FFE Service GmbH

Quantitative Separation of Protein Isoforms by Free Flow Electrophoresis

C. Timm[1], R. Wildgruber[1], K. Herzig[1], S. Madren[2], G. Weber[1]

[1]*FFE Service GmbH, Feldkirchen, Germany , www.ffeservice.com,* [2]*Biogen, RTP, North Carolina*

CE in the BIOTECHNOLOGY & PHARMACEUTICAL Industries

Introduction

Protein isoforms are defined as variants of a single polypeptide which generally alter its function and ensure the functional diversity of a proteome. About 90% of all isoform differences are due to alternative post translational modifications (PTMs), while less than 10 % results from differences in mRNA splicing. Due to the sometimes tiny physical/chemical differences between alternative isoforms, separation is challenging, and yet such separation is critical if characterization of isoforms is to be performed.

Recent developments in capillary electrophoresis (CE) and especially imaged capillary isoelectric focusing (iCIEF) have emerged as powerful charge based separation techniques within the biopharmaceutical industry. CE techniques offer raid and high-resolution separation. However, the very small volume of input sample per separation limits CE as a strictly analytical tool, which is not very useful for purifying sufficient amounts of isoforms for further identification and characterization.

We have developed a novel new separation protocol for IEF using Free Flow Electrophoresis (**IEF-FFE**), capable of extremely fast separations, using ultra-flat pH gradients that result in resolution of < 0.02 Δ-pH (< 0.04 Δ-pI) between individual separated fractions. The method runs entirely in native state, allowing structural and functional characterization of the separated isoforms (e.g. protein complexes, enzyme activity, binding studies, etc). The new method is capable of separating multiple isoforms from input quantities of up to 100 mg, and can be combined with rapid UV characterization.

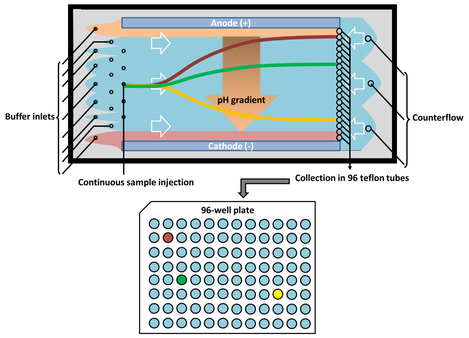
To demonstrate the utility and resolution of this new **IEF-FFE** approach, we used FFE to isolate 100 milligram amounts of individual isoforms of monoclonal antibodies (mABs). The native conditions of the separation enable further characterization of the binding properties of the individual antibody isoforms, specifically relevant for developing immunoassays or characterizing mAB drug preparations.

Material and Methods

Protein throughput:	2,5 mg/h (max. 3 mg/h)
Voltage:	2900 V
Field strength:	490 V/cm
Standard buffer flow rate:	33 ml/h
Transit/Separation time of sample:	20 minutes
pH-gradient:	8-9
Temperature:	5 °C
Resolution:	0.02 Δ-pH, 0.04 Δ-pI
IEF-gels	Serva Focus gel pH 6-11

Protocols for commercial ampholytes of different suppliers are available. The Antibody samples for this study were supplied by Biogen, RTP, North Carolina, USA.

Principle of IEF Free Flow Electrophoresis



Workflow

Gel electrophoresis directly from the FFE-run, fast CBB staining

Removal of polymer and ampholytes

Direct UV-detection within the FFE-fractions

Analysis of FFE-fractions with MS

Re-electrophoresis of FFE-fractions

Analysis of FFE-fractions with Capillary-IEF

ICE data

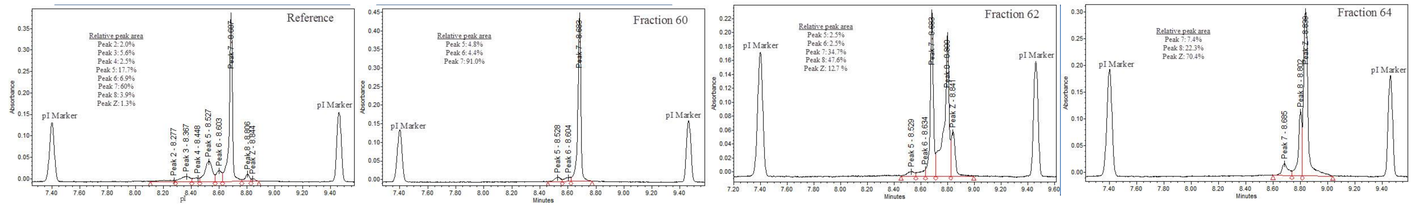
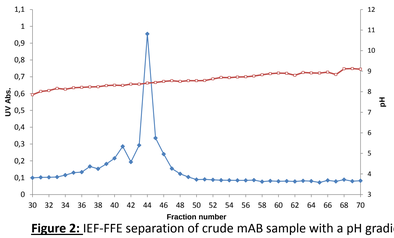
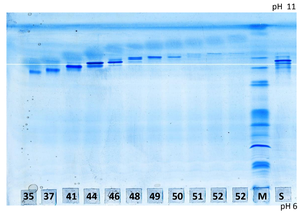


Figure 1: iCE analysis of crude mAB sample (Reference) and FFE purified mAB isoforms from different fractions. The ICE-data were provided by Biogen from a previous experiment and protocol.

IEF-FFE Pherogram



Native IEF PAGE



Re-electrophoresis of main peak

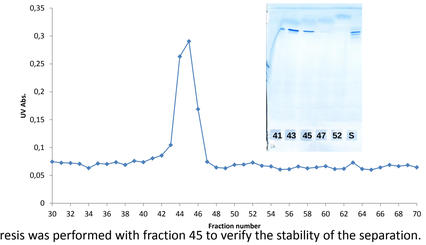
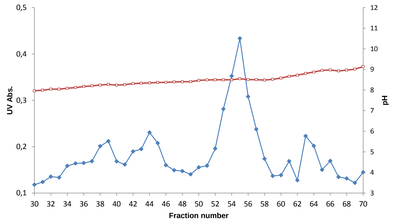
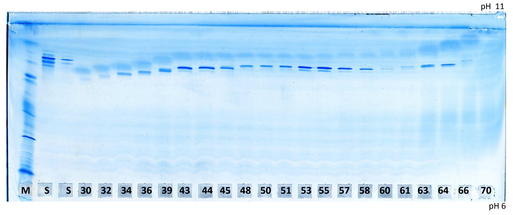


Figure 2: IEF-FFE separation of crude mAB sample with a pH gradient of 8-9, with subsequent IEF-PAGE analysis of the individual FFE fractions. FFE-Re-electrophoresis was performed with fraction 45 to verify the stability of the separation.

IEF-FFE – Zoomed pH-gradient





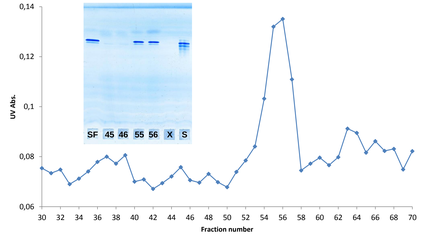


Figure 3: IEF-FFE separation of crude mAB sample on pH an ultraflat pH-gradient from 8 to 8.5, with subsequent IEF-PAGE analysis of the indiviual FFE fractions. FFE-Re-electrophoresis was performed with fraction 56 to verify the stability of the separation.

Unique benefits of this new FFE protein isoform separation method:

- Native separation conditions: Separation of intact and biochemically functional enzymes and antibodies.
- Continuous separation mode: preparative sample separation up to 0,5 - 3,0 mg/h.
- Direct compatibility for downstream analytics: PAGE, ELISA, enzyme assays, western blotting, flow cytometry, LC-MS, etc. with little or no manipulation of the FFE fractions.
- Separation of individual isoforms by FFE: purity of individual fractions > 90 %, even in cases of extremely small differences in the isoform properties.
- Customizable protocols: Variable modes of operation are possible, including straightforward fine-tuning of protocols for specific isoform properties and different commercial ampholytes.

Alternative separation method without ampholytes
=> Please see poster # 108