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Research Article

Analysis of monoclonal antibody by a novel CE-UV/MALDI-MS interface

mAbs are highly complex proteins that present a wide range of microheterogeneity that requires multiple analytical methods for full structure assessment and quality control. As a consequence, the characterization of mAbs on different levels is particularly product- and time-consuming. CE-MS couplings, especially to MALDI, appear really attractive methods for the characterization of biological samples. In this work, we report the last instrumental development and performance of the first totally automated off-line CE-UV/MALDI-MS/MS. This interface is based on the removal of the original UV cell of the CE apparatus, modification of the spotting device geometry, and creation of an integrated delivery matrix system. The performance of the method was evaluated with separation of five intact proteins and a tryptic digest mixture of nine proteins. Intact protein application shows the acquisition of electropherograms with high resolution and high repeatability. In the peptide mapping approach, a total number of 154 unique identified peptides were characterized using MS/MS spectra corresponding to average sequence coverage of 64.1%. Comparison with NanoLC/MALDI-MS/MS showed complementarity at the peptide level with an increase of 42% when using CE/MALDI-MS coupling. Finally, this work represents the first analysis of intact mAb charge variants by CZE using an MS detection. Moreover, using a peptide mapping approach CE-UV/MALDI-MS/MS fragmentation allowed 100% sequence coverage of the light chain and 92% of the heavy chain, and the separation of four major glycosylated peptides and their structural characterization.

Keywords:
Capillary electrophoresis / CE/MALDI-MS / Intact protein / Monoclonal antibody / Peptide mapping
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1 Introduction

The characterization of complex protein and peptide mixtures represents one of the biggest challenges in many research fields such as biological [1–3] or biopharmaceutical sciences [4, 5]. Since 1986 and with the approbation of Muromonab-CD3 by the Food and Drug Administration (FDA), mAbs have taken a major market share in the pharmaceutical industry, and their development is constantly increasing [6, 7]. mAbs are particularly interesting because they have good therapeutic efficiency, favorable pharmacokinetic and pharmacodynamics, and lead to reduction of side effects [8]. mAbs are highly complex glycoproteins potentially displaying many naturally occurring molecular microheterogeneities combined with imperfect processing. There is a continuous need for analytical method improvement that can ultimately provide a fast and accurate characterization. Today, the method of choice for the identification of proteins and peptides is the MS [9] generally coupled with separation techniques such as HPLC or gel electrophoresis. Technically, HPLC-MS is the most widely used coupling because of its high reproducibility and great resolution, but also especially for the relative ease of coupling with a fraction collector device or directly in the source of a mass spectrometer. As an alternative to HPLC, CE has been demonstrated to be a useful and powerful separation method for the characterization of charged and neutral molecules [10–12]. Advantages of CE are fast separation, high efficiency, and high resolution. However, properties of CE such as an ultralow flow rate, a BGE that can be highly salted, and the necessity of maintaining the electric field during the separation make the CE-MS coupling more difficult to implement [13, 14]. Nevertheless, despite these technical aspects, the hyphenation of CE to MS appears as a very attractive coupling that performs significant advances in many research areas such as proteomics [15, 16], metabolomics [17], and intact protein characterization [18, 19]. Since the end of the 1980s, a large number of CE-MS interfaces have been developed. Due to the direct coupling property, most innovation has been based on the coupling between CE and ESI [20] by sheath-flow [21, 22],

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Abbreviations: αLac, α-lactalbumin; **βCas**, β-casein; **CAII**, carbonic anhydrase II; **CHCA**, α-cyano-4-hydroxycinnamic acid; **Cyt c**, cytochrome c; **EACA**, ε-amino-caproic acid; **HC**, heavy chains; **HPC**, hydroxypropylcellulose; **Ins**, insulin; **LC**, light chains; **LE**, leading electrolyte; **Lys**, lysozyme; **Myo**, myoglobin; **RNase A**, ribonuclease A

Colour Online: See the article online to view Figs. 1 and 5 in colour.

liquid-junction [23,24], or sheathless [25,26] interfaces. However, the nature of the BGE (presence of salts, volatility) can affect the stability of the spray and then limit the number of BGE that does not prevent the performances of MS. MALDI-TOF-MS is an MS technology that presents a greater tolerance to the presence of salts or surfactants [22]. Moreover, hyphenation of electrophoretic methods with MALDI-MS allows to easily detect positive and negative modes in MS. Then, as an interesting alternative to CE/ESI-MS, a great number of CE/MALDI-MS interfaces have been developed [27–43]. The first strategy to hyphenate electrophoretic method with MALDI-MS consists of developing online interface [30–32]. However, because sample is first dried on a solid surface before insertion into the MALDI source, heavy instrumental development appeared as one of the most significant issues for the implementation of this interface. The second option consisted promoting the off-line coupling using CE collection fractions on a MALDI plate. This strategy opens the way to sample treatment directly on the plate such as enzymatic digestion [44] or enrichment [45]. Concerning the deposit process, majority of the existing interfaces used a T-junction with sheath flow [33–39]. More recently, sheathless interfaces have been developed. In 2008, Wang et al. have developed an interface based on the use of a junction liquid that allows to eliminate the addition of the sheath liquid [42]. In 2009, Busnel et al. have designed an iontophoretic fraction collection approach to avoid the sheath flow and the possible suction effect [43]. However, one of the interface drawbacks is to fill little reservoir on the plate before deposit process. This prevents automation and high-throughput analysis. Concerning fraction collection devices, only one commercial system is compatible with CE/MALDI-MS [37]. However, this interface is originally designed for HPLC/MALDI-MS coupling and presents some geometric drawbacks for CE/MALDI-MS, such as the minimum length of the capillary. At the same time, homemade robotic systems, such as electrospray [40] or inkjet spotting [41], have been developed. Meanwhile, since 2001 interfaces between MALDI-MS and microfluidic devices have been developed [46]. The easiest strategy was reported to carry out CE separation in an open channel, where the separated analytes were directly ionized from the open channel to a mass spectrometer [47,48]. However, the open channel was filled by gel to suppress EOF and reduce analyte dispersion after the CE separation. In that case, only CGE is applicable. Then this strategy cannot regularize free-solution CE modes including CZE.

The efficiency and robustness of these interfaces have been reported, but none of these systems allows the automation. Most off-line CE/MALDI-MS coupling does not possess a second detection system to control the separation. Few interfaces used an additional external UV system that made the methodology more expensive and automation with the development of synchronized software difficult [35,36].

In this work, we have developed the first totally automated off-line CE-UV/MALDI-MS/MS with integrated delivery matrix system. Based on different homemade modifications performed on a commercial CE device and a fraction collection system, we introduced a totally automated approach to make protein separation and peptide mapping strategy. In order to control the CE separation, the original UV cell of the CE apparatus was deported to allow the simultaneous UV detection and fraction collection without external UV system. Modification of the spotting device geometry allowed to reduce the total capillary length. Based on the properties of the CE apparatus to apply simultaneously a pressure and power supply, and the modification on the external cartridge, an integrated homemade delivery matrix system was developed, which is universally compatible in terms of MALDI deposition types. The performance of the method was evaluated with the separation of five intact proteins and a tryptic digest mixture of nine proteins. Finally, in order to confirm the performances of the system for the characterization of mAbs, application to the separation of intact mAb and bottom-up approach has been developed using the CE-UV/MALDI-MS interface.

2 Materials and methods

2.1 Chemicals and materials

All chemicals used in this study were of analytical grade or high purity grade and purchased from Sigma-Aldrich (Saint Louis, MO, USA). Water used for the preparation of buffers and sample solutions was obtained using an ELGA purelab UHQ PS water purification system (Bucks, UK). Cytochrome c (Cyt c), ribonuclease A (RNase A), α-lactalbumin (αLac), insulin (Ins), myoglobin (Myo), BSA, lysozyme (Lys), β-casein (βCas), carbonic anhydrase II (CAII) were obtained from Sigma-Aldrich. Trypsin (modified, sequencing grade) was purchased from Promega AG (Dübendorf, Switzerland).

2.2 Standard protein mixtures

For intact protein study, a standard protein solution of αLac (14.2 kDa), RNase A (13.6 kDa), Cyt c (12.3 kDa), Lys (14.3 kDa), and Myo (16.9 kDa) was prepared in water at 120, 50, 40, 40, and 50 nM, respectively.

2.3 Tryptic digestion procedure

Tryptic digests of protein solution were prepared using sequence-grade trypsin (1:10 w/w). Before digestion, each protein was diluted to a concentration of 3 μM in 1% formic acid. DTT was then added to the protein solution at a final concentration of 10 mM. The mixture was incubated for 5 min at 95°C, then cooled to ambient temperature. Then, iodoacetamide 100 mM was added at a final concentration of 20 mM and the mixture was placed in the dark for 20 min, 10 μL of trypsin was added and the mixture was incubated at room temperature for 3 h; additional 10 μL of trypsin was added to the sample for overnight digestion at room temperature. This solution was then analyzed directly by CE/MALDI-MS without any further purification step. Protein tryptic digests with 300 fmol of each proteins were injected.

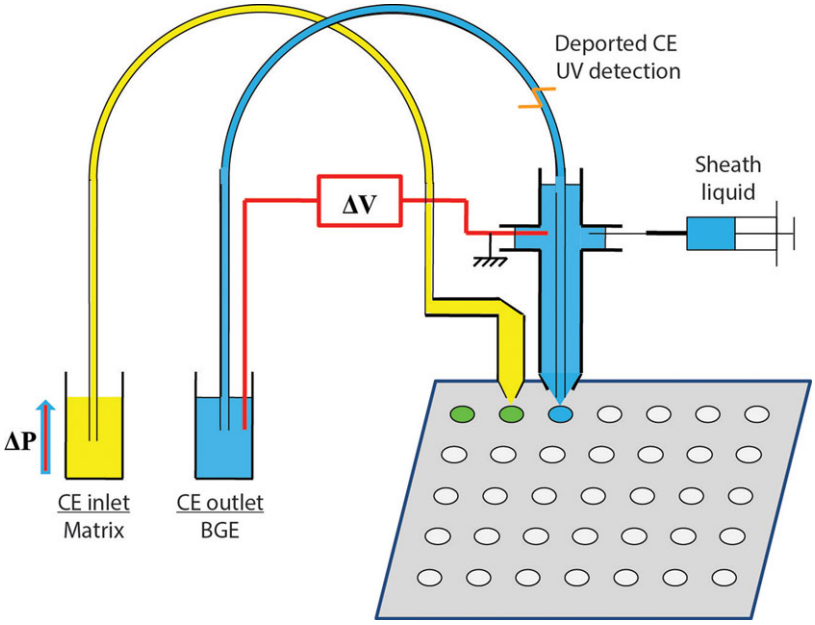

Figure 1. Schematic representation of the experimental setup of the CE-UV/MALDI-MS interface including the deported UV detection and integrated delivery matrix system.

2.4 Capillary electrophoresis

The CE experiments were carried out on a P/ACE MDQ™ CE system from Beckman Coulter (Brea, CA) equipped with a temperature-controlled autosampler and a power supply that delivered up to 30 kV. A 32 Karat (Beckman Coulter) was used for instrument control, data acquisition, and data handling. Polymicro bare fused-silica capillaries of 50 μm id were obtained from Photonlines (St-Germain-en-Laye, France). Considering modified capillaries, capillaries were coated in laboratory with hydroxypropylcellulose (HPC) following the protocol described by Shen et al. [49]. For model protein separation, 83.3 mM ionic strength ammonium acetate (pH 4.0) has been used as BGE. Samples were introduced into the capillary by electrokinetic injections (3 kV, 8 min), and the separation of proteins was performed by applying 20 kV across the capillary. For intact mAb separation, 400 mM ε-amino-caproic acid (EACA) acetic acid, 0.05% m/v, pH 5.7 has been used as BGE. For peptide mapping approach, solution of 1% formic acid and ammonium acetate (pH 9.3, 935 mM ionic strength) was used in CE as BGE and leading electrolyte (LE), respectively [50]. Except for the model protein separation, injection volumes have been calculated using CEToolbox application (Pansanel, GooglePlay).

2.5 Nano-HPLC

Protein digests were analyzed using an UltiMate 3000 nano-HPLC system (Dionex, Germering, Germany) coupled to a Proteineer FC (Bruker Daltonics, Bremen, Germany). A Pepmap100 C18 column (Dionex) was used. The gradient (solvent A, 0.1% TFA; solvent B, 0.1% TFA in 80% ACN) was as follows: 0–4 min, 4% B; 4–32 min, 4–55% B; 32–34 min, 55–90% B; 34–40 min 90% B; 40–45 min, 90–4% B; 45–65 min, 4% B. A flow rate of 300 nL/min was applied. The sample injection volume was 1 μL.

2.6 CE/MALDI-MS interface

Automated off-line coupling of CE to MALDI-MS was performed by using a homemade-modified automatic spotting device, Proteineer FC (Bruker Daltonics) for the sheath flow assisted spotting from the CE capillary end onto a MALDI target. The capillary outlet was positioned inside a fixed steel needle, being a modified part of a robotic *x*–*y*–*z* axis motion system. The original setup delivered from Bruker Daltonics was modified by us in order to reduce the distance between CE output and the MALDI target. This change comprises shifting of the original steel needle close to CE output and reduction of the minimum total capillary length from 110 to 60 cm. At the upper end of the needle, a cross-connection was fitted. On one side of the cross, the sheath liquid—which is the separation BGE—was supplied by means of the syringe pump included in the Proteineer FC (Bruker Daltonics) and on the other side, the ground was connected. The steel needle functioned as an electrode for the CE and the current was maintained by a liquid junction between the steel needle and end of the capillary (Fig. 1). During CE separation, the emerging droplet of CE effluent and sheath liquid made contact with the MALDI target. The original setup of the UV cell in the P/ACE MDQ™ (Beckman Coulter) was modified in order to allow the simultaneous UV detection near the outlet of the capillary and fraction collection (Fig. 1). A homemade UV window compatible with P/ACE MDQ system (Beckman Coulter) was mounted at the upper end of the cross-connection. Using three optical fibers with one couple connected in pairs, UV detection was deported out of the CE instrument without instrumental modification and

additional UV spectrometer. Therefore, for CE with fraction collection, a capillary with the UV detection window positioned 10 cm away from the CE capillary end was used.

In order to be universally compatible in terms of MALDI deposition types, a homemade delivery matrix system was developed to add the matrix just after sample deposition allowing dried droplet fraction collection directly onto the MALDI target. The matrix interface consisted of a second steel needle surrounding the matrix capillary (100 cm total length, 100 μm id) in the form of a coaxial tube. Matrix capillary end was placed at a distance of 5 mm from CE capillary end corresponding to the distance between two consecutive spots of a MALDI target provided by Bruker Daltonics. To avoid the use of an additional syringe pump to deposit the matrix, a modification was made on the external cartridge delivered by Beckman Coulter. The external extremity of the cartridge was pierced to allow the setup of a second capillary. Separation and matrix capillaries were then placed in the external cartridge and positioned in the CE inlet for matrix capillary and the CE outlet for separation capillary. Properties of P/ACE MDQ™ (Beckman Coulter) applied at the same time allowed pressure and power supply to perform CE separation simultaneously while using delivery matrix. The interface is also compatible with a thin-layer deposition type. In that case, matrix capillary was removed. Hystar 3.2 (Bruker Daltonics) was used for Proteineer FC control.

2.7 MALDI-TOF-MS and MALDI-TOF-TOF MS/MS

For intact protein and intact mAb separations, the matrix was prepared by dissolving 2,5-dihydroxybenzoïque (2 g/L) in 0.1% TFA/ACN (50/50). Fraction collection was performed using ground steel MALDI target (Bruker Daltonics). For peptide mapping approach, α-cyano-4-hydroxycinnamic acid (CHCA) (5 g/L) matrix was prepared following the classical AnchorChip protocol provided by Bruker Daltonics. Fraction collection was performed using AnchorChip MALDI target (Bruker Daltonics). When the samples were directly collected on a MALDI target with 0.6 μL sheath liquid, 0.6 μL of the matrix solution was automatically deposited on the same spot.

Mass spectra of the CE fractions were recorded using an Autoflex III MALDI-TOF (Bruker Daltonics), operating in reflector mode, and with FlexControl software. Positively charged ions were detected and sums of 1500 single-shot spectra were acquired automatically from each sample by using the AutoXecute software. For peptide mapping study, WarpLC 1.2 software was used. Data processing was performed using FlexAnalysis 3.0 and Biotools 3.2 provided by the mass spectrometer manufacturer. All spectra were calibrated according to an external calibration using protein calibration standard I (Bruker Daltonics) for intact protein separation and Peptide calibration standard II (Bruker Daltonics) for peptide mapping study. Data obtained from CE-MALDI-MS/MS experiments were processed using MASCOT search algorithm developed by Matrix Science (Boston, MA, USA).

3 Results and discussion

3.1 Evaluation of the CE-UV/MALDI-MS interface

CE-UV/MALDI-MS interface has been evaluated in terms of repeatability and robustness following the optimized separation conditions described by the team of Prof. Girault [51]. As a first step, we have evaluated the external UV detection (Fig. 1) by studying the repeatability in terms of migration time, peak area, and resolution of a standard protein separation in CE/MALDI-MS configuration. This is a significant parameter to determine if the developed system is adapted to CE collection. Indeed, especially for the coupling between CE and MALDI-MS, the off-line nature requires a second detection system to control the separation and then to approve the fraction collection. Moreover, an electropherogram obtained with the external UV detection as a control allowed optimizing the spotting process in terms of deposit window and deposit time interval. A neutral coated capillary has been used with a standard protein mixture containing αLac, RNase A, Cyt c, Lys, and Myo at 120, 40, 50, 50, and 40 nM, respectively. Samples were introduced into the capillary by electrokinetic injections (3 kV, 8 min), and the separation of proteins was performed by applying 20 kV across the capillary. No fraction collection has been made (Fig. 2A); CE capillary tip was positioned in the external BGE vial placed on the Proteineer FC (Bruker Daltonics). In order to avoid Joule heating effect during the separation, the length of the cartridge tubing was optimized to perform thermostated capillary in the UV cell. The UV detection window is located at 10 cm from the outlet when the total length of the capillary is 60 cm. In terms of migration time, peak area, and resolution, obtained RSD ($n = 4$) is less than 0.5, 2.5, and 3%, respectively. In addition, we have calculated S/N ratio ranging from 6 to 52. These results confirm that the externalization of the UV detection allows acquiring electropherograms with good quality, high repeatability, and a good robustness. In addition, this confirms the good stability of the HPC coating during the separation. Information concerning migration times allows defining the window of fraction collection, and assessing time interval for each deposit. These results confirm the possibility of obtaining CE-UV/MALDI-MS coupling without heavy instrumental development and without using an additional external UV detector. In addition, due to the use of single software to control CE apparatus, this UV modification contributes to strengthening the totally automated aspect of the coupling.

As a second step, in order to evaluate the impact of the spotting process on the separation, we have studied, using the external UV detection, the evolution of resolution and efficiency of the same standard protein mixture separation with fraction collection. For direct deposit on a MALDI plate, fraction collection window has been defined between 15 and 36 min, and a spotting time interval of 30 s has been chosen.

To prove the automated spotting process, Fig. 2 shows the UV electropherograms with (Fig. 2B) and without (Fig. 2A) fraction collection, and in the case of collection the

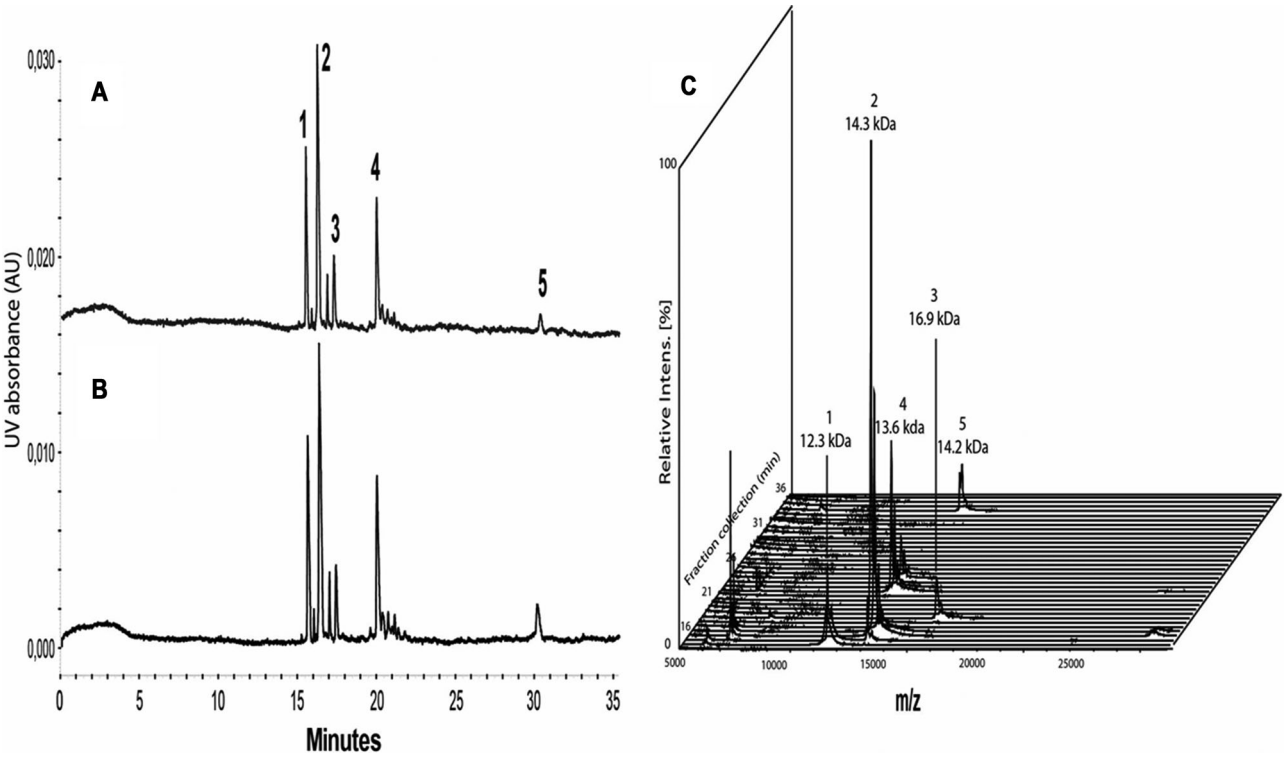

Figure 2. UV electropherogram without (A) and with fraction collection (B) and the corresponding MS spectra of each fraction (C) of a five-protein sample by CE-UV/MALDI-MS. Experimental conditions: HPC-coated capillary, 50 μm id × 60 cm (detection cell, 60 cm); BGE: 83.3 mM ionic strength ammonium acetate (pH 4.0); voltage: 20 kV; temperature: 25°C; UV detection: 200 nm; injection: 3 kV, 8 min; sample: (1) 40 nM Cyt c, (2) 40 nM Lys, (3) 50 nM Myo, (4) 50 nM RNase A, and (5) 120 nM αLac in water, MS experimental conditions: see Section 2.

representation of the experimental fraction using MS spectra of each spotting position.

Concerning the deposition process, UV electropherogram (Fig. 2B) shows a preservation of the resolution and efficiency as compared to the separation without fraction collection (Fig. 2A). The steel needle served as outlet electrode for the CZE. Liquid junction between steel needle and capillary tip maintained electrical current. Then, even if the steel needle moves on the *z*-axis to reposition the capillary tip from a spotting position to the next, no current breakdown takes place throughout the separation, and then electromigration never stops during the analysis. The localization of each protein, presented in Fig. 2C, shows a time shift between the UV detection and spotting process. This is totally in agreement with the fact that UV detection is located at 10 cm away from the capillary outlet. Moreover, offsets in time, between 1 min for Cyt c and 4–5 min for αLac, can be explained by the difference of protein effective mobilities described in the equation:

$$\mu_{\text{eff}} = \frac{Ll}{tV} \quad (1)$$

where μ_{eff} are the effective mobilities, L and l the total capillary length and length to detection window, respectively, V the applied voltage, and t is the migration time.

For example, for RNase A, a time shift of 3 min between detection time (20 min) and spotting time (23 min) corresponds to a value of $\mu_{\text{eff}} = 12.9 \times 10^{-5}$ cm^2 V^{-1} s^{-1}. Good agreement between time shift values and effective mobilities showed that the interface does not induce any significant suction effect. This confirms the use of a sheath liquid junction. Under these experimental conditions, it appears that the automated interface supports intact protein separations. In addition, flexibility of spotting parameters (spotting interval, sheath liquid flow rate) combined with UV control electropherogram allows high possibility of optimization as a function of the sample complexity.

3.2 Evaluation of the CE-UV/MALDI-MS/MS for peptide mapping approach

Peptide mapping is essential for the characterization of a complex mixture of digested proteins. In comparison to PMF, MS/MS peptide identification is not only based on digested peptide m/z ratio measure, but also on the fragmentation of those peptides, increasing the confidence of the identification. WarpLC 1.2 (Bruker Daltonics) is a software initially dedicated to perform peptide mapping strategy using off-line coupling between HPLC and MALDI-MS/MS. This software is fully compatible and could be easily adapted to the CE-UV/MALDI-MS/MS approach. Due to the complexity of biological samples, a low carry-over may involve

Table 1. Number of unique identified peptides and sequence coverage for a nine-protein tryptic digest using CE-UV/MALDI-MS-MS, NanoLC/MALDI-MS/MS, direct MALDI-MS, and the combination of the three deposition modes

Protein	CE-UV/MALDI-MS/MS		NanoLC/MALDI-MS/MS		Direct MALDI-MS		Combination of the three deposition modes	
	Identified peptides	Sequence coverage (%)	Identified peptides	Sequence coverage (%)	Identified peptides	Sequence coverage (%)	Identified peptides	Sequence coverage (%)
βCas	3	22.3	4	20.5	3	14.7	7	34.4
CAII	20	58.8	11	34.2	5	37.7	28	59.6
Cyt c	13	58.1	7	43.8	1	10.5	16	74.3
αLac	9	40.8	12	51.4	1	7.0	18	53.5
Lys	28	79.6	24	83.0	12	58.5	41	85.7
BSA	55	73.0	68	77.3	13	23.6	113	90.1
Myo	15	85.1	6	44.2	0	0	19	85.1
Ins	1	58.8	4	100.0	2	43.1	5	100.0
RNase A	10	100.0	25	93.5	8	54.4	32	100.0
	Total of id. pep.	Average seq. cov. (%)	Total of id. pep.	Average seq. cov. (%)	Total of id. pep.	Average seq. cov. (%)	Total of id. pep.	Average seq. cov. (%)
	154	64.1	161	60.8	45	27.7	279	75.9

CE experimental conditions: HPC-coated capillary, 50 μm id × 80 cm (detection cell, 70 cm); BGE: 1% formic acid, pH 2.1; voltage: –25 kV; temperature: 25°C; UV detection: 200 nm; injection: 1 psi, 76 s (100 nL injected); NanoLC and MS experimental conditions; see Section 2; sample: digests of βCas, RNase A, CAII, BSA, Lys, Ins, Cyt c, αLac, and Myo.

the presence of the same m/z ratio in two or three adjacent spots. In that case, the software selects the spot with the most intense m/z parent ion to perform MS/MS. After MS/MS spectra acquisition, WarpLC 1.2 proceeds to the exclusion of the same m/z parent ion present in the adjacent spots. The software allows a drastic reduction in the possible redundant peptides and improves the number of selected m/z ratios that increase the number of possible unique peptides.

To further characterize the fraction collection interface, 100 nL of a peptide mixture sample containing the tryptic digests of βCas, RNase A, CAII, BSA, Lys, Ins, Cyt c, αLac, and Myo (each at 3 μM) has been analyzed. Amounts of protein tryptic digests with 300 fmol of each protein were injected. In this study, a neutral coated capillary has been used and transient ITP preconcentration step has been implemented. For its compatibility with MALDI-MS, we used ammonium acetate (pH 9.3; 935 mM ionic strength) as the LE as in the Busnel et al. study [50]. Prior to the sample injection, a short zone of LE has been injected to allow transient ITP phenomena. A first separation without collection fraction was allowed with UV electropherogram to fix the spotting parameters. At the beginning of the separation, for 25 min, the capillary tip was positioned in the external BGE vial placed on the Proteineer FC (Bruker Daltonics). According to electropherogram, during this time no peptide has been detected by the UV. Fraction collection began after 25 min with spotting intervals of 30 s. In order to perform MS/MS, a dried droplet deposition protocol has been involved. Using the homemade delivery matrix system, CHCA matrix was placed in the inlet position of P/ACE MDQ™ system (Beckman Coulter) and delivered by a second capillary with a flow rate of 1.2 μL/min in accordance to deposit of 0.6 μL per spot. To determine the exact value of the pressure to perform 1.2 μL/min, we followed the protocol described by Gahoual et al. [3]. The separation was stopped after 65 min. A total of 80 fractions have been collected.

In order to evaluate the strength of the present CE-UV/MALDI-MS/MS coupling, the same nine-protein digest loading amounts (300 fmol of each protein) were applied for both NanoLC/MALDI-MS/MS and direct MALDI-MS. For HPLC-MS/MS analysis, NanoLC coupled with the same spotting device was used. Sample was separated onto an RP C18 analytical column for a 65 min gradient and deposited on the MALDI target with a 30 s spotting intervals. Dried droplet deposition protocol has been performed, which is similar to CE-MS/MS analysis. For direct MALDI-MS deposition, a nine-protein mixture (each at 300 fmol) without separation process has been deposited in the same MALDI target using the same dried droplet protocol. Table 1 summarizes the number of unique identified peptides and sequence coverage for each protein using CE-UV/MALDI-MS/MS, NanoLC/MALDI-MS/MS, direct MALDI-MS, and the combination of the three deposition modes.

When the number of peptides identified with the three techniques is compared, CE-UV/MALDI-MS/MS and NanoLC/MALDI-MS/MS appeared to obtain identical performances and are superior to direct MALDI-MS. While the direct MALDI-MS analysis allowed the identification of 45 peptides, CE-MS/MS analysis and NanoLC-MS/MS analysis resulted, respectively, in the identification of a total number of 154 different peptides ranging from 1 (Ins) to 55 (BSA) corresponding to average sequence coverage of 64.1%, and in the identification of a total number of 161 different peptides ranging from 1 (Cyt c) to 68 (BSA) corresponding to average sequence coverage of 60.8%, with a mass accuracy of 20 ppm

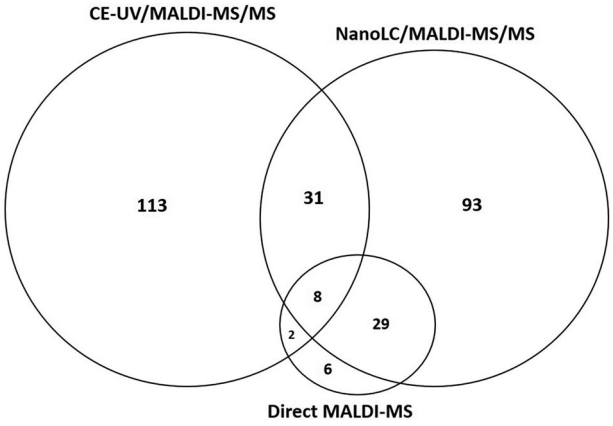

Figure 3. Venn comparisons representing the overlap of identified peptides by CE-UV/MALDI-MS/MS, NanoLC/MALDI-MS/MS, and direct MALDI-MS. Data originate from Table 1.

in MS and 0.5 Da in MS/MS. These results confirmed the usefulness of integrating a separation step prior to MS analysis. The results of the combination of the three deposition modes (Table 1) show a significant improvement of the total number of identified peptides, and of the average sequence coverage. Furthermore, we represented the merger of peptides from CE-MS/MS, NanoLC-MS/MS, and direct MALDI-MS analysis (Fig. 3). Results from the Venn comparison clearly show that CE-UV/MALDI-MS/MS and NanoLC/MALDI-MS/MS were complementarity methods at the peptide level with an increase of 42% in terms of unique identified peptides from 161 (NanoLC analysis only) to 276 (NanoLC and CE analysis). This complementarity also was useful for improvement of sequence coverage (Table 1). Using the combination of both NanoLC and CE analysis, we obtained an average sequence coverage of 75.9% as compared to 60.8% for single NanoLC analysis. This represented an increase of 20% in terms of average sequence coverage. This resulted in more confident protein identification.

Due to the ability of CE to separate a wide variety of peptides regardless of their chemical nature, these results unambiguously show the possibility of using CE-UV/MALDI-MS/MS to perform MS/MS peptide mapping characterization. Moreover, the automated setting up of a dried droplet deposition protocol, which is essential in MALDI for MS/MS acquisition in the case of complex protein mixture, demonstrates the full compatibility of the system for peptide mapping approach.

3.3 Analysis of mAb using CE-UV/MALDI-MS/MS

mAbs are tetrameric glycoproteins having molecular mass of approximately 150 kDa. They are composed of two heavy chains (HC) and two light chains (LC) linked to each other by several disulfide bonds. This type of compound presents a large number of microheterogeneities commonly found in proteins such as PTMs, including glycosylations and small chemical modifications [52]. In this work, we performed the analysis of a model commercially marketed humanized mAb following two strategies: the separation of intact mAb and peptide mapping approach, both using CE-UV/MALDI-MS interface.

The usage of CZE as a tool for the analysis of charge heterogeneity of intact therapeutic mAbs was investigated by several groups [53–55]. However, due to the drastic condition in terms of salt concentration, no MS detection has been investigated. Indeed, CZE separation of intact proteins is highly affected by the pH and ionic strength of the BGE. This represents a major drawback especially for the ESI-MS detection. Due to a greater tolerance to the presence of salts [22], MALDI-MS represents a good alternative. To demonstrate the CE-UV/MALDI-MS/MS fraction collection approach, an intact model humanized mAb has been considered. These experiments have been performed following the optimized condition described by the group of Dr. Ruesch [53]. BGE contained 400 mM EACA-acetic acid, 0.05% m/v, pH 5.7 was used. He et al. demonstrated the influence of the pH and EACA concentration on the mAb separation. Since charge variants of mAbs have different acid–base dissociation constants but similar Stokes' radii, pH buffer has to be selected such that the analytes exhibit significant relative difference in charge, increasing relative difference in electrophoretic mobility. Concerning EACA concentration, they demonstrated that increasing EACA concentration may improve separation efficiency and resolution. To avoid any protein adsorption to the capillary wall, HPC neutral capillary coated has been performed [55]. In addition, concentrated EACA buffer also was used for suppressing protein adsorption to the capillary wall and interacting with residual silanol groups that are not covered by HPC [53]. According to electropherogram represented in Fig. 4A, tip was positioned at the external BGE vial placed on the Proteineer FC (Bruker Daltonics) for 45 min, then fraction collection began with spotting intervals of 30 s. Figure 4B represents the reconstruction of the separation using MS spectra of each fractions.

First of all, these experiments showed the availability of the MALDI-MS to detect intact mAb using highly salted conditions. Moreover, the CE/MALDI-MS profile (Fig. 4B) confirms the good agreement between the UV detection and deposition time. This confirms the absence of carry-over effect and diffusion phenomenon. Unfortunately, limitation of MALDI-MS resolution for molecules up to 100 kDa does not allow the measurement of the exact mass of the charge variants and characterization of these glycoproteins. But this study is the proof of principle for the first analysis of intact mAb charge variants by CZE using an MS detection. This opens new pathways on the characterization of intact mAbs by CE-MS such as fraction collection, enrichment, or top-down approach.

The second strategy comprised characterization of mAb by bottom-up approach. As discussed previously, peptide mapping is commonly used for determining protein amino acid sequence. Moreover, this method allows to locate and/or quantify PTM. This methodology is quite important in early

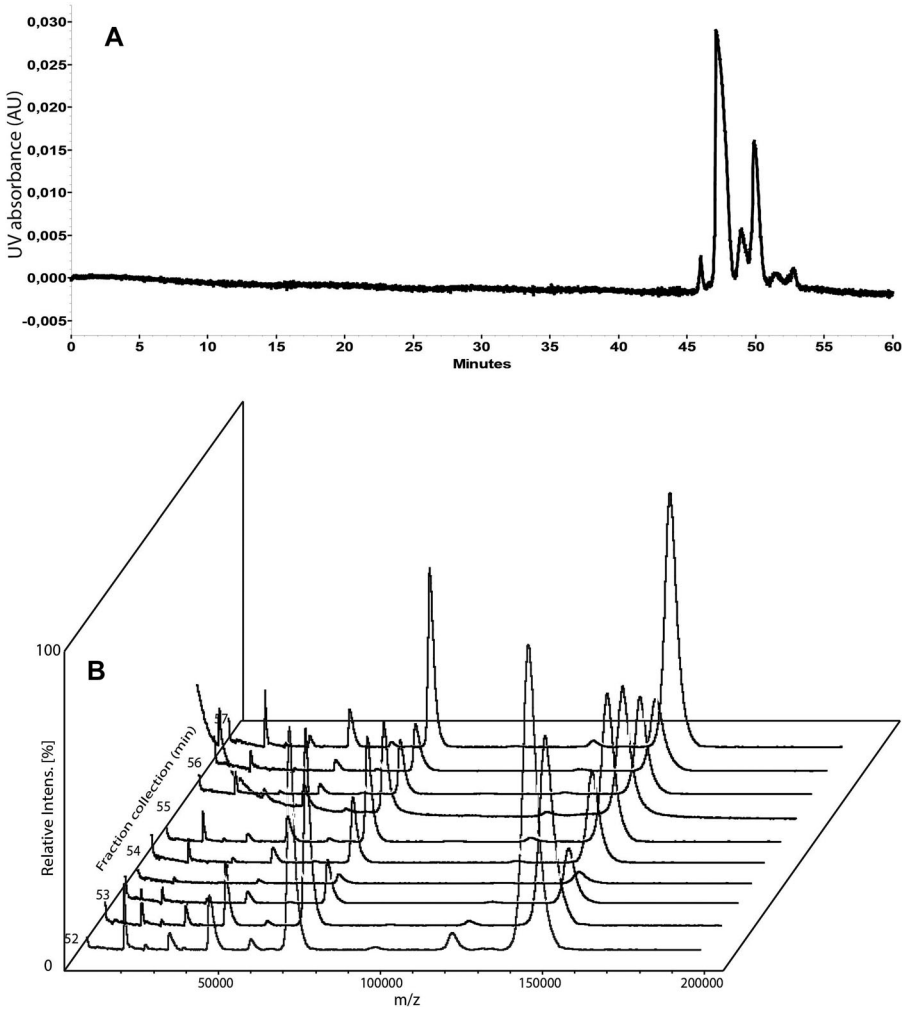

Figure 4. UV electropherogram (A) and corresponding MS spectra of each fraction (B) of intact mAb charge variants by CE-UV/MALDI-MS. Experimental conditions: HPC coated capillary, 50 μm id × 80 cm (detection cell, 70 cm); BGE: 400 mM EACA-acetic acid, 0.05% m/v, pH 5.7; voltage: 30 kV; temperature: 25°C; UV detection: 200 nm; injection: 2 psi, 30 s; sample: mAb at 4 g/L, MS experimental conditions: see Section 2.

development of therapeutic antibodies as well as during long-term life-cycle management of the biopharmaceutical products. As part of this work, one of the objectives was to investigate the peptide mapping of the same model humanized mAb by CE-UV/MALDI-MS/MS. A sample of mAbs was digested with trypsin enzyme following the same protocol previously described. Figure 5A shows a separation obtained for the tryptic digest of mAb (500 fmol injected). The total duration of the separation was less than 50 min with a detection window of 20 min between 25 and 45 min.

In this work, peptide identification was automated and based on MS/MS data, which means that identification was performed on parent ion mass measure and fragment identification. Results of MASCOT search showed sequence coverage of 92% for the HC and 100% for the LC of mAb corresponding to a total of 92 unique identified peptides (Fig. 5B). Some peptides were selected and fragmented several times during the analysis leading to the same identification. These phenomena reinforce the confidence of the identification by enabling peptide overlapping.

mAbs are heterogeneous by nature due to different structural modifications that the protein may experience during the lifetime. Those modifications can be critical quality attributes, as they can change the protein conformation and therefore influence the antibody activity. mAb glycosylations represent a class of PTMs that can significantly influence its structure, immunogenicity, and stability [56]. Results are obtained on the same set of data allowed to characterize the four major N-glycosylation carried out by the mAb (G0F, G1F, G2F, and Man5). As expected by the work of Gahoual et al. [5], we detected glycans still linked to the corresponding peptide backbone of the HC and confirmed the ability of CE migration mechanism to separate glycopeptides having a difference of only one galactose (Fig. 5C). The glycopeptide was detected between 34.1 and 36.1 min. Figure 5C represents the MS/MS fragmentation spectra of the glycopeptide bearing G1F. As expected, product ions observed in MS/MS mainly correspond to the fragmentation of the glycan moiety. Study of the fragmentation spectra led to deduction of the glycan G1F structure. Such advanced characterization of digested mAb peptide mixture in a 50 min analysis shows the potential of CE-UV/MALDI-MS/MS as a fast and sensitive method in mAb characterization.

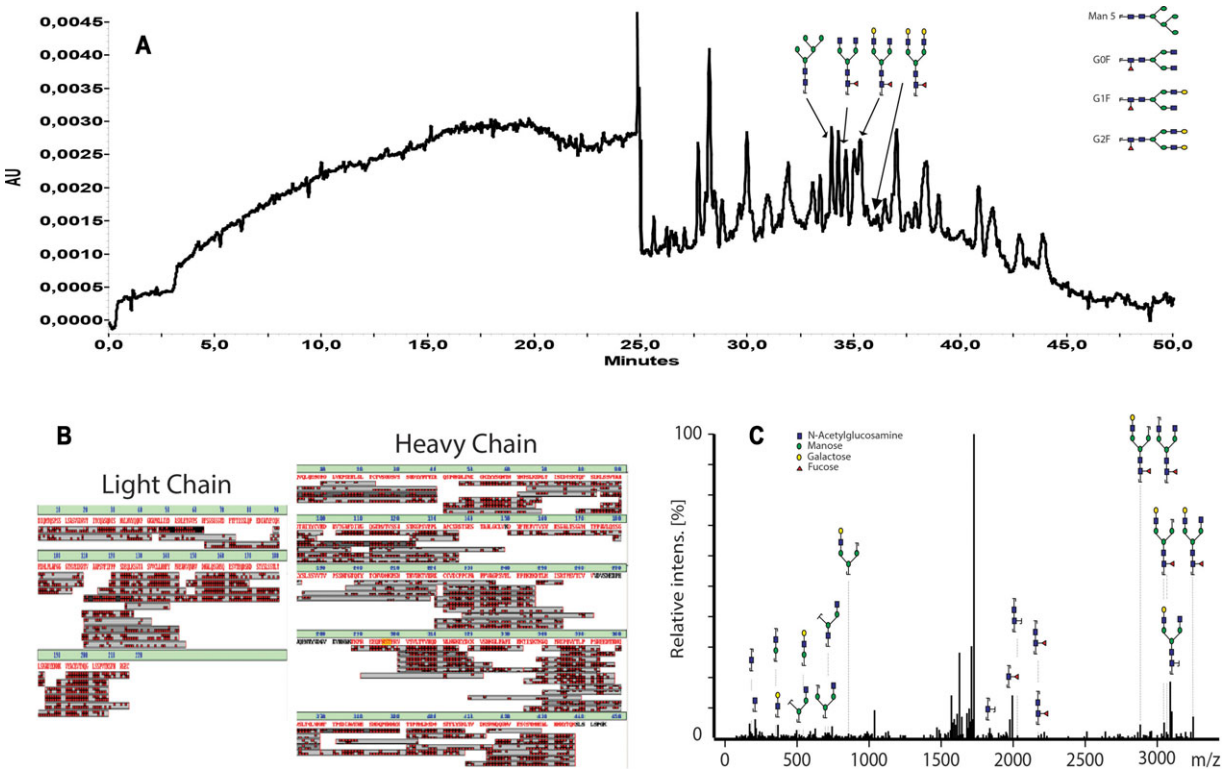

Figure 5. UV electropherogram corresponding to the analysis by CE-UV/MALDI-MS/MS of mAb tryptic digest (A), sequence coverage obtained for mAb HC and LC (B), and MS/MS fragmentation spectra of G1F glycopeptide (C). CE experimental conditions and MS experimental conditions: see Table 1 and Section 2.

4 Concluding remarks

In this work, we have developed the first automated off-line CE-UV/MALDI-MS/MS with integrated delivery matrix system. The system is based on different homemade modifications performed on P/ACE MDQ™ CE system (Beckman Coulter) and Proteineer FC automatic spotting device (Bruker Daltonics). In order to control the CE separation, the original setup of the UV cell in the CE apparatus was deported to allow the simultaneous UV detection near the outlet of the capillary and fraction collection. In order to reduce the total capillary length, the spotting device was modified with a shift of the original steel needle close to the CE output. Minimum total capillary length can be 60 cm. Finally, in order to be universally compatible in terms of MALDI deposition modes, a homemade delivery matrix system was developed. Neutral coated capillary can therefore be used for the achievement of intact protein or peptide separation. The evaluation of the system on the separation of five proteins showed the acquisition of electropherograms with high resolution and high repeatability. Migration times allow defining the window of fraction collection, and assessing time interval for each spot. Concerning peptide mapping approach on a nine digested protein mixture, 154 unique identified peptides were characterized using MS/MS spectra corresponding to average sequence coverage of 64.8%. On the basis of the results obtained and as compared to NanoLC/MALDI-MS/MS, CE-UV/MALDI-MS/MS can be considered as a complementarity method to conventional LC-MALDI/MS, especially, to improve the confidence in protein identification. Finally, this system has been used for the characterization of a humanized mAb following intact protein separation and peptide mapping strategies. In spite of the lack of resolution of the MALDI-MS, this work represents the first analysis of intact mAb charge variants by CZE using an MS detection and opens new ways of research for the characterization of intact mAbs. Moreover, the CE-UV/MALDI-MS/MS fragmentation allowed 100% sequence coverage of the LC and 92% on the HC, and the separation of the four major glycosylated peptides and their structural characterization in the same analysis data set. These result analyses demonstrate the power of CE-UV/MALDI-MS/MS as a sensitive method in mAb characterization and, more generally, in bottom-up proteomic approach.

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