁰.

b) *ORF6*: ORF6 is a membrane-associated protein. ORF6 expression has been confirmed in virus-infected Vero E6 cells, as well as in the lung and intestinal tissues of patients. In expressing cells and virus-infected cells, it is primarily located in the ER and Golgi compartments¹⁰¹.

c) *ORF7a*: ORF7a is a type I transmembrane protein that includes a signal peptide sequence, a luminal domain, a transmembrane domain, and a short C-terminal tail. Conversely, the ORF7b is an integral membrane protein expressed in SARS-CoV-2 infected cells, where it remains localized in the Golgi compartment. ORF7b has been found to be closely associated with intracellular virus particles, further underscoring its significance and importance¹⁰²⁻¹⁰³.

d) *ORF8*: ORF8 exhibits low homology to SARS-CoV-2 due to a deletion. Its structure resembles an immunoglobulin (Ig)-like fold, primarily due to the β -strand core. ORF8 has been observed to interact with the major histocompatibility complex I, thus facilitating their degradation in cell culture and potentially contributing to immune evasion¹⁰⁴.

e) *ORF10*: ORF10 is predicted to be located downstream of the N gene. Although its corresponding single guide RNA is rarely detected, the ORF10 has been found in infected cells¹⁰⁵.

1.3 SARS-CoV-2 life cycle

SARS-CoV-2 interacts with cellular receptors such as human ACE2 and host proteases, which activate the S protein. Below are seven stages describing SARS-CoV-2 life cycle (Figure 1.4).

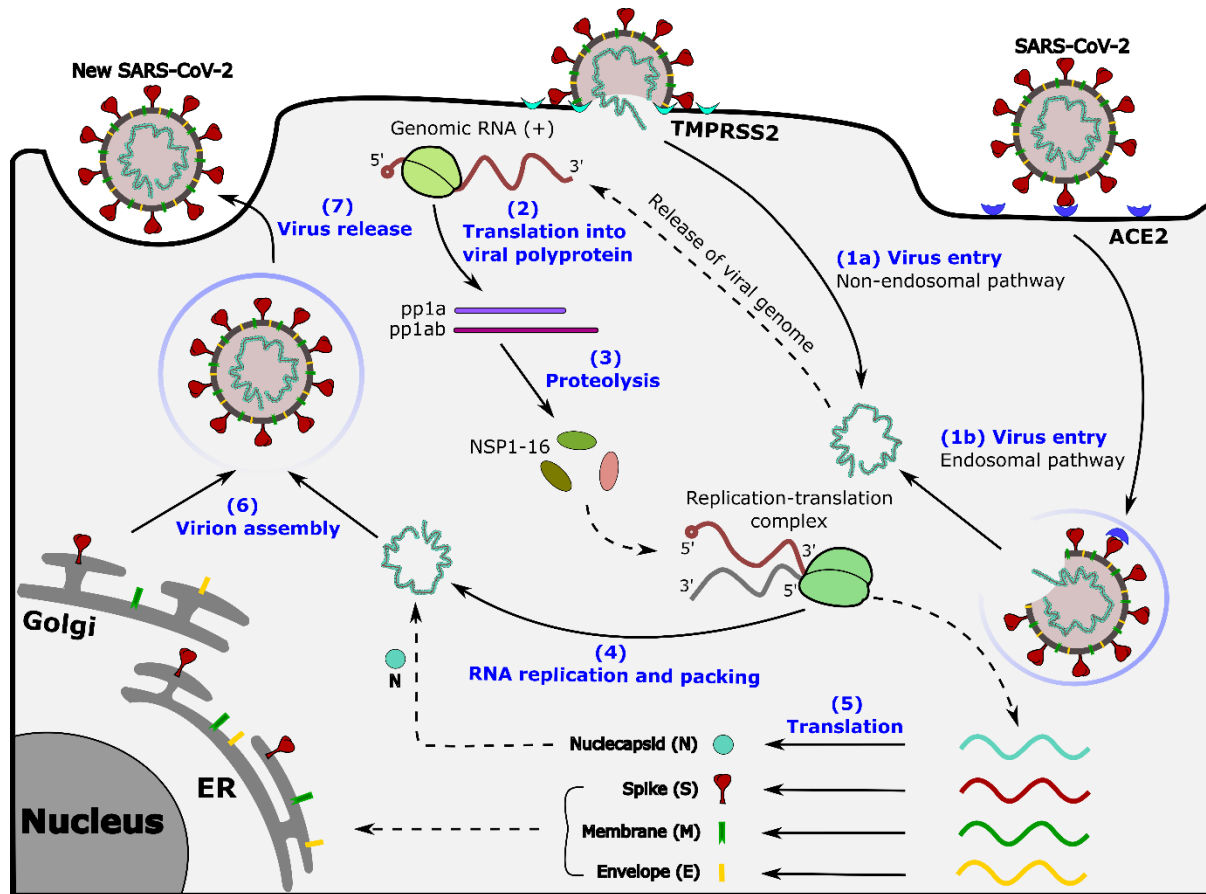


Figure 1.4: SARS-CoV-2 life cycle encompasses seven stages required for the creation of new SARS-CoV-2 particles and their release from human cells ¹⁰⁶.

Stage (1): SARS-CoV-2 can enter the cell cytoplasm through two mechanisms: (1a) The viral particle is endocytosed before fusing with the endosomal membrane. (1b) The viral membrane fuses directly with the cell membrane at the cell surface ¹⁰⁷.

Stage (2): Once inside the host cell and after releasing its RNA, translation begins immediately using the host cell ribosomes. Translation results in the formation of two large polypeptide chains: pp1a and pp1ab.

Stage (3): These polypeptide chains undergo proteolysis to produce individual NSPs, which then assemble to form the viral replication and transcription complex ¹⁰⁸⁻¹⁰⁹.

Stages (4 and 5): This complex transcribes a series of subgenomic mRNAs through a process of discontinuous transcription. Subsequently, these subgenomic mRNAs are translated into viral structural proteins. The N protein forms a complex with genomic RNA,

while the S, E, and M proteins are inserted into the viral envelope of the intermediate compartments within the intermediate compartments of the ER and Golgi.

Stages (6 and 7): Newly assembled viral particles are formed and released from infected cells through exocytosis ¹¹⁰.

1.4 SARS-CoV-2 variants

Since the outbreak of SARS-CoV-2, the virus has undergone mutations in its genes, some of which have been found to change its virulence and transmissibility. As a result, multiple variants have emerged, each with distinct characteristics compared to the original strain. According to the WHO, there have been five variants of concerns (VOCs) of COVID-19: Alpha (B.1.1.7), Beta (B.1.351), Gamma (P.1), Delta (B.1.617.2), and Omicron (B.1.1.529) ¹¹¹⁻¹¹² (Figure 1.5), as well as two variants of interest: Lambda and Mu ¹¹³. VOCs are known to exhibit increased transmissibility and resistance to therapeutic agents, resulting in high rates of hospitalization and mortality ¹¹⁴⁻¹¹⁵. Each variant can be further classified into several sublineages. For instance, Omicron has been identified to have many sublineages: BA.1, BA.2, BA.3, BA.4, BA.5 and others ¹¹⁶. Below is information on SARS-CoV-2 variants of concerns.

1.4.1 Alpha variant

The Alpha variant (B.1.1.7) is one of the first mutated strains derived from the original SARS-CoV-2. It was initially identified in the United Kingdom in November 2020, and infections surged in December 2020 ¹¹⁷. This VOC has acquired key mutations, including several S protein mutations located on RBD at N501Y, and specific deletions in N-terminal domain of S protein (NTD) at positions 69-70 and 144. Additionally, some non-S mutations were associated with this variant ¹¹⁸⁻¹¹⁹.

1.4.2 Beta variant

The Beta variant (B.1.351) was initially identified in South Africa at the end of 2020 and spread to other countries. This VOC has nine S protein mutations located on RBD with some key mutations at positions K417N, E484K, and N501Y, and specific deletions in NTD at positions 242-244 ¹²⁰.

1.4.3 Gamma variant

The Gamma variant (P.1) was initially identified in Brazil in November 2020 ¹²¹. Genetic sequencing of numerous virus samples from infected individuals revealed that the Gamma variant has accumulated over 22 mutations, including approximately 12 mutations in the S protein, without deletions. This variant has been associated with a significantly higher rate of hospitalization and morbidity, exceeding that of previously discovered variants by 3 to 4 times ¹²².

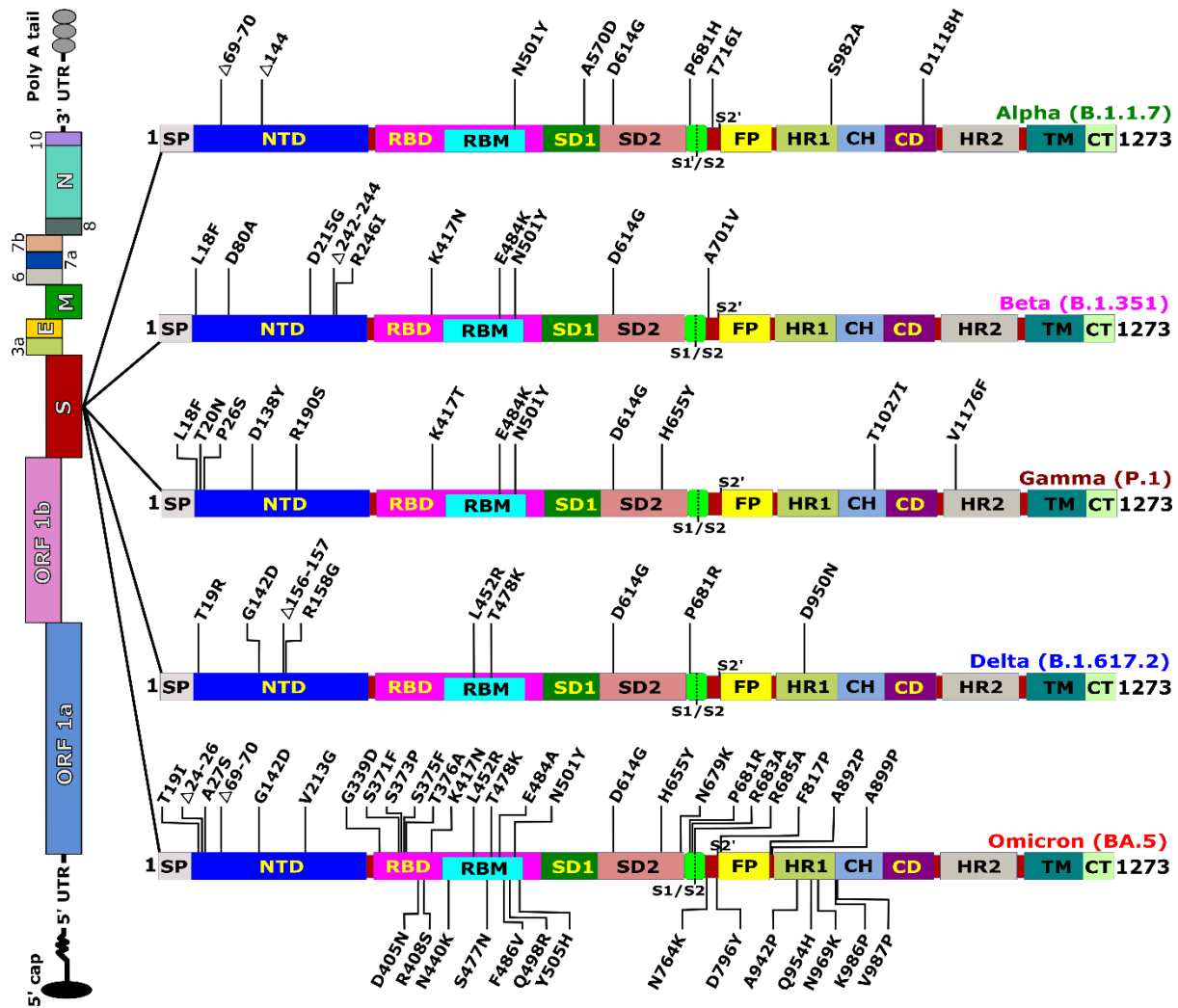


Figure 1.5: The key mutations and deletions on SARS-CoV-2 S protein of the five variants of concern: Alpha, Beta, Gamma, Delta, and Omicron (BA.5).

1.4.4 Delta variant

The Delta variant (B.1.617.2) was initially identified in India in late 2020 and rapidly became the dominant strain in many countries. It possesses 23 mutations, including approximately 9 mutations and deletions in the S protein. Key mutations of this variant include L452R and T478K mutations on RBD and P681R mutations on the cleavage site. This variant exhibits higher transmission and infection rates compared to other variants¹²³⁻¹²⁴.

1.4.5 Omicron variant and its sublineages

The Omicron variant (B.1.1.529) was first identified in Botswana in southern Africa in late November 2021, and cases rapidly began to appear and spread in other countries. It was classified as a variant of concern due to several worrisome characteristics. This variant harbors an extensive number of mutations exceeding 50 points, many of which contribute to immune evasion and enhanced transmissibility. In particular, the S protein alone has accumulated at least 32 genetic changes, with no apparent connection to previous variants¹²⁵⁻

¹²⁷. The emergence of the Omicron variant led to a rapid surge in the number of daily cases in the United States, exceeding one million cases. In 2022, it gave rise to several subvariants, including BA.5, BQ.1, BQ.1.1, and others ^{116, 128}. By 2023, new Omicron subvariants known as XBB.1.5, JN.1, and others had become the predominant cause of infection ¹²⁹.

1.5 COVID-19 antiviral medicines

COVID-19 medication helps manage symptoms, prevents the virus from spreading in the body, and regulates the body's immune response. There are several antiviral drugs for COVID-19, such as nirmatrelvir-ritonavir (Paxlovid), remdesivir (Veklury), molnupiravir (Lagevrio), and others.

1.5.1 Nirmatrelvir-ritonavir (Paxlovid)

Nirmatrelvir is a protease inhibitor that treats COVID-19 by blocking a specific protease, the Mpro, which the virus needs to replicate and continue infecting. Ritonavir is also a protease inhibitor, and low-dose ritonavir is used to slow the breakdown of nirmatrelvir. This allows nirmatrelvir to remain in your body longer, enhancing its effectiveness against COVID-19. Nirmatrelvir-ritonavir is approved to treat mild to moderate COVID-19 in people aged 12 and older who are at increased risk of serious illness. Nirmatrelvir-ritonavir was first authorized for use in December 2021 and became fully food and drug administration (FDA) -approved for adults in May 2023 ¹⁵.

1.5.2 Remdesivir (Veklury)

Remdesivir is an RNA polymerase inhibitor that acts as a nucleotide analog against SARS-CoV-2. It blocks the activity of RdRp in SARS-CoV-2, which is required for the virus to replicate and grow. Specifically, remdesivir resembles one of the building blocks of RNA in SARS-CoV-2. When remdesivir is present, it can be incorporated into the virus's RNA during replication, preventing the virus from spreading by using the incorrect building material. Remdesivir was first authorized for use in May 2020 for inpatient use. It became fully FDA-approved in October 2020 for people aged 12 and older. In January 2022, its use was expanded to outpatient treatment for all ages. In April 2022, it became fully approved for certain children aged 28 days and older ¹³⁰.

1.5.3 Molnupiravir (Lagevrio)

Molnupiravir is a nucleoside analog that treats COVID-19 by targeting the RdRp in SARS-CoV-2. It disrupts the process the virus uses to replicate, preventing it from making additional copies and spreading the infection. Molnupiravir was first authorized by the FDA in December 2021 ¹³¹.

1.6 Antibodies and nanobodies: Pioneering in SARS-CoV-2 treatment

1.6.1 Therapeutic antibodies targeting SARS-CoV-2

Ab is a component of the immune system, produced and secreted primarily by differentiated B cells, which include plasma cells and memory B cells. Ab is structured as a pair of polypeptide chains that form a flexible Y-shape. The stem of the Y is composed of the ends of two identical heavy chains, while each arm consists of the remainder of the heavy chain and a smaller protein known as a light chain. In specific Ab classes, the stem and the lower portions of the arms exhibit significant similarity and are collectively referred to as the constant region. In contrast, the tips of the arms exhibit significant sequence diversity, allowing them to bind to a wide range of antigens¹³²⁻¹³³. In essence, each Ab comprises two fragments: one fragment is the antigen binding site (Fab), located at the end of each arm, allowing the immune system to recognize a diverse range of antigens, and the other fragment is the crystallizable region (Fc), formed by two heavy chains¹³⁴ (Figure 1.6A).

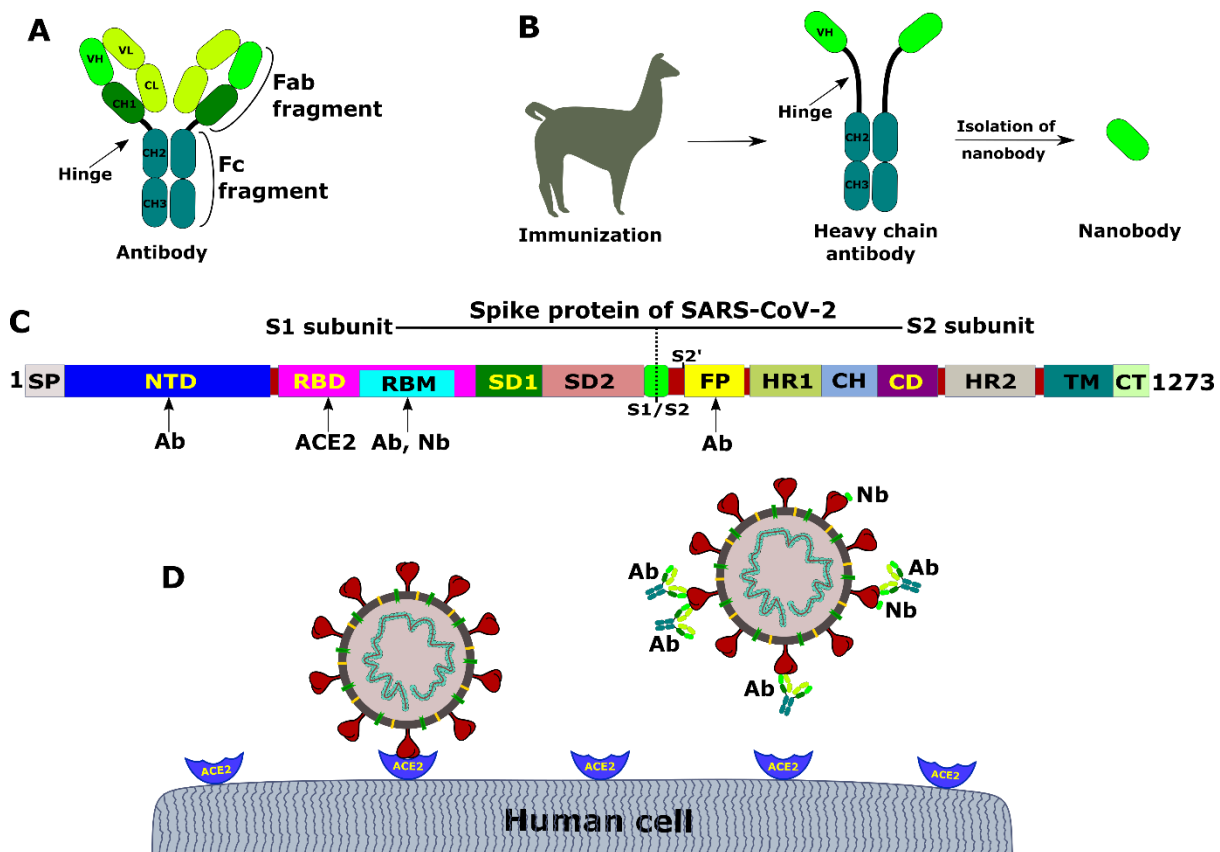


Figure 1.6: A) Ab structure contains antigen binding sites (Fab) and crystallization region fragments (Fc), which include VH (heavy chain variable domain), VL (light chain variable domain), CH (heavy chain constant region), and CL (light chain constant region). B) Nb isolated from the heavy chain of Ab that is extracted from the Camelidae members. C) The S protein structure; ACE2, Ab and Nb bind to RBD while Ab also binds to NTD and FP. D) SARS-CoV-2 binds to ACE2 to infect a human cell; Ab and Nb bind to the S protein to prevent SARS-CoV-2 from entering the human cell.

A monoclonal antibody (mAb) is a laboratory-produced protein designed to specifically bind to particular targets within the body, such as antigens present on the surface of cancer cells. It has been developed using hybridoma technology, recognized as the initial reliable source of Ab therapy¹³⁵⁻¹³⁶. The therapeutic and preventive potential of mAb against various conditions, including cancer, neurological disorders, and infectious viruses (such as HIV, Ebola, MERS-CoV, SARS-CoV, SARS-CoV-2, *etc.*) has already been well-established¹³⁷⁻¹⁴⁰. However, they can lead to certain side effects, mainly associated with immunomodulation and therapeutic Ab. These side effects may include phenomena such as Ab-dependent enhancement and cytokine storms, which could be linked to infection¹⁴¹⁻¹⁴³.

As mentioned in the preceding section, the S protein comprises two functional subunits: S1, responsible for the attachment to host cells, and S2, which facilitates the fusion of the virus with host cell membranes¹⁴⁴⁻¹⁴⁵. Although Ab targets both S1 (specifically RBD and NTD) and S2 (more specifically fusion peptide (FP)), it is noteworthy that most Ab is found to primarily target RBD¹⁴⁵⁻¹⁴⁷ (Figure 1.6C). Note that Ab can bind to RBD in closed and open states, but SARS-CoV-2 cannot interact with host cell ACE2 when in the down state¹⁰⁶. Therefore, Ab binding to the inactive RBD conformation is not relevant for further discussion.

Ab has proven to be a promising class of therapeutics against SARS-CoV-2 infection¹⁴⁸⁻¹⁵². While convalescent plasma from recovered patients contains Ab generated by the adaptive immune response, its impact on improving survival rates remains a topic of ongoing debate¹⁵³⁻¹⁵⁴. Moreover, the large-scale production of plasma-based therapies poses substantial challenges, primarily due to high costs. Therefore, the search for potent Ab on an industrial scale is emerging as one of the most viable strategies to combat COVID-19. In particular, the combination of Ab with another (either Ab or a nanobody (Nb)) can enhance neutralizing activity, providing a more effective approach to SARS-CoV-2 therapy¹⁵⁵.

Numerous Abs targeting RBD, such as REGN-CoV (REGN10933 + REGN10987), S309, LY-CoV555, LY-CoV016, AZD7442 (AZD8895 + AZD1061), CT-P59, LYCoV1404, P2C-1F11, *et al.* have received emergency use authorization as therapeutic agents. These Abs have demonstrated potential for treating SARS-CoV-2, effective against the wild type and various variants, including Alpha, Beta, Gamma, Delta, Omicron, and others. For example, REGEN-CoV, a mAb cocktail composed of REGN10933 and REGN10987, effectively reduces viral load and the number of COVID-19 patients. REGN10933 binds to the top of RBD, while REGN10987 attaches to the side. The binding domain of REGN10933 significantly overlaps with the ACE2 binding site on RBD, whereas the binding region of REGN10987 has a slight overlap with the RBD-ACE2 interface. Consequently, when these Abs bind to the S protein, they occupy the RBD-ACE2 interaction interface, fully blocking ACE2-S interaction. These Abs have demonstrated *in vitro* activity against various variants of SARS-CoV-2. The Beta and Gamma variants are fully resistant to REGN10933 and weakly resistant to REGN10987 in neutralization, whereas the Alpha and Omicron variants are not resistant to the neutralizing activity of REGN-CoV. The combination of REGN10933 and REGN10987 demonstrates both prophylactic and therapeutic efficacy against SARS-CoV-2 variants, including Alpha, Beta, and Gamma, but not against Omicron. Although

REGN10987 shows reduced neutralizing ability against the Delta variant, REGN10933 alone and in combination with REGN10987 can still effectively block the S protein from entering host cells ¹⁵⁶⁻¹⁵⁸.

Various Abs targeting NTD have been identified, such as 4A8, FC05, DH1050.1, DH1052, and others. Some of these Abs can block SARS-CoV-2 infection, while others may unintentionally increase viral infectivity and are associated with severe cases of COVID-19. Although NTD-targeting Abs do not prevent the virus from binding to ACE2, they are of significant interest due to their potential to neutralize SARS-CoV-2. These Abs induce conformational changes in the S protein that hinder the transition of RBD from its "down" to "up" position, thereby reducing the virus's ability to infect cells, even without directly blocking ACE2 binding. This underscores the S protein as a critical focus for vaccine and drug development ¹⁵⁹⁻¹⁶¹.

For Abs targeting FP, the S2 subunit is more conserved among coronaviruses compared to S1, resulting in greater cross-reactivity. However, Abs targeting S2 exhibits weaker neutralizing activity than those targeting S1. Abs COV91-27, COV44-62, VN01H1, C13B8, and others can neutralize wild type and multiple variants, including Alpha, Beta, Gamma, Delta, and Omicron (BA.2 and BA.4/5), though their neutralizing activity is limited compared to Abs targeting RBD and NTD. Abs targeting S2 offers insights into immune defenses and potential targets for vaccine development based on the conserved S2 subunit. S2-specific Abs may inhibit the conformational changes necessary for membrane fusion. FP is also considered a candidate epitope for next-generation coronavirus vaccines ¹⁶²⁻¹⁶⁴.

Numerous experimental studies have examined the binding of Abs to the S protein, but computational studies using molecular dynamics (MD) simulations remain limited. Nguyen *et al.* ¹⁶⁵ recently employed MD simulations to estimate the binding affinities of Abs CR3022 and 4A8 to RBD and NTD, respectively. Their findings indicated that CR3022 has a stronger affinity for RBD compared to 4A8 for NTD, suggesting that CR3022 may be a more effective candidate for COVID-19 therapy. In a separate study, Gigon *et al.* ¹⁶⁶ used a combination of constant-pH Monte Carlo simulations and the PROCEEDpKa method to map electrostatic epitopes for certain mAbs and ACE2 on RBDs of both SARS-CoV-1 and SARS-CoV-2. They proposed structural modifications to CR3022 that could enhance its binding affinity for SARS-CoV-2. Beshnova *et al.* ¹⁶⁷ developed a computational method named SARS-AB for predicting the binding interactions between the S protein and mAbs. They validated this approach using existing structures from the protein data bank (PDB) and demonstrated its effectiveness in predicting Abs-S protein interactions. It was shown that SARS-AB can be used to design potent Abs against emerging SARS-CoV-2 variants that may evade current Ab protections. SARS-AB could greatly speed up the discovery of neutralizing Abs against SARS-CoV-2 and its variants. Although several studies have highlighted the role of Abs in treating SARS-CoV-2, a comprehensive understanding of how a combination of Abs can prevent SARS-CoV-2 infection is still required. An experimental study recently showed that combining REGN10933 Ab with REGN10987 Ab significantly enhances neutralizing capacity ^{156, 168}. However, the investigation of the exact mechanisms underlying

this synergistic effect still needs to be completed. This thesis filled this gap by testing the phenomenon using full-atom and coarse-grained MD simulations ¹⁶⁹.

1.6.2 Therapeutic nanobodies targeting SARS-CoV-2

Nb is a recombinantly produced, antigen-specific, single-domain Ab segment derived from camelid heavy chain Ab. Nb is a relatively recent addition to recombinant Ab and is derived from animals such as camels, llamas, and alpacas ¹⁷⁰⁻¹⁷¹ (Figure 1.6B). While Nb lacks a light chain, which can be seen as a disadvantage in terms of antigen binding, they possess intriguing properties. These properties include higher solubility, smaller size, greater resistance to denaturation under certain conditions, and increased thermal and chemical stability compared to conventional Ab. Nb can be administered directly into the respiratory tract, the most common site of SARS-CoV-2 infection, using an inhaler ¹⁷²⁻¹⁷³. For example, camelid Nb composed solely of heavy chains, known as VHH, could provide a cost-effective and straightforward method for producing antiviral agents for passive immunization. Furthermore, Nb exhibits better tissue penetration and extravasation compared to classical Ab, enhancing its therapeutic potential ¹⁷⁴. Nb is classified as naïve or synthetic. Naïve Nb is extracted from camelids, including llamas, alpacas, camels, and dromedaries ¹⁷⁵. Synthetic Nbs are produced through various methods and are available in different libraries, such as the humanized synthetic Nb library and the display of synthetic Nb on the yeast surface ¹⁷⁶⁻¹⁷⁷.

Nb therapy has emerged as a promising approach for treating COVID-19 ¹⁷³. Nb targets RBD, disrupting the interaction between the S protein and ACE2, and thus inhibiting the virus's entry into host cells ¹⁷⁸⁻¹⁸⁰ (Figure 1.6D). Nb exhibits a high-affinity neutralization of SARS-CoV-2, making RBD an attractive target for vaccine development ¹⁸¹. Although synthetic Nb shows a high affinity for the prefusion S glycoprotein and exhibits strong neutralizing activities ¹⁸², it has certain limitations that can hinder its ability to meet therapeutic requirements. Nb can bind to two RBD domains: one with a binding epitope that overlaps with ACE2 binding region and another with a non-overlapping binding epitope that does not intersect with ACE2 binding region. Like Abs, Nbs also targets both open and closed RBDs. Additionally, Nb provides a rapid avenue for exploiting avidity, thus enhancing affinity and efficacy in the treatment of COVID-19 ¹⁸³⁻¹⁸⁴. Given that SARS-CoV-2 is prone to rapid mutations that can evade most potential Abs, Nb stands out as a promising candidate to address dangerous variants, such as Alpha, Beta, Gamma, Delta, and others ¹⁸⁵⁻¹⁸⁷. When combined with Nb or Ab, Nb can significantly enhance neutralizing activity, offering a more effective approach to the treatment of SARS-CoV-2 ¹⁷⁶.

Many Nbs have been identified, but only a few, such as HH1-H4, H11-D4, and Ty1 have demonstrated significant potential for COVID-19 treatment. These Nbs target RBD and disrupt its interaction with ACE2. Through experimental studies, Huo *et al.* ¹⁷⁶ disclosed that Nbs H11-D4 and H11-H4, bind to RBD with high affinity, preventing the S protein from attaching to ACE2. In another study, Hanke *et al.* ¹⁸⁸ found Ty1, RBD-specific Nb that effectively neutralizes SARS-CoV-2. Ty1 offers several practical advantages, including high-yield bacterial production, low cost, and scalability. Ty1 blocks the binding of RBD to ACE2 through steric exclusion by overlapping with the ACE2 binding site, while H11-H4 and H11-

D4 attach to different regions of RBD and do not overlap with the ACE2 site. Using MD simulation, Golcuk *et al.*¹⁸⁹ showed that H11-H4 can displace ACE2 from RBD due to repulsive electrostatic interactions, as both H11-H4 and ACE2 have similarly charged residues in close proximity when bound to RBD. H11-D4 also inhibits ACE2 binding, although to a lesser extent. Thus, H11-H4 and Ty1 disrupt ACE2 binding through different mechanisms, while H11-D4 is the least effective inhibitor among them. For SARS-CoV-2 variants, the ability of H11-H4 to disrupt ACE2 binding was diminished by the N501Y/E484K/K417N mutations in the Beta variant RBD, while H11-D4 was much less effective in preventing ACE2 binding to RBD with these triple mutations. In contrast, Ty1 binds to the Beta variant with twice the strength of ACE2, allowing it to neutralize this variant by sterically blocking ACE2 binding. For the Delta variant, the L452R mutation at H11-H4, H11-D4, and Ty1 binding interface may not affect their binding affinity^{188, 190-191}. While the impact of H11-H4 on RBD has been observed, theoretical studies have yet to explore the combination of H11-H4 with CR3022 Ab for treating SARS-CoV-2. To address this, we hypothesized that combining H11-H4 with CR3022 Ab could enhance the neutralizing ability against SARS-CoV-2, potentially leading to a new treatment for COVID-19. This hypothesis was validated through all-atom and coarse-grained MD simulations¹⁹².

1.7 Impact of SARS-CoV-2 on protein synthesis process

Upon entering host cells, ORF1a and ORF1b undergo translation and subsequent proteolytic processing mediated by virus-encoded proteinases. This process yields a functional NSP, which plays a pivotal role in viral infection and replication of the RNA genome¹⁹³. NSP encompasses numerous indispensable enzymes involved in RNA processing and viral replication¹⁹⁴⁻¹⁹⁵.

40S ribosome is responsible for mediating the interaction between mRNA codons and transfer RNA anti-codons, which facilitate the transfer of amino acids to form polypeptides¹⁹⁶. In the absence of NSP1, mRNA translation proceeds normally, leading to protein synthesis. In the presence of NSP1, it binds to the mRNA entry channel, folds into two helices, and interacts with the 18S ribosomal RNA (rRNA) at h18 as well as with the 40S ribosomal protein (rprotein) uS3 in the head and uS5 and eS30 in the body, where SARS-CoV-2 NSP1 would partially overlap with fully accommodated mRNA¹⁹⁷. Here, only the C-terminal domain binds to the 40S subunit of the human ribosome, leading to inhibition of mRNA translation⁵⁴ (Figure 1.7). Although this finding provides valuable information on the role of SARS-CoV-2 in invading and subverting human cells, the precise impact of binding of NSP1 to the 40S ribosome on mRNA translation remains unclear.

Some studies reported that several mutations in NSP1 can alter its structural and functional characteristics concerning SARS-CoV-2. The double mutation K164A/H165A within the C-terminal domain eliminates its ability to bind to the 40S ribosome¹⁹⁸⁻¹⁹⁹. Mutations such as Y154A/F157A and R171E/R175E also result in the loss of ribosome binding capability. In the linker domain, mutations R124A/K124A impair mRNA endonucleolytic cleavage guided by NSP1. The R99A mutation, located in the N-terminal domain, not only abolishes NSP1 evasion but also hinders NSP1-guided mRNA cleavage⁵⁵.

Even a minor deletion of essential amino acids within NSP1 is sufficient to nullify its evasion function²⁰⁰⁻²⁰¹. In addition, some drugs targeting NSP1 were found and have shown promise as potential candidates for antiviral therapy against SARS-CoV-2. For example, montelukast sodium hydrate, an FDA-approved drug, binds to the C-terminal of NSP1, reducing its inhibitory effect on host protein synthesis²⁰²⁻²⁰³.

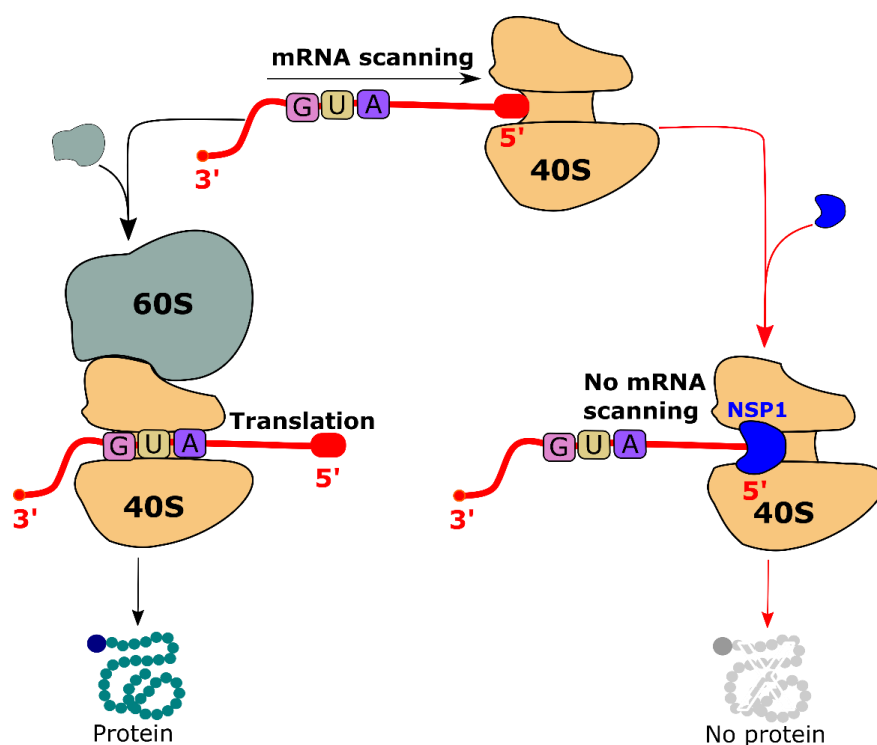


Figure 1.7: A schematic depicting (left) an mRNA translation process occurring in normal human ribosomes to synthesize protein, and (right) NSP1 action to suppress mRNA translation.

Recently, Borišek *et al.*¹⁹⁷ used MD simulation to investigate the interaction of SARS-CoV-2 NSP1 and SARS-CoV NSP1 with the 40S subunit of the ribosome. They found that binding of SARS-CoV-2 NSP1 and SARS-CoV NSP1 to the 40S subunit causes a critical switch in the residues Gln158/Glu158 and Glu159/Gln159. This switch remodels the pattern of interaction between NSP1 and neighboring rproteins (uS3 and uS5), as well as rRNA (h18) that surrounds the mRNA entry tunnel. This finding provides a clear picture of how SARS-CoV-2 invades human cells. However, the effect of SARS-CoV-2 NSP1 binding to the 40S ribosome on mRNA translation has not been theoretically studied, which prompted us to investigate this issue²⁰⁴. We hypothesized that NSP1 binding increases the binding affinity of mRNA to its entry channel, leading to the arrest of its translation and hence protein synthesis. This hypothesis was confirmed by our all-atom and coarse-grained MD simulations²⁰⁴.

Chapter 2: Computational methods

2.1 A general introduction to molecular dynamics simulation

Computer simulations serve as a bridge between microscopic length and time scales and the macroscopic world. Currently, two primary families of simulation techniques are prevalent: MD and Monte Carlo simulations. MD simulations play a crucial role as a tool for studying biomolecules and biomaterials. In the following sections, we characterize the classical MD simulation by describing its two main components: (1) Numerical schemes for integrating equations of motion are used to obtain the classical trajectories of the studied system in phase space, and (2) force fields are simplified energy expressions that enable the rapid evaluation of forces acting within the system during the simulation.

2.1.1 Dynamics of a molecular system

MD simulation is a computational method for studying molecular systems using computers. In MD simulation, trajectories of atoms are obtained using the Langevin equation along with numerical methods to simulate mainly many body systems. These simulations are of particular interest to chemists and biologists²⁰⁵.

The stochastic differential Langevin equation is similar to Newton's equation, but the friction and noise terms are added as follows.

$$m \frac{d^2 \vec{r}}{dt^2} = \vec{F}_c - \gamma \frac{d\vec{r}}{dt} + \vec{\Gamma} \equiv \vec{F} \quad (1)$$

m is mass of atom, γ is the friction coefficient, and $\vec{F}_c = \nabla V$, here V is potential of a biomolecular system. Random force $\vec{\Gamma}$ related to random interactions between atoms of the system and environment is noise, which is described as below.

$$\begin{aligned} \langle \Gamma(t) \rangle &= 0 \\ \langle \Gamma(t) \Gamma(t') \rangle &= 2\gamma k_B T \delta(t - t') \end{aligned} \quad (2)$$

where k_B is a Boltzmann's constant, T is absolute temperature, and $\delta(t - t')$ is the Dirac delta function.

The motion Eq. (1) can be solved by using the leap-frog algorithm, or the original Verlet, or the velocity Verlet algorithms²⁰⁶⁻²⁰⁸. During MD simulation, the length of all bonds associated with hydrogen atoms can be constrained by the SHAKE or the LINCS (or P-LINCS) algorithms²⁰⁹⁻²¹¹. The temperature is maintained through the Langevin thermostat²¹² with a collision frequency (often 2 ps^{-1}). A cutoff point is chosen to calculate van der Waals (vdW) and electrostatic interactions, in which the particle mesh Ewald method is applied for electrostatic interaction²¹³. The simulation box is chosen large enough to avoid interaction with the periodic images, and size effects are minimized by applying periodic boundary conditions. Counterions are added to neutralize the system.

2.1.2 Force fields for all-atom and coarse-grained models

In this thesis, both all-atom and coarse-grained models are utilized to explore the kinetics and thermodynamics of biomolecular systems.

a) All-atom model: The all-atom model is used to investigate the structures and properties of biomolecules at the atomistic level (Figure 2.1A). This approach allows for direct exploration of time scales ranging from tens to thousands of nanoseconds and length scales of up to tens of nanometers. In all-atom MD simulations, interactions between particles are calculated using an energy function known as a force field, which encompasses bonded and non-bonded interactions²¹⁴. Some force fields commonly used in all-atom MD simulations of biomolecules include OPLS, AMBER, CHARMM, and GROMOS²¹⁵. The general form of the force field for MD simulation is as follows^{211, 216}.

$$V = \sum_{bonds} k_b(r - r_0)^2 + \sum_{angles} k_\theta(\theta - \theta_0)^2 + \sum_{n=1, dihedrals}^3 k_n[1 + \cos(n\omega - \gamma_n)] + \sum_{i,j} \left[\left(\frac{A_{ij}}{r_{ij}^{12}} \right) - \left(\frac{B_{ij}}{r_{ij}^6} \right) \right] + \sum_{i,j} \frac{q_i q_j}{4\pi\epsilon_0 r_{ij}} \quad (3)$$

Here the first two terms of the Eq. (3) describe the bonded potential between two and three particles, which are modeled using harmonic functions with force constants k_b , k_θ and equilibrium values of r_0 , θ_0 , respectively. The third term represents the dihedral potential between four points, where k_n is the dihedral force constant, n is dihedral periodicity and γ_n is a phase of the dihedral angle. The last two terms describe the non-bonded potentials, including the vdW interaction represented by the 6-12 Lennard-Jones (LJ) function and the electrostatic interactions modeled by Coulombic interactions. The choice of different parameters in Eq. (3) depends on the force fields developed by different groups.

b) Coarse-grained models: Coarse-grained models have been developed to address certain challenges of larger biomolecular systems. They consolidate multiple atoms into a single interaction center, which not only saves computational time and resources but also often produces results that agree well with all-atom MD simulations and experimental data²¹⁷. Several common models, such as multiscale²¹⁸⁻²¹⁹, C_α -based²²⁰⁻²²¹, Martini^{219, 222-223}, UNRES coarse-graining²²⁴⁻²²⁵, and others are widely employed to investigate the dynamical properties of biomolecular systems.

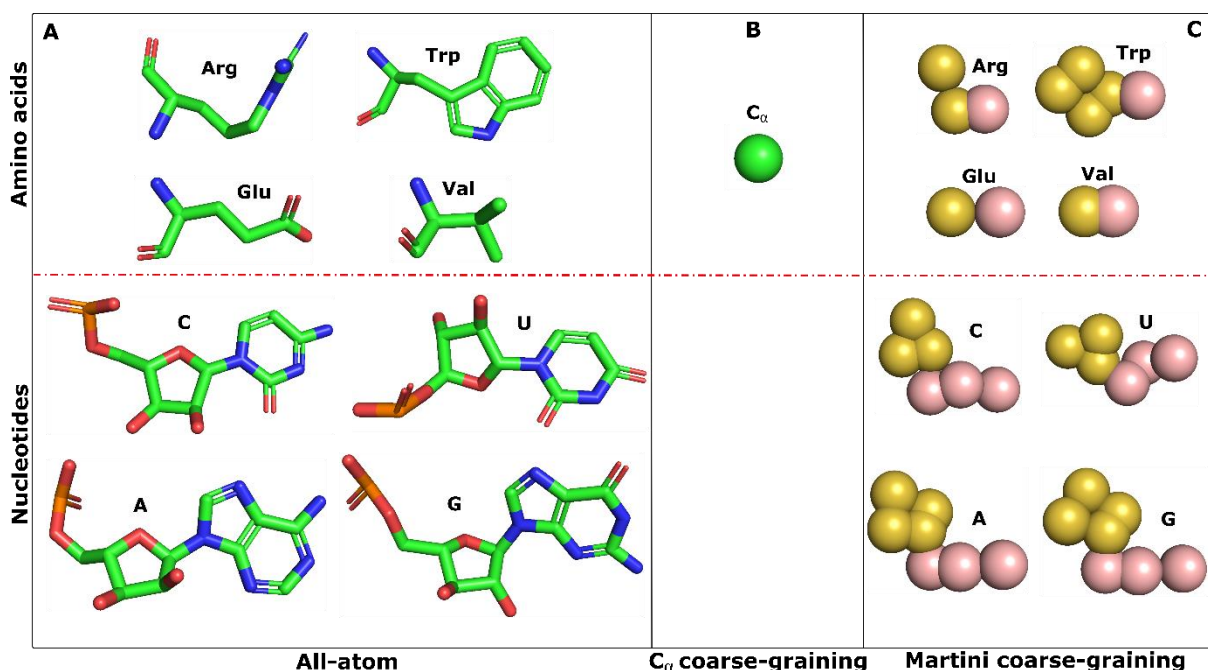
- *C_α coarse-grained model:* In this model, each residue is presented by one interaction site centered on the C_α atom (Figure 2.1B). There are many coarse-grained models, but in this dissertation, we used a Go-like model in which the potential energy for a given configuration is given by the following expression²²⁰⁻²²¹.

$$\begin{aligned}
V = & \sum_i k_b (r_i - r_0)^2 \\
& + \sum_i -\frac{1}{\gamma} \ln \left\{ \exp[-\gamma(k_\alpha(\theta_i - \theta_\alpha)^2 + \varepsilon_\alpha)] + \exp[-\gamma k_\beta(\theta_i - \theta_\beta)^2] \right\} \\
& + \sum_i \sum_j^4 k_{D_j} (1 + \cos[j\varphi_i - \delta_j]) + \sum_{ij} \frac{q_i q_j e^2}{4\pi \varepsilon_0 \varepsilon_r r_{ij}} \exp\left[-\frac{r_{ij}}{l_D}\right] \\
& + \sum_{ij \in \{NC\}} \varepsilon_{ij}^{NC} \left[13 \left(\frac{\sigma_{ij}}{r_{ij}}\right)^{12} - 18 \left(\frac{\sigma_{ij}}{r_{ij}}\right)^{10} + 4 \left(\frac{\sigma_{ij}}{r_{ij}}\right)^6 \right] \\
& + \sum_{ij \notin \{NC\}} \varepsilon_{ij}^{NN} \left[13 \left(\frac{\sigma_{ij}}{r_{ij}}\right)^{12} - 18 \left(\frac{\sigma_{ij}}{r_{ij}}\right)^{10} + 4 \left(\frac{\sigma_{ij}}{r_{ij}}\right)^6 \right] \tag{4}
\end{aligned}$$

These terms correspond to the energy contributions of $C_\alpha - C_\alpha$ bonds, bond angles, dihedral angles, electrostatic interactions, and LJ like attractive and repulsive interactions for both native and non-native contacts. Specifically, the bond potential between two adjacent interaction sites is described by a harmonic potential with a bond force constant k_b , an equilibrium bond length r_0 , and a pseudo bond length r_i for the i^{th} bond. The angle potential is modeled by a double-well potential, which describes the bond angle associated with bond α -helix and β -sheet conformations²²⁶. The constants of the double-well angle potential include γ , k_α , θ_α , ε_α , k_β , and θ_β . The k_{D_j} and δ_j are the dihedral force constant and the phase at periodicity j , respectively. The φ_i is the i^{th} pseudo dihedral angle. Electrostatics are treated using the Debye-Hückel theory with a Debye length l_D and a dielectric constant of 78.5. Lysine and arginine C_α sites are assigned $q = +e$, glutamic acid and aspartic acid are assigned $q = -e$, and all other interaction sites are uncharged²²⁷. The contribution from native interactions is computed using the 12-10-6 potential of Karanicolas and Brooks²²⁸, with the depth of the energy minimum for a native contact $\varepsilon_{ij}^{NC} = n_{ij}\varepsilon_{HB} + \eta\varepsilon_{ij}$, where ε_{HB} and ε_{ij} represent energetic contributions arising from hydrogen bonding and vdW contacts between residues i and j identified from the crystal structure of the protein, respectively. n_{ij} is the number of hydrogen bonds formed between residues i and j . The value of ε_{ij} is set based on the Betancourt-Thirumalai pairwise potential²²⁹, while the scaling factor η is determined for each protein based on a previously published training set to reproduce realistic protein stabilities for different structural classes. In this work, the values of η for intra-protein interactions in Abs (REGN10933 and REGN10987) and RBD domain (Table 2.1), were obtained using a procedure described in a previously published training set²³⁰. Collision diameters σ_{ij} between C_α interaction sites involved in native contacts are set equal to the distance between the C_α of the corresponding residues in the crystal structure divided by $2^{\frac{1}{6}}$. For non-native interactions, ε_{ij}^{NN} , and σ_{ij} are set to the average of the radii of the residues involved²²⁸. NC and NN stand for native contact and non-native contact, respectively²²⁰.

Table 2.1: The η values determined for the stability of protein domains and interfaces, as well as for interactions between Abs and RBD in C_α coarse-grained simulations.

	REGN10933	REGN10987	RBD	REGN10933-RBD	REGN10987-RBD
η	Chain L: 2.480 Chain H: 2.480 Interface: 2.124	Chain L: 2.480 Chain H: 2.480 Interface: 2.124	1.916	1.9	1.9

**Figure 2.1:** 3D structures of several amino acids (Trp, Arg, Glu, and Val) and nucleotides (C, U, A, and G). A) All-atom model with N atoms in blue, O atoms in red, C atoms in green, and P atoms in orange; B) C_α coarse-grained model for only amino acids, highlighting the C_α atom in green; and C) Martini coarse-grained model with backbone beads in light pink and side chain beads in wheat. The number of side chain beads varies depending on the type of amino acid and nucleotide.

- *Martini coarse-grained model:* The Martini model uses a four-to-one mapping, where, on average, four heavy atoms are represented by a single interaction center, except in the case of ring-like molecules. The general four-to-one mapping rule is insufficient for capturing the geometric specificity of small ring-like fragments or molecules. Therefore, ring-like molecules are mapped with a higher resolution. The model accounts for four primary types of interaction sites: polar (P), nonpolar (N), apolar (C), and charged (Q). Subtypes within a primary type are differentiated either by a letter indicating hydrogen-bonding capabilities or by a number representing the level of polarity²³¹⁻²³².

In the coarse-grained model for proteins, most amino acids are mapped to specific bead types. Apolar amino acids (Leu, Pro, Ile, Val, Cys, and Met) are represented by C-type

beads, while polar uncharged amino acids (Thr, Ser, Asn, and Gln) are mapped to P-type beads. Amino acids with small negatively charged side chains, such as Glu and Asp, are modeled as Q-type beads. Positively charged amino acids, Arg and Lys, are represented by a combination of a Q-type bead and an uncharged bead. Larger, ring-based side chains (His, Phe, and Tyr) are depicted using three ring-class beads, and Trp is represented by four. Gly and Ala residues are only represented by the backbone bead. The type of backbone varies depending on the secondary structure of the protein: in solution, or in a coil or bend, the backbone is strongly polar (P-type), whereas in a helix or β -strand, the inter-backbone hydrogen bonds greatly reduce its polarity (N-type) (Figure 2.1C) ²³¹.

Concerning the coarse-grained model for RNA, the RNA parameters were developed following the Martini parameterization strategy, which integrates top-down and bottom-up approaches ²³². Each nucleotide is represented by six or seven coarse-grained beads. The backbone is modeled using three beads, with the phosphate mapped to one bead and the sugar mapped to two beads. The pyrimidines (cytosine and uracil) are represented as three-bead rings, and the purines (adenine and guanine) as four-bead rings. For each nucleotide, the beads are divided into backbone beads (BB1, BB2, and BB3) and side chain beads (SC1, SC2, SC3, and SC4 for the purines). The first backbone bead (BB1) is the phosphate, and the last one (BB3) is the 3' end of the sugar. For the side chains, the beads are defined in cyclical order so that SC1 is attached to the backbone, and in dsRNA, the SC2 and SC3 beads would be base pairs with the opposing strand (Figure 2.1C) ²³²⁻²³³.

The energy of the Martini model is given by the following formula ^{219, 222-223}.

$$\begin{aligned}
 V = & \frac{1}{2} \sum_{ij} k_b (r_{ij} - r_b)^2 + \frac{1}{2} \sum_{ijk} k_a (\cos(\theta_{ijk}) - \cos(\theta_a))^2 \\
 & + \sum_{ijkl} k_d [1 + \cos(n\varphi_{ijkl} - \varphi_d)] + \sum_{ijkl} k_{id} (\varphi_{ijkl} - \varphi_{id})^2 \\
 & + \sum_{ij} 4\varepsilon_{ij} \left[\left(\frac{\sigma_{ij}}{r_{ij}} \right)^{12} - \left(\frac{\sigma_{ij}}{r_{ij}} \right)^6 \right] + \sum_{ij} \frac{q_i q_j}{4\pi\varepsilon_0\varepsilon_r r_{ij}}
 \end{aligned} \quad (5)$$

It includes the two-body harmonic, three-body angular, and four-body dihedral angle potentials. The non-bonded interactions in the Martini force field are based on the shifted and truncated 12-6 LJ and Coulomb potentials. For bonded interactions, the forces act between bonded sites i, j, k, l with equilibrium distance r_b , angle θ_a , and dihedral angles φ_d and φ_{id} . The force constants k_b , k_a , k_d and k_{id} are generally weak, including molecular flexibility at the coarse-grained level as a result of collective motions at the fine-grained level. The bonded potential is used for chemically bonded sites, and the angle potential represents the stiffness of the chain. Proper dihedral angle potential is currently used only to impose the secondary structure of the peptide backbone, while improper dihedral angle potential is used to prevent out-of-plane distortions of planar groups. For non-bonded interactions, the strength of LJ interactions of all particle pairs i and j at distance r_{ij} determines by the well-depth ε_{ij} , depending on the interacting particle types. The effective size of the particles is governed by the LJ parameter σ_{ij} . In addition to the LJ interaction, charged groups (type Q) bearing a

charge q_i and q_j interact via a Coulombic energy function with a relative dielectric constant ϵ_r for explicit screening^{223, 231-232}.

The Martini model enables long simulations of large systems by reducing the number of degrees of freedom compared to all-atom models. However, one limitation of the Martini model is its use of an elastic network model for keeping the tertiary structure of protein/RNA, which is incapable of folding protein/RNA, which may introduce artificial stiffness that could affect free energy calculations. This issue requires further study, but despite this limitation, the free energy estimates obtained with the coarse-grained Martini model generally agree well with experimental results²³⁴⁻²³⁵.

2.2 Experimental and computational methods for estimation of binding affinity

The determination of binding affinity in biomolecular systems is of great interest, primarily because it can be used to characterize the stability of biomolecular complexes²³⁶. Currently, several experimental methods have been developed to investigate the binding affinity of two interacting molecules, including atomic force microscopy (AFM)²³⁷, surface plasmon resonance (SPR)²³⁸, biolayer interferometry (BLI)²³⁹, fluorescence flow cytometry (FFC)²⁴⁰, dynamic force spectroscopy (DFS)²⁴¹, *et al.* In parallel, various computational methods have been developed to evaluate the binding affinity, such as thermodynamic integration (TI)²⁴², free energy perturbation (FEP)²⁴³, molecular mechanics with Poisson-Boltzmann or generalized born and surface area (MM-PBSA and MM-GBSA)²⁴⁴, linear interaction energy (LIE)²⁴⁵, steered molecular dynamics (SMD)²⁴⁶, umbrella sampling (US)²⁴⁷, and others.

2.2.1 Experimental methods for estimating binding affinity

a) AFM: AFM is extensively utilized to study the structure and function of biomolecules and their interactions in response to external forces. AFM is particularly effective for examining the binding properties of protein-ligand and protein-protein complexes, as the mechanical force required for their dissociation is approximately 100 pN. When an external force is applied to the target protein through the cantilever, the force experienced by the protein is calculated as $k\delta x$, where k represents the stiffness of the cantilever, and δx is the bending of the cantilever detected by the laser. The stability of the complex is characterized by the rupture force ($F_{\max}$), observed in the force-extension/time profile obtained with a constant pulling speed. A higher $F_{\max}$ indicates greater binding affinity²³⁷.

b) Other experimental methods: Several experimental techniques, such as SPR²³⁸, BLI²³⁹, FFC²⁴⁰, and DFS²⁴¹, have been employed to examine the stability of protein-protein complexes. These methods are used to determine dissociation constant (K_D), where a lower K_D indicates a higher binding affinity and greater stability of the complex. Both SPR and BLI methods are commonly used to estimate the K_D .

- *SPR:* SPR is an optical technique used to measure molecular interactions in real-time. It occurs when plane-polarized light hits a metal film under conditions of total internal reflection. The SPR signal depends directly on the refractive index of the medium on the

sensor chip. The binding of biomolecules alters the refractive index on the sensor surface. In an SPR experiment, one molecule (protein or ligand) is immobilized on a sensor chip, and binding to a second molecule (analyte) is measured under flow conditions. The response is measured in resonance units and is proportional to the mass on the surface; for any given interactant, the response is proportional to the number of molecules bound to the surface. This response is recorded and displayed as a sensorgram in real-time. SPR experiments can be used to measure kinetic binding constants²³⁸.

- *BLI*: BLI is an optical method used to measure macromolecular interactions by examining the interference patterns of white light reflected from the surface of a biosensor tip. BLI experiments are used to determine the kinetics and affinity of molecular interactions. In a BLI experiment, one molecule is immobilized on a dip and read biosensor, and binding to a second molecule is measured. A change in the number of molecules bound to the end of the biosensor tip causes a shift in the interference pattern, which is measured in real-time²³⁹.

2.2.2 Computational methods for estimating binding affinity

a) *SMD simulation*: SMD²⁴⁶ was designed to capture single-molecule force spectroscopy experiments, including AFM²⁴⁸, laser optical tweezers²⁴⁹, and magnetic tweezers²⁵⁰. In SMD simulations, an external force is applied to a dummy moving with constant speed v in the pulling direction (Figure 2.2). This dummy atom is connected with the pulled atom of the studied system through a spring with a spring constant k . If we define Δz as the displacement of the pulled atoms from its initial position, then the external force experienced by the system F is.

$$F = k(\Delta z - vt) \quad (6)$$

A typical force-displacement or force-time profile has the $F_{\max}$ (Figure 2.2), which can be used to characterize the mechanical stability of the biomolecular complex. It has been shown²⁵¹ that the non-equilibrium work (W) performed by an extended chain characterizes the mechanical stability better than $F_{\max}$. Using the force-displacement profile obtained from the SMD simulations, W is estimated using the trapezoidal rule.

$$W = \int F dz = \sum_{i=1}^N \frac{F_{i+1} + F_i}{2} (z_{i+1} - z_i) \quad (7)$$

where N is the number of simulation steps, F_i and z_i are the force experienced by the target and position at step i , respectively.

To estimate the non-equilibrium binding free energy (ΔG), we can use Jarzynski's equality²⁵² extended to the case of when the applied external force grows at a constant speed v ²⁵³.

$$\exp\left(\frac{-\Delta G}{k_B T}\right) = \left\langle \exp\left(\frac{W_t - \frac{1}{2}k(z_t - vt)^2}{k_B T}\right) \right\rangle_N \quad (8)$$

here $\langle \dots \rangle_N$ is the average over N trajectories, z_t is the time-dependent displacement, and W_t is the non-equilibrium work at time t determined by the Eq. (7).

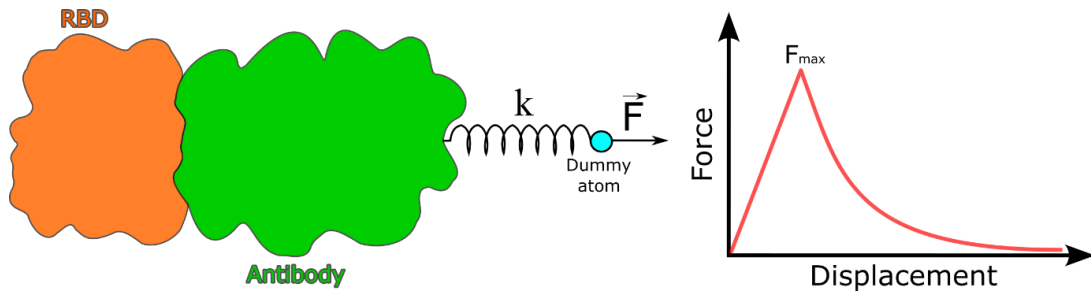


Figure 2.2: (Left) Schematic of SMD simulations of pulling Ab from RBD. (Right) Force-displacement profile from SMD simulations.

From the Eq. (8), we can extract the equilibrium free energy provided that the number of SMD trajectories is sufficiently large and the pulling is conducted at a slow enough rate. This approach proves to be feasible for small systems²⁵⁴ but not for larger ones. Nevertheless, we can estimate the non-equilibrium binding and unbinding barriers that delineate the transition state (TS) from the bound state at $t = 0$ and the unbound state at t_{end} ²⁵⁵. This estimation enables us to distinguish between weak binding and strong binding.

b) US simulation: US is a technique employed to compute the potential of mean force along a predefined reaction coordinate ξ . The primary objective of the US is to surmount the limitations commonly encountered in conventional MD simulations, which are often constrained by limited simulation time and face challenges in exploring infrequent events due to elevated energy barriers. The US method involves the introduction of a harmonic biasing potential that generates a series of configurations along the reaction coordinate. Some of these configurations serve as initial states for different US windows, each executed through independent simulations (Figure 2.3). In this work, we utilized coarse-grained US simulations to estimate the binding affinities of biomolecular systems.

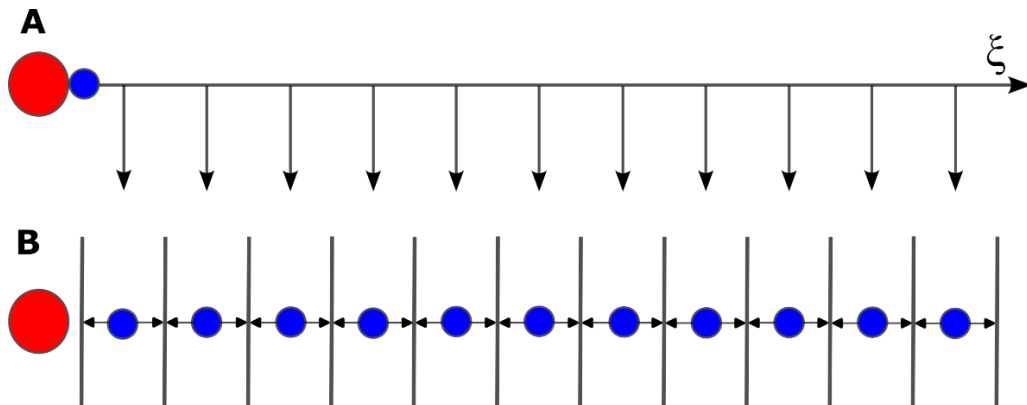


Figure 2.3: Schematic of US simulations to calculate the potential of mean force along the reaction coordinate. The first molecule (red) is kept fixed. A) The reaction coordinate is divided into small regions, and B) each region is sampled independently. To keep the second molecule (blue) to move within a spatial window, a harmonic potential is applied.

Subsequently, the weighted histogram analysis method procedure²⁵⁶ is used to obtain the one-dimensional potential of mean force (1D-PMF) as a function of the reaction coordinate. Standard error calculations and auto-correlations in the sampled time series are also taken into account.

- *Estimation of K_D value from US simulations:* K_D is calculated as^{165, 257}.

$$K_D = \frac{P_u}{P_b} [A] \quad (9)$$

$[A]$ represents the concentration of the free cofactor, in unbound state. It is defined as.

$$[A] = \frac{P_u}{V(r^*)} C_0 \quad (10)$$

where $C_0 = 1660$ is standard concentration used to normalize $[A]$ to the units of molarity, $V(r^*) = (4/3)\pi r^{*3}$ is the simulation volume in which we found free monomers in the unbound state. P_u is the probability of the system being in the unbound state, $P_u = 1 - P_b$. The bound state probability P_b is calculated from numerical integration of the 1D-PMF profile as.

$$P_b = \frac{\int_0^{r_b} 4\pi r^2 e^{-\beta G_{1D}(r)} dr}{\int_0^{r^*} 4\pi r^2 e^{-\beta G_{1D}(r)} dr} \quad (11)$$

here $G_{1D}(r)$ is the 1D-PMF, r_b is the distance threshold separating bound and unbound states, $\beta = 1/k_B T$, and r^* is the maximum distance between unbound cofactors found during the simulation process.

- *Estimation of binding free energy (ΔG_{bind}) from US simulations:* ΔG_{bind} is defined as the difference between the free energies in the bound and unbound states²⁵⁸.

$$\Delta G_{bind} = \left(-k_B T \ln \int^{bound} e^{\frac{-G_{1D}(r)}{k_B T}} \right) - \left(-k_B T \ln \int^{unbound} e^{\frac{-G_{1D}(r)}{k_B T}} \right) \quad (12)$$

here $G_{1D}(r)$ is the 1D-PMF as a function of r , k_B is the Boltzmann constant, and T is the absolute temperature. The symbols $\int^{bound}$ and $\int^{unbound}$ refer to summation over the bound and unbound regions, respectively.

c) Alchemical free energy calculations: Alchemical free energy calculations represent typical approaches that operate at the highest level of theoretical rigor and are also feasible with current computational capabilities. The alchemical method, known as the TI or FEP, relies on a derived nonphysical thermodynamic cycle. This cycle involves the calculation of the binding free energy as the sum of multiple steps during which the ligand/protein/DNA/RNA is "inserted" or "removed" from different environments, such as transitioning from the bound to the unbound state or vice versa²⁵⁹⁻²⁶⁰.

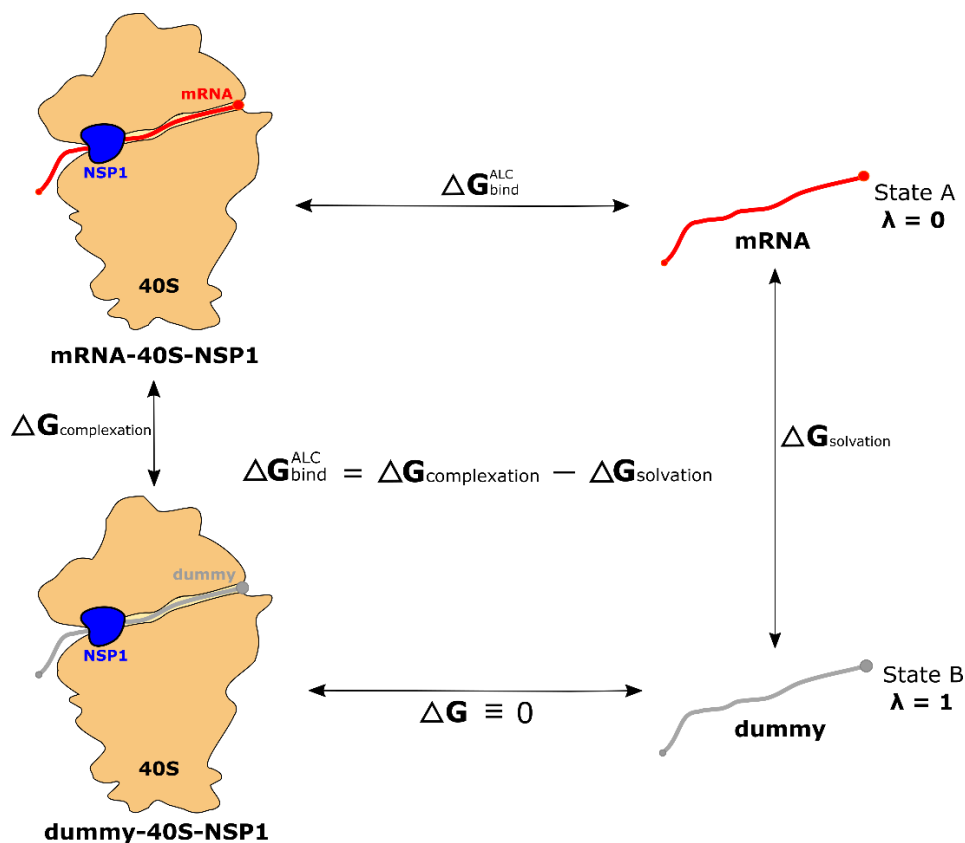


Figure 2.4: Thermodynamic cycle to calculate the binding free energy of mRNA to 40S-NSP1. State A ($\lambda = 0$) describes the full interaction between mRNA and 40S-NSP1, while state B ($\lambda = 1$) presents mRNA (dummy) without interaction with 40S-NSP1. Alchemical simulations are used in the Martini coarse-grained model.

The utilization of alchemical simulations has streamlined the process of conducting free energy calculations, making them more accessible than manually setting them up within MD simulations. Furthermore, this approach has demonstrated success in determining free energy differences in various scenarios: (1) Estimating the partition of a compound between different environments; (2) Assessing binding affinities of various biomolecular complexes (protein-ligand, protein-protein, protein-DNA/RNA) while modifying or mutating the protein or DNA/RNA; (3) Investigating how one or more mutations at the interface impact the binding of protein-protein or protein-DNA/RNA interactions ²⁶¹. In summary, alchemical methods allow for the computation of free energy differences, either in terms of relative free energies of binding or absolute free energies of binding ²⁶².

In this thesis, to evaluate the free energy associated with the binding of mRNA to the 40S ribosome both with and without NSP1, we formulate the thermodynamic cycle illustrated in Figure 2.4. Through this thermodynamic cycle, we can obtain the binding free energy $\Delta G_{\text{bind}}^{\text{ALC}}$ (the superscript ALC refers to alchemical simulation).

$$\Delta G_{\text{bind}}^{\text{ALC}} - \Delta G = \Delta G_{\text{complexation}} - \Delta G_{\text{solvation}} \quad (13)$$

where $\Delta G \equiv 0$ as is related to non-interacting ($\lambda=1$) mRNA being dummy and dummy-40S-NSP1. Then, the binding free energy has the following form.

$$\Delta G_{\text{bind}}^{\text{ALC}} = \Delta G_{\text{complexation}} - \Delta G_{\text{solvation}} \quad (14)$$

For alchemical transformations, we used a set of λ -values ranging from $\lambda = 0$ to $\lambda = 1$, where $\lambda = 0$ and $\lambda = 1$ correspond to a system with and without full interaction, respectively.

2.3 Analysis tools

We used Inkscape²⁶³ and Grace²⁶⁴ to plot figures from the data. PYMOL²⁶⁵ and VMD²⁶⁶ were utilized to visualize the biomolecular complexes. Modeller²⁶⁷ was used to add missing residues for protein and missing nucleic acids for RNA. SwissPDB Viewer²⁶⁸ and PDB Tools²⁶⁹ were used to add missing atoms for protein and RNA.

Chapter 3: Antibodies and nanobodies bind to SARS-CoV-2 spike protein, preventing SARS-CoV-2 from entering human cells

3.1 Cocktail of REGN antibodies binds more strongly to SARS-CoV-2 than its components, but the Omicron variant reduces its neutralizing ability

3.1.1 Introduction

mAb therapies for the treatment of COVID-19 have been shown to be highly effective in reducing virus load and alleviating symptoms when administered shortly after diagnosis²⁷⁰⁻²⁷¹. mAbs bind to the virus by targeting the S protein, which comprises the S1 and S2 subunits, thereby preventing SARS-CoV-2 from binding to the ACE2 receptor and inhibiting infection³⁴. The S1 subunit is often the primary target for the binding of mAb to both RBD and NTD²⁷². RBD-specific mAbs can be classified into four main classes, while NTD-specific mAbs typically focus on regions distant from RBD. It should be noted that ongoing research is actively exploring the discovery of mAbs that target the S2 subunit^{146, 273}.

Ab cocktails consisting of combinations of mAbs have shown promise in preventing viruses from evading neutralization *in vitro*^{138, 274}. One such example is a dual Ab cocktail called REGN-COV2, developed to combat SARS-CoV-2. This cocktail consists of two mAbs, REGN10933 and REGN10987, and has advanced to phase 2/3 clinical trials. REGN-COV2 is a therapeutic approach developed by Regeneron Pharmaceuticals, where both mAbs target RBD (Figure 3.1A)^{156, 158}. REGN10933 binds to the upper region of RBD, significantly overlapping with ACE2 binding site. In contrast, REGN10987 binds to a lateral region of RBD, distinct from REGN10933 epitope, and has no significant effect on ACE2 binding site¹⁵⁶. *In vitro* studies have shown that combining these two non-competing Abs provides protection against the rapid viral escape observed with either Ab alone¹⁵⁸. Subsequent studies have confirmed the efficacy of the combination-based approach, highlighting that REGN-COV2 retains its neutralization potency against SARS-CoV-2^{156, 158}.

Numerous experimental studies on SARS-CoV-2 variants, such as Alpha, Beta, Gamma, Delta, Lambda, Omicron, *etc.*, have demonstrated their increased ability to infect host cells and evade host immunity. This increased infectivity is often associated with enhanced binding affinity to ACE2 and reduced neutralizing capacity against most SARS-CoV-2's Abs^{114, 125, 275-281}. There are specific Abs capable of recognizing and binding to the S protein of these variants, thereby preventing the virus from entering human cells. One such promising approach involves the use of a mAb cocktail comprising REGN10933 and REGN10987, which has shown potential in neutralizing various variants of SARS-CoV-2, including Alpha, Gamma, Delta, and others²⁸²⁻²⁸³. However, the emergence of the Omicron variant in November 2021 has raised concerns about the effectiveness of mAb cocktails in treating COVID-19^{125, 280}. Therefore, gaining a comprehensive understanding of the molecular mechanisms underlying the activity of SARS-CoV-2 variants remains imperative for identifying appropriate and timely therapies for COVID-19.

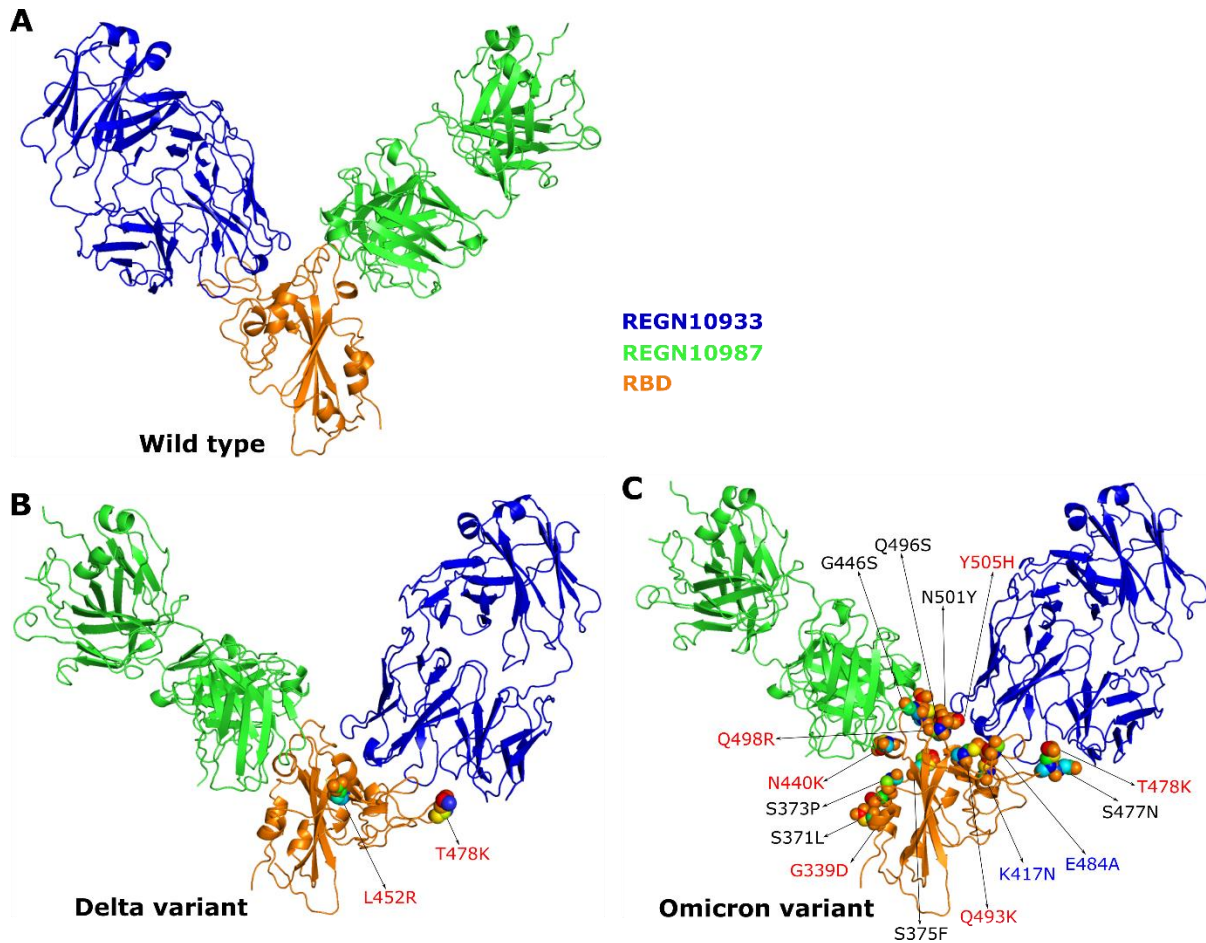


Figure 3.1: REGN10933 and REGN10987 in complex with RBD of A) wild type, B) Delta variant, and C) Omicron variant. Notably, mutations in the Omicron variant RBD are located in the binding regions for both components of REGN-COV2 cocktail. Mutations in the Delta variant RBD specifically affect REGN10933 binding site. Residues that carry a charge in the wild type RBD are shown in blue, while those that gain a charge after mutation are highlighted in red.

Despite the recognized importance of REGN-COV2 cocktail in the treatment of COVID-19, there is a noticeable lack of atomic-level studies on the structure and binding mechanisms of REGN-COV2 cocktail to RBD. In this work, we applied all-atom SMD and C_{α} coarse-grained US simulations to assess the binding affinity of REGN10933, REGN10987, and REGN10933+REGN10987 combination to RBD.

For SMD simulations, a pulling speed $v = 0.5$ nm/ns is used to complete the dissociation of REGN10933 or REGN10987 or RBD from the binding region. We considered only this pulling speed because the relative binding affinity of the complexes does not change between the different pulling speeds, as demonstrated by Nguyen *et al.*¹⁶⁵. The pulling direction of three systems is determined as follows: For REGN10933-RBD and REGN10987-RBD cases, an external force is applied to a dummy atom connected to the C_{α} atom closest to the Ab center of mass (CoM). The pulling direction is parallel to the vector connecting the CoMs of RBD and Ab. For the REGN10933+REGN10987-RBD complex, the pulling

direction is along the line connecting the CoM of RBD perpendicular to the line connecting the CoMs of REGN10933 and REGN10987. These complexes were then rotated to align the unbinding pathway of REGN10933-RBD, REGN10987-RBD, or REGN10933+REGN10987-RBD along the z-axis¹⁶⁹. We obtained $F_{\max} = 411.0$ and 318.3 pN for REGN10933-RBD and REGN10987-RBD, which are lower than that of REGN10933+REGN10987-RBD ($F_{\max} = 511.3$ pN). This results in a ranking of the binding affinities of REGN-COV2 Abs to RBD as follows: REGN10987 < REGN10933 < REGN10933+REGN10987. The association of REGN10987 with RBD was shown to be driven by vdW interactions, while electrostatic interactions dominate in the cases of REGN10933 and REGN10933+REGN10987.

For C_{α} coarse-grained US simulations, a total of 200 umbrella windows were generated by translating Ab in increments of 0.05 nm away from RBD along the vector connecting their two interface CoMs. A harmonic restraint was applied to maintain the Ab and RBD domain at target distances. The K_D values estimated from this approach for REGN10933 and REGN10987 binding to the RBD domain were 1.73 and 16.38 nM, respectively. This confirms that the binding affinity of REGN10933 is stronger than that of REGN10987, consistent with the all-atom SMD results.

Given the essential role of Delta and Omicron variants (Figure 3.1B&C) in viral infection, we further examined their interaction with REGN-COV2. Our all-atom SMD studies revealed that the binding affinity of REGN10933, REGN10987, and REGN10933+REGN10987 to the Delta variant remained nearly unchanged compared to the wild type. However, a significant decrease in interaction with the REGN-COV2 cocktail was observed for the Omicron variant, consistent with experimental observations^{125, 280}. This comprehensive analysis provides important mechanistic insights into the stability of these complexes, which may be instrumental in the development of Ab cocktail therapies for COVID-19.

3.1.2 Publication

a) Abstract: A promising approach to combat COVID-19 infections is the development of effective antiviral antibodies that target SARS-CoV-2 spike protein. Understanding the structures and molecular mechanisms underlying the binding of antibodies to SARS-CoV-2 can contribute to quickly achieving this goal. Recently, a cocktail of REGN10987 and REGN10933 antibodies was shown to be an excellent candidate for the treatment of COVID-19. Here, using all-atom steered molecular dynamics and coarse-grained umbrella sampling, we examine the interactions of the receptor-binding domain (RBD) of SARS-CoV-2 spike protein with REGN10987 and REGN10933 separately as well as together. Both computational methods show that REGN10933 binds to RBD more strongly than REGN10987. Importantly, the cocktail binds to RBD (simultaneous binding) more strongly than its components. The dissociation constants of REGN10987-RBD and REGN10933-RBD complexes calculated from the coarse-grained simulations are in good agreement with the experimental data. Thus, REGN10933 is probably a better candidate for treating COVID-19 than REGN10987, although the cocktail appears to neutralize the virus more efficiently than

REGN10933 or REGN10987 alone. The association of REGN10987 with RBD is driven by van der Waals interactions, while electrostatic interactions dominate in the case of REGN10933 and the cocktail. We also studied the effectiveness of these antibodies on the two most dangerous variants Delta and Omicron. Consistent with recent experimental reports, our results confirmed that the Omicron variant reduces the neutralizing activity of REGN10933, REGN10987, and REGN10933+REGN10987 with the K417N, N440K, L484A, and Q498R mutations playing a decisive role, while the Delta variant slightly changes their activity.

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STATEMENT

I declare that I am the co-author of the publication:

- Nguyen, H.; Pham, D. L.; Nissley, D. A.; O'Brien, E. P.; Li, M. S. **Cocktail of REGN antibodies binds more strongly to SARS-CoV-2 than its components, but the Omicron variant reduces its neutralizing ability.** J. Phys. Chem. B. **2022**, *126*(15), 2812-2823.

My contribution consisted of designing the research, performing all-atom simulations, analyzing the results, preparing figures, and writing the manuscript.

Warsaw, August 2024

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My contribution consisted of performing coarse-grained simulations, analyzing the results, preparing figures, and writing the manuscript.

Ho Chi Minh, July 2024

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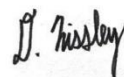
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My contribution consisted of interpreting the results and helping to write the manuscript.

Miami FL, July 2024



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My contribution consists of interpreting the results and assisting in writing the manuscript.

PA USA, July 2024

A handwritten signature in black ink that reads "Edward P. O'Brien".

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My contribution consisted of designing the research, interpreting the data, writing the manuscript, and supervising the overall project.

Warsaw, August 22 2024

Mai Suan Li

c) Publication for this section:

Cocktail of REGN antibodies binds more strongly to SARS-CoV-2 than its components, but the Omicron variant reduces its neutralizing ability

Hung Nguyen, Pham Dang Lan, Daniel A. Nissley, Edward P. O'Brien, and Mai Suan Li

J. Phys. Chem. B, 2022, 126, 2812-2823

Dostęp do artykułu „Cocktail of REGN Antibodies Binds More Strongly to SARS-CoV-2 Than Its Components, but the Omicron Variant Reduces Its Neutralizing Ability”

znajduje się tutaj: <https://doi.org/10.1021/acs.jpcb.2c00708>

3.2 Antibody-nanobody combination increases their neutralizing activity against SARS-CoV-2 and nanobody H11-H4 is effective against Alpha, Kappa and Delta variants

3.2.1 Introduction

mAb has emerged as a highly promising therapeutic class against SARS-CoV-2 infection¹⁴⁸. Numerous studies have provided evidence that convalescent plasma obtained from individuals who have recovered from SARS-CoV-2 infection, containing neutralizing Ab generated as part of the adaptive immune response, can significantly enhance patient survival rates²⁸⁴⁻²⁸⁶. However, plasma-based therapies based on plasma encounter significant limitations, primarily related to scalability. Therefore, the pursuit of potent Ab-based therapies on an industrial scale is increasingly recognized as one of the most viable strategies in the fight against SARS-CoV-2. As discussed in the previous chapter, the S protein plays a pivotal role in viral infection. This multifunctional molecular machine interacts with the ACE2 receptor in human cells and serves as a prime target to neutralize Ab. Consequently, it has become the focal point of therapeutic and vaccine development efforts²⁸⁷. Within the S protein, RBD and NTD located in the S1 subunit, along with FP in the S2 subunit, are considered particularly significant targets for combating SARS-CoV-2 infection.

Ab can neutralize SARS-CoV-2 by binding to various regions, such as RBD, or NTD, or FP. However, the majority of Ab has been found to primarily target RBD²⁸⁸⁻²⁸⁹, rendering it a pivotal focus. Unfortunately, varying experimental methods, conditions, and calibrations across different studies have yielded biased results concerning the binding affinity of Ab. This discrepancy has posed challenges in the development of Ab-based therapies for SARS-CoV-2²⁸⁸. For example, CR3022, an Ab, that originates from a convalescent patient with SARS-CoV-2, has shown potential due to its robust binding to RBD, with $K_D = 6.3$ nM, as reported by Tian *et al.*²⁹⁰, however, contrasting results have been reported in another study, indicating a much higher $K_D = 115$ nM, as documented by Yuan *et al.*²⁹¹. This disparity underscores the need for a comprehensive understanding of Ab interactions with components of SARS-CoV-2 to facilitate the development of effective therapies.

Nb offers an alternative to conventional Ab for applications in diagnostics and structural biology²⁹². Recently, they have gained prominence as therapeutic agents against SARS-CoV-2²⁹³⁻²⁹⁴. Notably, H11-H4, an Nb derived from llamas, demonstrates binding to RBD with $K_D = 11.8$ nM¹⁷⁶. This K_D value is higher than that reported by Tian *et al.*²⁹⁰ for CR3022, suggesting that H11-H4 exhibits weaker binding to RBD compared to CR3022. However, in comparison to the K_D value reported by Yuan *et al.*²⁹¹, it becomes evident that H11-H4 binds to RBD more strongly than CR3022. In addition, it is important to note that Nb can be used either alone or in combination with Ab for the treatment of severely ill COVID-19 patients (Figure 3.2A)¹⁷⁶. Although the binding affinity of Ab to SARS-CoV-2 has been computationally studied¹⁶⁵⁻¹⁶⁶, the binding free energy of Nb has not been calculated, despite molecular modeling studies exploring its interaction with RBD. Furthermore, no theoretical investigation of how the combination of Ab and Nb affects their neutralizing ability has been conducted.

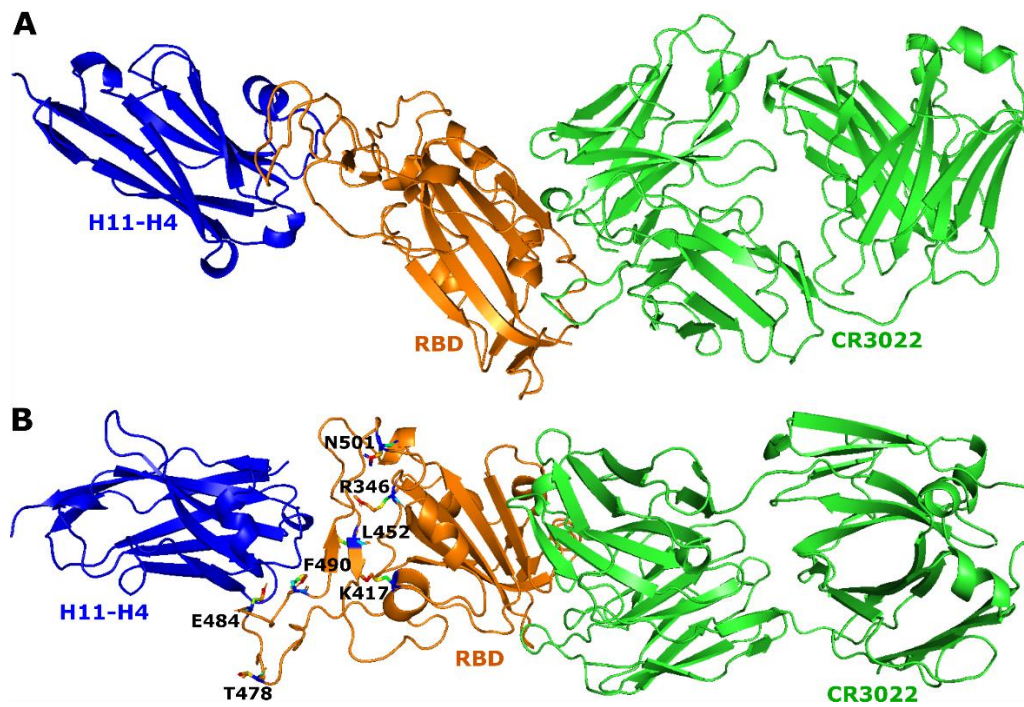


Figure 3.2: 3D structures of A) H11-H4 Nb and CR3022 Ab bound to RBD, and B) RBD mutations of variants Alpha, Beta, Gamma, Kappa, Delta, Lambda and Mu. They have contact with H11-H4, but not with CR3022.

Numerous experimental studies have been conducted on SARS-CoV-2 variants, including Alpha, Beta, Gamma, Kappa, Delta, Lambda, Omicrons, and others (Figure 3.2B) ^{114, 275-278, 295-296}. These studies have shown that many Abs and Nbs lose their neutralizing ability against SARS-CoV-2 due to these variants ^{279, 297}. However, recent studies showed that certain Ab and Nb can effectively neutralize most of these variants. For example, REGN10933 and REGN10987 Abs have demonstrated the ability to neutralize the Lambda variant, or Nbs derived from llamas have shown promise in combating the Delta variant ²⁹⁸⁻²⁹⁹.

In this study, we utilized all-atom SMD and Martini coarse-grained US simulations to estimate the binding affinity of CR3022 and H11-H4 with RBD, both individually and together.

For all-atom SMD simulations, the direction of H11-H4 or CR3022 pullout from the RBD binding region, as well as the direction of RBD pullout from the H11-H4 and CR3022 binding region, were determined. H11-H4–RBD and CR3022–RBD cases, an external force is applied to a dummy atom, which is linked to the C_{α} atom closest to the CoM of H11-H4 or CR3022. The pulling direction is aligned with the vector connecting the CoMs of RBD and Nb or Ab. For the H11-H4 + CR3022–RBD case, the pulling direction is selected differently due to the involvement of three molecules. An external force is applied to a dummy atom that is bonded to the C_{α} atom closest to RBD CoM, and the pulling direction is along the line connecting RBD CoM perpendicular to the line connecting CoMs of H11-H4 and CR3022.

¹⁹². At $v = 0.5$ nm/ns, the force-time profiles for the three complexes show that CR3022 ($F_{\max} = 1214.2 \pm 21.2$ pN) binds to RBD more strongly than H11-H4 ($F_{\max} = 925.6 \pm 15.2$ pN). When RBD is extracted simultaneously from both CR3022 and H11-H4, $F_{\max} = 2034.9 \pm 27.7$ pN, which is approximately twice as much as for CR3022–RBD and H11-H4–RBD individually. Note that although the rupture force $F_{\max}$ appears quite high due to fast pulling, the relative binding affinities of the complexes do not change with different pulling speeds ¹⁶⁵. This combination of CR3022 and H11-H4 is expected to enhance the binding affinity of RBD, thus increasing its neutralizing activity. The SMD results agree with the experimental results presented by Tian *et al.* ²⁹⁰ and Huo *et al.* ¹⁷⁶ for H11-H4 and CR3022 that interact with SARS-CoV-2. Still, for CR3022–RBD complex, they contradict Yuan *et al.* ²⁹¹. The variation in binding affinity is attributed to differences in the experimental conditions of the two groups, as discussed by Yuan *et al.* ²⁹¹. Our all-atom SMD results also showed that the binding of H11-H4 to RBD is mediated by vdW interactions, while the binding of CR3022 and H11-H4+CR3022 to RBD is driven by electrostatic interactions.

Martini coarse-grained US was used to estimate the binding free energy between CR3022 and H11-H4 to RBD. Binding free energy was obtained at -19.8 and -21.4 kcal/mol for H11-H4–RBD and CR3022–RBD, respectively. The lower binding free energy of CR3022 indicates that this Ab binds more tightly to RBD, which is consistent with previous computational studies ¹⁶⁵. For H11-H4 + CR3022–RBD, we obtained a binding free energy of -23.9 kcal/mol, consistent with the SMD results, indicating that the combination of H11-H4 and CR3022 enhances binding affinity and improves their ability to neutralize SARS-CoV-2.

Additionally, all-atom SMD was also employed to assess the binding affinity between H11-H4 and various SARS-CoV-2 variants, including Alpha, Beta, Gamma, Kappa, Delta, Lambda, and Mu. We found that H11-H4 exhibits effective neutralization of Alpha, Kappa and Delta variants, positioning it as a highly promising therapeutic for COVID-19.

3.2.2 Publication

a) *Abstract*: The global spread of COVID-19 is devastating health systems and economies worldwide. While the use of vaccines has yielded encouraging results, the emergence of new variants of SARS-CoV-2 shows that combating COVID-19 remains a big challenge. One of the most promising treatments is the use of not only antibodies, but also nanobodies. Recent experimental studies revealed that the combination of antibody and nanobody can significantly improve their neutralizing ability through binding to the SARS-CoV-2 spike protein, but the molecular mechanisms underlying this observation remain largely unknown. In this work, we investigated the binding affinity of the CR3022 antibody and H11-H4 nanobody to the SARS-CoV-2 receptor binding domain (RBD) using molecular modeling. Both all-atom steered molecular dynamics simulations and coarse-grained umbrella sampling showed that, consistent with the experiment, CR3022 associates with RBD more strongly than H11-H4. We predict that the combination of CR3022 and H11-H4 considerably increases their binding affinity to the spike protein. The electrostatic interaction was found to control the association strength of CR3022, but the van der Waals interaction dominates in the case of H11-H4. However, our study for a larger set of nanobodies and antibodies showed

that the relative role of these interactions depends on the specific complex. Importantly, we showed Beta, Gamma, Lambda, and Mu variants reduce the H11-H4 activity while Alpha, Kappa, and Delta variants increase its neutralizing ability, which is in line with experiment reporting that the nanobody elicited from the llama is very promising for fighting against the Delta variant.

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My contributions consisted of designing the research, performing simulations, analyzing the results, preparing figures, and writing the manuscript.

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My contribution consisted of designing the research, interpreting the data, writing the manuscript, and supervising the overall project.

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Chapter 4: Effect of SARS-CoV-2 non-structural protein 1 on protein synthesis

4.1 Binding of SARS-CoV-2 non-structural protein 1 to 40S ribosome inhibits mRNA translation

4.1.1 Introduction

SARS-CoV-2 is an enveloped, positive-sense, single-stranded RNA virus closely related to species known to infect a wide range of vertebrates³⁰⁰⁻³⁰¹. Its genome, approximately 30 kb in length, is a 5'-capped and 3'-polyadenylated RNA component of coronavirus particles. This genome encodes two large overlapping open reading frames in gene 1 (ORF1a and ORF1b) and includes various structural and NSPs at the 3' end^{293, 302}. After entering host cells, the viral genomic RNA is translated by the cellular protein synthesis machinery to produce NSPs that create favorable conditions for viral infection and mRNA synthesis^{193, 303}. Among these proteins, NSP1 is particularly enigmatic²⁰¹. NSP1, produced from the N-terminus of ORF1a, acts as a host shutoff factor, suppressing host gene expression and immune response, and playing a crucial role in the viral life cycle³⁰⁴.

All viruses rely on cellular ribosomes for protein synthesis and compete with endogenous mRNA to access this translation machinery, which serves as the focal point of control³⁰⁵. A common viral strategy to limit host gene expression is by redirecting translational resources toward viral mRNA. This phenomenon, known as host shutoff, enhances the access of viral transcripts to ribosomes and promotes evasion of the innate immune system³⁰⁶⁻³⁰⁷. Host shutoff is a hallmark of coronavirus infections, significantly contributing to the suppression of innate immune responses in several pathogenic coronaviruses, including SARS-CoV, MERS-CoV, and pandemic SARS-CoV-2³⁰⁸⁻³¹⁰. SARS-CoV-2-induced host shutoff is multifaceted, involving the inhibition of host mRNA splicing by NSP16, restriction of cellular cytoplasmic mRNA accumulation and translation by NSP1, and disruption of protein secretion by NSP8 and NSP9³¹¹⁻³¹⁴.

NSP1 does not interact with the 60S ribosomal subunit, it exclusively binds to the 40S ribosomal subunit and stalls canonical mRNA translation at various stages during initiation^{199, 315}. NSP1 is composed of 180 amino acids, organized into three distinct domains: the N-terminal domain, the linker domain, and the C-terminal domain⁵⁴. An early model of NSP1 lacked the C-terminal domain because it remains disordered in solution, forming an ordered helix-loop-helix structure only upon binding to the small ribosomal subunit^{54, 316}. In contrast, the N-terminal and linker regions do not directly bind to the 40S mRNA entry channel but instead stabilize NSP1's association with the ribosome and mRNA^{55, 316}.

Schubert *et al.*¹⁹⁸ demonstrated that the C-terminal domain interacts specifically with the 40S subunit of the human ribosome, inhibiting mRNA translation. The C-terminal domain binds to the mRNA entry channel, folds into two helices, and interacts with h18 of 18S rRNA, as well as with the 40S ribosomal proteins uS3, uS5, and eS30. These interactions cause NSP1 to partially overlap with the fully accommodated mRNA. NSP1 suppresses all cellular antiviral defense processes that depend on the expression of host factor expression,

including the interferon response, acting as a ribosome gatekeeper to stop translation and inhibit host cell protein synthesis. This shutdown of key parts of the innate immune system facilitates efficient viral replication and immune evasion³¹⁷⁻³¹⁸. Due to its crucial role in dampening the antiviral immune response, NSP1 is considered a potential therapeutic target^{57, 198, 319}. However, the precise atomistic mechanism by which NSP1 interactions with a conserved region in the 5' untranslated region of viral mRNA suppress viral protein expression remains unclear¹⁹⁸.

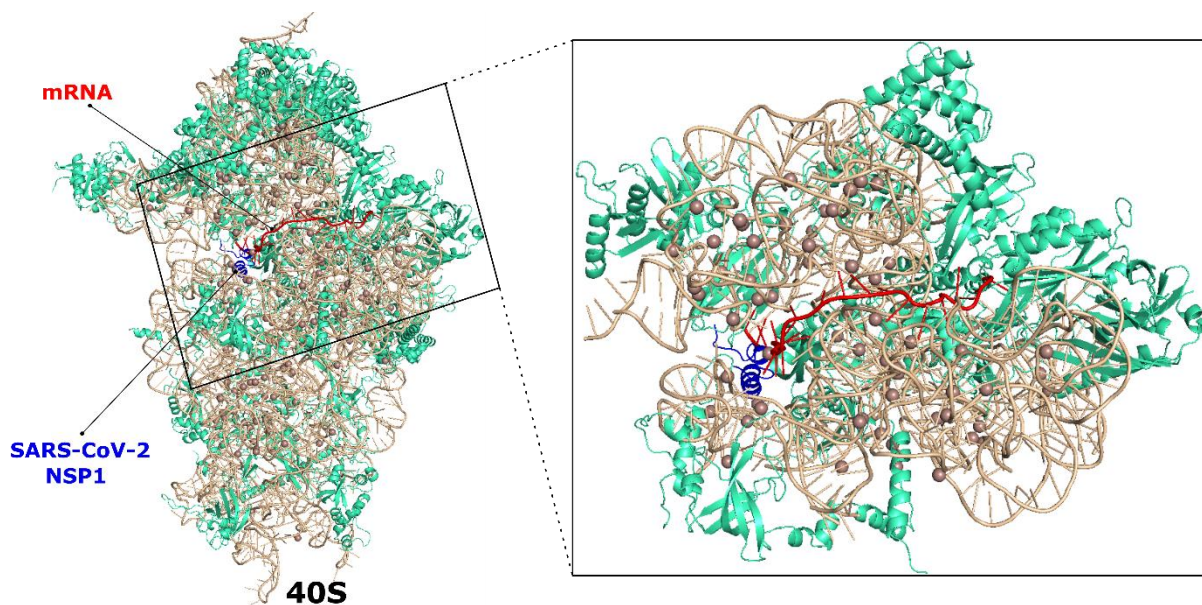


Figure 4.1: The 3D structure of the mRNA-40S-NSP1 complex, including 40S ribosome (ribosomal proteins: green-cyan, ribosomal RNA: wheat), mRNA (red), NSP1 (blue), and Mg^{2+} and Zn^{2+} ions (dark-salmon).

In this study, we used all-atom SMD and coarse-grained alchemical simulations (for the full 40S and truncated 40S ribosome (Figure 4.1)) to examine the impact of NSP1 binding on the 40S ribosome and its inhibition of the mRNA translation process²⁰⁴.

To calculate the binding affinity of mRNA to the ribosome in the presence and absence of NSP1, SMD simulations were conducted by pulling mRNA along its entry channel for both full 40S and truncated 40S complexes. An external force was applied to a dummy atom connected to the 5'-mRNA (O5' atom) via a spring with stiffness k . The pulling direction was along the mRNA entry channel. The complexes were rotated so that the exit direction was parallel to the z-axis²⁰⁴. We applied a pulling speed of $v = 0.5$ nm/ns, which is about 10 orders of magnitude greater than in experiments; however, previous work has shown that this choice does not affect the relative binding affinity, allowing us to distinguish strong binders from weak ones¹⁶⁵. The force-time profile shows that mRNA binds to 40S-NSP1 ($F_{\max} = 5023.3 \pm 232.1$ and 4501.3 ± 227.5 pN for the full 40S and truncated 40S ribosome, respectively) more strongly than to the 40S ribosome ($F_{\max} = 1832.9 \pm 127.4$ and 1763.6 ± 103.3 pN for the full 40S and truncated 40S ribosome). Here, the mRNA translation

process is primarily driven by the electrostatic interactions between mRNA and the 40S ribosome.

We also applied coarse-grained alchemical simulations with the Martini model to calculate the binding free energy of mRNA to the 40S ribosome with and without NSP1. For alchemical transformations, an optimal set of λ -values ranging from $\lambda = 0$ to $\lambda = 1$ was used, where $\lambda = 0$ and $\lambda = 1$ correspond to a system with and without full interaction, respectively. The optimal set of 30, 30, and 20 windows of λ -values was selected for the mRNA-40S, mRNA-40S-NSP1, and mRNA, respectively. The free energy of mRNA-40S binding is -13.1 ± 1.1 and -8.6 ± 1.2 kcal/mol for the full 40S and the truncated 40S ribosome, respectively, which is very close to the experimental value of -10.7 ± 0.1 kcal/mol³²⁰. In the presence of NSP1, the binding free energy of mRNA-40S-NSP1 is reduced to -37.1 ± 2.2 and -28.2 ± 2.6 kcal/mol for the full 40S and truncated 40S ribosome, respectively. This indicates that NSP1 significantly increases the binding affinity of mRNA to the 40S ribosome, suggesting that its attachment to the mRNA entry channel obstructs translation. This observation is consistent with the findings from all-atom SMD simulations, and our calculated results closely match the experimental data of earlier studies^{198, 316}.

4.1.2 Publication

a) Abstract: Experimental evidence has established that SARS-CoV-2 NSP1 acts as a factor that restricts cellular gene expression and impedes mRNA translation within the ribosome's 40S subunit. However, the precise molecular mechanisms underlying this phenomenon have remained elusive. To elucidate this issue, we employed a combination of all-atom steered molecular dynamics and coarse-grained alchemical simulations to explore the binding affinity of mRNA to the 40S ribosome, both in the presence and absence of SARS-CoV-2 NSP1. Our investigations revealed that the binding of SARS-CoV-2 NSP1 to the 40S ribosome leads to a significant enhancement in the binding affinity of mRNA. This observation, which aligns with experimental findings, strongly suggests that SARS-CoV-2 NSP1 has the capability to inhibit mRNA translation. Furthermore, we identified electrostatic interactions between mRNA and the 40S ribosome as the primary driving force behind mRNA translation. Notably, water molecules were found to play a pivotal role in stabilizing the mRNA-40S ribosome complex, underscoring their significance in this process. We successfully pinpointed the specific SARS-CoV-2 NSP1 residues that play a critical role in triggering the translation arrest.

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My contribution consisted of designing the research, performing MD simulations, analyzing the results, preparing figures, and writing the manuscript.

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My contribution consisted of designing the research, and analyzing the results.

Ho Chi Minh, July 2024

A handwritten signature in blue ink, appearing to be 'HLN'.

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My contribution consisted of designing the research, writing the manuscript, and supervising the overall project.

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Hung Nguyen, Hoang Linh Nguyen, and Mai Suan Li

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Chapter 5: Conclusions and future work

5.1 Conclusions

The results presented in this dissertation concern the mechanisms by which Ab and Nb bind to SARS-CoV-2 RBD, preventing the virus from entering human cells. They shed light on potential treatment strategies not only for wild-type SARS-CoV-2 but also for various variants of concern. In addition, we investigated how SARS-CoV-2 NSP1 interacts with the mRNA entry channel, which ultimately leads to the inhibition of the protein synthesis process. Our studies employ a range of theoretical and computational methods, including all-atom and coarse-grained models combined with MD methods. The main findings are as follows:

- 1) Using all-atom SMD and coarse-grained US simulations, we investigated the binding affinities between REGN10933 Ab, REGN10987 Ab, and the combination of REGN10933 and REGN10987 with RBD. Our results indicate that REGN10933 exhibits stronger binding affinity to RBD compared to REGN10987. Moreover, the combination of REGN10933 and REGN10987 demonstrates even greater binding strength to RBD. The stability of both REGN10933-RBD and REGN10933+REGN10987-RBD complexes is mainly governed by electrostatic interactions, whereas the stability of REGN10987-RBD complex relies on van der Waals interactions. In particular, REGN10933 and REGN10933+REGN10987 exhibit similar potency against both the Delta variant and the wild type. However, their effectiveness against the Omicron variant is reduced, which is in line with recent experimental findings.
- 2) We investigated the concurrent binding of H11-H4 Nb and CR3022 Ab to RBD using all-atom SMD and coarse-grained US simulations. Our results revealed significantly enhanced binding affinity compared to their individual associations with RBD. The combined action of H11-H4 and CR3022 resulted in increased neutralizing capacity against SARS-CoV-2. The stability of H11-H4-RBD complex is primarily governed by van der Waals interactions, while electrostatic interactions play a more significant role in the stability of CR3022-RBD and H11-H4+CR3022-RBD complexes. CR3022 has emerged as a promising candidate for COVID-19 treatment, especially against the wild-type strain. H11-H4 exhibits strong neutralizing abilities against Alpha, Kappa, and highly dangerous Delta variants, consistent with recent experimental results.
- 3) We investigated the interaction between mRNA and the 40S ribosome in the presence and absence of NSP1. Using full-atom SMD and coarse-grained alchemical simulations, our analysis revealed that mRNA exhibits significantly stronger binding affinity for the 40S-NSP1 complex compared to the 40S ribosome alone. This suggests that upon entry into host cells, NSP1 binds to the 40S ribosome, thereby hindering the translation process. These results are consistent with experimental observations. Our studies have shown that electrostatic interactions between mRNA and the 40S ribosome play a key role in driving the mRNA translation process.

5.2 Future work

Through a combination of all-atom and coarse-grained MD simulations, this dissertation has focused on two key problems: (1) the binding of Ab and Nb to SARS-CoV-2 RBD to prevent SARS-CoV-2 from entering the host cell, considering both the wild type and variants of concern and (2) How NSP1 binds to the mRNA exit tunnel to inhibit the mRNA translation process and disrupt protein synthesis in the human ribosome. In the near future we plan to work on the following issues:

- 1) Although our study has shown the importance of Ab-Ab or Ab-Nb combinations in the treatment of SARS-CoV-2 for both wild type and variants, it is important to note that the number of such pairs examined in our study was still limited. Further investigations are needed to determine whether these findings can be generalized to other Ab-Ab or Ab-Nb pairs.
- 2) Our study confirmed the inhibitory effect of NSP1 on the protein synthesis process when mRNA translation occurs in human cells. However, an important question remains about how NSP1 affects m7G-cap mRNA at the initiation stage of the translation process. Therefore, it should be considered to investigate the effect of NSP1 on m7G-cap mRNA in eukaryotic cells for confirmation through MD simulations.
- 3) NSP16 was experimentally shown to bind to U1/U2 small nuclear RNA upon SARS-CoV-2 entry into the host cell. It disrupts mRNA splicing, resulting in decreased host protein and mRNA levels, triggering nonsense decay of misspliced mRNAs. NSP16 binds to the 50-splice site recognition sequence of U1 and the branchpoint recognition site of U2. The disruption of mRNA splicing is consistent with the significant drop in steady state mRNA levels observed during SARS-CoV-2 infection. This effect reduces the host cell's innate immune response to virus recognition. However, the exact mechanism by which NSP16 binds to U1/U2 snRNAs and interferes with mRNA splicing remains unclear, necessitating further studies to fully understand this process.
- 4) NSP8 and NSP9 interfere with protein trafficking to the cell membrane by binding to signal recognition particle RNA (7SL RNA) in the signal recognition particles (SRPs). NSP8 binds to 7SL RNA in the region associated with SRP54 protein, while NSP9 binds to 7SL RNA in the region associated with SRP19 protein. This binding causes a failure in the translocation of nascent peptides into the ER lumen, leading to protein mislocalization, degradation in the cytoplasm, and ultimately protein secretion. The detailed mechanisms by which NSP8 and NSP9 impair protein trafficking by displacing SRP54 and SRP19 proteins from 7SL RNA are still unknown and require further investigation.

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