Supplementary Materials for

Structure-based design of prefusion-stabilized SARS-CoV-2 spikes

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Materials and Methods

*Design scheme for prefusion-stabilized SARS-CoV-2 spike variants*

The SARS-CoV-2 S-2P variant was used as the base construct for all subsequent designs (18). The S-2P base construct comprises residues 1-1208 of SARS-CoV-2 S (GenBank: MN908947) with prolines substituted at residues 986 and 987, “GSAS” substituted at the furin cleavage site (residues 682–685), and C-terminal foldon trimerization motif, HRV3C protease recognition site, Twin-Strep-tag and octa-histidine tag cloned into the mammalian expression plasmid pαH. Using this plasmid as a template, desired mutations were introduced at selected positions within the SARS-CoV-2 S2 subunit. Based on SARS-CoV-2 S-2P cryo-EM structure (PDB ID: 6VSB), pairs of residues with Cβ atoms less than 4.6 Å apart were considered for disulfide bond designs. We particularly targeted the regions that move drastically during the pre- to postfusion transition such as the fusion peptide, connector region and HR1. Salt bridge variants required that the charged groups of the substituted residues were predicted to be within 4.0 Å. For residues in loops, a slightly longer distance than 4.0 Å was allowed. Core-facing residues with sidechains adjacent to a pre-existing internal cavity were examined for potential substitutions to bulkier hydrophobic residues. Proline substitutions were designed in the FP, connector region, or HR1 and placed either in a flexible loop or at the N-terminus of a helix. Combinations were chosen to test whether pairs of the same type of design (e.g. disulfide/disulfide) or different types of designs (e.g. disulfide/proline) could result in additive effects on spike expression and stability.

*Protein expression and purification*

Plasmids encoding S variants were transiently transfected into FreeStyle 293-F cells (Thermo Fisher) using polyethyleneimine, with 5 μM kifunensine being added 3h post-transfection. Cultures were harvested four days after transfection and the medium was separated from the cells
by centrifugation. Supernatants were passed through a 0.22 µm filter and then over StrepTactin resin (IBA). Spike variants were further purified by size-exclusion chromatography using a Superose 6 10/300 column (GE Healthcare) in a buffer composed of 2 mM Tris pH 8.0, 200 mM NaCl and 0.02% NaN₃. For initial purification and characterization, single-substitution and combination spike variants were purified from 40 mL cultures. For the 1L HexaPro purification, the size-exclusion column used was a Superose 6 16/600 column (GE Healthcare).

ExpiCHO cells were transiently transfected with a plasmid encoding HexaPro using Expifectamine, and cells were grown for six days at 32 °C according to the manufacturer’s High Titer protocol (Thermo Fisher). Supernatants were then passed through a 0.22 µm filter, flowed over Strep-Tactin resin, and subsequently purified by size-exclusion chromatography using a Superose 6 10/300 column (GE Healthcare) in a buffer composed of 2 mM Tris pH 8.0, 200 mM NaCl and 0.02% NaN₃.

Differential scanning fluorimetry

In a 96-well qPCR plate, solutions were prepared with a final concentration of 5X SYPRO Orange Protein Gel Stain (Thermo Fisher) and 0.25 mg/mL spike. Continuous fluorescence measurements (λ<sub>ex</sub>=465 nm, λ<sub>em</sub>=580 nm) were performed using a Roche LightCycler 480 II, using a temperature ramp rate of 4.4 °C/minute increasing from 22 °C to 95 °C. Data were plotted as the derivative of the melting curve as a function of temperature.

Negative stain EM

Purified SARS-CoV-2 S variants were diluted to a concentration of 0.04 mg/mL in 2 mM Tris pH 8.0, 200 mM NaCl and 0.02% NaN₃. Each protein was deposited on a CF-400-CU grid (Electron Microscopy Sciences) that had been plasma cleaned for 30 seconds in a Solarus 950 plasma cleaner (Gatan) with a 4:1 ratio of O₂/H₂ and stained using methylamine tungstate.
(Nanoprobes). Grids were imaged at a magnification of 92,000X (corresponding to a calibrated pixel size of 1.63 Å/pix) in a Talos F200C TEM microscope equipped with a Ceta 16M detector (Thermo Fisher). Stability experiments with S-2P and HexaPro were performed by imaging samples as described above after 3 rounds of snap freezing with liquid nitrogen and thawing, after storing samples at room temperature for 1-2 days, or after incubating at 50 °C, 55 °C, or 60 °C for 30 minutes in a thermal cycler.

**Biolayer interferometry for quantification of protein expression**

Plasmids encoding spike variants were transfected into FreeStyle 293-F cells (Thermo Fisher) in 3 mL of medium and harvested four days after transfection. After centrifugation, supernatant was diluted 5-fold with buffer composed of 10 mM HEPES pH 7.5, 150 mM NaCl, 3 mM EDTA, 0.05% Tween 20 and 1 mg/mL bovine serum albumin. Anti-foldon IgG was immobilized to an anti-human Fc (AHC) biosensor (FortéBio) using an Octet RED96e (FortéBio). The IgG loaded biosensor was then dipped into wells containing individual spike variants. A standard curve was determined by measuring 2-fold serial dilutions of purified S-2P at concentrations ranging from 10 μg/mL to 0.16 μg/mL. The data were reference-subtracted, aligned to a baseline after IgG capture and quantified based on a linear fit of the initial slope for each association curve using Octet Data Analysis software v11.1. Transfections and subsequent measurements of spike concentration were carried out in triplicate.

**ELISA**

To examine the antigenicity of spike variants, S-2P and HexaPro were coated on a Costar® 96-well assay plates (High Binding polystyrene, Corning) overnight at 4°C. After blocking with 2% milk for 2 hours, serial dilutions of the positive sera, mAb CR3022, or negative serum control (GNEG) were added and incubated for 1 hour at room temperature. After three washes with
PBS-T, anti-human IgG Fab HRP (Sigma cat # A0293) (1:5000 dilution) was added to the plate for 30 minutes. Finally, 1-step Ultra TMB ELISA substrate (Thermo Fisher) was applied to develop the colorimetric signal. The reactions were stopped by H2SO4 and absorbance was read on a microplate reader at 450 nm.

**Human sera**

Plasma from K2-EDTA anti-coagulated whole blood specimens was acquired from two recovered COVID-19 patients (approximately 50 days post-onset of symptoms) who had tested positive for SARS-CoV-2 by RT-PCR assay during acute-phase infection. Donors provided informed consent for blood collection which was performed using standard techniques approved by the University of Texas at Austin institutional review board (protocol number 2020-03-0085).

**Surface plasmon resonance**

His-tagged HexaPro was immobilized to a NiNTA sensorchip (GE Healthcare) to a level of ~500 response units (RUs) using a Biacore X100 (GE Healthcare) and running buffer composed of 10 mM HEPES pH 8.0, 150 mM NaCl and 0.05% Tween 20. Serial dilutions of purified hACE2 were injected at concentrations ranging from 250 to 15.6 nM. Response curves were fit to a 1:1 binding model using Biacore X100 Evaluation Software (GE Healthcare).

**Cryo-EM sample preparation and data collection**

Purified HexaPro was diluted to a concentration of 0.35 mg/mL in 2 mM Tris pH 8.0, 200 mM NaCl, 0.02% NaN3 and applied to plasma-cleaned CF-400 1.2/1.3 grids before being blotted for 6 seconds in a Vitrobot Mark IV (Thermo Fisher) and plunge frozen into liquid ethane. 3,511 micrographs were collected from a single grid using a FEI Titan Krios (Thermo Fisher) equipped with a K3 detector (Gatan). Data were collected at a magnification of 81,000x, corresponding to
a calibrated pixel size of 1.08 Å/pix. A full description of the data collection parameters can be found in Table S3.

**Cryo-EM data processing**

Motion correction, CTF-estimation and particle picking were performed in Warp (28). Particles were then imported into cryoSPARC v2.15.0 for 2D classification, *ab initio* 3D reconstruction, heterogeneous 3D refinement and non-uniform homogeneous refinement (29). The one-RBD-up reconstruction was subjected to local B-factor sharpening using LocalDeBlur (30) and the two-RBD-up reconstruction was sharpened in cryoSPARC. Iterative model building and refinement were performed with Coot, Phenix and ISOLDE (31–33).
Figure S1. Negative-stain EM images of variants with left-shifted SEC peaks.
Figure S2. Negative-stain EM images of well-folded particles.
Figure S3. Characterization of a disulfide and cavity-filling combination variant (Combo23). (A) SEC traces of S-2P, Combo23, and the parental variants S884C/A893C (disulfide bond) and L938F (cavity filling). (B) DSF melting temperature analysis of S-2P, Combo23, and its parental variants. The black dashed line represents the Tm of S-2P, and the purple dashed line represents the Tm of S884C/A893C.
Figure S4. HexaPro exhibits enhanced expression and stability compared to S-2P. (A) SEC trace of HexaPro purified from a 1L culture of FreeStyle 293-F cells. (B) Negative stain electron
micrograph of HexaPro purified from FreeStyle 293-F cells. (C) SEC traces of S-2P and HexaPro purified from ExpiCHO cells. (D) Negative stain electron micrograph of HexaPro from ExpiCHO cells. (E-F) Binding of S-2P (E) and HexaPro (F) to human ACE2 assessed by surface plasmon resonance. Binding data are shown as black lines and the best fit to a 1:1 binding model is shown as red lines. (G-H) Assessment of protein stability by negative stain electron microscopy. The top row of micrographs in (G) and (H) corresponds to S-2P, the bottom row corresponds to HexaPro. Representative debris/aggregates are shown in circles.
Figure S5. Cryo-EM data processing workflow.
Figure S6. Cryo-EM structure validation. FSC curves and viewing distribution plots, generated in cryoSPARC v2.15, are shown for both the two-RBD-up (left) and the one-RBD-up (right) reconstruction. Cryo-EM density of each reconstruction is shown and colored according to local resolution, with a central slice through the density shown to the right.
Table S1. Expression summary of variants with single substitutions.

| Substitution(s) | Strategy | Fold change in expression relative to S-2P |
|----------------|----------|------------------------------------------|
| T547C, N978C   | Disulfide| 0*                                       |
| A570C, V963C   | Disulfide| 0*                                       |
| S659C, S698C   | Disulfide| 0.4*                                     |
| Replace (673-686) with GS | Remove flexible region | 0*                                    |
| Replace (673-686) with GS + A672C, A694C | Disulfide, Remove flexible region | <0.5*                                  |
| N703Q, V705C, A939C | Disulfide | <0.5*                                  |
| V705C, A893C   | Disulfide| <0.5*                                    |
| A713S          | H bond   | 1.0*                                     |
| T724M          | Cavity-filling | 1.3*                                  |
| L727C, S1021C  | Disulfide| <0.5*                                    |
| P728C, V951C   | Disulfide| 0*                                       |
| V729C, A1022C  | Disulfide| <0.1*                                    |
| S730L          | Cavity-filling | 0*                                     |
| S730R          | Salt bridge | 0.15*                                  |
| S735C, T859C   | Disulfide| <0.5*                                    |
| V736C, L858C   | Disulfide| 0*                                       |
| T752K          | Salt bridge | <0.5*                                  |
| A766E          | Salt bridge | <0.5*                                  |
| G769E          | Salt bridge | 3.0*                                   |
| I770C, A1015C  | Disulfide| <0.5*                                    |
| T778Q          | Hydrogen bond | 2.6*                                  |
| T791C, A879C   | Disulfide| 1.0*                                     |
| G799C, A924C   | Disulfide| 1.3*                                     |
| P807C, S875C   | Disulfide| 1.1*                                     |
| F817P          | Proline | 2.8*                                     |
| E819C, S1055C  | Disulfide| 0*                                       |
| E819C, Q1054C  | Disulfide| 0*                                       |
| L822C, A1056C  | Disulfide| 0*                                       |
| L828K          | Salt bridge | <0.5*                                  |
| L828R          | Salt bridge | 0.4*                                   |
| Δ(829-851)     | Remove flexible region | <0.5*                                  |
| T859K          | Salt bridge | 2.1*                                   |
| P862E          | Salt bridge | <0.5*                                  |
| L865P, Q779M   | Proline, cavity-filling | <0.5*                                  |
| T866P          | Proline | <0.5*                                     |
| I870C, S1055C  | Disulfide| 0*                                       |
| T874C, S1055C  | Disulfide| <0.5*                                    |
| S875F          | Cavity-filling | <0.5*                                  |
| S884C, A893C   | Disulfide| 1.5*                                     |
| G885C, Q901C   | Disulfide| 1.1*                                     |
| Q889C, L1034C  | Disulfide| <0.1*                                    |
| A890P          | Cavity-filling | 1.0*                                  |
| A892P          | Proline, cavity-filling | 1.4*                                  |
| A893P          | Proline | 1.5*                                     |
| A899F          | Cavity-filling | 0.9*                                  |
| A905F          | Proline | 2.1*                                     |
| I909C, Q901C   | Disulfide| 0*                                       |
| A899P          | Cavity-filling | 0.3*                                  |
| A901M          | Proline, Cav | 1.2*                                   |
| A903C, Q913C   | Disulfide| 0.82*                                    |
| V911C, N1108C  | Disulfide| 0*                                       |
| T912R          | Salt bridge | <0.5*                                  |
| T912P          | Proline, cavity-filling | 1.5*                                  |
| K921P          | Proline | 1.1*                                     |
| L922P          | Proline | 0.8*                                     |
| L938F          | Cavity-filling | 2.0*                                  |
| A942F          | Proline | 6.0*                                     |
| A944F          | Cavity-filling | 1.0*                                  |
| A944F, T724I   | Cavity-filling | 0.4*                                  |
| A944Y          | Cavity-filling | 1.9*                                  |
| G946P          | Proline | 1.0*                                     |
| Q957E          | Salt bridge | 1.0*                                   |
| T961D          | Salt bridge | 1.8*                                   |
| T961C, S758C   | Disulfide| 0*                                       |
| T961C, Q762C   | Disulfide| 0*                                       |
| V963L          | Cavity-filling | 1.8*                                  |
| Q965C, S1003C  | Disulfide| 3.8*                                     |
| A972C, Q992C   | Disulfide| 1*                                       |
| A972C, I980C   | Disulfide| 1.3*                                     |
| S974C, D979C   | Disulfide| 0.3*                                     |
| S975P          | Proline | 2.2*                                     |
| N978P          | Proline | 0.9*                                     |
| Residue | Interaction Type | Value |
|---------|-----------------|-------|
| I900C, Q992C | Disulfide | 2.0* |
| R1000Y | Cavity-filling + hydrogen bond | 0.3* |
| R1000W | Cavity-filling | 1.0* |
| S1003V | Cavity-filling | 1.9* |
| I1013F | Cavity-filling | 0.8* |
| R1039F | Charge removal, pi-pi stacking | 0.5* |
| V1040F | Cavity-filling | <0.5* |
| V1040Y | Cavity-filling | 0.3* |
| H1058W | Cavity-filling | <0.5* |
| H1058F | Cavity-filling | 0* |
| H1058Y | Cavity-filling | 0.3* |
| A1078C, V1133C | Disulfide | <0.5* |
| A1080C, I1132C | Disulfide | <0.5* |
| I1081C, N1135C | Disulfide | 0.3* |
| H1088Y | Cavity-filling | 1.6* |
| H1088W | Cavity-filling | 0.6* |
| F1103C, P1112C | Disulfide | 0.15* |
| V1104I | Cavity-filling | 0.7* |
| T1116C, Y1138C | Disulfide | 0* |
| T1117C, D1139C | Disulfide | 1.0* |
| D1118F | Charge removal, pi-pi stacking | 0.5* |
| I1130Y | Hydrogen bond | 0* |
| L1141F | Cavity-filling | 0.8* |
| ΔHR2 (Δ1161-1208) | Remove flexible region | 2.5* |

*aQuantified using the area under the curve of the size-exclusion trimer peak

*bQuantified using SDS-PAGE band intensity
Table S2. Expression summary of Combo variants.

| Combo # | Substitutions                          | Strategy                  | Fold change in expression relative to S-2P |
|---------|----------------------------------------|---------------------------|--------------------------------------------|
| Combo1  | A903C, Q913C, Q965C, S1003C            | Disulfide+Disulfide       | 2.2                                        |
| Combo2  | S884C, A893C, A903C, Q913C             | Disulfide+Disulfide       | 0.8                                        |
| Combo3  | T791C, A879C, A903C, Q913C             | Disulfide+Disulfide       | 0.5                                        |
| Combo4  | G799C, A924C, A903C, Q913C             | Disulfide+Disulfide       | 0.5                                        |
| Combo8  | T791C, A879C, S884C, A893C             | Disulfide+Disulfide       | 0.5                                        |
| Combo9  | G799C, A924C, S884C, A893C             | Disulfide+Disulfide       | 0.4                                        |
| Combo11 | A892P, A899P                           | Proline+Proline           | 1.9                                        |
| Combo12 | A892P, T912P                           | Proline+Proline           | 2.7                                        |
| Combo14 | A892P, A942P                           | Proline+Proline           | 6.2                                        |
| Combo16 | A899P, A942P                           | Proline+Proline           | 5.1                                        |
| Combo19 | L938F, A892P                           | Cavity-filling+Proline    | 3.0                                        |
| Combo20 | L938F, A899P                           | Cavity-filling+Proline    | 3.0                                        |
| Combo21 | F817P, L938F                           | Proline+Proline           | 3.9                                        |
| Combo22 | L938F, A942P                           | Cavity-filling+Proline    | 6.0                                        |
| Combo23 | S884C, A893C, L938F                    | Disulfide+Cavity-filling  | 2.9                                        |
| Combo24 | T791C, A679C, L938F                    | Disulfide+Cavity-filling  | 2.2                                        |
| Combo26 | L938F, A903C, Q913C                    | Cavity-filling+Disulfide  | 2.0                                        |
| Combo40 | F817P, S884C, A893C                    | Proline+Disulfide         | 2.0                                        |
| Combo42 | T791C, A879C, F817P                    | Disulfide+Proline         | 1.4                                        |
| Combo45 | A892P, A899P, A942P                    | 3X Proline                | 6.2                                        |
| Combo46 | F817P, A892P, A899P                    | 3X Proline                | 3.8                                        |
| Combo47 | F817P, A892P, A899P, A942P             | 4X Proline                | 9.8                                        |
Table S3. Cryo-EM data collection and refinement statistics.

| EM data collection and reconstruction statistics | SARS-CoV-2 S HexaPro One RBD Up | SARS-CoV-2 S HexaPro Two RBDs Up |
|-------------------------------------------------|----------------------------------|---------------------------------|
| Protein                                         | SARS-CoV-2 S HexaPro One RBD Up  | SARS-CoV-2 S HexaPro Two RBDs Up |
| EMDB                                            | EMD-22221                        | EMD-22222                       |
| Microscope                                      | FEI Titan Krios                  | FEI Titan Krios                 |
| Voltage (kV)                                    | 300                              | 300                             |
| Detector                                        | Gatan K3                         | Gatan K3                        |
| Magnification                                   | 81,000                           | 81,000                          |
| Pixel size (Å/pix)                              | 1.08                             | 1.08                            |
| Frames per exposure                             | 40                               | 40                              |
| Exposure (e^-/Å^2)                              | 45                               | 45                              |
| Defocus range (µm)                              | 0.8-2.3                          | 0.8-2.3                         |
| Micrographs collected                           | 3,511                            | 3,511                           |
| Particles extracted/final                       | 695,490 / 85,675                 | 695,490 / 90,274                |
| Symmetry imposed                                | n/a (C1)                         | n/a (C1)                        |
| Masked resolution at 0.143 FSC (Å)              | 3.21                             | 3.20                            |

| Model refinement and validation statistics       |                                  |                                 |
|-------------------------------------------------|----------------------------------|---------------------------------|
| PDB ID                                          | 6XKL                             |                                 |
| Composition                                     |                                  |                                 |
| Amino acids                                     | 2,920                            |                                 |
| Glycans                                         | 50                               |                                 |
| RMSD bonds (Å)                                  | 0.008                            |                                 |
| RMSD angles (°)                                 | 1.01                             |                                 |
| Mean B-factors                                  |                                  |                                 |
| Amino acids                                     | 37.9                             |                                 |
| Glycans                                         | 55                               |                                 |
| Ramachandran                                    |                                  |                                 |
| Favored (%)                                     | 95.8                             |                                 |
| Allowed (%)                                     | 4.1                              |                                 |
| Outliers (%)                                    | 0.1                              |                                 |
| Rotamer outliers (%)                            | 3.6                              |                                 |
| Clash score                                     | 6.39                             |                                 |
| C-beta outliers (%)                             | 0.0                              |                                 |
| CaBLAM outliers (%)                             | 2.57                             |                                 |
| MolProbity score                                | 2.07                             |                                 |
| EMRinger score                                  | 3.00                             |                                 |
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