A covalent, cationic polymer coating method for the CESI-MS analysis of intact proteins and polypeptides

Key Benefits:

- Easy 3-step covalent coating procedure
- Efficient, robust, reproducible separation of intact proteins, using positively-charged coating
- High efficiency electrospray ionization in the low-nanoflow regime
- CESI capillary can be re-coated

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Introduction

Both capillary electrophoresis (CE) and mass spectrometry (MS) are powerful techniques for intact protein analysis, particularly for the characterization of biologics and, more specifically, therapeutic monoclonal antibodies (mAbs). While comprehensive LC-MS and CESI-MS characterizations of proteins performed using a bottom-up proteomics (Level 3 peptide mapping) strategy are common and provide a wealth of information about sequence and post-translational modifications (i.e. quality attributes), crucial information about intact protein forms can be lost during the digestion process. Top-down proteomics and Characterization Levels 1 (intact mAb) and 2 (reduced mAb) in the biopharmaceutical industry provide critical information about intact proteins using MS, but benefit from or require a separation step prior to electrospray ionization (ESI)-MS due to sample complexity. 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.

Thus, CESI-MS is ideally suited for top-down proteomics and Characterization Levels 1 and 2 of mAbs.

Intact protein separations when using LC columns or bare-fused silica capillaries are known to suffer from the adsorption and poor surface desorption characteristics of some proteins. While poor protein desorption from chromatographic materials can plague LC-MS analyses of intact proteins, surface coatings to suppress protein-wall interactions in CE have been applied with success. For example, a positively-charged polymer like polyethyleneimine (PEI) can be covalently attached to a capillary's inner wall to eliminate protein-wall interactions at low pH and facilitate highly efficient CE separations of proteins for MS analysis. In this case, the cationic polyethyleneimine (PEI) coating is attached to the capillary wall through covalent siloxane bonds similar to those commonly used for attachment of LC stationary phases to chromatographic resins, providing a stable coating for MS analysis.

This technical note describes the necessary steps to covalently attach PEI to the surface of a bare fused-silica CESI cartridge capillary. Additionally, a suitability test using protein standards as a means to evaluate capillary performance for the separation of intact protein will be described.

Materials and Methods

The coating procedure requires three steps: (1) pre-conditioning of the capillary surface, (2) coating the capillary surface with PEI , and (3) purge unreacted polymer using a post-coating wash. All three steps are conveniently performed on the CESI 8000 using provided instrument methods.

Reagents needed:

Reagent	Catalog no.	Vendor
Methanol 0.2 mm filtered, Fisher Scientific Optima	A454-1	Fisher Scientific
N-trimethoxysilylpropyl)polyehyleneimine (PEI) 50% in isopropanol	SSP-060-100gm	Gelest Inc.
5 M Sodium Chloride – 500 mL	59222C-500 mL	Sigma Aldrich
Protein Test Mix	477436	Sciex
Methanol (MeOH) anhydrous 99.8%	322415-100 mL	Sigma Aldrich

Supplies needed from third party vendors:

Materials
15 and 50 mL conical bottom tubes
Assorted pipettes and corresponding tips
Microcentrifuge Vials
3 mL syringe with needle
0.5 mL centrifuge vials

Supplies needed from Sciex:

Material	Catalog no.
CESI 8000 vials	B11648
CESI 8000 caps	B24699
OptiMS Silica Surface Cartridge	B07367

Instrument methods settings used

The instrument parameters used to run the various methods are described below. These methods must be created using the CESI 8000 control software.

Go to Method Menu > Instrument Set up. The Initial Conditions Tab (Fig. 1), shows a set up that is common to all methods. Figures 2-6 and 8 show the time program for each method.

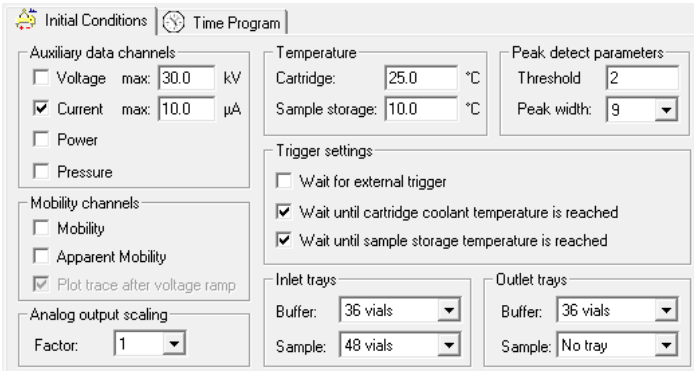
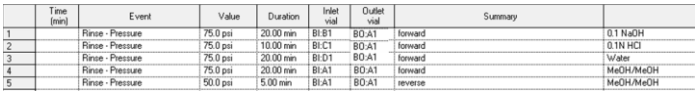


Figure 1. Initial Conditions tab

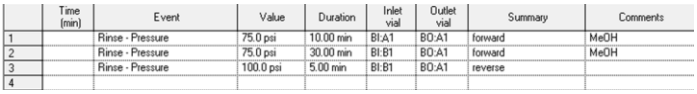


	Time (min)	Event	Value	Duration	Inlet vial	Outlet vial	Summary	
1		Rinse - Pressure	75.0 psi	20.00 min	BI:B1	BO:A1	forward	0.1 NaOH
2		Rinse - Pressure	75.0 psi	10.00 min	BI:C1	BO:A1	forward	0.1N HCl
3		Rinse - Pressure	75.0 psi	20.00 min	BI:D1	BO:A1	forward	Water
4		Rinse - Pressure	75.0 psi	20.00 min	BI:A1	BO:A1	forward	MeOH/MeOH
5		Rinse - Pressure	50.0 psi	5.00 min	BI:A1	BO:A1	reverse	MeOH/MeOH

Figure 2. Pre-conditioning method

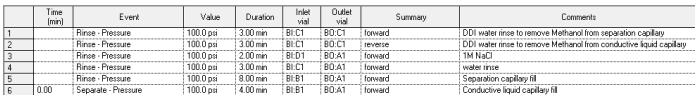
	Time (min)	Event	Value	Duration	Inlet vial	Outlet vial	Summary	Comments
1	0.00	Separate - Pressure	75.0 psi	30.00 min	BI:A2	BO:A1	forward	PEI coating
2								

Figure 3. Overnight coating method



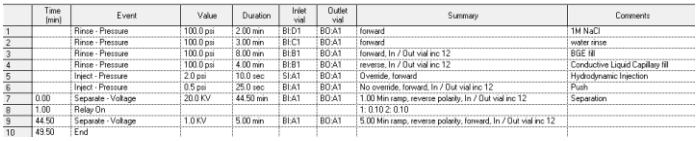
	Time (min)	Event	Value	Duration	Inlet vial	Outlet vial	Summary	Comments
1		Rinse - Pressure	75.0 psi	10.00 min	BI:A1	BO:A1	forward	MeOH
2		Rinse - Pressure	75.0 psi	30.00 min	BI:B1	BO:A1	forward	MeOH
3		Rinse - Pressure	100.0 psi	5.00 min	BI:B1	BO:A1	reverse	
4								

Figure 4. Post-coating purge method



	Time (min)	Event	Value	Duration	Inlet vial	Outlet vial	Summary	Comments
1		Rinse - Pressure	100.0 psi	3.00 min	BI:C1	BO:C1	forward	DDI water rinse to remove Methanol from separation capillary
2		Rinse - Pressure	100.0 psi	3.00 min	BI:C1	BO:C1	reverse	DDI water rinse to remove Methanol from conductive liquid capillary
3		Rinse - Pressure	100.0 psi	2.00 min	BI:D1	BO:A1	forward	1M NaCl
4		Rinse - Pressure	100.0 psi	3.00 min	BI:C1	BO:A1	forward	water rinse
5		Rinse - Pressure	100.0 psi	8.00 min	BI:B1	BO:A1	forward	Separation capillary fill
6	0.00	Separate - Pressure	100.0 psi	4.00 min	BI:B1	BO:A1	forward	Conductive liquid capillary fill

Figure 5. Capillary conditioning method



	Time (min)	Event	Value	Duration	Inlet vial	Outlet vial	Summary	Comments
1		Rinse - Pressure	100.0 psi	2.00 min	BI:D1	BO:A1	forward	1M NaCl
2		Rinse - Pressure	100.0 psi	3.00 min	BI:C1	BO:A1	forward	water rinse
3		Rinse - Pressure	100.0 psi	8.00 min	BI:B1	BO:A1	forward, In / Out vial inc 12	BGE fill
4		Rinse - Pressure	100.0 psi	4.00 min	BI:B1	BO:A1	reverse, In / Out vial inc 12	Conductive Liquid Capillary fill
5		Inject - Pressure	2.0 psi	10.0 sec	SI:A1	BO:A1	Overside, forward	Hydrodynamic Injection
6		Inject - Pressure	0.5 psi	25.0 sec	BI:A1	BO:A1	No overide, forward, In / Out vial inc 12	Push
7	0.00	Separate - Voltage	20.0 KV	44.50 min	BI:A1	BO:A1	1.00 Min ramp, reverse polarity, In / Out vial inc 12	Separation
8	1.00	Relay On					1: 0.10 2: 0.10	
9	44.50	Separate - Voltage	1.0 KV	5.00 min	BI:A1	BO:A1	5.00 Min ramp, reverse polarity, forward, In / Out vial inc 12	
10	49.50	End						

Figure 6. Capillary performance separation method

Note: To avoid premature capillary failure, it is strongly recommended that every voltage separation step is followed by a voltage ramp down with a 5 psi forward pressure. To set up this step properly ensure the separation step window is set up as shown in Fig. 7.

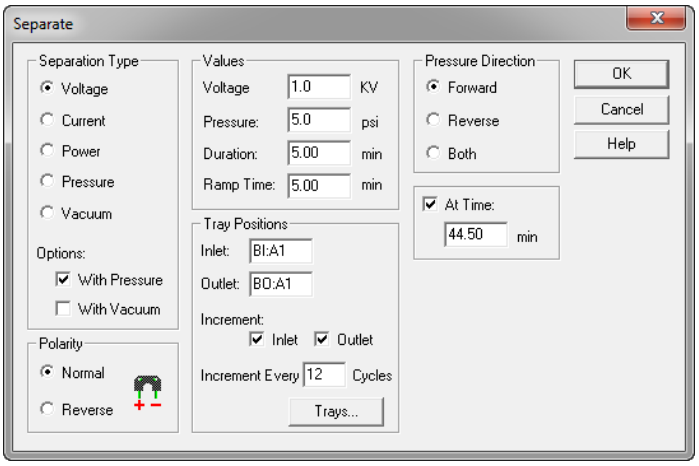


Figure 7. Separate step settings used in the ramp down of the separation method.

	Time (min)	Event	Value	Duration	Inlet vial	Outlet vial	S
1		Rinse - Pressure	75.0 psi	5.00 min	BI:C1	BO:C1	reverse
2		Rinse - Pressure	100.0 psi	10.00 min	BI:C1	BO:C1	forward
3		Rinse - Pressure	75.0 psi	5.00 min	BI:F1	BO:F1	reverse
4		Rinse - Pressure	100.0 psi	10.00 min	BI:F1	BO:F1	forward
5		Rinse - Vacuum	5.0 psi	30.00 min	BI:F2	BO:F2	reverse
6	0.00	Separate - Pressure	50.0 psi	5.00 min	BI:F2	BO:F2	reverse

Figure 8. Capillary storage/shutdown method

Reagents and Sample Preparation

1M Sodium Chloride (NaCl) - Positively coated capillary cleaning solution

High concentration sodium chloride is used to aid in the cleaning of the coated surface of anionic species present in the sample that may have adsorbed onto the surface. The mechanism for the removal of anionic contaminants from the cationic PEI surface is similar to anion exchange chromatography. The NaCl is removed from the capillary by subsequent water and BGE rinses. The NaCl solution can be prepared by direct dilution of the 5M stock solution. Dispense 2 mL of the 5M stock solution into a suitable flask and add 8 mL of DDI water. This can be kept refrigerated for up to 6 months.

Preparation of the BGE solution:

Step 1. Prepare 100 mM ammonium acetate (NH_4Ac) solution by diluting the stock solution of 7.5 M NH_4Ac. Dispense 666 µL of 7.5 M ammonium into a 50 mL volumetric flask and add DDI water for a final volume of 50 mL. This can be kept refrigerated for up to 6 months.

Step 2. Prepare 100 mM acetic acid (HAc) solution by diluting the glacial acetic acid. Dispense 287 µL of HAc into a 50 mL volumetric flask and complete to 50 mL with DDI water. This can be kept refrigerated for up to 6 months.

Step 3. Adjust the pH of the HAc solution with the NH4Ac solution to pH 3.1. This can be kept refrigerated for up to 6 months.

Preparation of system suitability stock solution:

The system suitability sample is a mixture of 3 proteins (Cytochrome C, Lysozyme, Ribonuclease A) at a total protein concentration of 3 mg/mL. The sample is provided lyophilized as a combination of 1 mg of each protein. Add 1.0 mL of DDI water to the vial and mix well to reconstitute the system suitability sample. This can be kept refrigerated for up to 1 month.

Preparation of system suitability sample (for injection):

Aliquot 10 µL of system suitability protein mix into a microcentrifuge vial and add 10 µL of 100 mM NH_4Ac pH 3.1 and 80 µL of DDI water and finger flick for proper mixing. This mixture results in 0.3 µg/µL of protein mix at a total volume of 100 µL in 10 mM NH_4Ac.

Description of the Coating Process

The coating process is best performed overnight since the coating reaction is complete only after 12 hrs. Therefore it is strongly recommended to begin the pre-conditioning method at least an hour before the end of your day. The post coating purge wash can be started the next day.

Step #1: Capillary pre-conditioning

In preparation for the coating, the silica surface must be cleaned of any impurities that may be present on the surface. The capillary surface preconditioning is comprised of a method in which the capillary undergoes a series of rinses with NaOH and HCl followed by water and MeOH. These steps serve to open siloxane groups and to deprotonate (during rinses with NaOH), protonate (during rinses with HCl), and wash (rinse with water and MeOH).

For this step, 1.5 mL of 0.1 N NaOH, 0.1 N HCl, DDI water and MeOH are dispensed in the CESI vials and capped. The vials must be placed in the appropriate position as described in Fig. 9.

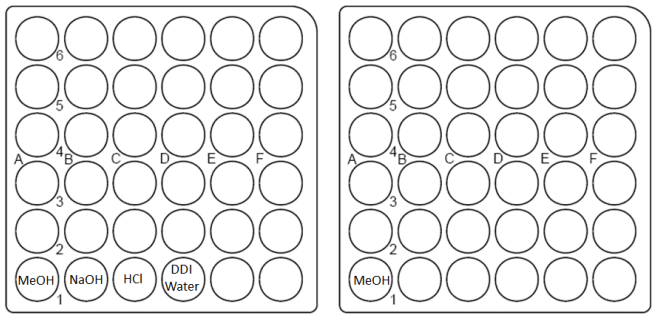


Figure 9. Reagent vials layout for capillary pre-conditioning procedure

Prepare two 50 mL conical bottom tubes each containing 4 mL of DDI water and 4 mL of filtered MeOH. Set the vial containing MeOH aside. Place the vial containing DDI water into the holster on the right side panel of the CESI 8000 by screwing it in until it is snug. Remove the protective sleeve from the sprayer housing and rest it in the holster so the sprayer tip is immersed in the DDI water. Be careful not to expose the tip.

Important Note: Throughout the entire coating procedure, the porous sprayer must be immersed in either DDI water or MeOH depending on the step. During step 1 or capillary pre conditioning, the sprayer end must be immersed in 4 mL of DDI water for the first 50 minutes of the method. Once the rinse with DDI water has completed, and the MeOH rinse has begun, remove the 50 mL tube containing DDI water from the holster, carefully and gently dab the sprayer housing dry with a tissue paper, and install the 50 mL tube containing MeOH into the holster. There is no need to stop or pause the method while switching tubes. **Under no circumstances should you touch the sprayer tip.**

Step #2: Capillary coating

After the capillary surface has been properly conditioned and activated, it is ready to be covalently coated with PEI. This method will rinse the capillary for only 30 minutes; however the polymer solution should remain inside the capillary overnight to complete the reaction.

Preparation of the coating solution requires a clean working area free of dust. If a gas line with dry Nitrogen, Helium, or Argon is available, have clean stainless steel tubing on hand to draw the methanol from the bottle. If gas lines are not available, set aside a 3 mL syringe with clean needle.

Draw 1.5 mL of methanol by piercing the septum of the anhydrous methanol bottle and dispense it into a 15 mL conical bottom tube and cap immediately. Using the stainless steel needle, fill the head space of the anhydrous methanol bottle with dry, inert gas (N, He, or Ar). If inert gas is not available, cap the bottle immediately and place it into a desiccator for storage. It is important to perform this step to keep the MeOH anhydrous.

Aliquot 300 µL of the PEI solution and dispense into the tube containing the anhydrous MeOH. The PEI solution is viscous and will deposit at the bottom of the vial and it may also adhere to the pipette wall. Pipette up and down repeatedly to ensure the polymer from the tip is dissolved into the methanol. Cap the tube and vortex vigorously until all of the PEI is dissolved into the MeOH. This may take several minutes.

TIP: If the PEI solution is kept refrigerated, it will be extremely viscous and drawing with a pipette tip will be very difficult. Simply cut the tip of the pipette tip (about 1 cm) with a clean razor blade prior to drawing the polymer solution.

Important note: The PEI solution must be prepared fresh every time and no more than 15 minutes prior to coating.

Dispense 1.5 mL of the coating solution into a CESI vial. Cap the vial and place it in the A2 position of the buffer inlet tray into the CESI 8000 instrument. Discard the remaining PEI solution.

Dispense 4 mL of filtered MeOH into a new 50 mL conical bottom tube and install this tube in the holster. It is important for the end of the sprayer to be resting in fresh, clean MeOH.

Start the coating method shown in Fig.3.

Following the overnight incubation period, the capillary is covalently coated with the cationic polymer and any unreacted PEI must be removed. To avoid unwanted carry-over of unreacted PEI into separation methods, it is strongly

recommended to clean up the instrument interface before proceeding with the post coating purge method.

Steps for instrument clean up:

1. Remove all previously used vials and discard appropriately. Place a fresh 50 mL tube of methanol into the holster to keep the porous tip immersed at all times to avoid it from drying out or plugging.
2. Carefully remove the CESI *inlet* vial ejector by pulling it straight downward to expose the capillary and electrode for cleaning.
3. Using a lab tissue soaked with methanol, gently wipe the inlet capillary and inlet electrode clean of PEI.
4. Remove the cartridge from the interface block and rest it on the transport rails (Fig 10). Clean the underside of the inlet interface block.

Important Note: ALWAYS remove the cartridge BEFORE removing the electrode. Failure to do so will break the separation capillary.

5. Remove the CESI electrode using the electrode removal tool.
6. Place both electrode and vial ejector in a beaker containing methanol (MeOH does not need to be high quality grade). Sonicate the beaker for 10 minutes.
7. Dry the electrode and ejector and re-install them into the CESI 8000.
8. Re-install capillary cartridge.

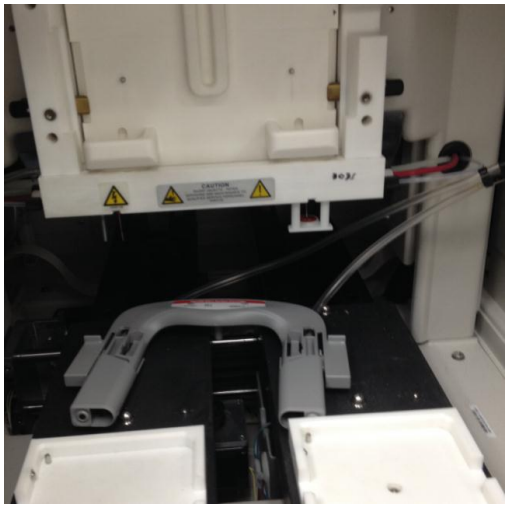


Figure 10. Rest the cartridge over the rails while keeping the porous tip submerged in methanol.

The instrument should now be cleaned of residual PEI and is ready for the post-coating purge wash step.

The post-coating purge procedure consists of a series of methanol rinses which allows for the removal of any unreacted PEI from inside the capillary.

Dispense 1.5 mL of filtered MeOH into 3 CESI vials and cap them. Dispense 4 mL of filtered methanol into a 50 mL conical bottom tube. Install the tube on the holster of the CESI 8000 and place the newly prepared CESI vials into the buffer trays as shown in the Fig. 11.

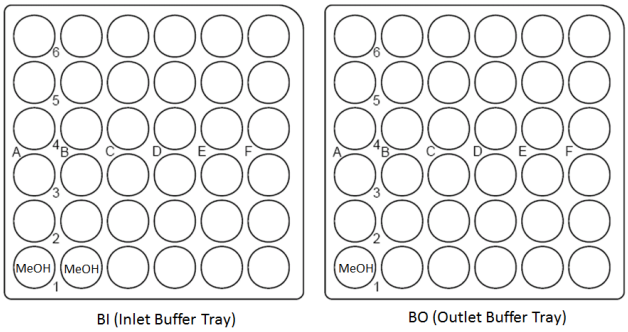


Figure 11. Reagent vials positioning for capillary post purge procedure

This step completes the coating and the capillary is now ready to be conditioned and then tested on the CESI 8000.

Notes:

- If the capillary isn't used right away, it can be stored using the storage/shutdown method provided in Fig. 8.
- When not in use, the PEI capillary must be stored dry.
- ***Always*** use vacuum to dry the separation capillary as shown in the storage/shutdown method in Fig. 8.

Conditioning of the positively-coated capillary

To obtain the best performance from the PEI capillary, it is necessary to condition prior to running a test sample. The conditioning is a simple procedure, consisting of applying -20 kV for 20 min. The optimum electrospray voltage is also determined at this point. For this procedure both separation and conductive liquid capillaries must be filled with BGE 100 mM NH_4Ac pH 3.1. Preparation of the BGE is detailed in a later section.

With the protective sleeve removed from the sprayer housing, install the sprayer into the adapter on the MS source.. Dispense 1.5 mL of the BGE, 1M NaCl, and DDI water into their respective CESI 8000 buffer vials and cap them properly. Place the vials in the proper positions as indicated in Fig.12.

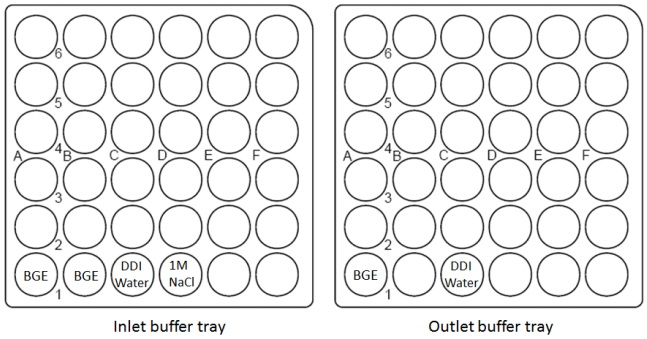


Figure 12. Layout of reagent vials required to condition and test performance of PEI-coated capillary

Use the capillary conditioning method as shown in Fig. 4. Upon completion of the conditioning method, align the tip of the sprayer with your specific mass spectrometer as described in the User Manual or Application Guide. Using the direct control screen in 32 Karat, apply 20 kV and reverse polarity with a ramp time of 1 min for a duration of 60 minutes (Fig. 13). Program the MS-ESI voltage to start with the 0 kV and increase slowly until spray is observed. Optimize the position of the tip and the spray voltage until a minimum ESI voltage needed for stable spray is determined. Allow the capillary to spray for 20 min to complete conditioning.

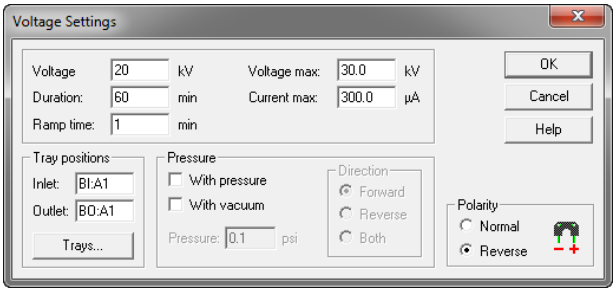


Figure 13. Direct Control settings for PEI capillary coating conditioning

Note: Confirm your ESI spray conditions in the last conditioning step with the information in the User Manual as it may be dependent on your MS instrument model.

Testing the PEI-coated capillary

Dispense 100 µL of the system suitability sample into a sample vial. Place the sample vial into the CESI buffer vial and cap it. Place the CESI vial into position A1 of the inlet sample tray.

The separation method used to test the performed is shown in Fig. 5.

Dispense 1.5 mL of the BGE, 1M NaCl, and DDI water into the CESI 8000 buffer vials and cap them properly. Place the vials in the proper buffer tray positions as indicated in Fig.12.

Important Notes:

- Because the surface of this capillary is positively charged, the electroosmotic flow (EOF) generated when applying voltage in *normal polarity* would flow in opposite direction relative to the MS. For this reason the separation method is done under *reverse polarity* so EOF now flows towards the MS. Reverse polarity is programmed into the CESI method provided for PEI-based separations.
- The separation step in the method (line 9 of the method shown in Fig.6) is for a voltage ramp down. Please ensure this step is performed with 5 psi forward pressure. The voltage ramp down is programmed into all of the CESI separation methods.
- To ensure a stable spray throughout the separation, always add 100 V to the minimum ESI voltage found during the capillary conditioning procedure for the MS acquisition method.

Fig. 14 shows the expected base peak electropherogram and corresponding spectra.

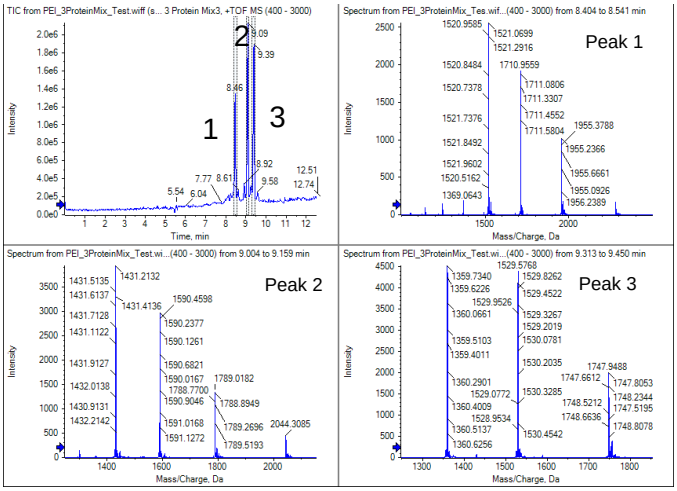


Figure 14. Base peak electropherogram and spectra expected for the three protein mix separated using a PEI-coated capillary. Peak 1 is Lysozyme, peak 2 is Ribonuclease A and peak 3 is Cytochrome C.

Storage of the positively-coated capillary

Capillary storage following use requires running the shutdown method as shown in Figure 8. Dispense 1.5 mL of DDI water and filtered MeOH into the CESI 8000 vials and cap them. Place the vials in the buffer tray positions shown in Fig.15.

Note: It is not recommended to set up the shutdown method following a long batch run as the methanol may evaporate before completion. To circumvent this, add or fill the methanol vial immediately before the shutdown method is run.

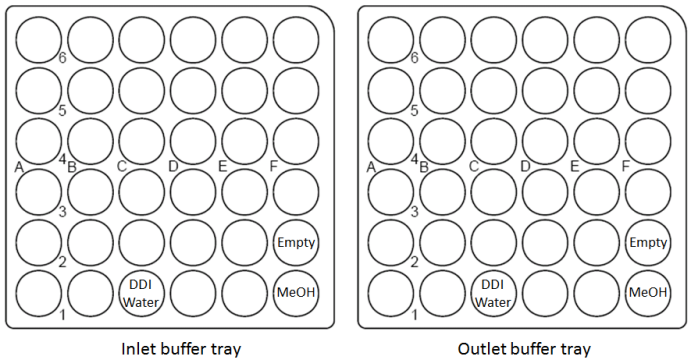


Figure 15. Vial layout used in the shutdown method

Conclusion

This technical note describes a simple method for coating bare fused-silica OptiMS capillaries for intact protein and polypeptide separation applications. Use of the PEI coating creates a robust and reproducible means to eliminate protein adsorption onto the capillary surface when performing CESI-MS. The covalent nature of the siloxane-linkage allows for very stable PEI coating at acidic pH. The coating can be stripped off the surface and recoated by using the same procedure described in this note. When used in CESI-MS, the PEI coating procedure provides a powerful tool for analysis of intact or complex protein cohorts associated with biotherapeutic characterization or top down proteomics.

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