Dissecting the heterogeneous glycan profiles of recombinant coronavirus spike proteins with individual ion mass spectrometry

Alyssa Q. Stiving¹, David J. Foreman¹, Zachary L. VanAernum², Eberhard Durr³, Shiyi Wang¹, Josef Vlasak¹, Jennifer Galli³, Jared O. Kafader⁴, Xuanwen Li¹, Hillary Schuessler¹, Douglas Richardson¹

Affiliations
1. Analytical Research and Development, Merck & Co., Inc., 770 Sumneytown Pike, West Point, PA 19846, USA
3. Infectious Diseases and Vaccines Discovery, Merck & Co., Inc., 770 Sumneytown Pike, West Point, PA 19846, USA
4. Departments of Chemistry and Molecular Biosciences, the Chemistry of Life Processes Institute, the Proteomics Center of Excellence at Northwestern University, Evanston, IL 60208, USA

Corresponding author:
Alyssa Q. Stiving: alyssa.stiving@merck.com

Abstract
Surface-embedded glycoproteins, such as the spike protein trimers of coronaviruses MERS, SARS-CoV, and SARS-CoV-2 play a key role in viral function and are the target antigen for many vaccines. However, their significant glycan heterogeneity poses an analytical challenge. Here, we have utilized individual ion mass spectrometry (I2MS), a form of charge detection mass spectrometry (CDMS) that uses a commercially available Orbitrap analyzer, to directly produce heterogeneous glycan mass profiles of these three coronavirus spike protein trimers under native-like conditions. Analysis by I2MS shows that glycosylation contributes to the molecular mass of each protein trimer more significantly than expected by bottom-up techniques. This highlights the importance of obtaining complementary intact mass information when characterizing glycosylation of such heterogeneous proteins. Enzymatic dissection to remove sialic acid or N-linked glycans demonstrates that I2MS can be used to better understand the glycan profile from a native viewpoint. Deglycosylation of N-glycans followed by I2MS indicates that the SARS-CoV-2 spike protein trimer contains glycans that are more difficult to remove than its MERS and SARS-CoV counterparts and differences in glycan removal are correlated with solvent accessibility. I2MS technology enables characterization of protein mass and intact glycan profile and is orthogonal to traditional protein mass analysis methods such as size exclusion chromatography-multiple angle light scattering (SEC-MALS) and field flow fractionation-multiple angle light scattering (FFF-MALS). An added advantage of I2MS is low sample use, requiring 100-fold less than other methodologies. This work highlights how I2MS technology can enable efficient development of vaccines and therapeutics for pharmaceutical development.
Introduction
Coronavirus (CoV) outbreaks from Middle East respiratory syndrome coronavirus (MERS-CoV) in 2003, severe acute respiratory syndrome coronavirus (SARS-CoV) in 2012, and, more recently, SARS-CoV-2 in late 2019 (the cause of coronavirus disease 2019, COVID-19), demonstrate a significant risk to global human health. In a race against nature, scientists across the globe have worked fervently to develop vaccines and therapeutics to prevent, treat, and shorten COVID-19 infection rates and symptoms. While coronaviruses have four major structural proteins, the surface-embedded spike (S) protein has been the primary antigen target for vaccines and therapeutics due to its key role in viral entry and infectivity. Coronavirus spike protein monomers range from 180-200 kDa (fully glycosylated) and consist of an extracellular N-terminus, transmembrane domain, and intracellular C-terminus. The S1 subunit, which includes the N-terminus and receptor binding domain (RBD), is responsible for receptor binding and the S2 subunit, which includes the C-terminus, is responsible for membrane fusion. The spike protein assembles into a homotrimer to decorate the virus surface. Existing analytical methodologies for characterizing and understanding of the structures of MERS and SARS-CoV spike proteins have been critical in the rapid development of vaccines and therapeutics for SARS-CoV-2.

Surface proteins of enveloped viruses are often heavily glycosylated and the spike proteins of coronaviruses are no exception. Glycosylation of proteins involves processing by enzymes in the endoplasmic reticulum and Golgi apparatus. Assembly of glycan structures occurs in a non-templated manner that is heavily dependent on the host cells, resulting in heterogeneous occupancy of each glycan site (macroheterogeneity) and variation in glycoforms at each site (microheterogeneity). The glycosylation on these viral surface antigens contributes to viral entry, immune evasion, and protein folding. Past comparisons of the glycan profiles for the MERS, SARS-CoV, and SARS-CoV-2 spike glycoproteins show clustered oligomannose-type glycans on the MERS spike protein likely due to processing enzyme accessibility and conservation of glycans in the S2 subunit regions of the SARS-CoV and SARS-CoV-2 spike proteins. Aligning glycan heterogeneity with that of the wild-type virus can take advantage of glycosylation-dependent mechanisms, targeting glycans as epitopes for improved vaccine efficacy. Despite their impact on vaccine antigen development, complete understanding of the complexity and heterogeneity of these spike protein glycan profiles remains a significant analytical challenge.

Traditional techniques to screen mass following small scale expression of proteins for pharmaceutical development include sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), size exclusion chromatography (SEC) or field flow fractionation (FFF) coupled to multiple-angle laser light scattering (MALS). All methods can provide protein molecular mass information and can work with heterogeneous or aggregated species. Additionally, when combined with UV and refractive index (RI) detectors, SEC-MALS and FFF-MALS can provide...
inherent measurements of the mass contribution from glycans.\textsuperscript{28} A limitation of each of these techniques is the requirement of significant amounts of protein (~100 µg) per acquisition, which poses a challenge when characterizing proteins produced at smaller scales. Native mass spectrometry is a tool that can be used for protein screening which requires small amounts of sample (~µM concentrations, ~µL volumes) and retains native-like protein structure upon transfer into the gas phase, allowing for interrogation of higher-order protein structure.\textsuperscript{29,30} Typical native MS experiments involve buffer exchange of the protein into volatile electrolyte solutions, commonly ammonium acetate. This buffer exchange step, while often done manually, has also been automated to enable rapid screening of proteins in an effort to increase throughput.\textsuperscript{31} The use of volatile electrolyte solutions with near-neutral pH combined with soft ionization methods (such as electrospray ionization (ESI) or nanoESI) generates ions of inherently lower charge states and narrower charge state distributions when compared to denaturing conditions. Therefore, native MS often results in higher charge state resolution and less signal overlap of heterogeneous species.\textsuperscript{32} Importantly, resolution of charge states is necessary for ensemble MS measurements in which isotopic patterns cannot be resolved in order to properly deconvolute the $m/z$ spectra to determine molecular mass. The practical upper mass limit for accomplishing this with homogeneous ions has been proposed to be around 20 MDa, but the upper limit significantly declines when any heterogeneity is introduced.\textsuperscript{33}

One mass spectrometric method to enable molecular mass determination without the need for resolving individual charge states in $m/z$ is charge detection mass spectrometry (CDMS). Traditional electrostatic linear ion trap CDMS methods measured ions one at a time as opposed to traditional ensemble measurements which measure hundreds to thousands of ions in one acquisition event. In this electrostatic linear ion trap setup, the time it takes the ion to travel through the trap (or half of the measured frequency of the ion as it oscillates back and forth within the trap) is proportional to the square root of the ion’s $m/z$.\textsuperscript{34–36} The integer charge of the ion can be directly determined from the height, or amplitude, of the current induced on the detection electrode as the ion passes through it.\textsuperscript{34,35,37,38} By directly measuring the ion’s integer charge alongside its $m/z$, mass can be calculated directly instead of relying on isotopic or charge state spacing, facilitating measurements of highly heterogeneous and/or large molecules. Due to this advantage, CDMS has been utilized to determine the molecular mass of numerous species such as virus capsids, virus-like particles, and bacteriophages that otherwise would not be possible using traditional ensemble MS measurements.\textsuperscript{35,39–41}

Kelleher, et al. described a method similar in principle to CDMS called Individual ion mass spectrometry (I2MS), utilizing commercially available Q-Exactive UHMR instruments.\textsuperscript{42,43} By taking advantage of the Orbitrap mass analyzer’s single-ion sensitivity, I2MS multiplexes single ion collection for subsequent CDMS analysis and enables simultaneous measurement of single ions that are required for CDMS analysis.\textsuperscript{42–46} This advantageously relieves the data acquisition burden compared to traditional CDMS techniques. The principles of operation for Orbitrap I2MS have been described in detail previously.\textsuperscript{42–44} Briefly, each ion signal within I2MS spectra is independently processed, generating information about the ion’s charge and $m/z$. A selective temporal overview of resonant ions (STORI) process is used to plot the current induced by the ion on the Orbitrap detection electrode as a function of acquisition time.\textsuperscript{47} The slope of this STORI plot is proportional to the ion’s charge.\textsuperscript{47} Protein standards of a broad mass range and charge state distribution can be measured using I2MS to create a STORI slope-to-charge calibration function using the known integer charges of the calibrant ions. Unknown ions are measured using I2MS to generate STORI plots where the resulting STORI slope of each ion is used to calculate the integer charge based on the calibration function. The measured ions are then binned to create a mass histogram to provide information on abundance and mass distribution of the unknown species.\textsuperscript{42–44}
Fast and efficient analysis of protein reagents is critical to enable swift preventative or therapeutic responses to evolving, rapidly spreading infectious agents such as SARS-CoV-2. While traditional native MS measurements generate molecular mass information quickly using minimal sample, ensemble MS measurements are not always a reasonable approach for highly heterogeneous proteins such as complex glycoproteins that coat the surface of many viruses. Herein, various forms of molecular determination techniques were compared to determine mass distributions of MERS, SARS-CoV, and SARS-CoV-2 spike protein trimers. Using traditional protein characterization techniques of SEC-MALS and FFF-MALS show comparable molecular masses to I2MS but require 100-fold more sample. Further investigation shows a greater glycan mass contribution observed by intact I2MS analysis compared with bottom-up glycoproteomics, in line with previous observations about the SARS-CoV-2 spike protein\footnote{15} and expanding these findings to the MERS and SARS-CoV spike proteins. Finally, enzymatic dissection to remove sialic acids or N-linked glycans reveals differences in higher order structure and solvent accessibility of N-linked glycans among these proteins.

**Methods/Experimental**

**Protein Expression**
The SARS-CoV1 prefusion-stabilized spike protein plasmid was generated to encode SARS-CoV1 Spike protein ectodomain residues 1–1190 (amino acid 1 denotes the starting methionine in the signal peptide) two proline substitutions at K968P and V969P, a C-terminal T4 fibritin trimerization domain, an HRV3C cleavage site, an 8x His-tag as described previously.\footnote{48} The SARS-CoV2 prefusion-stabilized spike protein plasmid was generated to encode SARS-CoV2 Spike protein ectodomain residues 1–1208 two proline substitutions at K986P and V987P, a “GSAS” substitution at the furin cleavage site (residues 682–685, RRAR), a C-terminal T4 fibritin trimerization domain, a thrombin cleavage site, and an 8x His-tag similar to previously described.\footnote{16} The MERS prefusion-stabilized spike protein plasmid was generated to encode MERS Spike protein ectodomain residues 1–1291, two proline substitutions at V1060P and L1061P, an “ASVG” substitution at the furin cleavage site (residues 748-751, RSVR), a C-terminal T4 fibritin trimerization domain, a thrombin cleavage site, and an 8x His-tag similar to previously described.\footnote{49} All gene-encoding regions were mammalian codon-optimized and subcloned into a eukaryotic-expression vector under the control of the CMV promotor. Plasmids were transiently transfected into Expi 293F cells (ThermoFisher) using Expifectamine (ThermoFisher) following manufacturer’s recommended protocol. Cell supernatants were harvested 72 hours post-transfection and clarified by centrifugation at 10,700 x g at 20°C for 30 minutes. PS-20 was added at a final concentration of 0.01% to the clarified supernatants to mitigate aggregation. Clarified supernatants were aliquoted into 250 mL Corning bottles and transferred to -70°C for storage until purification. Clarified supernatant was thawed in a 37°C shaking water bath and carried forward into purification. Spike protein was purified from cell culture supernatant using affinity chromatography on a HisTrap Ni Sepharose column (GE Healthcare) and eluted with 300 mM imidazole. His-tag was removed by overnight digestion at room temperature with either thrombin or HRV3C protease as appropriate. Spike protein was further purified by a second, subtractive affinity chromatography step to remove protease, IMAC contaminants, cleaved His-tags and uncleaved Spike. Final purification of the sample was achieved with size exclusion chromatography using a Superdlex 200 column (GE Healthcare) for SARS-CoV-2-PreS and MERS-PreS and using a Sephacryl S-300 column (GE Healthcare) for SARS-CoV-PreS-3C. Prominent protein containing fractions were pooled, concentrated and dialyzed into 50 mM HEPES, pH 7.5 and 300mM NaCl. Final protein concentration was determined by A280 analysis using protein-specific reduced extinction coefficients calculated from amino acid sequence. Protein purity was estimated by Coomassie-stained SDS-PAGE gel.
Field flow fractionation (FFF)
Asymmetric flow field-flow fractionation (AF4) was carried out using Eclipse Dualtech system (Wyatt Technology) equipped with RI (Optilab Tr-EX), MALS (Dawn Heleos-II), and UV detectors (Agilent). Long FFF channel was used with 10 kDa regenerated cellulose membrane. The channel was kept at room temperature (20°C). PBS mobile phase was run at a detector flow of 1 mL/min. Approximately 80 µg of each sample was injected. A constant cross flow of 2 mL/min was run for 40 min. Protein molecular weights were calculated using Astra 6 software using the conjugate analysis algorithm with the following parameters: protein dn/dc – 0.185 mL/g, glycan dn/dc – 0.135 mL/g, MERS UV extinction coefficient – 1.076 mL/(mg cm), SARS-CoV UV extinction coefficient – 0.973 mL/(mg cm), SARS-CoV-2 UV extinction coefficient – 1.002 mL/(mg cm).

Size Exclusion Chromatography – Multiple Angle Laser Light Scattering (SEC-MALS)
Separation of SARS Spike protein was performed on an Agilent 1100 HPLC system consisting of a quaternary pump, a vacuum degasser, a thermostated autosampler and column compartment with a Superose 6; 10/300GL Increase column (Cytiva Life Sciences). The column was kept at 20°C. The mobile phase (PBS) was delivered at 0.5 mL/min. To reduce baseline noise in the light scattering detector, a 25 mm high pressure filter with 0.1 µm pores (Millipore) was used for in-line filtration of the mobile phase. Detection was carried out using the Agilent HPLC DAD UV-detector G1315B (280 nm) and Wyatt Technology’s (Santa Barbara, CA) DAWN Heleos II light-scattering and T-Rex refractive index detectors. The Dawn Heleos II detector was calibrated with toluene according to the manufacturer’s instructions. 100 µg of protein complex was injected for each analysis. Determination of protein mass content and molecular weight was performed using the ASTRA 6.1 software (Wyatt Technology) using the conjugate analysis algorithm and the following parameters: protein refractive index values (dn/dc) – 0.185 mL/g, glycan dn/dc – 0.134 mL/g. Protein UV extinction coefficients were calculated based on the primary sequence using Vector NTI Advance 11 (Invitrogen). More details on the use of three detector systems for the analysis of protein complexes are reported elsewhere. 28,50

Individual Ion Mass Spectrometry (I2MS)
For native mass spectrometry I2MS experiments, proteins were buffer exchanged into 200 mM ammonium acetate (VWR International, Bridgeport, NJ) using Micro Bio-Spin P-6 columns (Bio-Rad, Hercules, CA). For desialylation experiments, the purified recombinant protein was mixed with sialidase (Sigma Aldrich, St. Louis, MO) at an enzyme:protein ratio of 0.25 mU per 9 pmol protein (equivalent to 0.5 mU per ~microgram of protein) and incubated at 37°C for 4 hours following manufacturer recommendations. Following incubation, the sample was buffer exchanged into 200 mM ammonium acetate using the same procedure as described above. For deglycosylation experiments, the purified recombinant protein was incubated with PNGase F (Promega, Madison, WI) at an enzyme:protein ratio of 1 unit PNGase F per 9 pmol protein (equivalent to 2 units of enzyme per ~microgram of protein) at 37°C for 1, 4, or 48 hours following manufacturer recommendations under native conditions. Following deglycosylation for each specified time point, the sample was buffer exchanged into 200 mM ammonium acetate using Micro Bio-Spin P-30 columns (Bio-Rad) with a 40 kDa MWCO following the manufacturer recommendations in order to remove most of the PNGase F protein and released glycans. Using the same starting sample for PNGase F incubation and drawing an aliquot for analysis at each time point compared to preparing a separate sample for each time point showed no substantial differences in mass by I2MS measurements. All mass spectrometry experiments were conducted using a Thermo Q-Exactive UHMR with modified software capabilities, enabling STORI files to be collected. Proteins were ionized using static spray via nanoelectrospray ionization (nESI) from coated pre-pulled capillaries (New Objective, Littleton, MA) by applying a spray voltage of 0.8-2.0 kV. Various instrument tune settings are included in the Supplemental Information. Trapping gas pressure was minimized to maximize the ion survival times. In-source trapping was utilized to
assist with desolvation of the proteins but was balanced in each case to prevent dissociation/fragmentation.

Proteins were first analyzed at concentrations of approximately 5 μM for collection of ensemble mass measurements. For I2MS measurements, the proteins were diluted 10-fold or more, the resolution was increased to 200,000 (at m/z 400), the number of microscans was decreased to 1, and the maximum injection time was lowered to ensure that only single-ion events were occurring at each m/z (typically 20-100 ms). Analysis of standard proteins with clearly resolvable charge states was used to calibrate for charge as described previously. Carbonic anhydrase, NIST mAb, pyruvate kinase, and beta-galactosidase tetramer and octamer (all purchased from Sigma Aldrich, St. Louis, MO) were used for generating a calibration curve that spans from 29.1 to 932 kDa and 9+ to 75+ charge states with an R² of 0.999. I2MS data was analyzed using software supplied by Thermo Fisher. Standard spectra processing settings were used along with an R-squared threshold of 0.998 and duration threshold of 0.6 (equating to a minimum of 1.2 s before ion death). Data was exported to .csv and subsequently visualized using GraphPad Prism 9.

Results & Discussion

Molecular mass characterization of highly heterogeneous protein reagents

The molecular masses of the three coronavirus spike protein trimers were measured using I2MS, SEC-MALS, and FFF-MALS techniques (Figure 1). Each protein contained a fibritin trimerization domain and analyses confirmed the presence of each spike protein trimer without any monomer or dimer peaks present. Masses of the MERS, SARS-CoV, and SARS-CoV-2 trimers were determined to be 576, 538, and 540 kDa, respectively, by I2MS as shown in Figure 1A, 1B, and 1C, respectively. The solid-colored vertical lines represent the theoretical masses based on the respective amino acid sequence of each trimer. There is a significant discrepancy in observed (black traces) vs. theoretical sequence mass, amounting to 127.5, 110.7, and 126.8 kDa of “extra mass” for the MERS, SARS-CoV, and SARS-CoV-2 spike protein trimers, respectively.

With 23, 23, and 22 N-linked glycan sites per monomer, extreme heterogeneity is expected within each spike protein trimer, as has been confirmed in the literature from analytical characterization of these proteins. This heterogeneity is also demonstrated by the broad mass profiles observed by I2MS, with FWHM of 17, 15, and 12 kDa, respectively, for MERS-SARS-CoV, and SARS-CoV-2. We used site-specific occupancy and glycan structure results from bottom-up glycopeptide analysis to determine the average expected mass contribution from N-linked glycans which suggests an “extra mass” having an order of magnitude of approximately 100 kDa. This will be discussed in more detail later. While nonspecific salt and solvent adducts may contribute to this additional mass, such contributions are not anticipated to be near the magnitude observed. In addition, in-source trapping to remove adducts with collisional activation did not result in any significant shifts in observed masses. Previous work analyzing the intact SARS-CoV-2 spike protein trimer using CDMS technology showed significant mass contributions from glycosylation as well and the work herein expands on this observation for additional coronavirus spike protein trimers. For these reasons, we expect that this extra mass is primarily due to glycosylation, as indicated by orthogonal SEC-MALS and FFF-MALS experiments. However, because I2MS does not inherently measure the mass contribution from glycans, an assumption of glycosylation as the exclusive source of “extra mass” does not account for other factors such as clipping and post translational modifications.

SEC-MALS and FFF-MALS are additional technologies commonly employed to determine mass and, when used in conjunction with UV and refractive index (RI) detectors, enable inherent measurement of the glycan mass contribution. Proteins generate both UV and RI signal whereas glycans generate only RI signal. Glycan mass contribution determined by SEC-MALS analysis of
the MERS, SARS-CoV, and SARS-CoV-2 spike protein trimers showed glycan mass contributions of 128.1, 171.3, and 160.9 kDa, respectively (Figure 1D-F). FFF-MALS measurements of the same proteins showed glycan mass contributions of 132.6, 155.6, and 128.4, kDa, respectively (Figure 1G-I). Both SEC-MALS and FFF-MALS allow the direct measurement of glycan mass contribution when coupled with UV+RI detection, however they each require ~100 μg of protein. This relatively high sample requirement poses a challenge when characterizing proteins produced at a small scale. Alternatively, I2MS requires 100-fold less protein and results in inferred glycan mass contributions that we show are of a similar order of magnitude to those measured by SEC-MALS and FFF-MALS. All three techniques can generate mass information in approximately one hour. While SEC-MALS and FFF-MALS still have their place in protein characterization and require less costly instrumentation, this work establishes I2MS as an important analytical tool in characterizing the accurate molecular mass of highly heterogeneous protein reagents while requiring significantly less starting material than classical methods. The need to efficiently characterize heterogeneous protein reagents used in development of vaccines and therapeutics highlights the importance of using native MS.

![Figure 1](image.png)

**Figure 1.** Molecular mass determination of MERS, SARS-CoV-1, and SARS-CoV-2 Spike glycoprotein trimers using (A-C) CDMS, (D-F) SEC-MALS, and (G-I) FFF-MALS and the corresponding amount of protein required for analysis using each technique. Vertical colored lines in I2MS plots (A-C) represent the theoretical molecular mass of each spike protein trimer as determined from the amino acid sequence (unglycosylated).

**Complementary data from bottom-up glycopeptide analysis and I2MS intact mass**

Given the important role that glycans play in virus function, understanding the detailed glycan heterogeneity and occupancy at each site is critical in preventing and treating infection. Mass spectrometry has contributed heavily toward this goal within the field. Analysis of bulk released glycans enables characterization of the glycan structures present on the protein, but lacks site specificity from which the glycan originated. Alternatively, analyzing glycopeptides via MS analysis of digested proteins without prior removal of glycans has been achieved for many viral glycoproteins but similar to any bottom-up method, does not provide the combinatorial nature of the glycans present on the intact protein. The theoretical mass contribution from glycans at the intact level can be determined by multiplying the mass of each glycan structure by its occupancy at each respective site, as determined from bottom-up experiments, and combining
across all occupied sites. When comparing the mass contribution of glycosylation as determined from intact, native I2MS in this work with that determined from bottom-up N-linked glycopeptide analysis in the literature\(^\text{24}\) (Figure 2A-B), discrepancies were observed for the MERS and SARS-CoV spike protein trimers. Previous work has shown a similar discrepancy in N-glycan mass contribution as determined from CDMS analysis\(^\text{15}\) of the SARS-CoV-2 spike protein trimer compared with that from bottom-up glycopeptide analysis\(^\text{13}\), which was again confirmed here (Figure 2C) by I2MS. Given that few O-linked glycans have been observed on the SARS-CoV-2 spike protein and those that have been observed have low occupancy\(^\text{51,53,54,57}\), the mass contribution from O-linked glycans alone is not expected to make up this difference.

It is clear in the literature that glycan patterns and complexity differ when comparing protein expressed as subdomains (e.g. receptor binding domain (RBD), S1, or S2) versus full-length spike protein both for N-linked and O-linked glycans.\(^\text{53,54,58}\) However, given that the comparisons made here between bottom-up and intact all use full-length spike proteins with the same trimerization domains, this is not expected to be the root cause of such a discrepancy. More likely, the characterization of spike protein glycan profiles by LC-MS/MS analysis of glycopeptides is not fully representative of the intact protein structure, particularly for glycans that are more complex or difficult to fragment, resulting in under-sampling of glycan microheterogeneity. Differences in glycopeptide ionization efficiency which result in inaccurate glycan occupancy values or lack of solvent accessibility may impede efficient digestion in certain regions of these proteins, resulting in incomplete characterization. Additionally, even when using the same cell lines, differences in expression systems or cell culture conditions may have impacts on the glycan patterns. Combining bottom-up glycopeptide analysis with intact native MS measurements can help to bridge the gap observed from bottom-up glycan profiling. Again, while nonspecific salt and solvent adducts may contribute to a slightly larger than expected mass for the intact mass measurements, such contributions are not expected to be near this magnitude.\(^\text{55}\) Additional complementary information that can be generated from the intact level via enzymatic dissection of the N-linked glycans is discussed later.

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**Figure 2.** I2MS mass spectra (solid black traces) of the MERS, SARS-CoV, and SARS-CoV-2 spike protein trimers alongside dashed lines representing the theoretical molecular mass of each respective glycosylated trimer. Theoretical mass was calculated by combining the mass contribution from the amino acid sequence in combination with the average glycan mass (weighted by abundance where given) as determined from bottom-up N-linked glycopeptide LC-MS/MS analysis in the literature.\(^\text{13,19}\) Significant differences between theoretical and observed molecular masses suggest that bottom-up glycopeptide analysis may be undersampling the glycans present.

*Interrogating spike protein trimer sialylation using I2MS*

Sialic acid plays a vital role in stabilizing molecules, receptor binding, and immune evasion.\(^\text{59,60}\) The heavily sialylated glycans of coronavirus spike proteins and sialylation found on the surface...
of cells can influence receptor-mediated viral binding.\textsuperscript{59,60} In addition, their relatively strong electronegative charge and requirement for lectin recognition may also impact purification strategies.\textsuperscript{61} Previous work studying N-linked glycans on the spike proteins derived from SARS-CoV-2 vaccine candidates categorized glycan profiles into high-mannose or complex-type glycans via truncation of glycans with sequential enzymatic glycosidase treatment.\textsuperscript{62,63} To accomplish this, mass shifts were characterized in deglycosylated peptides following glycan release from sequential enzymatic glycosidase treatment, first with Endoglycosidase H (cleaving oligomannose- and hybrid-type glycans) followed by PNGase F treatment (removing remaining complex-type glycans). While this method is advantageous in increasing signal intensity due to homogenization of deglycosylated peptides into oligomannose/hybrid- and complex-type categories, it lacks detailed information about glycan structures and terminal glycan processing such as sialylation.

To interrogate the extent of glycan sialylation from the intact trimer protein level and in an effort to reduce spectral complexity\textsuperscript{32}, we performed intact native mass measurements using I2MS for the three spike protein trimers following incubation with sialidase (Figure 3). Mass shifts of 16.4, 7.3, and 14.6 kDa corresponding to 12.9\%, 6.6\%, and 11.5\% of the total glycan mass contribution (as inferred based on “extra mass” beyond the unglycosylated protein sequence mass, determined from I2MS measurements) were observed for the MERS, SARS-CoV, and SARS-CoV-2 spike protein trimers, respectively. Interestingly, I2MS analysis of the SARS-CoV desialylated protein showed the additional presence of monomer and dimer, albeit at lower relative intensity than the intact trimer, thus suggesting instability of the spike protein trimer following removal of sialic acids. A control incubation of the SARS-CoV protein trimer under the same conditions as sialidase treatment but without sialidase enzyme present showed no dissociation into monomer and dimer (data not shown), suggesting this was not an artifact of sample preparation. MERS and SARS-CoV-2 spike protein trimers showed no evidence of dissociation into monomers or dimers following sialidase incubation, despite losing a greater amount of sialic acid (both by mass and relative percent) compared to the SARS-CoV spike protein.
Figure 3. Mass spectra determined by I2MS for (A) MERS, (B) SARS-CoV, and (C) SARS-CoV-2 Spike protein trimers following incubation with sialidase to remove terminal sialic acids. Solid black traces indicate the control spectrum (- sialidase) while solid colored traces indicate the mass spectrum of each respective protein following incubation with sialidase for 4 hours (+ sialidase).

Interrogating spike protein structure following enzymatic glycan dissection
We enzymatically dissected the coronavirus spike proteins by incubating with PNGase F under native conditions for variable amounts of time to interrogate the N-linked glycans from the intact level and to aid in bridging the gap between bottom-up and intact native I2MS results. From I2MS analysis of these deglycosylated spike protein trimers (Figure 4, solid traces) and comparing to the theoretical sequence masses of the trimers (Figure 4, dashed lines), it is clear that a complete shift to the theoretical sequence mass does not occur, even after extended incubation times. Potential contributions to this extra mass include N-linked glycans not removed by PNGase F in addition to O-linked glycans, post-translational modifications (PTMs), and sequence variants. An effort to remove all N-linked glycans by incubating for extended periods of time (72 hours and greater) was made, but measurement by I2MS was unsuccessful due to instability in nESI spray, likely caused by protein instability following significant glycan removal that resulted in protein aggregation or crashing out of solution.
Figure 4. Mass spectra determined by I2MS for (A) MERS, (B) SARS-CoV, and (C) SARS-CoV-2 Spike protein trimers following incubation with PNGase F for 1, 4, or 48 hours (solid colored traces). The control (not incubated with PNGase F) I2MS mass spectra and the amino acid sequence mass of the trimer are shown in solid black and dashed lines, respectively, for each protein.

Plotting the average masses of the deglycosylated spike protein trimers as a function of incubation time with PNGase F (Figure 5A) shows that each spike protein loses a significant mass from removal of N-linked glycans following short incubation (1-4 hours) and the mass loss begins to level off at extended incubation times (>4 hours). Comparing the absolute mass lost from removal of N-glycans shows a decrease from MERS to SARS-CoV to SARS-CoV-2, corresponding to a final mass loss of 67 kDa, 56 kDa, and 48 kDa, respectively, after 48 hours of incubation with PNGase F (Figure 5B). However, given that the total assumed mass contribution of glycosylation differs between the three proteins, the mass loss expressed as a percentage of the total glycan mass contribution (as inferred based on “extra mass” beyond the theoretical sequence mass, calculated from I2MS measurements) is shown in Figure 5C. Here we demonstrate that for MERS and SARS-CoV, 53% and 50% of the total “extra mass” is removed following incubation with PNGase F for 48 hours, respectively. In contrast, the SARS-CoV-2 spike protein trimer only loses 38% of the “extra mass” after incubation for this same extended time period.

Following incubation with PNGase F for 48 hours, 60.6, 54.9, and 78.5 kDa of “extra mass” (relative to sequence mass) for MERS, SARS-CoV, and SARS-CoV-2, respectively, was observed. The three most likely contributors to the extra mass left over after deglycosylation with PNGase F are: non-glycan post translational modifications, O-linked glycosylation, and/or the incomplete removal of N-glycans. It is likely that all three of these contribute to some extent to the extra mass. Considering that post translational modifications do not add significant extra mass, it can be expected that they are not a major contributor to the “extra mass” observed here. Overall, O-linked glycans are far less characterized than N-linked glycans in the literature regarding coronavirus spike proteins. However, taking SARS-CoV-2 spike protein as an example, O-linked glycans have been observed in numerous studies but is generally lacking in consensus, with anywhere from two to nine reported O-linked glycopeptides. Even accounting for the study that demonstrated the greatest number of occupied O-glycan sites, the additional mass would still only account for <10 kDa of the observed “extra mass.” For these reasons, it is believed that the primary contributor of the extra mass is from incomplete removal of the N-glycans.

Working with this assumption, we next considered additional factors that may influence the lack of apparent complete (or near-complete) N-linked deglycosylation. Protein architecture plays a key role in the processing of glycans and, conversely, the enzymatic removal of glycans under non-denaturing conditions may provide insight into protein structure. Because of this, we compared the solvent-accessible surface area (SASA) of the MERS (PDB: 5x59), SARS-CoV (PDB: 6crz), and SARS-CoV-2 (PDB: 6vsb) coronavirus spike protein trimers as determined from GetArea. Interestingly, the SASA of the SARS-CoV-2 spike protein trimer is lower than both the MERS and SARS-CoV proteins, which may in part explain the apparent lower extent of N-linked glycan removal (Figure 5C). Given that the PNGase F incubations were carried out under
native conditions, removal of buried glycans may occur more slowly than for those that are surface exposed.

The correlation between SASA and enzymatic removal of N-linked glycans shown here, suggest that deglycosylation and mass measurement by I2MS have the potential to provide information on structural differences between the convoluted spike proteins of coronaviruses. While intact, native I2MS analysis of stepwise-deglycosylated proteins shows the potential to provide a global overview of accessible vs. buried glycans, future work combining this information with bottom-up analysis of glycan composition at varying time points of PNGase F incubation may provide complementary structural information based on the accessibility of specific glycan sites or types. Furthermore, although we demonstrate a clear correlation between SASA and removal of N-linked deglycosylation, it is likely that PTMs and O-linked glycosylation play a minor role in the observed “extra mass” as well. Future work will interrogate potential differences in O-linked glycosylation between MERS and SARS-CoV proteins at a global level.

**Figure 5.** Results from I2MS measurements of MERS (blue), SARS-CoV-1 (green), and SARS-CoV-2 (purple) spike protein trimers by CDMS with respect to incubation time with PNGase F for removal of N-linked glycans. A break in the x-axis between 7 and 44 hours is shown for each plot for better visualization of the data. (A) Measured average masses of the trimers (solid data points) and amino acid sequence mass of trimers (dashed lines). Error bars show standard deviation from duplicate measurements but are often occluded by the data point size. (B) Change in trimer mass as compared to the respective control (fully glycosylated) protein. (C) Change in trimer mass as compared to the respective control (fully glycosylated) protein expressed as a percentage of the total mass contribution from glycosylation (as inferred based on “extra mass” beyond the naked protein, determined from I2MS measurements) alongside the solvent accessible surface area (SASA) of each protein trimer (determined using GetArea65; MERS PDB: 5x5964, SARS-CoV PDB: 6crz46, SARS-CoV-2 PDB: 6vsb19) in dashed lines.

**Conclusions**
We have used I2MS technology to characterize the highly heterogeneous glycan profile of three coronavirus spike protein trimers and compared our results to literature to demonstrate the importance of generating complementary bottom-up LC-MS/MS and intact mass MS to better characterize glycoproteins. We demonstrated that intact mass measurements using native mass spectrometry and I2MS technology uses significantly lower sample amounts (<1 μg) compared to traditional techniques such as SEC-MALS and FFF-MALS. Thus, the use of I2MS provides a distinct advantage in characterization of highly heterogeneous protein reagents produced at a small scale that are needed in rapid pharmaceutical development for prevention or treatment of severe disease. Comparing complementary data from intact mass measurements with bottom-up glycopeptide analysis from the literature suggests that bottom-up methods may be underestimating the mass contribution of glycans due to under-sampling of complex-type glycans, inaccurate occupancies, differences in ionization efficiency, or inefficient fragmentation for the three coronavirus spike proteins tested here. Further interrogation of the intact, native coronavirus
spike proteins by enzymatic dissection using sialidase provides insight into terminal glycan processing that may be missed using traditional categorical grouping of glycan types and showed greater amounts of sialic acid on the MERS and SARS-CoV-2 spike protein trimers. Finally, deglycosylation of the spike protein trimers using PNGase F incubation across various time points and analysis of mass changes by I2MS showed correlation between N-linked glycan removal and solvent-accessible surface area, highlighting the interplay between protein architecture and glycan accessibility.

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