Automated High-Throughput SEC Native Mass Spectrometry for mAb Screening

Investigating available methods for biotherapeutic development

Brock Juliano
Researcher
University of Michigan

Peter Rye, Ph.D.
Senior Application Engineer
Agilent Technologies

INTRODUCTION

Rapid and automated analysis of thousands of samples is key to many workflows, including the screening of monoclonal antibodies (mAbs). Automated size exclusion chromatography (SEC) combined with the power of ion mobility mass spectrometry (IM-MS) enables efficient biotherapeutic development. In this work, native mass spectrometry (nMS) and collision induced unfolding (CIU) are utilized for the analysis of mAbs at a rate of 3.1 seconds per sample.

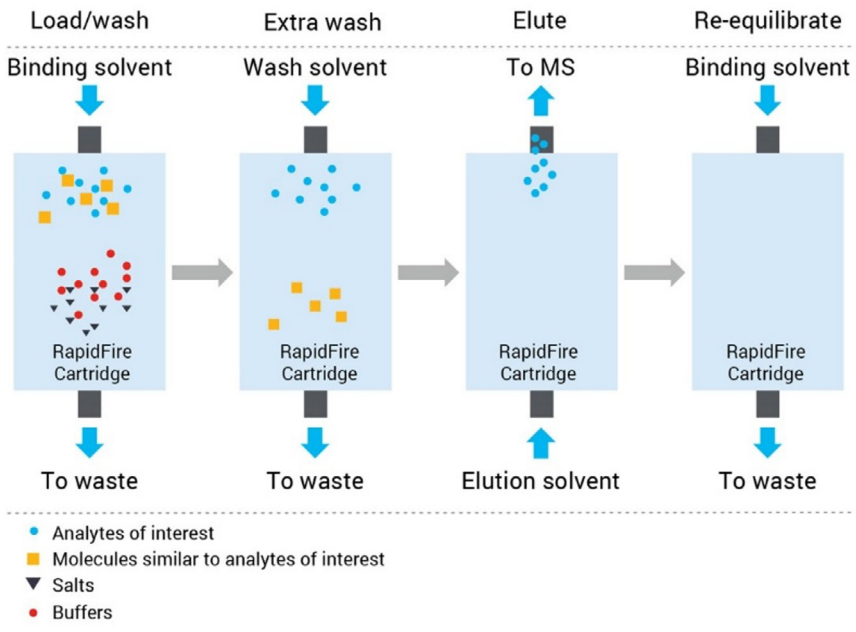
RAPIDFIRE 400 HIGH-THROUGHPUT MASS SPEC SYSTEM

Laboratories are being asked to handle more samples, collect more data, and do it all faster. The classic approach is to add more equipment but that comes at a cost; for instruments, operators, and space. Technologies including ultra-high performance liquid chromatography (UHPLC) and StreamSelect have been developed to increase the throughput of mass spec analysis, but the RapidFire approach presents an opportunity to reduce data acquisition times to as little as 2 seconds per sample.

The RapidFire 400 is an ultrafast autosampler and on-line solid phase extraction (SPE) system that connects to a mass spectrometer in place of a liquid chromatograph. The system can accomplish desalting and sample cleanup for thousands of samples with the average analysis time of 10 seconds per sample.

With RapidFire, samples are aspirated from a well plate and delivered onto an SPE cartridge (**FIGURE 1**). The sample is loaded onto the cartridge and washed to flush out the salts and buffers while the analytes of interest bind to the cartridge. An optional second wash can remove weak binding materials and further purify the sample of interest. Finally, the sample is eluted to the mass spectrometer for analysis. The system then re-equilibrates to the starting point.

FIGURE 1: RapidFire Online SPE



RapidFire 400 includes a variety of features to support its high-throughput capabilities. A 12-position cartridge handler automatically changes cartridges at predefined intervals or as different chemistries are required. With support for 96-, 384-, and 1536-well plates, the system can store over 130,000 samples. Temperature-controlled sample storage and the ability to work with sealed plates protect sample integrity and ensure quality results that can be trusted.

RapidFire integrates seamlessly with Agilent TQ, TOF, and QTOF mass spectrometers allowing for a system that is customized to the needs of your laboratory. Flexible data analysis allows for RapidFire data to be processed just like traditional LC/MS data in the MassHunter Software Suite, including tools like BioConfirm.

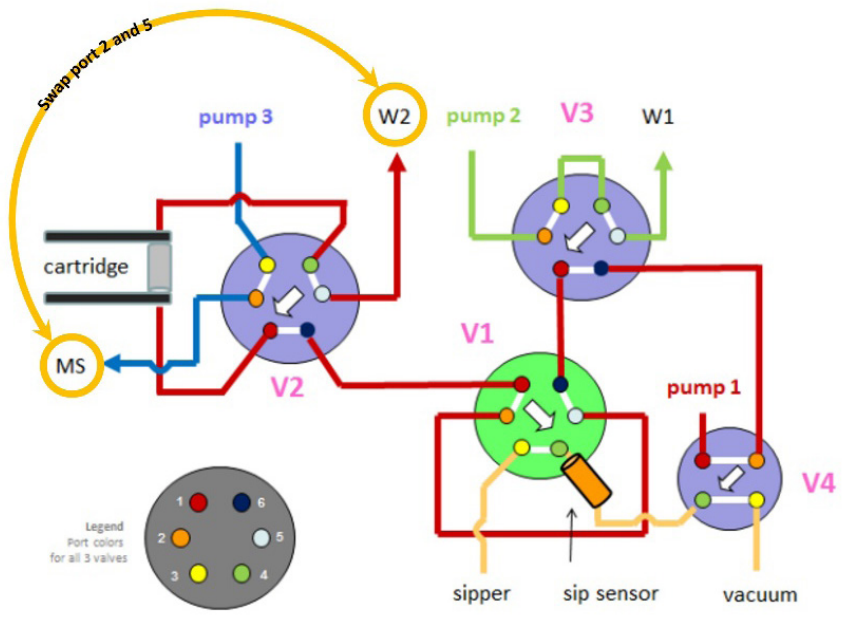
AUTOMATING NATIVE MS

A number of tools are available to characterize proteins, but sample requirements often make it less than ideal to study proteins in their native states. An ideal technique would be tolerant of heterogeneity, sensitive over a broad mass range, have a fast acquisition and analysis time, be label free, and provide structural insights. Native Mass Spec (nMS) meets most of these requirements, but the traditional implementation of nMS is difficult to automate and slow. By coupling RapidFire with an Agilent 6560 Ion Mobility Q-TOF mass spectrometer (IM-MS), all these goals are achieved.

The first step in developing a high-throughput nMS platform with RapidFire and IM-MS was to test it on a standard peptide mix. A test mixture of simple proteins yielded good results but highlighted some issues. The major issue was the system is configured for small molecule cleanup by default. By switching to a cartridge using size exclusion chromatography (SEC) packing material and a simple plumbing change, the desired results were achieved.

In the standard configuration, the sample is loaded onto a cartridge, interfering compounds are washed to waste, and then the analytes of interest are eluted to the MS. Since SEC

FIGURE 2: Plumbing Changes for SEC



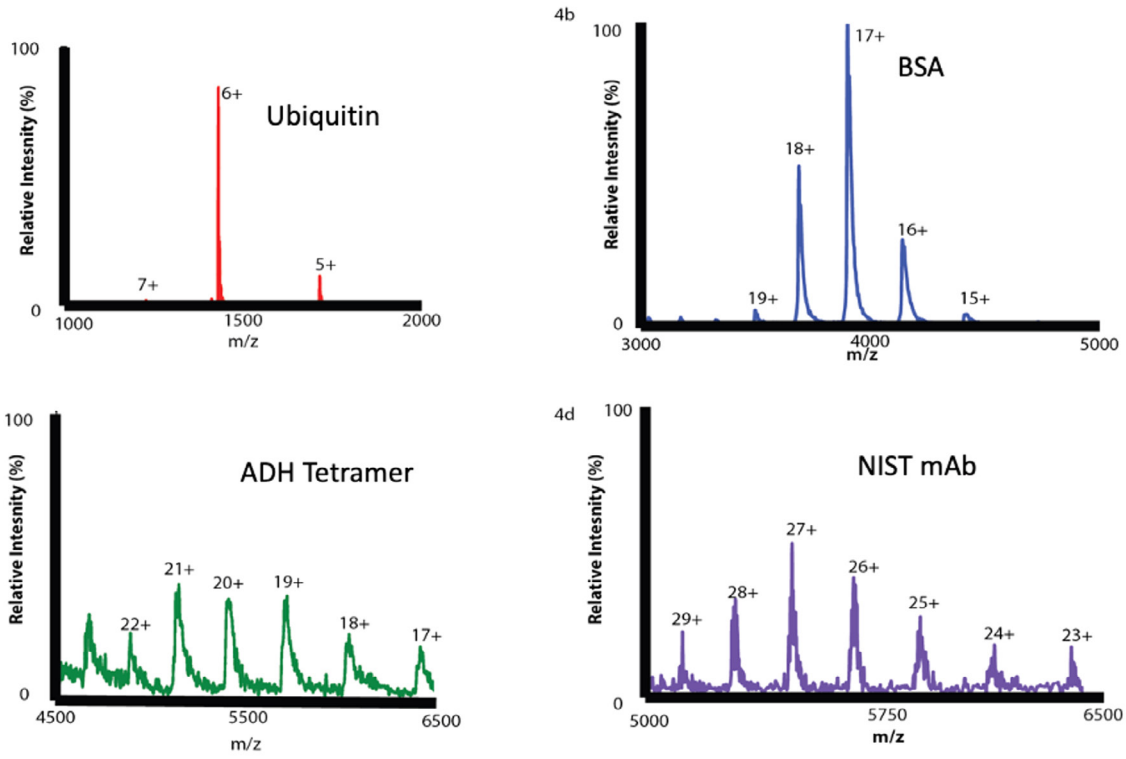
separates the sample components based on size, with the largest molecules eluting first, simply replacing a standard SPE cartridge with an SEC cartridge would have the analytes of interest go to waste. **FIGURE 2** shows the change made to the flow path that allow early eluting proteins to be analyzed by the mass spectrometer. The entire injection cycle, from aspiration through mass spec data acquisition, takes 3 seconds per sample.

The flow rate of a RapidFire system is higher than nESI can tolerate, making the Agilent Jet Stream Ion Source (AJS) a better choice.

Nano-electrospray ionization (nESI) is a common choice for nMS applications. The smaller emitter tip creates smaller droplets that require less energy to desolvate, which helps preserve the native structure of proteins. However, the flow rate of a RapidFire system is higher than nESI can tolerate, making the Agilent Jet Stream Ion Source (AJS) a better choice. The additional desolvation power provided by the AJS heated sheath gas is able to handle the flow rate of a RapidFire system without denaturing proteins.

IM-MS enables the separation of isomass analytes—same m/z but different size and shape—by combining drift time and m/z information. Furthermore, the drift times can be converted into collision cross sections (rotationally averaged measurements of a proteins size) that can then be used to compare to other structural biology techniques, such as NMR or cryo-EM.

FIGURE 3: RapidFire MS is Native MS

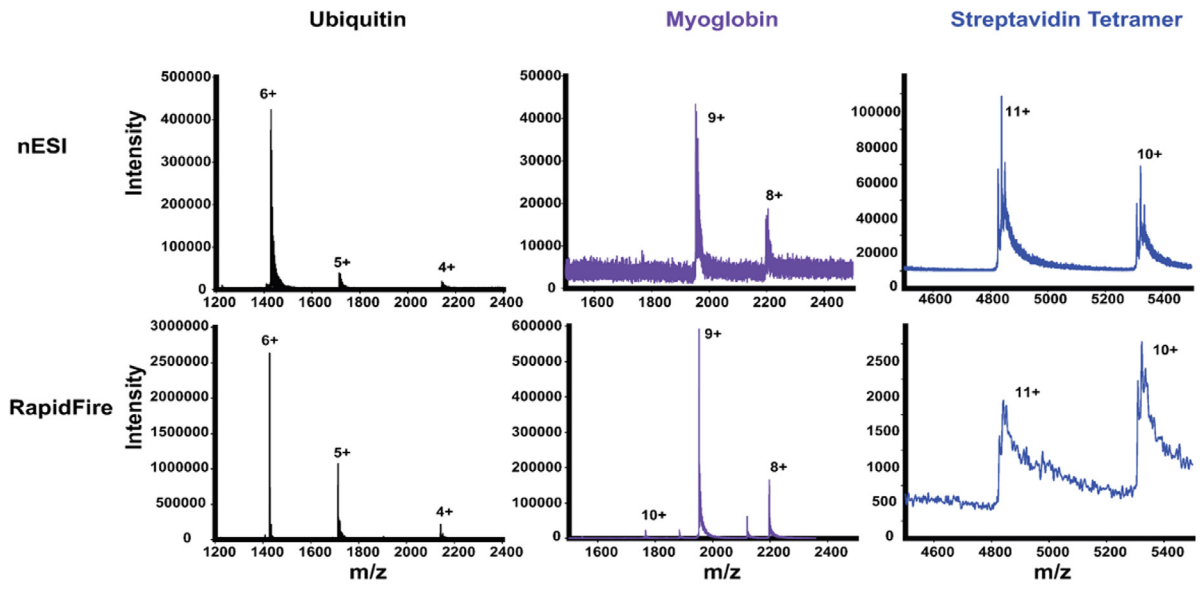


To assess the performance of the RapidFire methodology, a selection of well characterized proteins were analyzed. **FIGURE 3** shows the mass spectra from four proteins, ranging from 8 kDa to 150 kDa: ubiquitin, bovine serum albumin, alcohol dehydrogenase tetramer, and the NIST monoclonal antibody standard. The spectra contain the same charge states one would expect to see in a traditional native MS experiment. Notably, the tetramer remains visible in the spectra for the alcohol dehydrogenase tetramer, indicating the oligomeric states were not disrupted. Another set of proteins—streptavidin tetramer, insulin, β-lactoglobulin and guanidinium membrane transporter (GDX)—were also analyzed to show that even membrane proteins like GDX produce native like charge states with this RapidFire methodology.

To verify that the RapidFire data is in fact comparable to nESI-MS, **FIGURE 4** shows the results comparing the two methodologies. The charge states in the spectra produced by RapidFire are identical to those produced by nESI-MS.

The standard AJS nebulizer was used for all the work discussed so far, and while performance is acceptable, a micro-nebulizer has been shown to produce better native ion populations. The major difference between the two nebulizers comes down to internal diameter, the recommended flow rates, and source parameters (**FIGURE 5**). The standard nebulizer has an internal diameter of 120 µm and uses a flow rate over 50 µL/min, while the micro-nebulizer has an internal diameter of 50 µm and uses flow rates of 1-50 µL/min. Typical flow rates on a RapidFire system are around 1500 µL/min, so the flow

FIGURE 4: Comparisons between nESI-MS and RF-MS



rate at the nebulizer needs to be reduced to be compatible with a micro-nebulizer. There were three attempts to achieve this: reducing the flow rate of the system to 50 µL/min, using a 1:100 splitter, and reducing the flow rate of the system to 250 µL/min. Several experiments showed that lowering the flow rate to 250 µL/min—six times slower than the standard RapidFire flow rate but five time higher than recommended for a micro-nebulizer— was the best solution and produced quality data. Importantly, after months of use the micro nebulizer did not show signs of damage. The trade-off to a reduced flow rate was increasing the cycle time of the RapidFire from 3 seconds to 18 seconds, but this is still much faster than a typical nMS experiment.

Another capability of the RapidFire IM-MS system is to create collision induced unfolding (CIU) fingerprints. To implement CIU, a collision voltage ramp is applied to the ionized proteins, causing them to unfold before entering the drift tube. This generates structural intermediates with higher drift times. The changes in drift time can be tracked as a function of collision voltage, creating a CIU fingerprint.

FIGURE 5: Selected Tuning Parameters for 6560c

Parameter	Setting for AJS Nebulizer
Gas Temperature	140-250°
Drying Gas	5 L/min
Nebulizer	20 psi
Sheath Gas Temperature	140°
Sheath Gas Flow Rate	11 L/min
Capillary Voltage	3 kV
Fragmentor	400 V
Max Drift Time	90 ms
Trap Fill Time	80000 µs

Several proteins used in the initial experiments were also used to conduct CIU experiments. Each fingerprint required two sips on the RapidFire and the data was collected over 30 seconds. When comparing the features from the RapidFire CIU experiment to the traditional approach, the number of features and the fingerprints were similar. In some instances, there were differences which we can attribute to transient

features that may indicate the RapidFire approach is a bit more activating. In another set of experiments with the NIST mAb sample, the slight differences in the data may indicate that desolvation in the RapidFire methodology may not have been as effective as with the traditional approach. Further tuning of the gas temperatures may be able to resolve this difference. Overall, the CIU fingerprints were highly comparable between methodologies.

CONCLUSION

This work demonstrates that the RapidFire is an excellent approach to native mass spectrometry and capable of significantly reducing data acquisition time for these types of experiments. Looking forward, there are opportunities to develop RapidFire methodologies for monoclonal antibody stress screens using samples that have been deglycosylated, heat stressed, or oxidized. Using CIU data on these types of samples will allow for comparison of stability differences among them. There is also potential to combine RapidFire with additional types of proteomic experiments and expand to other classes of molecules such as RNA.

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DE93214607
5994-6220EN
Printed in the USA, June 1, 2023