# Boosting mass spectrometry-based analytics for biopharma

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# Chapter 7

## *Sheathless CE-MS as a tool for monitoring exchange efficiency and stability of bispecific antibodies*

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## 7.1 Abstract

Bispecific monoclonal antibodies (BsAbs) are receiving great attention due to their extensive benefits as biopharmaceuticals and their involvement in IgG4 mediated autoimmune diseases. While the production of BsAbs is getting more accessible, their analytical characterization remains challenging. We explored the potential of sheathless CE-MS for monitoring exchange efficiency and stability of in-house produced bispecific antibodies. Two IgG4 bispecific antibodies with different molecular characteristics were prepared using controlled Fab (Fragment antigen binding) -arm exchange. Separation of BsAbs from their parent monospecific antibodies was achieved using a PEI-coated capillary and acidic background electrolytes permitting reliable assessment of the exchange efficiency. This was especially valuable for a Fab-glycosylated BsAb where the high glycan heterogeneity resulted in an overlap of masses with the monospecific parent antibody, hindering their discrimination by MS only. The method showed also good capabilities to monitor the stability of the generated BsAbs under different storage conditions. The levels of degradation products were different for the studied antibodies indicating pronounced differences in stability. Overall, the proposed method represents a useful analytical tool for exchange efficiency and stability studies of bispecific antibodies.

## 7.2 Main

Current advances in antibody engineering have provided effective tools for the production of bispecific antibodies (BsAbs) [1]. The dual-targeting capacity of BsAbs has been especially beneficial for therapeutic purposes permitting *e.g.* redirecting of immune effector cells to tumor cells, targeting tumor angiogenesis or blocking signalling pathways [2]. Next to the therapeutic intervention, the use of BsAbs is highly relevant in many other clinical areas [3]. For instance BsAbs have been exploited for diagnostic purposes due to their capacity to bind simultaneously to a specific antigen and a detection moiety [3]. BsAbs also occur *in vivo* as human and cyno IgG4 antibodies are uniquely capable of exchanging half-antibodies with other IgG4 molecules through a stochastic process called Fab (Fragment antigen binding) -arm exchange [4]. Due to the large heterogeneity in circulating IgG4, it is estimated that ~99% of IgG4 molecules are bispecific in humans [5]. The formation of bispecific antibodies alters their effector functions as they for example cannot crosslink antigens nor form immune complexes. Bispecific functionally monovalent antibodies have furthermore been associated with (IgG4-mediated) autoimmune diseases and in some cases may increase the pathogenicity of autoantibodies [5-7]. Therefore, well-characterized BsAbs are important tools to facilitate their application as therapeutics, diagnostic tools or to study their role in health and (autoimmune) diseases.

Production of BsAbs has considerably improved over the last years and several ways of production, including quadroma technology, chemical conjugation and genetic approaches have been developed [1,2]. In particular, recombination of antigen-binding arms between individually expressed monospecific antibodies containing two matching point mutations (known as controlled Fab-arm exchange), has permitted generation of BsAbs not only in specialized production plants but also in different academic research labs [8].

The successful production of BsAbs has to be monitored using proper analytical techniques to confirm efficient heterodimer formation and assess occurring modifications and potential degradation products. In particular, presence of some monospecific, homodimeric antibody versions in BsAb preparations may confound *in vivo* and *in vitro* experiments and represents a risk factor for eventual therapeutic applications. Current methods for assessing exchange efficiency in the formation of BsAbs mainly focus on separation techniques such as cation-exchange chromatography (CEX) and hydrophobic interaction chromatography (HIC) or the use of mass spectrometry. However, when parent monospecific antibodies feature similar molecular properties (*e.g.* mass, charge variant distribution or hydrophobicity) the assessment of the levels of heterodimer formation versus homodimer becomes challenging. Capillary electrophoresis is a separation technique that has proven high potential for the characterization of mAbs, providing resolution based on charge, mass and also shape of the protein [9-11]. Furthermore, in contrast to conventional CEX and HIC using salt buffers, CE permits direct hyphenation with MS providing confident confirmation of the identity

of the separated species. Especially, when combined with nanoelectrospray ionization via sheathless CE-MS higher ionization efficiency and sensitivity can be obtained compared to sheath-liquid approaches, which is fundamental to determine low abundant species [12].

In this study we explored for the first time the capabilities of CE-MS to assess the heterodimer formation versus homodimer as well as degradation products of BsAbs. Two IgG4 BsAbs with different molecular properties were included in the study. To this end, monospecific IgG4 antibodies mAb1 and mAb2 were independently combined with a control antibody (mAb-Ct) resulting in bispecific functionally monovalent antibodies BsAb1 and BsAb2. The BsAbs were prepared in-house based on the protocol described by Labrijn et al. [8], but adapted for IgG4 antibodies by Vergoossen et al. [7]. Mutated monospecific IgG4 antibodies (S228P) were expressed in HEK293 cells and CHO cells. mAb-Ct contained, in addition to the S228P mutation, two matching point mutations at the CH3 domain (F405L and R409K) and was added in 30% excess compared with mAb1 and mAb2 to obtain a high exchange efficiency of the BsAb1 and BsAb2 and minimize contamination with the monospecific parental clone.

BsAb1 as well as the corresponding monospecific versions were desalted in Milli-Q water using Vivaspin filters (GE Healthcare, Munich, Germany) and analysed under acidic conditions using a BGE consisting of 10% acetic acid (pH 2.1) (acetic acid from VWR Chemicals, Radnor, PA). CE separations were performed in a CESI 8000 system (Sciex, Brea, CA). Bare fused silica capillaries with a porous tip (91 cm x 30 µm i.d) were employed (OptiMS, Sciex, Brea, CA). To avoid adsorption of the positively charged antibodies to the wall of the capillary, the surface was coated in-house with polyethylenimine (PEI) (Gelest, Morrisville, PA). The PEI coating was performed and conditioned following the protocol described by the manufacturer [13]. At the beginning of each run the separation capillary was flushed for 4 min with BGE (100 psi forward pressure) and the reverse line for 2 min (75 psi reverse pressure). The antibodies (1 ug/uL) were injected hydrodynamically by applying 2.5 psi for 15 s. Separation was performed using a reversed polarity of 20 kV at 20°C for 45 min. After each run the capillary was ramped down from 20 kV to 1 kV with 50 psi forward and reverse pressure in 5 min. To obtain good ionization efficiency and sensitivity for low abundance species CE was hyphenated with nano-ESI-MS via a sheathless interface (Sciex) to a Qtof-MS (Bruker Daltonics, Bremen, Germany). Detailed detection parameters are described elsewhere [14]. **Figure 1** shows the electropherograms obtained for the BsAb1 as well as the parent antibodies mAb1 and mAb-Ct. Both monospecific antibodies were baseline separated as consequence of their different overall charge (pI 6.5 for mAb1 and 8.5 for mAb-Ct calculated via Protpi.ch). The BsAb1 heterodimer formed of ½ mAb1 and ½ mAb-Ct migrated between them reflecting its hybrid nature. BsAb preparations contained around 30% excess of mAb-Ct which was clearly detected in the electropherogram (H3N4F1/H3N4F1 mAb detected at 150236.8 Da; calculated mass 150235.8 Da). Regarding the parent mAb1 only a minimal signal (~0.8%; H3N4F1/H3N4F1 mAb detected at 146137.7 Da; calculated mass 146139.9 Da) was detected indicating a high exchange efficiency of BsAb1 formation. The deconvoluted

mass spectra of the main peak revealed a mass of 148188.9 Da which is consistent with the theoretical mass calculated for the H3N4F1/H3N4F1 glycoform of BsAb1 (148187.9 Da) excluding the formation of artefacts in the protein during the exchange procedure (**Figure 1**).

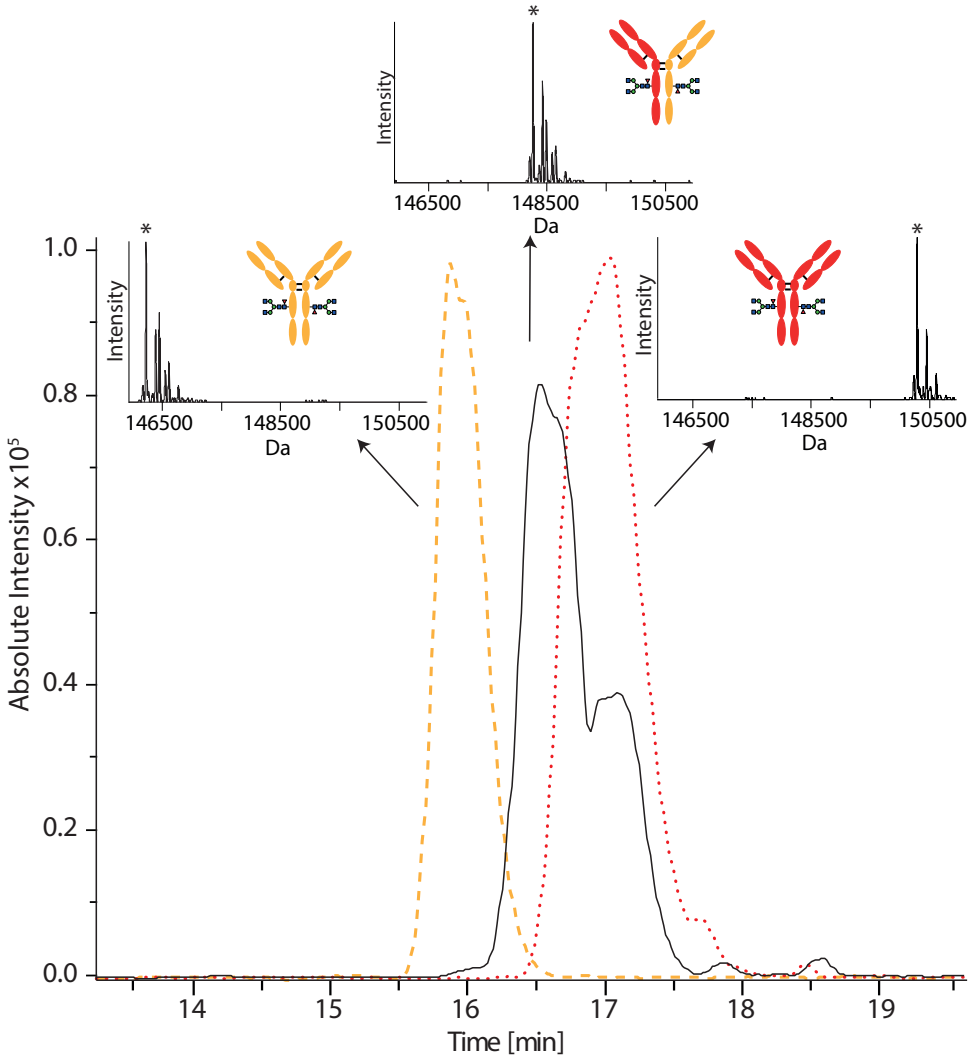


**Figure 1.** Base peak electropherogram (BPE) obtained after analysis of BsAb1 (solid line), mAb1 (dashed line) and mAb-Ct (dotted line). For each peak the deconvoluted mass spectra is depicted. Signals marked with * represent the H3N4F1/H3N4F1 Fc glycoform. Separation conditions: PEI-coated capillary; BGE, 10% AA; -20 kV, 20 °C.

BsAb2 was generated using a Fab glycosylated antibody mAb2 and mAb-Ct. In this case, some of the glycoforms of the parental and the bispecific antibody have very similar molecular masses. The Fab glycosylation of mAb2 introduced additional heterogeneity

resulting in a plethora of glycoforms. This complexity results in an overlap of signals in the deconvoluted mass spectrum when acquired by stand-alone MS hampering interpretation (**Figure S1**). For instance, for mAb2 a signal at 150606.2 Da was observed which corresponds to a glycan composition of H3N4F1/H3N4F1 for the Fc portion and H3N4/H3N5F1 (or H3N5/H3N4F1) for the Fab portion (calculated mass 150603.1 Da). This mass, however overlaps with another glycoform from BsAb2 with the glycan composition of H3N4F1/H3N4F1 for the Fc portion and H4N5F1 for the Fab detected at 150611.7 Da (calculated mass 150611.3 Da) not permitting to assess the presence of the mAb2 in the bispecific preparation. In these cases, a good separation of all the species proved to be crucial for assessing exchange efficiency. Using a BGE consisting of 10% acetic acid, overlap of the different species was observed in sheathless CE-MS (**Figure S2**). This is most probably a consequence of the highly similar calculated pI for mAb-Ct (8.5) and mAb2 (7.9). To increase resolution between the antibodies, different BGEs and separation temperatures were evaluated. The addition of MeOH (Actu-All Chemicals, Oss, The Netherlands) to the BGE (10 and 20%) resulted in a partial separation of the different species due to the increase on viscosity and consequent decrease on the EOF. Increasing the concentration of acetic acid (10-30%) further improved the resolution between peaks. Finally, using a BGE consisting of 30% acetic acid and 10% methanol, the decrease in the separation temperature from 20 to 15 °C allowed the baseline separation of both parental antibodies (**Figure 2**). This permitted to determine the identity of the separated antibodies with high confidence. Due to the Fab glycosylation, mAb2 showed a relatively lower ionization efficiency in the mass spectrometric measurement than mAb-Ct. This resulted in a similar signal for mAb-Ct (~30%) compared to BsAb2 (~70%) (**Figure 2**). Therefore, the amount of remaining mAb2 (~1 %) was estimated by spiking BsAb2 with mAb2. The addition of 0.85% of mAb2 was still detectable in the MS indicating that we can detect mAb2 amounts below 0.5%.

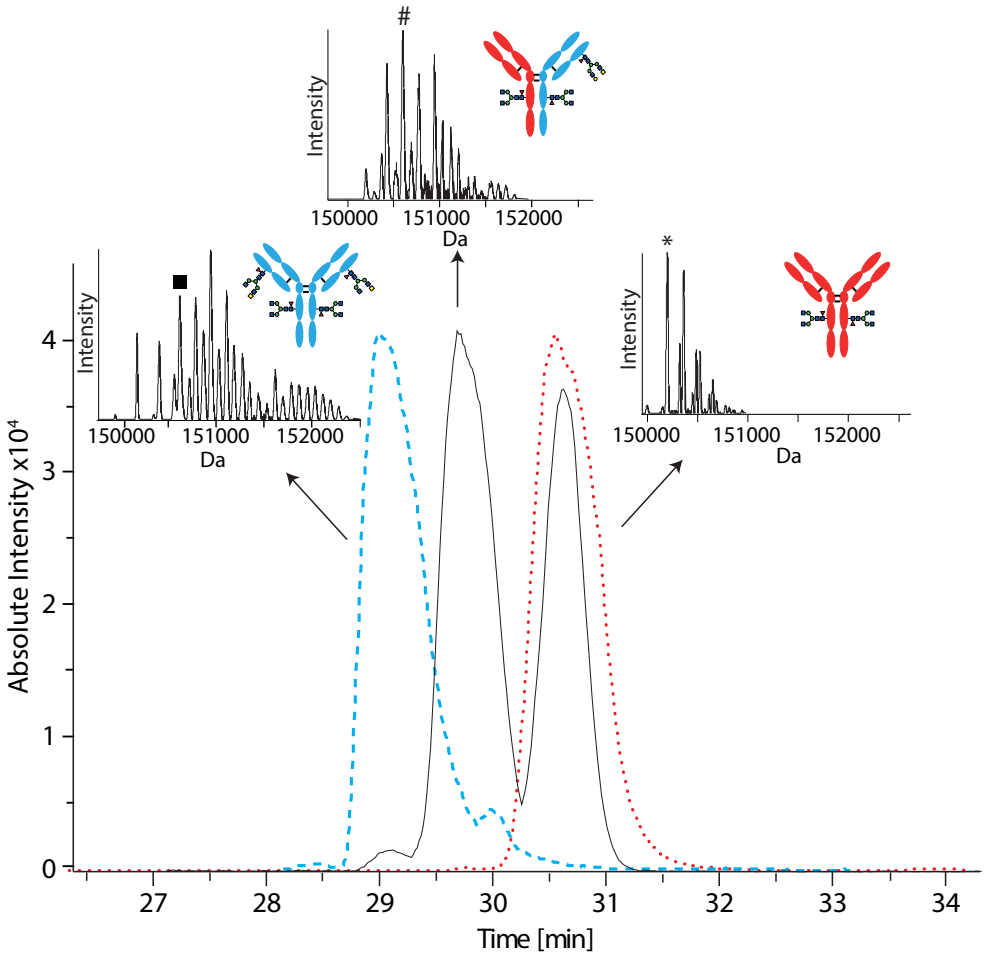


**Figure 2.** Base peak electropherogram (BPE) obtained after analysis of BsAb2 (solid line), mAb2 (dashed lines) and mAb-Ct (dotted line). For each peak the deconvoluted mass spectra is depicted. Signal marked with * represent the H3N4F1/H3N4F1 Fc glycoform without Fab glycosylation. Signal marked with # carry next to the Fc glycosylation one H3N5F1 glycan on one Lc. Signal with  carry next to the Fc glycosylation additional H3N5F1 glycans on both Lcs. Separation conditions: PEI-coated capillary; BGE, 30% AA + 10% MeOH; -20 kV, 15 °C.

Stability of these molecules is another key aspect in BsAb characterization. Degradation products such as antibodies missing a light chain, half antibodies or light chain dimers are often reported for these type of molecules [15]. Therefore, we also explored the ability of sheathless CE-MS to monitor the stability of BsAbs. To this end, antibodies were stored before and after exchange for different periods of time (0-6 months) at 4 °C or at -20 °C in 1 x PBS solution. Before analysis the samples were desalted in Milli-Q water using Vivaspin filters. For BsAb1, the analysis of the reference antibody already revealed low abundance

signals, at a migration time of 20 min. The masses of these signals were around 50 kDa and were assigned to various Fab fragments of mAb-Ct of different length (**Table S1**). No evidence of degradation products coming from mAb1 nor BsAb1 were observed in the freshly prepared samples. Storage of the sample for 2 months resulted in an increase in the levels of mAb-Ct-Fab fragments. The increase was more notable in the sample stored at 4 °C than in the one stored at -20 °C and got more pronounced after 6 months of storage (**Figure S3**). For BsAb2 the same type of Fab fragments coming from mAb-Ct were observed in the reference sample (not stored) (**Table S1**). In this case, the use of a different BGE (30% acetic acid + 10% MeOH) resulted in comigration of Fab fragments with the intact mAb-Ct. Still, it was possible to separately detect these species in the mass spectra and to determine their relative amount (**Figure S4**). In contrast to BsAb1, the amount of Fab fragments did not seem to increase during the storage of the sample. The same experiments were performed with the monospecific versions of the antibodies. For mAb-Ct, the same Fab fragments were observed in the reference sample. In line with the observations made for BsAb2 the storage conditions did not result in a noticeable increase of the amount of Fab fragments (**Figure S4**). For the parent molecules mAb1 and mAb2 we could not detect any degradation products in the reference standard, nor in the stored samples (data not shown). We hypothesize that the Fab fragments in the BsAb2 sample come from the remaining mAb-Ct (~30%) whereas in the BsAb1 they could also proceed from the degradation of the BsAb, which could indicate a lower stability of this BsAb. However, we could not find antibodies missing the Fab portion to confirm this theory. For BsAb1 and BsAb2 no evidence of further degradation products (*i.e.* additional PTMs or fragments) were observed suggesting that both BsAbs are stable over time. In vivo stability was also addressed by injecting the BsAb in mice and analyzing the circulating antibodies after 11 days by CE-MS (data available in [7]). No signs of additional species or degradation products were observed nor rearrangements of antibody arms indicating stability of the BsAbs in vivo.

To summarize, in this work we studied the potential of sheathless CE-MS to monitor exchange efficiency and stability of in-house produced BsAbs. The use of a positively coated capillary in combination with an acidic BGE provided adequate peak efficiency and quality of the mass spectra for the intact antibodies. BsAbs were separated from the parents monospecific antibody versions permitting reliable determination of the exchange efficiency even for BsAb2 where masses of the different species overlapped. The methods also allowed to monitor degradation products after prolonged storage and in vivo experiments. The method may exhibit limitations for the assessment of the exchange efficiency of BsAb where the parent mAbs exhibit similar calculated pIs but could be still valuable for their stability assessment. Although here this method has been applied to IgG4 monovalent BsAbs, it should be translatable to a variety of BsAb including bivalent BsAbs and BsAb from other subclasses often employed with therapeutic purposes (*i.e.* IgG1).

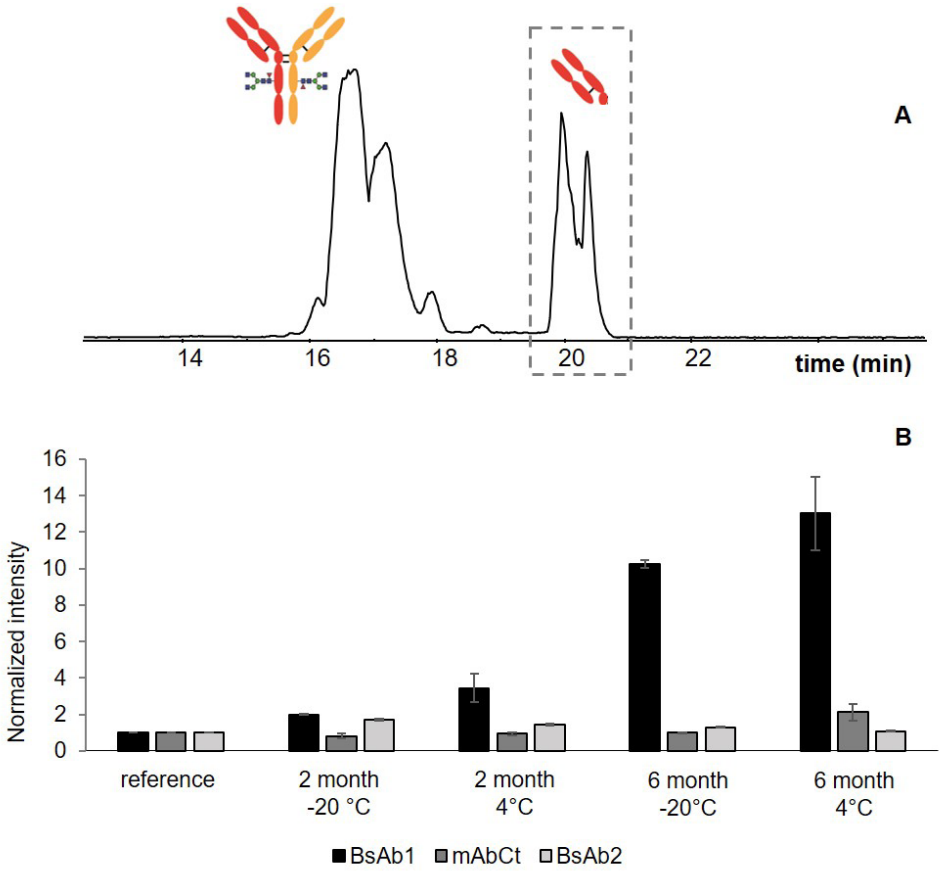


**Figure 3**. Base peak electropherogram (BPE) obtained after analysis of BsAb1 stored for 6 months at 4 °C (A). Normalized intensity obtained for the extraction of mAb-Ct-Fab (LC+Fd' (1-240)) storaged for different time and temperature (B).

## 7.3 Supporting information

Supplementary information is available free of charge on the Electrophoresis website: DOI: 10.1002/elps.202000166

## 7.4 Acknowledgments

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0040). MH receive royalties from licensed patent applications on MuSK-related research.

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