ABSTRACT: Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) utilizes an extensively glycosylated surface spike (S) protein to mediate host cell entry, and the S protein glycosylation plays key roles in altering the viral binding/function and infectivity. However, the molecular structures and glycan heterogeneity of the new O-glycans found on the S protein regional-binding domain (S-RBD) remain cryptic because of the challenges in intact glycoform analysis by conventional bottom-up glycoproteomic approaches. Here, we report the complete structural elucidation of intact O-glycan proteoforms through a hybrid native and denaturing top-down mass spectrometry (MS) approach employing both trapped ion mobility spectrometry (TIMS) quadrupole time-of-flight and ultrahigh-resolution Fourier transform ion cyclotron resonance (FTICR)-MS. Native top-down TIMS-MS/MS separates the protein conformers of the S-RBD to reveal their gas-phase structural heterogeneity, and top-down FTICR-MS/MS provides in-depth glycoform analysis for unambiguous identification of the glycan structures and their glycosites. A total of eight O-glycoforms and their relative molecular abundance are structurally elucidated for the first time. These findings demonstrate that this hybrid top-down MS approach can provide a high-resolution proteoform-resolved mapping of diverse O-glycoforms of the S glycoprotein, which lays a strong molecular foundation to uncover the functional roles of their O-glycans. This proteoform-resolved approach can be applied to reveal the structural O-glycoform heterogeneity of emergent SARS-CoV-2 S-RBD variants as well as other O-glycoproteins in general.

INTRODUCTION

The novel zoonotic 2019 coronavirus disease (COVID-19) global pandemic has led to more than 102 million reported cases and over 3 million deaths as of June 2021. The causative pathogen of COVID-19, severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), utilizes an extensively glycosylated spike (S) protein that protrudes from the viral surface to bind receptor angiotensin-converting enzyme 2 (ACE2) for cell entry. The evolution of the S protein is particularly important as it initiates pathogenesis and is the main target of vaccination and antibody therapeutic design, and a substantial number of predicted mutations in the S protein regional-binding domain (RBD) can potentially enhance ACE2 binding. Moreover, the more than 1 900 000 viral genomic sequences generated from global molecular epidemiology studies (as of June 9, 2021, www.gisaid.org) reveal the emergence of a number of variants in the S protein, indicating that SARS-CoV-2 may be evolving to acquire selective replication advantages, drug insensitivity, and immunological resistance, thereby underscoring the need for new technologies capable of rapid and deep structural profiling of the virus.

IMPORTANTLY, glycans flank the polybasic furin cleavage site of the S protein necessary for S stability during biogenesis and influence the conformational dynamics by masking protein regions from cleavage during ACE2 binding. How these different sites are glycosylated and how they influence ACE2 binding are likely to affect cell infectivity and could shield certain epitopes from antibody neutralization. The SARS-CoV-2 S protein carries 22 N-glycosylation sequons per protomer, which have been characterized in detail. However, characterization of O-glycosylation is a major challenge, and only putative O-glycosites have been reported. The presence and exact molecular structures of these O-glycans remain cryptic due to the immense glycan microheterogeneity.
and the lack of intact glycoprotein analysis. Importantly, individual glycosites can give rise to many glycan structural variants, i.e., glycoforms, and these differences in glycan structures, even at a single glycosite, can have critical implications on biological functions. It is crucial to decipher the intact O-glycoforms of the S-RBD because they are expected to play a role in viral function and viral binding with ACE2. Therefore, new approaches capable of the comprehensive structural elucidation of O-glycans are essential to provide new avenues for rational therapeutic development and to investigate S protein alterations in the emerging SARS-CoV-2 variants.

Conventional methods to analyze protein glycosylation rely on the bottom-up MS approach of analyzing glycopeptides obtained through enzymatic digestion. Although the bottom-up MS approach is a high-throughput method for global proteome identification of glycosites and N-glycan heterogeneity from complex samples, the relative abundance of various intact glycoforms and the overall post-translational modification (PTM) compositions of different coappearing “proteoforms” are lost. Moreover, O-glycan heterogeneity and structure characterization have not been achieved by the current methods. In contrast, by combining glycoproteomics with the top-down MS approach, which preserves the intact glycoprotein enabling high-resolution proteoform-resolved analysis, we could achieve simultaneous characterization of the molecular structures, the site specificity, and the relative abundance of various glycoforms. By further integrating native MS, which has recently emerged as a powerful structural biology tool to study protein structure–function relationships, we could investigate the gas-phase structural heterogeneity to achieve the direct quantification of individual glycoproteoforms.

Here, we developed a hybrid native and denaturing top-down MS method for the structural analysis of intact O-glycan proteoforms of the S-RBD (Figure 1). We harness the capabilities of a hybrid TIMS quadrupole time-of-flight (QTOF) mass spectrometer (timsTOF Pro) (Figure 1C), which provides high resolving power and sensitivity for both selective and comprehensive ion mobility separations of various native protein conformers and analyze their gas-phase structural heterogeneity, and the ultrahigh-resolution of a 12T Fourier transform ion cyclotron resonance (FTICR)-MS (Figure 1D), to comprehensively characterize the O-glycoforms of the S-RBD, including the exact glycan structures and relative molecular abundance (Figure 1E). We demonstrate that this hybrid native top-down MS approach can provide a high-resolution and wholistic proteoform-resolved landscape of diverse O-glycoforms of the S-RBD.

### RESULTS AND DISCUSSION

Native TIMS-MS Analysis of the S-Protein RBD. S-RBD protein expressed from HEK293 cells was used to perform native top-down MS for comprehensive glycoform analysis (Figure 1). We leveraged a native TIMS-MS approach to uncover multiple levels of information related to the intact protein structure, the intact glycoforms, and the native structural heterogeneity arising from the complexity and structural diversity of the S-RBD glycans. After ensuring reproducible native S-RBD protein sample preparation (Figure S1), we used the TIMS, the front-end of the timsTOF Pro (see Materials and Methods for details), to achieve high-resolution ion mobility separation of protein conformers. TIMS is a relatively recent high-resolution ion mobility method in which multiple ions are held in place against a moving gas under an electric field and then ejected at characteristic elution voltages from the TIMS cell enabling the determination of ion mobility ($1/K_e$) for structural analysis. The mobility measured in TIMS allows collision cross-section (CCS) determination of protein conformers, which relates to their overall size and shape, and consequently can be used to evaluate changes in the three-dimensional structure. However, ion mobility mapping and MS$^1$ analysis reveal enormous complexity in the native S-RBD sample (Figure S2). The diffuse spread of the ion mobility spectrum suggests multiple coexisting S-RBD proteoforms potentially arising from the multiple glycans (Figure S2A and S2C). The native S-RBD without any deglycosylation could not be isotopically resolved due to multiple overlapping ion signals from all of the diverse glycoforms/proteoforms carrying different charges (Figure S2C and S2D), as observed in complex glycoproteins previously.

To elucidate the O-glycans of the S-RBD, we completely removed the N-glycans from the S-RBD using a native PNGase F treatment (see Materials and Methods for details) to minimize the interference posed by the enormous N-glycan heterogeneity (Figure 2A). The removal of N-glycans constitutes $>10$ kDa of molecular weight loss compared to the fully glycosylated S-RBD, as expected from the multiple proteoforms detected by native TIMS-MS analysis (Figure S2). Fortunately, we found that after complete removal of N-glycans, the remaining O-glycans on the S-RBD were fully reproducible by native MS (Figure 2B). Although the remaining PNGase F was found to be coexisting with the S-RBD, further optimization of the deglycosylation treatment yielded highly reproducible native S-RBD protein sample preparation (Figure S1), we used the TIMS, the front-end of the timsTOF Pro (see Materials and Methods for details), to achieve high-resolution ion mobility separation of protein conformers. TIMS is a relatively recent high-resolution ion mobility method in which multiple ions are held in place against a moving gas under an electric field and then ejected at characteristic elution voltages from the TIMS cell enabling the determination of ion mobility ($1/K_e$) for structural analysis. The mobility measured in TIMS allows collision cross-section (CCS) determination of protein conformers, which relates to their overall size and shape, and consequently can be used to evaluate changes in the three-dimensional structure. However, ion mobility mapping and MS$^1$ analysis reveal enormous complexity in the native S-RBD sample (Figure S2). The diffuse spread of the ion mobility spectrum suggests multiple coexisting S-RBD proteoforms potentially arising from the multiple glycans (Figure S2A and S2C). The native S-RBD without any deglycosylation could not be isotopically resolved due to multiple overlapping ion signals from all of the diverse glycoforms/proteoforms carrying different charges (Figure S2C and S2D), as observed in complex glycoproteins previously.

![Figure 1. High-resolution glycoform characterization of intact S-RBD by hybrid top-down MS. (A–D) Illustration of the top-down glycoproteomics workflow for the comprehensive analysis of the S-RBD glycoforms. (A) SARS-CoV-2 coronavirus features a surface S protein that possesses a glycosylated RBD (highlighted in the dashed box in B). (B) Intact glycoprotein analysis proceeds by directly infusing solubilized S-RBD and electrospraying S-RBD protein ions into either (C) a hybrid trapped ion mobility spectrometry (TIMS) MS device (timsTOF) for native ion mobility MS/MS analysis or (D) an ultrahigh-resolution Fourier transform ion cyclotron (FTICR)-MS for denaturing top-down MS/MS. (E) Specific isolation of S-RBD proteoforms by top-down MS analysis, illustrating raw MS$^1$ and corresponding deconvoluted protein spectrum, for structural characterization of glycoforms. PDB: 6VXX (A) and 6M0J (B and E).](https://doi.org/10.1021/jacs.1c02713)
resolved charge states of the S-RBD (Figure 2C). Deconvoluted MS analysis of the isotopically resolved charge states revealed multiple O-glycoforms with molecular weights ranging from 25.5 to 26.8 kDa (Figure 2C, inset).

We next used TIMS to separate and analyze the various S-RBD O-glycoforms after N-glycan removal by taking full advantage of the TIMS front end of the timsTOF Pro instrument. MS parameters were optimized to preserve the intact S-RBD O-glycoforms for native MS analysis (Figure S3). Moreover, we found that native-like conformers of the S-RBD could be resolved and analyzed under sufficiently soft TIMS cell activation parameters (Figure S4). After careful additional TIMS tuning and optimization of native desolvation parameters (Figures S5 and S6), the timsTOF was sufficiently sensitive to allow high-resolution ion mobility separation of intact S-RBD O-glycoforms (Figure 3). Both native S-RBD monomers (z = 8+ to 12+) and noncovalent dimers (z = 14+ to 16+) were resolved by the native TIMS-MS analysis (Figure 3A). TIMS mobility separation revealed two distinct protein gas-phase native conformers separated in regional mobility between 0.95 and 1.25 1/K0 (Figure 3B). By interrogating each of the resolved mobility regions, native MS1 analysis revealed that the two gas-phase conformers correspond to the S-RBD monomer (Region 1) and dimer (Region 2) (Figure 3C).

TIMS enables the isolation of specific charge states corresponding to the ion mobility regions of the monomer (Figure S7) and dimer (Figure S8), thereby enabling the structural analysis of these native-like protein conformers. We note that the S-RBD dimer formation was persistent under various MS sample preparation conditions (Figure S9) and was also observed for the native S-RBD sample without deglycosylation (Figure S1). Recently, Brun et al. reported the presence of free recombinant S1 subunit found in HEK293 cell supernatants following viral infection. Thus, the native S-RBD dimer formation we observe suggests the potential for S1 dimer formation through RBD association and may point to functionally relevant roles during viral infection.

TIMS CCS distributions (determined using N2 as the TIMS buffer gas and hereafter referred to as TIMSCCSN2) of the most abundant ions corresponding to the native S-RBD monomer (10+ charge state, 2625 m/z isolation, 1.10 1/K0 peak) and S-RBD dimer (15+ charge state, 3484 m/z isolation, 1.08 1/K0 peak) corresponding to the ion mobility Regions 1 and 2 in B and C obtained under nitrogen drift gas (TIMSCCSN2). (E) Plot of TIMSCCSN2 values as a function of charge state for the ion mobility regions 1 and 2 corresponding to B and C. Data are representative of n = 3 independent experiments with error bars indicating the standard deviation of the mean.
additional O-glycans. Moreover, the CCS distribution for the native S-RBD O-glycoform dimer (Region 2, 15+ charge state, TIMSCCSN2 3246 ± 7 Å²) obtained from the native S-RBD MS analysis with glycosite localized to Thr323 corresponding to Core 1 GalNAcGalNeuAc, GalNAcGal(GalNeuAc)₂, and GalNAcGal glycoforms and Core 2 GalNAcGalNeuAc, GalNAcGal(GalNeuAc)₂, and GalNAcGal glycoforms. Theoretical ion distributions are indicated by the red dots, and mass accuracy errors are listed for each fragment ion. (C) Normalized extracted ion mobility spectra (EIMSs) of various ion mobility regions isolated from the native monomer (z = 10+). O-Glycoform structures corresponding to the various EIMSs are shown (compounds 1−3). MS isolation and ion mobility peak value are included for each EIM. Broad features suggest diverse structural heterogeneity between the various glycoforms. (D) Collision cross section of the S-RBD monomer (10+ charge state) obtained under nitrogen drift gas (TIMSCCSN2) for the various EIM compounds shown in C. Compounds 1, 2, and 3 (Comp 1, 2, and 3) correspond to GalNAcGalNeuAc, GalNAcGal(GalNeuAc)₂, and GalNAcGal(GalNeuAc)₂(GalNAcGalNeuAc) glycoforms, respectively. Nonglycosylated S-RBD (no glycan) X-ray structure (PDB: 6M0J) with disulfide bonds highlighted in yellow is used as the reference. Data are representative of n = 3 independent experiments with error bars indicating the standard deviation of the mean.

Figure 4. Native top-down MS/MS and native TIMS-MS of intact S-RBD O-glycoforms after PNGase F treatment. (A) Isolation of native monomer (z = 11+, centered at 2388.3 m/z) from MS followed by MS/MS analysis of isolated precursor. (Inset) Associated ion mobility heat map after isolation of the precursor and after CAD fragmentation. (B) Representative CAD fragment ions (b₁₄⁺, b₁₄⁺, b₁₄⁺, b₁₄⁺, b₁₄⁺, b₁₄⁺, b₁₄⁺, and b₁₄⁺) obtained from the native MS/MS analysis with glycosite localized to Thr323 corresponding to Core 1 GalNAcGalNeuAc, GalNAcGal(GalNeuAc)₂, and GalNAcGal glycoforms and Core 2 GalNAcGalNeuAc, GalNAcGal(GalNeuAc)₂, and GalNAcGal glycoforms. Theoretical ion distributions are indicated by the red dots, and mass accuracy errors are listed for each fragment ion. (C) Normalized extracted ion mobility spectra (EIMSs) of various ion mobility regions isolated from the native monomer (z = 10+). O-Glycoform structures corresponding to the various EIMSs are shown (compounds 1−3). MS isolation and ion mobility peak value are included for each EIM. Broad features suggest diverse structural heterogeneity between the various glycoforms. (D) Collision cross section of the S-RBD monomer (10+ charge state) obtained under nitrogen drift gas (TIMSCCSN2) for the various EIM compounds shown in C. Compounds 1, 2, and 3 (Comp 1, 2, and 3) correspond to GalNAcGalNeuAc, GalNAcGal(GalNeuAc)₂, and GalNAcGal(GalNeuAc)₂(GalNAcGalNeuAc) glycoforms, respectively. Nonglycosylated S-RBD (no glycan) X-ray structure (PDB: 6M0J) with disulfide bonds highlighted in yellow is used as the reference. Data are representative of n = 3 independent experiments with error bars indicating the standard deviation of the mean.
GalNAcGal(GalNeuAc)(GlcNAcGalNeuAc) glycan structures. The most abundant glycoform identified for the dimeric S-RBD (15+ charge state) corresponds to a deconvoluted mass of 52 257.22 Da, consistent with the expected sequence mass of dimeric S-RBD with Core 1 GalNAcGalNeuAc (52 257.54 Da, protein sequence map provided in Figure S10). All of the detected S-RBD O-glycans were confidently assigned solely to Thr323 (Figure S10). Coincidentally, this Thr323 residue is also \( n + 1 \) adjacent to a proline at residue 322, which is consistent with previous reports of increased O-glycosylation frequency near proline residues.63 Importantly, the precise localization of the S-RBD O-glycoforms to Thr323 reported here addresses the O-glycosite ambiguity in the previous studies on HEK293-derived S-RBD encountered using bottom-up MS approaches.20,21 We found that some glycoforms were sufficiently separated in ion mobility and \( m/z \) to enable CCS determination (Figure 4C and Figure S11). Compared to the nonglycosylated S-RBD (\( \sim 2180 \text{ Å}^2 \)), the O-glycosylated S-RBD showed a notable CCS increase (\( \sim 60 \text{ Å}^2 \)) for the GalNAcGal(GalNeuAc)(GlcNAcGalNeuAc) glycan, which is consistent with a previous report on the effects of single-extended O-glycan structure elongation on protein CCS (Figure 4D).61 The ability to connect between specific S-RBD O-glycoforms and their gas-phase structural heterogeneity enables deeper insights into the structural diversity of these complex O-glycoforms. We note that the broad feature shown in the extracted ion mobility spectra for the various glycoforms suggests diverse structural and conformational heterogeneity that hinder the ion mobility resolution by the timsTOF Pro (Figure 4C). In addition, due to the presence of multiple disulfide bonds and the low MS/MS signal abundance corresponding to the lower abundance S-RBD proteoforms, we found that CAD fragmentation on the timsTOF Pro alone was not sufficient for complete characterization of all S-RBD O-glycoforms.

To achieve in-depth glycoform analysis, we further utilized an ultrahigh-resolution 12T FTICR-MS equipped with CAD and electron capture dissociation (ECD) fragmentation.
modes and capable of baseline isotopically resolving the S-RBD O-glycoforms for MS/MS analysis (see Materials and Methods for details, Figure S12). Following further MS analysis, individual glycoforms were directly visualized with high resolution (Figure 5A and 5B). Following specific quadrupole isolation centered at appropriate m/z widths to capture individual S-RBD proteoforms (Figure 5C), the resolved intact glycoforms can be characterized by MS/MS using a combination of fragmentation strategies to achieve confident protein sequence assignments (Figure 5D).

By applying this method on all detectable S-RBD proteoforms, a suite of MS/MS spectra was generated for sequence analysis (Figure S13). This approach allowed for in-depth characterization of the O-glycoforms to reveal multiple additional S-RBD glycoforms with Core 1 and Core 2 O-glycan structures (Figure S14). The majority of the O-glycan identifications in this previous study focused on a specific site (T678) and other potential sites outside of the RBD. Moreover, the GalNAcGal(GalNeuAc)(GlcNAcGalFuc) glycan structure without terminal NeuAc was detected as a fragment ion byproduct during MS/MS analysis (Figure 5 and Figure S14A). The preservation of intact glycoform structures by this hybrid top-down MS approach underscores the importance of intact proteoform-level analysis to unambiguously assign complex O-glycan structures. In addition, the GalNAcGal(GalNeuAc)(GlcNAcGalFuc)-related proteoform without N-terminal acetylation (15+ most abundant charge state, centered at 1757.7 m/z) was also characterized (Figure S14). Therefore, the high-resolution
glycoform mapping presented in Figure 5 illustrates a unique strength of this native top-down MS approach to achieve isotopically resolved and high-accuracy characterization of highly heterogeneous glycoproteins, a major challenge in intact glycoprotein analysis.44,66

We also identified and characterized Core 1 (Galβ1–3GalNAc-Ser/Thr) O-glycan structures such as GalNAcGal-Gal (15+ most abundant charge state, centered at 1723.8 m/z) (Figure S15) and GalNAcGal( NeuAc)2 (15+ most abundant charge state, centered at 1743.2 m/z) (Figure S16). Interestingly, these two Core 1 O-glycans were previously reported for the S-RBD as potential modifications; however, the previous studies were not able to resolve the exact glycoforms and O-glycosite due to the challenges arising from inferring intact glycoprotein structures from peptide digests and the low signal abundance of O-glycans under conventional MS analysis.20,21,33 However, our native top-down MS strategy enables us to unambiguously reveal the exact glycoform corresponding to each of these Core 1 O-glycans. We detected an additional proteoform centered at 1745.5 m/z that could not be comprehensively characterized on both timsTOF Pro and FTICR due to low fragment ion abundance and close proximity to other glycoforms (Figure S17). However, this particular proteoform was found to only represent a minor ~4% of the total S-RBD proteoform abundance (Figure 6). Three additional Core 2 glycan structures were identified: GalNAcGalGalNAcGal (15+ most abundant charge state, centered at 1748.1 m/z, with additional N-terminal acetylated proteoform found at 1750.5 m/z) (Figures S18 and 19), GalNAcGalGalNAcGal (GalNAcGal + S) (15+ most abundant charge state, centered at 1756.2 m/z, with N-terminal acetylation and additional sulfonation (S)) (Figure S20), and GalNAcGalGalNAcGal (GalNAcGal + NeuAc) (centered at 1767.6 m/z) (Figure S21).

This hybrid native top-down MS approach enables the proteoform-resolved characterization of S-RBD O-glycoforms to identify and characterize multiple S-RBD O-glycoforms as well as a novel fucose-containing glycoform with Core 2 GalNAcGalGalNAcGal (GalNAcGalFuc) glycan structure (Figure 6A). Figure 6A shows all eight identified O-glycoforms of the S-RBD, which are also listed in the table in Figure 6B. As a unique advantage of the top-down MS approach, the molecular abundance of each intact protein glycoform can be relatively quantified. We found that the relative abundance of Core 1 to Core 2 S-RBD O-glycan structures was roughly 67:27, with the Core 1 GalNAcGalGalNAcGal being the most abundant O-glycoform (~65% relative abundance) (Figure 6B). This ability to unambiguously elucidate the structure of a specific S-RBD glycoform with high accuracy and quantify its relative abundance demonstrates the distinct advantages of this native top-down MS strategy over glycotope-based bottom-up MS approaches.44,64 Together, this proteoform-resolved intact glycoprotein analysis strategy enables the simultaneous characterization of O-glycan structures of the S-RBD and its microheterogeneity, including structure and relative molecular abundance (Figure 6B,C). Importantly, the accurate determination of the relative abundance of intact glycoforms provides the technical foundation to understand the functional significance of these distinct glycoforms of the S protein in the future. We note that the S-RBD studied here is recombinant rather than virus derived or extracted after viral egress in cell culture models of infection and thus will require additional investigation from viral models to establish the functional roles of these O-glycoforms. Although these identified O-glycoforms are specifically for S-RBD expressed from HEK293 systems, the HEK293 cell expression system is known to reflect the glycosylation sites expected for the virus,16,20,21,67 and the S-RBD is currently one of the antigens used for vaccine candidates and virus functional studies.68,69

**CONCLUSION**

In summary, the combination of native TIMS-MS and MS/MS from the timsTOF Pro and top-down MS from ultrahigh-resolution 12 T FTICR-MS enables the deep and comprehensive analysis of the intact glycoforms of the S-RBD. The native TIMS-MS analysis establishes direct connections between specific S-RBD proteoforms and their structural heterogeneity to provide new insights into the structural diversity of the S-RBD O-glycoforms. Importantly, we report the complete structural characterization of eight S-RBD O-glycoforms along with a Core 2-fucosylated glycan structure not previously reported. Moreover, the relative molecular abundances of each of these intact glycoforms can be quantified, providing new insights into the complex landscape of S-RBD O-glycosylation. Our proteoform-resolved method provides complete structural characterization of O-glycan microheterogeneity and complex glycostructures. This hybrid top-down mass spectrometry approach not only enables the comprehensive analysis of the S-RBD and the molecular signatures of emerging S-RBD variants of SARS-CoV-2 toward structure–function studies in the future but also can help catalyze the studies of other O-glycoproteins in general.

**MATERIALS AND METHODS**

**Materials and Reagents.** All chemicals and reagents were purchased from MilliporeSigma (Burlington, MA, USA) and used as received without further purification unless otherwise noted. Aqueous solutions were made in nanopure deionized water (H2O) from Milli-Q water (MilliporeSigma). Ammonium acetate (AA) was purchased from Fisher Scientific (Fair Lawn, NJ). Bradford protein assay reagent was purchased from Bio-Rad (Hercules, CA, USA). A 10% gel (10 or 15 comb well, 10.0 cm × 10.0 cm, 1.0 mm thick) for SDS-PAGE was purchased from ThermoScientific (Burlington, MA, USA). 3,3′-Diaminobenzidine (DAB) was purchased from ThermoScientific (Burlington, MA, USA). A 0.2 M Tricine solution with 1× Tris-buffered saline (TBS) was purchased from Sino Biological Inc. (cat. 40592-VNAH). Antibodies were purchased from MilliporeSigma. The recombinant SARS-CoV-2 spike regional-binding domain protein expressed in the HEK 293 cell line was purchased from Sino Biological Inc. (cat. 40592-VNAH).

**Complete N-Glycan Removal for O-Glycan Protein Analysis.** Peptide-N-glycosidase F (PNGase F) (New England Biolabs Inc., cat. P0704S) was used for complete removal of N-linked oligosaccharides. Brieﬂy, 20 μg of protein sample was buffer exchanged into 150 mM AA (pH = 7.4) solution by washing the sample five times through a 10 kDa Amicon ultra centrifugal filter (MWCO) of 10 kDa were purchased from MilliporeSigma. The recombinant SARS-CoV-2 spike regional-binding domain protein expressed in the HEK 293 cell line was purchased from Sino Biological Inc. (cat. 40592-VNAH).

**Sample Preparation.** Native protein samples were prepared by buffer exchanging into 150 mM AA solution by washing the sample five times through a 10 kDa Amicon ultra centrifugal filter (MilliporeSigma, Burlington, MA, USA). The protein sample was then diluted to 20 μM in 150 mM AA prior to native top-down MS analysis. Denatured protein samples were reduced using 20 mM tris-(2-carboxyethyl)phosphine hydrochloride (TCEP) and followed by buffer exchanging into 0.1% formic acid solution by washing the sample five times through a 10 kDa Amicon ultra centrifugal filter (MilliporeSigma, Burlington, MA, USA). The protein sample was then diluted to 20 μM in a 1:1 acetonitrile:water solution with 0.1% formic acid.
formic acid prior to top-down MS analysis. Protein samples subjected to N-glycan removal were prepared similarly following PNGase F reaction.

**Top-Down MS Analysis.** Intact protein samples were analyzed by nanoelectrospray ionization via direct infusion using a TriVersa Nanomate system (Advion Biosciences, Ithaca, NY, USA) coupled to a solvent XR 12-T Fourier Transform Ion Cyclotron Resonance (FT-ICR) mass spectrometer\(^{4,6,63}\) (FTICR-MS; Bruker Daltonics, Billerica, MA, USA). For the nanoelectrospray ionization source using a TriVersa Nanomate, the desolvating gas pressure was set at 0.45 PSI and the voltage was set to 1.2–1.6 kV versus the inlet of the mass spectrometer. The source dry gas flow rate was set to 4 L/min at 124 °C. For the source optics, the capillary exit, deflector plate, funnel 1, skimmer voltage, funnel RF amplitude, octopole frequency, octopole RF amplitude, collision cell RF frequency, and collision cell RF amplitude were optimized at 190 V, 200 V, 100 V, 35 V, 250 Vpp, 2 MHz, 490 Vpp, 2 MHz, and 2000 Vpp, respectively. Mass spectra were acquired with an acquisition size of 4M-words of data (with a resolution of 30 000 at 400 m/z) in the mass range between 200 and 4000 m/z, and a minimum of 250 scans were accumulated for each sample. Ions were accumulated in the collision cell for 0.1 s, and a time-of-flight of 1.5 ms was used prior to their transfer to the ICR cell. For collisionally activated dissociation (CAD) tandem MS (MS/MS) experiments, the collision energy was varied from 10 to 30 V, ion accumulation was optimized to 400 ms, and acquisition size was 4M-words of data. For electron capture dissociation (ECD) MS/MS experiments, the pulse length, ECD bias, and ECD lens were set to 0.1 s, 0.6 V, and 15 V, respectively, and the ion accumulation was optimized to 1800 ms. Tandem mass spectra were output from the DataAnalysis 4.3 (Bruker Daltonics) software and analyzed using MASH Explorer.\(^{62\text{-}64}\)

**Native Top-Down MS Analysis.** A timsTOF Pro mass spectrometer (Bruker Daltonics, Bremen, Germany) was coupled to a Bruker nanoElute LC system (Bruker Daltonics, Bremen, Germany). Samples were directly infused using the nanoElute, injecting 5 μL of native protein sample with a flow rate of 1 μL/min. For the MS inlet, the end plate offset and capillary voltage were set to 500 and 3800 V, respectively. The nebulizer gas pressure (N2) was set to 1.5 bar with a dry gas flow rate of 6 L/min at 180 °C. The tunnel out, tunnel in, and TOF vacuum pressures were set to 0.8584, 2.577, and 1.752 × 10^{-6} mBar. To calibrate the MS and trapped ion mobility spectrometry (TIMS) device, Agilent tune mix was directly injecting 5 μL of native protein sample with a flow rate of 1 μL/min. The MS/MS fragmentation data for each S-RBD O-glycoform; S-RBD proteoform characterization illus-

Native S-RBD without PNGaseF treatment; native TIMS-MS optimization and analysis of the S-RBD O-glycoforms; S-RBD proteoform characterization illustration by top-down MS; detailed analysis of top-down MS/MS fragmentation data for each S-RBD O-glycoform; table summarizing CCS values determined for the native S-RBD (PDF)

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**Supporting Information**

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacs.1c02713.

**ASSOCIATED CONTENT**

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The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacs.1c02713.

Native S-RBD without PNGaseF treatment; native TIMS-MS optimization and analysis of the S-RBD O-glycoforms; S-RBD proteoform characterization illustration by top-down MS; detailed analysis of top-down MS/MS fragmentation data for each S-RBD O-glycoform; table summarizing CCS values determined for the native S-RBD (PDF)

**Data Analysis.** All data were processed and analyzed using Compass DataAnalysis 4.3/5.3 and MASH Explorer.\(^{62\text{-}64}\) Maximum Entropy algorithm (Bruker Daltonics) was used to deconvolute all mass spectra with the resolution set to 80 000 for the timsTOF Pro or the instrument peak width set to 0.05 for the 12T FTICR. The sophisticated numerical annotation procedure (SNAP) peak-picking algorithm (quality factor 0.4; signal-to-noise ratio (S/N) 3.0; intensity threshold 500) was applied to determine the monoisotopic mass of all detected ions. The relative abundance for each protein isoform was determined using DataAnalysis. To quantify protein modifications, the relative abundances of specific modifications were calculated as their corresponding percentages among all of the detected protein forms in the deconvoluted averaged mass. MS/MS data were output from the DataAnalysis software and analyzed using MASH Explorer\(^{62}\) for proteoform identification and sequence mapping. All of the program-processed data were manually validated. For peak picking, the sophisticated numerical annotation procedure (SNAP) algorithm from Bruker DataAnalysis 5.3 was used with a quality threshold of 0.5 and an S/N lower threshold of 3. All fragment ions were manually validated using MASH Explorer. Peak extraction was performed using a signal-to-noise ratio of 3 and a minimum fit of 60%, and all peaks were subjected to manual validation. All identifications were made with satisfactory numbers of assigned fragment (>10), and a 25 ppm mass tolerance was used to match the experimental fragment ions to the calculated fragment ions based on the amino acid sequence. For ion mobility analysis by timsTOF Pro, the collisional cross section (CCS) in Å² for a species of interest was determined via the Mason–Schamps equation (eq 1)

\[
\text{CCS} = \frac{2\pi}{16} \frac{ze}{\mu} \frac{\Delta N}{kT} \text{N}_0 \text{K}_0
\]

where \(\mu\) is the reduced mass of the ion–gas pair (\(\mu = \frac{mM}{m + M}\), where \(m\) and \(M\) are the ion and gas particle masses), \(k_0\) is Boltzmann’s constant, \(T\) is the drift region temperature, \(z\) is the ionic charge, \(\epsilon\) is the charge of an electron, \(N_0\) is the buffer gas density, and \(K_0\) is the reduced mobility. Equation 1 was selected to agree with previously published CCS calculations.\(^{54,55,70}\) Theoretical CCS values were determined using the IMPACT method.\(^{59}\)
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