Characterization of Small Immunoconjugates (< 40 kDa) Using Capillary Electrophoresis–Mass Spectrometry

Application Note

Authors

Suresh Babu C.V.

Agilent Technologies India Pvt. Ltd,

Bangalore, India

Anne Basler, Sina Bunzendahl, and

Rainer Kneuer

Novartis Institutes for

Biomed. Research

Basel, Switzerland

Introduction

Use of monoclonal antibodies (mAb) as therapeutic drugs has increased considerably over the past years. Moreover, due to the success of mAbs, high efforts flow towards immunoconjugates. The full antibody or the antibody fragments are conjugated with small molecules such as drug or reporter molecules (for example, dyes and metal chelates) for use in therapy and diagnosis. However, random conjugation usually leads to a heterogeneous mixture of conjugates with broad labeling distribution and batch-to-batch variation. Therefore, reliable analytical methods are necessary to characterize the drug-protein ratio or degree of labeling of immunoconjugates. Capillary electrophoresis (CE) based separation is widely employed for bioconjugate analysis[1]. As conjugation of small molecules to proteins results in an electrophoretic mobility difference, CE poses a suitable technique to resolve the different degrees of conjugated species in the mixture by applying a current. Furthermore, coupling of CE to mass spectrometry (MS) provides fast and accurate identification of the immunoconjugates[2]. This Application Note demonstrates the use of the Agilent 7100 CE system coupled to an Agilent 6520 Accurate-Mass Quadrupole Time-of-Flight (Q-TOF) MS system with sheath-flow interface, for the characterization of small immunoconjugates (< 40 kDa). Single-chain variable fragment (scFv) conjugated to two small molecules (Cy5.5 and Desferal) were investigated in this study. Capillary electrophoresis provided separation of the conjugated products while the mass measurements allowed identification of the varying degrees of labeling of the immunoconjugate applied.

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Experimental

Samples

ScFv conjugated to Cy5.5 and Desferal were obtained from Novartis, Basel, CH. These two samples are referred to as scFv-A (3.32 mg/mL) and scFv-B (1.12 mg/mL), respectively. The samples were buffered in PBS and subjected to CE/MS analysis without any further processing.

Instrumentation/software

CE

Agilent 7100 CE

MS

Agilent 6520 Accurate-Mass Quadrupole Time-of-Flight (Q-TOF)

Interface

Orthogonal triple tube sheath liquid interface (G1607B)

Software

MassHunter Acquisition Software (B.06) for data acquisition and BioConfirm Software for data analysis. The raw MS spectra were subjected to maximum entropy deconvolution algorithms to generate the zero charge state spectra.

CE and MS parameters

Capillary Electrophoresis (CE)	
CE	7100 CE
Sample	Immunoconjugates
Injection	different time points
Capillary	PVA, 100 cm total length, 50 µm id
Buffer	50 mM acetic acid
Voltage	25 kV
Temperature	20 °C
Mass Spectrometry (MS)	
MS	6520 Q-TOF
Ionization mode	ESI
Acquisition mode	MS (mass range 300/500, 3,200 *m/z*)
Sheath liquid	0.5 % acetic acid in 50 % methanol, 5 µL/min
Drying gas flow	5 L/min
Nebulizer	10 psi
Drying gas temperature	200 °C
Fragmentor	250 V
Vcap	3,500 V

Results and Discussion

CE/MS is an ideal technique for the characterization of small immunoconjugates. Initial experiments were carried out to optimize the background electrolyte, resulting in 50 mM acetic acid used in this study. Figure 1A shows the deconvoluted mass spectrum for scFv-A. The base peak electropherogram (BPE) and the overlay of extracted ion electropherograms (EIE) are shown in Figure 1B. The mass distribution spectrum shown in Figure 1A indicates that the conjugated scFv incorporated 1-3 conjugated products. In addition, the presence of unconjugated scFv format (29304.80 Da) was observed. The mass difference (898 Da) measured between the conjugated species is in agreement with the expected theoretical masses. The unconjugated scFv format migrates to 23 minutes, while conjugated scFv-A species migrate between 23.5 and 26 minutes. As depicted in the BPE and EIE, strong separation of unconjugated and conjugated species was observed, which is consistent with the published reports on CE/MS[2]. Based on migration time and deconvoluted mass, the species were assigned according to their extent of conjugation. Similarly, the scFv-B immunoconjugates were analyzed, and the representative deconvoluted mass spectrum and the electropherograms are shown in Figure 2. There were 1-4 conjugated species identified for the scFv-B sample. A minute amount of unconjugated scFv was observed, indicating the efficiency of the conjugation reaction. The expected theoretical mass difference of reporter molecule B (753 Da, Desferal) between the conjugated species are shown in deconvoluted mass spectrum Figure 2.

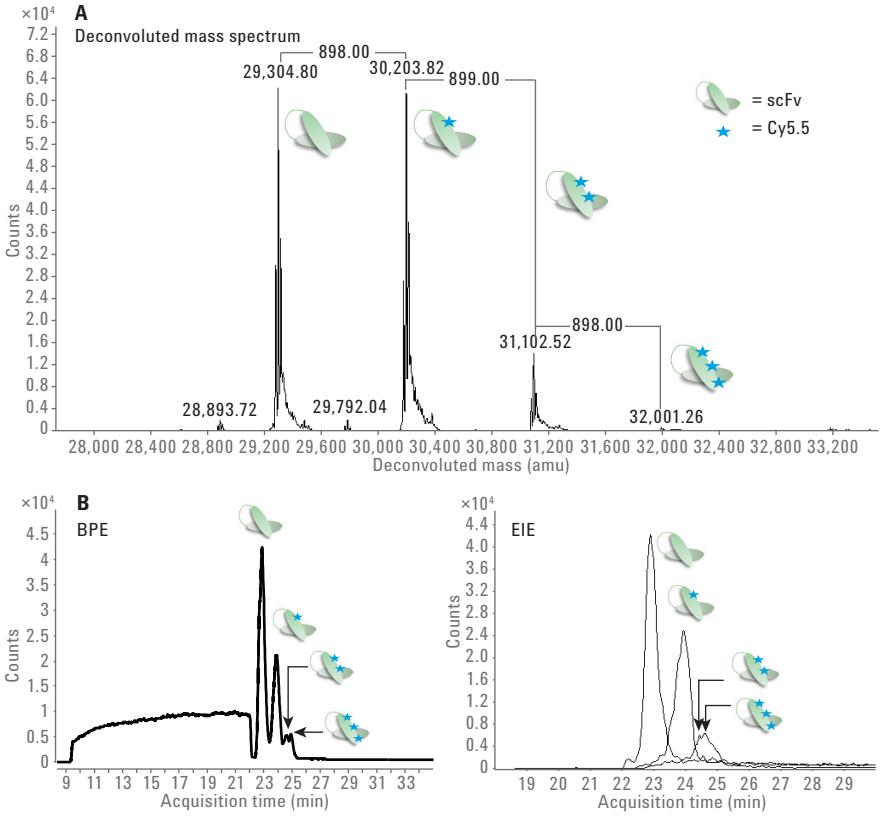

Figure 1. A) Deconvoluted mass spectrum of scFv-A conjugate. The assigned structures are based on deconvoluted mass. B) Electropherograms for separation of different scFv-A conjugate species.

Conclusion

In this study, a CE-Q-TOF MS method for the characterization of scFv conjugates labeled with two selected small reporter molecules was developed. Under the CE conditions employed, separation between the various degrees of conjugation of the two immunoconjugates applied was obtained. The accurate mass measurement combined with MassHunter BioConfirm Software enabled assignment of the specific conjugates. The results suggest that CE/MS is a potential tool for the characterization of immunoconjugates.

References

1. Wakankar, A., *et al.* Analytical methods for physicochemical characterization of antibody drug conjugates. *MAbs*. **2011**, *3(2)*, pp 161–172.

2. Haselberg, R., *et al.* Characterization of drug-lysozyme conjugates by sheathless capillary electrophoresis–time-of-flight mass spectrometry. *Anal. Chim. Acta*. **2011,** *698*(1-2), pp 77-83.

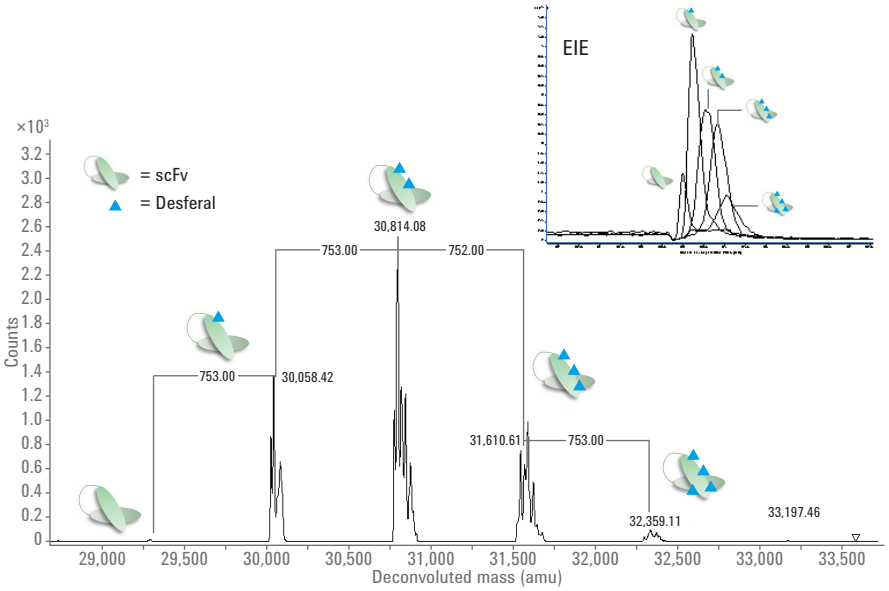

Figure 2. Deconvoluted mass spectrum of scFv-B conjugate. The assigned structures are based on deconvoluted mass. Insert depicts the electropherograms for separation of different scFv-B conjugate species.

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