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APPLICATION NOTE

Using LC-MS to Identify Clipping in Self-Assembled Nanoparticles During Vaccine Development

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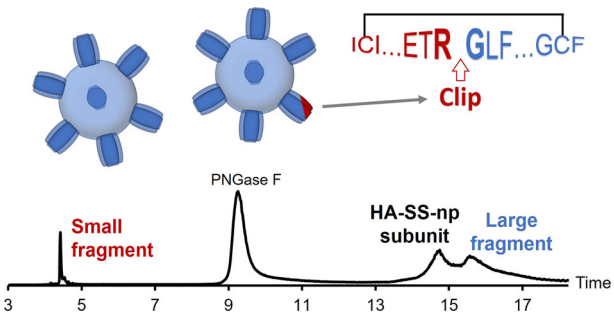

Abstract. A hemagglutinin stabilized stem nanoparticle (HA-SS-np) that is designed to provide broad protection against influenza is being developed as a potential vaccine. During an early formulation screening study, reducing gel (rCGE) analysis indicated product degradation in a few candidate buffers at the first-week accelerated stability point, whereas no change was shown in the size exclusion chromatography (SEC) measurement. A LC-MS workflow was therefore applied to investigate the integrity of this large HA-SS-np vaccine molecule (≈ 1 MDa). Application of LC-MS was critical to rationalize the conflicting results from the rCGE and SEC assays and led to the discovery that (1) an unexpected sequence clipping in the HA-SS-np subunits occurred, explaining the atypical reducing gel profile, and (2) an undisrupted disulfide bond held the two fragments together, explaining the unchanged SEC profile. This analytical case study led to a formulation buffer redesign, which mitigated the issue.

Keywords: LC-MS, Characterization, Vaccine, Nanoparticles, Clipping, Influenza

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Introduction

It is well known that clipping of fusion proteins and antibody therapeutics can occur at different stages of scale-up production [1–4]. Protein clipping is often discovered by high-throughput analytical methods, such as size exclusion chromatography (SEC), SDS-PAGE, or capillary gel electrophoresis-SDS (CGE) [1]. When high-throughput assays cannot conclusively identify product clipping or degradation though, LC-MS can be a powerful tool to further assess the integrity of the molecule. An example of such a case is reported using a nanoparticle-based vaccine candidate for influenza.

Hemagglutinin (HA) is a major glycoprotein on the surface of the influenza A virus and is a primary target for vaccine design [5]. By removing the head region of HA, enhanced exposure of the conserved HA stem region may allow broad protective immunity against influenza and eliminate the need for seasonal vaccination [5–8]. Following this strategy, stem-only HA immunogens have been genetically fused to *H. pylori* ferritin subunits that self-assemble into 24-mer nanoparticles (HA-SS-np) [7]. This glycosylated vaccine molecule has a particle size of approximately 25 nm and a molecular mass of ≈ 1 MDa.

During early development, a batch of formulation buffers was screened for one of the HA-SS-np vaccine candidates. An unexpected peak was observed in the reducing gel (rCGE) analysis for certain formulation buffer conditions, but the SEC profiles remained unchanged. LC-MS analysis of HA-SS-np was therefore critical to understand the findings of these two stability-indicating assays while pinpointing clipping within the subunits of the large self-assembled nanoparticle. This

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analytical investigation quickly prompted reformulation of HA-SS-np which ultimately resolved the clipping issue.

Experimental

HA-SS-np materials were produced at the Vaccine Production Program Laboratory (Gaithersburg, MD). HA-SS-np formulated in one of the five formulation buffers (containing sodium phosphate, sodium chloride, and sucrose, pH 7.2) was used for investigational purposes, and a HA-SS-np interim reference material (in 1x PBS) was used as a control. Reagents included LC-MS grade 0.1% formic acid in water (mobile phase A), 0.1% formic acid acetonitrile (mobile phase B), and ammonium bicarbonate that were purchased from JT Baker (Phillipsburg, NJ). RapiGest™ surfactant and [Glu1]-Fibrinopeptide B lock mass standard were purchased from Waters (Milford, MA). Formic acid, urea, and Zeba™ desalting columns (7K MWCO) were purchased from Pierce (Rockford, IL). Dithiothreitol (DTT) and iodoacetamide (IAM) were purchased from ThermoFisher Life Technologies (Grand Island, NY) and Sigma Aldrich (St. Louis, MO), respectively. Chymotrypsin and PNGase F glycosidase were purchased from Promega (Madison, WI); Lys-C was purchased from New England Biolabs (Ipswich, MA). Method details of the stability-indicating analyses (rCGE and SEC) are described in the online supporting information.

LC-MS Analysis

All LC-MS analyses were performed using an ACQUITY H-Class UPLC system (Waters) coupled with MS detection on a SYNAPT G2 QTof (Waters) and operated in positive-ion polarity with a lock mass correction being applied. For subunit LC-MS analysis, samples were denatured with 6 M urea, reduced with 40 mM DTT for 30 min at 60 °C, and alkylated with 80 mM IAM for 30 min. The samples were then buffer-exchanged into 50 mM ammonium bicarbonate, pH 7.8, and treated with PNGase F (1 unit/µg protein) overnight at 37 °C. LC separation was performed at a flow rate of 0.3 mL/min on a reversed-phase UPLC BEH C4 column (2.1 × 100 mm, 1.7 µm, Waters) at 80 °C using a linear gradient of 25%B to 40%B over a 15-min period, followed by a column wash and re-equilibration. The MS system operated in sensitivity mode and 600–4500-*m/z* acquisition range. The tune page settings were the following: 3.0 kV capillary, 35 V cone, 100 °C source, 350 °C desolvation, and 800 L/h desolvation gas. Data acquisition and processing were performed using MassLynx software, v.4.1 (Waters). Deconvolution of the mass spectra for the HA-SS-np peaks (based on the retention time window for each peak) was performed by MassLynx using the MaxEnt1 algorithm.

For reduced peptide mapping analysis, samples were denatured using 0.5% RapiGest™ in 50 mM ammonium bicarbonate (pH 7.8), reduced with 40 mM DTT, alkylated with 80 mM IAM, and incubated for 4 h with chymotrypsin and Lys-C in the same buffer (pH 7.8) at 37 °C using an enzyme/protein ratio of 1:50 (*w/w*). The samples were then heated to 70 °C for 30 min and treated with PNGase F overnight at 37 °C. For the non-reduced peptide mapping analysis, the same sample procedure was used, except no DTT was added. All digests were quenched and centrifuged, and the supernatants were subjected to LC-MS analysis. A flow rate of 0.2 mL/min on a reversed-phase UPLC peptide BEH C18 column (2.1 mm × 150 mm, 1.7 µm, Waters) at 65 °C was used for peptide separation. The gradient was the following: 0 min (3%B), 1 min (3%B), 91 min (57%B), 91.5 min (85%B), 102 min (85%B), 103 min (3%B), and 105 min (3%B). The MS system operated in resolution mode and 100–2000-*m/z* acquisition range. A collisional energy ramp of 30 to 45 V at a 0.5-s scan time was applied for the MS^E mode. The tune parameters were the following: 3.0 kV capillary, 35 V cone, 120 °C source, 350 °C desolvation, and 800 L/h desolvation gas. BiopharmaLynx software v.1.3 was used for data processing. Search parameters included up to 2 missed cleavages and the following post-translational modifications were considered: deamidation N/Q, oxidation M/W, carbamidomethylation C, and N-glycosylation. The precursor ion mass tolerance was set to 10 ppm and 20 ppm for the fragment ion mass tolerance.

Results and Discussion

HA-SS-np consists of 8 well-folded HA stem trimers (including ferritin) self-assembled into a 24-mer nanoparticle with each subunit/monomer about 41 kDa in size and containing 3 N-glycosylation sites. During formulation screening, rCGE and SEC release/stability assays were applied to evaluate the HA-SS-np under accelerated, stability conditions. At timepoint 0, an expected peak at 70 kDa (corresponding to the 41-kDa subunit) was observed in the reducing gel for both the development samples and the control; the larger apparent molecular size by gel migration was compounded by the glycoprotein interaction with the gel matrix. However, for the week-1 timepoint at holding temperatures ≥ 5 °C, an additional peak was observed at 37 kDa for certain development samples. Figure 1 shows a representative reducing electropherogram of HA-SS-np in one of the candidate formulation buffers in question that was overlaid with the control.

Interestingly, no change was observed for the SEC data (without denaturation or reduction) at the same timepoint (Fig. S-1). These observations were inconclusive; therefore, LC-MS characterization was performed to further evaluate the HA-SS-np molecule.

Since the glycosylated HA stems are stabilized with ferritin and presented as a large nanoplatform, denaturation and reduction followed by deglycosylation was required to reduce its size and heterogeneity prior to LC-MS analysis. As a result, two additional peaks were observed in the total ion chromatogram of the HA-SS-np sample in the candidate formulation buffer in comparison with the control (Figure 2). The corresponding mass spectra are shown in Figure 3 for peak identification.

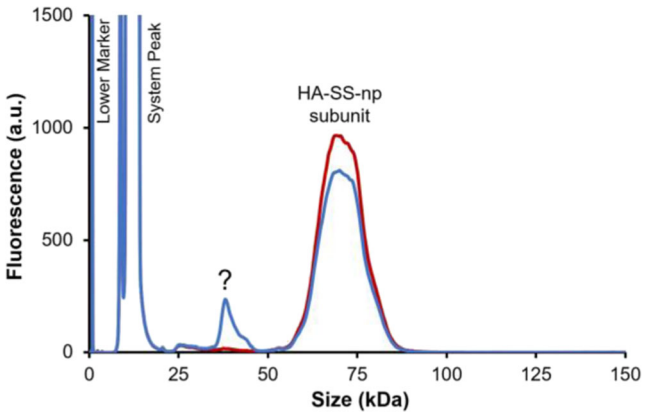

Figure 1. Electropherogram overlays of the HA-SS-np subunit (under reducing conditions) in a candidate formulation buffer incubated at 5 °C for 1 week (blue trace) and the interim reference material (red trace), which was used as a control for the analysis

The peak at 14.3–15.0 min corresponded to the HA-SS-np subunit (theoretical mass 41,394.9 Da accounting 5 Cys alkylated). However, the masses corresponding to the peaks extracted at 4.4–4.5 min and 15.5–16.2 min aligned with two complementary fragments of the HA-SS-np subunit where a clip occurred between Arg51 and Gly52. The small fragment corresponded to the first 51 amino acids (5657.2 Da accounting for 1 alkylation and two glycosylation sites). Congruently, the large fragment corresponded to amino acid residues from Gly52 to the end of the C-terminus of the protein (35,698.7 Da accounting 3 of 4 possible alkylation modifications and 1 glycosylation site). For the control sample, only the intact HA-SS-np subunit was observed, as expected.

These LC-MS results indicated an unexpected clipping event occurred when HA-SS-np was formulated in one of the candidate formulation buffers. Clipping has been observed in antibodies and fusion proteins [1–4], but limited cases have been reported for nanoparticle-based vaccine products or other emerging modalities. As shown in this case study, subunit LC-MS characterization proved to be vital for identifying the clipped species. The cutoff detection size for reducing gel analysis prevented detection of the small HA-SS-np fragment, while LC-MS analysis discovered and confirmed clipping of the HA-SS-np subunit with unambiguous detection of both

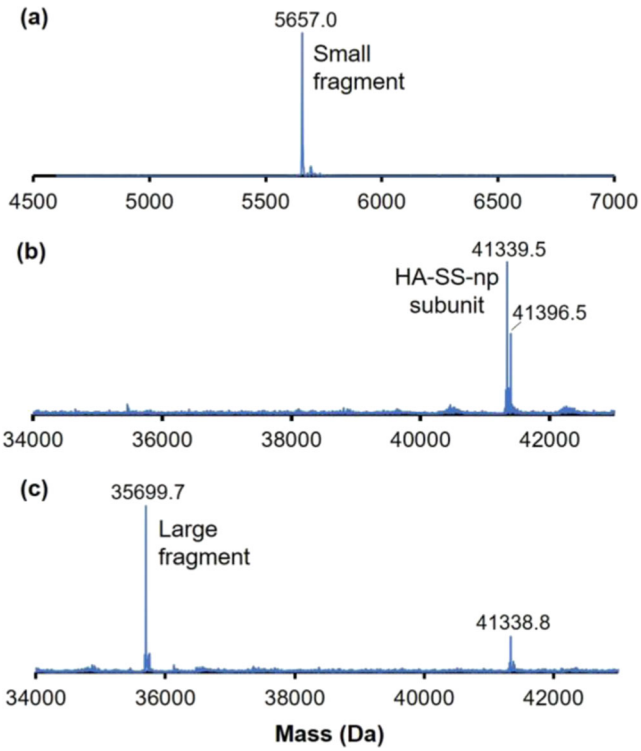

Figure 3. Deconvoluted mass spectra from HA-SS-np in the candidate formulation buffer showing (**a**) the small fragment (≈ 5.6 kDa) eluting between 4.4 and 4.5 min, (**b**) the intact HA-SS-np monomer (≈ 41.3 kDa) eluting between 14.3 and 15.0 min, and (c) the large fragment (≈ 35.7 kDa) eluting between 15.5 and 16.2 min. Mass errors were less than 40 ppm. A mixture of under- and complete alkylation of HA-SS-np monomer was observed in both the sample and control materials

fragments. Since pinpointing the source of contamination or degradation pathway was not the purpose of the study, the formulation buffers in question were eliminated from the candidate list.

Complementary to the subunit LC-MS analysis, peptide mapping was further applied to understand the nature of clipping, including disulfide bond linkage to help interpret the unchanged SEC profile. Each HA-SS-np subunit contained 2 disulfide bonds and one unpaired cysteine residue (total 5 Cys). From the non-reduced peptide mapping analysis, DTIC[4]IGY

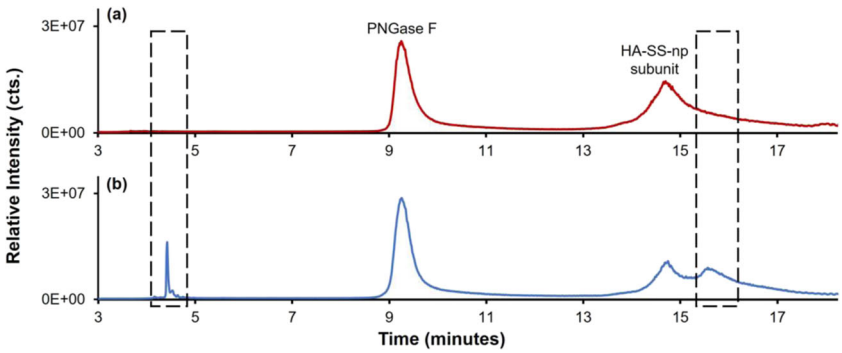

Figure 2. LC-MS total ion chromatogram of (**a**) the interim reference material (control) and (**b**) HA-SS-np stored in a candidate formulation buffer at 5 °C for 1 week

was found to be connected with EIGNGC^{161}F, which helped explain why clipping was not observed under native conditions by SEC (Fig. S-2 and Fig. S-3).

In addition, the disulfide-bridged fragments within the HA-SS-np subunit did not cause the self-assembled nanoparticle to fall apart. The other intramolecular disulfide bond within the same Lys-C-digested peptide C^{168}NNEC^{172}MESVK was also confirmed. However, once HA-SS-np was reduced, both the large fragment and unfragmented subunit species could be observed in the reducing gel. This LC-MS study helped conclude that site-specific clipping in the HA-SS-np subunits occurred and that a disulfide bond was responsible for masking the clipping, along with other non-covalent forces that held the self-assembled nanoparticle intact under native conditions. Overall, this work highlighted the importance of using LC-MS to quickly probe an atypical analytical observation during vaccine development and helped comprehend the conflicting results generated by the other assays.

Conclusion

To assist a formulation screening investigation, a LC-MS workflow was launched to assess the integrity of HA-SS-np and to understand the discrepancy between rCGE and SEC results. By deglycosylated subunit LC-MS analysis, two fragmented species of the HA-SS-np subunit were easily identified, and the clipping site was proposed. Disulfide bond peptide mapping analysis further confirmed the clipping location, as well as an intra-subunit disulfide bond that linked the two clipped fragments. Altogether, this case study highlighted that clipping in the subunits of self-assembled nanoparticles can be partially (only 1 fragment observed by rCGE) or fully disguised (no fragments observed by SEC) by bioseparation assays. By integrating LC-MS with rCGE and SEC analyses, conclusive and timely bioanalytical results were achieved during vaccine development, which triggered a formulation buffer replacement and ultimately eliminated the clipping issue.

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