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Current Opinion in Structural Biology

Protein engineering responses to the COVID-19 pandemic

Ching-Lin Hsieh and Jason S. McLellan

Abstract

Antigen design guided by high-resolution viral glycoprotein structures has successfully generated diverse vaccine candidates for COVID-19. Using conjugation systems to combine antigen design with computationally optimized nanoparticles, researchers have been able to display multivalent antigens with beneficial substitutions that elicited robust humoral immunity with enhanced neutralization potency and breadth. Here, we discuss strategies that have been used for structure-based design and nanoparticle display to develop COVID-19 vaccine candidates as well as potential next-generation vaccine candidates to protect against SARS-CoV-2 variants and other coronaviruses that emerge into the human population.

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Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the causative agent of COVID-19, has caused more than 258 million infections and more than 5 million deaths worldwide. Global development of vaccines and therapeutics against SARS-CoV-2 has proceeded at an unprecedented pace in efforts to slow the ongoing pandemic. Attributed to the technological advances of structural and computational biology, researchers have been able to rapidly determine SARS-CoV-2 spike structures and leverage this information to engineer novel spike-based antigens as vaccine candidates. Understanding the structural basis of antibody–antigen interactions also helped epitope-driven antigen design, aimed at focusing humoral immunity toward sites of vulnerability on the SARS-CoV-2 spike glycoprotein. The spike is a trimeric class I viral fusion protein, and each protomer comprises an S1 and S2 subunit [1,2]. The receptor-binding domain (RBD) in the S1 subunit hinges between a 'down' conformation and a receptor-accessible 'up' conformation. By contrast, the fusogenic S2 subunit adopts a metastable prefusion conformation that is capped by the S1 subunit. Binding of the RBD to the angiotensin-converting enzyme 2 (ACE2) receptor triggers S1 dissociation and a large-scale rearrangement of S2 that facilitates fusion of the viral and host-cell membranes as S2 transitions to a stable postfusion conformation [3–5].

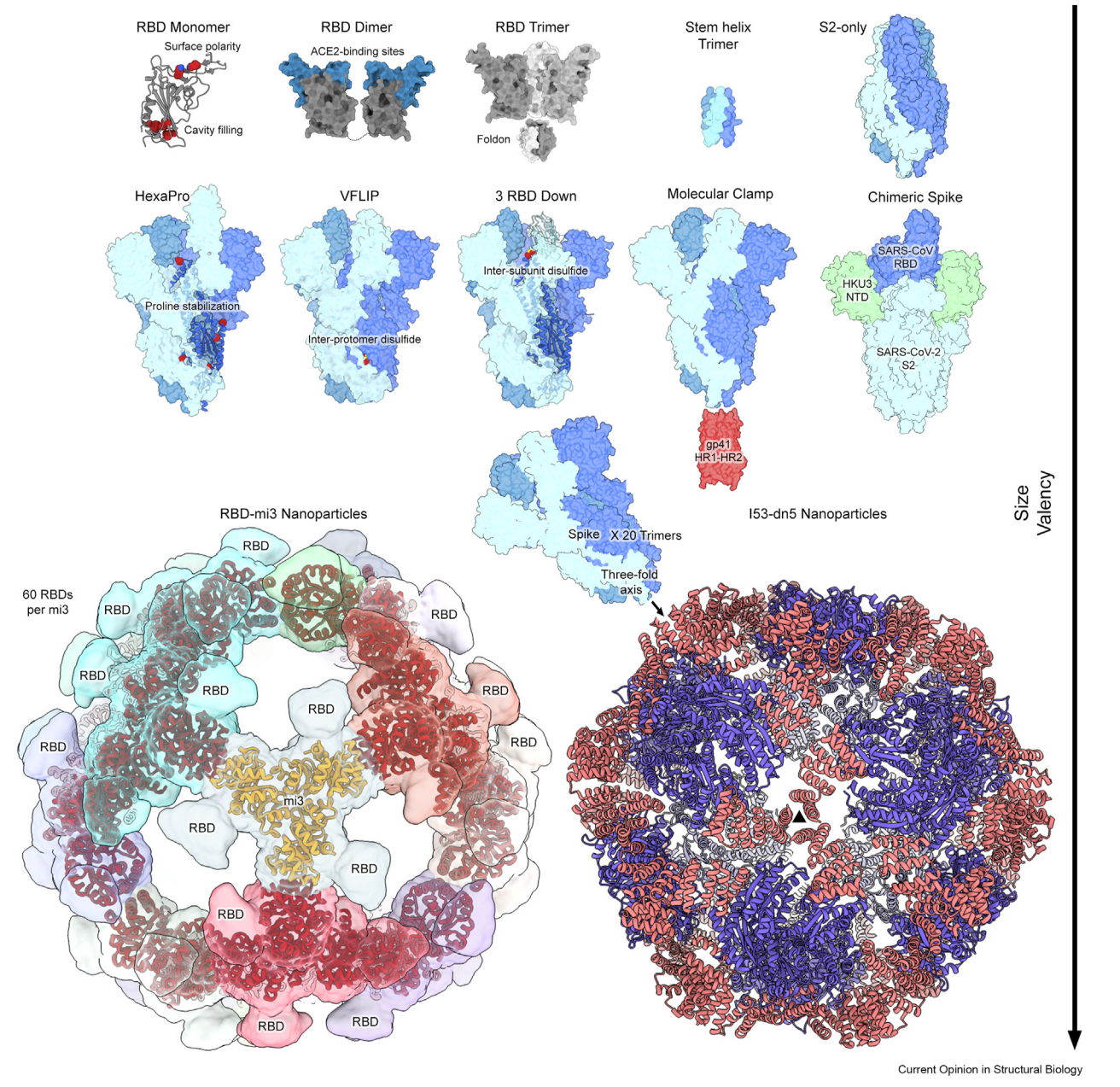
In this review, we first discuss the benefits of using structure-based design to optimize the RBD and stabilize the spike in the prefusion conformation, which has accelerated COVID-19 vaccine development. Secondly, we address the advantages of using chimeric spikes and nanoparticles to increase the breadth of the elicited immune response, counteracting potential spillover of SARS-like viruses from the subgenus Sarbecovirus.

RBD antigens

In early 2020, when the COVID-19 pandemic was just beginning, researchers quickly determined that the majority of neutralizing antibodies isolated from COVID-19 patients target the RBD of the spike glycoprotein [6,7]. As the compact size of the RBD lends itself to robust production in yeast, insect and mammalian cells, the RBD has been considered a prime target for vaccine development. Many studies have shown that RBD-directed antibodies can block binding of the ACE2 receptor, either by directly binding to the ACE2 binding site or by locking the RBD in a receptor-inaccessible down conformation [8–11]. Neutralizing RBD-directed antibodies were also shown to function through receptor mimicry, triggering premature S1 shedding and S2 transition to the postfusion conformation prior to viral attachment to cells [12–15].

In addition to eliciting a strong immune response, a good RBD subunit vaccine candidate should express well and have high physicochemical stability. Starr et al. applied deep mutational scanning (DMS) to the RBD using a yeast-surface-display platform to assess expression and ACE2-binding ability [16]. This led to the discovery of a mutation hotspot in proximity to a pocket that was

Figure 1



Examples of structure-based design and nanoparticle display of SARS-CoV-2 spike-based antigens. Schematic representation of monomeric, dimeric, trimeric RBDs, stem-helix trimer, S2-only antigen (top row), engineered trimeric spikes in the prefusion conformation (middle) and nanoparticles displaying 60 RBDs or 20 spikes (bottom). RBD monomer is depicted as a ribbon diagram with stabilizing substitutions [19,20] highlighted as spheres. RBD dimer, RBD trimer, stem helix trimer [58] and S2-only antigen are shown as molecular surfaces. For the RBD dimer [23], ACE2-binding sites (or receptor-binding motifs) are colored dark blue. All spike ectodomains are shown as molecular surfaces with proline substitutions [47], inter-protomer disulfide bond [49], inter-subunit disulfide bond [50,51,52] highlighted as spheres. The molecular clamp [59] derived from HIV gp41 HR1-HR2 (PDB ID: 1SZT) is depicted as a red molecular surface. The example of a chimeric spike [61] shown here comprises an NTD from HKU3-1 (green), an RBD from SARS-CoV (dark blue) and an S2 subunit from SARS-CoV-2 (light blue). One example of nanoparticles shown here is RBD-mi3 [72] (PDB ID: 7B3Y), wherein 20 copies of engineered 2-keto-3-deoxy-phosphogluconate (KDPG) aldolase trimer (ribbons) self-assemble to form a nanoparticle. 60 RBDs are conjugated to the mi3 surface via SpyTag:SpyCatcher system. The other example of nanoparticle display is I53-dn5 [77] (PDB ID: 6VFJ), wherein 20 copies of the trimeric component (light red) are used to display trimeric spikes.

previously demonstrated to bind linoleic acid [17]. Interestingly, I358F and F392W—the stabilizing substitutions that boosted expression—both introduce bulkier hydrophobic side chains that fill the loosely packed hydrophobic pocket (Figure 1). Using a similar yeast-surface-display system, Zahradník et al. independently discovered that the same substitution, I358F, is beneficial for RBD expression at elevated temperatures in yeast [18]. Based on structures of monomeric RBDs and trimeric spike ectodomains, Ellis et al. further computationally optimized the residues near the linoleic-acid-binding pocket [19], resulting in their identification of the Y365F and V395I substitutions (Figure 1), which lead to higher expression, less aggregation and improved stability of the RBD. Dalvie et al. transplanted conserved residues from other Sarbecoviruses into the SARS-CoV-2 RBD, generating two monodisperse RBD variants with lower tendency to aggregate under heat treatment [20]. In these variants, the substitutions L452K and F490W facilitated higher expression and stability of the RBD, likely by reducing surface hydrophobicity near the ACE2-binding site (Figure 1). Although the wild-type monomeric RBD is effective in protecting non-human primates from SARS-CoV-2 challenge [21], the engineered RBD-L452K—F490W protein elicited higher neutralizing titers against SARS-CoV-2 pseudoviruses than did the wild-type RBD [20].

Non-native dimerization motifs have been used to display divalent antigens and enhance immunogenicity and vaccine efficacy. For instance, Pan et al. demonstrated that the Fc (fragment crystallizable) region of IgGs could be fused with the RBD to facilitate dimer formation [22]. Notably, even when the Fc region was subsequently removed, a stable RBD dimer remained via an inter-molecular disulfide bond formed between the unpaired Cys538 from each monomer. Similarly, Dai et al. found that a portion of MERS-CoV RBD can spontaneously dimerize in solution [23]. The crystal structure of the MERS-CoV RBD dimer showed that each receptor-binding motif faced outward in a fully accessible orientation with the C-terminus of one RBD in close proximity to the N-terminus of the other. This inspired their design of a tandem RBD construct by covalently linking one protomer to the other without introducing any non-native residues [23]. Notably, this "tandem-RBD single-chain dimer" strategy was broadly applied to MERS-CoV, SARS-CoV and SARS-CoV-2 RBDs (Figure 1), all of which outperform their monomeric counterparts as antigens by eliciting higher neutralizing antibody responses in mice.

Trimerization motifs such as the T4 fibritin "foldon" domain have been widely used to stabilize class I viral fusion proteins in a biologically relevant trimeric form [24—26]. Researchers applied this strategy to the RBD by using glycine—serine linkers to genetically fuse the RBD with foldon (Figure 1), resulting in a trimeric RBD antigen that was evaluated in the COVID-19 vaccine candidate BNT162b1 [27]. Yu et al. also used foldon to form trimeric RBD and S1 antigens, both of which protected rhesus macaques against SARS-CoV-2 challenge [27,28]. Similarly, lambdoid phage 21 capsid-stabilizing protein (PDB ID: 1TD0) has been used to trimerize RBD and spike ectodomains [29]. While foldon is primarily composed of β-sheets, the GCN4 trimerization motif is α-helical, and can be fused in phase with the C-terminal heptad repeats of class I viral fusion proteins [24,30]. Hauser et al. further engineered an inter-protomer disulfide bond between GCN4 protomers to covalently stabilize the trimer and introduced three N-linked glycans on each GCN4 protomer to divert the immune response away from the trimerization motif [31]. A cocktail containing engineered GCN4-fused RBDs from SARS-CoV, SARS-CoV-2 and WIV-1-CoV induced polyclonal sera exhibiting broad neutralization activity, distinguishing it as a promising pan-Sarbecovirus vaccine candidate.

Stabilized spike antigens

Given that both NTD- and S2-targeted antibodies are neutralizing *in vitro* and protective *in vivo* [32—37], spike (S) immunogens may be advantageous in eliciting a broader immune response against coronaviruses than RBD alone. Full-length spike or spike ectodomain antigens also have more T-cell epitopes than RBD antigens [38]. Introducing two prolines at the short loop connecting the heptad repeat 1 (HR1) region and the central helix (CH) effectively stabilized the MERS-CoV and SARS-CoV spikes in the prefusion conformation [39,40]. This led several groups to introduce the same two proline substitutions at residues 986 and 987 to stabilize the SARS-CoV-2 spike [41—43]. This prefusion-stabilized construct, referred to as S—2P, facilitated rapid COVID-19 vaccine development, and is the antigen used in the Pfizer-BioNTech BNT162b2, Moderna mRNA-1273, and Janssen/J&J Ad.26.COV2.S vaccines [44—46]. Using the SARS-CoV-2 S—2P cryo-EM structure as a guide, our team designed and characterized 100 spike substitutions based on four strategies—proline, disulfide bond, salt bridge, and cavity-filling substitutions [47]. Specifically, in making proline substitutions, proline is preferentially placed in a loop or at the beginning of an α-helix to stabilize the spike in the prefusion conformation. For the disulfide bond strategy, a pair of cysteines are placed in regions that undergo conformational changes during the pre-to-post fusion transition. For the salt bridge strategy, an oppositely charged residue is introduced to counteract internal charge imbalance. Finally, for the cavity-filling strategy, a hydrophobic or aromatic residue is introduced to fill an adjacent cavity. After identifying a subset of beneficial substitutions, we characterized various

combinations to identify those with additive effects. Among these designs, the most promising antigen was HexaPro (Figure 1), which is composed of six proline substitutions throughout the region between the fusion peptide and HR1. HexaPro exhibited 10-fold higher expression than S−2P via transient transfections and was more resistant to both heat and physical stress. Interestingly, Juraszek et al. identified two of the same stabilizing prolines —A899P and A942P— which are part of the HexaPro substitutions [48]. Building on the base construct of HexaPro, Olmedillas et al. further introduced two cysteine substitutions—Y707C and T883C—to form an inter-protomer disulfide bond, and reverted Pro986 to Lys, fortifying the trimeric spike in a predominately closed conformation (Figure 1) [49]. Moreover, three groups independently engineered an inter-subunit disulfide bond (Cys383−Cys985) to covalently anchor the RBD to the apex of the neighboring S2 subunit (Figure 1) [50−52], which locks all RBDs in the down conformation. The addition of this inter-protomer disulfide bond (Cys383−Cys985) markedly improves spike thermostability, however, it does decrease spike expression levels.

Glycine is often observed in flexible loops or turns, and has been used as an alternative strategy to stabilize class I viral fusion proteins [53]. He et al. introduced two glycines at the same positions (residues 986 and 987) where the two prolines were introduced in the S−2P spikes, and also deleted the heptad repeat 2 (HR2) region, resulting in a stable construct named S2GΔHR2 [29]. Powell et al. deleted even more residues from the C-terminus of the ectodomain, leaving no stem helix (residues 1144−1213) at the base of S2 [54]. Removing these flexible regions seems to generate a more homogenous spike, but at the expense of losing important epitopes that have been shown to elicit neutralizing antibodies in mice and humans [36,37,55−58]. Instead of using foldon or the GCN4 trimerization motif, Watterson et al. re-purposed the HR1-HR2 six-helix bundle from the postfusion conformation of HIV-1 gp41 to clamp the SARS-CoV-2 spike in a trimeric conformation (Figure 1) [59,60]. Unfortunately, antibodies toward the gp41 six-helix bundle were elicited by vaccination with the molecular clamp spike, which resulted in false-positive HIV-1 tests in clinical trial participants, halting further clinical development.

To prepare for the potential spillover of additional coronaviruses from animal reservoirs, particularly for Sarbecoviruses that could use human ACE2 to enter and replicate in human cells, Martinez et al. designed four different chimeric spike constructs (Figure 1), each of which is composed of a combination of the NTD, RBD and S2 derived from either SARS-CoV-2, SARS-CoV-1 or bat Sarbecoviruses [61]. Priming and boosting mice with a cocktail of four chimeric spikes elicited strong neutralizing antibodies against SARS-CoV-2 variants of concern (VOCs) and bat Sarbecoviruses, and more importantly, protected the mice from heterotypic WIV-1-CoV infection.

Multivalent nanoparticle antigens

The use of multivalent protein nanoparticles to display viral antigens is an effective approach to enhancing the immunogenicity and breadth of the elicited antibody response [62]. Natural proteins that can form higher-order oligomers, such as ferritin (Fer) and lumazine synthase (LuS), were chosen to display RBDs or spike ectodomains [29,54,63−65]. Both Fer- and LuS-fused antigens can spontaneously self-assemble into an oligomeric nanoparticle. In addition, N-linked glycans can be introduced to the surface of Fer or LuS to improve the protein solubility and expression [64]. With the development of the SpyTag:SpyCatcher system [66], SpyTagged antigens can be easily linked to the surface of a nanoparticle that contains symmetry-matched SpyCatcher [64,67]. SpyTag and SpyCatcher were derived from the CnaB2 domain of the FbaB adhesion protein from *Streptococcus pyogenes* (Spy), a common human pathogen [66]. Thus, modifying SpyTag:SpyCatcher to avoid recognition by pre-existing Spy-targeting antibodies is beneficial for nanoparticles that use this "plug-and-display" system. Keeble et al. used proline substitution to stabilize a long loop in SpyCatcher and introduced negatively charged residues to balance the arginines present in engineered SpyTag, resulting in an optimized system named SpyTag003:SpyCatcher003 [68]. These substitutions reduced the nanoparticle's reactivity to preexisting human antibodies [67] and facilitated the rapid conjugation between the antigen and the nanoparticles. This optimization allows for complete conjugation within 30 min even when both protein partners are at low nanomolar concentrations, which is particularly useful for industrial production of large quantities of spike nanoparticles to fight the COVID-19 pandemic.

Advances in computational biology and protein design, led by the King and Baker laboratories, have had a major impact on designing and engineering artificial protein nanoparticles with a variety of symmetry axes to display antigens in their biologically relevant forms. I3-01 and its variant mi3 are both single component, self-assembled non-native nanoparticles [69,70] that have been widely used in combination with the SpyTag:SpyCatcher system [20,29,63,67,71,72]. Two surface-exposed cysteines from I3-01—Cys76 and Cys100—were substituted with alanine to generate a more stable mi3 nanoparticle that was less prone to aggregation [70]. This highly versatile system allowed Cohen et al. to conjugate 4 or 8 different RBDs from human, bat and pangolin Sarbecovirus spikes to mi3, generating mosaic RBD nanoparticles (Figure 1) [71] that elicited an antibody response capable of recognizing multiple zoonotic RBDs and neutralizing heterologous Sarbecoviruses. Notably, a single injection

of mosaic RBD nanoparticles elicited higher neutralizing antibody titers against heterotypic SARS-CoV than homotypic SARS-CoV-2 RBD nanoparticles. Considering the weak neutralizing activity of plasma IgGs from COVID-19 patients to zoonotic Sarbecoviruses and the fast adaptivity of this plug-and-display system, using nanoparticles to display newly emerging RBDs might be key to future pandemic preparedness.

Finally, two-component nanoparticles such as I53-50 provide an alternative way to display 60 RBDs or 20 trimeric spikes per particle [73,74]. The interface of two components, I53-50A and I53−50B, is stabilized by a chain of hydrophobic interactions and reinforced by salt bridges at the periphery [75]. Negatively charged residues were introduced to the interior surface of I53-50A to improve the overall antigen expression prior to assembly [76]. Both RBD-I53-50 and spike-I53-50 (Figure 1) elicited robust neutralizing antibodies and protected non-human primates from SARS-CoV-2 challenge [73,74]. One potential concern to displaying a high density of antigens on a nanoparticle is the restricted epitope accessibility for the antibodies, imparted by steric hindrance of neighboring antigens [77]. However, I53-50 nanoparticles displaying 60 RBDs induced sera exhibiting higher neutralization activity than did Fer displaying 24 RBDs [63]. Another independent study also showed that spike-I3-01 nanoparticles elicited higher neutralizing antibody responses than did spike-Fer [29]. These findings demonstrate that nanoparticles displaying more spikes per particle are attractive vaccine candidates.

Next generation pan-coronavirus antigens

While several antigen designs such as chimeric spikes and mosaic RBD nanoparticles have provided broader protection against SARS-CoV-2 VOCs and Sarbecoviruses, a major goal is to design a pan-coronavirus vaccine that protects against all existing human coronaviruses as well as coronaviruses that may emerge in the future. Identification of a conserved stem helix at the base of the S2 subunit may provide a new direction for epitope-focused designs (Figure 1) [36,37,55−58], which are likely to extend host immunity to all major betacoronaviruses. Moreover, a handful of comprehensive studies profiling the antibody response to the full spike using peptide-binding assays have discovered a few neutralization-sensitive epitopes in close proximity to the fusion peptide in the S2 subunit [78−81]. Interestingly, some cross-reactive and preexisting antibodies recognize cryptic sites that only become accessible when S1 dissociates from S2 [78]. Given the high sequence conservation of S2 among betacoronaviruses and the fact that neutralization-sensitive S2 epitopes could be cryptic [78,82], prefusion-stabilized S2-only antigens (Figure 1) may be promising universal coronavirus vaccine antigens.

Conflict of interest statement

J.S.M. is an inventor on U.S. patent no. 10, 960, 070 ("Prefusion Coronavirus Spike Proteins and Their Use") and U.S. patent application no. 62/972,886 ("2019-nCoV Vaccine"). C.-L.H. and J.S.M. are inventors on U.S. patent application no. 63/032,502 ("Engineered Coronavirus Spike (S) Protein and Methods of Use Thereof") and U.S. patent application no. 63/188,813 ("Stabilized S2 Beta-coronavirus Antigens").

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