Multivalent nanoparticle-based vaccines protect hamsters against SARS-CoV-2 after a single immunization

Shiho Chiba1,6, Steven J. Frey2,6, Peter J. Halfmann1, Makoto Kuroda1, Tadashi Maemura1, Jie E. Yang3,4, Elizabeth R. Wright3,4, Yoshihiro Kawaoka1,5✉ & Ravi S. Kane2✉

The COVID-19 pandemic continues to wreak havoc as worldwide SARS-CoV-2 infection, hospitalization, and death rates climb unabated. Effective vaccines remain the most promising approach to counter SARS-CoV-2. Yet, while promising results are emerging from COVID-19 vaccine trials, the need for multiple doses and the challenges associated with the widespread distribution and administration of vaccines remain concerns. Here, we engineered the coat protein of the MS2 bacteriophage and generated nanoparticles displaying multiple copies of the SARS-CoV-2 spike (S) protein. The use of these nanoparticles as vaccines generated high neutralizing antibody titers and protected Syrian hamsters from a challenge with SARS-CoV-2 after a single immunization with no infectious virus detected in the lungs. This nanoparticle-based vaccine platform thus provides protection after a single immunization and may be broadly applicable for protecting against SARS-CoV-2 and future pathogens with pandemic potential.
The COVID-19 pandemic continues to rage worldwide with more than a million estimated fatalities already and global economic costs in the hundreds of billions of dollars. Without an effective vaccine, SARS-CoV-2 will continue to strain the world’s economies and devastate many facets of our society. Various vaccines such as nucleic acid-based vaccines, viral vector-based vaccines, subunit vaccines, and inactivated vaccines are in different stages of clinical trials. The contemporary vaccine candidates focus on stimulating protective immune responses to the spike (S) protein of SARS-CoV-2, the protein that facilitates viral entry by binding to the angiotensin-converting enzyme 2 (ACE2) receptor on the surface of host cells. Neutralizing antibodies that target the spike protein could, therefore, play a role in protecting the host from this viral infection.

Although these standard vaccine platforms may provide the first generation of vaccines against SARS-CoV-2, nanotechnology has the potential to offer new and improved vaccine platforms against diseases caused by emerging viruses including SARS-CoV-2. Nanoparticles such as virus-like particles (VLPs) are ideal scaffolds for antigen display, because they emulate many of the properties of natural viruses including their size and geometry. Moreover, the multivalent display of antigens from nanoscale scaffolds can result in the effective clustering of B cell receptors and greatly enhance their immunogenicity. Indeed, a recent report confirmed that the S protein displayed on a nanoscale scaffold was more immunogenic in mice than the S protein administered alone, but the study used a two sequential immunizations (prime + boost) and did not test protective efficacy in mice challenged with SARS-CoV-2. In the present study, we created a general platform for nanoparticle-based antigen display that could provide protection against SARS-CoV-2 after a single immunization.

Results and discussion

Generation and in vitro characterization of nanoparticle-based vaccines. We first sought to develop a general platform for the VLP-based multivalent display of the S protein of SARS-CoV-2. VLPs comprise coat proteins that self-assemble to form repetitive, dense arrays of antigen that emulate the size and geometry of natural viruses. We generated VLPs coated with streptavidin (SA) that display biotinylated antigens, such as biotinylated SARS-CoV-2 S protein (Fig. 1a), based on the very high-affinity biotin-streptavidin interaction.

Specifically, we generated VLPs based on the coat protein of the RNA bacteriophage MS2 that assemble to form an icosahedral structure consisting of 90 homodimers. Peabody et al. generated a variant of the MS2 coat protein in which the two subunits of the dimer were genetically fused and found that a surface loop on this single-chain dimer could tolerate the insertion of a peptide. Accordingly, we generated a single-chain MS2 coat protein dimer wherein the second monomer had an AviTag inserted in this surface loop (Supplementary Note 1). The inserted AviTag allows for site-specific biotinylation by the enzyme BirA. DNA encoding this MS2-AviTag construct was co-expressed with BirA in BL21 (DE3) competent Escherichia coli (E. coli) cells. Following expression, the cells were lysed and the MS2-AviTag was purified by using HiScreen Capto Core 700 columns and size exclusion chromatography (SEC). The purified MS2-AviTag was partially biotinylated due to its co-expression with BirA. A commercially available kit was then used to further biotinylate the MS2-AviTag in vitro, which resulted in near 100% biotinylation. The MS2-Biotin was then added dropwise to an excess of SA, which had been expressed as inclusion bodies, refolded, and purified using Iminobiotin Affinity Chromatography (IBAC). The resulting MS2-SA VLPs were separated from the excess SA through SEC.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to determine that each purified MS2-SA VLP contained ~72 streptavidin molecules (Supplementary Fig. 1). The purified MS2-SA VLPs were further characterized by analytical SEC (Fig. 1b), dynamic light scattering (DLS) (Fig. 1c), and negative-stain transmission electron microscopy (NS-TEM) (Fig. 1d). Characterization by DLS indicated that the purified MS2-SA VLPs were ~50 nm in diameter, whereas characterization by NS-TEM indicated uniform particles with a diameter of ~30 nm. The larger average size indicated by DLS may arise because the scattering intensity is proportional to the sixth power of the radius, resulting in a disproportionately higher weighting to larger particles.

We next generated a biotinylated variant of the SARS-CoV-2 S protein that could be displayed on the MS2-SA VLPs. Wrapp et al. recently reported a prefusion-stabilized variant of the SARS-CoV-2 S protein, S-2P, which contains 2 proline substitutions. To make a version of this variant that was compatible with display on the MS2-VLPs, we created plasmids encoding the stabilized prefusion S ectodomain with a C-terminal AviTag and a his-tag, which we termed S2Pro (Supplementary Note 2). The AviTag allows biotinylation and subsequent conjugation to the VLPs, whereas the his-tag allows purification by use of immobilized metal affinity chromatography (IMAC). We expressed the S2Pro protein in Expi293F cells and purified the secreted protein from the cell culture media by using IMAC. The protein was then biotinylated enzymatically in vitro by BirA. Finally, the protein was separated from BirA and other impurities by using SEC and the purity was characterized by use of SDS-PAGE (Fig. 2a and Supplementary Fig. 2).

The purified, biotinylated S2Pro protein was then mixed with the MS2-SA VLPs to form VLP-S2Pro. SDS-PAGE was used to determine that each purified VLP-S2Pro particle contained ~18 S2Pro molecules (Supplementary Fig. 1). Further SDS-PAGE analysis of the VLP-S2Pro revealed the expected three distinct bands (Fig. 2a): the upper band runs alongside S protein alone and appears at ~140 kDa, which corresponds to the approximate molecular weight of a single monomer of the S trimer; the middle band appears at the molecular weight of an MS2 coat protein dimer (~29 kDa); and the lower band corresponds to the molecular weight of a monomer of SA (~14 kDa). This characterization indicates that the VLP-S2Pro is pure and consists of only S2Pro, SA, and MS2. The VLP-S2Pro construct was also characterized by using analytical SEC (Fig. 2b).

The UV trace of the VLP-S2Pro is represented by a solid line, which appears as a single peak with no trailing shoulder. The lack of a trailing shoulder suggests that there is little to no unbound S2Pro protein in the VLP-S2Pro solution, as the UV trace of the S2Pro protein alone results in a single peak that slightly trails the peak of the VLP-S and is represented by a dashed line. Furthermore, the locations of the peaks are consistent with the constructs’ size relative to the size of the molecular weight standard thyroglobulin (660 kDa). The location at which thyroglobulin elutes is represented by a vertical gray line.

We characterized the VLP-S2Pro constructs by DLS (Fig. 2c) and then by NS-TEM (Fig. 2d) to confirm the presence and coating efficiency of biotinylated S2Pro on the VLP. Consistent with biochemical characterization, VLP-S2Pro displayed clear three-component layers, from outside to inside, prefusion-stabilized variants of S2Pro, SA, and MS2 (Fig. 2d). Compared to the naked MS2-SA, glycoprotein S2Pro decorates the exterior of VLP-S2Pro (Fig. 2d, white arrowheads), forming a ~20 nm layer of a spike-containing protein shell. This result is consistent with expectations, as the S protein (with the trimerization domain and C-terminal AviTag) would theoretically be ~20 nm in length. Finally, to ensure that the S proteins remained properly folded after conjugation to the VLPs, we assessed the binding
Fig. 1 Assembly of VLP-S and characterization of MS2-SA VLP. a Scheme illustrating assembly of VLP-S, where biotinylated MS2 (yellow, PDB: 2MS2) is added to streptavidin (red, PDB: 3RY2) to create the VLP. S (green, PDB: 6VSB) biotinylated at the C-terminus is mixed with the VLP to create VLP-S. Biotinylated residues are colored blue. b Size exclusion chromatography trace for MS2-SA VLP. The column void volume is 7.2 mL. c Characterization of the MS2-SA VLP by dynamic light scattering. d Negative-stain transmission electron micrograph of MS2-SA VLPs.

Fig. 2 Characterization of S2Pro and VLP-S2Pro. a SDS-PAGE characterization of S\textsubscript{2Pro} and VLP-S\textsubscript{2Pro}. The VLP-S\textsubscript{2Pro} has been boiled to disrupt the streptavidin-biotin conjugation. The unprocessed gel is shown in Supplementary Fig. 3. b Size exclusion chromatography traces for S\textsubscript{2Pro} (dashed line) and VLP-S\textsubscript{2Pro} (solid line). The vertical gray line represents the peak elution volume of the molecular weight standard thyroglobulin (660 kDa). The column void volume is 7.2 mL. c Characterization of the VLP-S\textsubscript{2Pro} (solid line) by dynamic light scattering. d Negative-stain transmission electron micrographs of S\textsubscript{2Pro} incorporated on the surface of MS2-SA VLPs. Arrowheads (white) indicate the S\textsubscript{2Pro} proteins on the VLP surface. e Characterization of the binding of Fc-ACE2 (gray) and CR3022 (white) to S\textsubscript{2Pro} and VLP-S\textsubscript{2Pro} by ELISA (mean ± SD, n = 6: two independent assays, each with three technical replicates). Source data are provided in Supplementary Data 1.
of ACE2-Fc and the receptor-binding domain (RBD)—binding monoclonal antibody CR3022 to S2Pro (solid line). The vertical gray line represents the peak elution volume of the molecular weight standard thyroglobulin (660 kDa). The column void volume is 7.2 mL. c Characterization of the VLP-S6Pro (solid line) by dynamic light scattering. d Negative-stain transmission electron micrographs of S6Pro incorporated on the surface of MS2-SA VLPs. Arrowheads (white) indicate the S6Pro proteins on the VLP surface. e Cryo-EM of vitrified VLP-S6Pro. The inset shows a low-pass filtered to 10 Å volume of HexaPro structure (EMD: 22221, reported previously) for comparison. Arrowheads (black) indicate the representative S6Pro proteins on the VLP surface. f Characterization of the binding of Fc-ACE2 (gray) and CR3022 (white) to S6Pro and VLP-S6Pro by ELISA (mean ± SD, n = 6: two independent assays, each with three technical replicates). Source data are provided in Supplementary Data 1.

Fig. 3 Characterization of S6Pro and VLP-S6Pro. a SDS-PAGE characterization of S6Pro and VLP-S6Pro. The VLP-S6Pro has been boiled to disrupt the streptavidin-biotin conjugation. The unprocessed gel is shown in Supplementary Fig. 3. b Size exclusion chromatography traces for S6Pro (dashed line) and VLP-S6Pro (solid line). The column void volume is 7.2 mL. c Characterization of the VLP-S6Pro (solid line) by dynamic light scattering. d Negative-stain transmission electron micrographs of S6Pro incorporated on the surface of MS2-SA VLPs. Arrowheads (white) indicate the S6Pro proteins on the VLP surface. e Cryo-EM of vitrified VLP-S6Pro. The inset shows a low-pass filtered to 10 Å volume of HexaPro structure (EMD: 22221, reported previously) for comparison. Arrowheads (black) indicate the representative S6Pro proteins on the VLP surface. f Characterization of the binding of Fc-ACE2 (gray) and CR3022 (white) to S6Pro and VLP-S6Pro by ELISA (mean ± SD, n = 6: two independent assays, each with three technical replicates). Source data are provided in Supplementary Data 1.

We also generated VLPs displaying multiple copies of a second prefusion-stabilized variant of the S protein, called HexaPro, which was reported by Hsieh et al. to be more stable than S-2P and give a higher expression yield. We expressed a variant of HexaPro containing a C-terminal AviTag and a histag, which we termed S6Pro (Fig. 3a) (Supplementary Note 3). VLP-S6Pro were generated and characterized (Fig. 3 and Supplementary Fig. 1) as described above for VLP-S2Pro. In addition, to preserve the sample’s native integrity, minimize conformational changes possibly introduced during the negative stain process, and further confirm the incorporation of spike proteins, we performed cryo-electron microscopy (cryo-EM) on the VLP-S6Pro constructs. The MS-SA core was an approximately icosahedral sphere 30 nm in diameter and S6Pro spikes were studded on the core and formed the outer shell (Fig. 3c), the morphology of which was comparable to the previous reported structure of S6Pro (EMD: 22221).

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Protective efficacy and antibody response to a single immunization in Syrian hamsters. We next evaluated the antibody responses elicited by these nanoparticle-based vaccine candidates in Syrian hamsters. We and others have demonstrated that Syrian hamsters are highly susceptible to SARS-CoV-2 infection and present with pathological phenotypes similar to those of infected humans, making hamsters an ideal animal model to evaluate vaccine candidates. Hamsters (four groups; three animals/group) were immunized with VLP-S2Pro, VLP-S6Pro, MS2-SA VLPs alone, or PBS along with Alhydrogel, an aluminum hydroxide base adjuvant. The hamsters were bled 28 days after immunization to characterize their antibody responses (Fig. 4a).

Hamsters immunized once with the VLP-S conjugates had appreciable levels of IgG antibodies against the RBD of the S protein as determined by ELISA, with endpoint titers ranging from 320 to 640 (Table 1). In contrast, as expected, negligible anti-S antibodies were detected in hamsters immunized with the controls (VLPs alone or PBS). We have compared these titers with some previously published reports. We stress, however, that the assays used in the literature are not standardized and some reports have even used different animal models, and the differences must therefore be interpreted with caution. Tostanoski et al. characterized the immunogenicity of adenovirus serotype 26 (Ad 26) vector-based vaccines expressing a stabilized SARS-CoV-2 S protein in hamsters. Ad26.SPP, also termed Ad26.COV2.S, which has been evaluated in clinical trials, showed median endpoint antibody titers against the S RBD of up to 4757 four weeks after the first immunization. While neutralization assays used a pseudovirus, the median neutralizing antibody half-maximal inhibitory concentration (IC50) titers reported for the Ad26.SPP were as high as 375. Corbett et al. conducted a study in mice to test the mRNA vaccine called mRNA-1273, for which they reported geometric mean endpoint titers of 4479 (against S) 4 weeks after a single dose. Neutralizing antibody reciprocal IC50 geometric mean titers against a pseudovirus 4 weeks after a single immunization with mRNA-1273 at the highest dose (10 μg) were 775. Thus, our endpoint and neutralizing antibody titers compare well with those obtained using these other modalities that have been through clinical trials.

Four weeks after immunization, the animals were intranasally inoculated with 10^3 plaque-forming units of SARS-CoV-2/UT-NCGM02/Human/2020/Tokyo. While the animals in both control groups experienced significant weight loss, those immunized with VLP-S2Pro had recovered their average initial weight by day 3, and those immunized with VLP-S6Pro...
showed a slight increase in body weight over this 3-day period (Fig. 4b).

Three days after virus challenge, which is when virus levels in the lungs peak\(^1\), the animals were sacrificed and lung and nasal turbinate samples were collected. As expected, animals in both control groups (PBS and MS2-SA VLPs) had high viral loads in the lungs; however, in hamsters immunized with VLP-S\(_{2Pro}\) or VLP-S\(_{6Pro}\), no infectious virus was detected in the lungs (Fig. 4c). The lack of infectious virus in the lungs was consistent with the differences observed in body weight change between the vaccine and control groups. Moreover, despite the intranasal mode of challenge with SARS-CoV-2, the hamsters immunized with VLP-S\(_{2Pro}\) or VLP-S\(_{6Pro}\) had less virus in their nasal turbinate (Fig. 4d), with mean titers more than 150-fold lower (VLP-S\(_{2Pro}\)) and more than 700-fold lower (VLP-S\(_{6Pro}\)) relative to MS2-SA VLP controls.

In summation, we have developed a highly effective nanoparticle-based vaccine that provides protection in an animal model against SARS-CoV-2 after a single immunization. While these results are exciting, it is important to consider obstacles that might arise during clinical translation. One potential concern with subunit vaccines is the expression yield. Hsieh et al. designed the S\(_{6Pro}\) variant by introducing substitutions that improve both expression yield and stability and reported a yield of 10.5 mg/L in FreeStyle 293-F cells\(^18\). Expression in insect cells could also be evaluated for improving the expression yield. Further optimization would undoubtedly be needed to develop a cGMP (current Good Manufacturing Practices) compliant manufacturing process. Another potential concern is an immune response to the MS2-SA VLP scaffold. Antibody responses have been reported to other scaffolds, including viral vectors, and are certainly possible with our construct. The concerns are mitigated in part by the protective efficacy after a single dose. However, if necessary, the scaffold could be further shielded from the immune system by using techniques such as nanopatterning\(^23\).

Another important issue is the emergence of “variants of concern.” In this context, vaccine platforms that generate a robust immune response would be advantageous as they might still be able to provide some protection against resistant strains despite a reduction in neutralizing antibody titers. Our vaccine platform could also be readily adapted for the display of S proteins from variant strains. In the future, it would be particularly important to use this platform to display engineered antigens that provide broader—pan-sarbecovirus or pan-coronavirus—immunity. Given the number of people that must be immunized, and societal habits, an ideal vaccine against SARS-CoV-2 would offer protection after only one immunization. The development of multiple effective vaccine platforms\(^21,24,25\) that can offer such protection is critical because vaccines remain the best approach for protection from current and future pandemics. The nanoparticle-based vaccine platform described here should be broadly applicable for protecting against important pathogens including, but not limited to, SARS-CoV-2 and influenza viruses.

### Methods

**Expression and purification of MS2.** DNA encoding single-chain MS2 coat protein dimer with an AviTag inserted between the 14 and 15 residues of the second coat protein monomer was cloned into pET-28b between the Ndel and Xhol restrictions sites by GenScript Biotech Corporation (Piscataway, NJ). The MS2 dimer with the inserted AviTag was co-transformed with pAcm-BirA (Avidity LLC) into BL21(DE3) competent E. coli (New England Biolabs) according to the manufacturer’s instructions. The transformation was added to 5 mL of 2xYT media and grown overnight at 37°C. The 5-mL starter culture was then added to 1 L of 2xYT media, which was incubated shaking at 37°C until induction with IPTG (1 M; GoldBio) at an OD of 0.6. Immediately after induction, biotin (50 µM) was added to the culture and the incubator temperature was reduced to 30°C. After overnight incubation, the culture was centrifuged for 7 min at 7000×g and the supernatant was decanted. The cell pellet was resuspended in 25 mL of 20 mM Tris Base (pH 8.0) supplemented with lysozyme (0.5 mg/mL, Alfa Aesar), a protease inhibitor tablet (Sigma-Aldrich), and benzonase (125 units; EMD Millipore). The resuspended cells were then kept on ice and stirred intermittently for 20 min. Sodium deoxycholate (Alfa Aesar) was then added to a final concentration of 0.1%

| Animal # | PBS | VLP-S\(_{2Pro}\) | VLP-S\(_{6Pro}\) |
|----------|-----|-----------------|-----------------|
| Replicate | Traveling antibody endpoint titera | Neutralizing antibody titerb | |
| 1 | <10 | <10 | <10 |
| 2 | 20,480 | 640 | |
| 3 | <10 | <10 | <10 |

| Vaccine group | Replicate | Traveling antibody endpoint titera | Neutralizing antibody titerb |
|---------------|-----------|-----------------------------------|------------------------------|
| MS2-SA VLP    | 1         | 20,480                            | 20,480                       |
|               | 2         | 35,113                            | 35,113                       |
|               | 3         | 40,960                            | 40,960                       |
| VLP-S\(_{2Pro}\) | 1         | 81,920                            | 81,920                       |
|               | 2         | 81,920                            | 81,920                       |
|               | 3         | 81,920                            | 81,920                       |
| VLP-S\(_{6Pro}\) | 1         | 81,920                            | 81,920                       |
|               | 2         | 81,920                            | 81,920                       |
|               | 3         | 81,920                            | 81,920                       |
Expression, refolding, and purification of streptavidin (SA). SA was expressed, refolded, and purified essentially as previously described.26,27 Briefly, DNA encoding SA (Addgene plasmid #46367, a gift from Mark Howarth26) was transformed into BL21 (DE3) cells (New England Biolabs) according to the manufacturer's protocol. Inclusion body formation was confirmed by four culture tubes, each containing 5 mL of 2xYT media, which were incubated overnight at 37 °C. Each 5 mL culture was added to one of four 1 L flasks of 2xYT and grown at 37 °C. Upon reaching an OD of 0.6, expression of inclusion bodies was induced using IPTG (1 M; GoldBio) and the temperature of the incubator was reduced to 30 °C. After incubation, the culture was centrifuged at 77 min at 7000 × g (10000 × g) supernatant media was diluted twofold in PBS and run through a 1-mL MabSelect SuperX column (MiliporeSigma). The column was then washed with 20 column volumes of IBAC binding buffer. Then, 8 mL of wash buffer #1 (50 mM Tris, 100 mM NaCl, pH 8.0) was added to incubate at 4 °C for 1 h with occasional mixing. The supernatant was then homogenized, brought to a concentration of 0.1% (v/v) Triton X-100, pH 8.0, homogenized, and sonicated for 30 seconds at an amplitude of 35%. Each mix was then centrifuged at 27,000 × g for 20 min. The supernatant was discarded, and the two pellets were each again resuspended in 50 mL of resuspension buffer supplemented with lysozyme (1 mg/mL; Alfa Aesar) and benzonase (500 units; EMD Millipore) and the lysis procedure was repeated. This procedure resulted in two inclusion body pellets, which were then washed. Each inclusion body pellet was resuspended in 50 mL of wash buffer #1 (50 mM Tris, 100 mM NaCl, 100 mM EDTA, 0.5% (v/v) Triton X-100, pH 8.0), homogenized, and sonicated for 30 seconds at an amplitude of 35%. Each mix was then centrifuged at 12,000 × g for 10 min. The supernatant containing the folded SA was filtered by using a 0.45-µm bottle-top filter (VWR). The resulting filtrate was stirred vigorously, and ammonium sulfate was added to a concentration of 1.9 M to precipitate the protein. The precipitate was being stirred continuously overnight at 4 °C. In soluble protein was then pelleted by centrifugation at 15,000 × g for 15 min. The supernatant was drawn into a syringe, which was loaded onto a syringe pump, and added dropwise to a 1 L of chilled PBS. SA was being stirred continuously overnight at 4 °C. The solution of refolded protein was stirred continuously overnight at 4 °C. Insoluble protein was then pelleted by centrifugation at 7000 × g for 15 min and discarded. The supernatant containing the folded SA was filtered by using a 0.45-µm bottle-top filter. The resulting filtrate was stirred vigorously, and ammonium sulfate was added to a concentration of 1.9 M to precipitate the protein. The precipitate was being stirred continuously overnight at 4 °C. After being stirred for 3 h at 4 °C, the precipitate was removed by centrifugation for 10 min at 7000 × g. The supernatant was then filtered by using a 0.45-µm bottle-top filter. The ammonium sulfate concentration of the resulting filtrate was brought up to a total concentration of 3.6 M and stirred for 3 h at 4 °C to precipitate the SA. The SA precipitate was pelleted by centrifugation at 7000 × g for 20 min and resuspended in 20 mL of InmunoBioin Affinity Chromatography (IBAC) binding buffer (50 mM Sodium Borate, 300 mM NaCl, pH 11.0). This SA solution was then passed through 5 mL of Pierce Immobilin Agarose (Thermo Scientific) in a gravity flow column (G-Biosciences). The column was then washed with 40 mL of wash buffer (42 mM sodium bicarbonate, 8 mM sodium carbonate, 300 mM NaCl, 20 mM imidazole). The SA proteins were eluted from the column by incubating the Ni-NTA resin with 3 mL of elution buffer (42 mM sodium bicarbonate, 8 mM sodium carbonate, 300 mM NaCl, 300 mM imidazole) for 5 min before allowing for flow by gravity. This elution procedure was repeated twice, resulting in 9 mL of eluate. The eluate was concentrated by using a 10 kDa MWCO centrifugal filter (Millipore Sigma). S proteins were buffer exchanged into 20 mM Tris, 2 mM NaCl, pH 8.0 to allow for in vitro biotinylation and were quantified by using the BCA assay (Thermo Scientific).

In vitro biotinylation of AviTagged MS2 and SARS-CoV-2 S. Biotinylation was performed in vitro using a BirA biotin-protein ligase standard reaction kit (Aviday) following the manufacturer’s protocol. In brief, the protein solution (either MS2 or SARS-CoV-2 S) was buffer exchanged into a 20 mM Tris, 2 mM NaCl, pH 8.0 buffer and the protein concentration was adjusted to 45 µM BirA and a proprietary mixture containing biotin, ATP, and magnesium acetate (Biomix B) was added to the protein solution. This solution was shaken vigorously at 37 °C. After 2 h at 37 °C, more Biomix B was added, and the solution was nuated at 4 °C overnight. The proteins of interest were then purified through SEC with a Superdex 200 Increase 10/300 column (Cytiva) connected to an AKTA pure (Cytiva) and controlled by Unicorn 7.2 software (Cytiva). Biotinylated S proteins were quantified by using the BCA assay (Thermo Scientific).

Expression and purification of MS2-VA VLPs. MS2-SA was added dropwise to a molar excess of refolded SA solution that was stirred vigorously in a 5-mL glass vial. After a 30-minute incubation, the MS2-SA VLP was separated from the excess SA through SEC with a Superdex 200 Increase 10/300 column (Cytiva). The MS2-SA VLP was quantified by boiling a small aliquot at 90 °C in Nu-PAGE lithium dodecyl sulfate (LDS) sample buffer (Invitrogen) for 30 min and running the sample on a 12% Bis-Tris gel (Invitrogen). The gel was stained with SimplyBlue SafeStain (Invitrogen) and subsequently de-stained. Once sufficiently de-stained, the gel was imaged by using the ChemiDoc MP imaging system and Image Lab 5.2.1 software (Bio-Rad).

Preparation of VLP-S. MS2-SA and biotinylated S protein were mixed in a stoichiometric ratio found by using analytical SEC. We used analytical SEC to characterize mixtures consisting of 5 µg of biotinylated S protein and varying amounts of MS2-SA VLP. The ratio of the mixture that contained the least MS2-SA VLP was determined by using analytical SEC. We used analytical SEC to characterize mixtures consisting of 5 µg of biotinylated S protein and varying amounts of MS2-SA VLP. The ratio of the mixture that contained the least MS2-SA VLP was determined by using analytical SEC.
incubate for 1 h. Then, the plate was washed twice with PBST and developed with TMB substrate solution (Thermo Scientific) for 3 min; the reaction was then stopped with 0.16 M sulfuric acid. The absorbance of each well at 450 nm was read using a SpectraMax i3x plate reader (Molecular Devices) and Gen5 2.07 software (BioTek).

**Analytical SEC.** A Superdex 200 Increase 10/300 column (Cytiva) connected to an AKTA pure (Cytiva) and controlled by Unicorn 7.2 software (Cytiva) was equilibrated with PBS-T (0.1% Tween 20, 0.1% sodium azide) at 1 ml/column, and 50 µl of either VLP-S solution or S alone was loaded into the sample loop. Each sample included 5 µg of S protein. The sample loop was then flushed with PBS such that the sample was directed through the column at a flow rate of 0.5 ml/min. One column volume of PBS was run through the column. Unicorn 7 (Cytiva) was used to control the system and to output a chromatogram of UV absorbance at 210 nm.

**Dynamic light scattering.** A Vette (Eppendorf) containing 100 µl of VLP-S at a concentration of ~0.05 µg µl−1 was loaded into a DynaPro NanoStar Dynamic Light Scattering detector (Wyatt Technology). For each measurement, Dynamic software (Wyatt Technology) was used to allow the temperature to equilibrate to 25 °C, to collect ten acquisitions, and to output the results. Results were displayed by % Mass using the Isotropic Spheres model.

**Negative stain transmission electron microscopy.** Conventional negative-stain transmission electron microscopy (TEM) was performed, as described previously. Briefly, 4 µl of the diluted samples was applied onto glow-discharged 300 mesh copper grids (CF300-Cu; Electron Microscopy Sciences, PA), washed with PBS (1X), and stained in droplets of 1% phosphotungstic acid (PTA, PH 6–7) for 1 min. The grids were then drained from the grid backside and air-dried inside a petri dish for at least 30 min at room temperature to minimize the negative-stain artifacts of flattening and stacking.

**Plunge freezing and cryo-electron microscopy.** 4 µl of the VLP suspension was added to a glow-discharged copper grid (C-Flat 1.2/1.3, 400 mesh, Protechips). Grids were plunged frozen into liquid ethane by double-sided blotting using Vitrobot Mark IV (ThermoScientific) and stored in liquid nitrogen until imaging. Cryo-electron microscopy (cryo-EM) and cryo-electron tomography (cryo-ET) were performed as described previously on a Titan Krios (ThermoScientific Hillsboro, OR, USA) at 300 kV39. Images (defocus of ~5 µm) were recorded on a post-GIF Gatan K3 camera in EFTEM mode (4603 A/pixel) with a 20-eV s/Δt, CDS counting mode, using SerialEM 3.8.10. A total dose of 23.30 e/Å² was used and 34 frames were saved (1.14 e/Å² per frame). Frames were motion-corrected in MotionCOR2. Images were low pass filtered to 10 Å for better visualization and contrast in EMAN2.203.

**Viruses and titration assays.** The virus isolate SARS-CoV-2/UT-NCGM02/Human/2020/Tokyo was used in this study and was previously characterized in Syrian hamsters.

**Hamster immunization study.** The hamster immunization study in hamsters was performed after approval by the Institutional Animal Care and Use Committee at the University of Wisconsin. Golden Syrian hamsters (4-week-old females) were immunized with either 60 µg of SARS-CoV-2 S protein presented on the MS2-SA VLP. Antibody response was determined using sera from each hamster. The data are presented as the mean ± SD. For in vivo characterization, there were four groups (receiving either VLP-S, VLP-S, MS2-SA, or PBS) each with three hamsters (n = 3). To determine the resulting RBD IgG Endpoint Titers and Neutralizing Antibody titers (Table 1), three independent assays were conducted using sera from each hamster. The data are presented for each independent assay and also as the geometric mean with the geometric SD factor. Bodyweight after challenge with SARS-CoV-2 (Fig. 4b) was presented as the mean ± SD and significance was determined by a one-way analysis of variance (ANOVA) and Dunnett post-hoc multiple comparison between groups (p = 0.1). Assumptions of the normality of residuals and homogeneity of variance were validated by the Shapiro-Wilk test and the Brown-Forsythe test, respectively. All statistical analysis was carried out using Excel 2013 (Microsoft) and Prism 8 (GraphPad).

**Data availability**

The data needed to support the conclusions of this study have been included in the supplementary data. All other data supporting the conclusions of this study are available from the corresponding authors upon reasonable request.

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