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Structure-Based Design of Recombinant Spike Subunit Vaccine for Coronavirus Diseases

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Abstract*— The coronavirus disease 2019 (COVID-19) pandemic caused by severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) is still surging across the globe and has affected serious problems for both health and the global economy; therefore, the development of a vaccine with good efficacy becomes a must. To tackle the pandemic, numerous sectors of academia, industry, and the government collaborate to develop and investigate potential vaccine platforms. The recombinant subunit vaccine is one of the safest types of vaccine. However, its development has lagged behind other platforms, owing to the need for greater antigen manufacturability and immunogenicity. In this review, we outline several protein engineering strategies carried out in developing the recombinant COVID-19 vaccine, including the fusion of antigens with Fc fragment of human IgG, carrier proteins, trimerization domains, and stabilizing mutations. A systematic literature review was performed to summarize key takeaways from studies on developing recombinant subunit vaccines of SARS-CoV, MERS-CoV, and SARS-CoV-2, highlighting vaccine design and expression system, antigen structure, and *in vivo* and *in vitro* results of each protein engineering strategy. Several protein engineering strategies, particularly S protein and RBD, can improve the antigen's stability, manufacturability, and immunogenicity. Finally, novel protein engineering strategies are expected to be further developed to increase the vaccines' overall manufacturing, and the current recombinant vaccine candidates will be further processed into clinical stages to confirm their efficacy against pathogenic human coronaviruses.

***Keywords*—COVID-19 vaccine; protein subunit, manufacturability; immunogenicity.**

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I. INTRODUCTION

The COVID-19 pandemic is still engulfing the world and shows a trend of increasing infection cases getting closer to the highest level of the third global wave. The severe acute respiratory syndrome coronavirus (SARS-CoV-2) is the agent causing COVID-19 and is closely related to SARS-CoV, which caused the SARS epidemic between 2002 and 2004 [1]. New cases and deaths continue to rise at an alarming rate worldwide. Consequently, vaccinations must be developed quickly to prevent and block the present SARS-CoV-2 outbreak. Fortunately, earlier vaccine candidate development for SARS-CoV and MERS-CoV can provide valuable insights into vaccine design, potentially accelerating the SARS-CoV-2 vaccine development. Furthermore, the manufacturing process might be adapted from a previously used vaccine or vaccine candidate. The vaccine's preclinical and toxicological data can be used to develop a SARS-CoV-2 vaccine [2].

There are several criteria for an ideal SARS-CoV-2 vaccine, including protecting not only from severe disease but also preventing infection in all vaccinated populations, triggering a long-term memory immune response, being highly scalable, and having the potential to be easily accessible and inexpensive, affordable in limited time [3]. Based on World Health Organization (WHO) data, as of July 13, 2021, there are 107 vaccine candidates in clinical development and 184 vaccine candidates in preclinical development. Companies or research groups have developed or produced many vaccines to deal with this pandemic. These types of vaccines include living or inactivated virus vaccines using the conventional approach, recombinant protein and vector vaccines that are newly licensed, and RNA and DNA vaccines that have not yet become licensed vaccines. Each platform of the vaccine has both advantages and disadvantages properties [2].

Vaccines based on inactivated and attenuated viruses are developed in the traditional approach [4]. Inactivated virus vaccines induce a robust and safer immune response than live attenuated virus vaccines. The disadvantage of this platform

is the potential epitope alteration by the inactivation process. Then, a live attenuated virus vaccine can induce a more robust immune response and preservation of native antigens to mimic natural infection. However, the disadvantage of this platform is the risk of residual virulence, especially for immunocompromised people. These vaccine platforms can exacerbate infection through antibody-dependent enhancement (ADE), as reported in SARS-CoV infection [5]. Viral vector vaccines have the same advantages as live attenuated virus vaccines, but this platform has a quite problematic manufacturing process, bigger genomic integration risk, and response of immunity opposed to vectors [6].

Furthermore, other platforms have good safety and tolerance, such as RNA or DNA-based vaccines, viral-like particle vaccines, and vaccine subunits. The Viral-like particle (VLP) vaccine can mimic native virus conformation, leading to stronger immune responses. However, this platform has lower immunogenicity and a more complicated manufacturing process. DNA vaccines can be produced by a relatively straightforward manufacturing process that results in highly stable double-strand DNA molecules and can be freeze-dried for long-term storage [7]. Disadvantages of this platform include low immunogenicity range, challenging administration line, and higher genomic integration risk. RNA is also an example of an easily adapted vaccine to new pathogens and could express native antigens. However, this platform still has lower immunogenicity, requires a cold-transportation chain, and has a possible risk of RNA-induced interferon response [6].

Subunit vaccine development is based on synthesizing immunogenic fragments that could trigger a robust immune response. After culturing large quantities of pathogens, protein subunit vaccines can be produced using recombinant technology for protein antigen synthesis or protein isolation and purification methods [3]. In the last two decades, genetic engineering technology has provided the capacity to clone and enhance in vitro antigen production for recombinant subunit vaccines. Pure antigens offer the advantages of protein subunit vaccines, particularly in efficacy and safety. Synthetic peptides or recombinant proteins that only include certain immunogenic fragments make up the protein subunit vaccine components. However, negative effects from protein subunit vaccines are rare. This vaccine platform can be produced properly due to its stability and precise pathogen fragments. These good characteristics make protein subunit vaccines widely developed [4]. As of July 13, 2021, WHO data shows that 19 of the 107 candidate vaccines are in clinical phase III, and 42% of the total candidate vaccine is a subunit vaccine [8].

However, subunit vaccines, which are protein-based vaccines (PBV), also have some, such as the small size of the antigen, which can reduce its uptake by antigen-presenting cells (APCs). The low immunogenicity of this type of vaccine requires several booster and adjuvant doses. Then, the integrity of the antigen also needs to be confirmed not to reduce the vaccine's immunogenicity. Production of subunit protein vaccines is also limited in the scalability of antigen production [3]. Therefore, the structure-based vaccine design aims to create surfaces on immunogens that will generate protective immune reactions against the target pathogen [9]. Several strategies have been improved to overcome the limitations by modifying the PBV structure from primary to quaternary to improve target epitope recognition, and vaccine immunogenicity to avoid antigen-associated complications [9]. This study resumes several strategies in developing subunit vaccine utilizing the structure-based design of coronavirus spike protein to gain stronger vaccination effects by significantly enhancing neutralization antibody titer. The protein stability of recombinant subunit vaccines plays an important role in vaccine efficacy. Therefore, in this review, we also explain various modifications of spike protein used in the development of recombinant subunit vaccines to prevent coronavirus diseases and provide an overview of candidate recombinant protein vaccines against SARS-CoV-2.

II. Material and Methods

The source of this literature review was obtained from the results of studies related to the recombinant subunit vaccine development for SARS-CoV, MERS-CoV, and SARS-CoV-2. This paper determines several protein engineering strategies that can be used to design the COVID-19 recombinant subunit vaccine based on previous Coronavirus vaccine prototypes. We review essential features in each of these strategies, including vaccine design, expression system, antigen structure, and in vivo and in vitro results from each study.

III. Results and Discussion

A. Coronavirus Biology and Vaccine Development

First, we discuss Coronavirus (CoVs) biology that shapes our current understanding of CoVs structure and infection throughout the intracellular viral life cycle. This understanding will be useful in developing intervention strategies for public health emergencies caused by the Covs, especially in the development of subunit vaccines. Coronaviruses are RNA viruses with a high family diversity. Humans, other mammals, birds, cattle, and companion animals can all be the host range of CoVs. As a result, this pathogenicity virus raises high public health anxiety and concern for veterinarians, both of which have an economic impact [10]. For example, the COVID-19 pandemic significantly impacted transportation, tourism, trade, healthcare facilities, and other industries. Furthermore, some governments have adopted a "lockdown" strategy to restrict the spread of COVID-19, causing economic activity to be hindered and future global economic growth to be affected [11]. International Monetary Fund (IMF), the biggest world funding organization published Economic Outlook in October 2021 that the pandemic resulted in a global fall in economic growth with an annual rate -3.2% in 2020. This number is projected to recover in 2021 to 5.9% and 4.9% in 2022 [12].

Taxonomically, CoV is a member of the Nidovirales order and Coronaviridae family. Coronaviridae and Torovirinae are two subfamilies of the Coronaviridae family. The Coronaviridae subfamily consists of 4 genera (Alphacoronavirus, Betacoronavirus, Gammacoranavirus, and Deltacoronavirus) [6]. Alphacoronaviruses and Betacoronaviruses specifically infect humans and other mammals, whereas Gammacoronaviruses and Deltacoronaviruses have a larger host range, including bird

species. CoV infection can cause respiratory and enteric diseases in both humans and animals. Specifically, human Coronaviruses (HCoVs), including HCoV-229E, HCoV-OC43, HCoV-NL63, and HCoV-HKU1, have been spreading in the human population for long years and causing seasonal respiratory infections with mild symptoms commonly known as the "common cold" [13]. Furthermore, within the last 20 years, pathogenic CoVs such as SARS-CoV, MERS-CoV, and SARS-CoV-2 have emerged, especially in the human population. These viruses can potentially cause serious, life-threatening respiratory infections and lung injury [10].

The genome of CoV is an RNA molecule in single-stranded-positive-sense with a size of 30 kb, putting it the genome's largest RNA virus. Based on the CoV genome organization, there are two overlapping open reading frames ORFs) on the 5' terminus covering ORF 1a and ORF 1b, which make up two-thirds of the genome size (Fig. 1). ORF 1a and ORF 1ab will produce two polyproteins (pp) during translation, including pp1a and pp1ab. These proteins are cleaved by cellular proteolysis activity, resulting in 16 nonstructural proteins (Nsps). These nonstructural proteins play a critical role in CoV's genome replication and synthesis of subgenomic mRNA [14]. Furthermore, the CoV genome encodes four main protein structures, namely nucleocapsid (N), membrane (M), envelope (E), and spike (S) protein. These proteins are required to produce a fully functional viral particle [15].

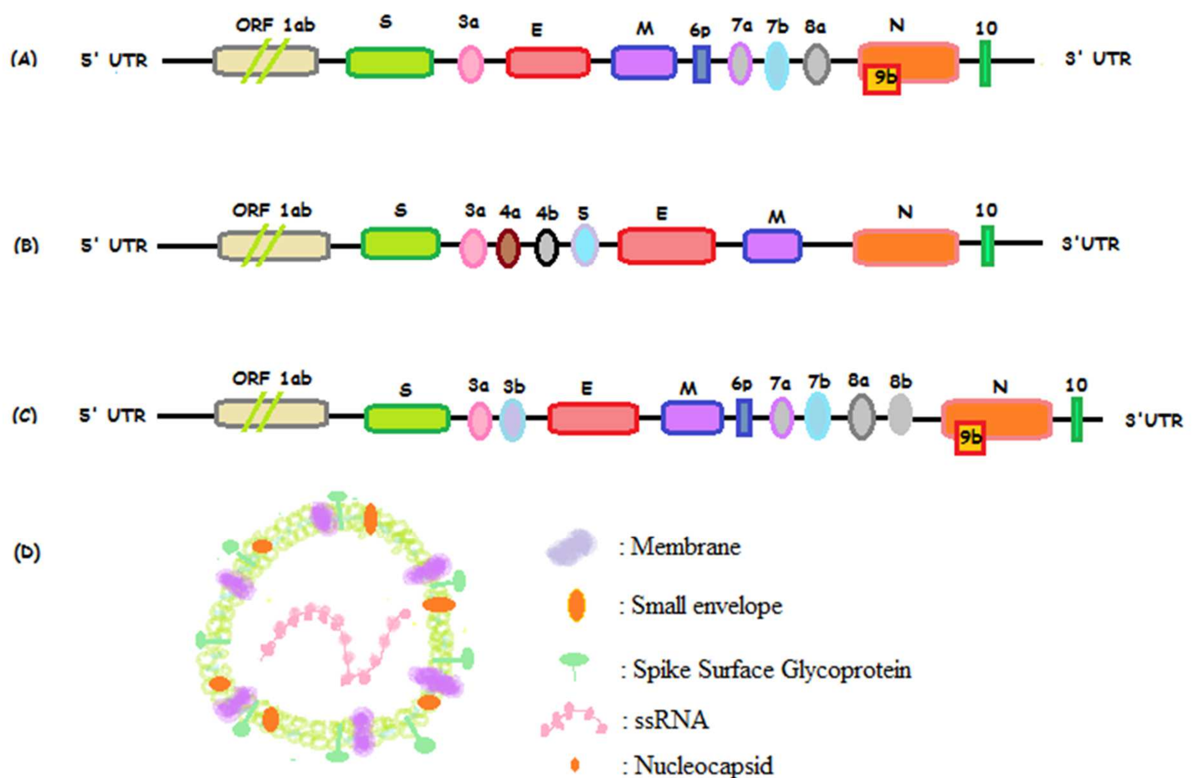

Fig. 1 Genomic structure and virion structure of CoV (a) SARS CoV-2, (b) MERS-CoV, (c)SARS-CoV, (d) Coronavirus encoding structural protein; Membrane, small Envelope, Nucleocapsid, Spike protein, and single-stranded RNA (ssRNA)

The outermost layer of CoV is the viral envelope, which is composed primarily of S and M proteins. Hemagglutinin Esterase (HE) is the third major component of the envelope protein present in certain coronaviruses but not all. The E protein is a minor component that has important structural functions [14]. The N protein is the only major structural protein that functions to form a nucleoprotein structure by binding to the viral RNA genome [15]. At the 3'-terminus of the viral genome, genes encode multiple accessory proteins. Although these proteins are not needed for CoV replication, they may provide biological benefits to CoV in the surrounding environment from infected cells. Some of the accessory proteins found in SARS-CoV have been shown to impact interferon signaling pathways and the production of pro-inflammatory cytokines [14]. Multiple accessory proteins include ORF 3a, 3b, 6, 7a, 7b, 8a, 8b, and 9b in SARS-CoV; ORF 3, 4a, 4b, 5, 8b in MERS-CoV; and ORF 3a, 6, 7a, 7b, 8, 10 in SARS-CoV-2 (Fig. 1) [6]. CoVs require many viral proteins to complete their life cycle. The S protein is a heavily glycosylated transmembrane protein with an amino acid sequence ranging from 1162 to 1452. The protein S monomer has a molecular size of 128-160 kDa before glycosylation, while the full-length monomer that has been glycosylated has a molecular mass of 150-200 kDa. After translation, the S protein will undergo folding, which results in a metastable prefusion conformation. Each monomer conformation will assemble a homotrimer that forms CoV's unique crown-like surface spike [14].

The S protein has 2 subunits, the S1 and S2 subunit. S1 will recognize the receptor and the S2 subunit involved in the cell membrane fusion, making this protein anchor into the viral membrane[16] (Fig. 2).

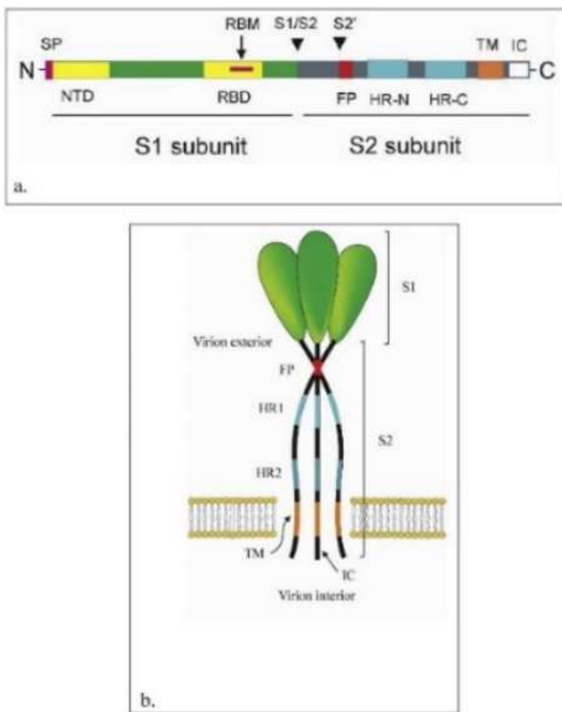

Fig. 2 Map (a) and structure (b) of CoVs spike protein [14].

S1-NTD (N terminal domain) and S1-CTD (C terminal domain) are the two primary domains of the S1 subunit, and both can fold independently. Both of these domains can bind to the receptor as a receptor-binding domain (RBD). The Coronavirus species determines which domain serves as an RBD. Other coronaviruses, such as SARS-CoV and MERS-CoV, have the RBDs at the S1-CTDs [17]. According to structural analysis, the RBD of SARS-CoV has the receptor-binding motif (RBM). RBM is a core and motif interacting with receptors directly [18]. In addition to being essential for virus-cell fusion, the S2 subunit is evolutionarily conserved among CoVs. This subunit consists of several regions, such as the fusion peptide (FP), two Heptad Repeat regions, and the highly conserved transmembrane domain. The Heptad Repeat regions are divided into Heptad Repeat region 1 (HR1 or HR-N) and Heptad Repeat region 2 (HR2 or HR-C) [14].

After RBD binds to receptors, S protein enters the cells to form the receptor-virus complex, which further proceeds to endosomes [20]. CoVs' S protein can bind to the same or different receptors depending on the virus. SARS-CoV and SARS-CoV-2 RBD, for instance, bind to Angiotensin-Converting Enzyme-2 (ACE-2), but MERS-CoV RBD binds to another receptor, which is Dipeptidyl Peptidase-4 (DPP4) [21]. Inside the endosome, the S protein undergoes proteolytic cleavage to form S1 and S2 subunits, with the S2 component facilitating viral envelope-host cell membrane fusion [20].

Once the virus enters the host cell, several Nsps are expressed. For instance, RNA-dependent RNA polymerase (Nsp12) mediates the replication of the CoV genome, and helicase (Nsp13) mediates the transcription of CoV mRNA [14]. CoV mRNA will produce a variety of Nsps and Sps during the translation process. The N proteins form viral nucleocapsids by attaching to CoV genomic RNA, whereas the S, E, and M proteins constitute CoV's envelope. Virions are assembled and exited from cells through exocytosis via the ER-Golgi pathway (Fig. 3) [19].

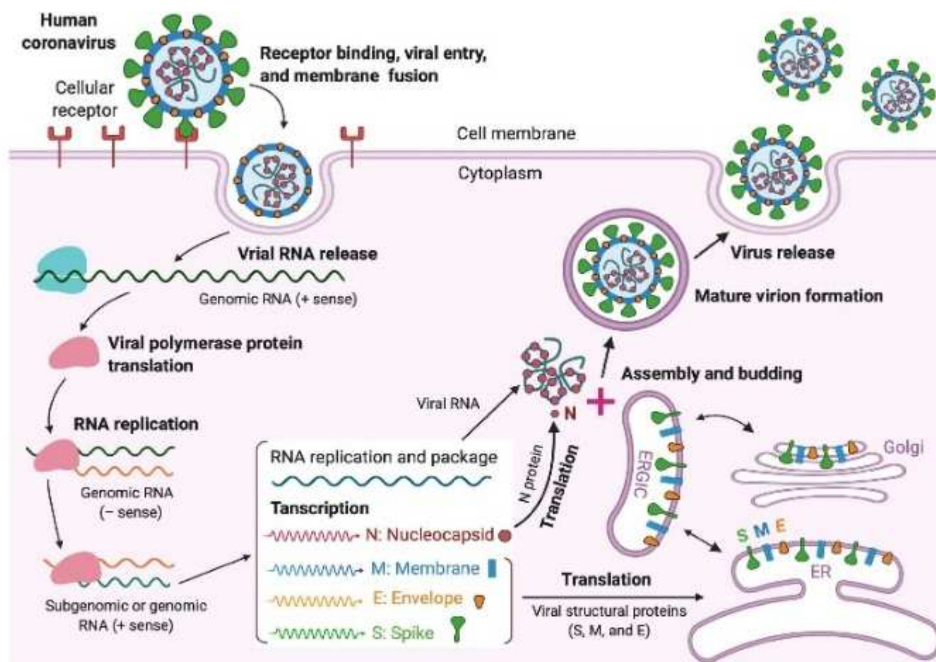

Fig. 3 CoVs Lifecycle[19]

The major antigen on the CoVs envelope is the S protein, which is essential for triggering host immunological responses. S-protein is the major target antigen for neutralizing antibodies during the infection. As a result, it has become a major target in vaccine development [16]. Development of CoV subunit vaccines could use several candidate antigens such as a full-length S and its antigenic components, including the S1 subunit, S2 subunit, RBD, and NTD [5], [17]–[19]. The first protein-based vaccine used Full-length S followed by RBD-based vaccines for SARS-CoV [6]. Although full-length S protein-based vaccines can induce robust immune responses and/or protection, studies have

demonstrated that antibodies elicited by some of these vaccines have been shown to increase viral infection in vitro, like in the case of SARS-CoV [20]. Based on previous SARS-CoV experiences, most of the MERS-CoV subunit vaccines are RBD-based. RBD-based vaccines showed greater immunogenicity and induced robust neutralizing antibodies, cellular immunity, and anti-MERS-CoV protection [21]. This finding also highlights safety concerns of vaccines with full-length S protein for SARS-CoV-2 infection. However, clinical evidence for Antibody-Dependent Enhancement (ADE) in human COVID-19 disease is still lacking. The high dosages of robust neutralizing antibodies are inducted or delivered to minimize ADE risk from immunotherapies, rather than lesser concentrations of non-neutralizing antibodies, which are more likely to cause ADE [22].

RBD-based vaccines constitute the major critical neutralizing antibody epitopes [23]. As a result, these vaccines may elicit robust neutralizing antibodies as well as significant antiviral protection. The difficulty of expressing spike protein will impact the yield and quantity of doses produced. RBD expression is easier on the expression system, but it only yields a relatively small protein; therefore, it does not have other neutralizing epitopes on the full-length S protein [24]. The antigenic drift was more prone to occur in RBD-based vaccines than vaccines that include the whole spike protein. So far, SARS-CoV-2 recombinant protein vaccine candidates are reportedly under preclinical studies, and some RBD-based and full S protein-based vaccines have reached clinical phase III [2].

Despite being smaller compared to the whole S protein, the S1 subunit either has the potential to trigger robust immunological responses or confer protective immunity to CoV infection [25], [26]. Thus, this region can be used as a potential target for developing subunit vaccines. NTD and S2 are less immunogenic as subunit vaccine targets, which can elicit fewer antibody titers, cellular immunity, and protective effects than other regions [27]. In considerations of efficacy and safety, both RBD or S1 subunits could be used as crucial targets for developing the hCoVs subunit vaccine candidates, particularly SARS-CoV-2. The S2 subunit is composed of some conserved sequences of amino acids. It has high homology among the diverse strain of the virus, so it can be used as a potential region for developing vaccines in universally, especially for different CoV strains [28].

B. Strategies for Recombinant Protein-Based Subunit Vaccine Design

We also discussed some of the strategies used in the development of CoVs subunit vaccines to support preparedness and future strategies in combating the Coronavirus infection. Stability and immunogenicity are the two most important factors influencing the ability of any vaccine candidate to induce protective immunity. Each antigen design and attribute must be able to maintain and enhance these two factors. Good antigen stability will affect the yield and immunogenicity of the antigen. Previous studies had developed designs to produce antigens for subunit vaccine candidates. As shown in Table I., we summarize some other essential strategies, including the expression system, antigen structure examination, and in vitro and in vivo studies used for developing recombinant CoVs subunit vaccines.

In developing CoVs subunit vaccines, S-protein is the main target antigen used to trigger the immune responses, particularly the production of neutralizing antibodies. However, the CoV spike proteins are large trimers with low stability [29]. The S protein is usually in the prefusion conformation, which is metastable and susceptible to converting to the postfusion conformation. This structural rearrangement removes the S1 component when synthesized as a recombinant protein. The S1 subunit consists of immunodominant sites for antigen recognition of immunity and neutralizing epitopes during CoV infections, especially RBD. Therefore, strategies for stabilizing the prefusion conformation of S protein and increasing perfusion S protein expression are needed. It also aims to improve the quality and quantity of antibodies that have target epitopes on the S1 subunit [21]. Increased S protein stability may enhance the potency of neutralizing antibody responses, mainly when antibodies are produced against conformational rather than linear epitopes. *Ex vivo*, a more stable S may make vaccine storage and dispersion of protein or virus-like particle (VLP) vaccines more accessible at immunization sites where cold-chain preservation is difficult [29]. In this review, we described the strategies to obtain stability and capability of the self-trimerization form of trimer S protein by developing antigen design with the fusion of protein fragments such as foldon [26], [30], molecular clamp [31], and trimer [32], [33] (Fig. 4). Other approaches to reach the prefusion-stabilized S protein involving two or more proline modifications or substitution at full-length spike protein and RBD with deleted N1-Linked glycosylated.

The most currently used protein fragment to achieve the trimeric form of S protein was done by fusion with Foldon. The Foldon domain consists of 27 amino acid sequences identified in the bacteriophage T4 fibritin protein [34]. The trimerized form of the S protein is generated by the fusion of the Foldon domain to the carboxy termini of the protein [35]. A study in SARS-CoV within the recombinant Baculovirus-expressed S protein resulted in high immunogenicity, indicating an excellent neutralizing response in both animals and human SARS-CoV variants. It is also revealed the major mechanisms of viral neutralization via RBD, which contain main neutralizing epitopes to block the receptor in the host cells [36]. However, when S protein was expressed in the eukaryotic system as a recombinant protein, the protein existed predominantly in the monomeric form[37]. S-Foldon is structurally similar to native spike protein with trimer conformation. The study, which included some protein design (Full S-Foldon, S1-Foldon, S1, S2 domain), showed S-Foldon significantly stronger potency to induce higher titer of neutralizing antibody than S1-Foldon (2.8-fold higher than sera from mice immunized with S1-Foldon) [26].

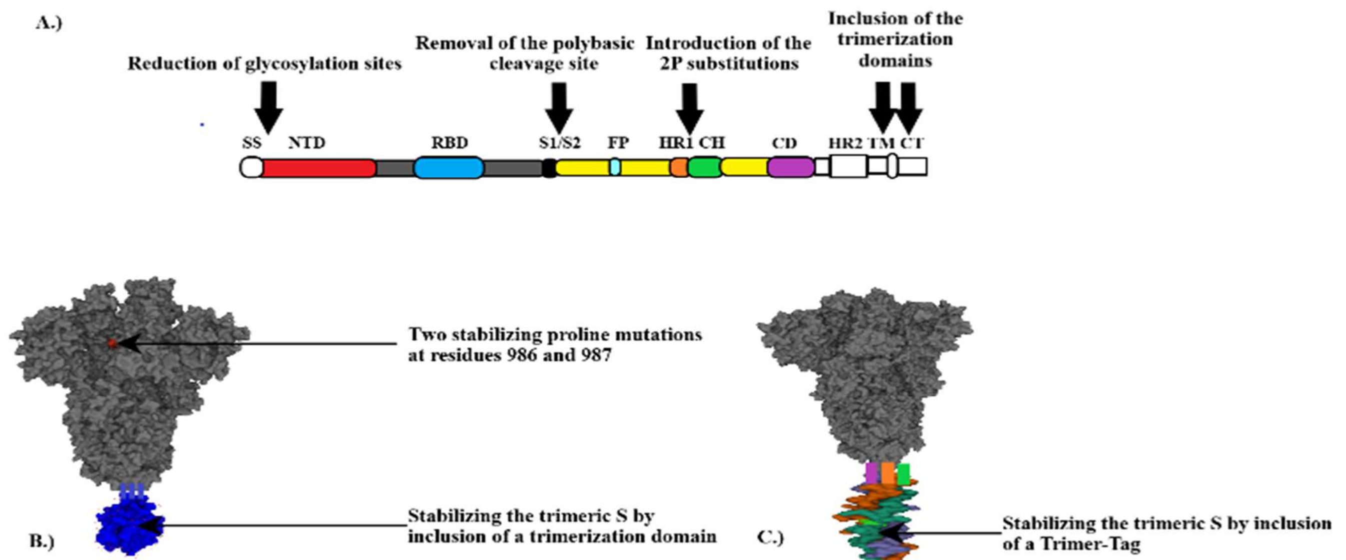

Fig. 4 Strategies to stabilize the conformation of Spike protein in CoVs vaccine development (A), such as the inclusion of stabilizing mutations or trimerization domain (B) and inclusion of Trimer-Tag (C). The prefusion structure of the SARS-CoV-2 spike was obtained from the Protein Data Bank (PDB ID: 6VSB), and the Collagen alpha-1(I) chain structure in the Trimer-Tag was obtained from UNIPROT (PRO_ 0000005721).

Furthermore, another study designed the trimerization strategy named Trimer-tag technology. Trimer-Tag technology, amino acids 1156 to 1406 coming from human Type1-alpha collagen, [38] has been described to express the S-Trimer antigen rapidly. The cDNA encoded Spike protein from WT SARS-CoV-2 (residue 1-1211) subsequently subcloned into pTRIMER mammalian expression vector (at two restriction sites *Hind III* and *Bgl II*) to allow in-frame fusion to Trimer-Tag. Trimer-Tag technology made S protein has its self-trimerization via disulfide bonds. A high expression level of S-Trimer with protein titer ~500 mg/L has resulted from developed fed-batch serum-free cell culture in a bioreactor [32]. The affinity purification scheme was also developed exploiting high binding affinity from Trimer-tag and Endo-180, collagen receptors capable of binding to C-terminal region of type 1 procollagen-19 and mature collagen [39]. Structures of S-Trimer determined by Cryo-EM indicated that it almost resembles the native prefusion state of the whole spike protein of CoV when it binds to the receptor, which confirms the structural integrity (determined at 3.2 Å and 2.6 Å) [33]. The stability analysis study also indicated that purified S-Trimer were stable in liquid solution formulations at the range temperature of 2- 8 °C up to 6 months [32]. An animal study also indicated that S-Trimer formulated with a proper adjuvant potentially enhances an excellent level of neutralizing antibodies and subsequently protects immunity in non-human primates (NHPs). Moreover, Trimer-Tag is considered to be safe since this protein is found abundantly in the human body. The C-propeptide or Trimer-tag is responsible for initiating the trimerization of collagen, which will cleave proteolytically, then end as a waste product. Data from the Trimer study indicated no ADA (Anti-Drug-Antibody) responses in patients consistently [38].

S-Clamp is also one of the methods being evaluated in clinical trials for alternate stabilization in subunit vaccines candidate [8]. SARS-CoV-2 S-Clamp as a vaccine candidate comes from incorporating recombinant S protein with Molecular Clamp (act as stabilization domain), performed in prefusion trimeric form. Codon-optimized S protein was introduced to the upstream of the Clamp trimerization motif. The Molecular Clamp trimerization domain consists of HR1 and HR2 of glycoprotein 41 (gp41), amino acid number 540-576, and amino acid number 619 to 656 from HumanImmunodeficiencyVirus-1 (HIV-1), which is commonly used as a standard fold in many viral families. This feature is purposed in S protein as a modular tag with the capability for stabilizing the prefusion trimeric viral antigen. The antigen structure then was decided by cryo-EM, resulting in the form of a smaller trimer peak that was resolved to 5 Å (Fourier shell correlation 0.143, as shown in Table I. and similar to previously defined trimeric-prefusion-conformation of SARS-CoV-2 Spike protein) and used in subsequent studies included mouse immunogenicity, hamster challenge, safety, and toxicology studies in rat models. The result showed that the S-Clamp vaccine elicits a high level of neutralizing antibody and broadly reactive and polyfunctional S-specific $CD4^+$ T cell and cytotoxic $CD8^+$ T cell *in vivo*. The SARS-CoV-2 S-Clamp is stable at 2-8°C. Therefore, the formulation with MF59 adjuvant can elicit neutralizing antibodies and cellular immune responses and provide a protective effect in immunized animal models [31].

The following strategy was the engineering of CoV S protein by proline substitution within the loop between the primary HR1 and CH, which has been demonstrated that could enhance both expression and conformational homogeneity of the prefusion-form of CoV S protein. The introduction of single proline substitutions dramatically increased the S protein's expression levels. The design of two consecutive proline substitutions in residue L1061 and V1060 MERS-CoV S2 subunit (called S-2P) resulted in an additional fifty times enhancement in S protein yield [40]. SARS-CoV-2 used a densely glycosylated spike protein, whereas the

method of structural rearrangement is triggered when S1 subunit binding to ACE-2, resulting in stable postfusion conformation. Two stabilizing proline mutation addition within the C-Terminal in the S2 subunit of SARS-CoV-2Sprotein could obtain ~0.5 mg/litre of the recombinant protein in prefusion stabilized form [41]. Furthermore *in vivo* study in NHPs showed that a higher dose, S-2P vaccine design candidate in 20µg doses, could elicit higher neutralization titers (up to 40 folds above the mean titers in convalescent COVID-19 subjects. This result in S-2P as SARS-CoV-2 S protein in stabilized trimer perfusion form may offer a potential candidate for recombinant subunit vaccine [35].

Further modification of 26 proline substitution has been described by Hsieh et al. (2021) [42]. The foremost promising design called Hexapro or Combo47, which contains all beneficial proline substitutions at F817P, A892P, A899P, A942P, and also two more proline substitutions in S-2P described before, showed higher expression compared to S-2P design by factor 9.8, an increased in melting temperature (~5°C) and vindicate the trimer prefusion conformation (Fig. 5). The investigation of Hexapro expressed in Expi-CHO cells could produce 35.5 mg/L well-folded protein. In contrast, the expression in FreeStyle 293-F cell only gained 10.5 mg/litre (representing an improvement by quite an order of magnitude than S-2P). The kinetic binding of HexaPro was also compared to S-2P and determined affinities of 13.3 nM and 11.3 nM. Further comparison showed the HexaPro design keeps being a folded-prefusion-conformation even after the three cycles of freeze-thaw and incubated at room temperature for 2 days long or 33 minutes at 55°C [43]. Meanwhile, S-2P, after the same number of freeze-thaw cycles, showed signs of aggregation and started to unfold in the next half-hour at 50° C [41], [42]. The well-preserved antigenicity of HexaPro and stability result indicated that HexaPromight is one of the potential candidates for the SARS-CoV-2 vaccine [42]. A recent study used the prefusion-stabilized HexaPro construct with six substitutions of proline (at amino acids 817, 892, 899, 942, 986, 987, and "GSAS" substitution at residues 682-685 (furin cleavage site), fusion with fold onto gain the trimerization motif and expressed in HEK293 cells. This design showed that HexaPro might elicit a robust neutralizing antibody when administered alum adjuvant [44].

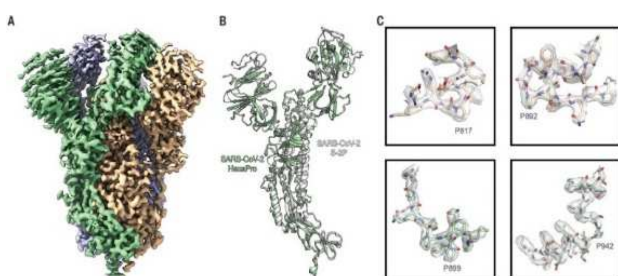

Fig. 5 High-resolution cryo-EM structure of HexaPro design [42].

Moreover, several protein designs have been reported to improve stability and manufacturability by increasing expression optimization [45]. The foremost promising design included 193 residues from SARS-CoV RBD (318-510) called RBD193, 219 residues (318-536) RBD219, and RBD219-N1(RBD219 with deleted N1-Linked glycosylated asparagine) (Fig. 6). Recombinant protein design using SARS-CoV RBD193 and RBD219 expressed in 293T cells and CHO-K1 cells demonstrated could elicit neutralizing antibody and protective immunity in mice vaccinated with those construct [43][44]. Further study associated with this construct showed that only expression RBD219-N1 within the yeast *Pichia pastoris X-33* might be expressed and purified in high yield and preserved its functionality and antigenicity. Subsequently, RBD219-N1 recombinant protein was successfully optimized in 10 L bioreactor to scale up the protein production process and increase the yield to 6-7 folds (from 60 mg/L to 400 mg/L) with up to 50% of purification recovery. Tag-free protein from the RBD219-N1 design has been successfully expressed as a highly purified and well-defined protein structure, making it suitable for vaccine candidates. Continuously, SARS subunit vaccine candidate with RBD219-N1 in high yielding yeast expression system and formulation with Alhydrogel adjuvant (1 RBD219-N1: 25 Alhydrogel) resulted in high neutralizing antibody titers, complete protection, and non-detectable viral loads (approximately none of eosinophilic pulmonary infiltrates). The vaccine formulation might be potentially developed for other and re-emerging Betacoronavirus [45].

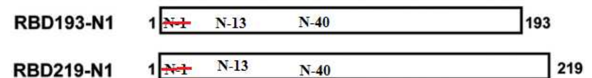

Fig. 6 Schematic Diagram of SARS-CoV-2 RBD193-N1 and RBD219-N1 construct

In addition to the previously discussed strategies for obtaining perfusion stabilization or trimer S protein, several other strategies have been developed to overcome the disadvantages of subunit vaccines that improve vaccine immunogenicity. These strategies include Fc-antigen fusion and carrier protein-antigen fusion. The Fc fusion protein has recently been used as a crucial method for developing more immunogenic subunit vaccines. Fc fusion protein also has the advantages of fast purification, a relatively long half-life, enhanced fusion protein folding, and improved binding to antigen-presenting cells [56][57]. Based on its mechanism of action, the Fc domain will help antigen bind with Fc receptors (FcR) on antigen-presenting cells (APCs), especially on dendritic cells (DCs). Only a few antigens not fused with Fc (soluble antigens) are internalized and not processed efficiently. On the other hand, the antigen complex with an antibody or fused with Fc will enter dendritic cells via the Fc receptor (FcR) and is rapidly processed. The adaptive immune response is increased and intensified [50].

In Fc-based fusion antigens, the Fc domain of the immunoglobulin fused with the protein of interest, especially the CoVsS protein or RBD. Showed that the SARS-CoV S protein consisting of 193 residues (amino acid 318–510) that fuses to human Fc-IgG1 had been shown to prevent S protein-mediated infection with IC50 less than 10 nm, whereas the S1 domain's IC50 was 50 nm [51]. Then, similar results were also observed in the immunized NHPs with the SARS-CoV-2 S1-Fc, including mice, rabbits, and monkeys. The fusion protein expressed in CHO cells was highly immunogenic, with high antibody titers detected at day 7. Using a pseudovirus neutralization assay, rabbits injected with S1-Fc showed

significant virus-neutralizing activity at day 14. In a live SARS-CoV-2 infection assay, two immunized monkeys with three injections of the S1-Fc developed higher viral neutralizing activities in <20 days than a recovered COVID-19 patient (Table I.) [52].

Considering RBD consists mainly of the S protein's neutralizing epitopes, it will be more effective in inducing the production of neutralizing antibodies and reducing the non-neutralizing antibodies production compared to the full-length S protein-based vaccine. Previously, Du et al. [53], [54](Table 1.) reported that RBD from SARS-CoV and MERS-CoV expressed from mammalian cell lines can evoke a long and robust humoral immune response in immunized mice. Using a similar approach, Liu et al.'s research [63] showed that mice immunized with RBD-Fc expressed on Expi293F cells had good neutralizing antibody responses. Elevated titers of RBD-specific antibodies and significant neutralizing activity were obtained based on the results of pseudotyped and live SARS-CoV-2 infection using hACE2 transgenic (hACE2-Tg) mice as an animal model. Furthermore, the RBD-Fc can induce and maintain neutralizing antibody responses relatively long term. The antisera collected from mice immunized can perform cross-neutralization against infection by pseudotyped SARS-CoV-2 RBD mutant. Then, the IgG of immunized mice can cross-neutralize the pseudotyped SARS-CoV and SARS-related coronavirus (SARSr-CoV). The SARS-CoV-2 RBD-Fc-based vaccine can be a feasible broad-spectrum subunit vaccine to hinder Betacoronavirus infection, especially for SARS-CoV-2 and its mutants, re-emerging SARS-CoVs or SARSr-CoVs that were probably emerging the world in the future.

Finally, the immunogenicity of RBD can also be enhanced by the fusion of RBD with a carrier protein, the P2 epitope (aa 830-844) of tetanus toxoid (TT) [55]. Tetanus toxoids are immunogenic in mice and humans and can be widely recognized in their interactions with many MHC class II molecules. An earlier study indicated that using the universal P2 epitope for $CD4^+$ T cells in a recombinant rotavirus subunit vaccine significantly improves immunogenicity [55]–[57]. The P2 has become a carrier as effective as the whole TT and can avoid epitope suppression [58]. As a vaccine development study for SARS-CoV-2, Hong et al. [69] evaluated the efficacy of RBD-P2 and the effect of using N protein on the efficacy of RBD-P2 in mice, rats, and NHPs. In this study, mice were immunized intramuscularly in 2 doses. Compared to those obtained with RBD-P2 with alum, mice vaccinated against N and RBD-P2 with alum had enhanced titer of neutralizing antibodies and cellular immune responses.

Furthermore, The NHPs immunized with RBD-P2/N and alum could slightly increase viral clearance than RBD-P2 + alum. However, this contrast was insignificant. This research provides essential information for developing the RBD-P2 as a SARS-CoV-2 vaccine candidate. It also elaborates on the usage of N in vaccine development, which can increase vaccine efficacy.

TABLE I
RECOMBINANT SUBUNIT PROTEIN VACCINE DESIGNS

Antigen Design	Type of Coronavirus	Expression System	Protein Structure	In Vivo and In Vitro Studies	Reference
Recombinant Subunit Protein Vaccine Design for Stability and Manufacturability					
Full S-Foldon, S1-Foldon, S1, S2 Domain	SARS-CoV	Baculovirus DNA into SF9 cells	S-foldon formed a trimeric state. S1-Foldon could also form a trimer.	The S-foldon protein elicited a significantly higher titer of neutralizing antibodies, while the S2 protein did not. Vaccination with S1, S1-foldon, full S, or S-foldon provides strong protection for vaccinated mice.	[26]
S-2P-Foldon	MERS-CoV	FreeStyle 293-F	The structure of the S2 subunit is similar to that of other CoV S2 subunits.	Elicited greater neutralization antibodies and significantly more robust neutralizing activity.	[40]
S-2P-Foldon	SARS-CoV-2	FreeStyle 293-F	The S protein structure is not different from the SARS-CoV S protein.	N/A	[41]
S-2P-Foldon	SARS-CoV	FreeStyle 293-F	The antigen structure is similar to other CoVs S proteins, especially in the Betacoronavirus genus.	N/A	[59]
S-2P-His Tag-Foldon	SARS-CoV-2	Hive Five cells (BTI-TN-5B1-4)	The S-2P has the same trimer structure as the similarly designed S protein produced in 293 mammalian cells.	The S-2P retains high immunogenicity at the lowest dose of 1 g with an aluminum adjuvant.	[35]
S-6P	SARS-CoV-2	FreeStyle 293-F cells	The HexaPro structure with one-RBD-up is similar to the S-2P structure.	HexaPro reacted with human convalescent serum and a monoclonal antibody (mAb) specific for RBD.	[42]
Sclamp	SARS-CoV-2	CHO cells	Sclamp structure is similar to the SARS-CoV-2 closed and opened trimeric S prefusion conformation.	Elicited significant neutralizing antibodies titers and S-specific $CD4^+$ and cytotoxic $CD8^+$ T cells that are widely reactive and polyfunctional.	[31]

RBD219-N1	SARS-CoV	*P. pastoris* X33	RBD219-N1 had retained defined secondary and tertiary conformation.	The high neutralizing antibody titers and antibody titers specific for RBD were obtained using aluminum adjuvant.	[48], [67]
S-Trimer (C Terminal Flag)	SARS-CoV	BHK-21 cells	The purified triSpike has properties like the native trimeric S-protein.	The immunized hamster with 2 g triSpike and aluminum adjuvant had a high antibody response.	[20]
S-Trimer	SARS-CoV-2	The GH-CHO (dhfr2/2) cell line	S-Trimer structure is mostly in closed prefusion conformation and resembles the native and full-length S.	The immunized animal models with this antigen S-Trimer and other essential components (AS03 or CpG 1018) and alum adjuvants had significant neutralizing antibody titers and Th1-biased cellular immune responses.	[32][33]
Recombinant Subunit Protein Vaccine Design for Efficacy					
S1-Ig	SARS-CoV	293T cells	The antigen structure is similar to the prefusion structures of Alpha- and Betacoronavirus S proteins that have been determined previously.	S protein (residues 318–510) are more efficient than the full-length S1 domain in blocking S protein-mediated infection.	[51]
S1-Fc	SARS-CoV-2	CHO cells	N/A	The immunized NHPs with S1-Fc produce a high titer of neutralizing antibodies.	[52]
RBD-Fc	SARS-CoV	HEK293T cells	RBD-Fc is conformationally and functionally proper.	In vaccinated mice, RBD-Fc induced robust and long-term humoral immune responses.	[54]
RBD-Fc	MERS-CoV	293T cells	N/A	Induced robust MERS-CoV S-specific antibodies in vaccinated mice.	[53]
RBD-Fc	SARS-CoV-2	Expi293F cells	RBD-Fc is conformationally and functionally proper.	Produced RBD-specific antibodies with high titers and strong neutralizing efficacy against pseudotypes and live SARS-CoV-2 infections.	[58]
RBD-P2	SARS-CoV-2	High Five insect cells	The expressed RBD-P2 and RBD-P2/N have long rod-like structures in a cluster.	Compared to using RBD-P2 plus alum, the immunization with N and RBD-P2 plus alum adjuvant boosted cellular immune responses in mice and increased the titer of neutralizing antibodies in the rat.	[55]

C. Future Perspective

Recombinant subunit vaccines have safer side effects than other vaccine platforms, such as inactivated or viral vector vaccines. However, the development of this vaccine platform may have some critical challenges, especially in the relatively low immunogenicity. Several strategies can be utilized to solve this problem, including combining recombinant protein with an appropriate adjuvant and stabilizing protein sequences, considering the use of fragment lengths. Several studies provided structure and epitope-based vaccines as promising strategies to enhance vaccine efficacy. In transgenic mice models, one of the construct designs used an RBD-based vaccine, significantly enhancing neutralizing antibody response and protecting against MERS-CoV infection [62]. We prospected that more structure guides for subunit vaccines will be developed to accomplish overall immunogenicity and efficacy challenges. Although most SARS-CoV and MERS-CoV subunit vaccines have been reported to have high immunogenicity and strong protection, these vaccine candidates are still in preclinical studies using animal models. Therefore, one or several promising subunit vaccine candidates are expected to be processed into clinical trials. It aims to verify the immunogenicity and protection against emerging CoVs.

Moreover, recombinant subunit vaccine offers distinct advantages over other platforms, such as mRNA and viral vector vaccines. The recombinant subunit vaccine could trigger a safe and robust immune response with much less production, storage, and transportation demand. It may become the main problem for transferring to low- and middle-income countries. The subunit vaccine does not carry a risk of impact from the use of vector backbone, as shown in the viral-vector vaccine, and it could be an ideal complement to obtain a prolonged immune response. In addition, several adjuvants have been formulated together with recombinant subunit vaccine, including Aluminum Hydroxide, CpG, Freund's complete adjuvant, AgnHB, AS01/AS03, and Matrix M. Advax [63]. The formulation with the suitable adjuvant resulted in higher immunogenicity and Th-1 Type cellular responses, which reduced the demand for recombinant protein doses [32].

The recent development of a recombinant subunit vaccine for the COVID-19 vaccine, called Novavax (NVX-CoV2373), which contains the full-length spike glycoprotein formulated with matrix-M adjuvant, elicited high titers and neutralizing antibodies after two doses of injection (with the inhibitory concentration greater than 99% titer response, 5 μg of formulated protein with matrix-M adjuvant, and 35 days after the first infection) [64][65]. So far, the recombinant vaccine

checks all the marks, which can offer a relevant contribution to overcoming the CoV pandemic worldwide.

IV. CONCLUSION

In conclusion, the drawback of subunit vaccine candidates, such as low immunogenicity, stability of antigen structure, limited scalability, and manufacturability, can be solved by re-designing recombinant subunit vaccines as described above. Structure-based vaccine design can provide several strategies used in developing Coronavirus subunit vaccines as the primary mitigation strategy to fight Coronavirus infection. Protein subunit vaccines can be produced through protein isolation and purification methods through recombinant technology after culturing large antigens. Low immunogenicity as the main problem for recombinant subunit vaccines could be solved by protein modifications, including stabilization of protein structure via mutations in particular sites and using purification techniques that might influence the elicited immune responses.

Furthermore, manufacturers' advantages in developing recombinant subunit vaccines are: First, specialized facilities such as BSL-3 are not required in the protein production step, which could save money in developing the vaccine. Moreover, the recombinant protein expression can be scaled up easily. Second, adding an adjuvant could lower the recombinant protein needed as well as boost immunogenicity. Third, no life component is used in the recombinant subunit protein vaccine, so this vaccine platform has a high safety profile; side effects and reactogenicity are mainly related to using adjuvants.

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