Using Electrokinetic injection to increase throughput and improve sensitivity in the detection of proteins by CE-MS

Stephen Lock[1], Christopher Loesnner[1], Esme Candish[2] and Edna Betgovargez[2]; [1]SCIEX, Warrington , UK and [2]SCIEX, Brea, California, USA

INTRODUCTION

Capillary electrophoresis (CE) is an orthogonal technique to LC separating compounds based on their charge. The properties of CE enable the reduction and often elimination of carryover and wall absorption which effects peak resolution and sensitivity of LC.

CESI (the integration of capillary electrophoresis (CE) and electrospray ionization (ESI) into a single process in a single device) are now enabling the easy connection of CE to mass spectrometers. In this work we will describe how a CESI-MS method has been developed to detect intact proteins between 15 -25,000 amu. We will compare electrokinetic versus pressure injections and discuss the factors that affect sensitivity of electrokinetic injection approach.

MATERIALS AND METHODS

Sample Preparation: Protein standards were made up to 1mg /ml concentration in water and diluted with an appropriate reconstitution solvent

Mass Spectrometry: For this development project a SCIEX QTRAP® 6500+ was fitted with the NanoSpray® III source. Gas 1 and 2 were not used and the temperature was set low (100 ºC) as ionization at these very low flow rates occurs by simply applying the ion-spray voltage. The curtain gas was set low (7 psi) and other MS conditions are shown in Figure 1 and 2. The MS method was split into 3 periods to cover the CE separation. In the first and last periods (1 minute each) the ionspray voltage was set to zero the middle period (13.5 minutes for electrokinetic and 32 minutes for a pressure injection) where the proteins were detected the ionspray voltage was set at 1800V

For Intact quantitation and protein detection the MS system was run in MRM mode for most of the target proteins this was typically a parent to parent transition (Figure 2) but for a IdeS cleaved antibody some dedicated product ions were available for LC and FC fragments shown in Figure 1.

The CE separation was dependent on the injection used and conditions are shown in Figure 3 and 4. Both methods used the same back ground electrolyte (BGE) which was 10% acetic acid other BGEs were not evaluated during this study. The conductive line solvent was 10% acetic acid and the capillary used was a neutrally coated capillary thermostatically controlled using recirculating liquid coolant at 25ºC.

Q1 Mass	Q3 Mass	Dwell (ms)	ID	DP (V)	CE (V)
1300.5	1511	150	IdeS LC1	220	15
1300.5	1322.1	150	IdeS LC2	220	15
1300.5	1341	150	IdeS LC3	220	15
1260.5	1316.1	150	IdeS FC1	220	15
1260.5	1307.8	150	IdeS FC2	220	15
1260.5	1369	150	IdeS FC3	220	15

Figure 1. MS conditions used for IdeS cleaved mAb fragments

Q1 Mass	Q3 Mass	Dwell (ms)	ID	DP (V)	CE (V)
848.2	848.2	200	MYO1	220	10
892.8	892.8	100	MYO2	220	10
942.4	942.4	100	MYO3	220	10
1669	1669	200	BetaLacto1	220	20
1836	1836	100	BetaLacto2	220	20
1530	1530	100	BetaLacto3	220	20
1430	1430	200	Lysozyme1	220	20
1589	1589	50	Lysozyme2	220	20
1589	1589	50	Lysozyme2	220	20

Figure 2. MS conditions used for intact test proteins.

Action	Time	Pressure (psi)	Direction	Voltage (kV)	Solution
Rinse	2.5 min	100	Forward	0	0.1 Molar HCl
Rinse	3 min	100	Forward	0	BGE
Rinse	0.75 min	75	Reverse	0	10% Acetic Acid
iTP injection	60s	5	Forward	10	Sample Vial
Separation	25 min	4	Forward and reverse ramp 2 minutes	25	BGE
Separation	2 min	100	Forward and reverse	10	BGE
Voltage Ramp down	2 min	10	Forward	1	BGE

Figure 3. The CESI method used for transient isotachophoresis (tITP) injections.

Action	Time	Pressure (psi)	Direction	Voltage (kV)	Solution
Rinse	2.5 min	100	Forward	0	0.1 Molar HCl
Rinse	3 min	100	Forward	0	BGE
Rinse	0.75 min	75	Reverse	0	10% Acetic Acid
EK plug	60s	0.5	Forward	0	Water or 50%Acetonitrile
EK injection	99s	0	Forward	10	Sample Vial
Separation	25 min	4	Forward and reverse ramp 2 minutes	25	BGE
Separation	2	100	Forward and reverse	10	BGE
Voltage Ramp down	2	10	Forward	1	BGE

Figure 4. The CESI method used for Electrokinetic injections.

Factors that effect sensitivity in CESI-MS detection of intact proteins

There are several factors that can have an impact on the sensitivity of any method developed for CESI-MS. One of the major concerns of most researchers in this area is the potential loading volume of CESI-MS which can be a couple of orders of magnitude lower than LCMS analysis. The first factor that reduces the impact of low loading volume is the fact that CESI-MS runs at flowrates typically over a 10,000 fold lower than conventional LC methods (10 -100 nL/min in CESI-MS compared to 100-1000 µl/min of LCMS methods). This dramatically lower flow rates improves de-solvation and ionization efficiency countering the effect of lower injection volumes.

To improve injection conditions further there are also injection modes which allow on-capillary focussing of proteins. Two typical ones used are a technique know as i transient isotachophoresis (tITP). In this approach the sample is prepared in a high ammonium acetate concentration (typically 50 - 250mM) and injected into a background electrolyte of acetic acid and a further small injection of acetic acid is placed behind the sample plug. This mode causing the proteins to stack up in a capillary and allows for larger injection plugs to be used. The second type of injection which can dramatically improve sensitivity is to inject a sample electrostatically by applying a voltage to the sample which causes ions of the same charge to move out of the sample into the capillary which contains a small plug of water. When the proteins move into the capillary in this way they stack up against the water : back ground electrolyte buffer junction. To decide on the most sensitive approach both these injection types were compared in Figure 5.

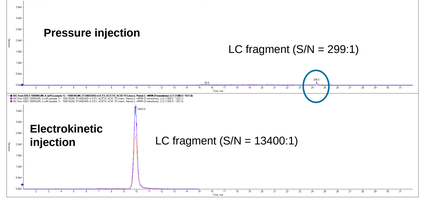

Figure 5. The effect of injection mode on a 25 kDa protein (an LC fragment of a mAb). In the top pane a 1 µg/mL standard containing 100 mM acetic acid was injected (60s, 5psi, 40 nL on capillary) which gave a signal to noise of 299:1. In the bottom pane the sample, in 75% Acetonitrile containing 0.05% acetic acid, in was injected by applying a voltage of 10kV to the sample for 99s this gave a signal to noise of 13400:1 (both peaks signal to noise were calculated automatically in the PeakView® software).

As the electrokinetic approach was over 40 times more sensitive the affect of sample solvent was investigated using model proteins of different isoelectric points.

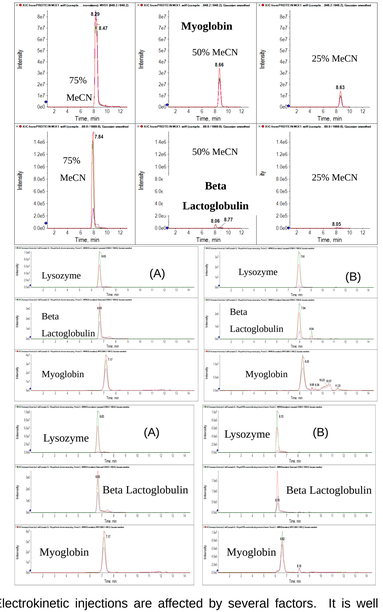

Figure 6. The effect of acetonitrile levels in the sample on two proteins (10 µg/mL) standards. The top pane shows the results for horse heart myoglobin (isoelectric point of 6.8) and the bottom pane is beta lactoglobulin (isoelectric point of 5.2).

Figure 7 The effect of the length of the focusing water plug in the capillary on the response of 3 different proteins at 10 µg/mL (Lysozyme, Beta Lactoglobulin and Myoglobin). Figure A is the result of a 60s,0.5 psi (4nL) water plug injected into the capillary before the electrokinetic injection and figure B uses a 60s, 5 psi (40 nL) water plug.

Figure 8. The effect of organic content of the focusing water plug in the capillary on the response of 3 different proteins at 10 µg/mL (Lysozyme, Beta Lactoglobulin and Myoglobin). Figure A is the result of a 60s,0.5 psi (4nL) water plug injected into the capillary before the electrokinetic injection and figure B uses a 60s,0.5 psi (4nL) water plug containing 50% acetonitrile

Electrokinetic injections are affected by several factors. It is well know that time and the voltage of an injection affect the flow of ions into the capillary and an increase in both increases the amount of ions injected so for these experiments we used the maximum permitted voltage and the same time for all injections (99s, 10kV). But what was unknown was the affect of organic content in the sample on the response observed so in figure 6 the amount of organic was varied and for all proteins a higher level of organic improved the response observed. The increase in response was different for different proteins and whether the response shifts with the isoelectric point or structure of the protein is still to be determined. Another factor that was investigated was the length of capillary focussing plug. What was shown in figure 7 was that if you increased the water plug volume 10 fold the response dropped and the peaks migrated more slowly to the MS detector with broader peaks observed. Similarly if you added acetonitrile to the water plug, as shown in figure 8, again the response dropped but this time the peaks migrated faster to the MS indicating with slightly sharper peaks which suggested that stacking had occurred but the protein transfer from the sample into the capillary was less.

Application of technique to IdeS cleaved antibody

To test the linear dynamic range a simply 3 orders of magnitude calibration line was tested ranging from a 0 – 1000 ng/ml concentration of protein, shown in Figure 9. In these preliminary tests we have also looked at sensitivity and carry over for the method Figures 10 and 11. What was observed that the CESI-MS respinse was linear over the range tested and the method provided excellent sensitivity with no carry over observed.

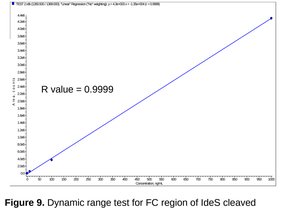

Figure 9. Dynamic range test for FC region of IdeS cleaved antibody

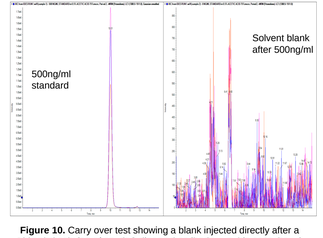

Figure 10. Carry over test showing a blank injected directly after a 500ng/ml solution of an IdeS digested antibody . In this case the LC fragment has been shown.

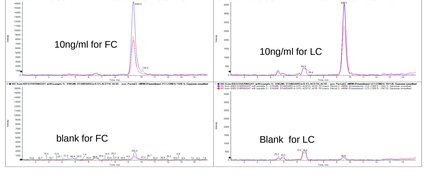

Figure 11. LOD assessment for LC and FC fragment of an IdeS cleaved antibody

CONCLUSIONS

- Electrokinetic injection of standards by CESI-MS was shown to be >40 times more sensitive than a pressure injection approach
- High levels acetonitrile in the sample were critical for low LODs for all proteins so far injected electrokinetically
- When linearity of this approach was tested using an IdeS cleaved mAb (0 – 1000 ng/mL) it was found to be linear and the resulting method produced very little carry over with excellent sensitivity.

.

TRADEMARKS/LICENSING

AB Sciex is doing business as SCIEX.

Document number: Related to RUO-MKT-10-6980-A