Determination of Antibody Population Distributions for Virus-Antibody Conjugates by Charge Detection Mass Spectrometry

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ABSTRACT: Virus-like particle (VLP) conjugates are being developed for biomedical applications; however, there is a lack of quantitative analytical methods to measure the extent of conjugation and modification of VLP based therapeutics. Charge detection mass spectrometry (CDMS) can measure mass distributions for large and heterogeneous complexes and is emerging as a valuable tool in the analysis of biologics. In this study, CDMS is used to characterize the stoichiometry and population distribution of antibodies covalently conjugated to the surface of a bacteriophage MS2 VLP. Initial CDMS analysis of the unconjugated MS2 particles suggested that they had packaged a broad distribution of exogenous genomic material. We developed procedures to remove the undesired genomic material from the VLP preparation and observed that, for the samples where the genomic fragments were removed, the antibody coupling reaction efficiency increased by almost a factor of 2. This meant there were (1) fewer VLPs with no antibodies bound, which is an important consideration for the efficacy of a targeted therapeutic and (2) fewer antibodies were wasted during the coupling reaction. CDMS could be employed in a similar manner as a tool to characterize coupling reaction product distributions and precursors and help inform the development of the next generation of conjugate-based therapies.

Virus-like particles (VLPs) have gained attention in recent years due to their potential applications in imaging diagnostics, drug delivery, gene therapy, and fundamental research.1−9 Viruses can be manipulated to encapsulate a non-native cargo and can be modified to alter their physical properties.7−9 As these systems are engineered to be increasingly complex, traditional analytical methods become unable to determine their composition due to their intrinsic heterogeneity.10,11 In particular, we set out to investigate a conjugate between full-length antibodies and the MS2 viral capsid to be used for imaging and drug delivery applications. Current methods to quantify the stoichiometry of antibodies bound to the surface of a capsid are scarce. SDS-PAGE can be used to confirm antibody attachment and provide a qualitative estimate of the modification; however, the different interactions between the stains and the proteins can limit the accuracy of this method. Recent advances in mass spectrometry (MS), particularly native MS, make it possible to analyze complex biological systems and gain important information about the stoichiometry, population distribution, and relative efficiency of antibody coupling reactions.

Native MS allows large noncovalently bound assemblies, such as VLPs, to be transferred from solution into the gas phase.12 Nevertheless, as size increases, it becomes increasingly difficult to resolve charge states in the m/z (mass to charge ratio) spectrum measured in conventional MS, and without charge state resolution, it is not possible to deduce the mass.10 Heck and co-workers have provided a convincing argument that this loss of resolution is not instrumental, but results from heterogeneity.10 There are two types of heterogeneity: intrinsic and extrinsic.11 Sources of extrinsic heterogeneity, such as incomplete desolvation, counterions, and salt adducts, can be minimized, if not completely removed, with extensive purification of the sample and through collisional activation of the ions in the mass spectrometer. However, many high molecular weight samples are intrinsically heterogeneous. This heterogeneity can result from a number of sources, including different post-translational modifications, and because the high molecular weight sample was assembled from different components or contains different numbers of components. Consequently, it is often not possible to obtain charge state resolution in the megadalton mass regime by conventional mass spectrometry methods.

Charge detection mass spectrometry (CDMS) is a method for measuring the masses of ions in the megadalton regime that circumvents the issue of m/z charge state resolution.13−15 CDMS is a single-particle technique where the masses of individual ions are determined from the simultaneous measurement of each ion’s m/z and charge. The ions are trapped in an electrostatic linear ion trap where they oscillate back and forth through a cylindrical detector. The charge induced by the oscillating ion is detected; the oscillation
frequency yields the ion’s m/z, and its charge is determined from the signal amplitude. CDMS is particularly well-suited to the analysis of high mass, heterogeneous analytes such as virus capsid conjugates.

In this work, CDMS is used to analyze MS2 VLPs with antibodies covalently bound to their surface. The MS2-antibody conjugates are very heterogeneous, with masses in the 2.5−5.0 MDa range. In addition to providing the average number of antibodies bound to the VLPs (which can be used to estimate the coupling efficiency), CDMS provides information on the distribution of bound antibodies. As we demonstrate here, this information can play a critical role in guiding improvements in the conjugation strategy. These improvements are especially important for future therapeutic applications involving expensive and difficult to produce reagents such as antibodies.

■ RESULTS AND DISCUSSION

Production of Empty MS2 Capsids. MS2 viral capsids engineered to have modification sites on the exterior (p-aminophenylalanin, pA) surfaces of the capsid were recombantly expressed in E. coli. Recent work in the Francis group has developed protocols for synthesizing MS2-antibody conjugates. However, the number of antibodies bound is unknown. Preliminary CDMS measurements for the unmodified capsid showed a broad peak with a center at 3.82 MDa (black line in Figure 1). This is significantly higher than the 2.482 MDa expected mass for an empty MS2 capsid (2.482 MDa), and the peak width (773 kDa) is broader than the 90 kDa expected mass and the elevated peak width both result from a small excess mass and broad peak width.

Two protocols, shown schematically in Figure 2, were tried in an effort to remove the extraneous nucleic acid. Taking advantage of the sensitivity of RNA to high pH and the stability of the MS2 capsid under these conditions, we first attempted to remove the RNA using a solution of phosphate buffer at pH 11.8. This “alkaline method” leads to capsids with significantly lower masses than the untreated samples as shown by the CDMS spectra in Figure 3 (the black line in Figure 3 shows the CDMS spectrum measured for untreated capsids and the red line shows the spectrum measured after the alkaline treatment). In addition, the intensity of the 260 nm peak in the UV spectra of the treated sample was slightly reduced relative to the intensity for untreated MS2 particles, which is consistent with at least partial removal of the genetic material (see Supporting Information, Figure S1A). Dynamic light scattering (DLS) measurements (see Supporting Information, Figure S1B) and transmission electron microscopy (TEM) images (see Supporting Information, Figure S1C) confirmed that the capsids remained intact following treatment by the “alkaline method”. However, the center of the peak in the CDMS spectrum following the alkaline treatment (3.152 MDa) is still much higher than expected for an empty MS2 capsid (2.482 MDa), and the peak width (773 kDa) is broader than in the spectrum for the untreated capsids (black line in Figure 3). The increased heterogeneity could result from incomplete removal of the RNA. We further hypothesized that some of the remaining mass could come from DNA fragments that are not hydrolyzed by the high pH conditions.

In order to remove the extraneous DNA and RNA from inside the capsid, we designed a “hybrid method” (see Figure 2) that included disassembly and purification to remove DNA and RNA, reassembly of the MS2 coat protein around yeast RNA, and subsequent alkaline treatment to remove the yeast RNA. A CDMS mass spectrum measured for capsids treated by the “hybrid method” is shown by the blue line in Figure 3. The main peak is centered on 2.65 MDa. While this is much lower than achieved with the “alkaline method”, it is still significantly (168 kDa) larger than the expected mass of empty MS2 capsids (2.482 MDa). The peak width (195 kDa) is much narrower than obtained with the “alkaline method” (773 kDa), but still broader than expected from the instrumental resolution (90−120 kDa). We suspect that the small excess mass and the elevated peak width both result from a small amount of nucleic acid retained by the capsids, which may contribute to its stability. The intensity of the 260 nm peak in the UV spectrum following the hybrid treatment is substantially reduced compared to intensity for the untreated MS2 particles (see Supporting Information, Figure S1A), confirming the reduction in extraneous nucleic acid. DLS (Figure S1B) and TEM (Figure S1C) confirm that the MS2 particles are intact following the hybrid treatment.

In addition to the main peak at 2.65 MDa in Figure 3, there is a smaller peak centered on 3.25 MDa. This could be due to oversized MS2 particles. For example, elongation of a T = 3 capsid along the 5-fold axis would lead to a prolate particle with 210 capsid proteins instead of the 180 expected for a T = 3 icosahedron. However, the peak at 3.25 MDa is consistent with 220 capsid proteins, and this number is difficult to explain by elongation of a T = 3 capsid along any axis.

Figure 1. CDMS mass spectra of untreated MS2 capsids with no antibody bound (black line) and after a coupling reaction with 3 equiv of antibody (red line).
peak could be due to a subpopulation of MS2 particles that have retained significantly more genomic material.

The hybrid method leads to MS2 capsids that are much more homogeneous. While the mass peak is still slightly higher than the mass expected for an empty MS2 particle, we suspect that some genomic material is retained for capsid stability. There is also a small high mass peak that could be due to reassembly products with a significant amount of encapsulated material or capsids which have not assembled correctly. Save for these two details, we have achieved the goal of removing residual genetic material, narrowing the capsid peak so that the mass shifts can be determined for antibody binding.

Synthesis of MS2-Antibody Conjugates. Empty MS2 capsids were reacted with aminophenol-antibodies, as described previously, to obtain MS2-antibody conjugates (MS2-Ab), shown schematically in Figure 4. The antibody chosen for these studies was a monoclonal anti-EGFR antibody (Ab) that is currently used for the treatment of breast cancer. Furthermore, the EGFR receptor is a target of interest since it is overexpressed in numerous other types of cancer, such as glioma and colon cancer. Two ratios of antibody to capsids were used to observe the extent of modification under differing coupling conditions.

Characterization of MS2-Ab Conjugates. MS2-Ab conjugates were first characterized using gel electrophoresis to confirm attachment of the antibody to the capsids (see Supporting Information, Figure S2). We observed that the migration of the capsids was consistent with the capsids having an antibody bound to their surface given the presence of bands corresponding to light and heavy chains of the antibodies and the corresponding modification with the viral coat protein.

CDMS Analysis of the Hybrid MS2-Ab Conjugates. Hybrid MS2 samples modified with 0, 3, and 10 equiv of antibody used during the coupling step were analyzed by CDMS and the results are shown in Figure 5. Because the peaks due to the addition of a specific number of antibodies are not well resolved in Figure 5, the measured peaks for the MS2-antibody conjugates were fit to extract the underlying distribution of bound antibodies.

CDMS Antibody Fitting Procedure. The CDMS spectrum for the bare hybrid MS2 capsids was fit using Origin2015 (OriginLab, Northampton, MA) to generate a peak fitting function. A function consisting of five Gaussians was found to provide an excellent fit ($R^2 = 0.9995$). The black line in Figure 5A shows the measured peak, and the red line shows the fit. The five Gaussians that make up the fitting function are shown by the green lines. These results should not be interpreted as indicating that there are five different subpopulations in Figure 5A. Five Gaussians is simply the minimum number that provides a good empirical fit to the...
measured spectrum. Inspection of Figure 5A shows that there are at least two components present.

The Gaussian parameters (relative intensities, positions, and standard deviations) were used to create a peak fitting function that was then used to fit the CDMS spectra of the MS2-antibody conjugates. The conjugate spectra were fit by series of peak fitting functions separated by integer values of the mass of the antibody (i.e., peaks centered on \( m_{\text{MS2}} + n \times m_{\text{Ab}} \) where \( m_{\text{MS2}} \) and \( m_{\text{Ab}} \) are the masses of the MS2 and the antibody, and \( n \) is the number of antibodies bound to the MS2). Note that by adopting this strategy we are assuming that all subpopulations present in the bare hybrid-MS2 capsids (Figure 5A) have the same reactivity in the antibody coupling reaction. The intensities of the overlapping peak fitting functions corresponding to the addition of 0, 1, 2, 3, and so on antibodies were adjusted using a least-squares procedure to generate the best overall fit to the measured spectrum. Figure 5B shows the fit (blue line) to the measured CDMS spectrum for MS2-antibody conjugates (black line) from a coupling reaction with 3 equiv of antibody. The red lines show the custom peak functions used to fit the measured spectrum and the intensities of the peaks corresponding to the addition of a specific number of antibodies (i.e., 0, 1, 2, 3, etc.) are shown by the orange bars in the histogram in Figure 5D. Figure 5C shows the fit (blue line) to CDMS spectra measured for MS2-antibody conjugates (black line) from a coupling reaction with 10 equivalents of antibody. The distribution of coupled antibodies derived from the fit is shown as the green bars in the histogram in Figure 5D.

Close inspection of the results in Figure 5C reveals that there is a poorly resolved structure that appears to be correlated with peak positions expected for specific numbers of antibodies. Poorly resolved features correlated with the expected peak positions are also evident in Figure 5B. Better resolution of peaks due to the addition of individual antibodies was not achieved partly because of the remaining heterogeneity of the hybrid MS2 particles.

The charge and mass determined for each ion by CDMS are independent variables. A correlation of charge and mass can reveal information about subpopulations that are not evident in the mass distributions. Scatter plots of charge versus mass for the three mass distributions given in Figure 5A—C are provided in Supporting Information (Figure S3A—C). The distributions in the scatter plots are broad and no additional subpopulations were resolved.

In the fitting procedure outlined above, the peak fitting function used to analyze the antibody conjugates was created from the mass spectrum of the bare particles. The underlying assumption is that all components that make up the mass distribution for the bare particles display the same reactivity in the antibody coupling reaction. TEM images and DLS data show that the antibody-capsid conjugates have similar shape, size, and morphology as the bare capsids. Antibody conjugation is not believed to impact the endogenous encapsulated material still present in the hybrid treated MS2 stock because the immunoglobulins are far too large to enter the capsids. If there are subpopulations of the bare particles with different reactivities in the antibody coupling reactions, then the values obtained here are averages.

The antibody population distributions for the hybrid-MS2 capsids shown in Figure 5D are relatively broad after coupling with both three and ten equivalents of antibody. With 3 equiv, the average number of bound antibodies is 1.72. With 10 equiv of antibody in the coupling reaction, the average number of attached antibodies increases to 5.18. In both cases it appears that around 45% of the antibody is not coupled.

To explore whether the number of attached antibodies is stochastic, we compared the measured distributions to Poisson distributions. The solid lines in Figure 5D show the Poisson distributions color coded to the distributions determined from

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**Figure 4.** Coupling scheme for producing antibody capsid conjugates. The MS2 capsid has an unnatural amino acid expressed on the surface of the particle that reacts with an aminophenol-antibody to couple the antibody to the MS2 capsid. For detailed information regarding the coupling reaction, see ref 19.
the experimental data (i.e., orange for 3 equiv of antibody and green for 10 equiv). The measured distributions follow the Poisson distributions, indicating that the coupling reactions are indeed stochastic and that there are no prominent subpopulations that are particularly reactive or unreactive.

We also determined the average number of antibodies attached to untreated MS2 (0.84 for 3 equiv of antibody) and for alkaline-treated MS2 (1.78 for 3 equiv). It appears that the coupling efficiencies for the alkaline-treated and hybrid-treated samples are approximately the same, while the untreated capsids have coupling efficiencies that are around a factor of 2 lower.

It is evident from Figure 5D that increasing the number of antibody equivalents in the coupling reaction from three to ten sharply reduces the number of MS2 capsids with no attached antibodies. Unconjugated capsids are not expected to be therapeutically active, and so information about their abundance is important, particularly in cases, such as an immune response, where their presence is detrimental to the patient.

**CONCLUSIONS**

We report the successful utilization of a high-mass single molecule mass spectrometry technique, CDMS, to characterize intact antibody-capsid conjugates. Using methods developed here, we have shown that information on the stoichiometry and population distribution of antibodies coupled to MS2 capsids can be obtained. The results provide information about the coupling efficiency and the fraction of capsids that have not been conjugated to an antibody.

In addition, to providing information on the stoichiometry and population distributions, we have shown that CDMS can be used to develop protocols to optimize antibody-capsid production. This is particularly important when considering the scalability of antibody conjugate therapies. Since CDMS can measure the coupling efficiency, it provides a valuable tool to optimize coupling reactions.

**EXPERIMENTAL METHODS**

General Experimental Procedures and Materials. Unless otherwise noted, all chemicals and solvents were of analytical grade and used as received from commercial sources. Wild-type antiepidermal growth factor receptor human IgG1 monoclonal antibodies were obtained from Eureka Therapeutics, Inc. (Emeryville, CA). Trimethylamine N-oxide (TMAO) and yeast tRNA were purchased from Sigma (St. Louis, MO). Spin concentrators with molecular weight cutoffs (MWCOs) of 30 and 100 kDa were purchased from Millipore (Billerica, MA).
Expression and Purification of T19paF MS2 Viral Capsid. The expression and purification of bacteriophage MS2 has been previously reported.42 A yield of 5–10 mg/L culture was obtained for T19paF MS2 following three rounds of purification. After the final resuspension, the concentration of the protein was determined by measuring the A280 on a NanoDrop spectrometer and using an extinction coefficient of ε = 18450 M⁻¹ cm⁻¹ (which was verified using a Bradford assay).

Alkaline Treatment. A solution of MS2 at 1 mg/mL (~72 μM capsid protein monomer) in 10 mM potassium phosphate buffer was mixed with a solution of 500 mM sodium phosphate at pH 11.8 in a 4:1 volume ratio. The mixture was incubated at room temperature for 2 h. Saturated ammonium sulfate was added to the reaction mixture in a 1:1 volume ratio and the mixture was incubated at 4 °C for 30 min. The protein was then precipitated by spinning at 15000 g for 10 min at 4 °C. The resulting precipitate was resuspended in saturated ammonium sulfate to the same volume as the previous step, and the procedure was repeated twice. To determine the final concentration, a serial dilution was performed and a protein gel was run in the presence of unmodified MS2 samples as controls (data not shown). The concentration was determined by staining with Coomassie dye and quantifying the intensities of the bands.

Hybrid Method Treatment of MS2 Viral Capsid. A 10 mg/mL solution of MS2 coat protein (~720 μM capsid protein monomer concentration) in 50 mM Tris and 100 mM NaCl pH 7.2 buffer was mixed 2:1 vol/vol with glacial acetic acid prechilled on ice. The reaction mixture was incubated on ice for 30 min, during which a white precipitate was observed. The solution was spun down using a microcentrifuge at 14,000 g for 20 min at 4 °C. The supernatant was loaded onto a NAP25 desalting column (Bio Rad Micro Bio-Spin 6 Columns). The pH of the final MS2 monomer concentration, a serial dilution was performed and a protein gel was run in the presence of unmodified MS2 samples as controls (data not shown). The concentration was determined by staining with Coomassie dye and quantifying the intensities of the bands.

General Procedure for Generating Aminophenol- Antibody Conjugates. An antibody solution in 25 mM phosphate buffer pH 8 was prepared by the addition of 100 mM pH 8 phosphate buffer to the antibodies supplied in PBS. To this solution is added 5 equiv of NHS-nitrophenol from a 5 mM DMSO stock. The mixture was incubated at room temperature for 1.5 h without agitation. The nitrophenol groups were reduced to aminophenol by addition of a stock solution of 100 mM sodium dithionite (Na₂S₂O₄) in water to reach a final concentration of 10 mM. The reduction was carried out at room temperature for 20 min, followed by removal of excess small molecules using 0.5 mL spin concentrators with a MWCO of 30 kDa with 10 mM pH 7.0 phosphate buffer. The concentration of the final product can be measured by UV absorbance at 280 nm using A280 of 1.4, yielding 1 mg/mL (~6.6 μM) as a conversion factor.

Generation of MS2-Antibody Conjugates. A solution of MS2 was added to the above solution of antibody-aminophenol conjugates at a 3:1 or 10:1 ratio of antibody to capsid. In general, the final concentration of the capsid was ~200–300 nM (corresponding to ~50 μM MS2 monomer), and the concentration of Ab was adjusted accordingly. To initiate the oxidative coupling, NaIO₄ was added to a final concentration of 1 mM, and the reaction was performed at room temperature for 5 min. Excess NaIO₄ was removed using a NAP5 desalting column equilibrated with 10 mM pH 7.0 phosphate buffer, followed by spin concentration with a MWCO of 100 kDa.

CDMS Instrument. The instrument, methods, and data analysis have been described in detail elsewhere.16,17,26–29 Briefly, ions are generated by a commercial nano-ESI source (Advion TriVersa Nanomate, Ithaca, NY) and introduced into the CDMS instrument via a heated metal capillary. The first vacuum region contains an ion funnel which transmits ions into an RF hexapole in the second region. The hexapole has a 100 V DC offset that sets the nominal ion kinetic energy per charge. The next vacuum region contains an RF quadrupole where the frequency was set to 120 kHz to transmit a broad m/z range. In the following vacuum region, ions that exit the quadrupole are focused into a dual hemispherical deflection analyzer (HDA). The HDA is an energy bandpass filter that transmits a narrow band of ion kinetic energies per charge into the final vacuum region. In the final region, the ions are focused into an electrostatic linear ion trap (ELIT). In the ELIT, trapped ions oscillate back and forth through a detection cylinder. When an ion is in the cylinder it induces a charge, which is detected by a charge sensitive amplifier. The resulting signal is amplified, digitized, and transferred to a computer for analysis using fast Fourier transforms. The oscillation frequency provides the m/z and the magnitude provides the charge. A trapping time of 95 ms was employed. The signal was optimized to maximize the number of single ion trapping events; multiple ion trapping events were discarded.

Preparation of Samples for Analysis Using CDMS. Prior to CDMS analysis, the samples were buffer exchanged into 100 mM ammonium acetate (Sigma-Aldrich, 99.999% trace metal basis) by size exclusion columns with a 6 kDa cutoff (Bio Rad Micro Bio-Spin 6 Columns). The pH of the resulting ammonium acetate solutions was around 6.8. Samples were prepared at a final concentration of about 1 mg/mL protein (72 μM MS2 capsid protein monomer).

ASSOCIATED CONTENT

Supporting Information

Brief discussion of the resolving power in CDMS. UV spectra, DLS data, and TEM images of MS2 capsids following treatment by the “alkaline method”. SDS-PAGE of MS2 capsids modified with antibody. Charge vs mass scatter plots for the CDMS mass distributions in Figure 5 (PDF)
The authors declare the following competing financial interest(s): The authors except MFJ declare no competing financial interest. MFJ is associated with a company that is developing charge detection mass spectrometry.

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