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Short Communication

Coupling imaged capillary isoelectric focusing with mass spectrometry using a nanoliter valve

Imaged capillary isoelectric focusing (iCIEF) is a powerful separation technique applied frequently for the analysis of biotherapeutics. However, direct mass spectrometric characterization is usually not possible. Here, focused peaks from an iCIEF system are transferred to a four-port nanoliter valve where peaks of interest are cut and transferred directly (flow injection) or via capillary zone electrophoresis to electrospray mass spectrometry. At first, flow injection coupling was tested with a mixture of peptides showing intraday precision (RSD) of 20.0 and 3.4% in area and 15.2 and 6.3% in intensity for angiotensin I and leucine-enkephalin, respectively. For the analysis of charge variants in an intact mAb, flow injection and CZE as second dimension were compared, demonstrating the usability of flow injection even for large proteins. However, improved spectra quality was achieved when CZE-MS was applied. In this way, accurate masses were obtained not only for the main isoform, but also for the main acidic and basic variants. These results demonstrate the power of iCIEF-CZE-MS for the analysis of biotherapeutics.

Keywords:
Antibody analysis / Capillary zone electrophoresis–mass spectrometry / Charge variants / Imaged capillary isoelectric focusing / Two-dimensional
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Based on the high resolving separation by their p*I*, IEF is an essential technique for the separation of proteins and peptides out of complex biological matrices or in biopharmaceutical samples. From conventional slab-gel IEF to the capillary format, this separation mode has evolved during years and has become a decisive analytical technique for product characterization, comparability studies and to monitor charge heterogeneity of therapeutic proteins [1–4]. Control of the charge heterogeneity of mAbs is highly important due to the potential impact of these variants on its biological activity [5].

Beyond separation and quantification, there is a great need for unequivocal identification of these variants. The evident benefits of CIEF and MS coupling regarding the high resolving power of p*I*-based separations and the unequivocal MS identification of individual peaks have promoted several investigations [6]. Our research group has previously shown the value of two-dimensional systems using standard CE instrumentation for CIEF, CZE-MS as second dimension and a nanoliter valve for the coupling [7, 8]. The four-port valve (VICI AG International, Schenkon, Switzerland) allows the direct coupling of two capillary-based techniques with high flexibility [9].

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Imaged CIEF (iCIEF) was introduced in 1992 [10] to avoid possible peak broadening during mobilization required by the single point detection of standard CIEF. By continuous detection over the whole capillary, iCIEF facilitates method development and allows studying of protein interaction and protein precipitation. CEInfinite (Advanced Electrophoresis Solutions Ltd., Cambridge, ON, Canada) recently introduces an iCIEF preparative equipment that incorporates the possibility of mobilization after focusing for fractionation and coupling with MS. By transfer of zones or peaks from the separation cartridge (200 or 320 μm id, 5 cm length, proprietary acrylamide coating for preparative iCIEF and iCIEF-MS, UV detection at 280 nm) to a transfer capillary with small id (50 μm), the resolution can be maintained.

In our setup (Fig. 1), iCIEF is connected by the transfer capillary to the nanoliter valve as interface for online coupling with MS. The valve allows cutting specific peaks and transporting them to the MS, reducing ESI interference and MS contamination derived from methylcellulose or carrier ampholytes from the CIEF. After peak focusing, the mobilization is performed by switching the 6-port valve of the CEInfinite and applying a defined flow with a syringe pump. During this time, the voltage is kept to help maintaining the resolution. The 50 μm id transfer capillary (approx. 11 cm length, fluorocarbon-based coating) from the iCIEF is connected to one-port of the nanoliter valve. A short 50 μm id capillary (fused silica, Polymicro Technologies, AZ, USA) is connected to the complementary port of the valve and connected to a waste vial. The time the peak of interest needs

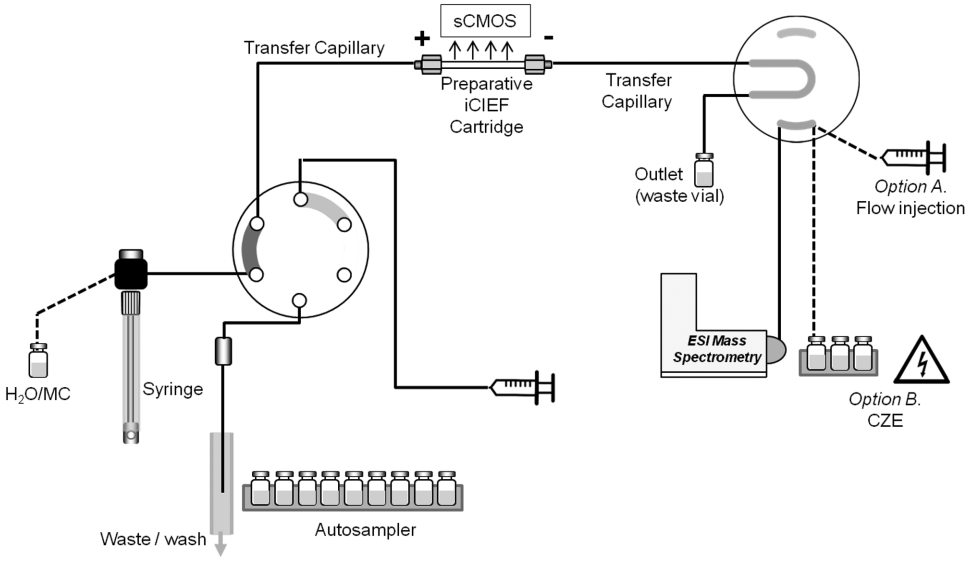

Figure 1. Schematic representation of the 2D system. Dashed lines represent the two options to transport the peak toward the MS: *Option A* flow injection and *Option B* CZE.

to arrive to the nanoliter valve is calculated from the exact distance between the end of the cartridge and the middle of the sample loop in the valve based on the defined speed of the syringe (approx. time 5 min).

When the iCIEF peak is in the sample loop, the valve is switched to position B where the loop is in the second dimension. PVA-coated capillaries [7] are connected to the valve for the second dimension. In this work, two different ways to transport the peaks from the sample loop to the MS were tested: flow injection (FI) (Fig. 1, *Option A*) or CZE (Fig. 1, *Option B*). For FI, a syringe pump (Cole-Parmer®, IL, USA) with 5 mL syringe (5MDF-LL-GT, SGE Analytical Science, Melbourne, Australia) is used to push the peak from the valve toward the MS. The connection to the MS is done by a micro-LC sprayer (G1946-67260, Agilent Technologies, Waldbronn, Germany) to fit with the flow delivered from the syringe pump (1 μL/min).

To initially test the coupling of iCIEF with MS, FI was chosen due to the simplicity of the approach. Before sample injection, the capillary was rinsed with water and with 0.5% methylcellulose to completely suppress the possible residual EOF. The catholyte and anolyte contained 0.1% methylcellulose with 100 mM sodium hydroxide and 80 mM phosphoric acid, respectively. A standard peptide mixture with Angiotensin I (p*I* 7.9) and Leucine-enkephalin (p*I* 5.5) with 2% AESlyte (pH 4–8) and 70% methylcellulose (0.5%) was injected hydrodynamically with pressure from a syringe. All carrier ampholytes, methylcellulose, and electrolytes were supplied by Advanced Electrophoresis Solutions Ltd. Figure 2 shows an example of the complete iCIEF-FI-MS workflow with the image from the end of the focusing time and the base peak obtained in the MS after cutting Angiotensin from the first dimension. Beside the peptide, methylcellulose can be observed with high intensity. To test the repeatability of the complete process, both peptides were cut in the same run in a multiple heart-cut approach. The RSD in peak area was 20.0 and 3.4% and in peak intensity 15.2 and 6.3% for Angiotensin I and Leucine-enkephalin, respectively, with the higher value probably due to the smaller peak width of Angiotensin and potential low cutting accuracy. The coupling of the preparative CEInfinite with the nanoliter valve and the ESI-MS was simple and uncomplicated and MS spectra of iCIEF separated peaks were easily obtained. However, clogging of the micro-LC sprayer occurred frequently after some runs, most likely due to the transfer of high amounts of methylcellulose and ampholytes from the IEF.

The iCIEF-FI-MS system was then tested by a hemoglobin solution containing four variants (HbA, HbS, HbF and HbC), being identical in the α-subunit but differing in the β-subunit. For all four iCIEF-separated variants, characteristic mass spectra were obtained using multiple heart-cutting (data not shown). Unequivocal assignation of the variants was complicated due to slight carry over and bubble formation, observed several times for this sample. The latter prevents proper cutting due to change of the velocity during mobilization.

Characterization of the main charge variants from Trastuzumab (Herceptin® from F. Hoffmann-La Roche Ltd.) was selected to test the potential of the coupling of iCIEF with MS by using the nanoliter valve. Figure 3B shows an example of the image for the mAb after the focusing step. The profile of Trastuzumab is in good agreement with the literature showing one small basic variant and a main acidic variant [4]. By using the iCIEF-FI-MS system, the main variant was cut and analyzed by MS (peak M, Fig. 3A**)**. It was possible to obtain meaningful mass spectra with good signal intensity; however, accurate mass determination is not provided due to the poor peak shape. Furthermore, methylcellulose from the sample and from the washing steps can be observed in the range from 1500 to 2500 *m/z* in Fig. 3A.

To improve mass spectra quality and to separate the methylcellulose from the peak, a CE equipment

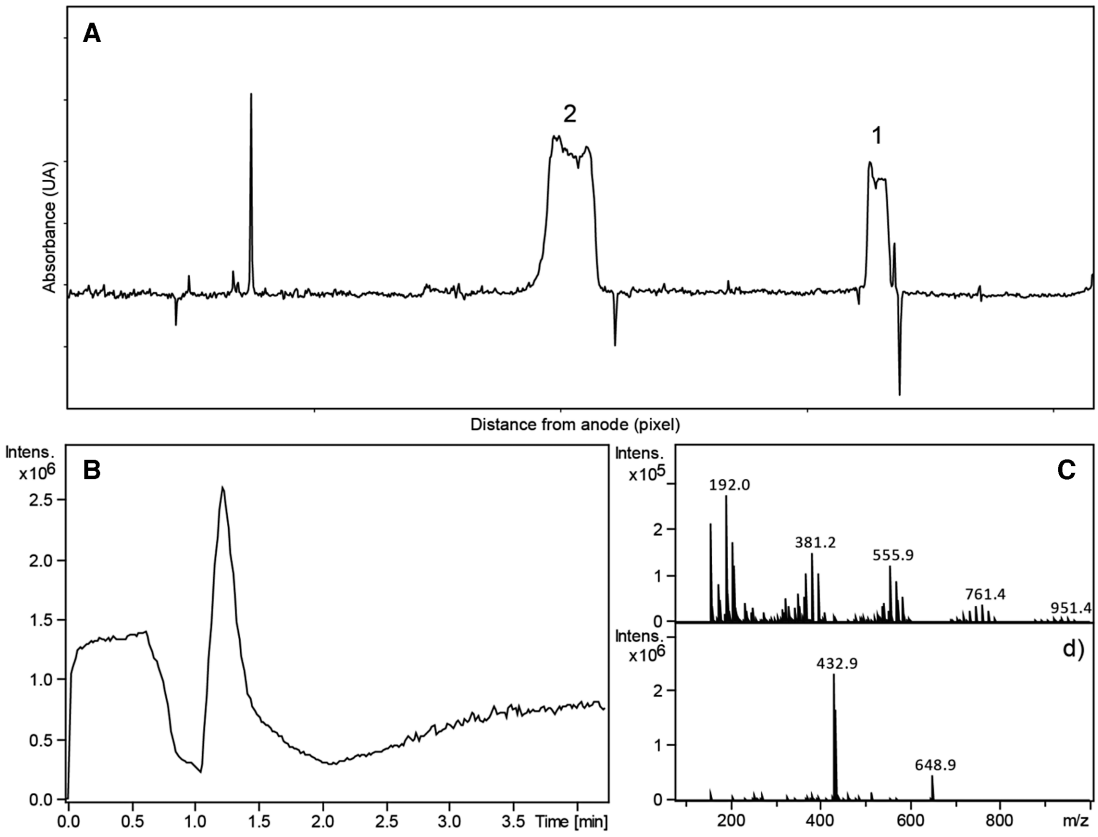

Figure 2. Workflow iCIEF-FI-MS (*Option A*) for a mixture of Angiotensin I (1) and Leucine-enkephalin (2) with (A) image after the focusing time in the iCIEF, (B) base peak from the cut of Angiotensin, (C) spectrum before the peak with methylcellulose, and (D) spectrum of the Angiotensin peak. Sample: 5% Angiotensin I and Leucine-enkephalin (50 μg/mL), 2% AESIyte (pH 4–8), 70% methylcellulose (0.5%). Focusing: 1 kV (1 min), 2 kV (1 min), 3 kV (6 min), interval time 15 s. Mobilization: 0.120 μL/min, 3 kV (25 min), 15 s interval time. FI-MS: 1 M HAc, 1 μL/min, micro-LC sprayer. MS conditions: positive polarity, 200–2000 *m/z*, 4500 V, dry gas 4.0 l/min, dry temperature 170°C.

(Agilent Technologies, Waldbronn, Germany) was placed as second dimension (Fig. 1, *Option B*). The connection with the valve was done in the same way using 50 μm PVA coatedcapillaries. For the MS connection, the micro-LC sprayer was exchanged for the Agilent CE-ESI-MS sprayer (G1607A, Agilent Technologies). Figure 3C shows the spectra obtained for the main variant M cut from a sample with the same composition as in Fig. 3A. For the most abundant glycoform G0F/G1F averaged mass of 148 221.1 ± 0.2 ($n = 3$) was determined. By introducing the separation step in the CZE, no methylcellulose is observed anymore in the raw spectra and the quality of the spectra is improved (compare Fig. 3C to 3A).

The variants in the iCIEF profile were also analyzed with the iCIEF-CZE-MS system using 320 μm id cartridges where high amounts of sample can be loaded to achieve sufficient sensitivity for these low abundant analytes. Figure 3D shows the mass spectrum cutting the most intensive acid variant (peak A, 3B). A slightly higher mass compared with the main variant was observed (148 222.5 ± 0.2, $n = 2$), which corresponds to a shift of +1.4 Da. This charge variant can be most likely attributed to deamidation, which is a known modification for Trastuzumab [11] and has been previously characterized by CZE-CZE-MS [12]. In the last-mentioned work the basic variant could not be characterized due to its lower abundance, similar to a recent work where CIEF is directly coupled with MS [13]. Here, the basic variant (peak B, Fig. 3B) was analyzed using the iCIEF-CZE-MS setup applying a sample concentration of 6.0 mg/mL (Fig. 3E). The deconvolution resulted in an exact mass of 148 201.3 ± 1.8 ($n = 3$). This shift of −19.8 Da compared with peak M, in combination with the shift in the p*I* value, can be explained by succinimide formation (−17 Da) or partial cyclisation of N-terminal glutamic acid (−18 Da), modifications forming basic species and occurring for this mAb [11, 14].

In conclusion, the CEInfinite preparative system in combination with the nanoliter valve as interface efficiently provides MS characterization of iCIEF separated peptides and proteins. Compared to our previous coupling with MS with the same nanoliter valve using conventional CIEF [7, 8] or other works of direct coupling CIEF-MS [6, 13], the benefits of imaged detection apply and no adaptation of the separation system is required making the coupling uncomplicated and method development easy. The iCIEF-FI-MS approach is straightforward and mass spectra can be obtained using any mass spectrometer with a standard sprayer for ESI.

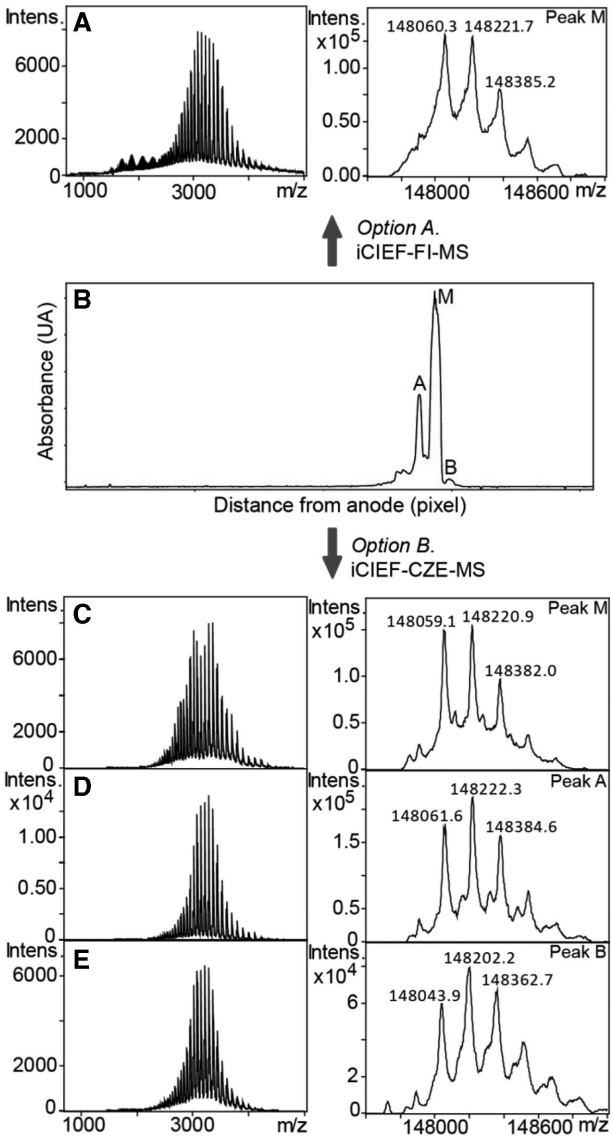

Figure 3. (A) Raw and deconvoluted spectra in MS using *Option A* for the main variant M (1.25 mg/mL). (B) Example of image after focusing for the mAb in 1% AESlyte pH 3–10, 2% AESlyte pH 8–10.5 HR, and 70% methylcellulose 0.5%. Raw and deconvoluted spectra in MS obtained using *Option B* for (C) the main variant M (1.25 mg/mL), (D) the main acidic variant A (2.0 mg/mL) and (E) the main basic variant B (6.0 mg/mL). Conditions for first dimension: focusing 1 kV (1 min), 2 kV (1 min), 3 kV (9 min), interval time 15 s; mobilization 0.120 μL/min, 3 kV (30 min), 10 s interval time. Conditions for second dimension *Option A*: methanol:water (50:50) with 1 % HAc, 1 μL/min, micro-LC sprayer. Conditions for second dimension *Option B*: 1M HAc as BGE, 13 kV, Agilent CE-ESI-MS sprayer, isopropanol:water (50:50) with 0.5% formic acid as sheath liquid. MS conditions: positive polarity, 700–5000 *m/z*, 5000 V, dry gas 3.0 L/min, dry temperature 170°C.

iCIEF-CZE-MS results in better spectra quality and less contamination of the MS and, thus, enables the characterization of minor charge variants of mAb.

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