Characterization of Intact Prostate Specific Antigen (PSA) and Its proteoforms by CESI-MS under Native and Denaturing Conditions.

Marcia R. Santos[1], Chitra K. Ratnayake[1], David M. Horn[2], Barry L. Karger[3], Alexander R. Ivanov[3], Rosa Viner[2]
[1]Sciex, Brea, CA, [2] Thermo Fisher Scientific, San Jose, CA, [3] Northeastern University, Barnett Institute of Chemical and Biological Analysis, Boston, MA

INTRODUCTION

Prostate Specific Antigen (PSA) is a ~30 kDa glycoprotein with one N-glycosylation site at Asn-69, secreted by the epithelial cells of the prostate gland. Traditionally, the most robust method to detect and quantify protein biomarkers is immunoaffinity analysis, despite the difficulties in validating antibodies and their possible cross-reactivity. In this work, we demonstrate a simple and fast method for the profiling of PSA proteoforms under native or denaturing conditions by using CESI-MS. Capillary Electrospray Ionization (CESI) is the integration of capillary electrophoresis (CE) and electrospray ionization (ESI) into a single process within the same device. CESI-MS operates at low nL/min flow rates offering several advantages including increased ionization efficiency and a reduction in ion suppression, see Fig. 1 A and B.
The limited choice of separation methods for intact proteins under native and denaturing conditions coupled with MS makes capillary electrophoresis an attractive approach for characterization of intact proteins and proteoforms. In this study we have detected more than 200 PSA proteoforms, using CE coupled with high sensitivity high resolution mass spectrometry (MS) compared to 127 proteoforms identified by direct infusion.

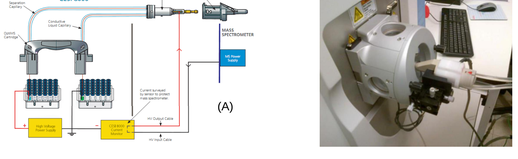
OVERVIEW

In this study we used a prototype hydrophilic neutral surface to enable the separation of proteoforms of PSA under both native and denaturing conditions aiming to identify and quantify how many proteoforms can be detected in comparison with direct infusion. The top 20 PSA proteoforms were identical in experiments performed under both native and denaturing conditions, where HexNac4Hex5dHex1NeuAc2 was identified as the most predominant glycan form agreeing nicely with ABRF Interlaboratory study from 2012 (1), which also revealed a total of 61 glycoforms were identified by bottom-up, top-down and PNGase F approaches. In our work, the combination of infusion (2) and CESI-MS experiments at both native and denaturing conditions resulted in identification and quantitation of 56 glycoforms. Total ion electropherograms acquired by CESI-MS performed under denaturing and native conditions resulted in detection of only 4-5 predominant peaks; however, the deconvolution analysis of our CESI-MS data under native conditions revealed a possible 236 proteoforms in contrast to 127 proteoforms identified by direct infusion. Under native conditions, due to the increased signal to noise ratio, we were able to identify additional proteoforms compared to the analysis under denaturing conditions. These hybrid proteoforms migrated noticeably later than the sialylated/complex glycan species.

METHODS

Sample Preparation:
Human PSA sample was purchased from Lee Biosolutions (St. Louis, MO) and diluted to 400 ng/µl in 10 mM ammonium acetate, pH 7.5 for native MS and into 10% acetic acid for all other experiments.

CESI-MS Conditions:
Intact PSA was separated using a CESI 8000 High Performance Separation and ESI Module (SCIEX) equipped with a prototype neutral coated capillary consisting of a porous sprayer operating in an ultra-low flow regime (**Figure 1A**), and analyzed on a LTQ Orbitrap Elite mass spectrometer (Thermo Fisher Scientific) at 120K resolution at *m/z* 400**.** Intact PSA under native conditions was analyzed on an Exactive Plus EMR (Thermo Fisher Scientific) at 140,000 FWHM resolution at *m/z* 200. **Figure 1B** shows the connection between CESI 8000 sprayer on Thermo's Nansource II.
Both types of separations (native and denaturing) were performed using a prototype neutral coated capillary. 10% acetic acid pH 2.2 and 40 mM ammonium acetate pH 7.5 were used as the background electrolytes for denaturing and native conditions, respectively. The data files were analyzed using Thermo Fisher Scientific Deconvolution 4.0 software.



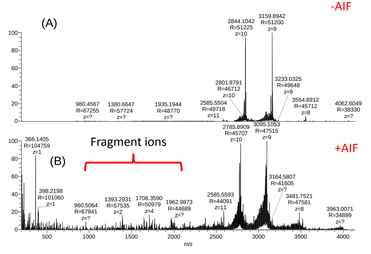
(B)

Fig. 1 A. Schematic representation of the CESI 8000 system. **B.** A Thermo Scientific™ nanospray source with an OptiMS sprayer adaptor

Direct Infusion under Native condition:
The protein solution 8 ng was directly infused at 20 nl/min via Advion nanochip into Exactive EMR instrument.

RESULTS

In first experiment we characterized the PSA sample by direct infusion. **Figure 2** shows the PSA analysis by direct infusion without (A) and with All Ion Fragmentation - AIF (B) using the Exactive Plus EMR. Fragment ions, mostly from N and C-termini generated by AIF enabled PSA identification. Observed b(red) and y(blue) ions are marked in Fig 2.C. Sequence coverage was only 10% due to several reasons:1) PSA contains 5 disulfide bonds; 2) analysis was done under native conditions. In this experiment we were able to detect and quantify 127 proteoforms by MS1.



(C)

IVGGWECEKHSQPWQVLVASRGRAVCGGVLVHPQWVLTAAH
CIRNKSVILLGRHSLFHPEDTGQVFQVSHSFPHPLYDMSLLKN
RFLRPGDDSSHDLMLLRLSEPAELTDAVKVMDLPTQEPALGTT
CYASGWGSIEPEEFLTPKKLQCVDLHVISNDVCAQVHPQKVTK
FMLCAGRWTGGKSTCSGDSGGPLVCNGVLQGITSWGSEPCA
LPERPSLYTKVVHYRKWIKDTIV

Fig. 2. PSA infusion analysis in native condition. A. MS1 spectrum. B. AIF spectrum of PSA. C. PSA sequence , identified b-ions are red and identified y-ions are blue.

CESI-MS under denaturing conditions

Figure 3A shows a separation of PSA at pH 2.2 of 10 % acetic acid as the background electrolyte and the corresponding ion density map (**Figure 3B**), illustrates the complexity of each peak. The amount of PSA injected was 4.2 ng representing only 1.6% of the total capillary volume. The base ion electropherogram clearly shows 4 predominant peaks nearly baseline resolved. However, the corresponding ion density maps (**Figure 3B**), raw and deconvoluted mass spectra (**Figure 4 A and B**) show numerous proteoforms and much higher complexity of the sample.
The analysis of the deconvoluted spectra (**Figure 4B**) corresponding to each CESI-MS peak (**Figure 4A**), showed the presence of over 202 proteoforms predominantly complex and hybrid structures. The data also indicates that the separation and migration order of PSA molecular species appears to be based on the hydrodynamic volume and charge of the proteoforms. In this experiment we confidently identified 36 glycoforms by HRAM MS1 spectra.

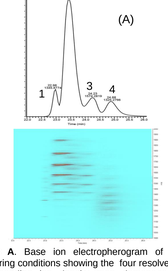


Fig. 3 A. Base ion electropherogram of PSA under denaturing conditions showing the four resolved peaks. **B.** Corresponding ion density map demonstrates the CE migration and m/z patterns of the detected proteoforms

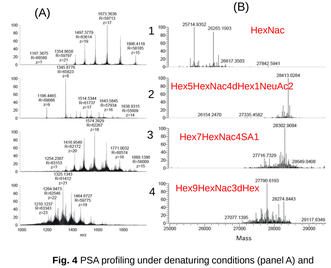


Fig. 4 PSA profiling under denaturing conditions (panel A) and corresponding deconvoluted spectra (panel B)

CESI-MS under native conditions

Figure 5A shows a separation of PSA using 40 mM ammonium acetate pH 7.5 as the background electrolyte and corresponding ion density map on **Figure 5B**. In this experiment the amount of PSA injected was 2.9 ng representing only 1.14% of the total capillary volume. The base ion electropherogram shows the presence of 5 main peaks.
The analysis of the deconvoluted spectra (**Figure 6B**), of each peak (**Figure 6A**) revealed the presence of over 236 proteoforms, with the main form, sialylated biantennary, eluting in peak 2. The overall separation profiles under native and denaturing conditions are very similar, strongly suggesting that this separation is based on the hydrodynamic volume and charge distribution of the protein isotope envelope. Under native conditions, we confidently identified 38 glycoforms by HRAM MS1 spectra.

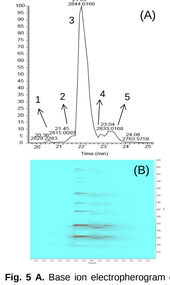


Fig. 5 A. Base ion electropherogram of PSA under native conditions, showing five distinct peaks. **B.** Density ion map of a separation of PSA under native conditions highlighting the CE migration patterns of proteoforms

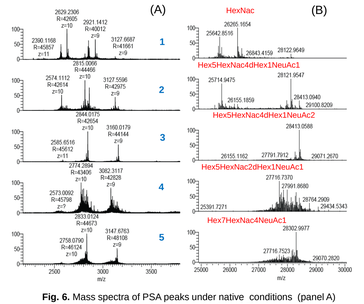


Fig. 6. Mass spectra of PSA peaks under native conditions (panel A) and corresponding deconvoluted spectra (panel B)

Venn diagram – Infusion, native and denaturing CESI-MS

The Venn diagram shown in **Figure 6** helps illustrate both common and unique glycoforms found among the three experiments. From the 56 identified main glycoforms, 19 species were common to the three approaches. By CESI-MS under denaturing and native conditions, we found 8 and 12 unique glycoforms, respectively, and with infusion, we detected 6 unique species. It is worth noting that even though we injected less PSA during the CESI-MS experiments we were able to detect more unique forms compared to infusion. However, all 3 approaches brought unique glycoforms to the picture. Additionally, no sample preparation was required other than dilution.

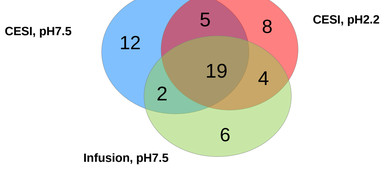


Fig. 6. Venn diagram showing the overlap between main PSA glycoforms observed at different conditions

Experiment	Amount Injected (ng)	# of proteoforms
CESI-MS Elite (denatured)	4.2	202
CESI-MS-EMR (native)	2.9	236
Infusion	8	127

Table 1. Injected amount in nanograms of PSA and number of proteoforms detected for each MS platform/experiment performed.

Quantitation of PSA proteoforms detected by CESI-MS compared to direct infusion

Figure 7 shows relative abundances of 15 main PSA glycoforms. All major glycoforms were consistently quantified in all 3 types of experiments , except Hex7HexNAc4Neu5Ac1 and Hex5HexNAc4dHex1Neu5Ac1 forms.

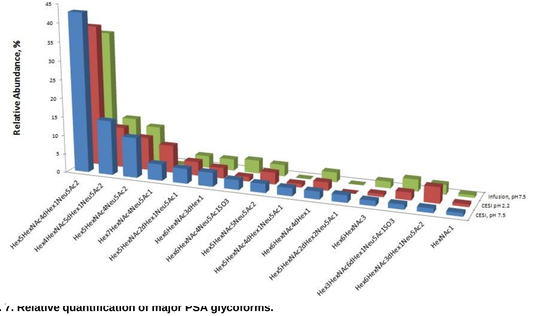


Fig. 7. Relative quantification of major PSA glycoforms.

Conclusions

- CE is a powerful technique for MS analysis of intact glycoproteins under both native and denaturing conditions.
- Prototype hydrophilic neutral coated capillary used in this work allowed for the separation of PSA proteoforms based on their structural heterogeneity.
- We demonstrated that CESI-MS under both native and denaturing conditions offer a significant improvement to the identification of glycoforms compared to a traditional technique as direct infusion.
- In this study, we confidently identified and quantified 56 PSA glycoforms via intact/top-down MS analysis.
- The amount loaded in the CESI-MS experiments were nearly half of the amount used in the infusion experiment and yet twice as many proteoforms were detected demonstrating the value of CESI as a front end separation/ionization for these molecules.
- In this work, we have shown that CESI-MS coupled with HRAM MS provides new and valuable information to established traditional approaches such as direct infusion ESI MS.

References

1. N. Leymarie et al, Mol Cell Proteomics. 2013 Oct; 12(10): 2935–2951.
2. R. Viner et al, From Qualitative to Quantitative: The Evolution of Glycoproteomics, HUPO 2013, Yokohama, poster 51

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