Determination of Ligand Number and Distribution in Intact Protein Conjugates with High Mass MALDI-TOF-MS

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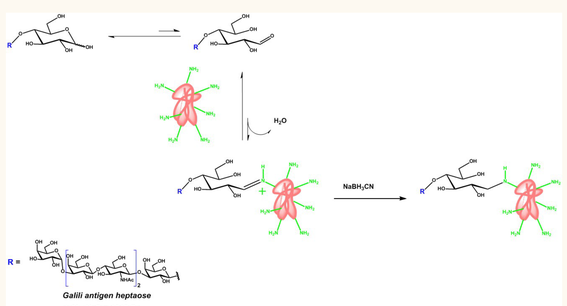
1. INTRODUCTION

MALDI-TOF fitted with a high mass sensitive detector allows for enhanced observation of intact proteins (e.g., BSA) and their conjugates with various ligands (e.g., glycans, fluorescent probes, drugs, etc.). For a protein like BSA or its glycan conjugates, the measured MALDI-TOF-MS signal at m/z 60-70 kDa, the MS resolution width (FWHM) is typically > 1 kDa. For BSA or its glycan conjugates, the measured MS signal becomes a broad MS peak. The conventional approach is to measure the centroid m/z of each wide MS peak and calculate a mass shift between the conjugated and the intact protein to estimate the average ligand number. This paper presents a novel approach to understand and analyze such MS data for the simultaneous quantitation of all possible conjugates and the calculation of their relative concentration distribution.

2. EXPERIMENTAL

Glycan-conjugated proteins (neoglycoproteins) were prepared with bovine serum albumin (BSA) by reductive amination of selected glycans to lysine[1,2]. Adjustments in coupling chemistry afforded different ratios of glycan to protein. Scheme 1 illustrates the synthetic pathway for the conjugates and the structures of the glycan ligands. Resulting neoglycoproteins were desalted by microdialysis before storage in 0.5X PBS. Dialyzed neoglycoproteins were analyzed directly or, if stored, desalted by Zeba spin columns or Millipore ZipTips. MALDI analysis was conducted on a Shimadzu Biotech Axima Confidence MALDI-TOF mass spectrometer equipped with a a CovalX HM4 high-mass detector. The instrument was operated in linear, positive ion mode with the HM4 ion-to-ion conversion dynode (HV2) set at -20.0 kV. Samples (1.0 μl; 3-15 μM) were spotted sandwich style using high-purity sinapinic acid matrix.



Scheme 1. Neoglycoprotein synthesis by reductive amination coupling of glycans to the lysine side-chains of a protein (e. g., BSA).

2. EXPERIMENTAL (Cont.)

The resulting data was calibrated to a mathematically defined peak shape with the native BSA as the calibration standard using Cerno Bioscience MassWorks Software (V5). The resulting peak shape calibration was then applied to the BSA conjugates and then quantitatively analyzed for each possible conjugate using the built in multivariant mixture analysis within the software. The fitting results and the glycan distribution plots were calculated in MatLab (R2016A).

3. RESULTS AND DISCUSSION

The observed MS signal for a macromolecule (see Figure 1) is a convolution of the intrinsic isotope distribution and the MS peak shape, where the former can be accurately calculated from the elemental composition of the molecule and the latter is determined by the mass analyzer along with its ionization and detection system. By measuring the MS signal with a known molecule such as BSA, it is possible to obtain the actual MS peak shape through a deconvolution operation. Once the actual MS peak shape is known, it is now possible to analyze an unknown (e.g., BSA-glycan conjugates), even if the measured MS signal comes from an unresolved mixture of conjugates with varying glycan numbers. This is accomplished by applying a least squares fit for each conjugate to yield their relative concentrations. The fitting residual also provides a critical diagnostic metric for evaluating the performance of the analysis and hence confidence in the results.

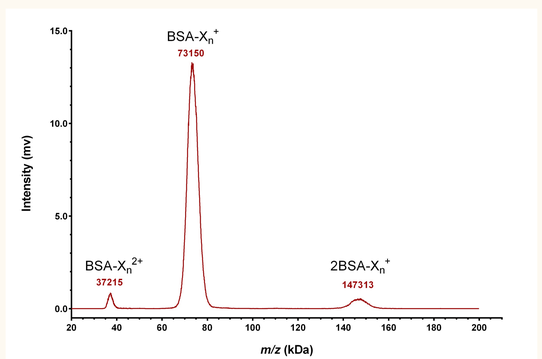


Figure 1. Linear MALDI mass spectrum of neoglycoconjugate product 168D-192-A2 from a second cycle of reductive amination using the ligand illustrated in Scheme 1. MALDI analysis was carried out at a minimal laser power of 80. A manually calculated average ligand density of 5.5 was determined.

3. RESULTS AND DISCUSSION (Cont.)

Initial results for initial samples showed poor fitting results which suggested the existence of components which had a molecular weight significantly less then the native BSA. It was hypothesized that at higher laser powers the “skewing” may be due to the sample decomposition from too high of a laser fluence. Indeed, when the laser power was reduced from 95 to 85 excellent fit results were obtained. It was determined other factors that need to be considered were the sample preparation steps - how much sample was loaded; how much matrix was used; how the matrix was spotted; salt content of the sample; etc. It was important that all the experimental and instrumental variables be consistent from measurement to measurement and particularly between the calibration standard (native BSA) and it’s conjugates.

Another issue to keep in mind is that the MALDI spectrum is always going to be an approximation of the protein since the chemical properties of the analyte will be changed by the addition of ligands. In this case, the molecule will lose basicity (e.g., be less likely to protonate) because the ligands are attached to a basic lysine by reductive amination. How much of an effect this is, is unknown since ionization in MALDI is a combination of processes involving protonation and cationization. So even the molecular cation of BSA itself is probably not homogeneous.

Whereas Figure 1 shows the unprocessed MS of a glycoconjugate product, Figure 2 illustrates the fitting results of the glycoconjugate using a 11 component mixture for the protein containing from 0 to 10 glycan's. The fit quality shows 99% spectral accuracy[3] indicating an excellent fit between the measured and calculated data.

The resulting relative concentration distribution shown in Figure 3 shows that the 5- and 6-glycan conjugates are the

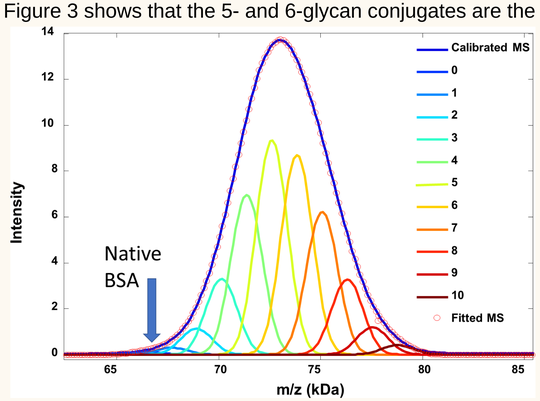


Figure 2. Least squares fitting results of the calibrated MALDI MS to the mixture of native and conjugate proteins. The blue envelope represents the calibrated MS and the circles show the sum of the calculated 11-component mixture of native protein and its conjugates. The fitting accuracy was determine to be >99% spectral accuarcy.[3]

3. RESULTS AND DISCUSSION (Cont.)

most abundant at 23% and 21% each, followed by the 4-glycan conjugate at 17% and 7-glycan conjugate at 15%, with further declining abundance on each side of the bell curve, consistent with what one would expect from the biochemical reaction perspective and the average number of ligands calculated from the simple mass shift. The BSA/glycan ratio calculated from the fit agrees with the value calculated from the peak shift at 5.5. While such complete concentration distribution of the BSA-glycan conjugates offers a lot more detailed information than the simple average number of ligands, future research will focus on validating the obtained quantitative and distributive results with other orthogonal measurements or through synthetic mixtures. This will help fully establish this novel method for direct analysis of any protein-conjugate systems.

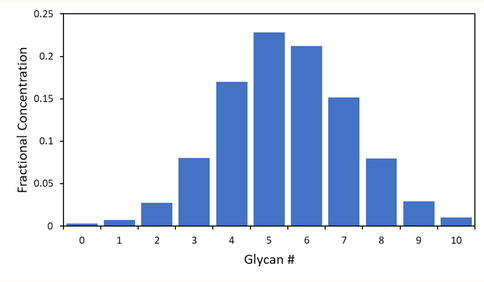


Figure 3. The relative concentration of each protein conjugate as calculated from the fitting results shown in Figure 2.

4. CONCLUSION

These initial results show great potential for the extensive application of this novel method of rapidly determining the ligand distribution in synthetic protein and antibody conjugates. While the spectral accuracy of the fit suggests excellent results, future work will focus on additional sample measurements to validate these results. However, it should be noted that the method has been quantitatively validated on other chemical systems including unresolved radio-labeled mixtures[4].

To obtain good results, care must be taken to control and optimize the experimental variables including laser power and sample preparation and it is vital the calibration sample (native protein) and the sample (conjugate proteins) be acquired under identical conditions in a timely manner. Spectral accuracy values provide a good tool for validating the quality of the measurements.

5. REFERENCES

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