June 2021

Prepared by Fasmatech & Karolinska Institute

Contents

1. D3.2 – Optimized HAB guns installed and tested

Efforts to develop a hydrogen atom source for activating ions stored in the Omnitrap platform were initiated before the TopSpec project andF numerous attempts have been undertaken throughout its course. Early efforts were focused on admitting pulses of molecular hydrogen through a hot polycrystalline cavity or tube (tungsten or tantalum) to form a free jet gas flow directed perpendicularly through the axis of the linear trap in segment Q5. At the highest temperatures tolerated by tungsten, the cracking efficiency of molecular hydrogen to generate radical hydrogen atoms, H•, was limited to <20% while the maximum kinetic energy of 5 meV achieved is dictated by the ~1000 m/s velocity of the under-expanding gas. **Figure 1** shows the latest version (v3) of the thermal hydrogen atom source and the high attachment efficiency of hydrogen atoms to free heme B and to heme B attached to cytochrome c. Hydrogen atom attachment to z radical fragment ions produced by ECD has also been observed with this design with the attachment efficiency shown to reduce as the length of the peptide increased.

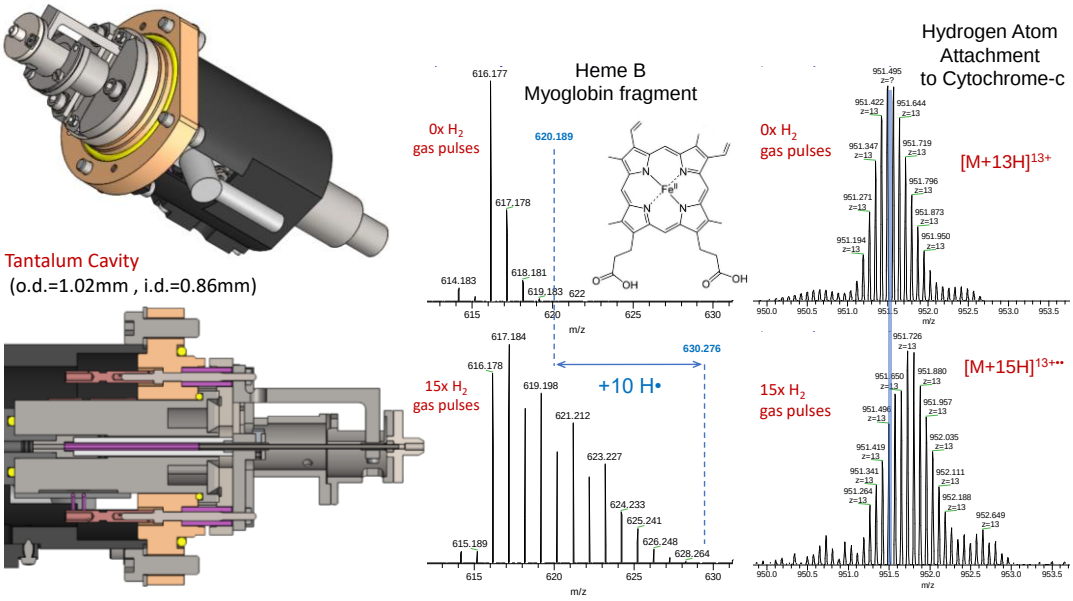


Figure 1. Design of the thermal hydrogen atom source using hot polycrystalline surfaces to crack molecular H_2 and mass spectra showing the attachment of H• to free heme B and also to heme B connected to cytochrome c.

The low attachment rate of thermal H• observed with the original design and the inability to generate fragments has led to the development of the hyperthermal source. Different methods have been considered to generate fast hydrogen atom beams, however, combining such a source of particles, whether charged or not, with the Omnitrap platform must satisfy a number of criteria to support the development of multiple-stage tandem ion activation-dissociation workflows. For example, advanced processing of ions in the Omnitrap platform relies on large sets of highly regulated instructions applied sequentially. Consequently, integration of the hyperthermal source requires a high level of synchronization to be achieved with all the additional analytical features currently available in the system. This is accomplished by generating and operating a low pressure discharge in pulsed mode. To satisfy the pressure tailoring approach adopted in the Omnitrap platform in order to optimize the different functions performed sequentially, as well as

to meet the stringent vacuum criteria imposed by the Orbitrap mass analyzer, the pulsed nature of the plasma is accomplished by driving a pulse valve at relatively high repetition rates to generate sequential pulses of gas while the DC discharge potential is always switched on. The gas load to the Orbitrap analyzer is therefore considerably lower compared to admitting hydrogen gas continuously, while a number of operations performed in the Omnitrap platform and optimized under collision-free conditions remain unaffected. Finally, a highly directional and focused beam of hydrogen atoms is necessary to maximize the efficiency of interactions with trapped ions. All these requirements are met by generating a pulsed beam of ionized hydrogen species formed in a discharge and originating from a pulsed source of gas. In principle, ions emitted from the discharge can be accelerated to the desired kinetic energy and subsequently neutralized en route to the trapping region filled with ions. Whist pulsed operation of this new source of particles in "ion mode" (production of a dense beam of hydrogen ions) was relatively straight forward to achieve, neutralization of the ions while maintaining the necessary density and the directionality of the beam was a very challenging task. After several design iterations and numerous experimental attempts an entirely new concept design was produced and tested successfully and a patent application was timely filed by Fasmatech.

1.1 Pulsed Hydrogen Ion Source Design & Evaluation

Before describing the new hybrid hyperthermal ion source and the direction that research efforts will focus in the near future, it is important to describe the precursor design of the pulsed source of hydrogen charged particles, excluding the neutralization stage, and also outline its basic features and performance characteristics.

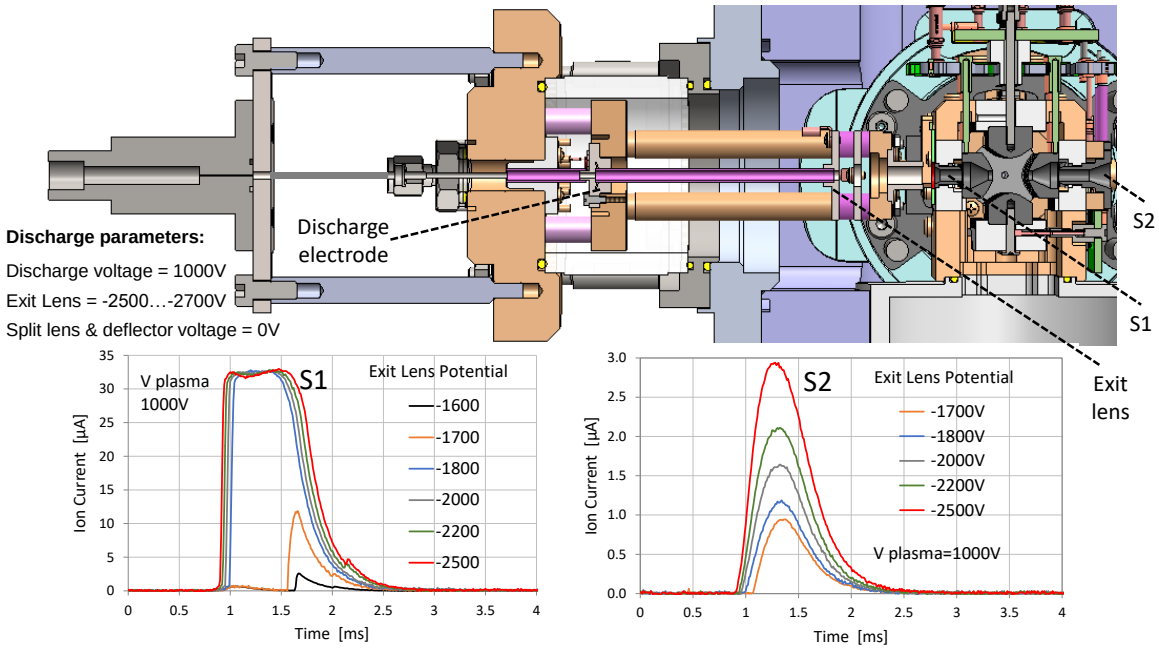


Figure 2. Cross sectional view of the pulsed ion source and time profiles of the H_n^+ ion current.

Figure 2 show a cross section of the Omnitrap platform and the side flange supporting the pulsed hydrogen ion source. A pulse valve is used to release pulses of hydrogen gas into a ceramic

cylindrical tube separated into an entrance and an exit part. A discharge electrode is installed between these parts forming a leak-tight conduit and supplied with a high voltage. The discharge is ignited at elevated pressure during passage of the gas pulse through the conduit. A series of electrodes are arranged downstream from the conduit to control beam divergence and focus charged particles through the trapping volume. Short (2 ms) pulses of ions with a peak current of 3 μA are measured on the skimmer lens S2 placed across segment Q5.

Injection of high kinetic energy hydrogen ions in the trapping region of the Omnitrap platform was first evaluated using ubiquitin and myoglobin ions. Mass spectra produced by bombardment of these proteins with ~1 KeV hydrogen ions and processed data using PeakFinder are presented in **Appendix I**. These results indicate that lower intensity fragment ions are generated from the more abundant charge-reduced and ionized precursor ions. A closer examination of the isotopic distributions in the higher m/z range of the MS2 spectrum shows that proton abstraction is also a key reaction in product ion formation. **Figure 3** shows a diagram highlighting possible formation pathways for the different charge states produced during activation of the protein precursors with hydrogen ions (H^+, H_2^+ and H_3^+). These pathways were observed in both experiments performed with ubiquitin and myoglobin. The ion formation map proposed in these experiments cannot be confirmed in experiments with intact transtuzumab since all ionized and charge-reduced product ions at the higher m/z range are not isotopically resolved.

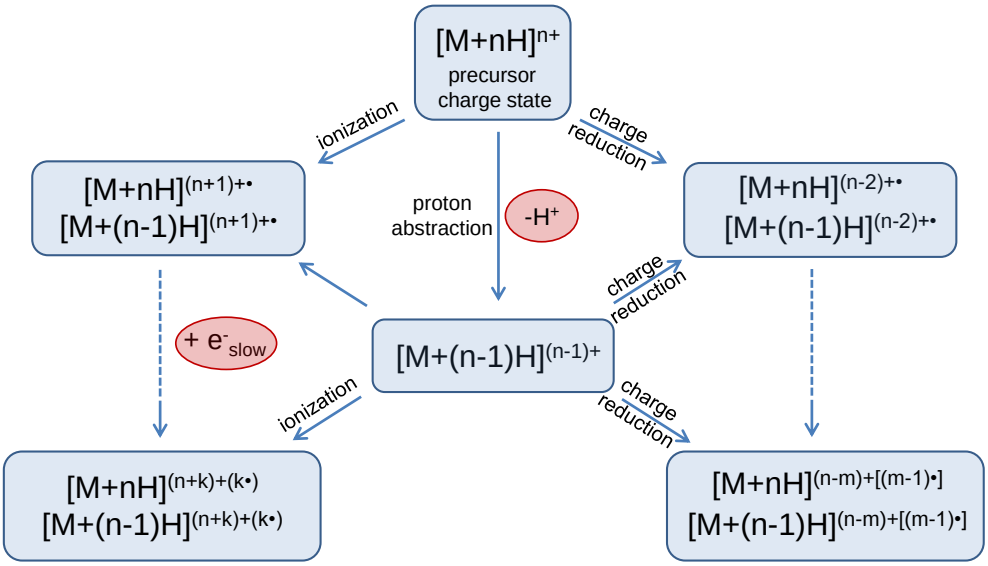


Figure 3. Proposed product ion and charge-state formation pathways of protein precursor species generated during activation with hydrogen ions emitted from a pulsed glow discharge source.

At the end of the reaction, the presence of charge-reduced product ions appears to be far greater compared that of the ionized counterparts and for the diagram present in **Figure 3**, the value of m is always greater compared to k, m>k. Nevertheless, in time resolved experiments it was also observed that at the onset of the reaction, the intensity of ionized product ions is always greater compared to the intensity of charge-reduced precursor ions, suggesting that the detachment of low energy electrons is likely a necessary step for the formation of the charge-reduced species, while fragments are formed via electronic excitation and by electronic-to-vibrational energy

transfer in a process similar to EID. Fragment ion formation via electron capture similar to ECD may also be one of the basic dissociation mechanisms.

Experiments performed with charge state 49+ of intact non-reduced trastuzumab ions sprayed under denaturing conditions are presented in the following **Figure 4 (a)**. Ions are stored in segment Q5 and irradiated by 20 consecutive gas pulses, each producing a mixture of H^+, H_2^+ and H_3^+ having a broad kinetic energy spread and a maximum kinetic energy dictated by the discharge voltage (~1 KeV). With the duty cycle of the pulse valve limited to ~80 ms, the total time of this part of the experiment within a single scan is approximately 1.6 s, while the actual activation time by hydrogen ions within this time window is only 50 ms. At the end of the activation period, the mass range of the charge-reduced product ions is extended up to 4000 Th while only the singly and doubly ionized species are observed. Together with the charge-reduced and ionized product ions, a series of fragments are formed in the lower m/z region of the mass spectrum with very high spectral density. Distribution plots shown in **Appendix I** indicate that the charge state for these lower m/z fragments are higher than those observed in the MS2 ECD spectrum of trastuzumab $[m+49H]^{49+}$ ions. This is yet another indication that fragmentation is directed primarily by electron ionization processes and to a lesser extent via electron capture, which is responsible for the formation of the charge-reduced precursors.

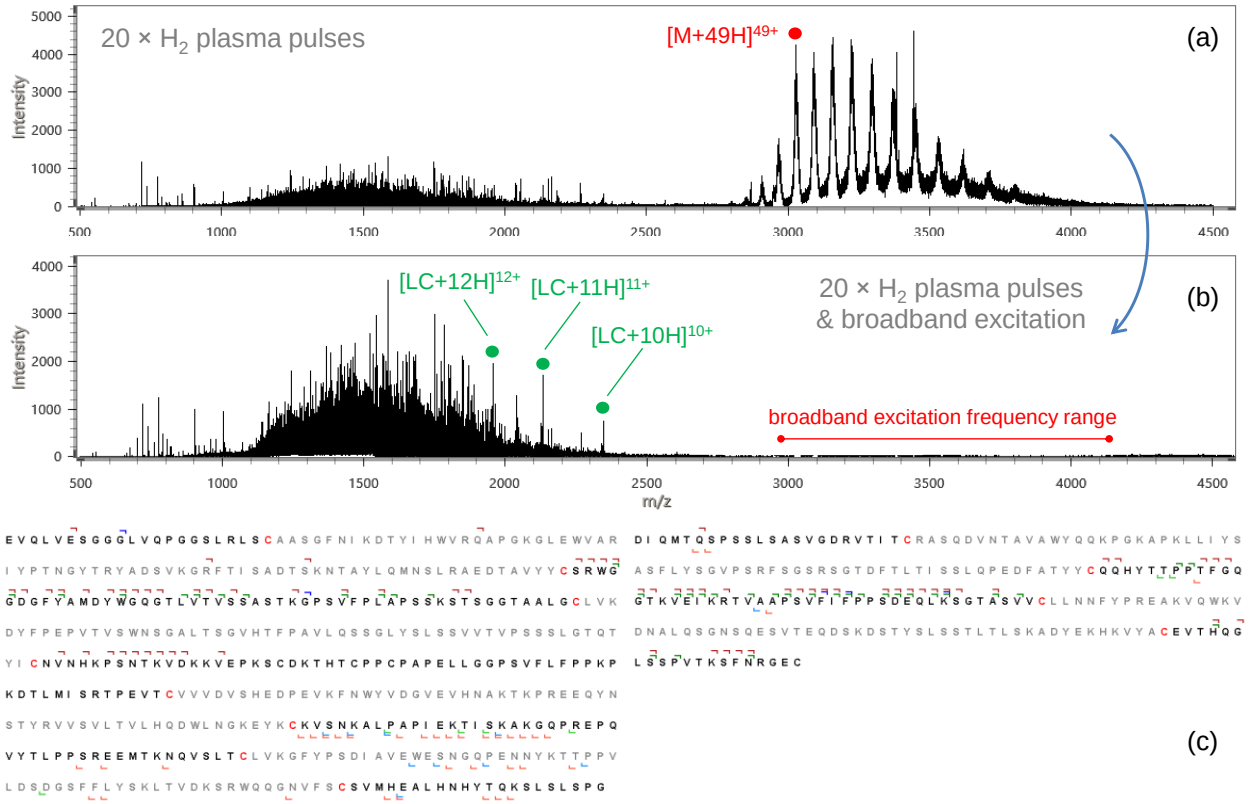


Figure 4. (a) MS2 mass spectrum produced by hydrogen ion activation of intact trastuzumab charge state 49+. (b) Two-step MS2 generated by supplemental collisional activation of the charge-reduced intact trastuzumab ions. (c) Sequence coverage of the 49+ intact non-reduced denatured trastuzumab ions obtained by hydrogen ion activation combined with broadband excitation.

The effect of collisional activation by the application of a broadband excitation signal across the high m/z region with frequencies corresponding to the secular frequency of the charge reduced product ions was also explored. This two-step MS2 mass spectrum is presented in **Figure 4 (b)** where intact light chain ions are produced with high efficiency. The sequence coverage obtained by processing the mass spectra manually in PeakFinder is also presented in **Figure 4**. Despite the lower sequence coverage compared to the other MS2 methods explored so far and carefully analyzed (CID, ECD), it is emphasized that the complexity of the spectrum is extremely higher and the number of identifications as well as the total ion signal assigned is relatively lower. Limited sequence coverage is therefore considered to be severely underestimated by the current processing method. The complexity of the mass spectrum is illustrated in **Figure 5**. In many cases, theoretical isotopic distributions cannot be matched to experimental isotopic distributions due to the complicated and partially unresolved isotopic patterns present through the mass range of interest. This MS2 workflow would greatly benefit by incorporating proton transfer reactions to concentrate the ion signal into a fewer and lower charge states located in the higher m/z region where isotopic distributions remain resolved.

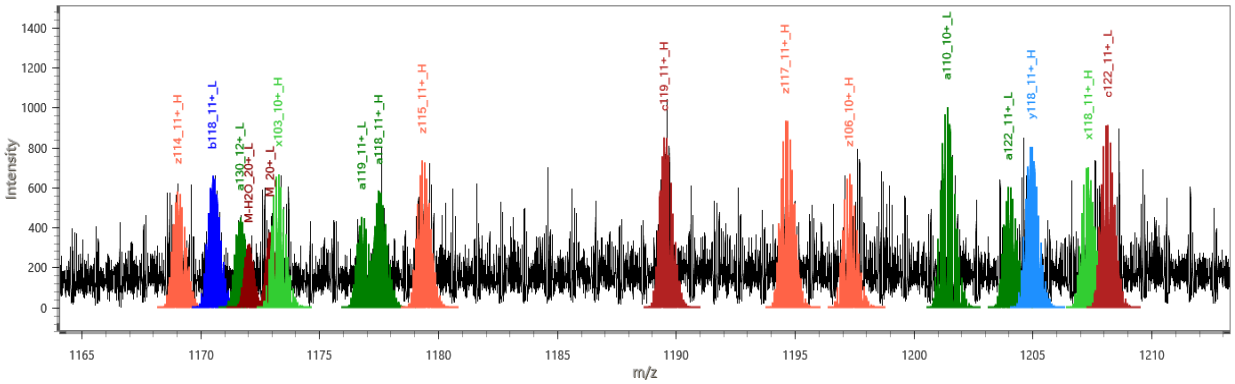


Figure 5. An example of the spectral congestion observed in the MS2 mass spectrum of the 49+ charge state of intact trastuzumab shown in **Figure 4 (b)**.

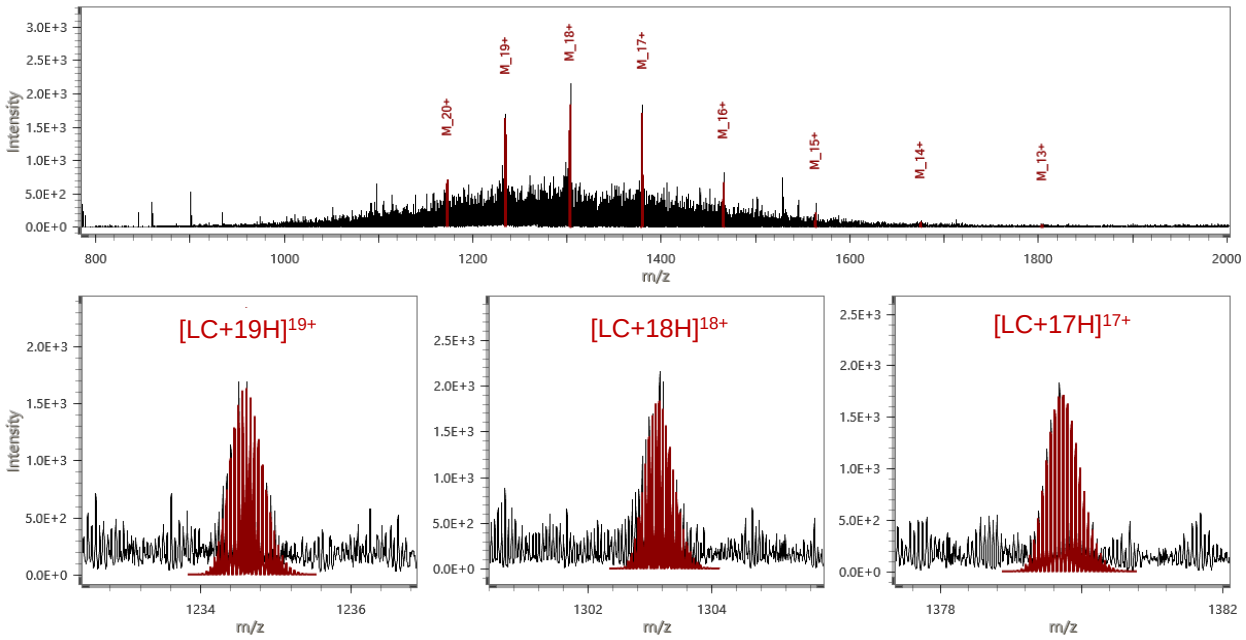


Figure 6. MS2 mass spectrum of the 49+ charge state of intact trastuzumab shown with experimental conditions favoring the production of high charge state light chain fragment ions.

Finally, another example of an MS2 experiment generated by hydrogen ion activation of intact denatured non-reduced trastuzumab is presented in **Figure 6** where intact light chain ions are produced with high efficiency and observed at much higher charge states (17+ to 19+) compared to those formed in CID or ECD described in deliverable D2.3. It is interesting to note that the light chain fragments appear to be even electron species, that is, the ions are of the form $[M+nH]^{n+}$. Future experiments will aim at identifying optimum conditions to generate these higher charge state light chains ions reproducibly to facilitate MS3/MS4 workflows further.

Overall, the higher charge state of the primary fragments suggest that ionization via electron detachment may play a significant role in fragment ion formation, however, proton abstraction and charge-reduction are also key mechanisms that contribute to the generation of fragments. Although the hydrogen ion source described here was a necessary intermediate step for the development of the hyperthermal configuration, the utility of this simple and robust system appears to be very useful and its full potential is yet to be explored. The Omnitrap platforms to be installed at Karolinska and Pasteur will be equipped with the hydrogen ion source and will be upgraded with the hybrid hyperthermal hydrogen atom-ion source describe below at a later stage.

The design of the Omnitrap platform and many of the additional mechanical sub-assemblies comprising the electron source and the hydrogen ion source incorporate PEEK material. Although PEEK is used extensively in high vacuum applications as an insulator, it was recently discovered that PEEK is outgassing strongly producing strong ion signals in the lower m/z range (<300 Th) when the electron source is switched on. Positive ion currents as high as 5 µA were measured inside the Omnitrap trapping volume during operation of a deuterium lamp employed for generating VUV photons. It was therefore decided to entirely eliminate PEEK from the Omnitrap design. The redesign process required considerable effort and resources to be completed and

details will be provided in the July 2021 Periodic Report. **Figure 7** shows the prototype hydrogen ion source and the new design constructed purely from metal and ceramic parts.

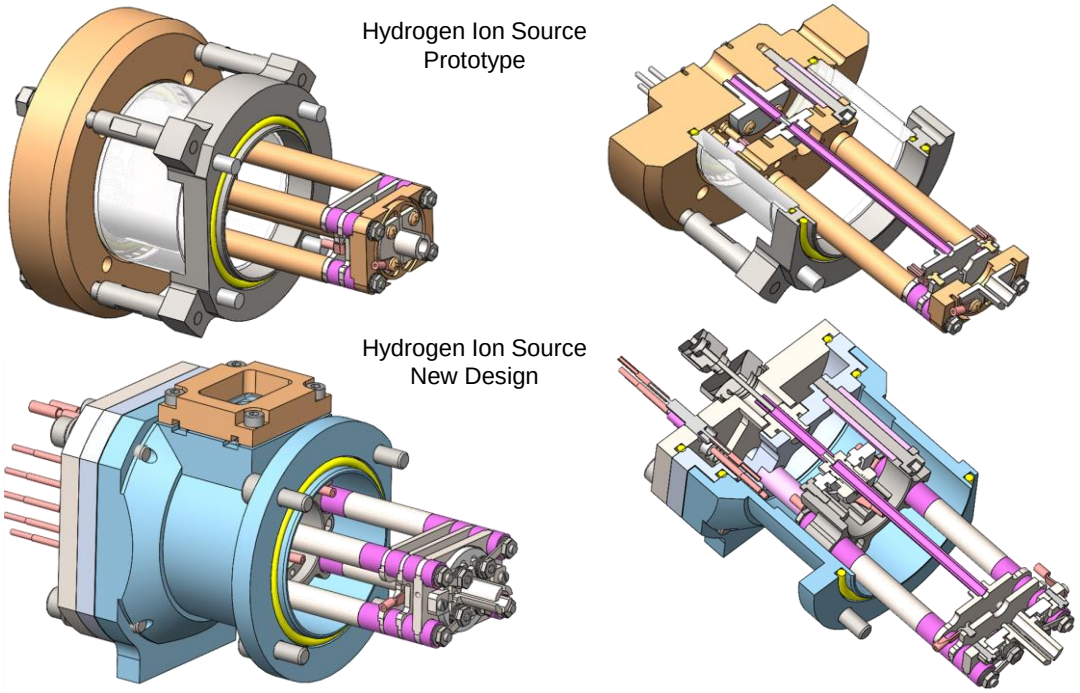


Figure 7. Mechanical models of the prototype and the new design of the hydrogen ion source.

1.2 Hybrid Hyperthermal Hydrogen Atom-Ion Source

The development of the hybrid hyperthermal atom - ion source is considered to be an essential addition to the ion-activation arsenal available in the Omnitrap platform and overall has the potential to be established as a major advancement in ion activation-dissociation methods. Following the completion of a first successful set of experiments demonstrating the conversion of fast hydrogen ions to hydrogen atoms, a patent application was prepared and timely filed. **Figure 8** is taken from the patent application and key components of the invention are highlighted. The concept relies heavily on the design of the pulsed hydrogen ion source (101) described in Section 1.1 above, which is driving a glow discharge in pulsed mode using gas pulses released from a pulse valve (103) and confining the flow of gas inside a conduit (106, 107) where a static DC potential applied to an electrode (108) is used to generate a dielectric barrier discharge (110) producing a pulsed beam of hydrogen ions (114) focused by a set of lens-electrodes (111, 112) in the direction of a neutralization stage (301). The neutralization stage comprises of a series of tungsten ribbon filaments (304) disposed along the axis of a cylindrical ceramic tube (303) to confine the gas flow and ions radially. Many different configurations were tested using cold metal surfaces (**Appendix II**) to convert ions into atoms without success. The ability to drive the ribbon filaments to incandescent temperatures was the key element for successfully converting atomic hydrogen ions into radical species.

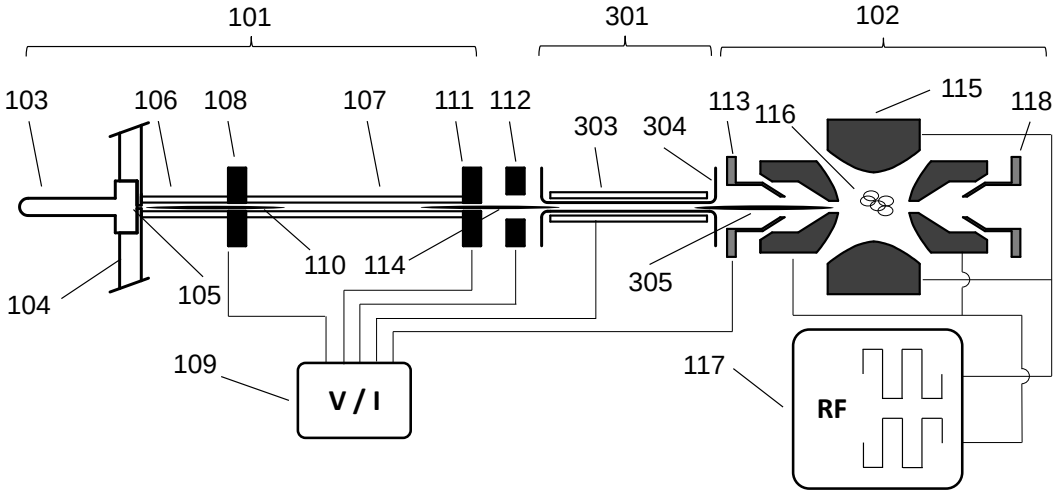


Figure 8. Schematic representation of the hybrid hyperthermal hydrogen atom-ion source disclosed in the patent application.

Figure 9 shows the attachment of hydrogen atoms to Heme B produced by collision induced dissociation of cytochrome c. Following the injection of consecutive pulses of a mixture of hydrogen ions and neutralized species, the isotopic distribution is shifted to the right. Currently, the design does not allow injecting neutral species alone since in the prototype design there was no provision to deflect the 1 KeV fast ions exiting the neutralization stage. As a result, extending the injection time to identify the maximum number of hydrogen atoms that can be attached leads to dissociation of the free heme B. Nevertheless, from these experiments it becomes evident that hydrogen atoms are formed abundantly and these can only be generated by neutralization of the hydrogen ions transported through the incandescent tungsten filaments. In the current design, switching off the heating current applied to the filaments will only generate fragments without the corresponding shift observed in the isotopic distribution when H• species are formed.

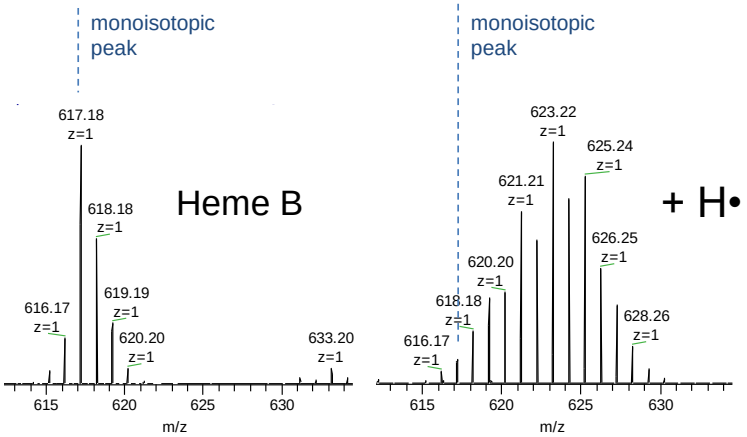


Figure 9. Free heme B generated by CID of cytochrome c and the shift in the isotopic distribution observed following injection of a mixture of hydrogen ions and atoms.

An essential aspect of the design is the ability to operate this new source of particles for ion activation - dissociation in three different modes. The first is concerned with the injection of the

hydrogen ions (H+, H_2^+ and H_3^+) alone. This is accomplished by maintaining the tungsten ribbon filaments at low temperature while applying a focusing potential to enhance transmission efficiency of the ions through the neutralization stage. In the second mode of operation, ions are converted to neutrals by reacting with electrons produced by thermionic emission in the incandescent filament source by applying a heating current to the tungsten ribbon assembly. A retarding potential is applied downstream from the incandescent filament source to deflect charged particles and only radical atoms or other neutrals in excited electronic states are injected in the ion trapping region. In the third and final mode of mode of operation, both neutrals and ions are injected in the trapping volume simultaneously. The results shown in **Figure 9** were obtained in the third mode of operation. **Figure 10** shows a cross section of the prototype hybrid source highlighting the major product species formed at the different stages of the system.

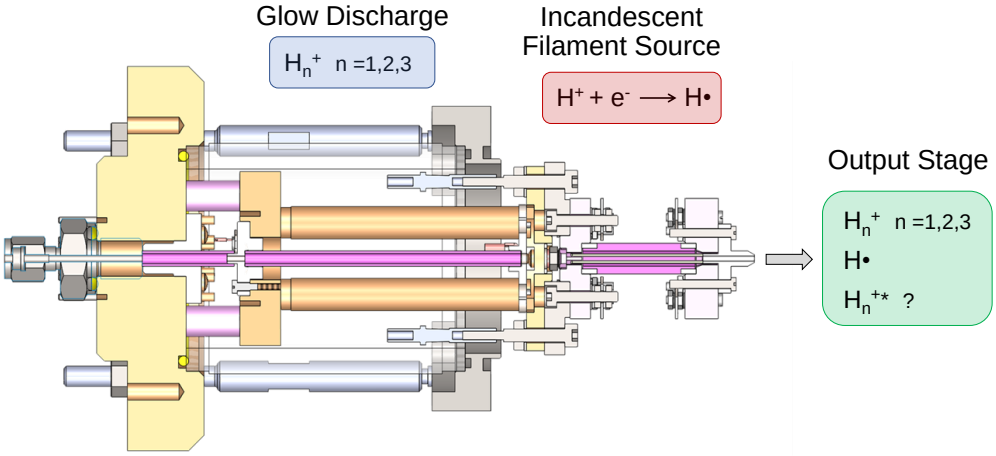


Figure 10. Cross section of the prototype CAD model of hybrid source.

The mechanical design of the hybrid source is nearly finalized and includes a cooling stage for sinking the heat produced by operating the tungsten ribbon filaments at high temperatures to promote the neutralization reaction between H^+ ions and electrons. PEEK has also been removed in this new design except the feedthru flange. Details of the system are highlighted in **Figure 11**.

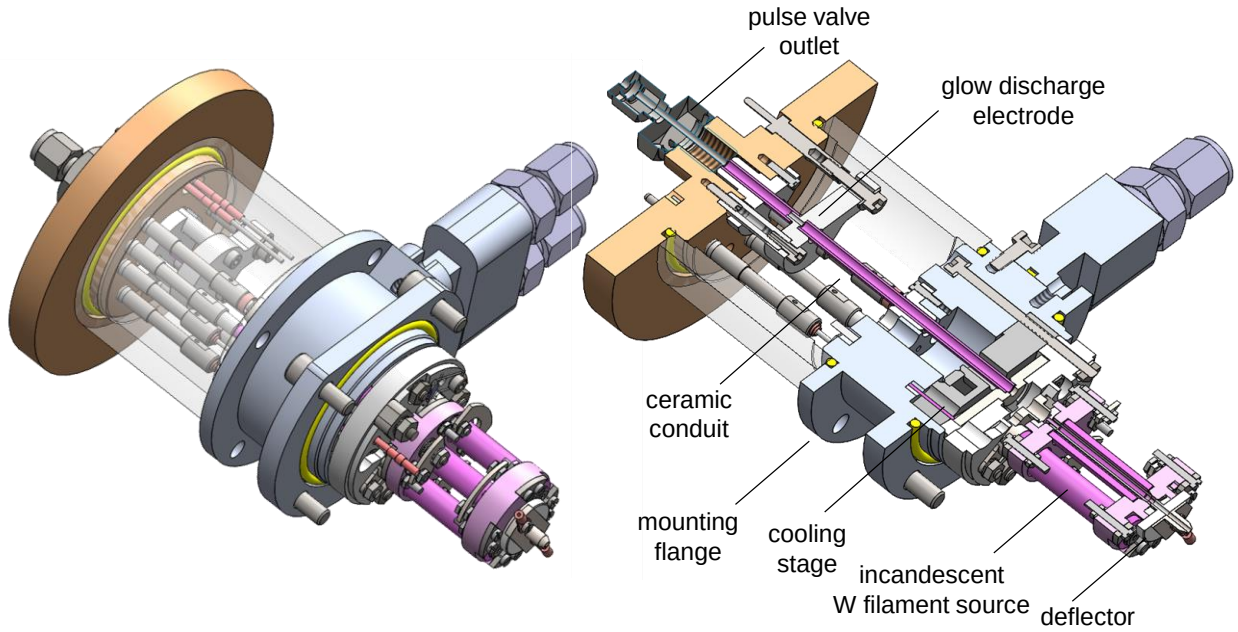
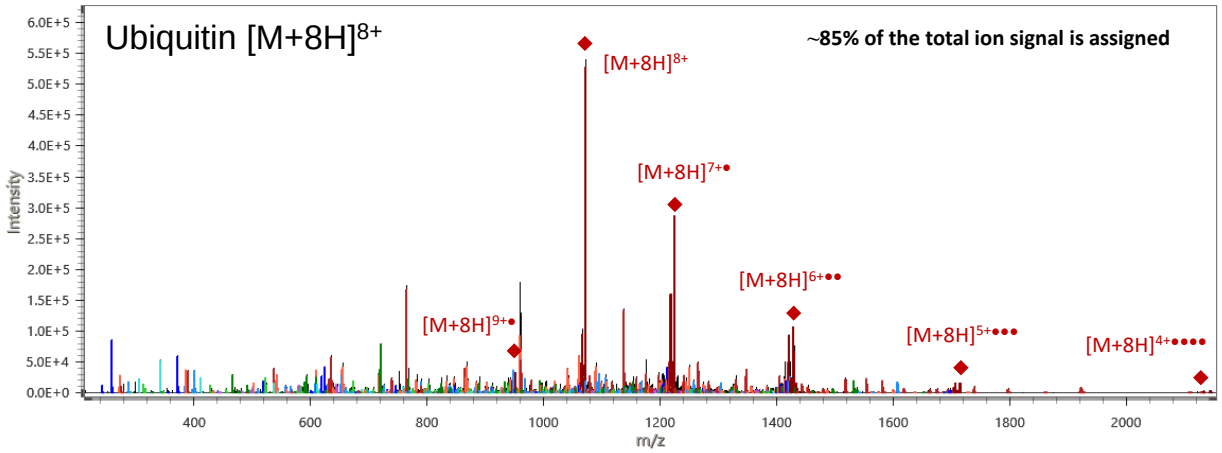
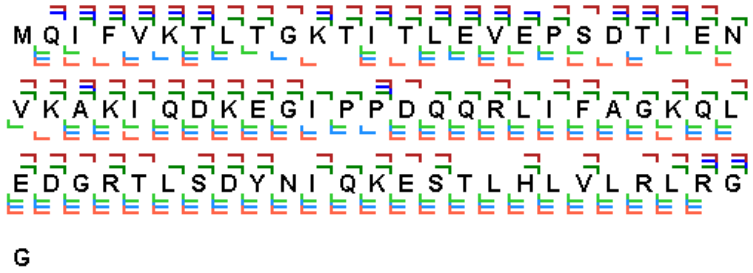


Figure 11. 3D model and cross section of the new hybrid hyperthermal hydrogen atom-ion source currently in the final stages of development.

Appendix I

[M+8H]$^{8+}$ Ubiquitin MS2 Hydrogen Ion Bombardent - *assignments in PeakFinder*

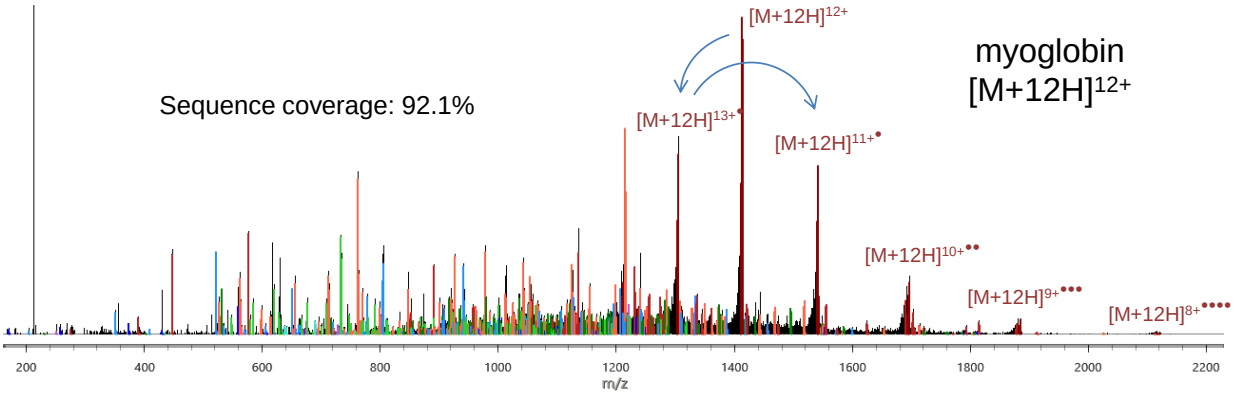


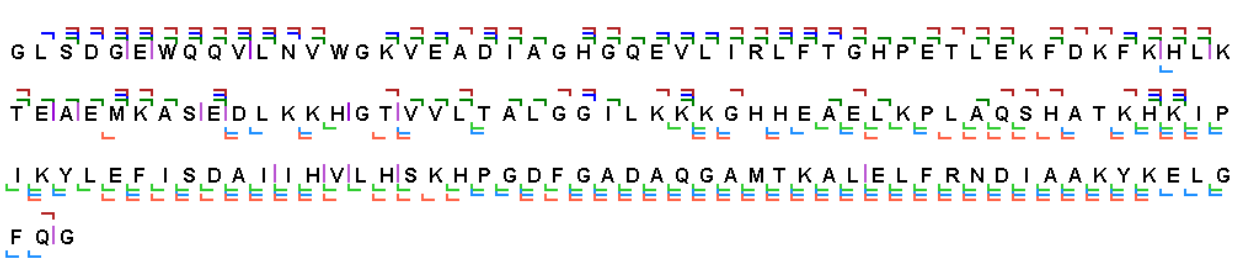


a : 80.3% x : 80.3%
b : 25% y : 75%
c : 75% z : 80.3%

Sequence Coverage : **100%**

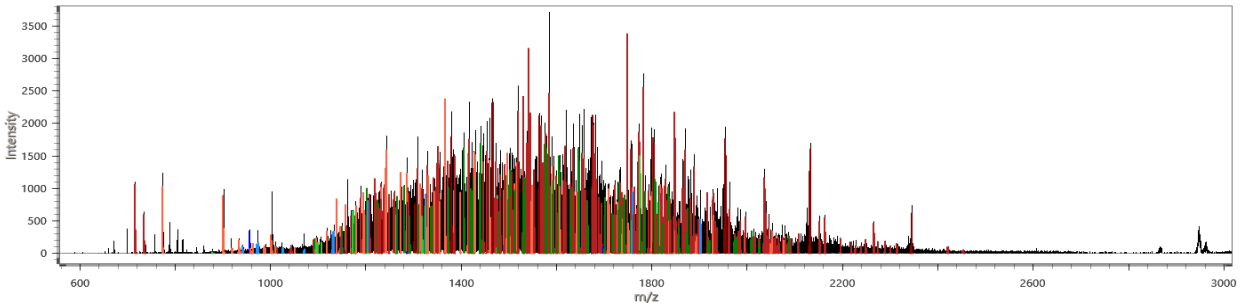
[M+12H]$^{12+\bullet}$ Myoglobin MS2 Hydrogen Ion Bombardent - *assignments in PeakFinder*



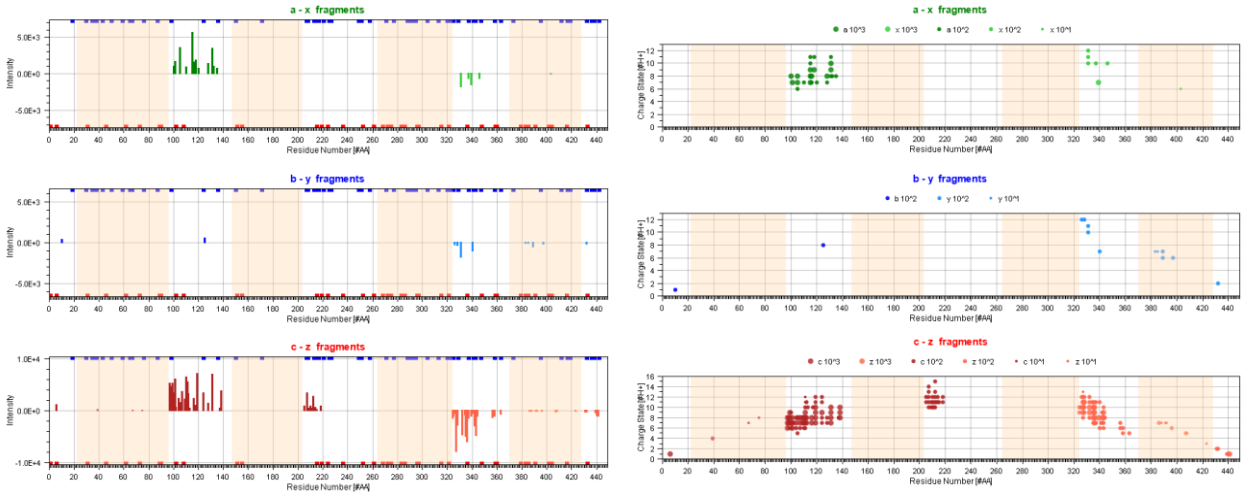


[M+49H]$^{49+}$ trastuzumab Hydrogen Ion Activation-Dissociation + Broadband Excitation

Assignments in PeakFinder

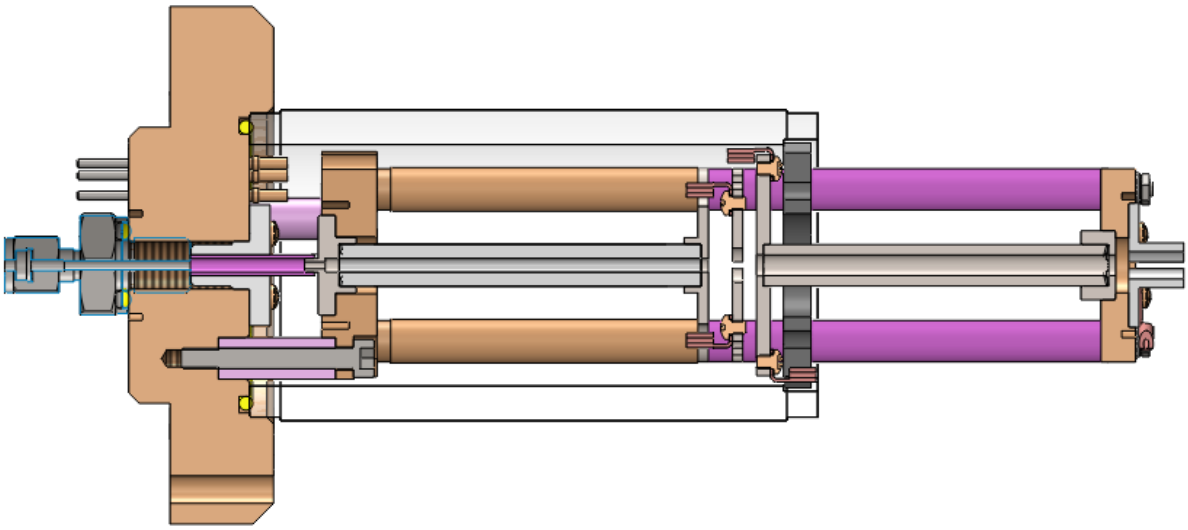


[M+49H]49+ Heavy Chain Fragments - Intensity and Charge State Distribution Plots



Appendix II

Test Version X



Test Version Z