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Top-down and middle-down approach by fraction collection enrichment using off-line capillary electrophoresis – mass spectrometry coupling: Application to monoclonal antibody $F_{c/2}$ charge variants

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ABSTRACT

The characterization of complex protein mixtures represents one of the biggest challenge in many research fields such as biological or biopharmaceutical sciences. Out of all categories, monoclonal antibodies (mAbs) and related products drawn the most interest due to their strong therapeutic potency and specificity. Because of their intrinsic complexity due to a large number of micro-heterogeneities, there is a crucial need for analytical methods to provide comprehensive in-depth characterization of these proteins. In this work, we developed a methodology using CE-UV/MALDI-MS to perform top-down or middle-down characterization after fraction collection enrichment applied to intact protein and mAbs samples. The performance of the method was evaluated with the rapid separation of three intact protein mixture. Good robustness of CZE separation and quality of MALDI-MS spectra and MALDI-ISD spectra of each protein confirms the usefulness of sample enrichment to obtain adequate quantity of deposed protein for top-down analysis and the proof of principle of the method. In a second step, the method was applied to the middle-down characterization of Fc/2 cetuximab variants. Identification of around 9% sequence coverage of Fc/2 cetuximab fragments allows to conclude on the feasibility of the strategy for middle-down characterization of Fc/2 cetuximab variants using CE-UV/MALDI-MS. Moreover, MALDI-ISD fragmentation of Fc/2 cetuximab variants confirm separation phenomenon based on the formation of Fc/2 dimers with and without C-terminal truncation.

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1. Introduction

One of the deeper challenge in many research fields such as biopharmaceutical [1,2] or biological sciences [3–5] is the characterization of complex protein mixtures. Since the middle of the 80s, monoclonal antibodies (mAbs) have taken a major market share in the pharmaceutical industry [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 micro-heterogeneities. There is a continuous need for analytical methods improvement to be able to give accurate characterization. Today, a plethora of separation technique based both on liquid chromatography and electrophoresis are used for antibody characterization and homogeneity assessment [9]. Concerning detection, the method of choice for the identification of proteins and peptides is mass spectrometry (MS) [10] generally coupled with separation techniques such as high-performance liquid chromatography (HPLC) or capillary electrophoresis (CE). Technically, HPLC–MS is the most widely used coupling thanks to its high reproducibility and its great resolution but also especially for the relative ease of coupling with a mass spectrometer. However, CE represent an alternative to HPLC due to its ability to separate intact protein [5]. Advantages of CE are fast separation, high efficiency and high resolution. However, properties of CE such as nature of background electrolyte (BGE) and the necessity of maintaining the electric field during the separation make the CE-MS coupling more difficult to

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implement [11,12]. Nevertheless, despite these technical aspects, CE-MS hyphenation appears as a very attractive coupling allowing to perform significant advances in many research areas such as proteomics [13–15], biotherapeutics [16–18] and intact protein characterization [19,20]. A large number of CE-MS interfaces has been developed preferably with electrospray ionization (ESI) [21–23]. However, the nature of the BGE (presence of salts, volatility) can affect the stability of the spray and then limits the choice of BGE to those that do not interfere with MS performance. This negative aspect is particularly observed for biotherapeutics characterization [24,25]. In the recent paper of our team, we developed an off-line CE-UV/MALDI-MS interface with integrated delivery matrix system to allow the separation of intact protein and biotherapeutics [26]. In this work, we performed the first analysis of intact mAb charge variants by CZE using a MS detection. However, limitation of MALDI-MS resolution for large molecules does not allow to measure the exact mass of the charge variants and does not give any structural information [26]. Top-down and middle-down approaches provide such alternative to obtain structural information using CE-UV/MALDI-MS. A "down"-type method is defined when the fragmentation happens primarily in the gas phase inside the mass spectrometer. Then, the mass spectrometer is used to analyze the entire molecule directly [27,28]. As top-down defined by characterization using fragmentation of intact protein, middle-down refers to MS/MS-based sequencing of large fragments of a protein after limited proteolysis [29]. There are number of mass spectrometric techniques that have been used to identify proteins by top-down approach including MALDI ion source decay (ISD) [30], electron-capture dissociation (ECD) [31] or electron-transfer dissociation (ETD) [32]. In the following report, MALDI-ISD will be used as fragmentation method. A well-known issue of top-down characterization is due to dynamic range limitation in regards to the complexity of the sample [27]. Top-down proteomic requires purified intact protein potentially provided by CZE separation prior to MS detection. Moreover to obtain good ISD spectra quality, MALDI-ISD requests quantity of sample which is not compatible with the miniaturized aspect of CE [33]. As a strategy to increase the level of sample, enrichment by fraction collection can be realized using the off-line CE-UV/MALDI-MS interface.

In this report, we used CE-UV/MALDI-MS system developed in house to allow top-down or middle-down characterization after fraction collection enrichment applied for intact protein and mAbs samples. As a preliminary study, the performance of the method was evaluated with the rapid separation of three intact protein mixtures. Repeatability and robustness of the method were confirmed to allow enrichment by fraction collection. The proof of principle of the method was assessed with comparison of top-down analysis of the different fractions after single or pooled multiple fractions from several repetition of separation of the same sample. Finally, to validate the strategy for the characterization of mAbs, middle-down characterization of Fc/2 cetuximab variant have been performed using CE-UV/MALDI-MS. The mAb selected was cetuximab which is human/murine chimeric IgG-1 directed against the epidermal growth factor receptor (EGFR) overexpressed in advanced-stage EGFR positive colorectal cancer. Cetuximab was approved in the US and EU in 2004 and 2005. Cetuximab contains two glycosylation sites both on the heavy chain (HC) [34]. Moreover, it has a large number of micro-heterogeneities such as PTMs and it also has one C-terminal lysine truncation. The knowledge of our group about the characterization of this mAb using MS [35–37] makes cetuximab an ideal sample for the assessment of the described method.

2. Experimental

2.1. Chemicals

Chemicals used were of analytical grade or high purity grade and purchased from Sigma-Aldrich (Saint Louis, MO, USA). Water used to prepare 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), lysozyme (Lys), were obtained from Sigma-Aldrich (Saint Louis, MO, USA). IdeS (Immunoglobulin-degrading enzyme of Streptococcus pyogenes) also named FabRICATOR was purchased from Genovis (Lund, Sweden). Cetuximab samples are the EMA/FDA approved formulation purchased Merck (Whitehouse Station, NJ, USA).

2.2. Middle-up sample preparation

Cetuximab was cleaved in the hinge region using limited proteolysis by IdeS (FabriCATOR, Genovis) to obtain two Fc/2 fragments (theoretical pI 7.74) and one $F(ab')_2$ fragment (theoretical pI 7.78). Sample was diluted using 147.25 μL of 50 mM sodium phosphate, 150 mM NaCl, pH 6.60, to a final concentration of 1 μg/μL. A volume of 2.25 μL of IdeS (67 units/μL) was added to the sample which was left at 37 °C for 30 min. After digestion completion, sample was desalted using Amicon centrifugal filters (cut off = 10,000 Da) in pure water at 10 °C and 14,000 g for 20 min. After desalting step, sample volume recovered was about 10 μL. Sample was finally diluted to a final concentration of 5 μg/μL in a total volume of 30 μL of pure water.

2.3. Capillary electrophoresis

The CE experiments were carried out on a P/ACE MDQ™ CE system from Sciex Separation (Brea, CA) equipped with a temperature controlled autosampler and a power supply able to deliver up to 30 kV. A 32 Karat™ (Siex Separation, Brea, CA) was used for instrument control, data acquisition and data handling. Polymicro bare fused-silica capillaries of 50 μm i.d., 375 o.d. were obtained from Photonlines (St-Germain-en-Laye, France). Concerning modified capillaries, capillaries were coated in laboratory with hydroxypropylcellulose (HPC) following the protocol described by Shen et al. [38]. For Cetuximab separation, inlet BGE 200 mM ε-Amino-caproic acid (EACA)-ammonium acetate 25 mM pH 5.7 and outlet BGE ammonium acetate 25 mM pH 5.7 have been used as separation condition. Injection volumes have been calculated using CEToolbox application (Pansanel, GooglePlay).

2.4. CZE/fraction collection interface

This interface have been described in a previous study [26]. Briefly, automated off-line coupling of CZE to MS was set up by using a homemade modified automatic spotting device Proteineer FC (Bruker Daltonics, Bremen, Germany) for the sheath flow-assisted spotting from the CZE capillary outlet onto a fraction collection target. The original set up of the UV cell in the P/ACE MDQ™ (Sciex Separation, Brea, CA) was delocalized outside the CE instrument in order to allow simultaneous UV detection and fractions collection. Hystar 3.2 (Bruker Daltonics, Bremen, Germany) was used for Proteineer FC control.

2.5. MALDI-TOF-MS

The matrix was prepared by dissolving 2,5-dihydroxybenzoïque (DHB) (2 g L^{-1}) in 0.1% trifluoroacetic acid/acetonitrile (TFA/ACN) (30/70). Fraction collection was realized using Ground Steel MALDI target (Bruker Daltonics, Bremen, Germany). Mass spectra of the

CE fractions were recorded using an Autoflex II MALDI-TOF (Bruker Daltonics, Bremen, Germany), operating in reflector mode and with FlexControl software. Positively charged ions were detected and sums of 2000 single-shot spectra were acquired automatically from each sample by using the AutoXecute software. For Top-down approach, sums of 15,000 single-shot spectra were acquired automatically from each sample. Data processing was performed with FlexAnalysis 3.0 provided by the mass spectrometer manufacturer. All spectra were calibrated according an external calibration using Protein calibration standard I (Bruker Daltonics, Bremen, Germany) for intact protein separation.

3. Results and discussions

3.1. Evaluation of enrichment by fraction collection using CE-UV/MALDI-MS

To enrich the fractions and achieve adequate sample amount for top-down analysis, we need to run multiple repetition of separation of the same sample without degradation or change in mobilities. As a preliminary study to evaluate CE-UV/MALDI-MS coupling to perform enrichment by fraction collection, the robustness and the repeatability of CE-UV/MALDI-MS interface has been confirmed following the optimized separation conditions described in precedent studies [26,39]. Briefly, a neutral HPC coated capillary has been used with a standard protein mixture, containing Cyt c, Lys and RNase A at 40 nM, 40 nM, 50 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. Electropherogram obtained with the external UV detection, located at 10 cm from the outlet when the total length of the capillary is 60 cm, allowed optimizing the spotting process concerning the deposit window and deposit time interval (Fig. 1a). In term of migration time, peak area and resolution, obtained RSD (n = 4) are less than 0.5%, 2.5% and 3% respectively. Moreover, calculated signal-to-noise ratio are ranging from 6 to 52. Values of migration times allows defining the window of fraction collection, and assessing time interval for each deposit which represent a primordial criteria to perform enrichment. In our study, fraction collection window has been defined between 15 and 23 min and a spotting time interval of 30 s has been chosen. Fig. 1 shows the UV electropherogram with fraction collection (Fig. 1a) and the representation of the experimental fraction using MS spectra of each spotting position (Fig. 1b). Concerning the deposition process, correlation between UV electropherogram and the corresponding MS spectra of each fraction of a three protein sample shows a preservation of the resolution and efficiency [26]. These results then approve that the CE-UV/MALDI-MS interface allows acquiring electropherograms and deposition process with great quality, high repeatability and a good robustness to realise enrichment by fraction collection.

To assess sample enrichment, comparison of CE-UV/MALDI-MS studies between a single deposit analysis and the collection fraction from repetition of three separation of the same sample have been realized. Time of one deposit analysis represents 35 min while total time of three repetition represents around 2 h. Deposit parameters are similar to those described above. Only spotting time interval have been change. The fraction collection process developed in this study based on the apparent mobility of each peak described by the equation:

$$\mu_{app} = \frac{Ll}{t_m V}$$

with μ_{app} the apparent mobility which is the sum of effective mobility and residual electroosmotic flow, L and l the total capillary length and length to detection window, respectively, V the applied voltage and t_m the migration time. Deposition time t_d is then calculated by the equation:

$$t_d = \frac{L^2}{\mu_{app} V}$$

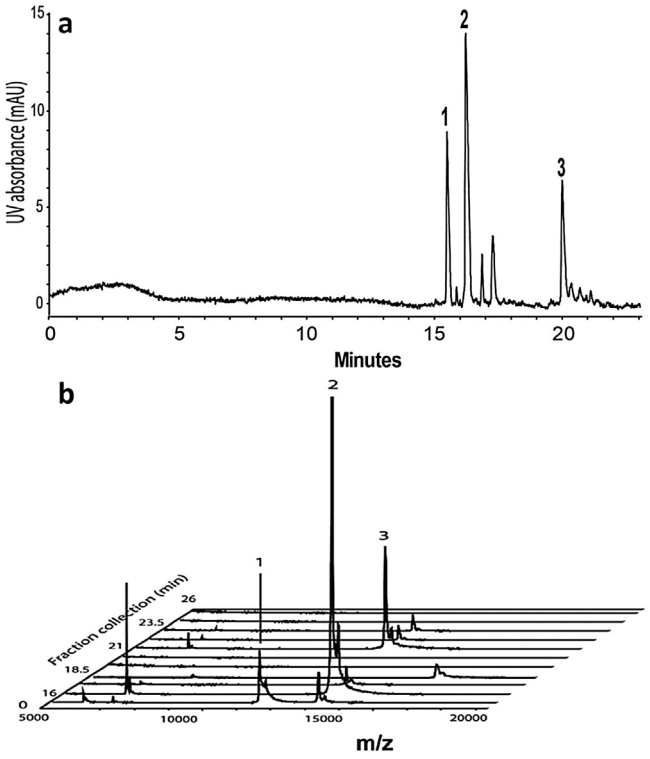


Fig. 1. (a) UV Electropherogram with fraction collection and (b) the corresponding MS spectra of each fraction of a three protein sample by CE-UV/MALDI-MS. Experimental conditions: HPC coated capillary, 50 μm d.i. x 60 cm (detection cell, 60 cm); BGE: 83.3 mM ionic strength ammonium acetate (pH 4.0); 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 and (3) 50 nM RNase A in water, MS Experimental conditions: See Section 2.

This strategy permits to collect three different fractions allowing theoretically to recover the total quantity of each protein present in each peak.

The results presented in Fig. 2 show the CE-UV separation of the three proteins and the corresponding MALDI-MS spectra of each peak with and without enrichment. For the three model protein, a significant increase of the signal have been observed between the single deposition experiment and the pooled fractions corresponding to three repetitions of separation of the same sample. This confirms the good enrichment process of the sample with the absence of carryover effect and diffusion phenomenon. Then, the presence of intact proteins in the different fractions and directly deposit in the MALDI target plate, allows for the analysis with a top-down proteomic strategy.

3.2. Top-down characterization of intact protein using CE-UV/MALDI-MS

As described below, the good robustness and repeatability of the CE-UV/MALDI-MS interface permit to perform separation and enrichment of sample in order to obtain fraction of pure and intact protein. To demonstrate the proof of principle of top-down characterization using CE-UV/MALDI-MS coupling, analysis of each fraction using ISD fragmentation process with MALDI-MS has been performed. DHB matrix in 0.1% TFA/ACN (30/70) was deposit on each fraction directly on the MALDI target plate. ISD experiments

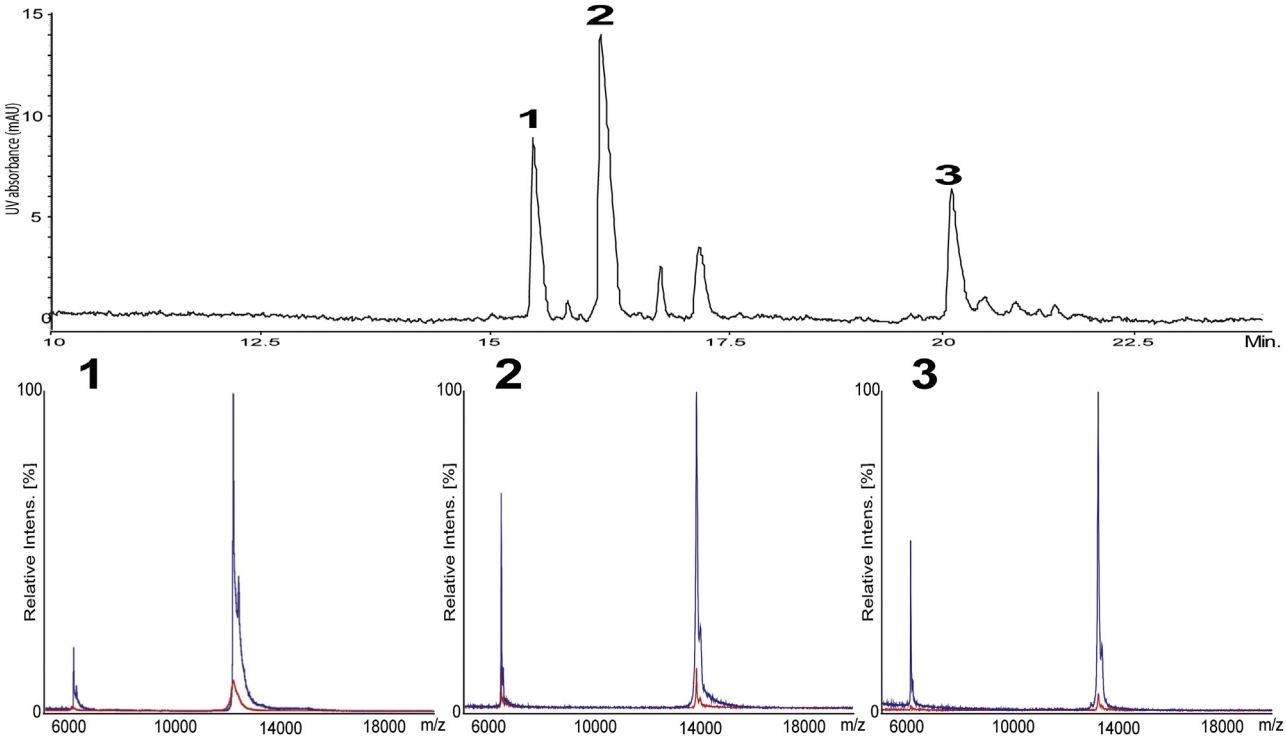


Fig. 2. UV Electropherogram with fraction collection and MS spectrum of (1) Cyt c, (2) Lys and (3) RNase A from CE-UV/MALDI-MS analysis. (Red spectrum) single deposition on the MALDI target-plate. (Blue spectrum) Enrichment of three repetitions on the same MALDI target-plate. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

were realized using the same Autoflex II (Bruker Daltonics, Bremen) previously employed for enrichment studies. Due to the age of the mass spectrometer (2006), the laser power significantly increase by a factor 1.5 as compared to the classical MS experiments. To assess the importance of sample enrichment to perform top-down analysis, ISD fragmentation have been realized in the deposit of a single separation and the collection fraction from repetition of three separation of the same sample. The results presented Fig. 3 show the ISD fragmentation of the three peaks of the CE-UV electropherogram (Fig. 1a) corresponding to Cytc, Lys and RNase A respectively. For each protein, ISD spectra have been done for one deposit (red) and for fraction collection of three repetition of separation (blue). Corresponding single deposit ISD spectra, results show no fragmentation process for each protein. This confirms that the amount of protein in each fraction are not sufficient to allow fragmentation by MALDI-ISD top-down approach. Concerning enrichment sample by three repetition of separation, ISD spectra shows protein fragmentation for each peak. As expected below, no carryover and diffusion phenomenon have been observed. These results confirm the usefulness of enriching the sample before top-down analysis to increase the amount of each protein. Regarding ISD spectra of each protein, the results presented in Fig. 3 allow to identify 17 amino acids (AA) for Cytc, 19 AA of Lys and 24 AA of RNase A corresponding to 17%, 14% and 20% sequence coverage respectively. The low percentage of sequence coverage obtained in this study can be explained by our MALDI instrument dating from 2006. Indeed, the performance of our MALDI does not allow us to obtain good ISD quality. Regarding literature and the result obtained with MALDI instrument newest with sequence coverage until 90% for 25 kDa protein [30,40], it is likely that sequence coverage would be comparable in our condition. Moreover, next generation MALDI instrument requires less quantity of sample by fraction to obtain ISD spectra. This can ease the sample enrichment step of our methodology and then decrease the sample consumption or the number of separation repetition. However, the novelty of this study was to show the proof of principle for the top-down characterization of intact protein after sample enrichment using CE-UV/MALDI-MS. The results presented Fig. 3 confirm the feasibility of this method on a rapid separation of three model proteins. To approve our CE-UV/MALDI-MS top-down approach on real sample, we assessed our system for the characterization of mAbs which represent a highly complex glycoproteins potentially displaying many naturally-occurring molecular micro-heterogeneities.

3.3. *Middle-down characterization of cetuximab Fc/2 variants*

Although mAbs were introduced as treatments against disease in the late 1980, they currently represent the most rapidly growing category of therapeutic molecule. The Fc region of mAbs is common to many new platforms as biosimilar, antibody drug conjugate or fusion protein. This region of the protein is conditioning the antibody isotype and particularly is responsible for effector functions such as antibody-dependent cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC) like phagocytosis [41]. The presence of an Fc region has consequences for Fc-mediated effector functions that might be desirable for therapeutic applications. Many levels of characterization are performed following different strategies as intact molecular weight (Mw) measurement, top-down, middle-down and bottom-up approaches [9,34]. Middle-down approach consists to characterize large portion of partially digested protein using different fragmentation mode (ISD, ETD...). This approach is particularly developed for biotherapeutic characterization [32,42]. Indeed, due to the complexity and the large Mw of mAbs and related products, middle-down proteomic may facilitate obtaining MS data and thus structural information. In our study, Fc/2 fragments of cetuximab has been selected to prove the feasibility of the methodology. Cetuximab is a chimeric mouse-human IgG1 known to bear 2 N-glycosylation sites on each HC. As every mAbs, cetuximab

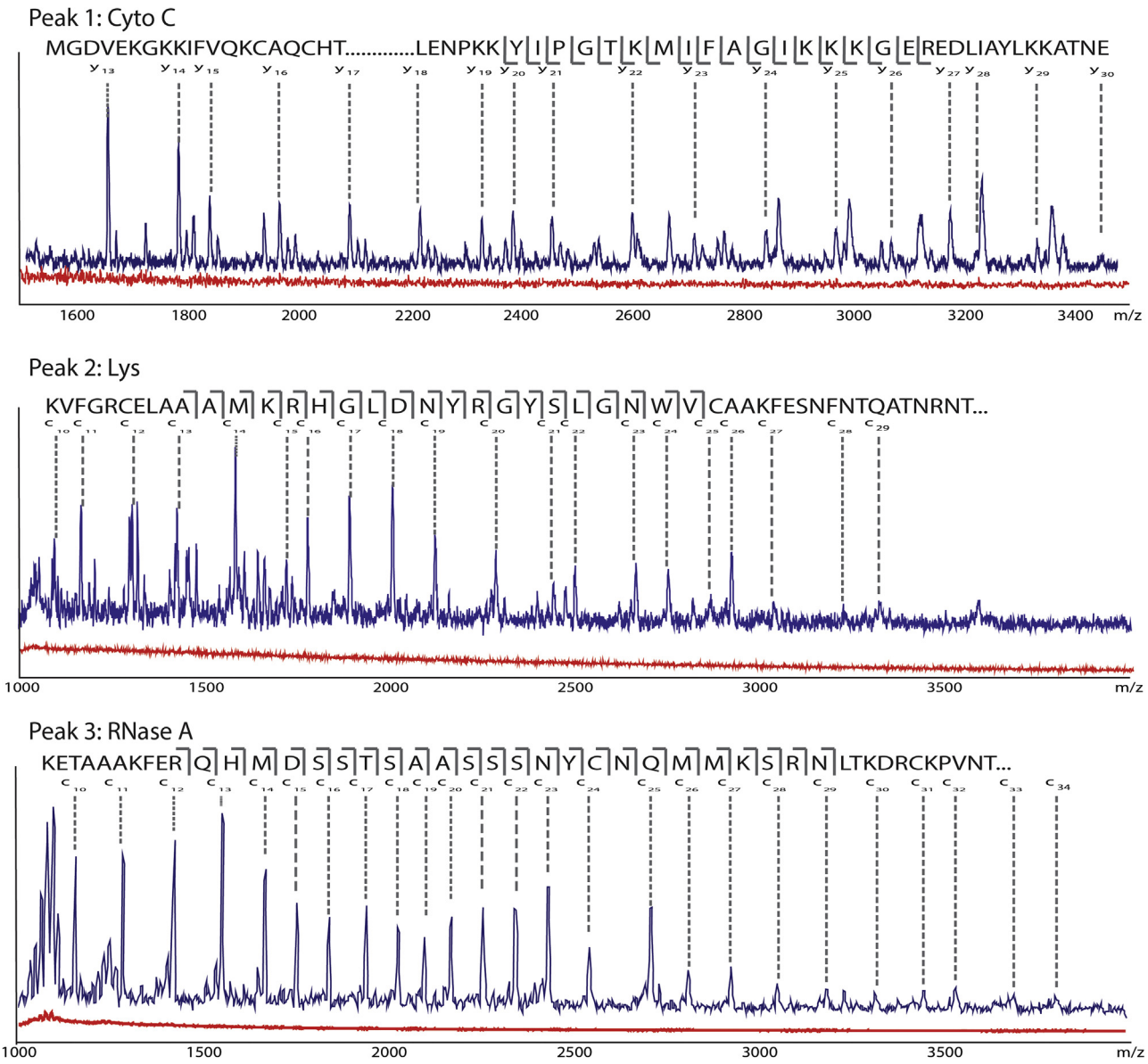


Fig. 3. Top-down MALDI-ISD spectra of Cyt c (peak 1), Lys (peak 2) and RNase A (peak 3) from CE-UV/MALDI-MS/MS analysis of a solution of three proteins with fraction collection (see Fig. 1). (Red spectrum) single deposition on the MALDI target-plate. (Blue spectrum) Enrichment of three repetitions on the same MALDI target-plate. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

can exhibit a wide variety of micro-heterogeneities particularly due to PTMs. Furthermore, this mAb can also experience HC C-terminal lysine truncation which affect Fc/2 variants. To obtain, Fc/2 fragments of cetuximab, IdeS digestion was performed. IdeS is a cysteine endopeptidase enzyme naturally secreted by *Streptococcus pyogenes* [43], IdeS enzymatic reaction cleaves cetuximab between the two consecutive glycine residues present in the hinge region to obtain two types of fragments (Fc/2 and F(ab')2). IdeS has demonstrated an exceptional specificity in IgG proteolysis on contrary to papain for instance, therefore it has been consequently used to ease and improve mAbs characterization [44]. In a previous work of our group, cetuximab IdeS digest was separated and characterized by offline CZE-UV/ESI–MS using an instrumental setting developed in house [36]. Briefly, assymetric CZE conditions was based on inlet background electrolyte (BGE) composed of ε-aminocaproic acid (EACA) 200 mM/ammonium acetate 25 mM, pH 5.7 and outlet BGE composed of ammonium acetate 25 mM, pH 5.7. HPC coating was used to minimize analyte adsorption on the capillary wall and reduce the electroosmotic flow (EOF). A separation of three peaks were identified as intact and with one lysine truncation Fc/2 variants. Moreover, Fc/2 dimers were characterized demonstrating the presence of three peaks in the electropherograms [36].

In our CE-UV/MALDI-MS methodology, pooled fraction collection of three repetitions of separation of the same sample have been performed to obtain enrichment fraction corresponding to 2.5 pmol of Fc/2 fragments in each fraction. Time of one deposit analysis represents 1 h while total time of three repetition represents around 3.5 h. Each peak has been collected based on apparent mobility as previously described. The collection of the three different fractions allow theoretically to recover the total quantity of each Fc/2 fragment present in each peak. MALDI-ISD spectra were acquired after deposition and complete evaporation of DHB matrix directly on the MALDI target plate. Fig. 4 shows the CZE-UV electropherogram and the ISD spectra of Fc/2 variants corresponding to the three peaks. Presence of fragmentation residues in each ISD spectra allows to

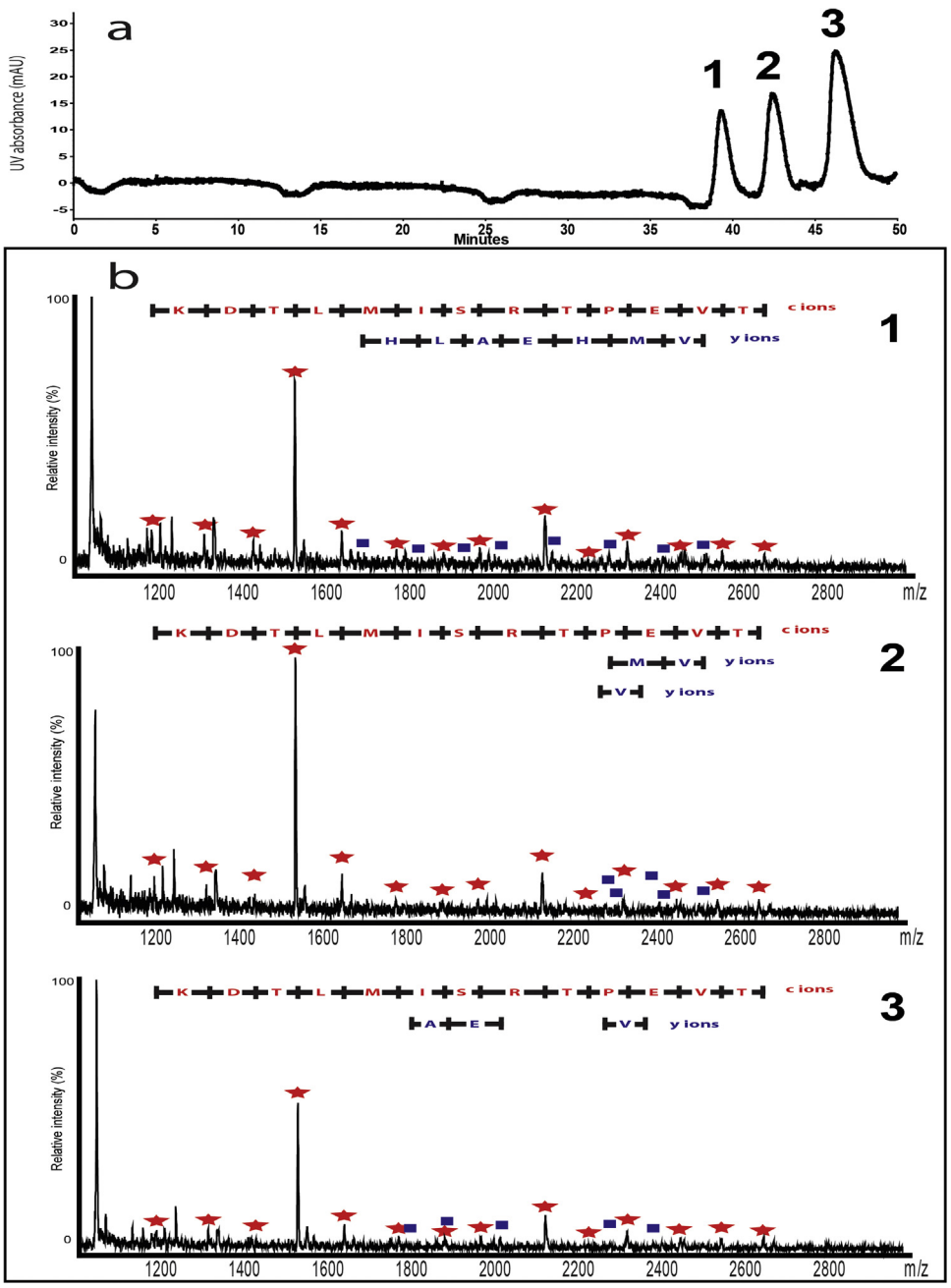


Fig. 4. CZE-UV off-line coupled to MALDI-MS via fractionation for a middle-up characterization of Cetuximab $F_{c/2}$ variants. (a) CZE-UV electropherogram. (b) Middle-down MALDI-ISD spectrum of $F_{c/2}$ variants corresponding to the three peaks. CE Experimental conditions: Experimental section; MS condition: See Experimental section; sample, IdeS digest of cetuximab (5 mg/mL); sample injection 0.5 psi 60 s.

conclude on the feasibility of the strategy for middle-down characterization of Fc/2 cetuximab variants using CE-UV/MALDI-MS.

Regarding ISD spectra of each fraction, Fig. 4b allows to identify 20 AA, 16 AA and 16 AA respectively corresponding to around 9% sequence coverage of Fc/2 cetuximab fragments. This result is in agreement with model protein characterization study and can be explained by our instrument setup. Indeed, due to the generation of our MALDI instrument, it is clearly not possible to obtain optimal ISD quality. However, as described in the literature, new generation of MALDI instrument allowed to obtain until 90% sequence coverage for 25 kDa protein [30,40]. Moreover, less quantity of sample by fraction to obtain ISD spectra are requested with newest MALDI instrument. This decreases the number of separation repetition and then can ease the sample enrichment step of our methodology.

However, more interestingly in this case, ISD fragmentation allows to observe mostly c-ions but also some low intense y-ions. Regarding c-ions, every ISD spectra indicates the same following AA sequence corresponding to Fc/2 N-terminal fragments. The complete similarity in terms of c-ions masses and sequence coverage were expected due to the similarity in terms of sequence for the Fc/2 variants in their N-terminal part. Concerning y-ions, ISD spectra corresponding to peak 1 (Fig. 4b-1) and peak 3 (Fig. 4b-3) show a difference of 128 Da between y-ion mass peaks of first detected valine residue. This mass difference corresponds to the loss of C-terminal lysine residue and then confirms the presence of Fc/2 and Fc/2-K

variants in peak 1 and peak 3 respectively. More interestingly, ISD spectrum of the peak 2 (Fig. 4b-2) leads to the same y ions series for the valine observed in peak 1 and peak 3 exhibiting the concomitant presence of Fc/2 and Fc/2-K variants in the second peak of the separation. These results confirm our previous work based on the presence of Fc/2 dimers to understand the separation process [36]. Indeed, ISD spectra approve the fact that peak 1 corresponds to Fc/2 homodimer, peak 2 corresponds to Fc/2/Fc/2-K heterodimer and peak 3 correspond to Fc/2-K homodimer. This observation proves the orthogonality of the methodologies and shows the ability of CE-UV/MALDI-MS coupling to be used to perform enrichment fraction followed by top-down analysis for mAbs characterization.

4. Conclusions

To summarize, we report here the proof of principle for the top-down characterization of intact protein after sample enrichment using CE-UV/MALDI-MS and the application to biopharmaceuticals. To validate the method, developed CZE offline instrumental setting was implemented to perform sample enrichment by multiple separation for direct deposition of collected fractions on a MALDI target plate. Three model protein mixture has been studied to assess the method. Good robustness of CZE separation and MALDI-MS spectra of each protein confirm the good enrichment process of the sample with the absence of carryover effect and diffusion phenomenon. Following CZE separation step, top-down approach by MALDI-ISD has been performed on each fraction with and without enrichment process. Results confirm the usefulness of sample enrichment to obtain adequate quantity of deposed protein for top-down analysis and the proof of concept for the method consisting to characterize intact protein mixture by top-down approach using CE-UV/MALDI-MS. Finally, to confirm the performances of this approach for the characterization of mAbs, middle-down characterization of Fc/2 cetuximab variant have been performed using CE-UV/MALDI-MS. After sample enrichment, MALDI-ISD spectra of the three fractions corresponding to the three main peaks of CZE separation were acquired. Identification of around 9% sequence coverage of Fc/2 fragments allows to conclude on the feasibility of the strategy for middle-down characterization of Fc/2 cetuximab variants using CE-UV/MALDI-MS. Moreover, mass difference for y-ions series present on each ISD spectra confirm the separation process based on the formation of Fc/2 dimers with and without C-terminal truncation. These results demonstrates the potential of enrichment fraction and top-down analysis by CE-UV/MALDI-MS as a sensitive method to in-depth mAbs characterization. Despite analysis time up to a few hours, the described analytical methodology appears to be a serious alternative to classical chromatographic methods especially in terms of intact protein characterization. Indeed, while top-down analysis allows to obtain crucial information on the primary structure of proteins, miniaturization properties of CE and geometry of the interface permit to reduce drastically the volume of analyte and open the ways to sample treatment directly on the plate such as desalting, purification or enzymatic digestion.

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